

# Effects of irreversible electroporation on cervical cancer cell lines *in vitro*

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**Abstract.** The effects of irreversible electroporation (IRE) on the proliferation, migration, invasion and adhesion of human cervical cancer cell lines HeLa and SiHa were investigated in the present study. HeLa and SiHa cells were divided into a treatment group and control group. The treatment group cells were exposed to electric pulses at 16 pulses, 1 Hz frequency for 100  $\mu$ sec with 1,000 V/cm strength. Cellular proliferation was determined 24 h after treatment using a Cell Counting Kit-8 (CCK-8) assay and carboxyfluorescein diacetate-succinimidyl ester (CFDA-SE) labeling assay. The different phases of the cell cycle were detected using flow cytometry. Wound healing, Transwell invasion and Matrigel adhesion assays were performed to evaluate the migration, invasion and adhesion abilities of HeLa and SiHa cells. The expression levels of metastasis-associated proteins were determined by western blot analysis. CCK-8 and CFSE labeling assays indicated that the inhibition of cellular proliferation occurs in cells treated with IRE. Additionally, cell cycle progression was arrested at the G1/S phase. A western blot analysis indicated that the expression levels of p53 and p21 proteins were increased, whilst those of cyclin-dependent kinase 2 (CDK2) and proliferating cell nuclear antigen (PCNA) proteins were decreased. However, wound healing, invasion and adhesion assays indicated that cellular migration, invasion and adhesion abilities were not significantly altered following exposure to IRE. IRE was not observed to promote the migration, invasion or adhesion capacity of HeLa and SiHa cells. However, IRE may inhibit the capacity of cells to

proliferate and their progression through the cell cycle *in vitro*. Preliminary evidence suggests that the underlying mechanism involves increased expression levels of p53 and p21 and decreased expression levels of CDK2 and PCNA.

## Introduction

Cervical cancer occurs frequently in females. In recent years, the incidence of cervical cancer has increased, particularly among young women below the age of 50 years (1). Currently, cervical cancer treatments include surgery, chemotherapy and radiation therapy. These are usually effective treatments, however, the structure and function of the reproductive system may become damaged as a result. The pregnancy rate of postoperative patients is low, and the risk of miscarriage and premature birth is increased (2). Loss of fertility is an undesirable side effect in the majority of young patients, therefore, further research is required to establish an effective treatment for cervical cancer that allows patients to retain full reproductive and sexual function.

The application of pulsed electric fields is a novel method used to treat tumors. Varied biological and cellular effects are observed following application of the different pulse widths of electric field, including reversible electroporation, irreversible electroporation (IRE) and intracellular electromanipulation (3,4). IRE has been widely studied and the main mechanism of its action on tumor cells is the irreversible breakdown of the cell membrane (5,6). Previous studies have confirmed the validity of the IRE treatment on tumor cells (7-9). IRE presents the following benefits: i) The treatment time is short; ii) the curative effect does not depend on the thermal effect (10); and iii) the tissue scaffolding, large blood vessels and other tissue structures surrounding target areas are not damaged (11,12). Therefore, IRE has broad application prospects in the field of tumor treatment.

Tumor tissue often does not share boundaries with the surrounding normal tissue due to its invasion; therefore, performing a treatment that targets only the tumor tissue is difficult. Residual tumor cells at the edge of the therapeutic range of the pulsed electric fields is inevitable. The behavior of

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the residual tumor cells is important in order to determine the progress of the disease and for the safe use of IRE as a clinical treatment. The behavior of residual tumor cells following IRE treatment has not been fully elucidated. Therefore, in the present study, HeLa and SiHa cells were treated with a sublethal dose of pulsed electric fields. The changes in cellular behaviors of the residual tumor cells following treatment was observed, including proliferation, migration, invasion and adhesive ability.

## Materials and methods

**Chemicals and reagents.** RPMI-1640 medium and fetal bovine serum (FBS) were purchased from GE Healthcare Life Sciences (Logan, UT, USA). A Cell Counting Kit-8 (CCK-8) was obtained from Guangzhou Yiyuan Biological Technology Co., Ltd. (Guangzhou, China). Propidium iodide (PI) and Carboxyfluorescein diacetate-succinimidyl ester (CFDA-SE) were obtained from Beyotime Institute of Biotechnology (Shanghai, China). Transwell inserts were purchased from Corning Incorporated (Corning, NY, USA). Matrigel was purchased from BD Biosciences (Franklin Lakes, NJ, USA). The primary antibodies against p53, p21, cyclin-dependent kinase 2 (CDK2), proliferating cell nuclear antigen (PCNA) and  $\beta$ -actin were obtained from BIOSS (Beijing, China).

