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Prognostic impact of atypical chemokine receptor expression in patients with gastric cancer

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ABSTRACT

Background: Atypical chemokine receptors (ACRs), which serve as a decoy receptor to attract chemokines, including DARC, D6, and CCX-CKR, have an important role in inhibiting invasion and metastasis of cancer cells; however, their expression in gastric cancer has not been characterized. The purpose of this study was to determine the predictive value of ACRs for overall survival in gastric cancer.

Methods: We performed immunohistochemical analysis on formalin-fixed, paraffin–embedded cancer tissue and used Western blot analysis on cell lines with an antibody against ACR protein. We investigated tumor material from total of 282 consecutive gastric specimens, composed of 101 normal gastric tissues, 181 peri-carcinoma tissues (2 cm away from the carcinoma), and their relationships to clinicopathologic features and survival, using a tissue micro-array.

Results: We found the expression of ACRs to be lower in gastric cancer cell lines or tissues than in normal cell line, peri-carcinoma, or normal tissues, respectively ($P < 0.05$). In univariate analysis, the three proteins and their co-expression were significantly associated with higher overall survival. In multivariate analysis, each of these molecules was not favorable for overall survival; however, their co-expression was an independently prognostic factor for overall survival (hazard ratio, 0.276; 95% confidence interval, 0.173–0.444; $P < 0.001$).

Conclusions: These findings highlight the possibility that the multiple loss of ACRs may occur during the development of tumorigenesis, and their co-expression in gastric cancer may be predictive of favorable outcomes.

1. Introduction

Chemokines are multifunctional secreted peptides that have a critical role in regulating leukocyte migration. They are also involved in malignancy progression and metastasis. Chemokines direct the migration of tumor cells to specific organs or tissues [1]; CXCL12/CXCR4 has been reported to be required for peritoneal metastasis of gastric cancer and ovarian cancer, and CCR7/CCL19 (CCL21) could promote the pathogenesis and progression of breast cancer, melanoma, non–small cell lung cancer, and gastrointestinal cancer, and act directly on tumor cells to control their malignancy-related functions [2,3]. How

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the function of these factors is regulated is not well understood. A new subfamily of chemokine receptors does not signal along classic G-protein–mediated pathways; they efficiently internalize their cognate chemokine ligands and act as scavengers instead. These so-called atypical chemokine receptors (ACRs) are specialized for chemokine sequestration and act to regulate chemokine bioavailability, and therefore influence responses through signaling-competent chemokine receptors [4]. Atypical chemokine receptors are composed at least three members subfamily of chemokine receptors: Duffy antigen receptor for chemokines (DARC), D6, and ChemocentryX chemokine receptor (CCX-CKR). These receptors do this through binding and/or internalizing their chemoattractant ligands without activating signal transduction cascades leading to cell migration [5].

These ACRs are promiscuous, and all correlate with human tumor. Studies have shown that DARC is a negative regulator of prostate cancer, lung cancer, and melanoma by sequestering chemokines [6]. D6 research has shown that it is expressed on malignant vascular tumors, T-cell large granular lymphocyte leukemia cells, and choriocarcinoma cell lines [7]. D6 is a potent chemokine scavenger and negative regulator of inflammatory responses; D6-deficient mice showed a higher number of gastric cancer incidence [8]. ChemocentryX chemokine receptor can decrease CCL19, CCL21, CCL25, and CXCL13 protein levels in breast cancer and significantly inhibit tumor growth and lung metastasis. A significant correlation between CCX-CKR and lymph node metastasis was observed in human breast cancer tissue [9]. Therefore, we hypothesized that the ACRs could inhibit the proliferation, invasion, and metastasis of human gastric cancer, particularly the potential of invasion and metastasis, which preliminarily demonstrate that the intra-tumor chemokine network may be at least partly regulated by ACRs.

