

# CXCL10 enhances radiotherapy effects in HeLa cells through cell cycle redistribution

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**Abstract.** Radiotherapy is a crucial treatment for cervical cancer, the second most common type of cancer in women worldwide. In this study, we investigated the effects of CXC chemokine ligand 10 (CXCL10) gene therapy combined with radiotherapy on cervical cancer using HeLa cells. TUNEL assay revealed that the apoptotic rate in the combined treatment of CXCL10 gene therapy and radiotherapy was greatly increased compared with that of CXCL10 or radiotherapy alone. Flow cytometry showed that CXCL10 overexpression in HeLa cells resulted in a prolonged G1 phase and shortened S phase at 72 h post-transfection. Western blot analysis revealed that p27<sup>Kip1</sup> was up-regulated in CXCL10-treated HeLa cells; however, cyclin E was down-regulated. These results indicate that the combination of CXCL10 gene therapy and radiotherapy is an effective strategy for the growth suppression of HeLa cells, and that CXCL10 enhances the radiotherapy effects through cell cycle redistribution. Our data provide new insight into the treatment of cervical carcinoma, involving an effective combination of gene therapy and radiotherapy against tumors.

## Introduction

Cervical cancer is the second most common cancer in women worldwide and a leading cause of cancer mortality in women in underdeveloped countries (1,2). Previous studies have demonstrated that high-risk human papillomavirus (HPV) infection is a necessary factor in the development of cervical cancer (3). As early gene products of the high-risk HPV type, E6 and E7 are consistently retained and expressed in cervical carcinoma cells, and inhibit the functions of the tumor suppressors, p53 and retinoblastoma protein (pRb), through ubiquitin-dependent proteolytic degradation (4). The consistent overexpression

of HPV E6 and E7 oncoproteins is required to maintain the malignant phenotype of cervical carcinoma cells, suggesting that they actively block the execution of a senescence program. Thus, these oncoproteins may be ideal targets for developing drugs against cervical cancer (5).

Radiotherapy plays a crucial role in the treatment of cervical cancer. Currently, approximately 80% of cervical cancer patients require radiotherapy (6,7). Radiotherapy has been used at various clinical stages in the treatment of cervical cancer; however, its efficacy is not completely satisfactory and requires further improvement. The 5-year survival rate of stage I/II cervical cancer patients is 65-85%, and that of stage III/IV patients is 20-50% (8,9). Hence, it is imperative to find alternative treatments which combine radiotherapy to form new comprehensive ways to improve the effectiveness of cervical cancer treatment.

Chemokines are a family of small cytokines secreted by cells, which participate in pleiotropic functions including cell migration, cell maturation and angiogenesis (10). Chemokines play significant roles in the biological processes of inflammation, immune surveillance and development (11). CXC chemokine ligand 10 (CXCL10)/IFN- $\gamma$ -inducible protein 10 (IP-10) is known to be one of these chemokines, and is secreted by certain cell types including monocytes, endothelial cells and fibroblasts (12). The primary functions of CXCL10 are regulating the migration of monocytes/macrophages, T cells and NK cells, eliciting potent thymus-dependent anti-tumor effects *in vivo* (13). Conversely, CXCL10 has been shown to induce significant anti-tumor activity (14). Furthermore, CXCL10 has been reported to inhibit angiogenesis (15). These studies indicate that CXCL10 is a potent agent in the treatment of carcinoma. In the present study, we investigated the effects of CXCL10 gene therapy combined with radiotherapy on cervical cancer using HeLa cells. The study aimed to provide new insight into the treatment of cervical cancer and to explore the potential of CXCL10 in this treatment.

## Materials and methods

**Cell line.** The cervical carcinoma cell line, HeLa, was obtained from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), maintained in a 37°C incubator with a humidified 5% CO<sub>2</sub> atmosphere.