**Cell culture and exposure to electric pulses.** HeLa and SiHa human cervical cancer cell lines (The Institute of Biological Engineering in Chongqing Medical University, Chongqing, China) were cultured in RPMI-1640 enriched with 10% FBS, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin at 37°C and 5% CO<sub>2</sub>. The cell suspensions were exposed to the electric pulse therapeutic system in the State Key Laboratory of Power Transmission Equipment and System Security and New Technology at Chongqing University (Chongqing, China) as presented in Fig. 1. The pulse parameters were selected as 16 electric pulses, 1 Hz frequency for 100  $\mu$ sec with 1,000 V/cm strength, based on a previous study (13). These parameters were used in order to ensure partial survival of the tumor cells, for their behavior to be examined.

**CCK-8 assay.** The following three groups were established: i) The treatment group, cells were treated with electric pulses; ii) the control group, cells without any treatment; and iii) the blank group, which did not contain cells. Cells were adjusted to a density of  $5.0 \times 10^4$  cells/ml and seeded in 96-well plates, in 100  $\mu$ l 10% FBS and cultured for 2, 4, 6, 8, 10, 12 and 24 h at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Next, 10  $\mu$ l CCK-8 reagent was added to each well for 2 h. The optical density (OD) value of each well was determined using a microculture plate reader at a wavelength of 450 nm. Each experimental group comprised of 6 wells and all experiments were repeated in triplicate. The survival rate of cells was calculated as follows: Cell survival rate =  $\frac{\text{OD}_{\text{experimental group}}}{\text{OD}_{\text{control group}}} \times 100$ .

**Cell proliferation assay.** CFDA-SE was used to examine the proliferative abilities of cells. It diffuses into the cytoplasm where it links to the target protein by covalent bonding and releases green fluorescence following hydrolysis. In the

process of cell proliferation, the fluorescence intensity reduces as the cells divide. Fluorescence is evenly distributed between the two daughter cells, therefore, cell proliferation can be measured from the cell fluorescence intensity.

HeLa and SiHa cells were divided into the control and treatment groups and labeled with 10 ml CFDA-SE at 37°C for 10 min in the dark, then washed twice with phosphate-buffered saline (PBS) containing 10% FBS to remove excess CFDA-SE. Cells were then plated in 6-well plates and incubated at 37°C with 5% CO<sub>2</sub>. Cells were collected and analyzed 24 h after seeding using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA). Proliferation index and precursor frequency were analyzed using ModFit LT, version 3.2 (Verity Software House, Inc., Topsham, MA, USA).

**Cell cycle analysis.** The control and treatment groups of HeLa and SiHa cells were collected 24 h after treatment and were cultivated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere, then fixed in 75% cold ethanol overnight. Following a wash with PBS, cells were incubated with 100 ml RNase A (100 mg/ml) and 400 ml propidium iodide for 30 min at 37°C. The cell cycle phase was determined by flow cytometry at 488 nm, and the relative ratios of the G1, S and G2 phases were analyzed using FlowJo, version 2.8 (FlowJo LLC, Ashland, OR, USA). The experiment was performed in triplicate.

**Wound healing assay.** The control and treatment groups of HeLa and SiHa cells were seeded in a 6-well plate. When the cells reached 80-90% confluence, the middle of the culture was scraped with a sterile pipette tip (10  $\mu$ l). The floating cells were removed by washing with PBS. Five fields of view were randomly selected and viewed with a microscope and photographed. Cells were then cultured in serum-free medium for 48 h and images were captured. The scratch width was determined using Image-Pro Plus, version 6 (Media Cybernetics, Inc., Rockville, MD, USA), and scrape repair rate =  $[(\text{width}_{0\text{h}} - \text{width}_{24\text{h}}) / \text{width}_{0\text{h}}] \times 100$ . The experiment was repeated three times.

**Cell invasion analysis.** The cell invasion ability of cells was determined by Transwell invasion assay *in vitro*. The upper chamber of 24-well Transwell plates with polycarbonate membrane (8-mm pore size) were covered with 40  $\mu$ l Matrigel (BD Biosciences; 1:4 dilution) and incubated for 24 h at 37°C. The lower chamber was filled with 500  $\mu$ l RPMI-1640 with 10% FBS. The non-invading cells were removed from the membrane using a cotton swab and the Transwell plate was fixed with 4% paraformaldehyde for 30 min. The cells were then stained with crystal violet for 10 min at room temperature. The cells that passed through the polycarbonate membrane were counted using a Leica microscope. The experiment was repeated three times for each group.

