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Original Article

Circulating low IL-23: IL-35 cytokine ratio promotes progression associated with poor prognosisin breast cancer

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Abstract: The interleukin (IL)-12 family, composed of heterodimeric cytokines including IL-12 (formed by IL-12p35 and IL-12p40 subunits), IL-23 (formed by IL-23p19 and IL-12p40 subunits), IL-27 (formed by IL-27p28 and EBI3 subunits) and IL-35 (formed by IL-12p35 and EBI3 subunits), establishes a link between innate and adaptive immunity that involves different immune effector cells and cytokines to tumors. However, the role of IL-12 family in breast cancer (BC) progression and prognosis remains unclear. In the present study, we demonstrated evidence indicating that EBI3, IL-12p35 and IL-12p40 but not IL-23p19 or IL-27p28 were highly expressed in BC tissues, suggested that tumor derived EBI3, IL-12p35 and IL-12p40 were associated with tumor progression. Circulating IL-12 and IL-23 low expressed, but IL-27 and IL-35 high expressed in BC patients, especially circulating IL-23 associated with IL-35 to mediate BC tumor resection. Ki-67, p53 and EGFR expression on BC tissues, as well as CA125, CA153 and CA199 levels on BC bloods increased when circulating IL-23: IL-35 ratio decreased. Together, for the first time, our data suggest that circulating IL-23: IL-35 ratio may be an important indicator association with BC progression and prognosis. However, further research should be carried out to assess the implications of circulating IL-23: IL-35 ratio in a larger sample size.

Keywords: Breast cancer, interleukin (IL)-12, IL-23, IL-27, IL-35

Introduction

Recently, breast cancer (BC) is the most prevalent cancer among women worldwide, withan incidence rate of approximately 1.7 million cases per year and 0.5 million deaths per year [1]. Traditional prognostic parameters such as histological type, lymph node stage, nottingham prognosis index and serum tumor biomarkers are used in the assessment of BC outcomes. However, the survival outcomes of BC patients are still not optimistic. Early-stage BC has a favorable prognosis with a 5-year survival rate up to 90%, while this rate declines to 20% upon tumor spreading to distant organs [2]. Therefore, it has been necessary to identify an effective biomarker for more accurately predicting the prognosis of BC patients. Inflammation within the tumor microenvironment correlates with increased invasiveness and poor prognosis in many types of cancer, including BC [3]. Moreover, lots of clinical and experimental evidences indicate that the outcome of an immune response, tumor rejection or promotion, toward an evolving BC is largely determined by the type of immune response , chronic inflammation or acute inflammation elicited [4]. In recent decades much attention has focused on the uncovering of the role of cytokines in BC.

The interleukin (IL)-12 family, which is composed of heterodimeric cytokines including IL-12, IL-23, IL-27 and IL-35, establishes a link between innate and adaptive immunity that

involves different immune effector cells and cytokines to tumors. However, the role of IL-12 family in BCprogression and prognosisremains unclear.

IL-12, formed by IL-12p35 and IL-12p40 subunits, is produced by activated antigen-presenting cells with an antitumor via inducing IFN-γ production by NK and T cells [5], shifting differentiation of naive Th0 cells toward the Th1 phenotype [6] and enhancing antibody dependent cellular cytotoxicity against tumor cells [7]. IL-12p35 subunit also can form IL-35 with Epstein-Barr virus-induced gene 3 (EBI3) subunit. However, IL-35 appears to have aprotumor rolethroughexpanding Tregs and inhibiting $CD4^+CD25^-$ effector T cells [8], stimulating IL-35-producing $CD1d^{high}CD5^+$ B cells mediated tumor cell proliferation [9], inhibiting apoptosis [10, 11] and enhancing myeloid cell accumulation [12]. Similarly, IL-12p40 subunit also can form IL-23 with IL-23p19 subunit. IL-23 has also been reported to play a protumor role by promoting tumor cell epithelial-mesenchymal transition (EMT) [13], enhancing anti-apoptotic and drug resistance [14] and inducing tumor cell migration and invasion [15]. Notablely, IL-27, build by EBI3 and IL-27p28, has pleiotropic functions in the regulation of immune responses with both pro-inflammatory and anti-inflammatory properties. Therefore, IL-27 acts with a double-edged sword, both antitumor and protumor effects of IL-27 are conceivably expected depending on the type of cells that IL-27 stimulates and the tumor context [16, 17].

Accordingly, to uncover the pleiotropic functions of IL-12 family cytokines within the BC tumor microenvironment and peripheral blood, our study was designed to evaluate prospectively the independent prognostic importance of circulating IL-12 family cytokines in patients with BC and the potential association with early cancer detection or disease monitoring.

