# Plasma Levels of Interleukin-35 and its Association with Clinical Features of Breast Cancer Patients at Assiut University Hospitals

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**Interleukin-35 (IL-35), is a recently identified cytokine that belongs to the IL-12 family, it is a potent anti-inflammatory and immunosuppressive cytokine which was first recognized to be produced by regulatory T cells (Tregs) cells, and recently was found to be produced by regulatory B cells (Bregs). The study aimed at determining whether plasma levels of IL-35 are associated with clinical characteristics of breast cancer (BC) patients. The study included 40 patients with breast cancer (BC), and 10 matched controls. The IL-35 cytokine was measured in plasma using ELISA. Results showed that plasma IL-35levels were significantly higher in BC than healthy controls (*P*< 0.05), and were significantly associated with BC grade 2 and HER-2 over expression level "3+", suggesting that plasma IL-35 levels may be associated with the development and progression of BC.**

Breast cancer (BC) is the most common cancer in women. According to American Cancer Society publication, 252,710 new cases of invasive breast cancer were diagnosed among women and 2,470 cases were diagnosed in men [1]. In Egypt, it is the most common cancer among women, representing 18.9% of total cancer cases (35.1% in women; 2.2% in men) in Egypt National Cancer Institute's (NCI) series of 10,556 patients during the year 2001, with an age-adjusted rate of 49.6 per 100,000 people [2]. According to Egypt's breast cancer incidence rates in 2014, the high frequency of breast cancer (33.8%, 26.8% and 38.7%) among females in Lower, Middle, and Upper Egypt respectively and expected to increase by 3-fold increase (253%) up to year 2050 [3; 4].

The system used in staging breast cancer is the TNM classification which uses information on tumor size and how far it has spread within the breast and to adjacent tissues (T), the extent of spread to the nearby lymph nodes (N), and the presence or absence of distant metastases (spread to distant organs) (M). Once the T, N, and M are determined, a stage of 0, I, II, III, or IV is determined [5]. The recent version to the TNM stage for breast cancer also incorporates biologic factors to further refine the breast cancer staging system and was implemented by oncology programs in 2018 [6]. There are two main types of *in situ* breast cancer: ductal carcinoma *in situ* (DCIS) which was found in 83% of *in situ* cases, and lobular carcinoma *in situ* (LCIS) which was found in 13% of *in situ* cases diagnosed during 2010-2014 [7; 8]. Also, There are four molecular subtypes of BC have been identified using routinely evaluated biological markers, including the presence or absence of hormone (estrogen or progesterone) receptors ($HR^+/HR^-$) and excess levels of human epidermal growth factor receptor 2 (HER2, a growth-

promoting protein) and/or extra copies of the HER2 gene ($HER2^+$/$HER2^-$) [9].

IL-35 cytokine belongs to the IL-12 family of cytokines, is a heterodimer of p35 subunit of IL-12 and the Epstein-Barr Virus (EBV)-induced gene 3 (EBI3) subunit, [10]. It is immune-suppressive cytokine as it plays a role in inhibiting effector T cell proliferation and down-regulation of Th17 cell development and differentiation. $CD4^+$ Tregs, $CD8^+$ Tregs was demonstrated to express IL-35 and mediate immune-suppression in patients with prostate cancer [11]. Also, Bregs have recently been shown to produce IL-35, mediating their regulatory immune response in cancer patients [12].

Due to the suppressive activity of IL-35, there has been interest in evaluating the role of IL-35 in the development of different diseases. Some diseases like multiple inflammatory diseases and cancers have been demonstrated to be associated with increased IL-35 expression [13]. In a study, it was found that human serum IL-35 levels and IL-35 expression in colorectal cancer cells are associated with the severity and clinical stage of colorectal cancer [14]. IL-35 mRNA expression in the peripheral blood mononuclear cells in patients with invasive ductal carcinoma (IDC) of breast cancer, is significantly up-regulated compared with age-matched healthy women [15]. Another study demonstrated that IL-35 expression in breast cancer tissue was associated with tumor progression and the circulating IL-23: IL-35 ratio might be an important indicator related to breast cancer progression and prognosis [16]. In the present study, we compared plasma IL-35 levels of patients with BC versus healthy control and further analyzed the association between IL-35 plasma levels in BC patients' clinical characteristics.

