Regulation of metastatic action through CXCR4 and HPV16 E6/7 in cervical carcinogenesis

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Cervical cancer is one of the most common malignancies affecting women, and infection with high-risk HPV type 16 is a major cause of this cancer. This study investigates the influence of C-X-C chemokine receptor type 4 (CXCR4) on cervical carcinogenesis associated with HPV16 E6/7 infection, using primary cancer cells isolated from Taiwanese cervical cancer patients as a model for cervical cancer. We investigated the functional interaction between CXCR4 in Cx cells and HPV16 E6/7 transfected Cx cells (CxWJ cells), according to cell cycle, invasive ability and MMPs and CXCR4 gene expression. Our results indicate that the up-regulation of CXCR4 gene expression in CxWJ cells partially enhance proliferation and the invasive ability provided by HPV 16 E6/7 stimulation. These data provide evidence of a functional interaction between HPV16 E6/7 in CXCR4, suggesting that cooperative stimulation of HPV E6/7 in the up-regulation of CXCR4 in human cervical cancer cells may be necessary to completely overcome the oncogenic function associated with the progression of cervical carcinogenesis.

Keywords: C-X-C chemokine receptor type 4 (CXCR4); HPV; Cervical cancer; Matrix metalloproteinases (MMPs)


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Introduction

Chemokines are a class of small inflammatory or homeostatic cytokines (8-10 kDa) sharing biological activity in stimulating the migration of lymphocytes, monocytes, neutrophils, endothelial cells, mesenchymal stem cells, and malignant epithelial cells [1]. Chemokines are also chemotactic molecules regulating immune cell trafficking, recently involved in multiple CNS activities [2]. Interestingly, it has been reported that several chemokine receptors including CCR2, CCR5, CXCR1, CXCR2, CXCR4, and CXCR7 can undergo homo- or hetero-dimerization upon ligand binding; a process that was proposed to regulate distinct intracellular signaling pathways [3]. C-X-C motif ligand 12 (CXCL12 or Stromal derived factor-1; SDF-1) plays a crucial role in the regulation of normal stem/progenitor-cell trafficking and...
homing [4], CXCL12, is the only ligand for CXCR4 (C-X-C motif receptor 4), and acts as autocrine/paracrine growth factor for several cancers [5]. CXCR4 expression has been reported in at least 23 epithelial, mesenchymal and hematopoietic cancers, CXCR4 participate in cell proliferation, angiogenesis, tumor aggressiveness and metastasis [6]. SDF-1 is a member of the CXC subfamily of chemokines that interacts with the 7 transmembrane G-protein-coupled receptor CXCR4 [7]. The role of the SDF-1/CXCR4 axis in prostate cancer has been experimentally demonstrated. It is known that CXCR4 mRNA and protein are expressed in prostate cancer cell lines [8]. CXCR4 activation regulates tumor cell proliferation, motility and survival, mainly through the Akt pathway [9]. The overexpressed CXCR4 in nude mice with invasiveness of the tumors and metastasis were significantly increased [10].

Cervical cancer is one of the most common forms of cancer in women and a leading cause of death among gynecological malignancies [11]. In Taiwan, cervical cancer is the sixth most common form of cancerous malignancy among females [12]. The majority of women diagnosed with cervical cancer exhibit advanced, widely disseminated malignancy and poor prognosis [13]. An overwhelming body of evidence has demonstrated that oncogenic types of human papillomavirus (HPV) play an important role in the development of cervical cancer precursors [14]. However, only a small fraction of females infected with HPV develop cervical cancer, indicating that other factors contribute to the progression of lesions in invasive cervical cancer [15]. The role of HPV as a cause of cervical cancer has been demonstrated, as has its association with other malignancies. The serotype most frequently found in HPV-positive cervical tumors is HPV 16 [16, 17].

In this report, we investigate whether CXCR4 up-regulation induced by HPV 16 E6/7 infection contributes to the tumorigenesis of malignant cells in human cervical cancer. The aim was to develop a scientific basis with which to provide technical support for cervical cancer therapy.