**Matrigel adhesion analysis.** The 96-well plate was covered with Matrigel for 1 h and other protein binding sites were blocked using 100  $\mu$ l bovine serum albumin (5 mg/ml) in DMEM for 1 h. A total of  $4.0 \times 10^5$  cells/well from each group were suspended in 100  $\mu$ l RPMI-1640 and cultured for 2 h. Non-adherent cells were washed away with PBS and adherent

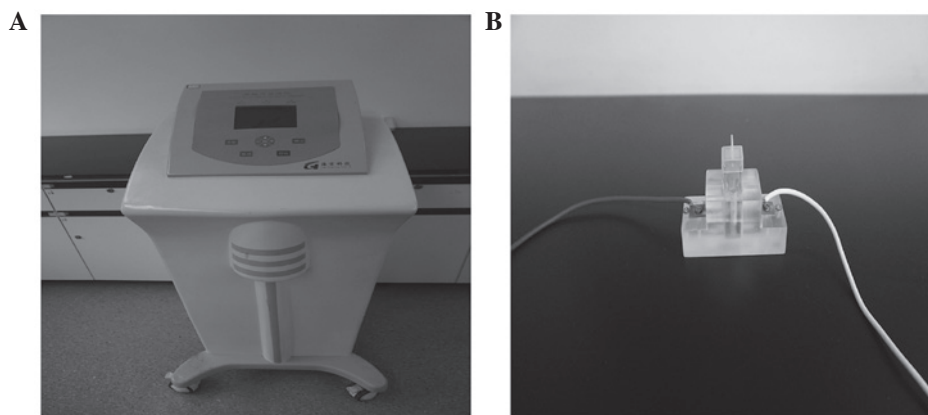


Figure 1. (A) Electric pulse therapeutic equipment. (B) Electric transfection cup, on which cell suspension was placed for electric pulse processing.

cells were treated with 20  $\mu$ l MTT in 200  $\mu$ l serum-free medium. The cells were cultured for 4 h, then the solution was removed carefully and replaced with 150  $\mu$ l dimethyl sulfoxide. The cells were oscillated in the dark for 15 min at a speed of 300 r/min. The OD value of each well was determined using a microculture plate reader at a wavelength of 490 nm. Each experimental group comprised 6 wells, and all experiments were repeated in triplicate.

**Western blot analysis.** Following IRE treatment, the cells were collected and lysed in cold radioimmunoprecipitation assay buffer (Cell Signaling Technology, Inc., Danvers, MA, USA). Proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked for 2 h at room temperature in PBS containing 5% non-fat milk, and then incubated overnight at 4°C with the following primary antibodies: Rabbit polyclonal anti-P53 (cat no. bs-8687R), anti-P21 (cat no. bs-10129R), anti-CDK2 (cat. no. bs-0757R), anti-PCNA (cat. no. bs-2007R) and anti- $\beta$ -actin (cat. no. bs-0061R) were obtained from BIOSS. Mouse anti-rabbit secondary antibody conjugated with horseradish peroxidase (bs-0295M; BIOSS) was used to visualize the stained bands with a Beyo Enhanced chemiluminescence Plus kit (Beyotime Institute of Biotechnology). Equal loading of protein was confirmed by stripping the blots and reprobing with  $\beta$ -actin antibody.

**Statistical analysis.** All data was processed with the statistical software SPSS, version 19.0 (IBM SPSS, Armonk, NY, USA). Student's t-test, one-way analysis of variance and  $\chi^2$  test were used to analyze differences between groups.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Growth inhibition of HeLa and SiHa cells.** CCK-8 assay was used to detect the effect of IRE on cellular growth. It was determined that IRE may inhibit the growth of the two cell lines (Fig. 2). The viability of HeLa and SiHa cells was decreased gradually. The survival rate of HeLa cells decreased from 62.95 $\pm$ 10.01% at 2 h to 39.69 $\pm$ 4.34% at 24 h after IRE. The survival rate of SiHa cells decreased from 61.58 $\pm$ 8.28 to 40.71 $\pm$ 6.48% over the same time period.

