

ORIGINAL ARTICLE

Hormonal-receptor positive breast cancer: IL-6 augments invasion and lymph node metastasis via stimulating cathepsin B expression [☆]

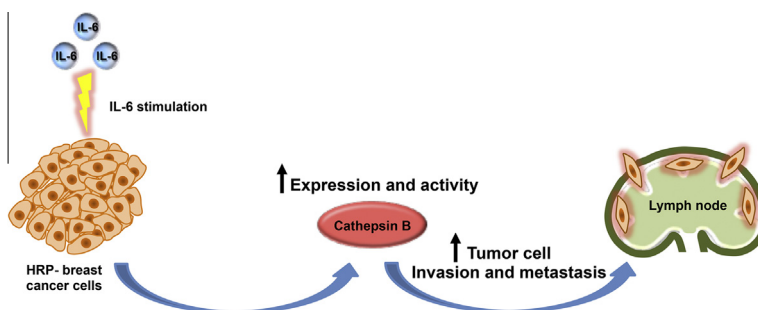


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GRAPHICAL ABSTRACT

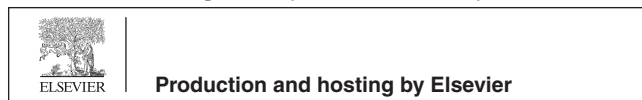


[☆] The Abstract (number A41) has been accepted in AACR special conference: Advances in Breast Cancer Research, October 17–20, 2015 Hyatt Regency Bellevue, Bellevue, Washington, USA.

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Peer review under responsibility of Cairo University.



ARTICLE INFO

Article history:

Received 12 March 2016

Received in revised form 18 June 2016

Accepted 24 June 2016

Available online 30 June 2016

Keywords:

Hormonal-receptor positive breast cancer

IL-6

Cathepsin B

Invasion

Lymph node metastasis

Epithelial-mesenchymal transition

ABSTRACT

Hormonal-receptor positive (HRP) breast cancer patients with positive metastatic axillary lymph nodes are characterized by poor prognosis and increased mortality rate. The mechanisms by which cancer cells invade lymph nodes have not yet been fully explored. Several studies have shown that expression of IL-6 and the proteolytic enzyme cathepsin B (CTSB) was associated with breast cancer poor prognosis. In the present study, the effect of different concentrations of recombinant human IL-6 on the invasiveness capacity of HRP breast cancer cell line MCF-7 was tested using an *in vitro* invasion chamber assay. The impact of IL-6 on expression and activity of CTSB was also investigated. IL-6 treatment promoted the invasiveness potential of MCF-7 cells in a dose-dependent manner. Furthermore, MCF-7 cells displayed elevated CTSB expression and activity associated with loss of E-cadherin and upregulation of vimentin protein levels upon IL-6 stimulation. To validate these results *in vivo*, the level of expression of IL-6 and CTSB in the carcinoma tissues of HRP-breast cancer patients with positive and negative axillary metastatic lymph nodes (pLNs and nLNs) was assessed. Western blot and immunohistochemical staining data showed that expression of IL-6 and CTSB was higher in carcinoma tissues in HRP-breast cancer with pLNs than those with nLNs patients. ELISA results showed carcinoma tissues of HRP-breast cancer with pLNs exhibited significantly elevated IL-6 protein levels by approximately 2.8-fold compared with those with nLNs patients ($P < 0.05$). Interestingly, a significantly positive correlation between IL-6 and CTSB expression was detected in clinical samples of HRP-breast cancer patients with pLNs ($r = 0.78$, $P < 0.01$). Collectively, this study suggests that IL-6-induced CTSB may play a role in lymph node metastasis, and that may possess future therapeutic implications for HRP-breast cancer patients with pLNs. Further studies are necessary to fully identify IL-6/CTSB axis in different molecular subtypes of breast cancer.