Materials and Methods

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**Table I**

<table>
<thead>
<tr>
<th>CXCR4</th>
<th>5'-GGAGGGGATCATGATATAC-3' (forward)</th>
<th>5'-GAAGATGATCGAGTAGTGGG-3' (reverse)</th>
<th>-125 bp product (expected amplified product)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5'-GTCTCTCTGACTTCAACAGCG-3' (forward)</td>
<td>5'-ACCACCCTGGTCTGTAAGC-3' (reverse)</td>
<td>-532 bp product (expected amplified product)</td>
</tr>
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**Cell culture.** Cervical cancer cells were obtained from patients admitted to the Department of Obstetrics and Gynecology of the National Cheng Kung University (NCKU) Medical Center to undergo surgery for benign uterine neoplasm [11, 12]. All patients underwent total abdominal hysterectomy. Specimens were removed only from typical and clinically clear-cut (Grade II) cases. Prior written informed consent was obtained from patients and all procedures were reviewed and approved by the ethics board at NCKU in adherence to Helsinki Principles.

Following surgery, cervical tissue was dissected and immersed in a culture medium for the preparation of normal cervical epithelial cells. The Cx cell is a cervical cancer cell line established by Prof. C.Y. Chou [22]. The cells were obtained from a 48 y/o Taiwanese female with squamous cell carcinoma of the uterine cervix, characterized as HPV-negative and p53-mutation-negative. CxWJ cells were established using a stable clone from Cx cells transfected with HPV 16 E6 and E7 [11, 12]. The cells were maintained on culture dishes, in a culture medium supplemented with 10% (v/v) FBS. The cells were cultured in an incubator with an atmosphere of 95% air and 5% CO₂ at 37 °C.

**Cell cycle analysis.** The method for cell cycle analysis using propidium iodide (PI), that is, using the fluorescent nucleic acid dye PI to identify the proportion of cells that are in one of the three interphase stages of the cell cycle. Cells were harvested and fixed in 1ml cold 70% ethanol at least 8 hours at -20°C. DNA was stained in PI/RNaseA solution and the cell cycle (At least 10,000 single cells) was detected by flow cytometry (FACSCalibur, Franklin Lakes, New Jersey, USA). Data was analyzed by WinMDI 2.8 free software (Franklin Lakes, New Jersey, USA).

**Confocal microscopy of CXCR4 expression.** Confocal microscopy was performed as described previously [23]. Briefly, the cells (2×10⁶ cells) were fixed on cover slips. After treatment, they were incubated with mouse anti-CXCR4-phycoerythrin antibody (FAB170P, R&D system, MN USA) for 30 minutes, and then washed with PBS. The cells were mounted onto microscope slides using mounting medium containing DAPI.

**Migration (in vitro scratch assay) and Invasive assay.** The migration assay was creating a scratch in a cell monolayer by Culture Insert Dish (ibidi Gmbh, DE), capturing cell migration images at 24 hours treatment by microscopy. The invasive assay of cells through modified Boyden chambers containing polycarbonate filters with 8-µm pores (24 well Millicell, Millipore, Germany). After incubation for 24 hours, invaded cells were stained with 0.1% crystal violet solution and photographed using a QImaging RETIGA Exi digital camera (Burlney, BC, Canada) under Olympus CKX41 microscope. The number of invaded cells was then counted and subjected to statistical analysis.
RT-PCR. A reverse transcriptase system (Promega, Southampton, UK) was used to synthesize cDNA from 1 microg of total RNA. Between 2 and 4 microL of cDNA were used for PCR analysis. PCR (50 microL) reactions were performed using 100 ng of each primer and 1 unit of Dynazyme II (Flowgen, Lichfield, UK). Thermal cycling was conducted for 30 cycles at the following temperature/durations: 94 °C for 40 s, 60 °C for 40 s, and 72 °C for 40 s using a Progene thermal cycler (Cambridge, UK). A final extension of 72 °C was performed for 10 min at the end of 35 cycles. The primers used by Human MMP multiplex PCR kit (Signosis). All primers were checked against all other MMPs sequences for specificity. PCR reactions were performed on 5% polyacrylamide/TBE minigels using the mini-Protean system (BioRad, Hemel Hempstead, UK) and stained using 0.5 microg/mL ethidium bromide. Gels were visualized using an Apligene UV CCD camera system.

Real-time PCR. Real-time PCR was conducted using SYBR Green PCR MasterMix according to the manufacturer’s instructions. Quantitative real-time PCR (qRT-PCR) was performed using approximately 200 ng of SYBR Green PCR MasterMix in an ABI Prism 7300 system (Applied Biosystems, Foster City, CA, USA). PCR conditions were 95°C for 5 s, 60°C for 30 s, and 72°C for 30 s for 40 cycles. The primers used for amplification of the human CXCR4 genes are listed in Table I. Sample cells from three plates were run in duplicate, using the threshold suggested by the software for the instrument to calculate Ct. To normalize readings, we used Ct values from 18s as internal controls (GAPDH) for each run, obtaining a delta Ct value for each gene.