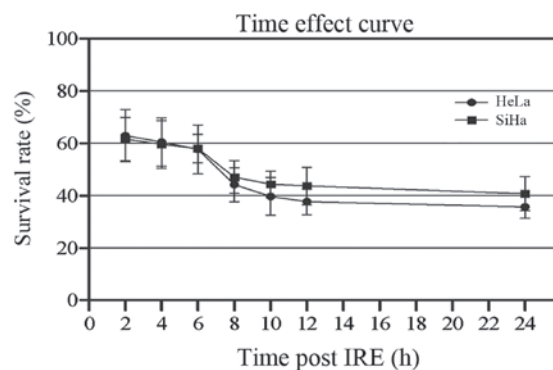


Figure 2. Cell proliferation assay of HeLa and SiHa cell lines at 2, 4, 6, 8, 10 and 24 h after exposed to electric field of 16 pulses, 1 Hz frequency for 100  $\mu$ sec with 1,000 V/cm strength. Data are presented as the mean  $\pm$  standard deviation of 3 independent experiments. IRE, irreversible electroporation.

**Proliferation suppression of HeLa and SiHa cells.** CFDA-SE fluorescence intensity was determined 24 h after IRE treatment to evaluate effects on cell proliferation (Fig. 3). Cell proliferation was inhibited in HeLa and SiHa cells treated with IRE for 24 h compared with the control group. The HeLa cells at generations 5 and 6 (7.38 $\pm$ 2.21%) were decreased significantly compared with the control group (69.77 $\pm$ 6.56%) ( $P = 0.03$ ); however, there was a greater number of cells in the treatment group at generations 1-4 compared with the control group ( $P = 0.009$ ). The SiHa cells at generations 5 and 6 (21.72 $\pm$ 3.99%) decreased significantly compared with the control group (86.08 $\pm$ 8.96%). Conversely, there was a greater number of cells at generations 1-4 compared with the control group ( $P = 0.008$ ). The results showed that IRE can inhibit cell proliferation.

**Cell cycle arrest in HeLa and SiHa cells.** The IRE inhibition of the proliferation of HeLa and SiHa cells may be due to abnormal cell cycle progression. Therefore, the cell cycle distribution was examined in each group using PI staining and flow cytometry. As presented in Fig. 4, HeLa and SiHa cells treated with IRE presented significantly higher percentages of cells in the G1 phase (59.91 $\pm$ 6.99%) compared with the control group (44.63 $\pm$ 5.79%) and significantly lower percentages of cells in the S phase compared with the control group (32.09 $\pm$ 5.2 vs. 45.13 $\pm$ 4.89%). Therefore, it is possible that IRE treatment leads to cell cycle arrest in the G1 phase.

