Role and mechanism of FLNa and UCP2 in the development of cervical cancer

AIHONG WANG[1,2], LU LIU[1], MIAO YUAN[1,3], SAI HAN[1], XUEWU YOU[1], HUI ZHANG[4], FUHUA LEI[5] and YOUZHONG ZHANG[1]

[1]Department of Obstetrics and Gynaecology, Qilu Hospital of Shandong University, Jinan, Shandong 250012; [2]Department of Obstetrics and Gynaecology, Feicheng Hospital Affiliated to Shandong First Medical University, Tai'an, Shandong 271600; [3]Department of Obstetrics and Gynaecology, The First Affiliated Hospital of Shandong First Medical University, Jinan, Shandong 250011; [4]Department of Obstetrics and Gynaecology, The Second Affiliated Hospital of Shandong First Medical University, Tai'an, Shandong 271000; [5]Department of Pathology, Feicheng Hospital Affiliated to Shandong First Medical University, Tai'an, Shandong 271600, P.R. China

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Abstract. Recent studies have reported that filamin A (FLNa) and uncoupling protein 2 (UCP2) are highly expressed in various types of cancer, but little is currently known about their roles in cervical cancer (CC). In the present study, immunohistochemical staining of paraffin sections of cervical tissues was performed in order to compare the differential expression of FLNa, UCP2, p16 and Ki67 between CC and high-grade intraepithelial neoplasia (HSIL). UCP2 and FLNa were knocked down in CC cell lines to investigate the effects on cell proliferation, cell cycle arrest, apoptosis, migration and invasion. In addition, the present study investigated the expression of cell-associated proteins [extracellular signal-regulated kinase (ERK), phosphorylated (p) ERK, protein kinase B (AKT), p-AKT and B-cell lymphoma-2 (Bcl-2)] and the mRNA levels of cellular proteins such as Ras, matrix metalloproteinase (MMP)-2 and MMP-9. FLNa and UCP2 expression levels were significantly higher in CC tissues than in HSIL tissues, with no significant differential expression of p16 or Ki67. UCP2 expression was significantly different in patients with clinical stage II or higher or lymph node metastasis compared with in other patients with cervical cancer. FLNa or UCP2 knockdown slowed or decreased SiHa and HeLa cell proliferation, migration and invasion, with no significant change in apoptosis, and downregulated the protein levels of p-ERK1/2, and the mRNA levels of Ras, MMP-2 and MMP-9. UCP2 knockdown arrested the cell cycle at the G_2 phase in SiHa and HeLa cells, while FLNa knockdown arrested the cell cycle at the G_2 phase in HeLa cells. The results of the present study revealed that FLNa and UCP2 play roles in the development and progression of CC via the Ras/MAPK/ERK signalling pathway. FLNa and UCP2 are superior to p16 and Ki67 for early prediction of CC, indicating that FLNa and UCP2 may be used for the early diagnosis of CC. UCP2 may be used to predict the prognosis of CC.

Correspondence to: Dr Youzhong Zhang, Department of Obstetrics and Gynaecology, Qilu Hospital of Shandong University, 107 Wenhua Xi Road, Jinan, Shandong 250012, P.R. China
E-mail: zhangyouzhong@sdu.edu.cn

Abbreviations: FLNa, filamin A; UCP2, uncoupling protein 2; CC, cervical cancer; NC, normal cervix; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade intraepithelial neoplasia; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; AKT, protein kinase B; MMP, matrix metalloproteinase; Bcl-2, B-cell lymphoma-2

Key words: FLNa, UCP2, cervical cancer, diagnosis, proliferation, apoptosis, migration, invasion

Introduction

Cervical cancer (CC) is a common malignant gynaecological tumour. In 2015, worldwide data showed that CC was the second most common type of cancer (second only to breast cancer) among women in developing countries (1). In 2018, 8,622,539 cases of cancer were newly diagnosed in women worldwide, and cancer-associated mortality among women reached 4,169,387 cases, where CC (7.5%) was the fourth leading cause of cancer mortality after breast cancer (15.0%), lung cancer (13.8%), and colorectal cancer (9.5%) (2). In China, the prevalence of CC is 28.2 per 1,000 women aged 30-44 years (3). Given the prevalence of CC, identifying improved treatment methods for patients at different stages is important. Early CC (stage IA) is usually treated with surgery, and advanced CC (stage IIB-IV) is usually treated with non-surgical options. For intermediate-stage CC (stages IB and IIA) (4), no consensus on the optimal treatment has been established, and the majority of patients undergo surgery or radiotherapy; however, currently available tests cannot reliably predict risk factors in these patients. If such patients undergo surgery and risk factors are confirmed during the operation, radiotherapy will be required after surgery. Numerous studies

have demonstrated a high incidence of complications following radical surgery combined with radiotherapy (5-9). Every effort should be made to avoid radical surgery combined with pelvic radiotherapy. These patients are not recommended to receive surgery, and at-risk patients may benefit more from chemo-radiotherapy. Proper preoperative selection of non-surgical treatment over unnecessary surgery will greatly improve the prognosis (6,8-10). Hence, high-risk predictors must be identified to enable stratified management. Filamin A (FLNa) can be used as a predictor of chemotherapy sensitivity and as a biomarker of prognosis (11), and uncoupling protein 2 (UCP2) can be used as an indicator of chemotherapy sensitivity in patients with advanced CC (12). The present study focused on FLNa and UCP2 and investigated their expression levels in CC, their roles in the development and progression of CC, and their value for early diagnosis and prognosis prediction of CC.

As a non-muscular actin-binding protein and an important scaffolding protein, FLNa interacts with a number of proteins involved in cell signalling and cytoskeletal reorganization, and is also regulated by phosphorylation (13). FLNa is widely distributed in the cytoplasm and is involved in the adhesion between cells. FLNa plays roles in signal transduction and protein sorting (14,15). Increased or decreased FLNa expression contributes to conditions such as inflammation and cancer (14,15). FLNa is aberrantly expressed or mutated in numerous different types of cancer, such as breast cancer, colon cancer, melanoma and prostate cancer (16-21).

UCP2 is a member of the mitochondrial UCP family, which is widely distributed throughout the body (22). As a transmembrane transporter of the inner mitochondrial membrane, UCP2 decreases the production of ATP and reactive oxygen species (ROS) by decoupling mitochondrial oxidation and phosphorylation, thereby protecting tissues and organs from oxidative stress (23). Previous studies have demonstrated increased UCP2 expression in numerous malignant tumours, including colon cancer, cholangiocarcinoma, breast cancer and leukaemia (24-27).

To the best of our knowledge, few studies have investigated the roles of FLNa and UCP2 in CC. To detect the mechanisms of FLNa and UCP2 in the development and progression of CC, the present study used paraffin sections of cervical tissues, including normal cervix (NC), low-grade intraepithelial neoplasia (LSIL), high-grade intraepithelial neoplasia (HSIL) and CC tissues, and performed immunohistochemical staining to determine the expression levels of FLNa and UCP2. The expression of FLNa and UCP2 was knocked down in CC cell lines in order to investigate the effects on cancer cell proliferation, cell cycle arrest, apoptosis, migration and invasion. In addition, the present study investigated the effects of interfering with FLNa and UCP2 expression on apoptosis-associated proteins [extracellular signal-regulated kinase (ERK), phosphorylated (p) ERK, protein kinase B (AKT), p-AKT and B-cell lymphoma-2 (Bcl-2)] and the mRNA levels of Ras, matrix metalloproteinase (MMP)-2 and MMP-9 in CC cell lines (SiHa and HeLa) with the aim of determining the mechanisms of action underlying FLNa and UCP2 in the development and progression of CC, and their roles in the early diagnosis and prognosis prediction of CC.