Statistical analysis. All data are reported as the mean (±SEM) of at least three separate experiments. Statistical analysis was performed using a t-test with significance set at $P < 0.05$.

Results

HPV16 E6/E7 infected cervical cancer cells increased cell cycle S-G2/M phase distribution. Cell-cycle distribution of with or without HPV 16 E6/E7 transfected cells was analyzed by flow cytometry, with the aim of determining whether the effects were a result of cell injury. As shown in Figure 1A, cervical cancer cells transfected with HPV 16 E6 and E7 demonstrated an increase in S-G2/M phase, compared with the untransfected samples (Figure 1B) ($^{*}P<0.05$ vs. Cx group). These observations suggest that the HPV 16 E6/E7 transfected cervical cells may have undergone hyperplasia.

HPV16 E6/E7 enhanced cell migration and Invasion. To explore the potential role of HPV16 E6/E7 in the migration and invasive activity of cervical cancer cell lines, we employed modified Boyden chambers and a digital camera under microscope to identify the activity of Cx and CxWJ cells (Figure 1C). HPV16 E6/E7 stimulation resulted in a significant increase in the invasive ability of CxWJ cells (Figure 1D) ($^{*}P<0.05$ vs. Cx cells group). The MMPs mRNA expression were determined by RT-PCR. The
Figure 1E shows that the MMP2, 9, 14, 3, 7 were expression in Cx cells but not MMP3 and 7 in CxWJ cells. The results indicate that HPV induces migration and invasive activity in cervical cancer cells but not by MMP3 and 7 gene expression.

Up-regulation of CXCR4 gene expression in HPV16 E6/7 infected cervical cancer cells. CXCR4 gene expression was analyzed in cervical cancer cells using confocal microscopy, flow cytometry and real-time reverse transcription-polymerase chain reaction (RT-PCR) assay. As shown in Figure 2A, CxWJ exhibited CXCR4 gene expression (red color) but Cx cells did not. Figure 2B shows CXCR4 mRNA expression assessed using qRT-PCR. CXCR4 mRNA expression was significantly induced in CXWJ cells compared with Cx cells (# P<0.01 vs. Cx cells group). By flow cytometry, CXCR4 protein expression was significantly induced in CXWJ cells compared with Cx cells (Figure 2 C and D) (* P<0.05 vs. Cx cells group), indicating that up-regulation may play an important role in cervical tumorgenesis. Taken together, these results suggest that HPV 16 E6/E7 transfection induced the CXCR4 gene expression in Cx cells and therefore plays an important role in cervical tumorgenesis induced by CXCR4 up-regulation.

Discussion
Cervical cancer remains a fatal disease despite considerable advances in treatment\(^\text{[18]}\). Upon infection with HPV, cervical epithelial cells develop from premalignant cervical lesions to malignant invasive cancer via a multistep process \(^\text{[19]}\) from normal, CIN (Cervical Intraepithelial Neoplasia) I (mild), to CIN II (moderate), CIN III (severe), and finally invasive cervical carcinoma. Fortunately, most cases of CIN do not develop into cervical cancer, and tend to develop only if appropriate treatment is not received \(^\text{[20]}\).

Studies have indicated that ovarian tumors produce CXCL12, a growth factor capable of binding to CXCR4, some of which are expressed in ovarian cancer \(^\text{[21]}\). Little is known about the role of the HPV and CXCR4 and MMPs production; however, the results are inconclusive. A loss of MMP3 and 7 noted in CxWJ cells results in a different response and an increase in migration and invasive ability. Further study concerning changes in the epithelial and mesenchymal characteristics will be required to elucidate the role of CXCR4 and MMPs alteration in cervical carcinogenesis.

Our results indicate that HPV 16 E6/7 transfection of CxWJ cells causes them to respond differently to CXCR4 production resulting in an increase in growth, migration and invasive ability. Further study regarding changes in epithelial and mesenchymal characteristics will be required.
to elucidate the role of HPV16 E6/7 in cervical carcinogenesis.

**Conflict of interest**

The authors declare no conflict of interest.

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**References**


