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Chitinase 3 like 1 is associated with tumor angiogenesis in cervical cancer

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ABSTRACT

Elevated serum levels of a secreted glycoprotein chitinase 3 like 1 (CHI3L1) are associated with poor prognosis and short survival time of patients with cervical cancer (CxCa). Our previous microarray data showed the increased expression of *CHI3L1* in invasive CxCa compared to normal tissue, implicating a potential role of CHI3L1 in CxCa. To establish the pathological role of CHI3L1 in the development of CxCa, this study focused on its expression in CxCa and angiogenic impacts in tumor vessel formation. CHI3L1 activated angiogenesis by promoting endothelial cell migration and tube formation *in vitro* but failed to protect CxCa cell lines, CaSki and HeLa against apoptosis induced by γ-irradiation. In addition, the capability of CHI3L1 to induce proliferation and migration of CaSki and HeLa cells was cell type specific. In an analysis of 103 specimens from CxCa patients, increased expression levels of CHI3L1 mRNA and protein in invasive CxCa were 4-fold ($P < 0.05$) and 2-fold ($P < 0.01$), respectively, stronger than those in normal subjects. The immunostaining of CHI3L1 was positively correlated with VEGF expression ($P = 0.0019$) and microvessel density ($P = 0.0110$). Moreover, CHI3L1 expression was also positively associated with cancer metastasis ($P = 0.011$). The data suggest the crucial role of CHI3L1 by promoting angiogenesis, which may contribute to the development and progression of CxCa. The findings help establish CHI3L1 as a prognostic biomarker and therapeutic target for CxCa patients.

1. Introduction

Cervical cancer (CxCa) represents the third most common cancer in women and the fourth leading cause of cancer mortality in women worldwide (Jemal et al., 2011). The infection of high-risk human papilloma virus particularly types 16 and 18, is well known to play a crucial role in the development of CxCa. However, many factors which affect CxCa development and progression remain obscure. Sakunjia et al. (2010) have demonstrated the differential gene expression profiling in invasive CxCa by microarray analysis. They showed that the chitinase 3 like 1 (*CHI3L1* or YKL-40) is one of the top ten most up-regulated genes and its increased expression level is related to the degree of the disease, suggesting its pivotal role in tumor development and progression.

CHI3L1 is a 40 kDa mammalian glycoprotein which is related in amino acid sequence to the chitinase protein family but contains no enzymatic property. CHI3L1 was found to be a major secreted protein of human articular cartilage chondrocytes and synovial cells (Ling and Recklies, 2004). In addition, CHI3L1 has been found in sera of patients with various diseases including inflammatory bowel disease (Bernardi et al., 2003), pulmonary sarcoidosis (Johansen et al., 2005), systemic sclerosis (Nordenbaek et al., 2005) and liver fibrosis (Lebensztejn et al., 2007). Several studies of human solid tumors including CxCa have shown the association of high serum CHI3L1 level with poor patient prognosis and short survival time (Cintin et al., 2002; Dehn et al., 2003; Dupont et al., 2004; Jensen et al., 2003; Johansen et al., 2004; Mitsuhashi et al., 2009).

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The pathophysiological functions of CHI3L1 have been studied in a broad type of human cancers such as breast cancer (Shao et al., 2009), glioblastoma (Francescone et al., 2011a) and colorectal cancer (Kawada et al., 2012). These studies have demonstrated that CHI3L1 can activate cell proliferation, migration, angiogenesis, and protect tumor cells from apoptosis after gamma irradiation, suggesting its multifaceted functions in tumor development and progression. Thus determining a potential role for CHI3L1 in CxCa would be interesting, as it may mediate the development and/or progression of this cancer. We investigated the functions of CHI3L1 *in vitro* including cell proliferation, migration, anti-apoptosis and angiogenesis. The expression of CHI3L1 was determined in CxCa, along with its role in tumor vascularization and metastasis. The results obtained from cell lines and clinical samples indicate that the key function of CHI3L1 in CxCa is associated with angiogenesis during CxCa development and progression. Moreover, CHI3L1 also plays an important role in tumor metastasis. Our present findings indicate the applicability of CHI3L1 as a prognostic biomarker for CxCa patients.

2. Materials and methods

2.1. Patients and tumor samples

Cervical tissues were collected from patients who attended the Tumor Clinic, Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Thailand from June 2005 to January 2012. This project was approved by the Ethical Committee of Khon Kaen University (HE531286). All tissue samples were histologically reviewed and confirmed by the pathologist. Cervical tissues obtained were 103 squamous cell carcinomas (SCCA); 6 stage I, 29 stage II, 62 stage III and 6 stage VI according to FIGO staging; and 53 normal cervixes which were derived from surgical resections of myoma patients. Of 103 patients, 55 patients regarding tumor metastasis data were available. Cancer metastasis involved regional lymph nodes and distant organs such as liver, lung, bone and vagina. Patients with HIV infection were excluded.