Materials and methods

Subjects

A total of 65 BC patients with pathologically confirmed were collected at the Department of Surgery of Xiaolan Hospital of Southern Medical University, between December 2010 and July 2013. According to the World Health Organization guidelines, the tumor-node-metastasis (TNM) system of tumor stage and histological grade were performed, 53 patients (81.5%) were in T2, 4 patients (6.2%) were in T1, 5 patients (7.7%) were in T3 and 3 patients (4.6%) were in T4, and the demographic and clinical characteristics of the selected subjects were summarized in Table S1. Both cancer and normal tissues (>2 cm away from cancer tissues) were obtained from operative, and fixed in 10% buffered formalin and/or frozen immediately in liquid N^2, stored at -80°C until use. Blood samples of BC patients were collected pre-operation and post-operation according Samy et al. reported [18]. For normal controls, 40 healthy volunteers (HV) were organized by the Medical Examination Center of Xiaolan Hospital of Southern Medical University, between December 2010 and July 2013. Serum aliquots (200 μL for each) were rapidly frozen and stored in a -80°C freezer until further analysis. There was no significant difference in age and gender between BC patients and control subjects. Patients who were male gender, diagnosed with no definitive surgery or excisional biopsy and received preoperative-blood transfusion, chemotherapy or hormonal therapy were excluded. The study was approved by the Internal Review and Ethics Boards of Guangdong Medical University, Xiaolan Hospital of Southern Medical University, and Dongguan Hospital Affiliated to Medical College of Jinan University, and informed consent was obtained from the parents.

Immunohistochemistry

Immunohistochemistry of 65 BC and matched normal tissues were performed to detect the expression of EBI3, IL-23p19, IL-27p28, IL-12p35, IL-12p40, Ki-67, p53 and EGFR. Briefly, tissue sections (4 μm) were prepared from formalin-fixed paraffinembedded tissue blocks were dewaxed in xylene and rehydrated in graded ethanols, and immersed in 0.3% Hydrogen peroxide solution for 10 min at room temperature to block the endogenous peroxidase activity, and washed by Phosphate Buffered Saline (PBS) solution. Antigenic epitopes were next retrieved by heating for 2 min in 10 mmol/L citrate buffer (pH 6.0). Then, the sections were incubated with the primary antibody for EBI3 (sc-32868, Santa Cruz Biotech, Inc.), IL-23p19 (sc-21083, Santa Cruz Biotech, Inc.), IL-12p35 (sc-7925, Santa Cruz Biotech,

Inc.), IL-27p28 (sc-27487, Santa Cruz Biotech, Inc.), IL-12p40 (sc-7926, Santa Cruz Biotech, Inc.), Ki-67 (NCL-L-Ki67-MM1, Leica Novocastra), p53 (NCL-L-p53-DO7, Leica Novocastra) and EGFR (NCL-L-EGFR-384, Leica Novocastra) for 30 min at room temperature as our previously reported. Next, the sections were washed with PBS and followed by a goat anti-rabbit and mouse IgG-HRP (Kit-0015, Maixin Biotech, Fuzhou, China) secondary antibody for one hour at room temperature at 1:500 dilutions. In the end, the sectionswere visualized using DAB Detection Kit (Enhanced Polymer) (Kit-0015, Maixin Biotech, Fuzhou, China) and chromogenic reaction was controlled under a microscope (Nikon). After immunostaining, sections were immersed into hematoxylin for nuclear staining, then dehydrated through gradient concentrations of ethanol, cleared with xylene, and covered with neutral balsam. The score of immunohistochemical sections were assessed by two pathologists in a blinded fashion to the clinical status of the patients by Michalski and colleaguesas previously reported [19]. The immunoreactive area (percentage of positive staining cells) and intensity scores of proteins were evaluated.

Western blotting

Western blot analysis was performed to determine the expression levels of IL-12p35, IL-12p40, IL-23p19, IL-27p28 and EBI3 in BC tissues and matched normal tissues. Frozen tissues were ground in liquid N^2 and lysed with RIPA lysis buffer (Beyotime, China) containing 0.5 M DTT, 0.1 M PMSF and 20× phosphatase inhibitor for 30 min on ice, then centrifuged at 12,000 g for 5 min at 4°C. The protein concentration in the supernatant was determined by BCA Protein Assay Kit (Beyotime, China). Equal amounts of total protein (~150 μg) were separated on 10% SDS-PAGE electrophoresis and transferred on to polyvinylidene fluoride (PVDF) membranes (Millipore, Massachusetts, USA). The membranes were blocked in 5% nonfat milk, and hybridized with primary antibody overnight at 4°C, identified with a secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature. Immunoblots were visualized by enhanced chemiluminescence detection reagents (Millipore, Massachusetts, USA), with β-actin as a loading control.

ELISA analysis

Serum samples were collected from venous blood at room temperature and stored at -80°C until use. Serum IL-12, IL-23, IL-27 and IL-35 levels were measured using the Human IL-12 (p70) ELISA MAX™ Deluxe (Biolegend, San Diego, CA, USA), Pre-coated LEGEND MAX Human IL-23 ELISA Kit (Biolegend, San Diego, CA, USA), Pre-coated LEGEND MAX™ Human IL-27 ELISA Kit (Biolegend, San Diego, CA, USA), Pre-coated LEGEND MAX Human IL-35 ELISA Kit (Biolegend, San Diego, CA, USA), respectively, according to the manufacturer's instructions. CA125, CA153 and CA199 were analyzed on UniCelDxI 800 immunoassay system (Beckman Coulter, Brea, CA).