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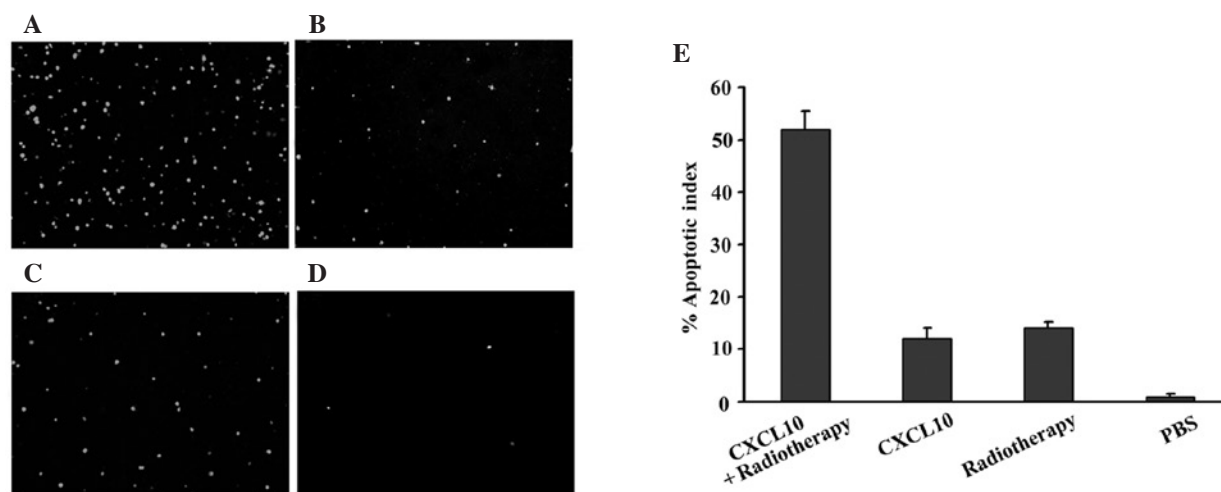


Figure 1. The combined treatment of CXCL10 gene therapy and radiotherapy increased cell apoptosis. The apoptotic cells were detected using TUNEL assay. (A) PBS, (B) CXCL10, (C) radiotherapy, (D) CXCL10 + radiotherapy, (E) quantitative measurement.

**Plasmid DNA construction and preparation.** The open reading frame of CXCL10 cDNA (GenBank accession: NM\_001565.2) was cloned into plasmid pcDNA3.1(+) (Invitrogen, USA) between *Eco*RI and *Xho*I sites to obtain recombinant plasmids expressing CXCL10. As a control, the pure pcDNA3.1(+) plasmid was used as an empty vector. The plasmids were purified through two rounds of passage over EndoFree columns (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. The expression of plasmid DNA was confirmed in the transfected cells by using RT-PCR.

**DNA transfection and radiotherapy.** The cells were seeded in 96-well plates at  $1 \times 10^4$  per well and transiently transfected with the recombinant vector of pcDNA3.1(+)-CXCL10 by lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. External beam radiotherapy using X-rays was introduced, and the seeded cells were administered a single radiation dose of 5 Gy.

**Quantitative assessment of apoptosis.** Cell apoptosis was determined using the DeadEnd™ fluorometric TUNEL system (Promega, Madison, USA) according to the manufacturer's instructions. The nucleus of apoptotic cells was observed under a fluorescence microscope (Axiovert 200; Carl Zeiss, Germany) at an excitation wavelength of 520 nm. Five random fields were randomly selected and analyzed. The apoptotic index was calculated as a ratio of the apoptotic cell number to the total cell number under each field.

**Flow cytometry.** Cell cycle progression was analyzed using a flow cytometer (ESP Elite; Coulter, USA). Briefly,  $1 \times 10^6$  cells were fixed in 80% cold ethanol for 30 min at 4°C and washed three times with phosphate-buffered saline (PBS). Then, the cells were incubated in the propidium iodide (PI) buffer [50 mg/ml PI, 0.1% Triton X-100, 0.1 mM EDTA(Na)<sub>2</sub> and 50 mg/ml RNase-A] for 30 min at 4°C in the dark until flow cytometry analysis.