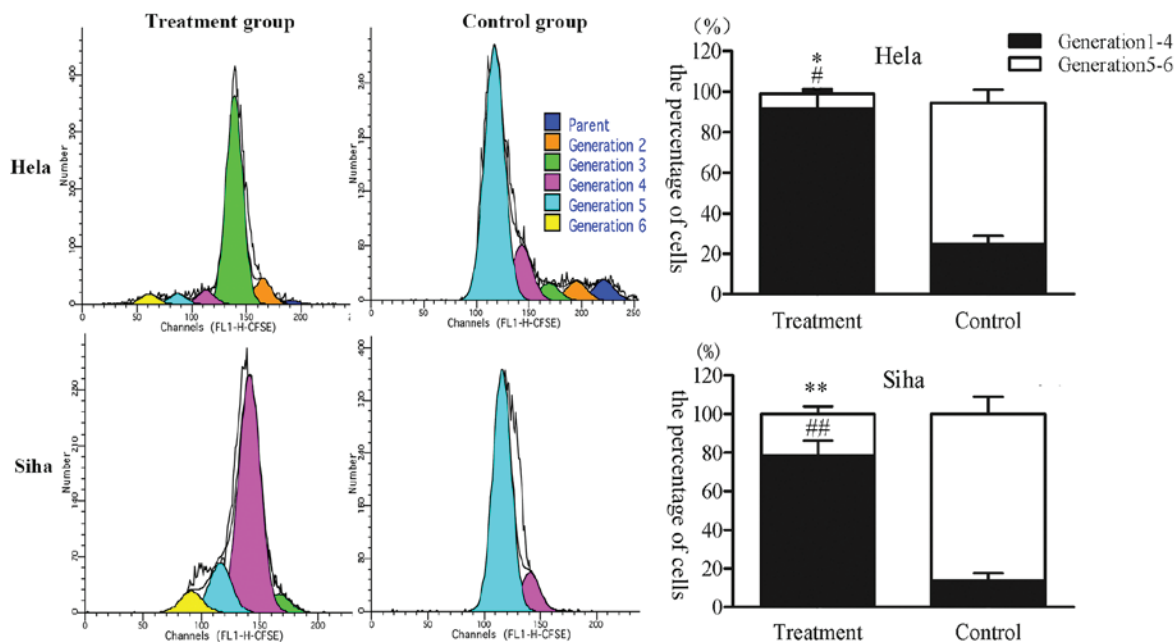


Figure 3. Effect of IRE on cell proliferation. Cell proliferation labeled with CFDA-SE were detected by flow cytometry. Cells at generations 5-6 decreased significantly vs. the control group ( $P<0.05$ ). Proliferation of HeLa and SiHa cells was suppressed following electroporation. \* $P<0.05$  and \*\* $P<0.05$  vs. the HeLa control group. \*\* $P<0.05$  and ## $P<0.05$  vs. the SiHa control group. CFDA-SE, carboxyfluorescein diacetate-succinimidyl ester.

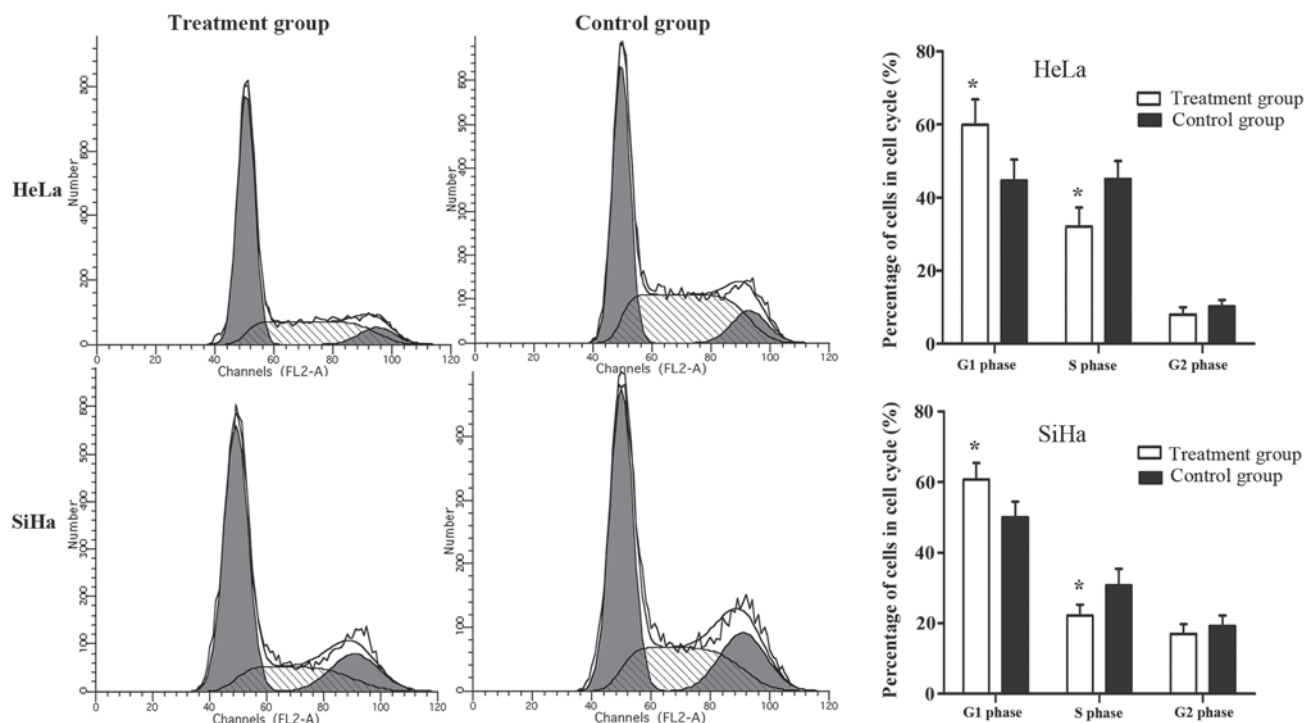


Figure 4. Effects of IRE on the cell cycle. Following a 24 h treatment with IRE, HeLa and SiHa cells were fixed and stained with propidium iodide. The DNA contents were subjected to flow cytometry to measure numbers of cells in the different stages of cell cycle. Column diagram analysis indicated that IRE increased cell cycle arrest in the G1 phase. \* $P<0.05$  vs. the control group. IRE, irreversible electroporation.

