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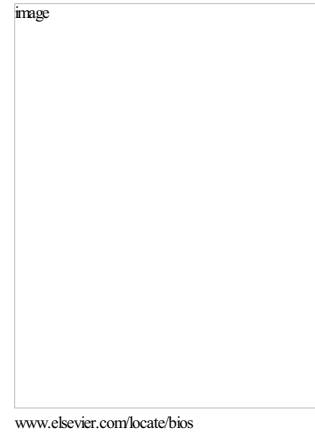
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IL-10 correlates with the expression of carboxypeptidase B2 and lymphovascular invasion in inflammatory breast cancer: the potential role of tumor infiltrated macrophages

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Abstract

Background:

Pro-carboxypeptidase B2 (pro-CPB2) or thrombin-activatable fibrinolysis inhibitor (TAFI) is a glycoprotein encoded by the *CPB2* gene and deregulated in several cancer types, including breast cancer. Thrombin binding to thrombomodulin (TM), encoded by *THBD*, is important for TAFI activation. *CPB2* gene expression is influenced by genetic polymorphism and cytokines such as interleukin 10 (IL-10). Our previous results showed that tumor infiltrating monocytes/macrophages (CD14$^+$/CD16$^+$) isolated from inflammatory breast cancer (IBC) patients' secrete high levels of IL-10. The aim of the present study is to test genetic polymorphism and expression of *CPB2* in healthy breast tissues and carcinoma tissues of

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non-IBC and IBC patients. Furthermore, to investigate whether IL-10 modulates the expression of *CPB2 and THBD in vivo* and *in-vitro.*

Materials and methods

We tested *CPB2* Thr325Ile polymorphism using restriction fragment length polymorphism, (RFLP) technique in healthy and carcinoma breast tissues. The mRNA expression of *CPB2, THBD* and *IL10* were assessed by RT-qPCR. Infiltration of $CD14^+$ cells was assessed by immunohistochemistry. We investigated the correlation between infiltration of $CD14^+$ cells and expression of *IL10* and *CPB2*. Furthermore, we correlated *IL10* expression with the expression of both *CPB2* and *THBD* in breast carcinoma tissues. Finally, we validated the role of recombinant IL-10 in regulating the expression of *CPB2* and *THBD* using different breast cancer cell lines.

Results:

Our data showed that *CPB2* genotypes carrying the high-risk allele [Thr/Ile (CT) and Ile/Ile (TT)] were more frequent in both IBC and non-IBC patients compared to control group. *CPB2* genotypes did not show any statistical correlation with *CPB2* mRNA expression levels or patients' clinical pathological properties. Interestingly, *CPB*2 and *IL10* expression were significantly higher and positively correlated with the incidence of $CD14^+$ cells in carcinoma tissues of IBC as compared to non-IBC. On the other hand, *THBD* expression was significantly lower in IBC carcinoma versus non-IBC tissues. Based on molecular subtypes, *CPB*2 and *IL10* expression were significantly higher in triple negative (TN) as compared to hormonal positive (HP) carcinoma tissues of IBC. Moreover, *CPB*2 expression was positively correlated with presence of lymphovascular invasion and the expression of *IL10* in carcinoma tissues of IBC patients. Furthermore, recombinant human IL-10 stimulated *CPB*2 expression in SUM-149 (IBC cell line) but not in MDA-MB-231 (non-IBC cell line), while there was no significant effect *THBD* expression.

Conclusion:

Carcinoma tissues of IBC patients are characterized by higher expression of *CPB*2 and lower expression of *THBD*. Moreover, *CPB*2 positively correlates with *IL10* mRNA expression, incidence of CD14^{+} cells and lymphovascular invasion in IBC patients. IL-10 stimulated *CPB*2 expression in TN-IBC cell line suggests a relevant role of *CPB*2 in the aggressive phenotype of IBC.

Keywords:

Inflammatory breast cancer, carboxypeptidase B2, thrombin-activatable fibrinolysis inhibitor, interleukin-10, thrombomodulin, macrophages, lymphovascular invasion.

Introduction:

Inflammatory breast cancer (IBC) is a particularly aggressive and highly metastatic form of breast cancer. The progression of IBC is associated with (lymph) angiogenesis, and a pronounced ability for lymphovascular invasion and formation of lymphatic tumor emboli that promote metastasis and dissemination to different organs [1-3]. In addition, the overall survival (OS) and breast cancer-specific mortality (BCSM) of IBC are higher among patients with a triple negative (TN) molecular subtype as compared to others [4]. In this regard, novel therapeutic options that target TN-IBC molecular subtype should be considered [5].

Case report studies from different countries showed that IBC may be associated with thrombosis. For instance, a 54-year-old woman initially diagnosed with spontaneous brachial, axillary, and subclavian venous thrombosis was also found to have late stage IBC [6]. Another study showed that an IBC patient presented early with arm lymphoedema and venous thromboembolism (VTE) [7]. Serra and colleagues reported that axillary vein thrombosis may be considered as the first clinical manifestation of IBC [8]. Indeed, VTE increases the rate of morbidity and mortality among cancer patients. The incidence of VTE

has increased in the past 2 decades [9], and 20% to 30% of VTE cases were found to be associated with the development of cancer [10]. Studying the relationship between thromboembolism and cancer may provide novel insights for early diagnosis of the disease [7].

