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Overexpression of gelsolin in human cervical carcinoma and its clinicopathological significance

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ABSTRACT

Objectives. Cervical carcinoma is the second most common cause of death from gynecological cancers worldwide. Knowledge of the molecular mechanisms underlying the tumorigenesis of cervical cancer cell, except human papilloma virus infection, is limited.

Methods. A microarray was used to study the differential expression of genes in cancerous tissues to identify new molecular markers for diagnosis and prognosis. Their differential expression was confirmed with Western blotting and immunohistochemical analyses. The clinical correlations and prognostic significance of the aberrantly expressed proteins were evaluated to identify novel biomarkers of cervical cancer.

Results. The expression of gelsolin was significantly upregulated in 78% of patients with cervical cancer, and gelsolin was selected for further study. Gelsolin expression was stronger in cervical tumor tissues than in the surrounding noncancerous tissues ($P<0.001$). Gelsolin expression in the plasma of cervical cancer patients was increased 2.2-fold compared with that of healthy control subjects ($P<0.001$). The levels of plasma gelsolin in the early and late stages were significantly different ($P=0.006$). According to immunohistochemical analysis, increased gelsolin expression was associated with histological type and FIGO stage II. The 5-year overall survival and recurrence-free survival rates for the low-expression group (cut-off = 115) were significantly higher than those of the high-expression group. Cancer cells with reduced gelsolin expression exhibited reduced migration and proliferation.

Conclusions. These results provide strong evidence that gelsolin plays an important role in cellular proliferation and migration in cervical cancer and suggest that gelsolin is a promising marker for cervical cancer screening and prognosis.

Introduction

Carcinoma of the uterine cervix is the most prevalent female genital cancer in the less-developed countries worldwide [1]. In Taiwan, 1828 cases of newly diagnosed cervical cancer were reported in the 2006 annual report of the Department of Health. The mortality rate for cervical cancer ranks sixth among women in Taiwan [2]. Surgery has long been considered the standard therapy for early-stage (stages I–IIa) disease, whereas concurrent radiotherapy and chemotherapy is the standard therapy for advanced disease [3]. The 5-year relapse-free survival and overall survival of all patients with invasive cervical cancer are 77.7% and 69.6%, respectively [4]. After successful primary treatment, the most important issue is the early detection of recurrence because advanced recurrence is always asymptomatic to curative treatment. Only a small improvement has been noted in the treatment outcomes of patients with cervical cancer in this decade. Known factors contributing to a poor prognosis are positive lymph-node metastasis, nonsquamous cell carcinoma, a tumor size larger than 4 cm at all stages, and a high-grade (moderately and poorly differentiated) tumor in stage I disease [5]. Although imaging studies (computed tomography, magnetic resonance image) are routinely used in the management of invasive cervical cancer, there are still limitations in the diagnosis of early lymph-node metastasis. Available serum tumor markers (squamous cell carcinoma antigen and carcinoembryonic antigen) are elevated in 30%–40% of patients with early-stage disease and in around 70% of patients with late-stage disease, but neither are prognostic factors. Patients with metastatic disease do not benefit from surgical treatment, and the field of irradiation and the outcome of radiotherapy are based on the detection of metastasis at diagnosis. Therefore, markers of early

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metastasis are helpful in selecting the treatment modality. For patients whose disease has been successfully treated and who only require follow-up, no markers that can identify an early relapse or that can define the site of relapse are available. Therefore, diagnostic and prognostic markers for carcinoma of the uterine cervix are required because of their potential importance both in basic research, to understand the characteristics of the tumor, and in its clinical management, to improve patient survival and quality of life.

Persistent infection with the oncogenic human papillomavirus (HPV) is necessary for the development of cervical carcinogenesis, but it is not sufficient to immortalize and transform the epithelial cells [6]. The transformed cells undergo further genetic alterations, which disrupt cell-cycle control and allow the tumor to escape the host immune response, thus generating an invasive phenotype. HPV "early" proteins, E6 and E7, are the major oncoproteins involved in cancer progression. Changes in key cellular pathways may provide useful biomarkers to improve the sensitivity of current cancer screening methods, such as the Papanicolaou test.

Gelsolin was previously identified from an Affymetrix microarray in our laboratory (Table S1), but its role in cervical carcinoma has not been determined. Gelsolin is a calcium-activated actin-binding protein involved in dynamic changes in the actin cytoskeleton. Gelsolin controls the length of actin polymers in vitro. The role of gelsolin during carcinogenesis is still controversial. Thus, we performed a clinicopathological study to explore the potential for gelsolin as a prognostic marker for cervical cancer.