**Effect of IRE on cell migration.** A wound healing assay was used to examine the effect of IRE on cell migration. No significant differences were observed between the control and treatment groups in HeLa and SiHa cells (Fig. 5). When the counted respective scrape repair rates were determined, similar results were

obtained (Table I). Therefore, IRE treatment was not observed to have a significant effect on HeLa and SiHa cell migration.

**Effect of IRE treatment on cell invasion.** Cell invasion ability is an important factor for determining tumor malignancy.



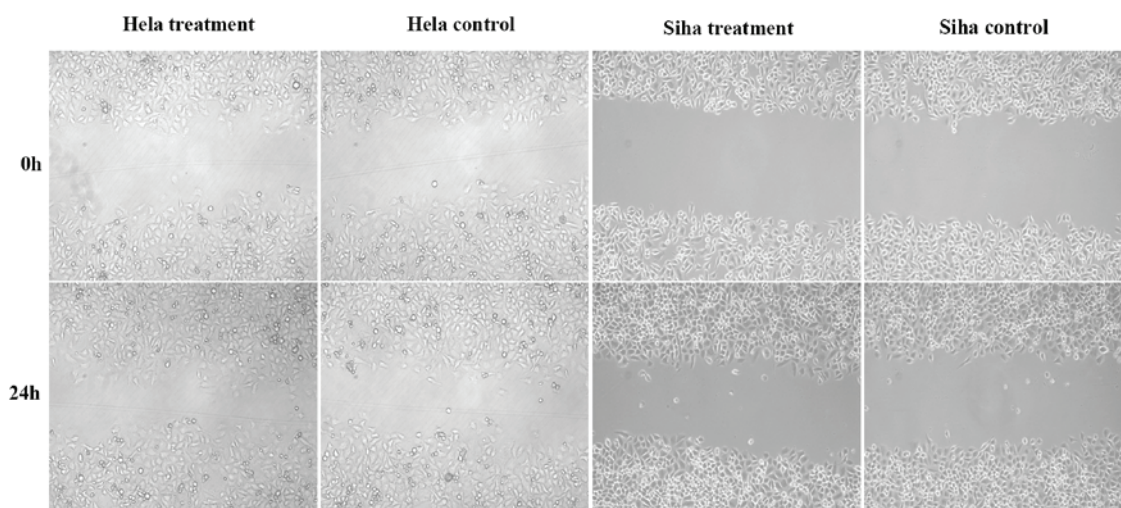


Figure 5. Effect of irreversible electroporation on cell migration. Following 24 h treatment with electric pulses, the wound healing rate in HeLa and SiHa did not differ significantly when compared with the control group.