One of the proteins associated with the formation of VTE is pro-carboxypeptidase B2 (pro-CPB2), also known as thrombin activatable fibrinolysis inhibitor (TAFI) [11]. pro-CPB2 is a glycoprotein, encoded by *CPB2,* synthesized mainly in the liver as a 60-kDa zymogen, and proteolytically activated by plasmin, thrombin or the thrombin-thrombomodulin complex [12]. The active form pro-CPB2, known as CPB2 or TAFIa, is a 35-kDa basic carboxypeptidase [13]. CPB2 inhibits lysis of the fibrin clot by removing C-terminal lysine residues from partially-degraded fibrin, attenuating plasminogen activation and ultimately fibrinolysis [14].

Previous studies showed that pro-CPB2 protein might be associated with poor prognosis of cancer. For instance, pro-CPB2 is highly secreted from lung carcinoma cells and plays a crucial role in the pathogenesis of thrombotic disorders in lung cancer patients [15]. Another study reported high pro-CPB2 levels in the advanced colorectal cancer patients as compared to those showing localization of cancer cells in the colon [16]. Serum pro-CPB2 levels are significantly higher in breast cancer patients as compared to healthy control. High levels of pro-CPB2 may be associated with thrombotic disorders in breast cancer patients [17]. A Chinese study suggested that *CPB2* Thr325Ile polymorphism might be considered a risk factor for development of breast cancer in Chinese Han populations [18]. A pilot study assessing pro-CPB2 level in plasma of Egyptian breast cancer patients showed an increase in pro-CPB2 plasma level that is correlated with advanced stages, poor prognosis and high risk of disease recurrence. Furthermore, *CPB2* Thr325Ile polymorphism was associated with the development of breast cancer in Egyptian population [19].

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The anticoagulant/antifibrinolytic factor thrombomodulin (TM) encoded by *THBD* gene is a type 1 transmembrane glycoprotein [20, 21] that has the ability to regulate inflammation and tumor promotion [22-25]. *THBD* expression in carcinoma tissues is associated with less advanced stage and a better prognosis in several cancers [26-28]. Thrombin binding to TM is important for the physiological activation of pro-CPB2 to CPB2 and has been shown to be responsible for the anti-metastatic effects of TM [29]. Furthermore, CPB2 has been shown to inhibit cell invasion and motility of breast carcinoma cells [30].

Interleukin 10 (IL-10) is "double-edged sword" cytokine as it displays both pro and anti-tumor activities [31]. It is suggested that IL-10 is expressed at a higher level by metastatic cancer cells to down regulate the inflammatory response of cell-mediated immunity [32]. IL-10 levels in serum of breast cancer patients were associated with disease initiation and progression [33]. A recent study reported higher expression level of IL-10 in the examined breast cancer tissue, and increased IL-10 expression associated with poor prognosis [34]. Indeed, the tumor microenvironment of IBC patients is characterized by the activation of different signaling pathways such as NF-κB, COX-2, and JAK/STAT, which induce the expression and secretion of various pro- and anti-inflammatory cytokines [35, 36]. Our previous studies showed that IL-10 is over expressed by tumor-associated macrophages isolated from IBC patients [35, 36]. Recently we found that the expression of *IL10* in IBC carcinoma tissues correlate with GCC haplotypes (-1082A/G, -819T/C, and -592A/C) in the gene promoter region [37].

Many studies have shown a differential role for cytokines including IL-10 in regulating the expression of *CPB2* in different cell lines. For example, the treatment of human hepatocellular carcinoma cell line, HepG2, with TNFα or IL-6 in combination with IL-1β, or bacterial lipopolysaccharide (LPS) for 24-48 h decreases expression of pro-CPB2 protein by two-fold. Additionally, IL-10 increases the expression of *CPB2* mRNA in HepG2 cell line

[38]. In the present study, we examined the mRNA expression level of *CPB2*, *THBD* and *IL10* in non-IBC and IBC carcinoma tissues, and whether the *CPB2* Thr325Ile polymorphism affects expression of *CPB2*. Furthermore, we investigated the effects of IL-10 on the expression of *CPB2 and THBD in vivo* and *in-vitro.*

Materials and Methods:

Patients and Samples

For patient enrolment we obtained an institutional Review Board (IRB) approval from the ethics committee of Faculty of Medicine, Ain Shams University, Egypt. Patients were diagnosed with non-IBC or IBC by clinical ultrasound examination, mammography and tru-cut biopsy [36, 39]. A total of 135 breast cancer patients (91 non-IBC and 44 IBC) and 50 healthy volunteers were enrolled in the present study. Carcinoma tissues were obtained during modified radical mastectomy (MRM). Normal breast tissues were obtained from healthy volunteers undergoing mammoplasty. All enrolled healthy volunteers and breast cancer patients signed informed consent that agrees with publication of anonymous data.

Quantitative real-time Polymerase chain reaction (RT-qPCR)

Total RNA was extracted from 30 mg of fresh breast tissues (normal and carcinoma) and breast cancer cell lines MDA-MB-231 and SUM-149 using GeneJET^{TM} RNA Purification Kit (Thermo scientific, ON, Canada). RNA concentration was measured using Infinite$^{®}$200 PRO NanoQuant (Tecan, Männedorf, Zürich, Switzerland) and 1 μg was reversed transcribed into complementary DNA using RevertAid First Strand cDNA Synthesis Kit (Thermo scientific, ON, Canada). Quantitative PCR was performed as described before [40, 41] via using StepOnePlus 96 well device (Applied Biosystems, San Francisco, CA, USA) and in a 25 μl total volume of 12.5 μl SYBR green master mix (Applied Biosystems, Darmstadt, Hesse, Germany), 0.5 μl of each *CPB2* upstream 5′-GCTGCCGGAGCGTTACAT-3′ and