Materials and methods

Tissue samples from patients. In the present study, paraffin sections were collected from 33 patients with NC, 33 patients with LSIL, 40 patients with HSIL and 45 patients with CC among patients (47.51±11.41 years) treated at Feicheng People's Hospital, (Tai'an, China), between January 2010 and December 2019. The tissues were cut into 4-μm sections and 0% neutral buffer formalin solution was used to fix the samples at room temperature for 24-48 h. The clinical stage was determined according to the 2018 International Federation of Gynecology and Obstetrics (FIGO) staging guidelines (4). Patient age, previous human papilloma virus (HPV) test results, thyrocalcitonin levels, colposcopy findings, and pathology findings were recorded. For each tissue collection, a written consent from the subject was obtained. The present study was approved by the Ethics Committee of Qilu Hospital (approval no. KYLL-2017-560).

Cell lines and cell culture. Human CC cell lines (C33A, SiHa, HeLa, CaSKi, ME-180 and HH-8) and HUCEC cells (normal human cervical epithelial cells), which were used as a control, were provided by Shanghai Meixuan Biotechnology Co., Ltd. Cells were cultured in 90% Dulbecco's Modified Eagle's Medium (DMEM) (high glucose; H) + 10% foetal bovine serum (FBS) (Hyclone; Cytiva). Cells were transferred under sterile conditions, seeded into 8-10 ml of complete medium (90% DMEM [H] + 10% FBS), and cultured at 37˚C and 5% CO_2 in an incubator. The SiHa and HeLa cell lines, which have high FLNa and UCP2 expression levels, were selected for the subsequent experiments.

Antibodies and reagents. The following antibodies were used for immunofluorescence staining: Anti-GAPDH (1:5,000; cat. no. Ab8245; Abcam); anti-UCP2 (1:100; cat. no. 11081-1-AP; ProteinTech Group, Inc.) and anti-FLNa (1:100; cat. no. ab76289; Abcam). The antibodies were used to detect UCP2 and FLNa expression in different CC cell lines.

The following antibodies were used for immunohistochemical staining: Anti-UCP2 (1:200; cat. no. 11081-1-AP; ProteinTech Group, Inc.), anti-FLNa (1:200; cat. no. ab76289; Abcam), anti-P16 (1:200; cat. no. 10883-1-AP; ProteinTech Group, Inc.), and anti-Ki67 (1:1,000; cat. no. ab92742; Abcam).

The following primary antibodies were used for western blotting of relevant proteins: Anti-UCP2 (1:1,000; cat. no. 11081-1-AP; ProteinTech Group, Inc.), anti-FLNa (1:1,000; cat. no. ab76289; Abcam), anti-GAPDH (1:5,000; cat. no. ab8245; Abcam), anti-ERK1/2 (1:1,000; cat. no. 4695; Cell Signaling Technology, Inc.), anti-P-ERK1/2 (1:1,000; cat. no. 4370; Cell Signaling Technology, Inc.), anti-AKT1 (1:1,000; cat. no. ab227100; Abcam), anti-P-AKT1 (1:10,000; cat. no. ab81283; Abcam), and anti-BCL-2 (1:1,000 cat. no. ab32124; Abcam).

Western blotting. After the cell samples were fully lysed using Lysis Buffer (Beyotime Institute of Biotechnology), they were centrifuged at 12,000 x g, 4˚C for 5 min, and part of the supernatant was collected for protein determination using the bicinchoninic acid (BCA) method.

For the western blotting, the protein was separated via SDS-PAGE (10 and 12% gels), and the concentration was adjusted to 1 mg/ml, the protein quality was 20 μg, and the volume was 20 μl per lane. Before separating the proteins on a gel, a standard curve was drawn according to the optical density (OD) value and the standard protein concentration to calculate the total protein concentration in the sample. After transferring the sample solution, the membrane was blocked at room temperature for 2 h., using 1% BSA (Roche Diagnostics). The membrane was removed and then washed with tris-buffered saline Tween-20 (TBST) on a shaking table for 5 min, 3 times. In an incubation bag, the primary antibodies (FLNa and UCP2) were diluted with sealing solution and incubated overnight at 4˚C. The film was washed with TBST for 5 min, 3 times, and then the sheep anti-rabbit secondary antibody (1:5,000; cat. no. SA00001-2 Jackson ImmunoResearch Laboratories, Inc.) horseradish peroxidase-conjugated was added and BCA Protein Quantitation kit (Beyotime Institute of Biotechnology) was used.

Immunofluorescence. The SiHa and HeLa cells were blocked with 3% BSA-PBS (Roche 735094) for 30 min at room temperature. The present study used anti-UCP2 and anti-FLNa as primary antibodies and FITC [Fluorescein (FITC)-conjugated Affinipure Goat Anti-Rabbit IgG; 1:50; cat. no. SA00003-2; ProteinTech Group, Inc.] as a secondary antibody. The cell samples were fixed in 4% precooled paraformaldehyde at room temperature for 15 min, washed with phosphate-buffered saline (PBS) 3 times (3 min each time), and incubated in 0.5% Triton X-100 for 20 min at room temperature. Next, diluted antibody solution (in PBS containing 5% bovine serum albumin) (Hyclone; Cytiva) was added (PBS 0.01 M, pH 7.4 was used in the blank control group), and the samples were incubated at 4˚C overnight. On the next morning, the samples were removed from the refrigerator, placed at room temperature for 15 min, and then washed with PBS (0.01 M, pH 7.4) 5 times (5 min each). After removal of any extra PBS, FITC was added, and then DAPI was added, followed by incubation in the dark for 2 min. The nucleus was identified by blue fluorescence microscope (Olympus Corporation; x10 magnification).

Immunohistochemical staining and evaluation. Antigen retrieval was performed using TE buffer pH 9.0, heating in the microwave on band 3 for 10-15 min (700 W oven), the retrieval solution was allowed to cool at room temperature. Endogenous peroxidase activity was blocked by incubating sections in 3% H_2O_2 solution in ddH_2O for 10 min at room temperature. Tissue sections (4 mm-thick) were stained with FLNa antibody (diluted in PBS, 1:200) and UCP2 antibody (diluted in PBS, 1:200). The expression of FLNa and UCP2 was evaluated in cancer cells.(DAB Detection Kit, Ventana Medical Systems, Inc)The expression scores were grouped as follows: 0, <10% positive cells; 1, 10-25% positive cells; 2, 26-50% positive cells; 3, 51-75% positive cells; and 4, >75% positive cells. The cells were stained with DAB for 40 sec and haematoxylin and eosin for 8 min, both at room temperature, and the staining intensity was rated as 0, none; 1, weak; 2, mild; and 3, strong. The final score was the product of the quantitative expression score and staining intensity and was denoted as negative (0-1) or positive (≥2). All immunohistochemical results were reported by the pathologists at Feicheng People's Hospital (Tai'an, China) and reviewed by another experienced pathologist. A confocal microscope was used to view the images (Olympus Corporation; x40 and x200 magnification).

Reverse transcription-quantitative PCR (RT-qPCR). The primers (Table I) were designed and synthesized by Shanghai Meixuan Biotechnology Co., Ltd. TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract the total RNA from SiHa and HeLa cells, which was stored at -80˚C. The total RNA (2 μg) was used for the synthesis of cDNA according to a PrimeScript RT Reagent kit with g DNA Eraser. Reverse transcription was performed as 50˚C for 10 min). The fluorophore was provided by Shanghai Meixuan Biotechnology Co., Ltd. (SYBR Premix Ex Taq). The PCR conditions were predenaturation at 95˚C for 30 sec, then 45 cycles of 95˚C for 5 sec and 60˚C for 30 sec. The data were analysed using the $2^{-\Delta\Delta Cq}$ relative-expression method (28).