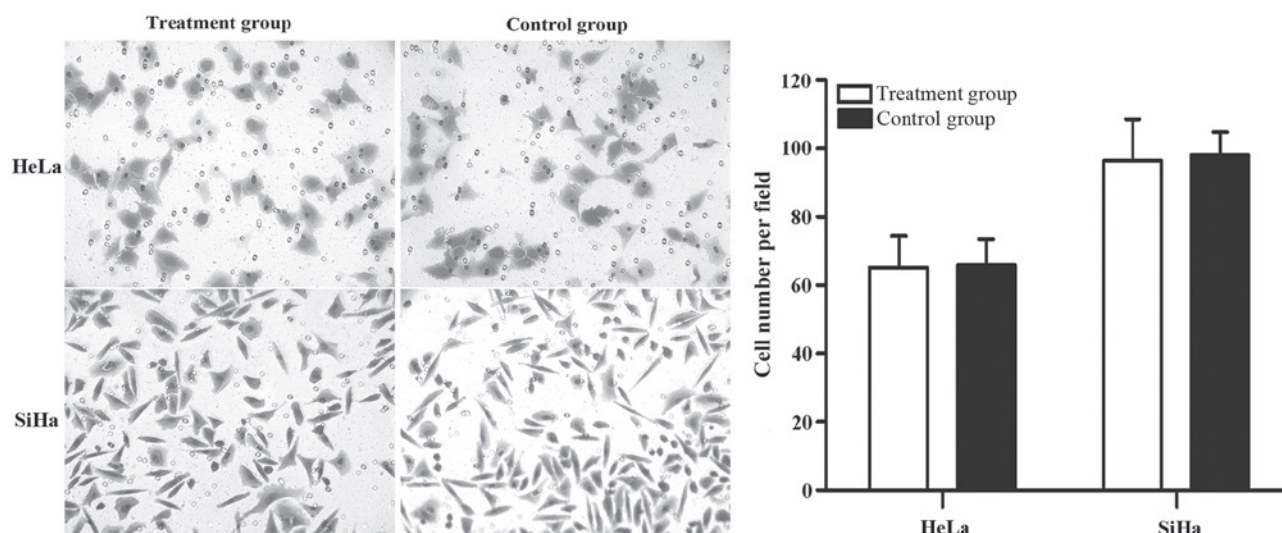


Figure 6. Effect of IRE on cervical cancer cells. Cells ( $1.0 \times 10^5$ ) were seeded in the upper Transwell chamber for 24 h and then stained with crystal violet. There was no significant difference in the invasive cells between the treatment group and the control group. IRE did not increase the invasive ability of HeLa and SiHa cells 24 h after treatment. Data are presented as the mean  $\pm$  standard deviation. IRE, irreversible electroporation.

Table I. Scrape repair rate of HeLa and SiHa cells subsequent to the pulse treatment at 24 h.

Repair rate	Treatment group (%)	Control group (%)
HeLa cells	20.53 $\pm$ 7.94	23.13 $\pm$ 3.53
SiHa cells	14.76 $\pm$ 6.30	17.39 $\pm$ 3.79

Data are presented as the mean  $\pm$  standard deviation.

The effect of IRE on cell invasion was investigated by Transwell assay. The number of HeLa and SiHa invading cells did not differ when the treatment group was compared with the control group (Fig. 6). Similar to the results of the migration assay, IRE has no clear effect on HeLa and SiHa cell invasion.

**Effect of IRE on cell adhesion.** The effect of IRE treatment on the adhesion ability of cells was verified by adhesion assay. MTT assay was used to detect the adhesive ability of cells. No significant difference was observed in the OD values of HeLa and SiHa cells when the treatment group was compared with the control group (Table II). This indicates that IRE treatment of cervical cancer cells does not affect the cell adhesion potential.

**Analysis of the expression level of proliferation-associated genes.** IRE treatment led to the reduction of cell proliferation and limited cell cycle progression. Therefore, the expression level of genes associated with cell cycle progression p53, p21, CDK2 and PCNA was investigated. The protein expression levels of p53 and p21 were significantly greater 24 h after IRE treatment. The protein expression levels of CDK2 and PCNA were significantly reduced compared with the control group (Fig. 7).

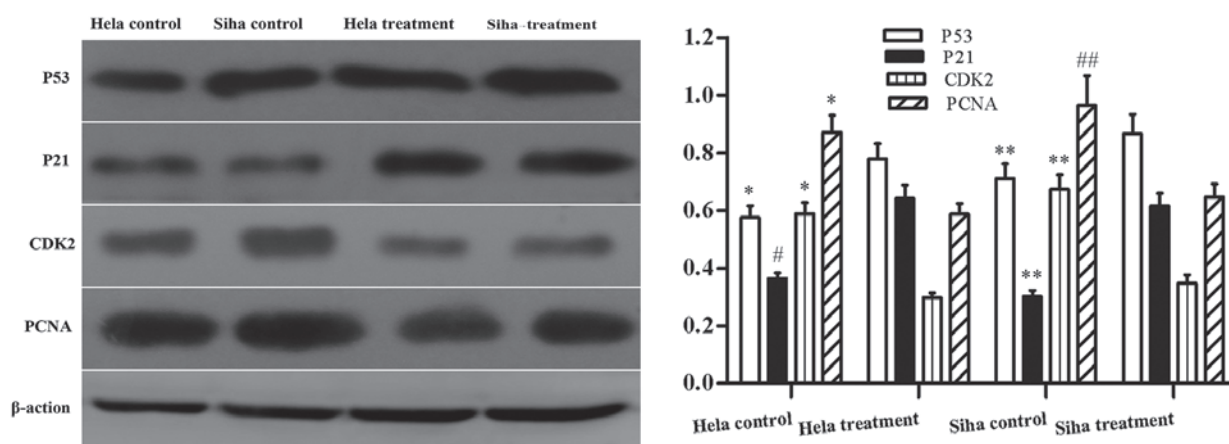


Figure 7. Expression of proliferation signaling pathway-associated genes. HeLa and SiHa cells were treated with irreversible electroporation. Western blot analysis performed 24 h after treatment indicated reduced expression levels of p53 and p21. Additionally, increased expression levels of CDK2 and PCNA were observed. CDK2, cyclin-dependent kinase 2; PCNA, proliferating cell nuclear antigen. \* $P < 0.01$  and # $P < 0.05$ , compared with the HeLa treatment group; \*\* $P < 0.01$  and ## $P < 0.05$ , compared with the SiHa treatment group.