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CPB2 downstream 5′-CATTCCTAATGACATGCCAAGCT-3′, *THBD* upstream 5′-CCCTGAACAAGAAT TGGAAGCT -3′ and *THBD* downstream 5′- GGAGCCTAGGATT CTGCATTTCTA-3′, and *IL10* upstream 5′- GCCACCCTGATGTCTCAGTT -3′ and *IL10* downstream 5′- GTGGAGCAGGTGAAGAATGC -3′ primers (10 pmol/µl), 2.5 µl of cDNA and 8 µl of RNase free water. Reaction thermal profile started with initial denaturation at 95°C for 10 min, then followed by 40 cycle of 94°C for 15 sec and 60°C for 1 min. Amplification specificity was verified using melting curve analysis and 2% agarose gel electrophoresis of the PCR products. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method after normalization to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Qiagen, Valencia, CA, USA) (10 pmol\µl).

Restriction Fragment Length Polymorphism (RFLP)-PCR assay for *CPB2* (Thr325Ile) polymorphism

DNA was extracted from breast tissues by using GeneJET^{TM} Genomic DNA purification Kit (Thermo scientific, ON, Canada). The RFLP-PCR assay was carried out in a 25 µl total volume using 1µl of each designed *CPB2* upstream 5′-TTCAAAGCTGCACATTAACTG-3′ and *CPB2* downstream 5′-CTTTAGTAGCTCAAA GTTCTC-3′ primers (10 pmol/µl), 3 µl of DNA extract (250 ng DNA), 12.5 µl of EmeraldAmp® MAX PCR green master mix and 7.5 µl of free RNase water. The PCR thermal profile started with an initial denaturation at 94 ℃ for 5 min, followed by 35 cycles at 94 ℃ for 45 sec, 54 ℃ for 1 min, and 72 ℃ for 45 sec, followed by terminal extension at 72 ℃ for 10 min. The PCR product has an expected size of 200 bp. Following PCR, the PCR product was digested by SpeI restriction enzyme (10U) (Takara, Dalian, China) for 2 h at 37 ℃ and subject to agarose gel electrophoresis. The digest PCR products migrated bands were visualized on 2% agarose gels by using iBox Scientia 615 Imaging System (UVP Upland, CA, USA). Samples with CC genotype showed digestion with SpeI was represented by a band at 100 bp. Samples with TT genotype did not show

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digestion with SpeI was represented by a band at 200 bp. Samples with both genotypes CT bands at 100 bp and 200 bp were detected as described before [42]. Twenty percent of samples were re-genotyped in separate runs to exclude the possibility of false genotyping.

Immunohistochemistry

Immunohistochemical (IHC) staining was performed on 4 μm thick paraffin embedded tissue sections as we described before [43] using antibodies against CD14 (1:50) (Chemicon, Temecula, CA, USA) and CD68 (1:50) (DakoCytomation, Glostrup, Denmark). Staining was achieved by adding 100 μl of DAB+ chromogen diluted 1:50 in substrate buffer [EnVision+ Dual Link System-HRP (DAB+)] for 10 min. Finally, tissue specimens were rinsed three times in phosphate buffer saline (PBS), the nuclei were counterstained with hematoxylin and mounted using Permount® for microscopic examination. The stained area fractions were calculated using image J software (National Institutes of Health, Bethesda, MD, USA).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting

Protein content of fresh breast carcinoma tissues lysates was assessed by Bradford assay [44]. Equal concentration of tissue protein lysates (30 μg/well) were subjected to 10% SDS-PAGE, followed by transfer to Polyvinylidene fluoride (PVDF) membrane (Millipore, Darmstadt, Germany). The membranes were blocked for 1 h with 5% non-fat dry milk in TBS-0.5% Tween 20, followed by incubation overnight at 4 $^{\circ}$C with anti-IL-10 (R&D systems, MN, USA) (1:100) then washed and incubated for 1 h with 1:10000 diluted peroxidase-labeled goat anti-rabbit secondary antibody. After washing, the bands were visualized by using Pierce ECL immuno-blotting substrate (Thermo Fisher Scientific, Rockford, IL, USA). Signals representing protein bands were captured with the iBox Scientia 615 Imaging System (UVP Upland, CA, USA). Visualized protein bands were analyzed by ImageJ (National Institutes of Health, USA.) software and quantified relativity to β-tubulin as loading control [44].

Effect of recombinant human IL-10 on the expression of *CPB2* by breast cancer cell lines

We studied the effect of recombinant human IL-10 on MDA-MB-231 (non-IBC) and SUM149 (IBC) cell lines (gift from Dr. Bonnie Sloane, Department of Pharmacology, Wayne State University, Detroit, MI, USA). Cell lines were cultured as previously described [36, 45]. MDA-MB-231 and SUM-149 cells were seeded at the density of (3.5×10^5/well) in six well plate and incubated at 37°C and 5% CO_2 for 24 h in appropriate complete media, Ham's F.12 containing 5% FBS and DMEM containing 10% FBS for SUM149 and MDA-MB-231, respectively [45, 46]. Cultured media were removed and cells incubated in serum free media conditioned with recombinant IL-10 (R&D systems, Minneapolis, Minnesota, USA) at concentrations of 100, 200 and 400 ng/ml for 24 h [36]. Then cells were washed twice with cold PBS and total RNA extracted.