**Western blot analysis.** Cells were dissolved in RIPA buffer on ice and centrifuged to obtain the supernatant. The extracted

proteins were subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Amersham Biosciences, USA). The membranes were blocked in 0.5% bovine serum albumin and incubated with primary antibodies. Then, the membranes were washed with PBST and incubated with secondary antibodies conjugated with horseradish peroxidase. After washing the membranes with PBST, the immunoblots were visualized by the enhanced chemiluminescence system SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA).

## Results

**The combined treatment of CXCL10 and radiotherapy increases cell apoptosis.** In this study, four types of treatment including PBS, CXCL10 (0.2 μg DNA), radiotherapy (5 Gy), and CXCL10 plus radiotherapy (0.2 μg DNA and 5 Gy respectively), were applied to the cervical cancer cell line, HeLa, *in vitro*. TUNEL assay revealed that the cellular apoptotic rate in the CXCL10 and radiotherapy groups alone was 12 and 14%, respectively, at 72 h post-treatment (Fig. 1). However, the apoptotic rate in the combined CXCL10 and radiotherapy group reached 52%, approximately two-fold the sum of CXCL10 and radiotherapy alone (Fig. 1). These results indicate that CXCL10 combined with radiotherapy achieves a more positive anti-tumor effect.

**Altered cell cycle induced by CXCL10 treatment.** In order to establish the reason why CXCL10 increases radiotherapy-induced apoptosis, we examined the cell cycle following the treatment of HeLa cells. Flow cytometry analysis revealed that CXCL10 overexpression in HeLa cells significantly induced a prolonged G1 phase and shortened S phase at 72 h post-transfection, resulting in 71% of cells in the G1 phase and only 8% of cells in the S phase (Fig. 2). However, there was no obvious difference in the cell cycle following the treatment with PBS, liposome and the empty vector (Fig. 2). These data demonstrate that CXCL10 overexpression causes cell cycle redistribution.

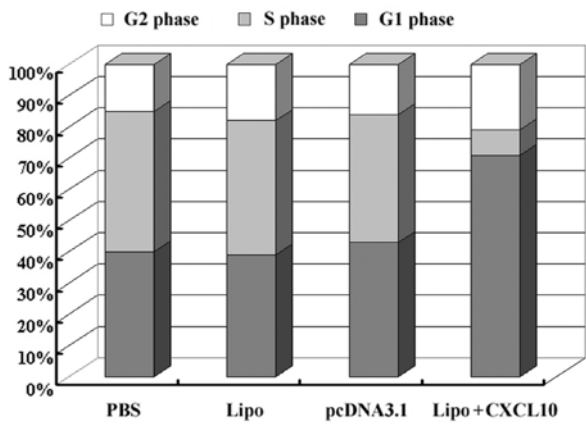


Figure 2. Altered cell cycle induced by CXCL10 treatment in HeLa cells. Flow cytometry revealed that CXCL10 overexpression in HeLa cells induced a prolonged G1 phase and shortened S phase at 72 h post-transfection.

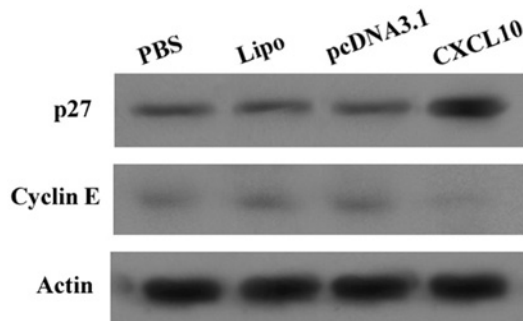


Figure 3. The changes in cell cycle proteins following CXCL10 treatment. Western blot analysis revealed that p27<sup>Kip1</sup> was up-regulated in CXCL10-treated HeLa cells compared with the controls (PBS, liposome and the empty vector). However, cyclin E was down-regulated in CXCL10-treated HeLa cells.