Silencing of the FLNa and UCP2 genes in SiHa and HeLa cells. Small interfering RNA (siRNA) sequences were designed and synthesized by Shanghai Meixuan Biotechnology Co., Ltd. Once the cells reached 40-50% confluency, the cells were seeded into 24-well plates and transfected with Lipofectamine® 2000 according to the manufacturer's instructions (Thermo Fisher Scientific, Inc.). First, 20 pmol siRNA was dissolved in 50 μl Opti-MEM culture medium with free FBS. Then, 1 μl Lipofectamine® 2000 was dissolved in 50 μl Opti-MEM culture medium for 5 min at room temperature. Finally, the aforementioned two solutions were mixed while resting for 20 min at room temperature, then 400 μl mixed solution was added to each well in the 24-well plates, and the transfection medium was changed to cultured medium (Hyclone; Cytiva) containing 90% DMEM high sugar and 10% FBS) after 4-6 h. The cells were extracted for the PCR experiment after 48 h. siRNAs were transfected into cells with Lipofectamine® 2000 for 48 h, and then cells were collected for qPCR to analyse the mRNA levels of UCP2 and FLNa. After 48 h, a sequence with good interference was selected for later experiments; a third siRNA was selected to knockdown UCP2, and a second siRNA was selected to knockdown FLNa in HeLa and SiHa cells. Each *in vitro* experiment was performed three independent times and in three replicates.

Cell proliferation assay. Cell Counting Kit-8 (CCK-8) (Beyotime Institute of Biotechnology) was used to detect cell survival. The absorbance at 450 nm was measured with a microplate reader (Multiskan 3; Thermo Fisher Scientific, Inc.). The measurement was $5x10^3$/100 μl for each sample. All experiments were repeated at least three times. The equation used for the calculation of the cell proliferation inhibition rate was as follows: Inhibition ratio=(OD value of control group-OD value of experimental group)/(OD value of control group-OD value of blank hole) x100%.

Migration and invasion assays

Scratch test. The transfected cells were digested and seeded into 6-well plates. Once the cells reached 100% confluency, the medium was replaced with serum-free medium to starve

Table I. Primers for mRNA detection of relevant genes.

Gene	Species	Forward, 5'-3'	Reverse, 5'-3'
hGAPDH	h	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
RAS	h	GAGTAGCGCAGTCGCCAAAG	GCTCGGGGTCCAATAGTAGC
MMP2	h	CGCATCTGGGGCTTTAAACA	GCACTGCCAACTCTTTGTCC
MMP9	h	TCTATGGTCCTCGCCCTGAA	CATCGTCCACCGGACTCAAA

h, human; MMP, matrix metalloproteinase.

the cells overnight. Different siRNAs were transfected into different groups of cells, and the medium was replaced with fresh serum-free medium after 4-6 h. A 100-μl pipette tip was used to scratch the plates, an image of which was captured and recorded as 0 h. After 48 h, another image was captured to analyse cell confluency relative to that at 0 h. The present study used Image Pro Plus software (version 6.0; Media Cybernetics, Inc.) to test and calculate the distance between the cells. The formula used for the calculation of the cell migration distance was as follows: Cell migration distance=0 h intercellular distance-48 h intercellular distance. A confocal microscope was used to detect the cells (Olympus Corporation; x100 magnification).

Transwell assay. A total of $5x10^4$ cells were inoculated into a Transwell chamber (BD Biosciences), and 500 μl of fresh medium containing 20% FBS was added to the lower chamber. The chamber was incubated at 37˚C and 5% CO_2 for 48 h. Next, the cells were fixed in 4% paraformaldehyde for 10 min, stained with crystal violet solution for 5 min at room temperature and washed with water until the base membrane was transparent. Lastly, images of the cells were captured using a confocal microscope (Olympus Corporation; x100 magnification), then the cells were counted and analysed.

Cell cycle and apoptosis assays. The transfected cells were washed twice using precooled PBS and then digested in 70% alcohol at 4˚C overnight. For cell cycle detection, a cell cycle detection kit was obtained from Beyotime Institute of Biotechnology. A FACSCalibur flow cytometer (BD Biosciences) was used to detect red fluorescence at an excitation wavelength of 488 nm. Appropriate analysis software (FlowJo; version 10; FlowJo LLC) was used for DNA content and light scattering analyses. For apoptosis detection, an Annexin V-FITC apoptosis assay kit was obtained from Beyotime Institute of Biotechnology. Cells were collected and treated as described in the kits and analysed by flow cytometry.

Statistical analysis. SPSS software (version 21.0; IBM Corp.) was used for immunohistochemical staining statistical analysis. The χ^2 and Fisher's exact test were performed. For the cell line experiments, all assays were performed in at least triplicate. The results are expressed as the mean ± SD and analysed by unpaired t-test or one-way ANOVA followed by Tukey's post hoc test, with SPSS software (version 21.0). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of FLNa/UCP2 in human cervical cancer tissues and cell lines. To detect the expression levels of FLNa and UCP2 in CC, the present study performed immunohistochemical staining on 33 NC, 33 LSIL, 40 HSIL and 45 CC tissues. Representative images are presented in Fig. 1A. The expression levels of FLNa and UCP2 in CC were rated as the product of the quantitative score and staining intensity (n=45). The scores ranged from 0 to 12, and scores of 0-1 were rated as negative, whereas scores ≥2 were rated as positive. The median score of each protein in the tissues was used as the threshold to separate low expression from high expression (Table II). The results show that FLNa and UCP2 were highly expressed in the CC tissues (Table III). The FLNa and UCP2 expression levels were detected by PCR and western blotting in six human CC cell lines (C33A, SiHa, HeLa, CaSKi, ME-180 and HH-8), and HUCEC cells (normal human cervical epithelial cells) as control cells. All experiments were repeated at least three times (Fig. 2A-C). The SiHa and HeLa cell lines, which had the highest FLNa and UCP2 expression levels, were selected for the subsequent experiments. To investigate the expression of FLNa/UCP2 in cells, the present study performed immunofluorescence staining in SiHa and HeLa cells. The immunofluorescence quantitative analysis is presented in (Fig. 2D). It was revealed that FLNa and UCP2 were mainly located in the cytoplasm of CC cells, as determined by immunofluorescence staining (Fig. 2D). According to the results of the present study, FLNa and UCP2 were highly expressed in CC cells. The immunohistochemical staining experiment showed the same results, and thus, SiHa and HeLa cells were selected for the subsequent experiments in order to determine the function of FLNa/UCP2 in CC cell lines.

Associations between the expression of FLNa and UCP2 in CC tissues and clinicopathological factors. To investigate the associations between FLNa/UCP2 expression in CC tissues and clinicopathological factors, the present study analysed age, tumour size, tumour differentiation, clinical stage, lymph node metastasis and histological type of the patients. No significant association was observed between the expression levels of FLNa in CC and age (P=0.714), tumour size (P=0.064), tumour differentiation (P=0.317), clinical stage (P=0.828), lymph node metastasis (P=0.737) or histology (P=0.060) (all P>0.05). No significant association was observed between the expression level of UCP2 in CC and age (P=0.276), tumour size (P=0.194), tumour differentiation (P=0.064) or histology

Table II. Expression of FLNa and UCP2 in cervical cancer.

		Low expression		High expression	
Protein	Total	Score	n	Score	n
UCP2	45	0-1	35	2-12	10
FLNa	45	0-6	17	9-12	28

FLNa, filamin A; UCP2, uncoupling protein 2.