Statistical Analysis

Data were expressed as mean ± standard deviation (SD). Statistical difference between groups was assessed by Student's t-test and Chi square test. P values < 0.05 were considered to be statistically significant. To determine the association between *CPB2* Thr325Ile polymorphism and the development of breast cancer we used Hardy-Weinberg equilibrium to compare the genotype frequencies in cancer patients and the healthy control group. Logistic regression was used to calculate the odds ratios (OR) and 95% confidence interval (CI) to estimate the relative association between breast cancer development and a particular allele and genotype using SNPstat software [47, 48]. Correlation was assessed by Pearson's correlation coefficient using SPSS 22.0 software.

Results:

Clinical and pathological characterization of breast cancer patients

Clinical and pathological characterization of non-IBC and IBC patients was described in Table 1. Statistical analysis showed that IBC patients were characterized with significantly larger tumor size ($p = 0.003$) in comparison with non-IBC patients. Moreover, the status of lymph node metastasis and lymphovascular invasion in IBC patients was significantly higher ($p = 0.002$ and 0.001, respectively) than non-IBC patients.

Incidence of *CPB2* genotypes carrying the high-risk allele [Thr/Ile (CT) and Ile/Ile (TT)] was higher in breast cancer patients compared to healthy volunteers

The genotypes and alleles distributions for the *CPB2* Thr325Ile polymorphism are presented in Table 2. The genotype distribution of both studied groups' fits the Hardy–Weinberg equilibrium ($p > 0.05$). The incidence of *CPB2* genotypes carrying the high-risk allele [Thr/Ile (CT) and Ile/Ile (TT)] was significantly higher ($p = 0.003$) in breast cancer patients in comparison with healthy volunteers (OR: 0.32; [95% CI: 0.16-0.64]; $P = 0.001$). We did not detect a statistically significant difference in the incidence of *CPB2* genotypes carrying the high-risk allele [Thr/Ile (CT) and Ile/Ile (TT)] between non-IBC and IBC patients. *CPB2* mRNA expression level did not show a statistical correlation with the incidence of *CPB2* genotypes carrying the high-risk allele [Thr/Ile (CT) and Ile/Ile (TT)] in each of healthy volunteers and breast cancer patients or even among patient non-IBC and IBC subgroups (Supplementary Table 1). In addition, there was no association between the incidence of *CPB2* genotypes carrying the high-risk allele [Thr/Ile (CT) and Ile/Ile (TT)] and patients' clinical pathological properties including tumor grade, tumor size, lymphovascular invasion, lymph node status, expression of ER, PR and Her-2 in each of non-IBC and IBC patient groups (Supplementary Tables 2 & 3).

***CPB2* mRNA expression level is higher in triple negative (TN) breast carcinoma tissues of non-IBC and IBC patients in comparison to hormonal positive (HP), and positively correlates lymphovascular invasion in IBC patients**

Carcinoma tissues obtained during MRM showed a significant (p= 0.001) higher *CPB2* mRNA expression level compared to normal breast tissues obtained from mammoplasty of healthy volunteers (Figure 1A). *CPB2* mRNA expression level was significantly (p= 0.003) higher in IBC compared to non-IBC tissue (Figure 1B). Interestingly, carcinoma tissues of TN breast cancer patients expressed significantly higher level of *CPB2* mRNA compared to HP patients in both non-IBC and IBC groups (p= 0.03 and 0.02, respectively) (Figure 1 C & D).

Low expression of *THBD* mRNA in IBC compared to non-IBC carcinoma tissues

Although there was a non-significant difference in *THBD* mRNA expression between healthy and breast tissues (Figure 1E), there was a significantly (p= 0.02) lower level in IBC compared to non-IBC tissue (Figure 1F). On the other hand, there was no significant difference in the expression of *THBD* mRNA expression level in carcinoma tissues of TN compared to HP patients in both non-IBC and IBC groups (Figure 1 G & H). The *THBD* mRNA expression level showed non-significant correlation with *CPB2* mRNA expression level in both non-IBC and IBC patients (Supplementary Table 4). With respect to the relationship between *THBD* mRNA expression and clinical pathological data, statistical analysis detected significant inverse correlations with tumor grade (r= -0.684, p= 0.02) and lymph node status (r= -0.711, p= 0.005) in both non-IBC and IBC patients.

Expression level of IL-10 is higher in triple negative compared to hormonal positive IBC patients and positively correlates with *CPB2* mRNA expression

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IL10 mRNA is significantly higher ($p= 0.01$) in breast carcinoma as compared to healthy breast tissues (Figure 2A). Furthermore, *IL10* mRNA expression was significantly ($p=0.02$) higher in IBC compared to non-IBC (Figure 2B). According to molecular subtypes of patients, we did not detect significant differences in *IL10* mRNA expression in TN compared to HP in the non-IBC sub-group (Figure 2C). On the other hand, in the IBC sub-group *IL10* mRNA expression level was significantly higher in TN ($p= 0.03$) as compared to HP carcinoma tissues (Figure 2D). In the same manner, IL-10 protein levels were significantly ($p= 0.03$) higher in IBC as compared to non-IBC tissue (Figure 2E). IL-10 protein levels did not differ between TN and HP in non-IBC carcinoma tissues (Figure 2F and 2G), but was significantly higher in TN as compared to HP IBC carcinoma tissues (Figure 2H and 2I). *IL10* mRNA expression do not correlate with mRNA expression of *CPB2* in non-IBC patients group. However, we detected a significantly positive correlation between mRNA expression of *CPB2* and *IL10* in TN (r= 0.783, $p= 0.004$) and HP (r= 0.625, $p= 0.01$) in the IBC patients patient subgroups (Supplementary Table 4).