## Material and Methods

Ethics Statement: Written informed consent was obtained from all patients and controls at the time of enrollment in the study. The study protocol was approved by the local Ethics Committee of the Faculty of Medicine, Assiut University.

### Subjects

This case-control study was conducted on a total of 40 Breast cancer patients without any therapeutic intervention, classified as stage I (early stage disease) and stages II – IV (advanced stage disease) attending Clinical Oncology and Nuclear Medicine department at Assiut University Hospitals from October 2017 to July 2018. Patients with breast cancer were diagnosed according to National Comprehensive Cancer Network (NCCN) clinical practice guidelines in oncology [17]. Additionally, this study included also, 10 age and sex matched healthy subjects. They were selected from patient`s relatives or health workers in Assiut University Hospitals during the study period.

Exclusion criteria included any breast cancer patients with previous chemotherapy or radiotherapy treatment. The practical part of the study was carried out at Medical Research Center, Faculty of Medicine, Assiut University.

### Methods

All patients underwent full medical of history taking: including age, family history of breast cancer and complete clinical examination according to NCCN guidelines.

- Blood samples collection and processing

About 5mL of venous blood was collected in two tubes (2.5ml each) labeled with the patient's name, sex, age and the date of collection. Heparinized blood collection tubes were used for separation of peripheral blood mononuclear cells (PBMCs) by Ficoll density gradient centrifugation and plasma separation. Plasma was stored at -80ºC for further IL-35 cytokine detection by ELISA. Peripheral blood mononuclear cells were isolated.

- Determination of plasma IL-35 levels by ELISA

Plasma collected from 40 patients and 10 controls were analyzed for IL-35 with an ELISA kit (Elabscience, USA) according to the manufacturer's instructions. The lowest detectable concentration of IL-35 was 0.08± 0.04 ng/ml.

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been

pre-coated with an antibody specific to Human IL-35. Standards and plasma samples were added to the micro ELISA plate wells and combined with the specific antibody, then a biotinylated detection antibody specific for Human IL-35 and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components were washed away. The substrate solution was added to each well. Only those wells that contain Human IL-35, biotinylated detection antibody and Avidin-HRP conjugate appeared blue in color. The enzyme-substrate reaction was terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value was proportional to the concentration of Human IL-35. The concentration of Human IL-35 in the samples was calculated by comparing the OD of the samples to the standard curve.

### Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 7.0 software (GraphPad Software Inc., SanDiego, CA, USA). Data were presented as the mean ± standard deviation (SD). In comparison between the different groups, the statistical significance was assessed with unpaired two-tailed Student's t test and one way ANOVA test. Chi square test was used to analyze the correlation between IL-35 expression levels and patients' clinic-pathological characteristics. *P* value < 0.05 was considered statistically significant.

## Results

A total of 50 subjects were enrolled in this study. They were classified into 2 groups; one group included 40 naïve breast cancer patients. The other group included 10 age matched healthy individuals as controls. Ages of patients ranged between 25-80 years old and the mean age was 51.53±1.95, while ages of controls ranged between 25-65 years old and the mean age was 50.7±3.14. No significance difference was found between the patients and control groups as regard age (*P*-value =0.76).

In this study, sandwich ELISA was used to examine the levels of IL-35 in plasma of 40 patients with BC and 10 healthy controls. As shown in Figure 1, the levels of IL-35 in plasma samples from BC patients were (368.4±27.03) pg/mL, which was nearly 15-fold higher than that in healthy controls (25.58 ± 5.42) pg/mL (*P*<0.0001).

The association of plasma IL-35 levels with clinico-pathological features of the patients of BC was evaluated. As shown in Table (1), and figures (2, 3), high levels of IL-35 were significantly associated with BC grade 2 (350±136 pg/mL, *P*=0.0002), and HER-2 over expression level "3+" (821±33.33 pg/mL, *P*<0.0001). However, other clinical characteristics, including age, BC histology, ER, PR levels, and TNM stage and stage grouping were not directly related to the high levels of plasma IL-35 as shown in Table (1), suggesting that plasma IL-35 levels may be associated with the development and progression of BC.

