Irreversible electroporation-mediated shRNA knockdown of the HPV18 E6 gene suppresses cervical cancer growth 

*in vitro and in vivo*

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Abstract. Irreversible electroporation (IRE) is a physical, non-thermal cancer therapy, which leads to cell death via permanent membrane permeability. This differs from reversible electroporation (RE), which is used to transfer macromolecules into target cells via transient membrane permeability. Given the electrical impedance of the electric field, RE co-exists outside the central zone of IRE ablation. In the present study, the feasibility of using IRE at a therapeutic dose to mediate short hairpin RNA (shRNA) knockdown of human papillomavirus (HPV)18 E6 in HeLa cervical cancer cells *in vitro* and *in vivo* was investigated. Experimental results indicated that the HeLa cells survived the combined treatment with IRE and shRNA plasmid transfection. Additionally, residual tumor tissue in a nude mouse model demonstrated green fluorescence. Subsequent studies showed that the combined treatment inhibited the growth of HeLa cells and tumors. Western blotting analysis showed marked changes in the growth-associated proteins between the combined treatment group and the control. It was concluded that a therapeutic dose of IRE was able to mediate the transfection of HPV18 E6 shRNA into HeLa cervical cancer cells *in vitro* and *in vivo*. This combined treatment strategy has promising implications in cancer treatment for the ablation of tumors, and in eliminating microscopic residual tumor tissue.

Introduction

Electroporation is a promising, minimally invasive technique that is able to increase the permeability of cell membranes and tissues located in the externally applied pulsed electric fields (1). The consequence of permeability varies as increasing voltage is applied. Reversible electroporation (RE) occurs under a relatively low voltage and causes the permeability of the cell membrane to increase temporarily, meaning the treated cells survive (2). By contrast, irreversible electroporation (IRE) happens when the pulsed electric fields exceed a certain threshold and the treated cells are killed (3).

Neumann *et al* (1) have demonstrated a medical application of electroporation by using pulsed electric fields to temporarily permeabilize cell membranes and deliver foreign DNA into cells. Strategies of employing a combination of pulsed electric fields and chemotherapeutic drugs or DNA (small molecules compared to usual plasmid sizes) emerged in the following decades, namely electrochemotherapy (ECT) (4) and gene electrotransfer (5). RE has been mainly used in combination strategies to temporarily increase permeability, whilst keeping the tissues and cells alive so the transfected small molecules (e.g., chemotherapeutic drugs, short hairpin RNA (shRNA) vector, or DNA vaccine) can bring about therapeutic benefits (6,7).

Until 2005, Davalos *et al* (8) proposed the term IRE to distinguish between cell destruction and RE by using electroporation as a monotherapy without employing any small molecules to destroy tissues. Subsequently in 2010, Pech *et al* (9) first reported a human clinical study (kidney tumor; n=6) where electroporation was applied as a means of soft tissue destruction. A number of pre-clinical tests have been reported in various other types of tumor including liver (10,11), lung (8), pancreatic (12) and prostate (13). Therefore, IRE has been considered as a novel, physical cancer treatment.
However, it is notable that the tissue heterogeneity in structure affects electric conductivity and electric field distribution, and thus cell survival upon IRE treatment, due to the ‘electric field sinks’ effect (14), and the volume of a single time ablation is <1 cm³ without repositioning the electrodes (15,16).

In a previous study, Joyce et al (17) hypothesized that outside the central zone of IRE ablation exists a peripheral zone of reversible electroporation, where gene transfer may occur. This was demonstrated by performing IRE in the liver of a Yorkshire pig model, and by administrating a green fluorescent protein (GFP)-labeled plasmid by bolus or primed infusion through the hepatic artery or portal vein. It is notable that this study used a high concentration of plasmid, delivered through the blood vessels and the study was conducted in the liver of a healthy pig model (17). Therefore, in the present study, the feasibility of using IRE to mediate human papillomavirus (HPV)18 E6 shRNA plasmid transfection into cervical cancer cells in vitro and in vivo was investigated, and the effect of this combined treatment on tumor growth was observed.