Expression of *CPB2* and *IL10* correlate with the presence of lymphovascular invasion in IBC

CPB2 mRNA expression level showed no correlation with patients' clinical pathological data including tumor grade, tumor size and lymph node status in both non-IBC and IBC patients. However, *CPB2* mRNA expression levels significantly (r= 0.766, $p= 0.004$) correlated with the presence of lymphovascular invasion in IBC but not non-IBC patients (Figure 3 A & B). In addition, mRNA expression of *IL10* was significantly (r= 0.831, $p= 0.002$) and positively correlates with lymphovascular invasion in and IBC patients (Figure 3C).

***CPB2 and IL10* mRNA positively correlate with infiltration of CD14⁺ cells in IBC carcinoma tissues**

Our previous results showed that CD14^{+} cells highly infiltrate the carcinoma tissues of IBC as compared to non-IBC patients. However, we did not detect a significant difference in the infiltration of CD68^{+} cells between these two patient groups. Furthermore, we showed that CD14^{+} cells isolated from tumor microenvironment of IBC patients highly secrete IL-10 as compared to non-IBC [36]. Since IL-10 regulates the expression of *CPB2* [38], we assessed the correlation between infiltration of CD14+ cells and the expression of *IL10 and CPB2* in IBC carcinoma tissues. Analysis of IHC staining revealed high infiltration of CD14^{+} in IBC carcinoma tissues as compared to non-IBC (Fig. 4 A & B). Expression of *CPB2 and IL10* significantly (r= 0.605, *p*= 0.05 and r= 0.753, *p*= 0.01, respectively) and positively correlate with the infiltration of CD14^{+} cells in IBC carcinoma tissues (Fig. 4 C & D). However, we did not find any correlation between *CPB2 and IL10* mRNA expression and infiltration of CD68^{+} cells in either non-IBC or IBC carcinoma tissues (data not shown).

Recombinant IL-10 stimulates the expression of *CPB2* mRNA in IBC cell line SUM149

To validate our findings using an *in vitro* system, we stimulated MDA-MB-231, a TN-non-IBC cell line, and SUM149, a TN-IBC cell line, with varying concentrations of recombinant IL-10 (100, 200 and 400 ng/ml) for 24h. The mRNA extracted from the treated cells was subjected to *CPB2* and *THBD* RT-qPCR analysis. Results showed that treatment with IL-10 did not significantly alter *CPB2* expression in MDA-MB-231 cells, while *CPB2* expression levels in SUM149 cells were significantly induced (*p*= 0.04, 0.03 & 0.01) in response to different concentrations of recombinant IL-10 (100, 200 and 400 ng/ml, respectively) (Figure 5A). On the other hand, recombinant IL-10 did not significantly alter *THBD* expression in either MDA-MB-231or SUM149 cell lines (Figure 5B).

Discussion:

IBC is the most lethal form of breast cancer and is characterized by phenotypes that differ from other breast cancer. Several studies including ours have shown that IBC disease displays unusual biological properties. These include rapid progression, metastasis and production of a unique repertoire of growth factors, cytokines and chemokines, high infiltration of macrophages/monocytes ($CD14^+$ cells), and detection of multiple viral DNAs compared to non-IBC [36, 49, 50]. Recent studies have also shown that VTE is more common in IBC patients [6, 8]. Moreover, high plasma levels of pro-CPB2, a zymogen encoded by *CPB2,* are a risk factor for VTE [11].

In the present study, we found that the incidence of *CPB2* genotypes carrying the allele [Thr/Ile (CT) and Ile/Ile (TT)] was significantly higher in breast cancer patients compared to control population. However, we did not detect any correlation between the incidence of genotypes carrying the high-risk allele [Thr/Ile (CT) and Ile/Ile (TT)] and tumor grade, tumor size, lymph node status, ER status, PR status and Her-2 status in either non-IBC or IBC patients. In agreement with this finding, a study conducted by Fawzy and colleagues found that incidence of *CPB2* genotypes carrying Thr/Ile (CT) and Ile/Ile (TT) allele was higher in breast cancer patients as compared to healthy patient group, and positively correlates with pro-CPB2 antigen levels in peripheral blood of Egyptian breast cancer patients. Moreover, they found that the (Thr/Thr) genotype corresponds to the highest level of plasma pro-CPB2 antigen and the (Ile/Ile) genotype corresponds to the lowest level of plasma pro-CPB2 antigen [19]. We found the *CPB2* mRNA expression was higher in breast carcinoma tissues as compared to normal breast tissues. In addition, *CPB2* mRNA expression was higher in IBC than non-IBC carcinoma tissues. We did not detect any correlation between the incidence of *CPB2* Thr325Ile different genotypes and *CPB2* mRNA in breast carcinoma tissues of either non-IBC or IBC patients.

The triple negative breast cancer (TNBC) patients currently have no targeted therapies and are characterized by poor disease prognosis and low survival rate [51].

We found that *CPB2* mRNA expression was higher in the carcinoma tissues of TNBC patients compared to HP patients. On the other hand, female sex steroid hormones were found to decrease expression of *CPB2* mRNA and pro-CPB2 protein in human hepatoma cell line, in a mechanism mediated by phosphatidylinositol 3-kinase and Akt signaling pathway and independent of the estrogen receptor [52].

Our results showed that *CPB2* expression in breast carcinoma tissues did not show any significant correlation with tumor size, tumor grade, lymph node involvement, expression of hormone receptors (ER/PR) and HER-2. However *CPB2* expression positively correlated with presence of lymphovascular invasion in IBC and not in non-IBC patients. Since lymphovascular invasion is essential for metastasis, this suggests a role for pro-CPB2 in metastasis. Previous studies have shown an association between pro-CPB2 and metastasis in Chinese breast cancer patients [18], and with poor prognosis in Egyptian breast cancer patients [19].