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Introduction

Breast cancer is the second leading cause of cancer death among women worldwide [1]. A study on 359 patients showed that approximately 44% of breast cancer patients in Egypt belong to luminal A molecular subtype which is considered to be the most prevalent subtype in both early- and advanced-stage disease [2]. The carcinoma cells of this subtype are characterized by positive estrogen receptor (ER^+) and/or progesterone receptor (PR^+) and negative human epidermal growth factor receptor ($HER2^-$) [2]. Lymph node metastasis status is considered as one of the important predictors for breast cancer progression and the increase in the number of positive metastatic lymph nodes contributes to poor survival outcomes. In addition, each positive metastatic lymph node was found to increase the risk of breast cancer death by approximately 6% [3]. A previous study [4] showed that about 70% of Egyptian breast cancer patients with luminal A molecular subtype also show positive metastatic lymph nodes. Although different therapeutic approaches exist, studies using large cohorts showed that patients diagnosed with 10 or more positive lymph nodes have a 70% chance of tumor relapse [4–6]. Therefore, understanding the mechanism(s) underlying how carcinoma cells invade axillary lymph nodes is highly warranted.

The multifunctional proteolytic enzyme cathepsin B (CTSB) plays an important role in cancer progression [7–9]. Because of its increased and redistributed expression pattern in human cancers, pro- or active CTSB is considered a marker for invasion and metastasis of various carcinoma cells [10].

The increases in CTSB expression level and activity as well as alterations in tracking, localization and secretion of the enzyme have been observed in many cancers such as oral carcinoma [11], colorectal [12], lung [13], ovarian [14] and endometrial [15]. Localization of CTSB within the cell surface has been shown in different types of invasive cancer cells, e.g. breast [16], ovarian [17] and prostate [18] cancer. Invasive carcinoma cells showed altered lysosomal localization of CTSB from perinuclear to peripheral regions and secretion of CTSB into the extracellular space [19]. CTSB was found to bind with the annexin II heterotetramer in the cell surface caveolae (lipid raft) of invasive carcinoma cells [20,21]. This binding seems to facilitate conversion of pro-CTSB to its active form [21,22]. In the caveolae active CTSB may convert the pro-form of urokinase-type plasminogen activator (pro-uPA) into active uPA [21]. Thus, CTSB plays an important role in activation of pericellular proteases and digestion of extracellular matrix (ECM) proteins which facilitate breast cancer motility and invasion [20]. A previous study conducted by the authors and colleagues showed proteolytic activity of CTSB and its co-expression with caveolin-1 induced motility and invasion of the inflammatory breast cancer (IBC) cells [8]. Furthermore, inhibition of CTSB using the enzyme specific inhibitor CA074 reduced the degradation of ECM proteins, invasion and motility of IBC cell lines [8].

Interleukin-6 (IL-6) has been shown to stimulate aromatase expression in adipose tissue thus stimulating estrogen synthesis and potentially contributing to breast cancer progression [23]. Moreover, IL-6 was found to induce expression of CTSB through different signaling pathways. For instance, the

complex form of IL-6 and soluble-IL-6-receptor (IL-6/sIL-6R) stimulated expression of CTSB and cathepsin L (CTSL) in human gingival fibroblasts through the c-Jun N-terminal kinase-activator protein-1 (JNK-AP-1) caveolae involved pathway [24]. In addition, IL-6 and cyclic adenosine monophosphate (cAMP) were found to stimulate the secretion of CSTB from human osteoblasts [25]. However, the impact of IL-6 stimulation on CTSB expression and activity in breast cancer has not yet been fully elucidated.

The aim of the present study was to investigate the effect of different concentrations of IL-6 on the invasive potential of the human HRP-breast cancer cell line MCF-7 and to evaluate expression and activity of CTSB as a downstream mechanistic cue for IL-6 stimulation. Furthermore, the association between IL-6 expression and CTSB levels in carcinoma tissues of HRP-breast cancer patients with positive lymph node (pLNs) vs negative lymph nodes (nLNs) was assessed. This study demonstrated the role played by IL-6 in regulating expression and activity of CTSB-mediated invasion and lymph node metastasis in HRP-breast cancer.

Material and methods

Materials

Culture media and tissue culture supplies were from Lonza (Lonza, Verviers, Belgium). CTSB antibody was a gift from Dr. Bonnie Sloane (Department of Pharmacology, School of Medicine, Wayne State University, Detroit, MI, USA). Recombinant human IL-6 was purchased from R&D Systems (R&D Systems, Wiesbaden, Germany). All other chemicals were from Sigma (Sigma-Aldrich, Deisenhofen, Germany) unless otherwise stated.