Statistical analysis

The results are presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism version 5.0 software (GraphPad Software Inc., San Diego, CA, USA). Student's t-test and Chi-square test were employed for analysis the differences of categorical variables. Pearson correlation was used to measure the degree of dependency between variables. A value of $P<0.05$ was deemed significant, and values of $P<0.01$ and $P<0.001$ were considered as highly significant.

Results

EBI3, IL-12p35 and IL-12p40 but not IL-23p19 or IL-27p28 are highly expressed in BC tissues

Our previously study showed that high expression of EBI3 and IL-12p35 are correlated to the severity of malignancy and the clinical stage of colorectal cancer [19]. However, the role of EBI3 and IL-12p35 expression on BC were not be elaborated. Therefore, we systemic evaluated EBI3 and IL-12p35 expression collaborative with IL-23p19, IL-27p28 and IL-12p40 expression on BC and matched normal tissues by immunohistochemical analysis. Results showed cancer sections from all patients displayed positive reactivity for EBI3, IL-12p35 and IL-12p40, but low or loss expression of IL-23p19 and IL-27p28 (**Figure 1A**). For quantitative analysis, the expression levels for EBI3, IL-12p35 and IL-12p40 in cancer tissues were shown signification higher expression than normal tissues (**Figure 1B**). However, there is no

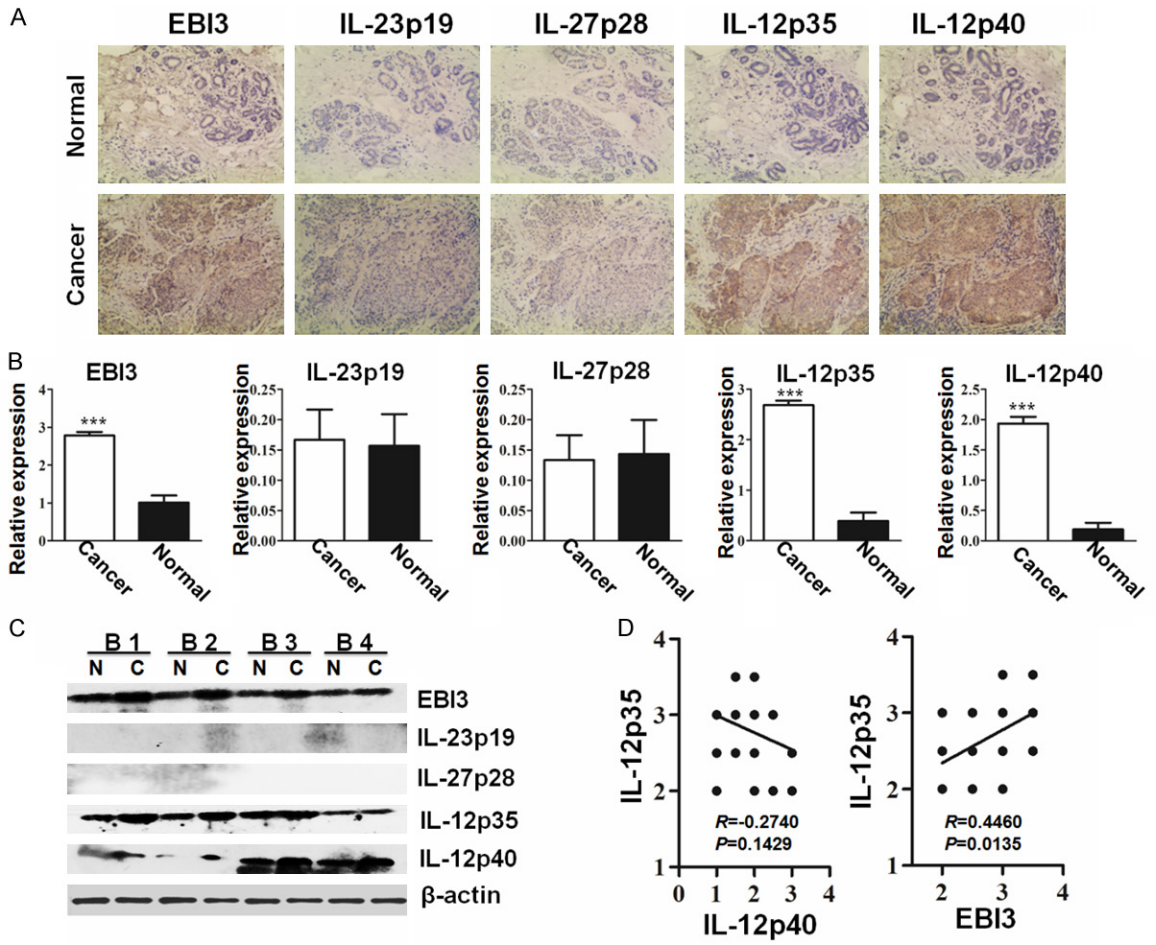

Figure 1. Quantitative analysis of EBI3, IL-23p19, IL-27p28, IL-12p35 and IL-12p40 expression in BC and normal tissues. Representative images (200×) for the immunohistochemical staining of EBI3, IL-23p19, IL-27p28, IL-12p35 and IL-12p40 in BC and normal tissues (A). Bar graphic figures showing the relative expression levels ofEBI3, IL-23p19, IL-27p28, IL-12p35 and IL-12p40 assessed in all tissues (B). The expression of EBI3, IL-23p19, IL-27p28, IL-12p35 and IL-12p40 on normal tissues, shown with "N", and cancer tissues, shown with "C" from representative BC patients (B1, B2, B3 and B4) were confirmed by western blot analysis (C). Results for correlation analysis of IL-12p35 with IL-12p40 and EBI3 in BC tissues (D). *, P<0.05; **, P<0.01; ***, P<0.001.