The activation of pro-CPB2 depends on the concentration of TM [53]. We found that mRNA expression of *THBD* was lower in the carcinoma tissues of IBC as compared to non-IBC patients. However, the level of *CPB2* mRNA was higher in the carcinoma tissues of IBC patients; although this may not result in functional CPB2 enzyme due to decreased expression of *THBD* in the tumor microenvironment. At the molecular level, *THBD* mRNA expression was not correlated with *CPB2* mRNA expression in the carcinoma tissues of either non-IBC or IBC patients. The *THBD* mRNA expression level found to be significantly and inversely correlated with tumor grade and lymph node status in both non-IBC and IBC patients. This is in agreement with a previous study of bladder cancer, which demonstrated that decreased TM expression level is a predictor of aggressiveness in advanced bladder cancer [54]. TM

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expression was also shown to be decreased in human prostate cancer cell lines, DU-145, LNCaP and PC-3 as compared to normal prostate epithelial cells PrEC [55].

Our results also demonstrate that *IL10* expression was higher in breast carcinoma tissue in comparison with healthy tissues. This is in agreement with a previous study showing higher expression levels of IL-10 in breast cancer tissues [34]. Herein, the expression of IL-10 was higher in IBC as compared to non-IBC carcinoma tissues. Moreover, the IL-10 expression level was higher in TN-IBC versus HP-IBC tissues. This, in part, supports previous study showing that IL-10 is over expressed in ER-negative breast tumors as compared to ER-positive carcinoma tissues [56].

It is well established that the tumor microenvironment plays an essential role in breast cancer progression. Higher infiltration of TAMs in breast carcinoma tissues of IBC is associated with the secretion of cytokines and growth factors such as IL-10, IL-8, tumor necrosis factor-alpha (TNF-α) and CC-chemokine ligand 2 (CCL2) that enhance motility, invasion and the aggressive properties of IBC [36, 57]. However, the source of *CPB2* mRNA in the breast cancer remains unclear. *CPB2* mRNA and pro-CPB2 protein found to be expressed by macrophages [42]. In the present study, *IL10* expression was positively correlated with infiltration of macrophages in carcinoma and lymphovascular invasion in IBC patients. This is in agreement with previous study on lung cancer showed that higher mRNA expression of *IL10* by TAMs was associated with advanced stage, tumor size, lymph node metastasis and lymphovascular invasion [58]. Our results showed that infiltration of CD14$^+$ cells positively correlates with mRNA expression of *IL10* and *CPB2* in IBC but not in non-IBC carcinoma tissues.

CPB2 gene expression is regulated by various cytokines. For instance in acute coronary syndrome (ACS) there is a positive correlation between IL-1β, IL-6, TNF-α and serum pro-CPB2 concentration [59]. TNF-α, IL-6 in combination with IL-1β and lipopolysaccharide

(LPS) decrease transcription of mRNA/pro-CPB2 protein levels by approximately two-fold, while IL-10 increased pro-CPB2 protein levels by two-fold in a mechanism modulated by binding of tristetraprolin (TTP) to the *CPB2* 3'-UTR, [38]. Similarly, we detected a positive correlation between *IL10* and *CPB2* expression in the carcinoma tissues of non-IBC and IBC patients as compared to normal breast tissues of healthy control. These results were validated *in vitro*. We found that IL-10 significantly upregulated expression of the *CPB2* in the IBC cell line SUM-149 but not in non-IBC cell line MDA-MB-231, while no significant effect appeared on *THBD* expression in either cell lines. Our results suggest that macrophage infiltration and expression of *IL10* in IBC carcinoma tissues may induce the expression of *CPB2*.

In conclusion, our study showed that *CPB2* and *IL10* expression are highly expressed in carcinoma tissues, and that this increase correlates with lymphovascular invasion in IBC patients. Furthermore, we demonstrated that the TN patients are characterized by higher expression of *CPB2* as compared to HP patients in each of non-IBC and IBC groups. *THBD* expression is down regulated in IBC as compared to non-IBC carcinoma tissues. A potential mechanism linking patient tumor characteristics with increased *CPB2* expression may be through the high infiltration of CD14^{+} cells and *IL10* in carcinoma tissues of IBC patients. Thus *CPB2* may be regulated by IL-10 *in vivo*, as demonstrated by the *in vitro* data. Further studies are needed to fully understand the role of *CPB*2 expression, and pro-CPB2 activation by the thrombin-TM complex, in the etiology of IBC.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Sources of support:

Research Ethical approval for Human Participants

For breast cancer patients and healthy volunteer enrolment in this study we obtained Ethical approval from the Institutional Review Board (IRB#00006379), Faculty of Medicine, Ain Shams University, Egypt.

Author's contribution:

HTM, EAG, NEH, SAI and MMM carried out the experiments. MSH recruited patients' provided the clinical diagnosis, grouped/sub-grouped patients and prepared of clinical pathological data. HTM, SAI, MSH, DCM and MMM performed data analysis and interpretation of results. HTM, SAI, ZAB, DCM, MBB and MMM made substantial contributions to concept and design of experiments. HTM and MMM drafted the manuscript. All authors have read, revised and approved the manuscript.