Table III. Positive expression of FLNa and UCP2 in NC, LSIL, HSIL and CC.

		FLNa expression					UCP2 expression				
		Positive		P-value			Positive		P-value		
Group	n	n	%	vs. NC	vs. LSIL	vs. HSIL	n	%	vs. NC	vs. LSIL	vs. HSIL
NC	33	8	23.3	N/A	N/A	N/A	0	0	N/A	N/A	N/A
LSIL	33	19	57.6	0.006	N/A	N/A	3	9.1	0.237	N/A	N/A
HSIL	40	24	72.5	0.002	0.834	N/A	2	5.0	0.498	0.823	N/A
CC	45	39	86.7	0.000	0.004	0.005	10	22.2	0.011	0.124	0.023

FLNa, filamin A; UCP2, uncoupling protein 2; NC, normal cervix; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade intraepithelial neoplasia; CC, cervical cancer.

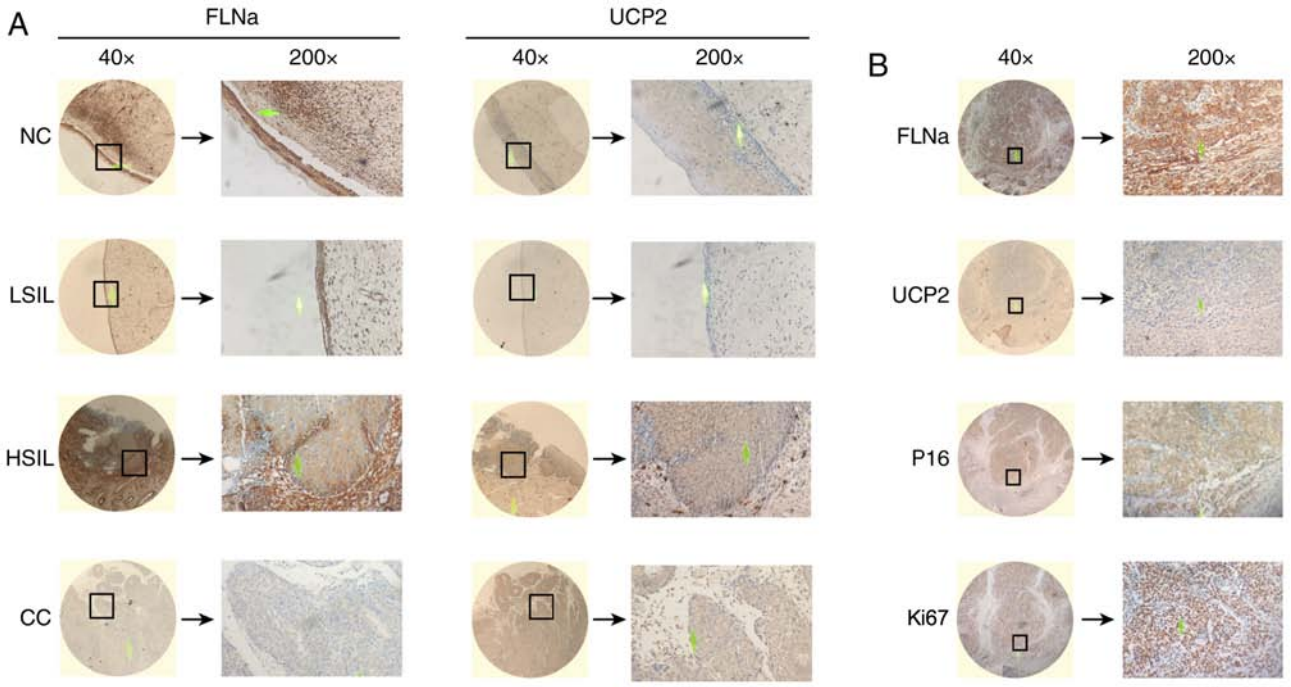

Figure 1. Immunohistochemical staining of FLNa, UCP2, p16 and Ki67 in different groups of cervical tissues. (A) Representative images for the immunohistochemical staining of FLNa and UCP2 in NC, LSIL, HSIL and CC tissues (magnification x40 and x200). (B) Representative images for the immunohistochemical staining of FLNa, UCP2, p16 and Ki67 in CC tissues (magnification x40 and x200). FLNa, filamin A; UCP2, uncoupling protein 2; NC, normal cervix; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade intraepithelial neoplasia; CC, cervical cancer.

(P>0.999). However, significant associations were observed for clinical stage (P=0.009) and lymph node metastasis (P=0.022) (both P<0.05) (Table IV).

Expression of four immunohistochemical indicators, FLNa, UCP2, p16 and Ki67, in CC tissues. To investigate whether the predictive function of FLNa/UCP2 was better than that of