**Changes in cell cycle protein levels following CXCL10 treatment.** To elucidate the molecular mechanism of the G1 phase arrest induced by CXCL10, the proteins involved in the cell cycle were detected by Western blot analysis. There was no obvious difference in protein levels of p27<sup>Kip1</sup> and cyclin E among cells treated with PBS, liposome and empty vector (Fig. 3). However, in CXCL10-treated cells p27<sup>Kip1</sup> was found to be up-regulated and cyclin E down-regulated compared with the controls (PBS, liposome and the empty vector) (Fig. 3). These results indicated that the levels of cell cycle proteins were changed following CXCL10 overexpression.

## Discussion

The present study was designed to examine whether the combined treatment of liposome-encapsulated CXCL10 gene therapy and radiotherapy inhibits the growth of cervical carcinoma cell line, HeLa. To our knowledge, this is the first study to demonstrate that CXCL10 gene therapy combined with radiotherapy leads to a greater inhibition of HeLa cell growth compared with CXCL10 or radiotherapy alone.

CXCL10 and radiotherapy both possess anti-cervical carcinoma efficacy; however, their anti-tumor mechanisms are different. CXCL10 induces cell apoptosis through the p53-dependent pathway initiated by the suppression of HPV

E6 and E7 expression (16). Radiotherapy induces cell apoptosis through the destruction of host genome DNA (17). We hypothesized that the combination of CXCL10 gene therapy and radiotherapy would have the following advantages, and therefore improve the efficacy of cervical carcinoma treatment. Firstly, radiation improves the efficiency of CXCL10 gene transfer into tumor cells, since the stability of the cell membrane can be weakened by radiation. Secondly, gene therapy induces cell cycle arrest in certain cases, leading to an increased sensitivity to radiation. It has been recognized that cell cycle redistribution is essential for radiation. For example, the S phase is least sensitive to radiation; however, gene synthesis of the S phase in tumor cells is very active (18). In the present study, the markedly increased G1 and decreased S phases of HeLa cells were observed following treatment with CXCL10, which can improve the anti-tumor efficacy of radiation.

In order to reveal the molecular process of G1 arrest induced by CXCL10, two cell cycle-related proteins, cyclin E and p27<sup>Kip1</sup>, were examined in our study. Cyclin E is one of the nuclear proteins and a significant regulatory factor of the cell cycle. Cyclin E binds CDK2 to form a complex that promotes cell cycle progression from the G1 to S phase and consequently induces the cell division process (19). p27<sup>Kip1</sup>, a cyclin-dependent kinase inhibitor, plays a significant role in limiting cell cycle progression. p27<sup>Kip1</sup> mainly inhibits the activity of cell cycle kinase complexes such as cyclin E-CDK2, which leads to G1 phase arrest (20). p27<sup>Kip1</sup> executes its inhibitory effects in two ways. First, it inhibits the phosphorylation of CDK2-Thr160, resulting in the retarded activation process of cyclin E-CDK2. In addition, p27<sup>Kip1</sup> also directly binds cyclin E-CDK2 complex to inhibit the activated kinase complex (21). In this study, the decreased cyclin E and increased p27<sup>Kip1</sup> expression could explain the prolonged G1 and shortened S phase, indicating that CXCL10 causes cell cycle redistribution in HeLa cells.

In summary, we demonstrated that the combination of CXCL10 gene therapy and radiotherapy is an effective treatment strategy for the growth suppression of the cervical carcinoma cell line, HeLa. CXCL10 enhanced the radiotherapeutic effects in HeLa cells through cell cycle redistribution. Our data provides new insight into the treatment of cervical carcinoma, involving an effective combination of gene therapy and radiotherapy against tumors.

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