Patients' samples

Patients diagnosed with HRP-breast cancer were recruited from the breast cancer clinic of Ain-Shams University hospitals during the period from December 2011 till December 2013. *The approval of ethics committee of Ain-Shams University hospital was obtained and all patients signed a consent form before enrolling in this study.* Depending on clinical and pathological diagnosis the patients were divided into 2 groups: the first group ($n = 14$) comprised of nLNs that showed no metastatic axillary lymph nodes and the second group ($n = 14$) was of pLNs patients with more than 4 metastatic axillary lymph nodes. Each tissue specimen was divided into two halves: the first half was fixed in 10% neutral formalin buffered for histopathological diagnosis and immunohistochemistry and the other half was snap frozen in liquid nitrogen for further studies.

Cell culture

The human breast cancer cell line MCF-7 was a gift from Dr. Bonnie F. Sloane (Department of Pharmacology, School of Medicine, Wayne State University, Detroit, MI, USA). MCF-7 cells were maintained in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 1% glutamine,

1% penicillin/streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C.

Invasion assay

Cellular invasion of breast cancer cells was assessed using BD BioCoat™ Matrigel™ Invasion Chamber (Becton Dickinson Labware, Franklin Lakes, NJ, USA). MCF-7 cells at a density of 2.5×10^4 per well were seeded in the upper chamber with RPMI-1640 serum free media. The lower chambers were filled either with 500 µL of RPMI-1640 media supplemented with 3% FBS (control), or with different concentrations of IL-6 (10 ng/mL, 25 ng/mL and 50 ng/mL) in RPMI supplemented with 3% FBS. After 72 h of incubation the experiments were stopped and cells were fixed and stained as described before [26]. Non-invasive cells that remained in Matrigel or were attached to the upper side of the filter were removed with cotton swabs. Cells on the lower side of the filter, which invade in response to different concentrations of IL-6, were stained using the Diff-Quik staining kit (Dade-Behring, Inc., Englewood Cliffs, NJ, USA) and counted using light microscopy. Invasive cells were quantified by counting three random microscopic fields and expressed as number of invaded cells.

Preparation of MCF-7 cell lysates

MCF-7 cells were grown at a density of 250,000 cell/mL in 30 mm Petri dishes containing RPMI-1640 supplemented with 10% FBS for 48 h at 37 °C in a humidified CO₂ incubator. After 48 h cells were washed with PBS and grown in RPMI-1640 media supplemented with 3% FBS and different concentrations of recombinant human IL-6 (10 ng/mL, 25 ng/mL and 50 ng/mL). Experiments were run in triplicate for each concentration of recombinant human IL-6. Control MCF-7 cells were grown in RPMI-1640 supplemented with 3% FBS. After 48 h of incubation cells were washed three times with PBS and incubated overnight in serum free media. Overnight media were collected and stored at -80 °C for future experiments. Cells were washed twice with PBS at 4 °C and collected by scraping with rubber policeman in lysis buffer (250 mM sucrose, 25 mM 2-(4-morpholino) ethanesulfonic acid (MES), pH 7.5, 1 mM EDTA, 0.1% Triton X-100). Cell lysates were sonicated on ice in a 50 W Ultrasonicator five times for five seconds each as described before [27].

CTSB activity assay

Activity of CTSB in MCF-7 cell lysates was assessed as described previously [27,28]. One part of cell lysates was diluted in 7 parts of activator buffer (5 mM EDTA, 10 mM dithiothreitol (DTT), pH 5.2) and the mixture was incubated for 15 min at 37 °C. 100 µL of this mixture was added to 200 µL assay buffer containing a fluorometric CTSB substrate (ZArg-Arg-NHMec). Fluorescence was measured at 1-min interval for 30 min with excitation at 360 nm and emission at 465 nm using a Tecan-Spectrafluor Plus plate reader. Data were expressed as picomoles of NHMec formed per minute per µg DNA. Concentrations of DNA were assessed using SYBR green method against a standard curve of salmon-sperm DNA.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of CTSB

