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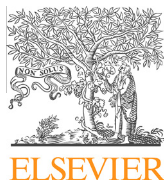

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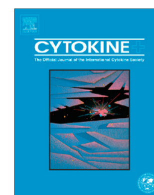

Increased interleukin-35 expression in tumor-infiltrating lymphocytes correlates with poor prognosis in patients with breast cancer

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ABSTRACT

Interleukin-35 (IL-35) is a recently discovered inhibitory cytokine, which is firstly discovered to be produced by regulatory T cells (Tregs) and proposed as a key effector molecule of Treg function. This study aims to analyze the correlation between IL-35 expression in tumor-infiltrating lymphocytes (TILs) of breast cancer tissue and patients' clinical characteristics. Plasma IL-35 was also determined in 60 patients with breast invasive ductal carcinoma (IDC) and 30 healthy women by enzyme-linked immunosorbent assay. IL-35 expression in the tissue specimens was analyzed by immunohistochemistry. It was shown that 39.1%, 40.0% and 19.1% of the 110 patients were absent, weak, and strong IL-35 expression in the TILs, respectively. Strong IL-35 expression in TILs was significantly associated with age >50 years, tumor size >2 cm, TNM stage III, and negative ER (All $P < 0.05$). Patients with elevated IL-35 expression in TILs had significantly worse progression-free survival (PFS) and overall survival (OS) than patients with weak or no IL-35 expression (All $P < 0.05$). High plasma IL-35 levels were significantly associated with TNM stage III and lymph node metastasis (All $P < 0.05$). Plasma IL-35 level and IL-35 expression in the TILs of breast cancer tissues may be a valuable biomarker in the development and prognosis of IDC.

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1. Introduction

The healthy immune system plays important roles in controlling the progression of malignant diseases. At the same time, immune suppression of cancer is the major cause of cancer development and progression [1]. Regulatory T cells (Tregs) have been found to play key roles in tumor escape and thus contribute to cancer progression [2]. Mammary tissues of high-grade breast cancers often contain large numbers of tumor-infiltrating lymphocytes (TILs) [3–5]. The TILs not only contain $CD8^+$ cytotoxic T lymphocytes and $CD4^+$ T helper lymphocytes, which can mediate antitumor responses, but also include large numbers of Treg cells, which can suppress the beneficial antitumor responses [6,7]. Interleukin-35 (IL-35) is a recently discovered inhibitory cytokine, which is produced by Tregs and proposed as a key effector molecule of Treg function [8].

IL-35, a heterodimer of p35 subunit of IL-12 and the Epstein-Barr Virus (EBV)-induced gene 3 (EBI3) subunit, belongs to the IL-12 family of cytokines [8,9]. In addition to the ability of suppressing effector T cell proliferation and downregulation of Th17 cell development and differentiation, IL-35 also enlarges regulatory responses by generating a potent population of IL-35-producing $CD4^+$ $Foxp3^-$ induced regulatory T cell population, defined as iTr35 cells [10]. In addition to $CD4^+$ Tregs, $CD8^+$ Tregs was also demonstrated to express IL-35 and mediate antigen-specific suppression in patients with prostate cancer [11]. Furthermore, regulatory B cells (Bregs) have recently been shown to produce IL-35 [12,13].

Abbreviations: IDC, invasive ductal carcinoma; IL-35, interleukin-35; Tregs, regulatory T cells; TILs, tumor-infiltrating lymphocytes; EBI3, Epstein-Barr Virus (EBV)-induced gene 3; Bregs, regulatory B cells; PFS, progression-free survival; OS, overall survival; ER, estrogen receptor; PR, progesterone receptor; HER2, human epithelial growth factor receptor 2; EGFR, Epidermal Growth Factor Receptor; NSCLC, non-small cell lung cancer.

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Given the direct suppressive activity of IL-35, there has been interest in evaluating the role of IL-35 in the development of a variety of diseases. Some diseases have been demonstrated to be associated with increased IL-35 expression, including multiple inflammatory diseases and cancers. In murine models of melanoma and colorectal carcinoma, the establishment of tumors leads to increased IL-35 expression in $CD4^+$ TILs, which are subsequently able to suppress T-cell proliferation [10]. In human, non-immunocytes have also been certified to express IL-35, including tumor cells [14–16]. Human serum IL-35 levels and IL-35 expression in colorectal cancer cells are associated with the severity and clinical stage of colorectal cancer [17]. In patients with invasive ductal carcinoma (IDC) of breast cancer, IL-35 mRNA expression in the peripheral blood mononuclear cells is significantly up-regulated compared with age-matched healthy women [18]. Another study demonstrated that IL-35 expressed in breast cancer tissue was associated with tumor progression and circulating IL-23:IL-35 ratio might be an important indicator association with breast cancer progression and prognosis [19]. In the present study, we compare plasma IL-35 levels of patients with IDC versus healthy women and further analyze the correlation between IL-35 expression of TILs in breast cancer tissue and patients' clinical characteristics.