Materials and methods

Study population

A list of consecutive patients who had undergone treatment for invasive cervical carcinoma of International Federation of Gynecology and Obstetrics (FIGO) stages I to IIB with primary radical surgery, between 2000 and 2008 at Chang Gung Memorial Hospital, Taoyuan, Taiwan, was retrieved from the hospital database and were included in the reported prognosis analysis. The medical records of the patients were retrospectively reviewed. The study protocol was approved by the Medical Ethics and Human Clinical Trial Committee of the Chang Gung Memorial Hospital.

Tumor samples

Fresh samples of the tumor tissues and adjacent noncancerous mucosa were harvested immediately after cervical carcinoma resection. Samples dissected from the surgical specimens were immediately snap-frozen in individual vials with liquid nitrogen. The frozen specimens were stored at −70 °C in a tumor bank until analysis.

RNA extraction and affymetrix oligonucleotide microarray

The total RNA from a paired cervical squamous cell carcinoma and adjacent noncancerous mucosa were extracted using TRIZOL reagent (Life Technologies, Rockville, MD), as described previously [7,8]. Total RNA (20 μg) was used for labeling and hybridization with the Affymatrix GeneChip Human genome U133A 2.0 array (Affymetrix, Santa Clara, CA) containing 14500 human genes. The slides were scanned, and the intensities were acquired with GenePix Pro 4.1 software (Axon Instruments Inc. Foster City, CA).

Immunoblot analysis

Total cell lysates from the tumors, adjacent noncancerous mucosa, and cultured cell were prepared and the protein concentrations were determined with the method described by Bradford [9,10]. Equal amounts of protein per lane were fractioned with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to a nitrocellulose membrane. The membrane was then blocked for 2 h at room temperature with 5% (w/v) nonfat dried milk in TBS. The membrane was washed three times with TBS, and incubated for 18 h with specific antibodies. After further washing, the membrane was incubated for 1 h with horseradish-peroxidase-conjugated, affinity-purified antibody directed against rabbit IgG (diluted 1:10,000 in TBS; Santa Cruz Biotechnology Inc., Santa Cruz, CA). The immune complexes were then visualized by chemiluminescence with an ECL detection kit (Amersham Inc. Piscataway, NJ) [11]. The antibodies used were gelsolin rabbit polyclonal antibody (dilution 1:8,000; Sigma Chemicals, St Louis, MO), cyclin D1 rabbit monoclonal antibody (dilution 1:3000; Epitomics, Burlingame, CA), and p21 mouse monoclonal antibody (dilution 1:1000; Thermo Scientific, Fremont, CA).

Plasma gelsolin determination

A quantitative enzyme-linked immunosorbent assay (ELISA) was used to measure plasma gelsolin. A microplate was precoated with a monoclonal antibody specific for gelsolin. Standards, normal plasmas, and patient plasmas (diluted 1:250–500) were pipetted into the wells. After the plates were washed, an enzyme-linked polyclonal antibody specific for gelsolin was added to the wells. The plates were washed again, a substrate solution was added to the wells, and color developed in proportion to the amount of gelsolin bound in the initial step. The color development was stopped, and the intensity of the color was measured according to the manufacturer's (Molecular Probes Systems, Eugene, OR) instructions.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissues were examined immunohistochemically using a monoclonal antibody directed against human gelsolin (diluted 1:800; Sigma) following the avidin–biotin complex method, as described previously [12]. Comparisons were made between the staining intensity of the carcinoma cells and that of benign superficial epithelium on the same slide. The negative group consisted of cancer cells with no detectable (−) or only trace staining for gelsolin (+1). The positive group consisted of cancer cells with moderate (+2) or high levels (+3) of gelsolin immunoreactivity. The numerical scoring (histoscore, Q) was confirmed by a second independent examiner, blinded to the initial score. The results were scored by multiplying the percentage of positive cells (P) by the intensity (I), according to the formula: $Q = P \times I$. For example, a section in which 10% of the tissue had a staining score of +1, 60% a score of +2, and 30% a score of +3, $Q = (10 \times 1) + (60 \times 2) + (30 \times 3) = 220$ [13].

Establishing a stable gelsolin knockdown HeLa cell line

Clones (TRCN0000029724-29728) of small interfering RNAs (siRNAs) targeting *gelsolin* were purchased from the National RNAi Core Facility (Institute of Molecular Biology, Academia Sinica, Taiwan). The transfection of siRNA targeting the endogenous *gelsolin* gene was performed with Lipofectamine Reagent (Invitrogen). After incubation for 24 h, the cells were transferred to medium containing puromycin for selection, and were then used in migration and proliferation assays. After 2 weeks of selection, specific repression of the targeted gene was confirmed by Western blot analysis.

In vitro migration assay

The effect of *gelsolin* knockdown on the migration of the HeLa cell line was assessed with a rapid in vitro assay (Transwell technique), as described previously [14,15]. Briefly, the cell density was adjusted to 2.5×10^5/mL, and 200 μL of this suspension was added to the wells of a