For SDS-PAGE, protein concentration of total MCF-7 cells and homogenized human breast cancer tissue lysates was first determined using the Bradford assay. Both lysates were reduced and separated by SDS-PAGE (12% acrylamide) at protein concentration of 20 µg/well. Gels were electrotransferred onto nitrocellulose membranes and detection was performed with primary antibody against CTSB (1:4000) and secondary antibody conjugated with horseradish peroxidase (1:10,000) in 5% (w/v) non-fat dry milk dissolved in Tris-buffered saline washing buffer [TBS, (20 mM Tris, pH 7.5, 0.5 M NaCl)] containing 0.5% Tween-20. After washing, specifically bound antibodies were detected by tetramethyl benzidine (TMB) chromogen/substrate and the reaction was stopped by immersing the membrane in water for 20–30 s. All the above-mentioned steps were analogously repeated for the detection of IL-6 levels in breast carcinoma tissue using anti-human IL-6 antibody (R&D Systems, Wiesbaden, Germany). Bands intensity was quantified with ImageJ software (National Institutes of Health, Bethesda, MA, USA) using β-actin as loading control.

Immunocytochemical staining

For immunocytochemical staining of CTSB, MCF-7 cells were seeded at a density of 20×10^3 on glass cover-slips in RPMI-1640 supplemented with 3% FBS and different concentrations of recombinant IL-6 (10 ng/mL, 25 ng/mL and 50 ng/mL) for 48 h. To immunolocalize intracellular CTSB, MCF-7 cells seeded in different concentrations of IL-6 were washed four times with PBS and fixed with methanol (−20 °C) for 5–10 min. Cells were then permeabilized with 2% saponin in PBS and blocked by incubating with 0.5% bovine serum albumin in PBS. CTSB intracellular staining was carried out as previously described [29]. The primary antibodies used were rabbit anti-human CTSB (5 µg/mL), mouse anti-human E-cadherin (BD Bioscience CA, USA) and rabbit anti-human vimentin (Santa Cruz Biotech, CA, USA). The secondary antibodies used were fluorescein-conjugated affinity-purified donkey anti-rabbit IgG and Texas red-conjugated affinity-purified donkey anti-mouse IgG (20 µg/mL) in 5% normal donkey serum. Cell nuclei were visualized by 4',6'-diamidino-2-phenylindole (DAPI) staining (Sigma, Deisenhofen, Germany). Control samples were run in parallel but the primary antibody was omitted. Coverslips were mounted with anti-fade reagent and were analyzed with a Zeiss Axiovert microscope (Zeiss, Oberkochen, Germany) at 40X magnification.

Immunohistochemical staining of CTSB of human HRP-breast cancer tissues

Immunohistochemical staining for CTSB in carcinoma tissues of breast cancer patients was performed as described previously [9]. Formalin-fixed and paraffin-embedded tumor tissues were briefly deparaffinized in xylene and rehydrated through graded ethanol. After antigen retrieval processing in a steamer, tissue sections were incubated for one hour at room temperature with the CTSB primary antibody (1:500) diluted in Dako antibody diluent. Detection was carried out by incubating