Table 1. Association between plasma IL-35 levels and clinico-pathological characteristics of BC patients

| Characteristics | Number of cases | IL-35 (pg/mL) | *P* value |
|---|---|---|---|
| Age: | | | |
| 25-44 | 11 | 326.8 ± 37.61 | |
| 45-64 | 23 | 368.3 ± 27.74 | NS |
| ≥ 65 | 6 | 380.4 ± 44.87 | |
| Histology: | | | |
| Ductal | 33 | 356.3 ± 28.62 | NS |
| Lobular | 7 | 425.4 ± 76.75 | |
| Grade: | | | |
| 1 | 1 | 146 | |
| 2 | 37 | 350±136 | 0.0002* |
| 3 | 1 | 854.3 | |
| 4 | 1 | 787.6 | |
| ER: | | | |
| Negative | 8 | 364.9 ± 54.69 | NS |
| Positive | 32 | 369.3 ± 31.31 | |
| PR: | | | |
| Negative | 9 | 349.6 ± 52.39 | NS |
| Positive | 31 | 373.9 ± 31.76 | |
| HER-2: (*n*=21) | | | |
| Non over expressed (0-2+) | 19 | 363.3 ± 27.16 | <0.0001* |
| Over expressed (3+) | 2 | 821 ± 33.33 | |
| T stage: | | | |
| 1 | 6 | 383.4±50.99 | |
| 2 | 21 | 343.1±25.23 | NS |
| 3 | 8 | 370.8±99.32 | |
| 4 | 5 | 542.5±1 | |
| N stage: | | | |
| 1 | 20 | 364.4±36.42 | |
| 2 | 12 | 374.5±50.77 | NS |
| 3 | 8 | 369±73.59 | |
| TNM stage group: | | | |
| II | 17 | 366.2 ± 24.04 | NS |
| III | 23 | 370.1 ± 44.07 | |

ER, estrogen receptor; PR, progesterone receptor; HER2, human epithelial growth factor receptor 2;
*P*>0.05 is not significant (NS).

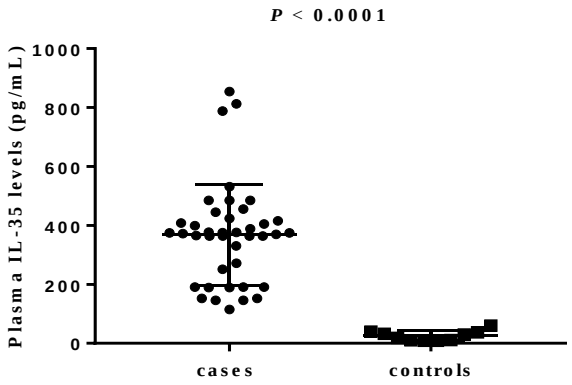


**Figure 1.** Plasma IL-35 concentrations in 40 patients with BC and 10 healthy controls detected by ELISA. Data are expressed as mean ±SD

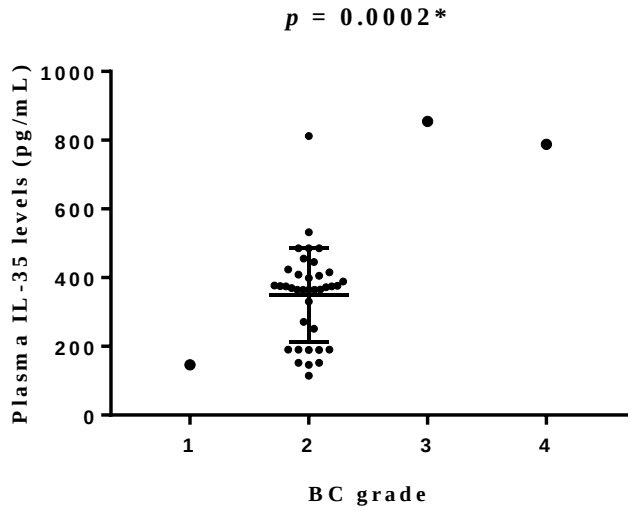


**Figure 2.** Plasma IL-35 concentrations in patients with BC at different grades. Plasma IL-35 levels were significantly higher in patients BC grade 2 (*P*=0.0002). Data are expressed as mean ±SD.