However, the relation of their expression in gastric cancer to progression and the prognosis of the patients is unclear. We examined the expression of DARC, D6, and CCX-CKR in different gastric cancer cell lines and specimens of human gastric tissue by Western blot and immunohistochemistry, to analyze their correlation to clinicopathologic characteristics and patients' outcome.

2. Materials and methods

2.1. *Patient characteristics with follow-up data and cell lines*

The present study included 282 patients with gastric cancer who underwent surgery between 2006 and 2008 at the Department of Surgical Oncology, First Hospital of China Medical University. We treated patients exclusively by total or subtotal gastrectomy with lymphadenectomy, according to tumor location. Adjuvant therapy was not administered to patients. We chose consecutive paraffin material from the archive of the Institute of Pathology and used it when adequate tumor tissue was available. Follow-up data were available from all patients, whom we assessed every 6 mo for 5 y or until death. Cancer-specific survival was calculated from the date of primary surgical resection to the date of gastric cancer–associated death or the date of recorded cancer progression. We collected all patient-derived specimens and archived them under protocols approved by the Institutional Review Board of the First Affiliated Hospital China Medical University. At least two pathologists confirmed the diagnoses; staging was based on pathologic findings according to the American Joint Committee on Cancer guidelines [10]. Table 1 lists patient details.

We purchased normal gastric cell line GES-1 and gastric cancer cell lines MKN28 (well differentiated), AGS, SGC-7901 (moderately differentiated), and MGC-803 (poorly differentiated) from the cell bank of the Chinese Academy of Sciences. We maintained GES-1 in RPMI 1640 (Hyclone, Logan City, UT) supplemented with 20% fetal bovine serum. Five cancer cell lines were maintained in RPMI 1640 (Hyclone) supplemented with 10% fetal bovine serum. All the cell lines were in a 5% CO_2 humidified atmosphere at 37°C.

2.2. *Tissue micro-array construction and immunohistochemistry*

We constructed a paraffin tissue micro-array (TMA) containing tissue from the tumor according to its hematoxylin-eosin slides. Briefly, we used 5-μm sections of formalin-fixed, paraffin-embedded tissue samples stained with hematoxylin-eosin to define representative areas of viable tumor tissue. We took 1.0-mm needle core biopsies from the corresponding areas of the paraffin-embedded tumor blocks using a tissue manual–arraying instrument. We then placed these needle core biopsies in recipient paraffin array blocks at defined coordinates. Three probes of each tumor were taken from central tumor areas. We incubated the cores in the paraffin block for 30 min at 37°C to improve adhesion between cores and the paraffin of the recipient block.

For immunohistochemical staining, we deparaffinized and rehydrated 5-μm sections from each paraffin block. For antigen retrieval, we boiled slides in 0.01 mol/L, pH 6.0, sodium citrate buffer for 15 min in a microwave oven. After blocking with the 5% normal rabbit serum, we incubated sections in a 1:500 dilution of goat anti-human DARC polyclonal antibody (ab40821; Abcam, Cambridge, UK), a 1:250 dilution of goat anti-human D6 (ab1656), and a 1:250 dilution of goat anti-human CCX-CKR (ab74806), which stayed overnight at 4°C. After incubation with a secondary antibody, the visualization signal was developed with diaminobenzidine. A pathologist who was blinded to outcome data independently evaluated sections pathologist three times. We scored the staining of ACRs from 0 to 3, considering only the cytoplasmic reaction; a score of 0 required that no staining was seen, 1 required 25% of cells to be positive, 2 required 25%–50% of cells to have been stained, 3 required 50%–75% of cells to be positive, and 4 required 75% of cells to have been stained.

2.3. *Western blot*

We washed cells twice with cold phosphate-buffered saline, lysed them on ice in radioimmunoprecipitation buffer with protease inhibitors, and quantified by bicinchoninic acid method. We resolved 50-μg protein lysates on 6% sodium dodecyl sulfate polyacrylamide gel, electrotransferred them to