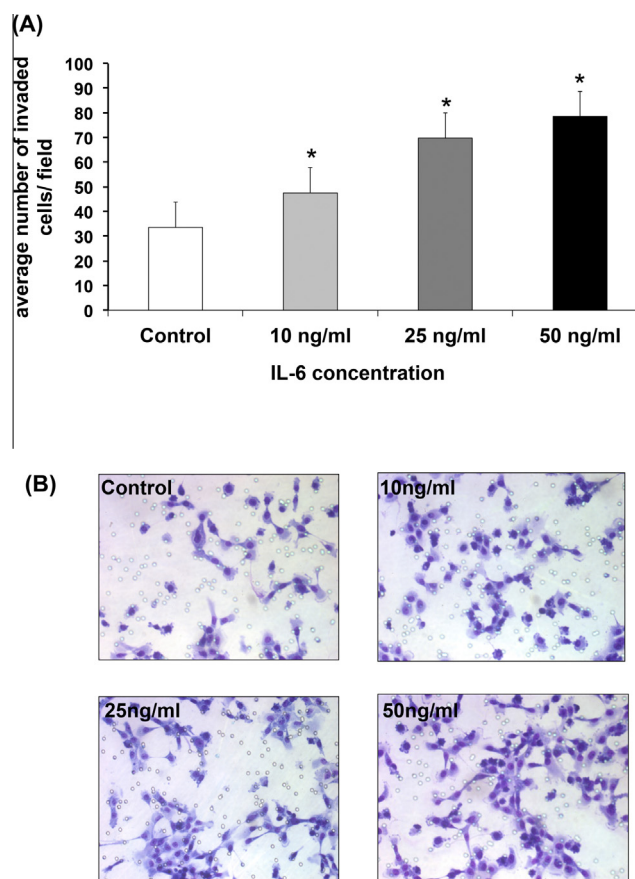


Fig. 1 IL-6 induces invasion of MCF-7 cells. (A) Equal numbers of MCF-7 cells were seeded and incubated with control media (RPMI-1640/3% FBS), 10, 25, and 50 ng/mL IL-6 for 72 h on BD invasion chambers. MCF-7 cells that had invaded through the filters were stained using Diff-Quik Stain Set (blue), and the number of cells was counted in three random microscopic fields. (B) Representative images of stained invasion filters. Results are representative of three independent experiments. Data are expressed as mean ± SD. * represents $P < 0.05$ as determined by Student's *t*-test, scale bar = 150 µm.

Table 1 Overview on the number of invaded MCF-7 cells upon stimulation with different concentration of IL-6.

Cells	Average number of invaded cells/field ± SD	<i>P</i> -value
Control	33.5 ± 0.479	
10 ng/mL IL-6	47.5 ± 0.46	0.0006
25 ng/mL IL-6	69.75 ± 0.77	0.0097
50 ng/mL IL-6	78.5 ± 0.67	0.002

Data are expressed as mean ± SD, $n = 3$.

P value was calculated by Student's *t*-test.

tissue sections with 100 µL of the secondary HRP-labeled rabbit antibody for 45 min and staining was developed with 3-3'-diaminobenzidine (DAB) for 15 min. Negative controls, in which the primary antibody was omitted, were run in parallel. The stained slides were analyzed semi-quantitatively using imageJ software (National Institutes of Health, Bethesda, MD, USA).

Enzyme-linked immunosorbent assay (ELISA) for IL-6

ELISA was performed according to the manufacturer's instruction with Human IL-6 Quantikine ELISA (R&D Sys-

tems). Equal protein concentration of total tissue homogenates of HRP-breast cancer with pLNs and nLNs patients was utilized.

Statistical analysis

Results were analyzed using SPSS (SPSS, Chicago, IL, USA), version 15.0. Differences among variables were evaluated using χ^2 analysis, Pearson's rank correlation test, Fischer's exact test and Student's *t*-test. Data were expressed as mean \pm SEM. Differences were considered statistically significant at $P < 0.05$.

Results

IL-6 enhances invasive phenotype of MCF-7 cells in a dose-dependent manner

BD-invasion chambers were used to test whether IL-6 as an exogenous stimulant may alter invasiveness of MCF-7 cells. Indeed, IL-6 showed chemotactic properties and induced invasion of MCF-7 cells through Matrigel-coated filters in a dose-dependent manner. The present results revealed that directional invasiveness was significantly enhanced by 42%, 107% and 134% in presence of 10, 25, and 50 ng/mL IL-6, respectively, as compared to control cells ($P < 0.05$) (Fig. 1A & B and Table 1).

IL-6 augments expression and activity of CTSB in MCF-7 cells

Influence of different concentrations of IL-6 (10, 25, and 50 ng/mL) on expression of CSTB protein level in MCF-7 cells was tested. IL-6 induced the expression and activity of CTSB in a dose-dependent manner. IL-6 at concentrations of 25 and 50 ng/mL resulted in an increase in the cellular activity