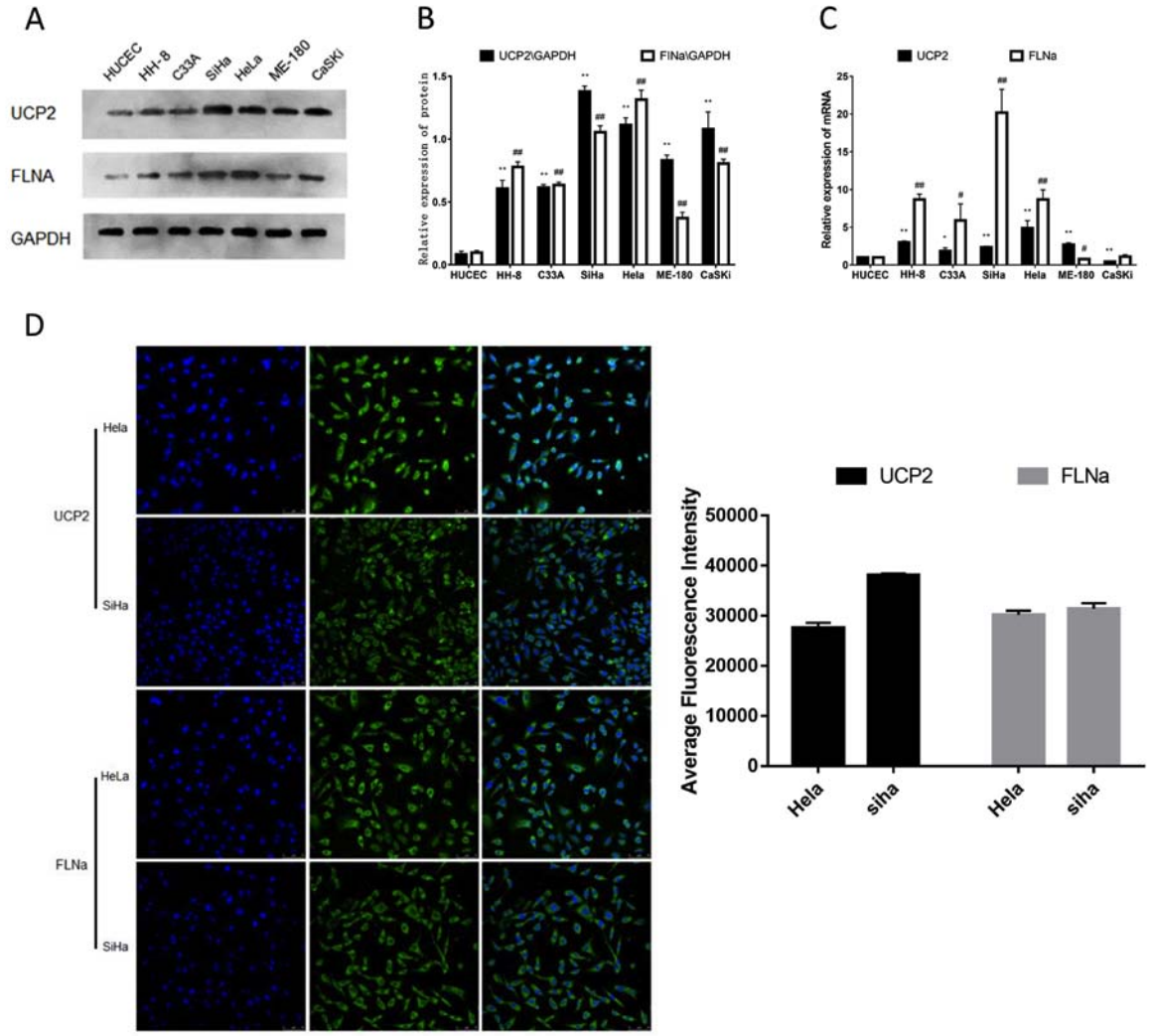

Figure 2. FLNa and UCP2 expression in different CC cell lines. (A) Relative expression of FLNa and UCP2 detected by western blotting in six human CC cell lines (C33A, SiHa, HeLa, CaSKi, ME-180 and HH-8) and HUCEC cells (normal human cervical epithelial cells) as control cells. (B) Quantification of the protein expression levels in (A) compared with the HUCEC cells. (C) Relative expression of FLNa and UCP2 detected by reverse transcription-quantitative PCR in six human CC cell lines (C33A, SiHa, HeLa, CaSKi, ME-180 and HH-8) and in HUCEC cells (normal human cervical epithelial cells) as control cells, compared with the HUCEC cells. (D) Immunofluorescence staining showing that FLNa and UCP2 were mainly located in the cytoplasm of HeLa and SiHa cells. Quantification of the immunofluorescence is shown. *P<0.05, **P<0.01, $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. FLNa, filamin A; UCP2, uncoupling protein 2; CC, cervical cancer.

p16 and Ki-67 in cervical HSIL progression to CC, the present study stained for FLNa, UCP2, P16 and Ki-67 as four CC markers in immunohistochemical staining. The expression levels of these four markers were observed under x200 and x40 magnification under a confocal microscope (Olympus Corporation). FLNa and UCP2 were primarily stained in the cytoplasm of CC cells as brown or dark-brown particles. The positive rate of FLNa was 86.7% (39/45), and the positive rate of UCP2 was 22.2% (10/45). p16 was expressed in the nucleus as brown or dark-brown particles, and the positive rate was 97.8% (44/45). Ki67 was expressed in the nucleus as brown or dark-brown particles, and the positive rate was 100% (45/45). Representative images are presented in Fig. 1B. The present study compared the positive rates of FLNa, UCP2, p16 and Ki67 between the CC and HSIL tissues (Table V). Significant differences in FLNa (P=0.005) and UCP2 (P=0.023) were observed between HSIL and CC tissues. No significant difference was observed in p16 (P=0.917) or Ki67 (P=0.471). FLNa and UCP2 were superior to p16 and Ki67 for early prediction of progression from HSIL to CC. The present study also analysed the associations between the expression levels of FLNa, UCP2 and p16 in CC and HSIL tissues (Table VI). It was revealed that in the CC tissues, both FLNa and p16 were positively expressed in 39 cases, and neither was expressed in one case (P<0.01). Both UCP2 and p16 were positively expressed in 10 cases, and neither was expressed in one case, indicating a positive association, though this result was not statistically significant (P>0.05). In HSIL tissues, both FLNa and p16 were positively expressed in 29 cases, and neither were expressed in two cases, but this was not statistically significant (P>0.01). Both UCP2 and p16 were positively expressed in two cases, and neither was expressed in two cases, but this result was not statistically significant (P>0.05). The aforementioned analysis indicated that there is an association between the expression levels of FLNa and p16 (P<0.01).

Table IV. Association between the expression levels of FLNa and UCP2 and the clinicopathological features of cervical cancer.

		FLNa, n			UCP2, n		
Group	Total	Low expression (n=17)	High expression (n=28)	P-value	Low expression (n=35)	High expression (n=10)	P-value
Age, years				0.714			0.276
≤45	17	7	10		15	2	
>45	28	10	18		20	8	
Diameter of tumour, cm				0.064			0.194
≤4	35	16	19		29	6	
>4	10	1	9		6	4	
Degree of differentiation				0.317			0.064
Low	28	9	19		19	9	
Moderate or high	17	8	9		16	1	
Stage				0.828			0.009
I	30	11	19		27	3	
II-III	15	6	9		8	7	
Lymph node metastasis				0.737			0.022
Yes	13	4	9		7	6	
No	32	13	19		28	4	
Pathological pattern				0.060			>0.999
SCC	40	13	27		31	9	
Adenocarcinoma	5	4	1		4	1	

FLNa, filamin A; UCP2, uncoupling protein 2; SCC, squamous cell carcinoma.

Table V. Positive expression of FLNa, UCP2, p16 and Ki67 in CC and HSIL.

		FLNa			UCP2			p16			Ki67		
		Positive			Positive			Positive			Positive		
Group	Total, n	n	%	P-value	n	%	P-value	n	%	P-value	n	%	P-value
HSIL	40	24	72.5	0.005	2	5	0.023	38	95	0.917	39	97.5	0.471
CC	45	39	86.7		10	22.5		44	97.8		45	100	

FLNa, filamin A; UCP2, uncoupling protein 2; CC, cervical cancer; HSIL, high-grade intraepithelial neoplasia.

Knockdown of FLNa/UCP2 influences cell proliferation, apoptosis and the cell cycle. To investigate the functions and signalling pathways of FLNa and UCP2 in the development and progression of CC, FLNa and UCP2 siRNAs (Table VII) were designed and synthesized to downregulate the expression of FLNa and UCP2 via plasmid transfection, and the knockdown effect was confirmed via western blotting HeLa and SiHa cells with stable, high expression levels of FLNa and UCP2 were used for subsequent experiments. All experiments were repeated at least three times. Based on the qPCR, a third siRNA was selected to knockdown UCP2, and a second siRNA was selected to knockdown FLNa in HeLa and SiHa cells (Fig. 3A and B). To investigate the functions and mechanisms of action underlying UCP2 and FLNa, UCP2 and FLNa expression was knocked down in HeLa and SiHa cells, and the CCK-8 assay was used to analyse cell proliferation. The present study used the cellular proliferation inhibition rate to present the results of the CCK-8 assays, which demonstrated that cell proliferation was inhibited (Fig. 3C). The present study also used flow cytometry to analyse cell apoptosis, which revealed that following UCP2 and FLNa knockdown in HeLa and SiHa cells, there was no significant difference in apoptosis compared with the control groups (Fig. 4A). Following UCP2 and FLNa knockdown in HeLa cells and UCP2 knockdown in SiHa cells, more cells were arrested at the G_2 phase. Following UCP2 knockdown in SiHa cells G2 cells content increased.

Table VI. Co-expression of FLNa, UCP2 and p16 in CC and HSIL.

				FLNa			UCP2		
Protein	Type	n	Group	Negative	Positive	P-value	Negative	Positive	P-value
p16	HSIL	40	Negative (n=2)	2	0	0.071	2	0	1
			Positive (n=38)	9	29		36	2	
	CC	45	Negative (n=1)	1	0	0.009	1	0	0.599
			Positive (n=44)	5	39		34	10	

FLNa, filamin A; UCP2, uncoupling protein 2; CC, cervical cancer; HSIL, high-grade intraepithelial neoplasia.

Table VII. FLNa and UCP2 interference sequences.