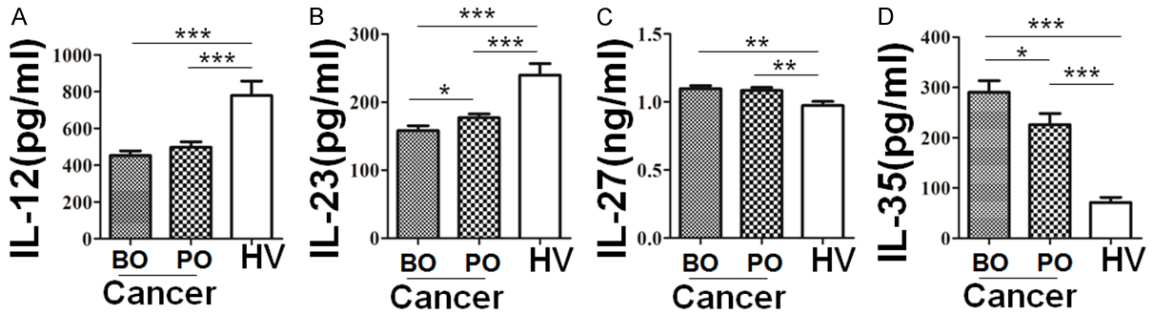

Figure 2. Circulating IL-12, IL-23, IL-27 and IL-35 were detected in BC and healthy individuals serum by ELISA. Bar graphic figures showing circulating IL-12 (A), IL-23 (B), IL-27 (C) and IL-35 (D) levels in serum of 65 cases preoperative (BO) and postoperative (PO) BC patients, and 60 healthy volunteers (HV). *, P<0.05; **, P<0.01; ***, P<0.001.

difference of IL-23p19 and IL-27p28 expression, respectively, between cancer and normal tissues. In order to further determine EBI3, IL-23p19, IL-27p28, IL-12p35 and IL-12p40