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Figures legends:

Figure 1: *CPB2* and *THBD* mRNA expression in non-IBC and IBC patients' breast carcinoma tissues. Bars represent *CPB2* mRNA expression measured by RT-PCR, (A) *CPB2* is down-regulated in normal compared to carcinoma tissues, (B) Carcinoma tissues of IBC, overexpress *CPB2* mRNA compared to non-IBC (C and D) Carcinoma tissues of TN patients overexpress *CPB2* in non-IBC (C) and IBC (D). Bars represent *THBD* mRNA expression in (E) normal breast and carcinoma breast tissues; (F) non-IBC compared to IBC carcinoma tissues, with significant down-regulation in IBC; (G&H) carcinoma tissues of TN and HP subgroups of non-IBC (G) and IBC (H). *indicates a significant *p* value, as determined by Student's t test.

Figure 2: mRNA and protein expression of IL-10 in non-IBC and IBC patients' carcinoma tissues. Bars represent (A) RT-PCR results showing down-regulation of *IL10* mRNA expression in normal breast tissues as compared to carcinoma breast tissues. (B) *IL10* mRNA highly expressed by IBC as compared to non-IBC carcinoma tissues. (C) Non-significant difference between the level of expression of *IL10* mRNA in TN and HP subgroups of non-IBC patients. (D) Significant increase in the expression of *IL10* by TN subgroup compared to HP subgroup in IBC patients. (E) Relative density value of IL-10 protein assessed by western blot and normalized against β-tubulin, statistical analysis

revealed that IL-10 protein was significantly over-expressed in IBC compared to non-IBC carcinoma tissues. (F) Representative IL-10 immunoblots of non-IBC carcinoma tissues (G) Statistical analysis of immunoblots revealed a non-significant difference in the IL-10 expression in TN and HP subgroups. (H) Representative IL-10 immunoblots in the carcinoma tissues of IBC patients. (I) Statistical analysis revealed high level of expression of IL-10 in TN compared to HP carcinoma tissues *indicates a significant p value, as determined by Student's t test.

Figure 3: *CPB2* **and** *IL10* **mRNA expression positively correlate with lymphovascular invasion in IBC tissues.** (A) Representative microscopic fields of H&E stained paraffin embedded tissue sections in IBC tissues showing dermal lymphovascular invasion (magnification: middle panel 10X, while left and right panel 40X). Bars represent the expression of *CPB2* (B) and *IL10* (C) in the presence of lymphovascular invasion. *indicates a significant p value, as determined by Chi square test.

Figure 4: *CPB2* **and** *IL10* **mRNA expression positively correlates with the infiltration CD14^{+} cells in IBC carcinoma tissues**. (A) Representative fields of microscopic images showing immunostaining (*brown color*) of higher expression of CD14 in IBC (n = 15) as compared to non-IBC (n = 15) carcinoma tissues. (B) Bars represent the infiltration of CD14^{+} cells in non-IBC and IBC carcinoma tissues. (C&D) Scatter plots showed that *CPB2* and *IL10* mRNA expression positively correlates with the infiltration CD14^{+} cells in IBC carcinoma tissues. *indicates a significant p value, as determined by Student's t test.

Figure 5: Different concentrations of recombinant IL-10 stimulate the mRNA expression of *CPB2* **in SUM149 IBC cell line**. RT-PCR analysis detected (A) Significant increase in the expression of *CPB2* mRNA by SUM-149 after stimulation with different concentrations of recombinant human IL-10 for 24 h. (B) Non-significant change in the

expression of *THBD* mRNA by MDA-MB-231 and SUM-149. *indicates a significant *p*

value, as determined by Student's t test.