	Sequence, 5'-3'
UCP2	
1	GCCUGUAUGAUUCUGUCAATT
	UUGACAGAAUCAUACAGGCTT
2	CCUGUAUGAUUCUGUCAAATT
	UUUGACAGAAUCAUACAGGTT
3	GGUAAAGGUCCGAUUCCAATT
	UUGGAAUCGGACCUUUACCTT
Control 1	ACGGGCUCUUAAAGGAUCATT
	UGAUCCUUUAAGAGCCCGUTT
FLNa	
1	GCACUUACAGCUGCUCCUATT
	UAGGAGCAGCUGUAAGUGCTT
2	GCUGGCAGCUACACCAUUATT
	UAAUGGUGUAGCUGCCAGCTT
3	GCACAUGUUCCGUGUCCUATT
	UAGGACACGGAACAUGUGCTT
Control 2	AGUAGUCGUAUCGGACAACTT
	GUUGUCCGAUACGACUACTTT

FLNa, filamin A; UCP2, uncoupling protein 2.

Following FLNa knockdown in SiHa cells, no significant cell cycle arrest was observed (Fig. 4B).

Knockdown of FLNa/UCP2 influences cell invasion and migration. The scratch test and Transwell assay were used to detect cell migration and invasion. The results revealed that cell migration (Fig. 3D) and cell invasion were significantly decreased (Fig. 3E) with knockdown of these two proteins.

Knockdown of FLNa/UCP2 influences cell signalling pathway proteins. Western blotting was performed in order to detect the expression levels of relevant proteins (Fig. 5A), including ERK, p-ERK, AKT, p-AKT and Bcl-2. The results showed no significant change in ERK/p-ERK, AKT1 or Bcl-2 levels (Fig. 5D), but p-ERK1/2 (Fig. 5B) and p-AKT1 (Fig. 5C) were downregulated. PCR showed that the mRNA levels of Ras (Fig. 5E), MMP-2 (Fig. 5F) and MMP-9 (Fig. 5G) were decreased.

Discussion

For decades, population-wide cytology screening for CC in Europe, North America, Australia and New Zealand has contributed to a rapid decline in the prevalence of CC (29,30). In Eastern Europe and Central Asia, however, CC-associated premature mortality continues to rise due to the lack of effective screening and treatment strategies (31). Given the scale of the patient population, prognostic indicators of CC must be identified to enable stratified management of patients.

The present study showed that the positive rate of FLNa was significantly higher in CC tissues than in NC tissues, contradicting the results of a previous study (32) showing that the positive rate of FLNa was higher in normal prostate tissue than in prostate cancer tissue. This discrepancy may be associated with the different roles of FLNa in cancer cells at different sites and to the different pathological types, and further research is required in order to investigate the mechanism underlying increasing or decreasing FLNa expression levels. The results of the present study showed that the positive rate of UCP2 was significantly higher in CC tissues than in NC tissues, which is consistent with a study by Horimoto *et al* (24). Furthermore, the present study revealed that the positive rate of FLNa was 86.7% (39/45) in CC tissues (positive, n=39; negative, n=6). Among the six tissue samples with negative FLNa expression, two had positive UCP2 expression, indicating that UCP2 was a useful complementary indicator to FLNa and may help to decreased missed diagnoses during screening. Immunohistochemical staining showed that FLNa may be expressed in both CC and HSIL tissues. Thus, a positive screening result suggests high-grade cervical lesions, and that there is an association between the expression levels of FLNa and p16, which is very important for precancer screening, although the absence of negative individuals in the present study was a limitation, and so more data are required. The positive rate of UCP2 was significantly higher in CC tissues than in NC tissues, with no significant difference between NC, LSIL and HSIL tissues. UCP2 is more specific with regard to clinical stage and lymph node metastasis, and positive screening may help predict at-risk patients or advanced clinical stages.

The necessity for surgical treatment should be evaluated in each patient. Non-surgical options (such as radiotherapy) may be more advantageous than surgery. In clinical practice, p16 and Ki67 have been widely used for CC screening. Among high-risk HPV-positive subjects, p16/Ki67 dual staining is

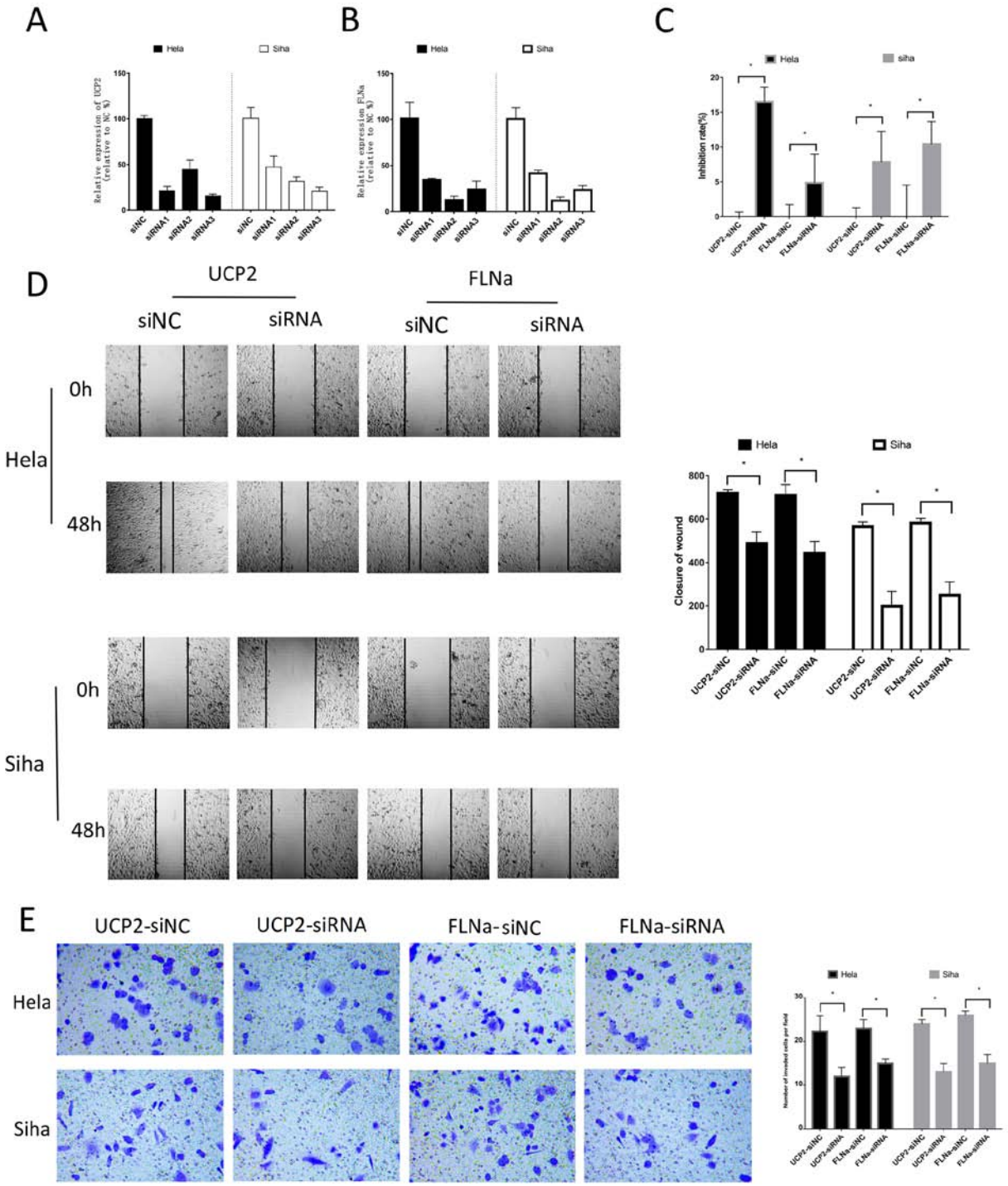

Figure 3. Investigation into the functions of UCP2 and FLNa. (A) Based on quantitative PCR, a third siRNA was selected to knock down UCP2 in HeLa and SiHa cells. (B) Based on quantitative PCR, a second siRNA was selected to knockdown FLNa in HeLa and SiHa cells. (C) CCK-8 analysis of cell proliferation. The present study used the cellular proliferation inhibition rate to present the results of the CCK-8 assays. Cell proliferation was inhibited. (D) Representative images of cell migration from the 0 and 48 h timepoints, which was significantly decreased following knockdown of FLNa/UCP2. (E) Representative images of cell invasion, which was significantly decreased following knockdown of FLNa/UCP2. *P<0.05. UCP2, uncoupling protein 2; FLNa, filamin A; siRNA, small interfering RNA; CCK-8, Cell Counting Kit-8; SiNC, small interfering normal control.