2.2. Cell culture

CxCa cell lines (CaSki and HeLa) were cultured in DMEM high-glucose (DMEM-HG) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (all from Invitrogen, Carlsbad, CA). Human microvascular endothelial cells (HMVECs) were cultured in EBM-2 medium supplemented with 10% FBS, 1% Penicillin/Streptomycin, 1 μg/mL Hydrocortisone and 10 ng/mL human epidermal growth factor (hEGF).

2.3. Generation of recombinant CHI3L1 protein

The full-length human CHI3L1 cDNA was generated from tissues of CxCa patients. This CHI3L1 cDNA with a 6x histidine tag was subcloned into a pFastBac1 vector (Invitrogen, Carlsbad, CA), which was then transformed and amplified in DH10Bac *E. coli* to generate bacmid DNA. The recombinant bacmid DNA was transfected into monolayers of Sf9 insect cells using Cellfectin reagent (Invitrogen) by which recombinant protein was produced. The recombinant CHI3L1 protein was subsequently purified by Ni-NTA (Invitrogen) and PD-10 desalting columns (Millipore, Billerica, MA).

2.4. Cell proliferation and MTS assay

Cell proliferation was assessed by measuring the viable cells using a MTS assay (Promega, Madison, WI). The cells were seeded in 96-well tissue culture plates at a density of 3×10^3 cells/well, and supplemented with 100 μL of serum-free DMEM-HG and 1% Penicillin/Streptomycin with or without recombinant CHI3L1 protein. After 24 h incubation, the MTS assay was performed according the manufacturer's instructions.

2.5. Cell viability and Live/Dead assay

Cervical cancer cell lines were exposed to 10 Gy γ-irradiation generated from a radioactive cesium source. Cell viability was assessed by the Live/Dead Assay (Invitrogen, Carlsbad, CA). After 72 h incubation, cells were assessed with the Live/Dead mixture (calcein AM and ethidium homodimer) according to the manufacturer's instructions. Fluorescent images of live (green) and dead (red) cells were analyzed and quantified for the percentages.

2.6. Cell migration

The role of CHI3L1 on cell migration was investigated by performing the transwell chemotaxis assay. Briefly, a total of 2×10^5 cells (in 0.1 mL serum-free DMEM-HG) were seeded into the upper chamber of the Transwell with 8 μm pore polycarbonate membrane insert (Corning, Tewksbury, MA). The lower chamber contained 0.6 mL DMEM-HG with or without recombinant CHI3L1 protein. After incubation for 6 h, chambers were disassembled and the membranes were stained with 5 mg/mL of DAPI for 10 min and placed on a glass slide. Then cells migrating across the membrane were counted in 4 random visual fields under the light microscope. The migrated cells were digitally imaged.

2.7. Tube formation assay

HMVECs (1×10^4 cells) were seeded in a 96-well plate containing 50 μL of growth factor-reduced Matrigel (BD Bioscience, San Jose, CA) in the presence of CHI3L1 or in serum-free medium. After 6 h incubation, tube numbers of each group were assessed at least for 6 fields under the light microscope with 200× magnification.

2.8. Western blotting analysis

Cells were collected and lysed in buffer containing 0.25 mM HEPES, 14.9 mM NaCl, 10 mM NaF, 2 mM $MgCl_2$, 0.5% NP-40, 0.1 mM PMSF, 20 μM pepstatin A and 20 μM leupeptin. After centrifugation, supernatant was collected and measured for protein concentration (Bio-Rad, Hercules, CA). The protein sample (100 μg) was separated on a 12% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane and immunoblotted with primary antibodies; anti-CHI3L1 and actin (Cell Signaling Technology, Boston, MA). Bound antibodies were detected, first by using appropriate peroxidase-coupled secondary antibodies and then by Super West Pico Chemiluminescent Substrate kit (Thermo Scientific, Rockford, IL).

2.9. Quantitative reverse transcription-polymerase chain reaction (QRT-PCR)

Total RNA was extracted from cervical tissues using TRIzol reagent (Gibco, Grand Island, NY) according to the manufacturer's instructions and then the first strand complementary DNA (cDNA) was prepared from 1 μg of total RNA with oligo d(T) primers using the Improm II™ Reverse Transcriptase System (Promega, Madison, WI). QRT-PCR was performed to quantify the level of gene expression of *CHI3L1* and *GAPDH* (reference gene), using a SYBR Green I assay (Amresco, Solon, OH). PCR was conducted on a LightCycler480 system (Roche Diagnostics, Indianapolis, IN) and a melting curve profile was analyzed for

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