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Original contribution

Role of gastrin-releasing peptides in breast cancer metastasis☆

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Summary The gastrin-releasing peptide, which is an unfolded protein response regulator and functions as a Ca^{2+}-binding molecular chaperone in the endoplasmic reticulum, is a regulatory human peptide that elicits gastrin release and regulates gastric acid secretion and enteric motor function. It has been shown to exhibit mitogenic activity in small cell lung cancer and plays a role in a lot of other human cancers including tumors in colon, stomach, pancreas, breast, and prostate. This study investigated the gastrin-releasing peptide expression in breast cancer to demonstrate the role of this biomarker in breast cancer metastasis. Gastrin-releasing peptide was analyzed in breast cancer tissue microarray specimens, including 200 primary breast cancer specimens and the corresponding lymph nodes from the same patients, through immunohistochemistry. The effect of gastrin-releasing peptide on the invasion ability of MCF-7 cells was evaluated using transwell assays. Gastrin-releasing peptide was highly expressed in breast cancer patients with lymph node metastasis. Besides, among the patients with lymph node metastasis, the ones with higher expression of gastrin-releasing peptide had shorter survival time. Overexpression of gastrin-releasing peptide significantly enhanced cell invasiveness. Conversely, a knockdown of gastrin-releasing peptide through the short hairpin RNA approach remarkably reduced MCF-7 cell invasion. Gastrin-releasing peptide expression may be associated with lymph node metastasis and may be used as an indicator of undesirable prognosis in patients with breast cancer.

1. Introduction

Breast cancer is the most common invasive cancer in women worldwide, thus attracting increased attention from researchers. One reason for the mortality of breast cancer patients is metastasis, which is related to poor prognosis. Axillary lymph node metastasis is the most frequently occurring metastatic disease and one of the most important prognostic signs. It can also guide the surgeon to the appropriate therapy [1]. In recent years, the neuropeptide gastrin-releasing peptide (GRP), progastrin-releasing peptide, has been reported as the most promising marker for small cell lung cancer (SCLC) [2]. It is the mammalian homologue of bombesin, a neuropeptide with diverse effects in the immune, pulmonary, and gastrointestinal systems. GRP binds to and activates a specific receptor, which is one of the 7 members of the transmembrane-spanning,

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G protein–coupled receptor superfamily. Its growth-stimulating properties, as well as its effects on morphogenesis, angiogenesis, cell migration, and cell adhesion, may be responsible for the more aggressive tumor behavior in cancer patients as well as their shorter survival. Some authors [2] have emphasized the clinical value of GRP as the tumor marker of choice in SCLC. However, limited and inconsistent data are available on the prognostic value of this marker in breast cancer. The present study aims to investigate the diagnostic and prognostic significance of GRP in breast cancer.

2. Materials and methods

2.1. Patient samples

Two hundred tissue specimens and clinical data of female primary invasive duct breast cancer, diagnosed between January 1997 and December 2004, were obtained at the General Hospital of Tianjin Medical University. The patients' records of these samples, including clinical data, histopathologic records, and treatment information, were all verified by pathologists. Paraffin-embedded tumor tissue samples and their lymph node tissues were collected from patients who had not been subjected to chemotherapy and radiotherapy before surgical resection but had received chemotherapy after their surgical operation. The use of these tissue samples in this study was approved by the institutional research committee. Tissue microarray (TMA) blocks consisting of 200 primary tumors and the corresponding lymph nodes (either metastatic or nonmetastatic) were constructed from the formalin-fixed, paraffin-embedded breast cancer samples mentioned above. The hematoxylin and eosin slides from the blocks were reviewed, and 2 representative tumor regions were chosen and marked to define the tissue of interest. Tumor TMA blocks were prepared by punching a cylinder using a hollow needle 2 mm in diameter. The blocks were obtained from 2 selected areas of each donor block before being inserted into an empty paraffin block. Thereafter, 4-μm sections were cut from the TMA blocks to generate the TMA slides for immunohistochemical (IHC) analyses.

2.2. Immunohistochemical analysis

The sections were deparaffinized and rehydrated before the endogenous peroxydase activity was blocked with 3% hydrogen peroxide in 50% methanol. The samples were then treated with citrate buffer saline (pH 6.0) for 15 minutes at 95°C in a microwave oven for antigen retrieval before being blocked with 10% normal goat serum for 30 minutes at room temperature. Thereafter, the sections were incubated with primary antibodies (1:100, goat antihuman GRP; Santa Cruz, Biotechnology, Santa Cruz, CA) for another 30 minutes at room temperature and subsequently incubated overnight at 4°C. The staining system used in this study was PicTure PV6000 (Zhongshan Chemical Co, Beijing, China). Staining was performed using 3,3′-diaminobenzidine. The sections were counterstained with hematoxylin, followed by dehydration and mounting. The negative controls were prepared by using phosphate-buffered saline instead of the first antibody.

2.3. Immunostaining assessment methods

The staining index (SI) was determined to investigate the difference in GRP expression in primary breast cancer tissues and the corresponding lymph nodes. Both the intensity and percentage of positive cells were considered. Five visual fields were randomly observed, with 100 cells in each field counted at 400× magnification. Positive cells from among the 100 tumor cells in each field were then counted. Tumor cells with brown cytoplasm were considered positive and then rated based on 4 classes, as follows: none (0), weak brown (1+), moderate brown (2+), and strong brown (3+). The average percentage of positively stained tumor cells was categorized into 5 classes, as follows: 0 for negative cells, 1 for 1% to 25%; 2 for 25% to 50%; 3 for 50% to 75%; and 4 for greater than 75%. The SI is equal to the product of the positive range score and the positive extent score. An SI of 6 or greater was defined as the high expression, whereas that less than 6 was defined as the low expression [3–5].

2.4. Cell culture and transfection

The Michigan Cancer Foundation-7 (MCF-7) cell lines used in this study were cultured in Dulbecco modified Eagle's medium medium supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA). The cells were randomly divided into 3 groups, as follows: control group (pGPU6/green fluorescent protein [GFP]/Neo plasmid), overexpression group (pGPU6/GFP/Neo-GRP), and silencing group (pGPU6/GFP/Neo-short hairpin RNA [shRNA]). The vectors were transfected into the cells using the Lipofectamine 2000 reagent (Invitrogen). The cells were then collected after 72 hours.

2.5. Total RNA isolation and real-time polymerase chain reaction

Total RNA was isolated from cultured MCF-7 cells using TRIzol reagent. The quantity of RNA was validated, and complementary DNA was synthesized through reverse transcription. Primers were designed and synthesized by TaKaRa Biotechnology Co Ltd. The Gene AMP PCR System 5700 Sequence Detector was used to amplify the complementary DNA in a 25 μL reaction mixture using the following conditions: denaturation at 94°C for 5 minutes, then 35 cycles of 94°C for 30 seconds, the optimal annealing temperature at 59°C for 45 seconds and 72°C for 40 seconds. The primer sequences used for GRP were as follows: forward