more sensitive and specific than thyrocalcitonin for cervical lesion screening (33). In the present study, the positive immunohistochemical staining rates of FLNa, UCP2, p16 and Ki67 were analysed in both CC and HSIL tissues. The results demonstrated significant differential expression of FLNa and UCP2, but not p16 or Ki67, between CC and HSIL. Furthermore, FLNa and UCP2 were superior to p16 and Ki67 for early prediction of progression from HSIL to CC. The positive rates of p16 and Ki67 were extremely high in CC tissues [p16, 97.8% (44/45); Ki67, 100% (45/45)], with no significant differential expression between patients with different clinical stages or between those with and without risk factors, potentially due to p16/Ki67 dual staining being advantageous for precancerous screening; however, it cannot be used to predict the prognosis.

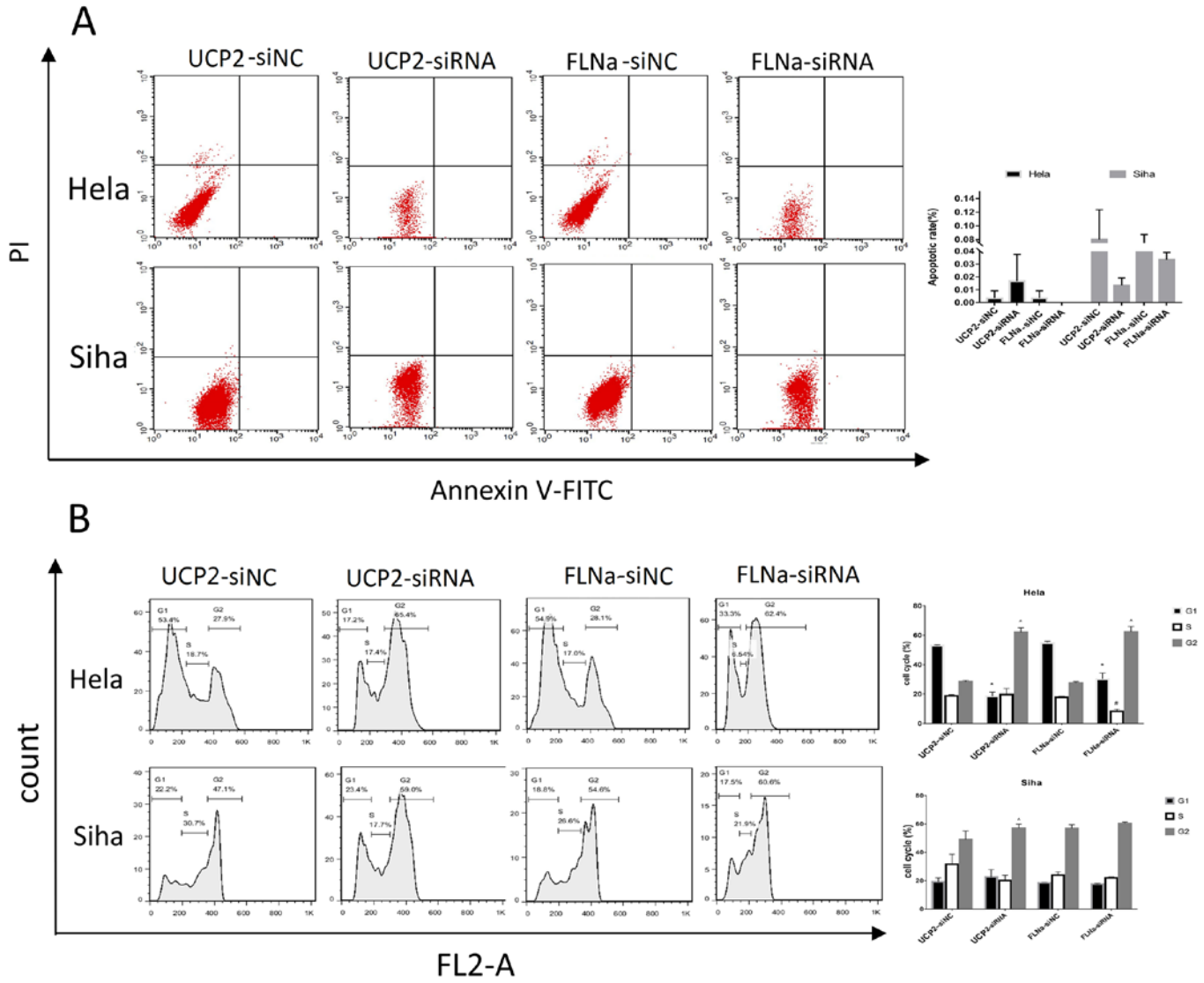

Figure 4. Representative images of flow cytometry. (A) Flow cytometry detection showed that there was no significant difference in apoptosis compared with the control groups. (B) Cell cycle detection. After UCP2 and FLNa knockdown in HeLa cells and UCP2 knockdown in SiHa cells, more cells were arrested at the G_2 phase. Following UCP2 knockdown in SiHa cells G2 cells content increased. Following FLNa knockdown in SiHa cells, no significant cell cycle arrest was observed. *P<0.05, **P<0.01, $^{\#}$P<0.05, $^{\#\#}$P<0.01; UCP2, uncoupling protein 2; FLNa, filamin A; SiNC, small interfering normal control; siRNA, small interfering RNA.

The present study showed that UCP2 was more advantageous for clinical staging and prediction of lymph node metastasis in patients with CC compared with p16/Ki67 dual staining.

Tumour development and progression depend on the activation state of signalling pathways that regulate cell proliferation and differentiation (34). Among the numerous signalling pathways associated with tumour development and progression, mitogen-activated protein kinase (MAPK) signalling is an important pathway. As the most important MAPK family member in mammalian cells, ERK is widely expressed in various tissues and is involved in the regulation of cell proliferation and differentiation. Abnormal activation of this signalling pathway leads to abnormal cell proliferation and differentiation, which are important steps in tumour development and progression (35,36).

Loss of integrity of the extracellular matrix and basal membrane (mainly collagen, laminin and fibronectin; lytic enzymes can destroy these components) is an important step in tumour metastasis (37). MMPs consist of a large family of proteolytic enzymes, and their expression is increased in a number of malignant tumour types, cultured tumour cells and oncogene-transformed cells. *In vitro* experiments have demonstrated that a high invasion capacity of tumour cells is associated with increased expression of MMP-9 (38,39). MMP-2 and MMP-9 are upregulated in CC and precancerous lesions, and studies have investigated the value of MMP-2 and MMP-9 overexpression in predicting cervical diseases (40).