Table II. OD values of HeLa and SiHa adhesive cells subsequent to the pulse treatment at 24 h.

OD value	Treatment group	Control group
HeLa cells	0.234±0.023	0.300±0.010
SiHa cells	0.241±0.037	0.283±0.049

OD, optical density. Data are presented as the mean ± standard deviation.

## Discussion

IRE is a potential novel therapy for the treatment of tumors. Previous studies have confirmed that the use of pulsed electric fields in cancer cells and animal xenograft may lead to the irreversible electroporation, necrosis and apoptosis of tumor cells (13,14). Additionally, IRE has been used to treat malignancy, with short-term success (15,16). Cervical cancer is the most common malignancy of the female reproductive system. Advance screening technology in cervical cancer allows for early-stage diagnosis of patients. The specific anatomical location of the cervical tumor may be exposed fully by a simple instrument, which provides the necessary basic conditions required for effective IRE ablation treatment.

Residual tumor tissue often occurs following IRE treatment. In the case of radio frequency ablation (RFA), it has been determined that RFA may promote cancer recurrence. Additionally, residual cancer cell proliferation in animal experiments and clinical treatment of liver cancer has been observed (17,18). Irreversible electroporation has therapeutic efficacy in the clinical treatment of prostate cancer and renal cancer. Although it has not been used clinically in the treatment of cervical cancer, experimental research has made significant progress (19). However it is limited by the lack of long-term follow-up observation of tumor recurrence and metastasis. Existing physical therapies, such as use of the electric knife

and laser are limited to the treatment of carcinoma *in situ*; and are not used for invasive lesions. This is because these therapies increase residual tumor cell proliferation and invasion. In the present study, HeLa and SiHa human cervical carcinoma cell lines were investigated. The behavior of residual tumor cells treated with sublethal doses of pulsed electric fields was determined.

Based on previous experimental results (14), a field strength of 1,000 V/cm, frequency 1 Hz and 16 pulses were selected as pulse parameters. The change of the proliferative capacity of cells was investigated. The results indicated that the proliferation of HeLa and SiHa cells was inhibited 24 h after IRE treatment and cell cycle progression was arrested at the G1 stage. The expression levels of cell cycle-associated proteins p53, p21, CDK2 and PCNA were also determined. The increased expression levels of p53 induce the expression of p21, which combines with the cyclin E/CDK2 complex to suppress the activity of CDK2. This leads to a halt of cell cycle progression at the G1 and S phases (20,21). The present study confirmed increased expression levels of p53 and p21, whilst recording decreased expression levels of CDK2. PCNA is closely associated with DNA synthesis processes in cells and is frequently used as an evaluation index for tumor cell proliferation (22). It is possible that the inhibition of the proliferation of residual tumor cells may be a result of PCNA expression and the P53-P21-CDK2 signaling pathway. Invasion and metastasis are important in determining the behavior of malignant tumor cells and may be one of the factors contributing to the difficulties in treating cancer. Initially, the invasion of tumor cells occurs by the adhesion of molecules to the extracellular matrix (ECM), and matrix metalloproteinases, including proteolytic enzymes, damage the integrity of the ECM. In conclusion, local invasion is dependent on tumor cell migration capacity through the basement membrane. Matrigel mainly contains laminin and collagen IV, so is able to serve as an *in vitro* ECM model. Measuring penetration ability through a membrane coated by Matrigel is able to indicate the invasion ability of tumor cells.

In the present study, Matrigel adhesion, Transwell chamber invasion and wound healing assays were used to investigate the effect of sublethal doses of pulsed electric fields on the invasive and metastatic properties of HeLa and SiHa cells, and to determine whether IRE ablation may promote the proliferation of residual cancer cells. The results indicated that the adhesive, invasive and migratory properties of tumor cells were not significantly altered. Therefore, it may be concluded that if complete ablation of the tumor cells is not achieved, it will not result in proliferation of the remaining cells, or induce the occurrence of distant metastases.

In conclusion, the present study confirmed that sublethal doses of pulsed electric field do not enhance the proliferation, invasion or metastasis of HeLa and SiHa cervical cancer cell lines *in vitro*. It supports the use of IRE ablation in cervical lesions, in which the growth of residual disease may be temporarily suppressed, and subsequent treatment by ablation may be used if required. However, as the present study was an *in vitro* investigation, further experiments, including those using animal models are required in order to confirm the effects of IRE treatment on the various cell behaviors of cervical cancer tumors *in vivo*, in addition to its mechanisms of action.

## Acknowledgements

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