Materials and methods

shRNA plasmids. The enhanced (E)GFP labeled pGenesil-1 plasmid (Shanghai GeneChem Co., Ltd., Shanghai, China) was used to construct the shRNA plasmid targeting the HPV18 E6 gene, as previously described (18). shRNA targeted the HPV18 E6 coding region at nucleotides 391-411 in intron 1 of the HPV18 bicistronic transcripts. A total of two pairs of DNA oligonucleotides (Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) were cloned into the BamHI/HindIII restriction site of the pGenesil-1 plasmid. The sequences for the sense and antisense strands were 5'-GATCCCTGGGTT ATACAATTATTAATACAGATATTAATAATTGTA TAAACCCAGTGA-3' and 3'-GGACCCAATATGTTAAATAA TTAAGTTCT CTAATATTATACATATGGGTACCT CTAAG-5', respectively. The negative control shRNA (Beijing Changguo Biotechnology Co., Ltd.) had limited homology to any known sequences in the human genome. The sequences for the sense and antisense strands were 5'-GATCCTGGGTT ATACAATTATTAATACAGATATTAATAATTGTA TAAACCCAGTGA-3' and 3'-GGACCCAATATGTTAAATAA TTAAGTTCT CTAATATTATACATATGGGTACCT CTAAG-5', respectively. The negative control shRNA (Beijing Changguo Biotechnology Co., Ltd.) had limited homology to any known sequences in the human genome. The sequences for the sense and antisense strands were 5'-GATCCTGGGTT ATACAATTATTAATACAGATATTAATAATTGTA TAAACCCAGTGA-3' and 3'-GGACCCAATATGTTAAATAA TTAAGTTCT CTAATATTATACATATGGGTACCT CTAAG-5', respectively. The negative control shRNA (Beijing Changguo Biotechnology Co., Ltd.) had limited homology to any known sequences in the human genome.

Cell culture. HPV18-positive HeLa cervical carcinoma cells (Shanghai Cell Bank, Type Culture Collection Committee, Chinese Academy of Sciences, Shanghai, China) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both from Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) at 37°C in a 5% CO₂ humidified incubator.

Treatment of cells with IRE and plasmid transfection. Exponentially growing HeLa cells were collected and resuspended in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) (without FBS), with a final concentration of 2x10⁶/ml. Cells were divided into 6 groups and subjected to the following treatments: Group A, CTL (untreated control); group B, IRE; group C, transfected with 6 μg CTL shRNA plasmid; group D, IRE + CTL shRNA; group E, E6 shRNA plasmid; and group F, IRE + E6 shRNA. Groups D and F were subjected to IRE treatment after 10 μg of the appropriate plasmids were added in cell suspension, as described previously (19). Briefly, IRE was performed on each 500 μl aliquot of HeLa cell suspension. Samples were placed in a parallel aluminum plated Gene Pulser Cuvette (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with an electric pulses therapeutic system (State Key Laboratory of Power Transmission Equipment and System Security and New Technology, Chongqing University, Chongqing, China) at a pulse parameter of 1 Hz and 800 V, and for 10 pulses at a duration of 100 μs for each pulse. Groups C and E were transfected with 6 μg of the appropriate plasmid using Lipofectamine 2000 reagent in OPTI-MEM medium (Invitrogen, Thermo Fisher Scientific, Inc.) according to the manufacturer’s instructions for 6 h. Group B was treated with IRE alone.

Total RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from cultured HeLa cells using the RNeasy kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. HPV18 E6 transcripts were detected using primers 5'-AGGCCG ATTAAGGGGTA-3' and 5'-CGGTAGGCGTACG TGG-3'. The housekeeping gene GAPDH was used as a reference gene for normalization. Gene expression relative to GAPDH was determined using the 2⁻ΔΔCq method (20). qPCR was performed using a 2X Brilliant SYBR-Green QPCR Master Mix (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA) as described previously (21). The qPCR cycling conditions included pre-incubation for 5 min at 94°C, followed by 30 cycles of denaturation for 30 sec at 94°C and annealing for 30 sec at 50°C, prior to an extension step for 30 sec at 72°C and a final extension step for 10 min at 72°C. PCR products were resolved and analyzed on 1% agarose gels containing 0.5% ethidium bromide (Beyotime Institute of Biotechnology, Haimen, China).

Western blotting. Protein extracts were prepared 48 h following transfection or IRE treatment and subsequently subjected to western blot analysis for HPV18 E6, p53 and proliferating microarray following IRE treatment or transfection with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA).
were performed within 10 min after 10 µg plasmid was injected at multiple points; iv) group 4, received both treatments (IRE received intratumoral injection of 10 µg isolated plasmid alone received only IRE (800 V; 100 µs; 1 Hz; 10 pulses); iii) group 3, the remaining mice (n=20) were randomly divided into four groups: i) group 1 (control), received no treatment; ii) group 2, received only IRE (800 V; 100 µs; 1 Hz; 10 pulses); iii) group 3, received intratumoral injection of 10 µg isolated plasmid alone at multiple points; iv) group 4, received both treatments (IRE was performed within 10 min after 10 µg plasmid was injected into the tumor tissue at multiple points). The tumors were measured every seven days with a caliper until the animals were sacrificed by the cervical dislocation method at day 28 following subcutaneous injection of HeLa cells suspension, since a subcutaneous transplant tumor with growth of >1 month or that is >1 cm in diameter is prone to necrosis. Tumor volume was calculated by the following formula: \(V=\frac{abc}{6}\), where \(V\) is the volume, \(a\) is the maximum diameter, and \(b\) and \(c\) are the other two perpendicular diameters.