In the present study, the effects and underlying mechanisms of FLN and UCP2 on the biological behaviour of cultured CC cell lines *in vitro* were investigated. Previous studies on FLNa, such as that by Zhu *et al* (41), demonstrated that FLNa regulates the Ras/ERK and Ras/guanine nucleotide exchange factor-1 pathways to decrease intracellular MMP-9, inhibit degradation of the extracellular matrix, and thereby prevent tumour cell migration, which is consistent with the findings of the present study. With regard to the mechanism underlying UCP2 in the

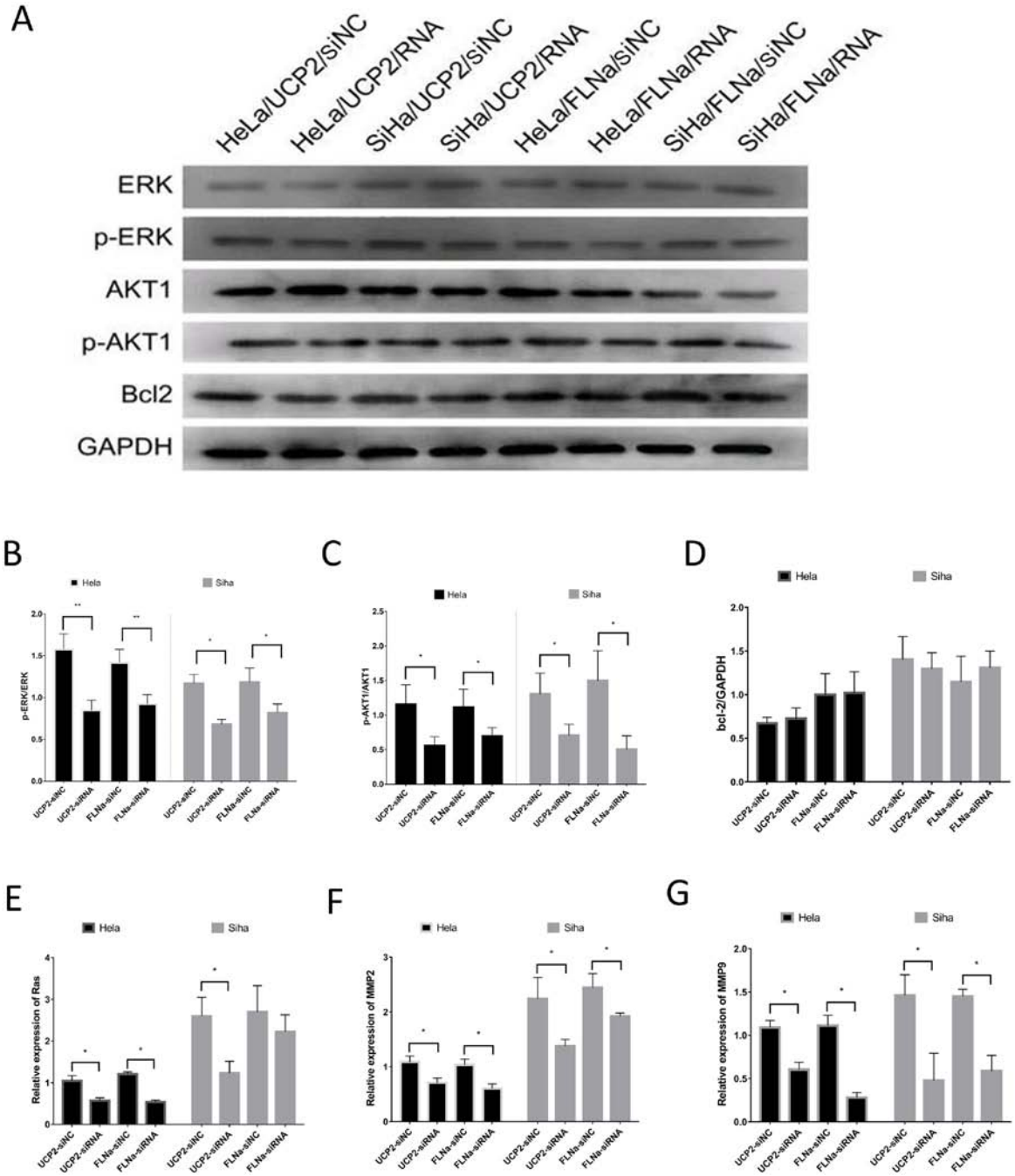

Figure 5. Investigation of the signalling pathways of UCP2 and FLNa. (A) Protein levels detected by western blotting, including ERK, p-ERK, AKT, p-AKT and Bcl-2. (B) After UCP2 and FLNa knockdown in HeLa and SiHa cells, p-ERK1/2 was downregulated; (C) p-AKT1 was downregulated; (D) there was no significant change in Bcl-2 expression; (E) the RAS mRNA levels were decreased in HeLa cells, but there was no significant change in SiHa cells; (F) MMP-2 mRNA levels were decreased; and (G) MMP-9 mRNA levels were decreased. *P<0.05; **P<0.01. UCP2, uncoupling protein 2; FLNa, filamin A; ERK, extracellular signal-regulated kinase; p, phosphorylated; AKT, protein kinase B; Bcl-2, B-cell lymphoma-2.

development of colon cancer, it has been suggested that UCP2 may be involved in the development and progression of colon cancer by regulating the production of ROS, which then activate ERK1/2 and JNK1/2, resulting in hyperproliferation of intestinal epithelial cells (42). These studies suggest that FLNa and UCP2 play roles in tumour development and progression via the Ras/MAPK/ERK signalling pathway. Our results from cultured CC cell lines *in vitro* show that FLNa and UCP2 were mainly distributed in the cytoplasm of CC cells and that knockdown of FLNa or UCP2 slowed cancer cell proliferation, arrested the cells at the G_2 phase, with no significant apoptosis, and decreased cell migration and invasion, suggesting that FLNa or UCP2 knockdown affected the biological behaviour of CC cells by decreasing the expression levels of cell-associated proteins (such as p-ERK1/2 and p-AKT1) and the mRNA levels of Ras, MMP-2 and MMP-9. Taken together, these data suggest that FLNa and UCP2 play a role in tumour development and progression via the Ras/MAPK/ERK signalling pathway, which is consistent with findings of previous studies (41,42).

Due to limitations of the experimental conditions, the present study did not analyse the tumourigenicity of FLNa or UCP2 *in vitro*. Several studies have demonstrated the roles of FLNa and

UCP2 in the development of colon and prostate cancer. In the present study, *in vitro* experiments were performed with cultured CC cells and showed that FLNa and UCP2 play roles in the development and progression of CC via the Ras/MAPK/ERK signalling pathway. FLNa and UCP2 are upregulated in CC cells and show relatively low expression levels in NC cells, reflecting the specificity of their high expression in CC. FLNa and UCP2 are superior to p16 and Ki67 for early prediction of the progression of HSIL to CC. FLNa expression levels were associated with and p16 expression levels, and may be used as indicators for this purpose. UCP2 is specific for clinical stage and lymph node metastasis and may be used as a prognostic indicator. Combined FLNa and UCP2 screening facilitates stratified management of patients. Particularly for patients who are UCP2-positive, the necessity for surgery should be re-evaluated.

The present study had a relatively small sample size; therefore, experimental data from studies with larger sample sizes are required to further validate the application of FLNa and UCP2 as clinical diagnostic strategies.

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Availability of data and materials

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

Authors' contributions

AW and YZ conceived and designed the experiments. AW performed the experiments, analysed the data and wrote the paper. LL, MY, SH and XY participated in the cell experiments. HZ and FL participated in the clinical research. All authors read and approved the final version of this manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Qilu Hospital (approval no. KYLL-2017-560). All patients provided written informed consent prior to the study start.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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