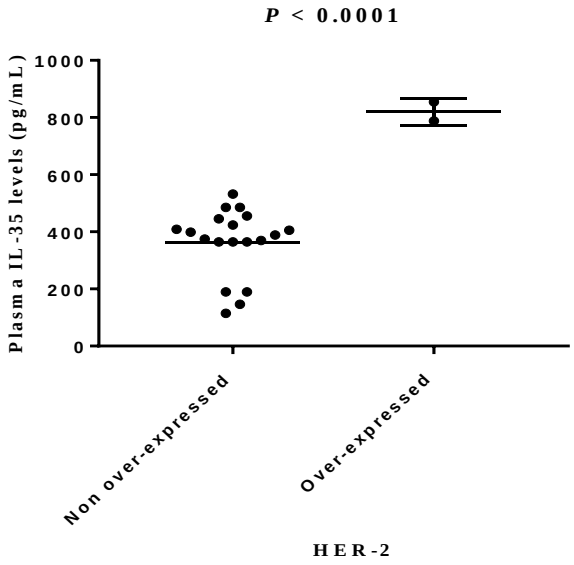


**Figure 3.** Plasma IL-35 concentrations in BC patients with different HER-2 expression levels. Plasma IL-35 levels were significantly associated with HER-2 expression levels in BC patients ($P$<0.0001). Data are expressed as mean ±SD.

## Discussion

Our study showed that the mean levels of IL-35 in plasma samples from BC patients were (368.4 ± 27.03) pg/mL, which was significantly higher than that in healthy controls (25.58 ± 5.42) pg/mL. These results agreed with a study on IL-35 expression levels in tumor- infiltrating lymphocytes (TILs) correlated with breast cancer which found that IL-35 was highly expressed in the TILs of part of patients with breast cancer [18].

Also, our results agreed with other study which found that circulating IL-35 was also of higher expression in BC patients than in controls, and IL-35 ratio was significantly lower in serum of BC patients versus healthy volunteers, and significantly rise by tumor resection [16]. These findings explained by the immune-suppressive nature of IL-35 cytokine which appears to have a pro-tumor role through expanding Tregs and inhibiting $CD4^{+}CD25^{-}$ effector T cells, stimulating IL-35-producing $CD1d^{high}CD5^{+}B$ cells mediated tumor cell proliferation, inhibiting apoptosis, and enhancing myeloid cell accumulation [19; 20].

This study evaluated the association of plasma IL-35 levels with clinical features of the patients of BC. It was found that high levels of IL-35 were significantly associated with BC grade 2 (350±136 pg/mL), and HER-2 over expression level "3+" (821 ± 33.33 pg/mL). However, other clinical characteristics, including age, BC histology, ER, PR levels, and TNM stage and stage grouping were not directly related to the high levels of plasma IL-35, suggesting that plasma IL-35 levels may be associated with the development and progression of BC.

As shown in a recent study, elevated IL-35 expression in the TILs was significantly correlated with more aggressive tumor phenotypes, including higher tumor grade, larger tumor size like our study [18]. In

another study about the relation between IL-35 and Prostate carcinoma (PCa), plasma IL-35 levels were found to be significantly positively associated with tumor stage and bone metastasis, two important indicators of poor outcomes. Given the roles of IL-35 in protecting tumor cells against immunity, these evidences further support our hypothesis that IL-35 might promote the progression of PCa [21].

Another study from non-small cell lung cancer (NSCLC) has demonstrated that patients with NSCLC have elevated serum level of IL-35 compared with healthy individuals and high serum IL-35 was significantly associated with high TNM classification and lymph node metastasis [22]. Zeng *et al.,* 2013 have found that elevated serum IL-35 levels were positively correlated to poor progression and higher number of peripheral Treg cells in colorectal cancer patients. Particularly, a significant reduction for serum IL-35 was noted in patients after surgical resection. All together those data suggest that IL-35 could be a valuable biomarker for assessing cancer progression and prognosis.

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