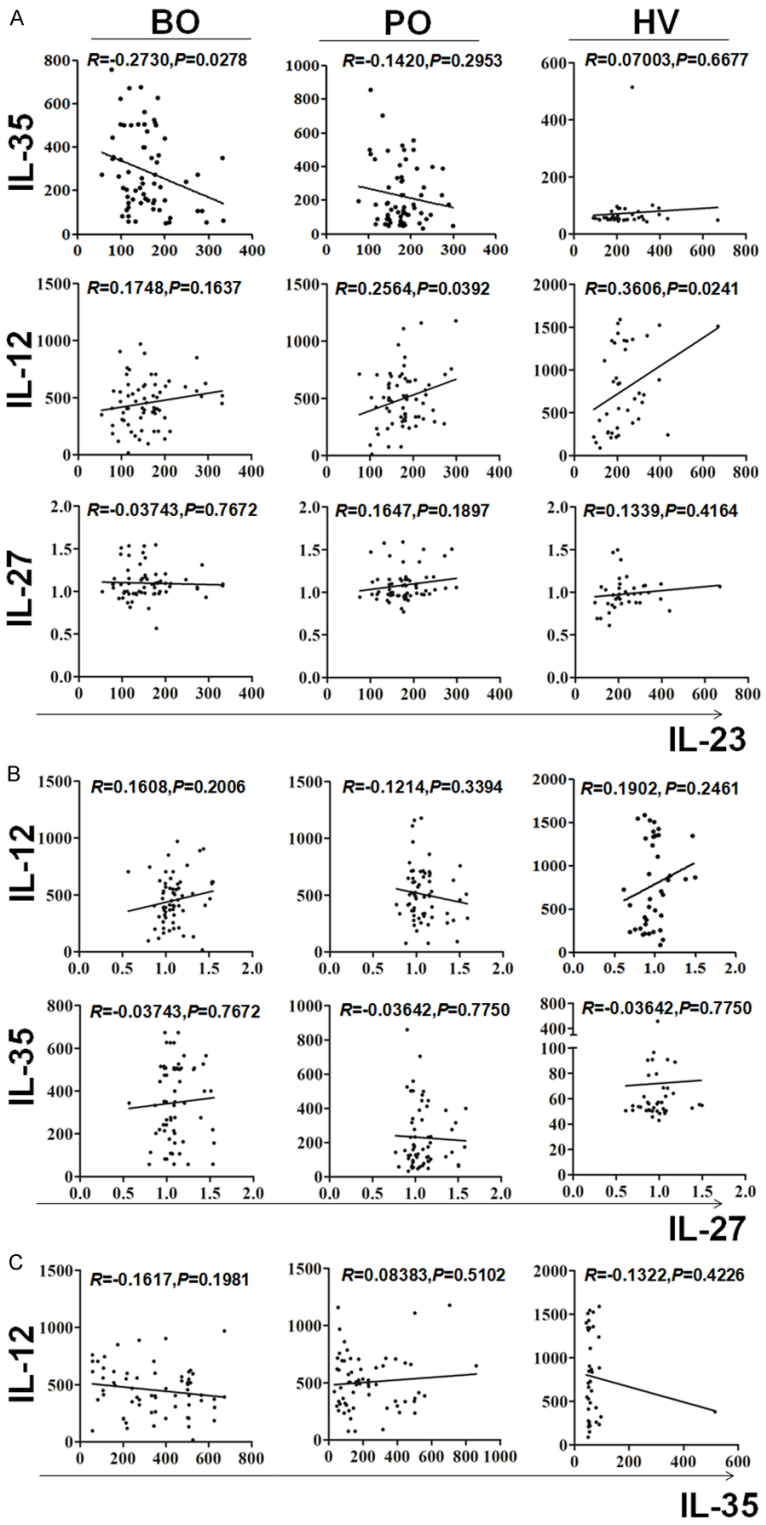

Figure 3. Relationship among the levels of IL-12, IL-23, IL-27 and IL-35 in serum of BC patientsand healthy individuals. Correlations among the levels of IL-23 with IL-12, IL-27 and IL-35 (A), the levels of IL-27 with IL-12 and IL-35 (B), and the levels of IL-35 with IL-12 (C) in preoperative (BO) and postoperative (PO) BC patients, and healthy volunteers (HV) were analyzed by Pearson correlation.

expression on cancer and normal tissues, western blot analysis was performed on 8 cases cancer and 8 cases matched normal tissues. In line with the above results, EBI3, IL-12p35 and IL-12p40 expression on cancer tissues were higher than matched normal tissues (**Figure 1C**). Of noted, no visible IL-23p19 and IL-27p28 expression were detected both on cancer and normal tissues (**Figure 1C**), suggesting tumor derived EBI3, IL-12p35 and IL-12p40, but not IL-23p19 or IL-27p28 associated with BC progression. Furthermore, IL-12p35 was noted to be positively correlated to EBI3 but not to IL-12p40 in BC tissues after Pearson correlation analysis (**Figure 1D**), suggesting tumor derived IL-35 but not IL-12 associated with BC progression. This results were consistent with the findings in colorectal cancer, pancreas cancer, gastric cancer that tumor derived IL-35 were associated with tumor progression [10, 12, 19-21].

Circulating IL-12 and IL-23 low expressed, but IL-27 and IL-35 high expressed in BC patients

The above expression data prompted us to examine IL-12, IL-23, IL-27 and IL-35 levels in BC patients. For this purpose, we conducted ELISA analysis of IL-12, IL-23, IL-27 and IL-35 using bloods. In line with the expression data, IL-12 and IL-23 levels were lower in BC patients than that of control (**Figure 2A** and **2B**), but IL-27 and IL-35 levels were higher in BC patients than that of health control (**Figure 2C** and **2D**). More importantly, a sig-

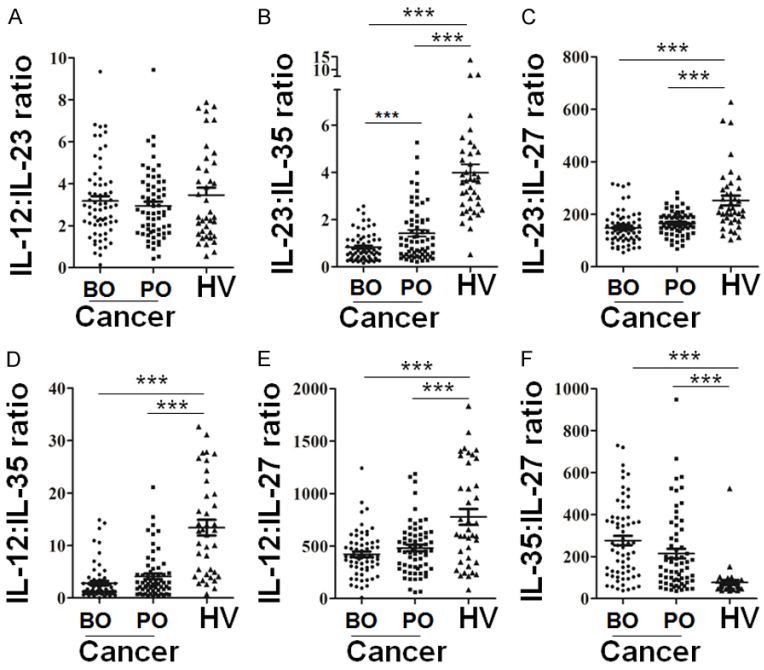

Figure 4. The expression ratio among IL-12, IL-23, IL-27 and IL-35 levels in BC patients and healthy individuals. The expression ratio of IL-12 to IL-23 (A), IL-23 to IL-35 (B), IL-23 to IL-27 (C), IL-12 to IL-35 (D), IL-12 to IL-27 (E), IL-35 to IL-27 (F) in preoperative (BO) and postoperative (PO) BC patients, and healthy volunteers (HV) were analyzed. *, P<0.05; **, P<0.01; ***, P<0.001.

nificantlyrise for serum IL-23 but reduction for serum IL-35 were noted in all patientsafter tumor resection (**Figure 2B** and **2D**). Altogether, those data suggested that serum IL-23 and IL-35 could be a valuable biomarker for assessing BC progression.