2. Materials and methods

2.1. Patients and normal donors

The association of IL-35 expression with breast cancer was assessed in two independent IDC cohorts. One cohort included 110 unrelated Chinese women with IDC who underwent lumpectomy or mastectomy in the Affiliated Hospital of Binzhou Medical College between January 2008 and May 2010, allowing the assessment of progression-free survival (PFS) and overall survival (OS). The second cohort included 60 patients with newly diagnosed IDC from March 2014 to December 2014 and 30 age-sex matched healthy donors. Blood samples were collected from healthy donors and patients before surgery. Pathological and clinical informations were obtained from their medical records. Clinicopathologic characteristics included age of onset, tumor size, histological subtype and grade, lymph node involvement, pathological TNM stage, survival time and the status of estrogen receptor (ER), progesterone receptor (PR) and human epithelial growth factor receptor 2 (HER2). Any patient who had received chemotherapy or radiotherapy before obtaining specimens was excluded from this study. This study was approved by the Institutional Review Board of Binzhou Medical College and informed consent was obtained from each study participant.

2.2. Treatment and outcome assessment

All the 110 patients underwent lumpectomy or mastectomy and then received adjuvant therapies including chemotherapy, radiotherapy and endocrine therapy according to the National Comprehensive Cancer Network Guideline. All the 110 patients were followed up every 3 months, every 6 months, or annually. The last follow-up examination was completed on October 31, 2015. Progression-free survival (PFS) was defined as the time from the date of the surgery to the date when locoregional recurrence, distant metastasis, and/or contralateral breast cancer occurred. Overall survival (OS) was defined as the time from the date of the surgery to the date of death or the last follow-up examination.

2.3. Immunohistochemical staining for IL-35

Formalin fixed paraffin embedded (FFPE) mammary tissue specimens were sectioned (4-μm thick). The tissue sections were then de-waxed with xylene and rehydrated with gradient ethanol. Endogenous peroxidase was quenched by treating the tissue sections with 0.3% H_2O_2 at 37 °C for 15 min. Antigen retrieval was performed by boiling the slides in the retrieval solution (Tris/EDTA, pH 9.0) for 20 min in a microwave oven. After serial blocking with 10% normal goat serum and then 4% bovine serum albumin in TBS (pH 7.5) for 30 min, the tissue sections were incubated with mouse anti-human IL-35 monoclonal antibody (Clone: 15K8D10, Imgenex, USA) at 1:100 dilution at 4 °C overnight. The tissue sections were washed thoroughly with PBS, and then incubated with biotinylated goat anti-mouse secondary antibody (1:500 dilution, Beijing Zhong Shan Jin Qiao Biotechnology, China) at 37 °C for 30 min. Color was developed by avidin–biotin-peroxidase complex (ABC) method according to the instructions from the ABC kit (Beijing Zhong Shan Jin Qiao Biotechnology, China). Nuclei were counterstained with Mayer's hematoxylin. The stained tissue sections were dehydrate by alcohols and xylene and mounted on slides. The slides were observed under the Olympus IX81 microscope.

2.4. Immunohistochemical staining score

All the stained tissue sections were evaluated independently by two pathologists, who were blinded to patients' clinical data. IL-35 expression levels in TILs were scored as 0, 1, and 2 representing no staining, weak staining, and strong staining, respectively. Tissue sections with inconsistent staining scores from the two pathologists were re-evaluated and discussed until a consensus was reached.

2.5. Determination of plasma IL-35 levels

Five milliliters of peripheral blood were withdrawn from the 60 patients and the 30 healthy individuals in the morning after overnight fasting. The blood samples were collected in EDTA-containing tubes. All the blood samples were processed within 2 h after collection. The blood samples were centrifuged at 2500 rpm for 15 min. The plasma were collected and analyzed for IL-35 with an ELISA kit (Biolegend, USA) according to the manufacturer's instructions. Each sample was run in duplicate. The lowest detectable concentration of IL-35 was 0.08 ± 0.04 ng/ml.

2.6. Statistical analysis

Statistical analysis software SPSS 19.0 (SPSS, Chicago, IL) and GraphPad 5 software were used. Chi-square test was used to analyze the correlations between IL-35 expression levels and patients' clinicopathological characteristics. The cumulative survival curves were estimated by Kaplan–Meier method. The plasma IL-35 level was presented as mean ± standard deviation (SD). Mann-Whitney *U* test was used to compare the plasma IL-35 levels of different groups. All statistical tests were two-sided and a $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression analysis of IL-35 in TILs of breast cancer tissue by immunohistochemistry stain

The age range of the patients was from 31 to 65 years old (median 46). Of the 110 patients, 43 (39.1%) showed no positive IL-35 staining, 44 (40.0%) had weak IL-35 staining and 21 (19.1%)

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