**Histology and microscopy.** Initially, the tumor that received combined treatment was harvested and cut in half to identify the IRE ablation effect, the feasibility of IRE-mediated plasmid transfer into tumor tissue and the expression of plasmid. One half of the specimen was fixed in 10% neutral buffered formalin for histopathology, and the other half was freshly frozen in optimal cutting temperature compound (Tissue-Tek; Sakura Finetek USA, Inc., Torrance, CA, USA) for fluorescence microscopy. Formalin-fixed tissues were processed routinely, sectioned at 4 µm thickness and stained with hematoxylin and eosin. Frozen tissues were cut into 10 µm sections and stained with DAPI, and observed under a fluorescence microscope (Nikon Eclipse TE300; Nikon Corporation, Tokyo, Japan) equipped with a GFP emission filter to detect green fluorescence as well as a TRIT-C filter to detect autofluorescence. Images were acquired on NIS-Elements Basic Research software (version 2.30; Nikon Corporation, Tokyo, Japan).

**Statistical analysis.** All experiments were performed in triplicate. \(P<0.05\) was considered to indicate a statistically significant difference. Results were statistically analyzed with one-way analysis of variance (ANOVA) and Post-hoc ANOVA Tukey's HSD test or unpaired t-test at 5% level of significance. Statistical analysis was performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA).

**Results**

**IRE induces the transfection of E6 shRNA plasmid into HeLa cells and results in E6 mRNA knockdown.** An EGFP labeled HPV18 E6 shRNA plasmid was successfully constructed and identified by DNA sequencing. To verify the feasibility of using IRE to transduce a plasmid into HeLa cells, the expression of GFP was observed 48 h after IRE (800 V; 100 µs; 1 Hz; 8 pulses) treatment under an inverted fluorescence microscope. Few HeLa cells survived 24 h after IRE combined with plasmid treatment. Strong green fluorescence was observed in the cells that survived (Fig. 1A-D).

HPV18 E6 mRNA expression was then measured by qPCR. The knockdown efficiency of HPV18 E6 mRNA level was up to 90%. There were statistically significant differences in HPV18 E6 mRNA expression between CTL group and IRE+E6 shRNA group (\(P<0.01\); Fig. 1E), the difference also appeared between IRE + CTL shRNA group and IRE+E6 shRNA group (\(P<0.05\), Fig. 1E).

**Combination of HPV18 E6 shRNA plasmid transfection and IRE inhibits HeLa cell proliferation in vitro.** A previous study demonstrated that tumor cell proliferation is positively dependent on E6 levels and HPV DNA load (21). The present study confirmed that IRE induced the transfection of interference...
IRE and shRNA plasmid transfer suppresses cervical cancer growth

IRE and shRNA plasmid transfer suppressed cervical cancer growth. The results of the experiment showed that the proliferation of HeLa cells was significantly inhibited by the combination of IRE and E6 shRNA. This effect was confirmed by various assays, including CCK-8 and fluorescence microscopy. The fluorescence microscopy results indicated that the combination of IRE and E6 shRNA plasmid inhibited the proliferation of HeLa cells. The results showed that the combination treatment with IRE and shRNA plasmid significantly inhibited the proliferation of HeLa cells compared to the control group (P<0.01; Fig. 4A and B). To investigate the effect of the combined treatment with IRE and shRNA plasmid transfection, the tumors were harvested and western blotting was performed. The HPV18 E6 oncoprotein level was significantly decreased in the IRE + plasmid group vs. the control group (P<0.05; Fig. 4C and D). An opposite trend was observed in the level of p53 protein, which is consistent with the mechanism of p53 degradation by E6 (22).

Discussion

IRE is a type of physical therapy for cancer based on electrical and biological effects of pulsed electric fields. The electric biological effects of electric pulse are known. In the 1970s, cell electroporation or electropermeabilization (defined as the permeabilization of the cell membrane induced by exposure to short and intense electric pulses) were used to increase the cellular uptake of normally non-permeable molecules (e.g., drugs, dyes or DNA) (23). Recently, electroporation-based treatments (based mainly on RE), including ECT and electrogene therapy (EGT) have been employed in clinical settings (24). By modulating the electric pulses and the parameters of electroporation, permanent permeabilization may be observed under transmembrane potential which eventually leads to cell death; this is termed IRE. IRE has been used in the food industry for sterilization for decades (24). By modulating the electric pulses and the parameters of electroporation, permanent permeabilization may be observed under transmembrane potential which eventually leads to cell death; this is termed IRE. IRE has been used in the food industry for sterilization for decades (24).