Circulating IL-23 associated with IL-35 to mediate BC progression

Above results suggested that serum IL-23 and IL-35 could mediate BC progression. Next, the correlation between serum IL-23 and IL-35, also and the correlation among serum IL-12, IL-23, IL-27 and IL-35 both on BC patients and health control were analyzed by Pearson correlation analysis. Results shown serum IL-35 levels was noted to be negatively correlated to serum IL-23 levels in patient blood before surgery. However, this relationship was broken after tumor resection (**Figure 3A**). Conversely, positively correlation of serum IL-12 levels with serum IL-23 levels was broken in preoperative BC patients (**Figure 3A**). Of noted, there was no significantly correlation between serum IL-12 levels and serum IL-27 levels, serum IL-27 levels and serum IL-23 levels, serum IL-27 levels and serum IL-35 levels, and serum IL-12 levels and serum IL-35 levels both on preoperative and postoperative BC patients or healthy volunteers (**Figure 3B** and **3C**). This results suggested that IL-23 may be associated with IL-35 to mediate BC progression.

IL-23: IL-35 ratio in serum maybe a prognostic factor on BC patients

Given that circulating IL-23 may be associated with IL-35 to mediate BC progression, value of IL-23: IL-35 ratio was further evaluated. Meanwhile, value of IL-12: IL-23 ratio, IL-23: IL-27 ratio, IL-12: IL-27 ratio, IL-12: IL-35 ratio and IL-35: IL-27 ratio were also evaluated (**Figure 4A-F**). Results showed IL-23: IL-35 ratio (**Figure 4B**), IL-23: IL-27 ratio (**Figure 4C**), IL-12: IL-35 ratio (**Figure 4D**) and IL-12: IL-27 ratio (**Figure 4E**) in peripheral blood of BC patients were lower, but IL-35: IL-27 ratio (**Figure 4F**) were higher, than that of in healthy volunteers, respectively. Notably, only IL-23: IL-35 ratio had a guiding change in peripheral blood of BC patients after tumor resection (**Figure 4B**), suggesting IL-23: IL-35 ratio may play animportant value in tumor treatment. In addition, Ki-67, p53 and EGFR expression on cancer tissues from 16 cases BC patients with IL-23: IL-35 ratio decreased, and 16 cases BC patients with IL-23: IL-35 ratio >1.6 and increased 2-fold were retrospective analyzed (**Figure 5A**). As expected, cancer tissues from patients with IL-23: IL-35 ratio decreased showed higher expression of Ki-67, p53 and EGFR than that from patients with IL-23: IL-35 ratio >1.6 and increased 2-fold (**Figure 5B**), suggesting IL-23: IL-35 ratio may relate to BC progression and prognosis. Besides, CA125, CA153 and CA199 levels on BC bloods also show a increasing trend when IL-23: IL-35 ratio decreased, especially for CA125 (**Figure 5C**).

Together, our data suggested that tumor derived IL-35 associated with BC progression, and circulating low IL-23: IL-35 ratio promotes