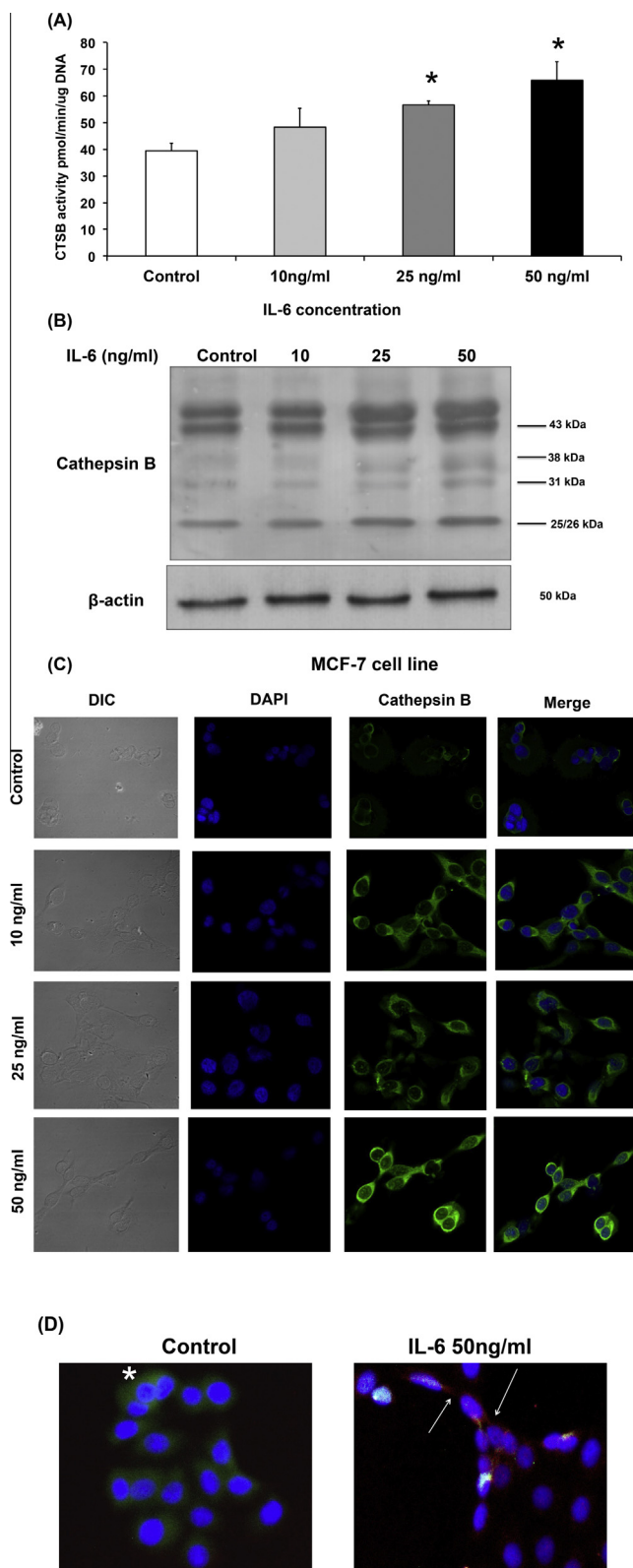


Fig. 2 Activity profile and expression of CTSTB are increased in MCF-7 cells stimulated by different concentrations of IL-6. (A) Concentrations of 25 and 50 ng/mL of IL-6 induced a significant increase in CTSTB activity ($P = 0.015129$ and $P = 0.003182$, respectively) as compared to control cells grown in RPMI-1640/3% FBS culture media. Data are expressed as mean \pm SD. (B) Immunoblotting of CTSTB expression in MCF-7 cells induced with different concentrations of IL-6. (C) Immunocytochemical staining of CTSTB in MCF-7 cells stimulated with different concentrations of IL-6. Intracellular CTSTB was detected by immunofluorescence using a CTSTB specific antibody. Control MCF-7 cells seeded in RPMI-1640/3% FBS media show weak expression of CTSTB (green fluorescence), which increases gradually with increasing IL-6 concentration. DIC shows a fusiform phenotype of MCF-7 cells with 50 ng/mL IL-6 treatment. Blue color indicates DAPI nuclear staining. (D) Immunofluorescence staining of E-cadherin and vimentin in control and 50 ng/mL IL-6-treated MCF-7 cells for 24 h. IL-6 treatment of MCF-7 cells decreases E-cadherin expression (green fluorescence, cells labeled with asterisk) and enhances expression of vimentin (red fluorescence, cells labeled with arrow), suggestive acquisition of migratory phenotype.

of CTSB ($P = 0.015129$ and $P = 0.003182$, respectively) (Fig. 2A). Moreover, the mature active forms of CTSB (single chain (31 kDa) and double chain (25/26 kDa)) were highly detected in cell lysates of MCF-7 cells stimulated by 25 and 50 ng/mL IL-6 (Fig. 2B).