IRE and RE co-exist and cannot be separated due to the existence of dielectric impedance (24). Given the inherent dielectric properties of the cell suspension or tumor tissue, the electric field strength decreases as the distance from needle electrode increases (24). A simplified example of this co-existence of IRE and RE is a shooting target of concentric circles with IRE in the middle and RE in the periphery.

IRE was considered as a major side effect in RE-based techniques (such as ECT or EGT). The cells in the target area survived following RE exposure. Electric field parameters were strictly controlled to avoid IRE, which could lead cell to death. Conversely, during the treatment of IRE, appropriate electric field parameters were selected to ensure that the target area was completely covered by IRE. The target area
was destroyed by IRE. During IRE treatment, all RE activity
in which reversible nanopores in the surface of the cells are
formed, and the treated cells are not killed, needs to be mini-
mized. In a Yorkshire pig model, Joyce et al (17) demonstrated
that a peripheral zone of reversible electroporation, where gene
transfer can occur, exists outside the central zone of IRE abla-
tion. IRE was performed in the liver of a Yorkshire pig model
with the administration of 7 mg GFP-labeled plasmid via bolus
or primed infusion directly through the hepatic artery or portal
vein. This study showed that liver ablation by IRE was clearly
demarcated on histology, and 31/36 liver specimens treated
with IRE and the GFP plasmids demonstrated strong green
fluorescence (17). In subsequent studies, it was observed that
IRE facilitated gene transfer of the granulocyte-macrophage
colony-stimulating factor plasmid and brought about a local
and systemic biologic response (26). This demonstrated that

Figure 2. Effect of IRE and E6-shRNA plasmid transfection on HeLa cell growth. (A) Cell proliferation was assessed by the cell-counting kit-8 method and
represented by relative cell number. (B and C) The protein levels of HPV18 E6, PCNA and p53 were examined by western blotting, and GAPDH served as
the internal reference. Data are depicted as the mean ± standard deviation from at least three separate experiments.  *P<0.05, vs. the control. CTL, control;
PCNA, proliferating cell nuclear antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPV18 E6, human papillomavirus type 18 E6 protein; IRE,
irreversible electroporation; shRNA, short hairpin RNA.

Figure 3. Incomplete ablation of IRE and the expression of GFP in the surviving tumor tissue. (A-C) Haematoxylin and eosin staining of representative IRE
ablated area from one half of the tumor showed incomplete ablation as expected. Frozen sections of tumor tissues were obtained from the other half of the
tumor and stained by DAPI, then observed by a fluorescence microscope. (D) DAPI staining. (E) GFP expression. (F) Merged images. GFP, green fluorescent
protein; IRE, irreversible electroporation;
the technique holds the potential for tumor eradication and immunotherapy of residual cancer.

In the present study, by modulating the electric pulse parameters of IRE, a situation of incomplete ablation was simulated within a certain area (in a cuvette or a tumor). A therapeutic dosage (≥IRE threshold of 667 V/cm (27)) of pulsed field was projected to the subjects (cells or tumors), in which the co-existence of IRE and RE following treatment was observable. IRE treatment killed the majority of the HeLa cells in the cell suspension and ablated part of the tumors. The cells that survived showed green fluorescence under an inverted fluorescence microscope. Frozen sections of the treated tumor showed that the peripheral margin was intact and demonstrated strong green fluorescence. These results indicated a therapeutic dose of IRE was able to mediate plasmid transfection into the tumor in vivo and in vitro. Further results confirmed that the plasmid was expressed in the surviving tumor cells, and the effect of the combined treatment with IRE and shRNA was greater than the single treatment with IRE or shRNA. Notably, the resultant changes in protein levels and cell growth were more significant in the combined IRE and shRNA treatment group compared to the changes in the E6 shRNA transfected group, although no statistically significant difference was observed. These results may be due to a co-effect of IRE and shRNA plasmid transfection on tumor cells. This notable observation may be explored in future studies.

In conclusion, the present study verified the feasibility of utilizing IRE to mediate HPV-18 E6 shRNA transfection into cervical cancer HeLa cells in vitro and in vivo. The shRNA plasmid was well expressed in HeLa cells in vitro and in vivo, and the interference effect was detected by PCR, western blotting and CCK-8 assay. This combined treatment strategy has promising implications in cancer treatment for the ablation of tumors and in eliminating microscopic residual tumor tissue.

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