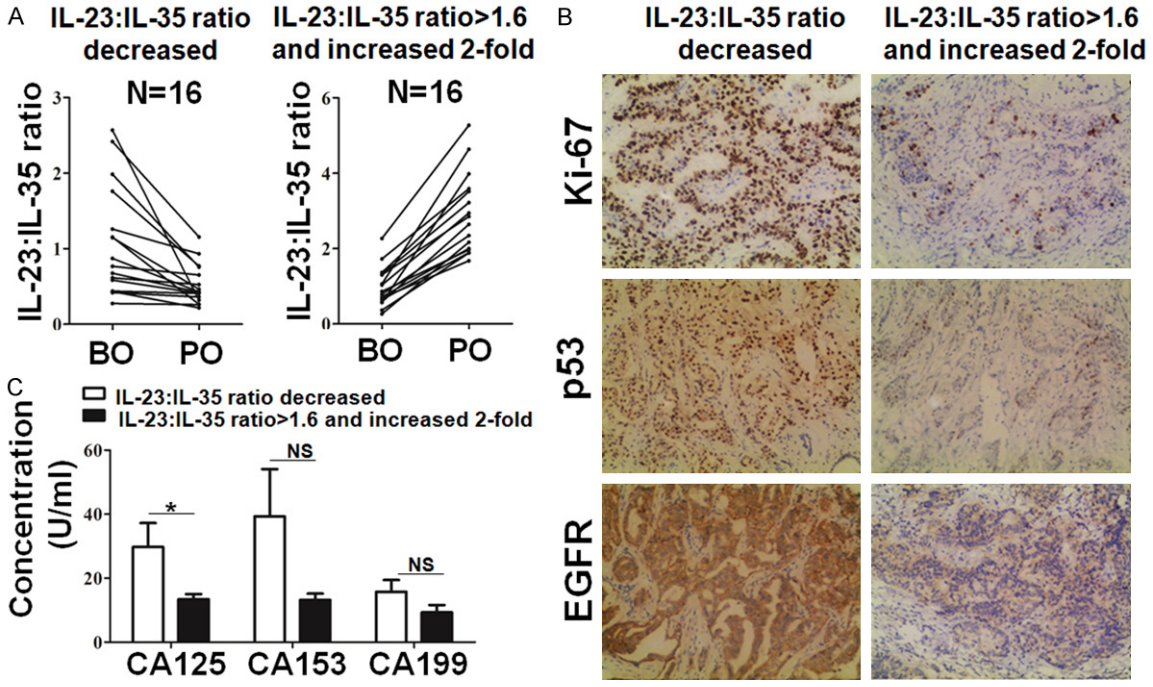

Figure 5. Ki-67, p53 and EGFR expression on BC tissues, as well as CA125, CA153 and CA199 levels on BC bloods increased when circulating IL-23: IL-35 ratio decreased. Before-after graph of 16 cases preoperative (BO) matched postoperative (PO) BC patients with IL-23: IL-35 ratio decreased, or with IL-23: IL-35 ratio >1.6 and increased 2-fold were used to display circulating IL-23: IL-35 ratio (A). Ki-67, p53 and EGFR expression between patients with IL-23: IL-35 ratio decreased (n=16) and patients with IL-23: IL-35 ratio >1.6 and increased 2-fold (n=16) were analyzed by immunohistochemical staining (B). CA125, CA153 and CA199 levels between patients with IL-23: IL-35 ratio decreased (n=16) and patients with IL-23: IL-35 ratio >1.6 and increased 2-fold (n=16) were also evaluated (C). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$: NS, no signification.

BC progression associated with poor prognosis.

Discussion

In the present study, we demonstrated evidence indicating that (1) EBI3, IL-12p35 and IL-12p40 but not IL-23p19 or IL-27p28 were highly expressed in BC tissues, suggested that tumor derived EBI3, IL-12p35 and IL-12p40 were associated with tumor progression. (2) Circulating IL-12 and IL-23 low expressed, but IL-27 and IL-35 high expressed in BC patients, especially circulating IL-23 associated with IL-35 to mediate BC tumor resection. (3) Ki-67, p53 and EGFR expression on BC tissues, as well as CA125, CA153 and CA199 levels on BC bloods increased when circulating IL-23: IL-35 ratio decreased.

Currently, the role of IL-23 in tumor progression is controversial [13, 22-25]. It has been reported that IL-23 was known to be essential for Th17 cell survival and expansion, and for making pathogenic Th17 cells [26, 27], which have antitumor effects by attracting CTL and NK cell migrating into the tumor suppressing tumor growth and metastasis [28]. On the other hand, it has also been reported that IL-23 contributed to EMT through the Wnt/β-catenin pathway [13], induced de novo gut tumorigenesis, through activation of innate lymphoid cells [29], promoting tumor migration and invasion [15, 25]. In here, we established IL-12p40, a subunit of the IL-23 was high expression on BC tissues. However, IL-23p19, another subunits of IL-23 was low expression or could not be detected on BC tissues, suggesting IL-23 may play an antitumor effects in BC. Similar result has been reported inhuman lung adenocarcinoma and oral squamous cell carcinoma [30, 31]. Low expression of circulating IL-23 in BC patients also supported this speculation. Meanwhile, Heckel et al. demonstrated that established breast tumor cell lines produced IL-12p40 to form monomer/homodimer, but not to form a heterodimer with the IL-12p35 sub-

unit to build IL-12 [32]. Of noted, we also found IL-12p35 was noted to be positively correlated to EBI3 but not to IL-12p40 in BC tissues, consistent with our found in colorectal cancer [19]. It is different from low expression of circulating IL-23 in BC patients of our demonstrated, Gangemi et al.found IL-23 was significantly higher expression in peripheral blood of BC patients compared with the healthy controls [33]. These differences may be associated with the choice of specimens. 26% (13/50) patients, Sebastian et al. selected, were prior to receive first line chemotherapy [33], but the patients who received chemotherapy or hormonal therapy were excluded, and 81.5% (53/65) patients were in T2 stage in our study. We have observed circulating IL-23 was significantly elevated after tumor resection. Besides circulating IL-23, we also found circulating IL-12 had significantly lower expression but no relationship to tumor resection.

Different from controversial IL-23, now all present evidence supported tumor derived IL-35 promoting tumor growth and angiogenesis [10, 12, 19-21]. Here, we demonstrated EBI3 associated with IL-12p35, both of subunits of IL-35, were highly expressed in tumor tissues and mediated BC progression. Circulating IL-35 were also higher expression, similar to our previous reported in colorectal cancer [19], Jin et al. reported in pancreatic ductal adenocarcinoma [34], and Gu et al. reported in non-small cell lung cancer [35]. Additional, in contrast to the IL-23, circulating IL-35 was significantly decreased after tumor resection. Conversely, high level of the circulating IL-27 was no significant differences between preoperative and postoperative. Notably, circulating IL-35 levels was noted to be negatively correlated to circulating IL-23 levels in patient blood before surgery, and this relationship was broken after tumor resection, suggesting IL-23 associated with IL-35 to mediate BC progression. The IL-23: IL-35 ratio was significantly lower in serum of BC patients versus healthy volunteers and significantly rise by tumor resection. Although this study also found that IL-23: IL-27 ratio, IL-12: IL-35 ratio and IL-12: IL-27 ratio in peripheral blood of BC patients were lower, and IL-35: IL-27 ratio were significantly higher than that of in healthy volunteers, respectively, all had nothing to do with surgery treatment.