A

Non-IBC | IBC

B

C

r= 0.605

D

r= 0.753

A

B

28
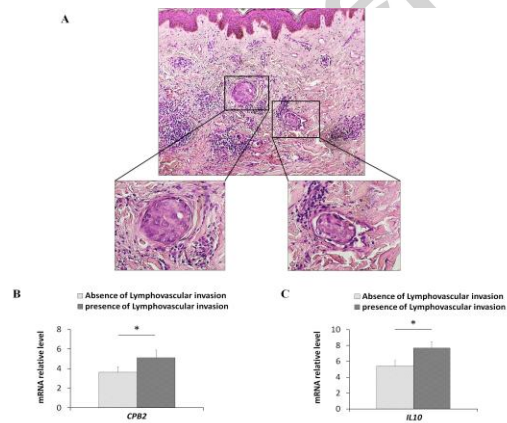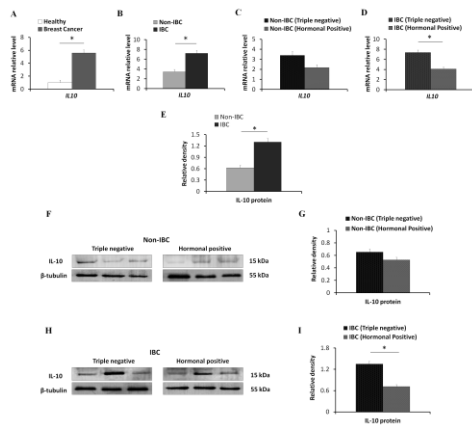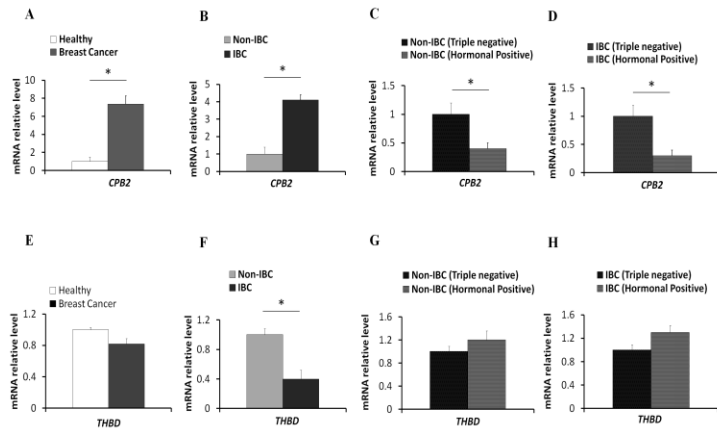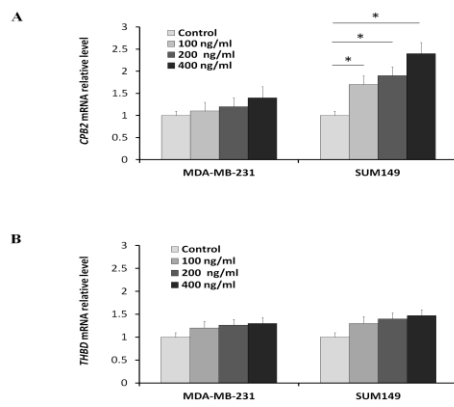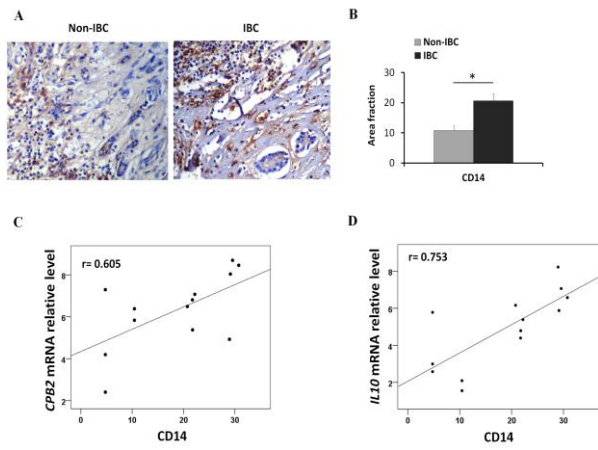
Tables:

Table 1: Clinical and pathological characterization of non-IBC versus IBC patients

Characteristic	Non-IBC (N = 91)	IBC (N = 44)	*P* value
Age [year]			
Range	27-78	29-72	
Mean ±SD	51.98 ±10.969	49.34 ±11.994	0.223[a]
NA	1	0	
Tumor Size [cm]			
Mean ±SD	4.6 ±3.5	5.73 ±2.71	
≤4	58 (63.8%)	16(36.4%)	0.003[*a]
>4	33 (36.2%)	28(63.6%)	
Pathology			
Ductal Carcinoma *In Situ*	10 (11%)	0 (0%)	
Invasive Ductal Carcinoma	73 (81.3%)	41 (93.2%)	0.069[a]
Invasive Lobular Carcinoma	7 (7.7%)	3 (6.8%)	
Tumor grade			
G1	4 (4.4%)	0 (0%)	
G2	74 (80.2%)	35 (79.5%)	
G3	14 (15.4%)	9 (20.5%)	0.304[b]
Axillary Lymph Node Metastasis			
≤4	64 (70.3%)	14 (31.8%)	0.002[*b]
>4	27 (29.7%)	30 (68.2%)	
Lymphovascular invasion			
Negative	75 (82.4%)	14 (31.8%)	0.001[*b]
Positive	16 (17.6%)	30 (68.2%)	
ER			
Negative	20 (45.5%)	35 (38.5%)	
Positive	15 (34.1%)	38 (41.8%)	0.668[b]
NA	9 (20.5%)	18 (19.8%)	
PR			
Negative	19 (43.2%)	42 (46.2%)	
Positive	15 (34.1%)	31 (34.1%)	0.913[b]
NA	10 (22.7%)	18 (19.8%)	
Her-2			
Negative	21 (47.7%)	44 (48.4%)	
Positive	13 (29.5%)	29 (31.9%)	0.915[b]
NA	10 (22.7%)	18 (19.8%)	
Data are reported as means ±SD [a] Student's t-test. [b] Chi square test. * Significant *p* value (*p*< 0.05) NA = not available			

Table 2: Genotype and allele frequencies of *CPB2* (Thr325Ile) polymorphism among studied groups using SNPstat software.

CPB2 Genotypes	Healthy (n=50) No (%)	Breast cancer (n=135) No (%)	Odds ratio (95% CI)	*P*-value	Non-IBC (n=50) No (%)	IBC (n=135) No (%)	Odds ratio (95% CI)	*P*-value
Thr/Thr (CC)	25 (50)	33 (24.4)	1.00		23 (25.3)	10 (22.7)	1.00	
Thr/Ile (CT)	17 (34)	59 (43.7)	0.38 (0.18-0.80)	0.003*	39 (42.9)	20 (45.5)	0.94 (0.41-2.17)	0.94
Ile/Ile (TT)	8 (16)	43 (31.9)	0.25 (0.10-0.61)	0.003*	29 (31.9)	14 (31.8)	1.11 (0.42-2.95)	0.94
Thr/Ile (CT) + Ile/Ile (TT)	25 (50)	102 (75.6)	0.32 (0.16-0.64)	0.001*	68 (74.7)	34 (77.3)	1.15 (0.49-2.69)	0.75
CPB2 alleles								
C	67 (67)	125 (46)		0.01*	48 (55)	97 (53)		0.86
T	33 (33)	145 (54)			40 (45)	85 (47)		