Interestingly, Ki-67, p53 and EGFR expression on cancer tissues from patients with IL-23: IL-35 ratio decreased were significantly higher than that from patients with IL-23: IL-35 ratio >1.6 and increased 2-fold. High expression of Ki-67, p53 and EGFR shows a more aggressive behavior and poor prognosis in BC [36-39]. Therefore, we demonstrated that circulating low IL-23: IL-35 cytokine ratio promotes progression associated with poor prognosis in BC. Meantime, tumor biomarkers CA125, CA153 and CA199 levels, for BC aided diagnosis on bloods [40], also increased when circulating IL-23: IL-35 ratio decreased in our observed.

Together, for the first time, our data suggest that circulating IL-23: IL-35 ratio may be an important indicator association with BC progression and prognosis. However, further research should be carried out to assess the implications of circulating IL-23: IL-35 ratio in a larger sample size.

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Table S1. The demographic and clinical characteristics of the selected subjects

NO.	Gender	Age	TNM
BC1	Female	44	T1N0M0
BC2	Female	49	T1N0M0
BC3	Female	48	T1N0M0
BC4	Female	57	T1N0M0
BC5	Female	39	T2N0M0
BC6	Female	41	T2N0M0
BC7	Female	46	T2N0M0
BC8	Female	57	T2N0M0
BC9	Female	53	T2N0M0
BC10	Female	55	T2N0M0
BC11	Female	68	T2N0M0
BC12	Female	42	T2N0M0
BC13	Female	44	T2N0M0
BC14	Female	51	T2N0M0
BC15	Female	49	T2N0M0
BC16	Female	50	T2N0M0
BC17	Female	39	T2N0M0
BC18	Female	54	T2N0M0
BC19	Female	30	T2N0M0
BC20	Female	60	T2N0M0
BC21	Female	27	T2N0M0
BC22	Female	42	T2N0M0
BC23	Female	49	T2N0M0
BC24	Female	50	T2N0M0
BC25	Female	53	T2N0M0
BC26	Female	41	T2N0M0
BC27	Female	59	T2N0M0
BC28	Female	38	T2N0M0
BC29	Female	59	T2N0M0
BC30	Female	44	T2N0M0
BC31	Female	53	T2N0M0
BC32	Female	45	T2N0M0
BC33	Female	45	T2N0M0
BC34	Female	68	T2N0M0
BC35	Female	40	T2N0M0
BC36	Female	44	T2N0M0
BC37	Female	33	T2N0M0
BC38	Female	47	T2N0M0
BC39	Female	29	T2N0M1
BC40	Female	43	T2N0M1
BC41	Female	81	T2N1M0
BC42	Female	59	T2N1M0
BC43	Female	48	T2N1M0
BC44	Female	42	T2N1M0
BC45	Female	55	T2N1M0
BC46	Female	27	T2N1M0
BC47	Female	63	T2N1M0
BC48	Female	48	T2N1M0
BC49	Female	39	T2N1M0
BC50	Female	48	T2N1M0
BC51	Female	74	T2N1M0
BC52	Female	42	T2N1M0

BC53	Female	61	T2N1M1
BC54	Female	47	T2N1M1
BC55	Female	42	T2N^{2}M0
BC56	Female	47	T2N^{2}M0
BC57	Female	59	T2N3M0
BC58	Female	29	T3N1M0
BC59	Female	27	T3N1M0
BC60	Female	31	T3N1M0
BC61	Female	45	T3N1M0
BC62	Female	53	T3N^{2}M0
BC63	Female	28	T4N0M0
BC64	Female	59	T4N0M0
BC65	Female	48	T4N^{2}M0
HV1	Female	51	-
HV2	Female	39	-
HV3	Female	44	-
HV4	Female	36	-
HV5	Female	35	-
HV6	Female	48	-
HV7	Female	57	-
HV8	Female	53	-
HV9	Female	67	-
HV10	Female	50	-
HV11	Female	55	-
HV12	Female	49	-
HV13	Female	33	-
HV14	Female	39	-
HV15	Female	36	-
HV16	Female	38	-
HV17	Female	55	-
HV18	Female	54	-
HV19	Female	50	-
HV20	Female	60	-
HV21	Female	57	-
HV22	Female	61	-
HV23	Female	52	-
HV24	Female	48	-
HV25	Female	48	-
HV26	Female	43	-
HV27	Female	46	-
HV28	Female	55	-
HV29	Female	44	-
HV30	Female	60	-
HV31	Female	39	-
HV32	Female	38	-
HV33	Female	41	-
HV34	Female	47	-
HV35	Female	45	-
HV36	Female	56	-
HV37	Female	47	-
HV38	Female	59	-
HV39	Female	48	-
HV40	Female	43	-

BC: breast cancer; HV: healthy volunteers; TNM: tumor-node-metastasis.