The effect of IL-6 on the EMT process and CTSB expression in MCF-7 cells was further evaluated. Immunostaining results indicate that upon increasing the concentration of IL-6, MCF-7 cells lost cell-cell contact and acquired fibroblastic shape with increased CTSB expression (Fig. 2C). This substantiates that IL-6 enhanced EMT as reflected by loss of E-cadherin expression and increased vimentin expression in MCF-7 cells upon 50 ng/mL IL-6 treatment as determined by immunofluorescence microscopy (Fig. 2D). Occasionally, nuclear localization of CTSB, which was obviously increased at the concentration of 50 ng/mL IL-6 (Fig. 2C) was observed, and although speculative this suggests a transcriptional activity function.

IL-6 and CTSB expressions are upregulated in carcinoma tissues of HRP-breast cancer with pLNs patients

The clinical and pathological characteristics of ER⁺, PR⁺, HER⁻ patients included in this study are represented in Table 2. The carcinoma tissues of pLNs patients are characterized by a statistically significant increase in lymphovascular invasion ($P = 0.034$) as compared to nLNs patients. No other clinical-pathological data showed statistical significance between both groups of patients.

As the HRP-breast cancer cell line MCF-7 exhibited an invasive phenotype via IL-6-mediated upregulation of CTSB expression, this finding was tested *in vivo* using carcinoma tissues of breast cancer patients. Therefore, the expression level of IL-6 and CTSB in human breast carcinoma tissues obtained

during modified radical mastectomy of HRP-breast cancer with nLNs and pLNs patients was assessed. Western blot data revealed a significant upregulation of IL-6 and CTSB expression in carcinoma tissues of pLNs as compared to nLNs patients ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 3A & B and Suppl. Table 1). The mature active forms of CTSB (single chain (31 kDa) and double chain (25/26 kDa)) were more prominent in pLNs as compared to nLNs breast cancer patients. To test whether a correlation between CTSB and IL-6 expression exists, Pearson's rank correlation in nLNs ($n = 10$) and pLNs ($n = 10$) was used. A significant positive correlation ($r = 0.78$, $P < 0.01$) between the expression of IL-6 and CTSB in carcinoma tissues of pLNs but not in nLNs breast cancer patients was found (Fig. 3C). ELISA results uncovered a 2.8-fold increase in IL-6 protein levels in carcinoma tissues of HRP with pLNs compared with those nLNs patients ($P < 0.05$) (Fig. 3D). IHC staining for CTSB in nLNs and pLNs carcinoma tissues confirmed Western blot results: carcinoma tissues of pLNs patients expressed higher CTSB than those of nLNs (Fig. 3E). The present results support the hypothesis of the potential role that may be played by IL-6 in regulating CTSB expression and activity in breast cancer tissues.

Discussion

Cytokines are key regulatory molecules secreted by stromal cells as well as tumor cells in the breast tumor microenvironment [30,31]. IL-6 is one of the major cytokines secreted within the tumor microenvironment by breast cancer cells-associated monocytes [27] and adipocytes [32]. IL-6 was found to have multiple tumor promoting functions [33] via stimulating proliferation and inhibiting apoptosis [31,34]. For example, IL-6 secreted from adipose stromal cells was found to induce inva-

Table 2 Clinical and pathological characterization of nLN and pLN patients.

Characteristics	nLNs (ER ⁺ , PR ⁺ , HER2 ⁻) (N = 14)	pLNs (ER ⁺ , PR ⁺ , HER2 ⁻) (N = 14)	P-value
<i>Age</i>			
Range	27–78	31–70	0.352 ^a
Mean ± SD	56.64 ± 14.383	51.71 ± 13.076	
NA	0	0	
<i>Tumor size</i>			
≤4	1(7.14%)	3 (21.43%)	0.472 ^b
>4	13 (92.86%)	11 (78.57%)	
NA	0	0	
<i>Tumor grade</i>			
G1	1 (7.14%)	0	0.513 ^b
G2	1 (7.14%)	2 (14.29%)	
G3	12 (85.71%)	12 (85.71%)	
G4	0	0	
NA	0	0	
<i>Lymphovascular invasion</i>			
Negative	14 (100%)	11 (78.57%)	0.034 ^{a,b}
Positive	0	3 (21.43%)	
NA	0	0	

Data are expressed as mean ± SD.

NA: Data not available.

* Significant P value was calculated by ^aStudent's *t*-test or ^bFisher's exact test.

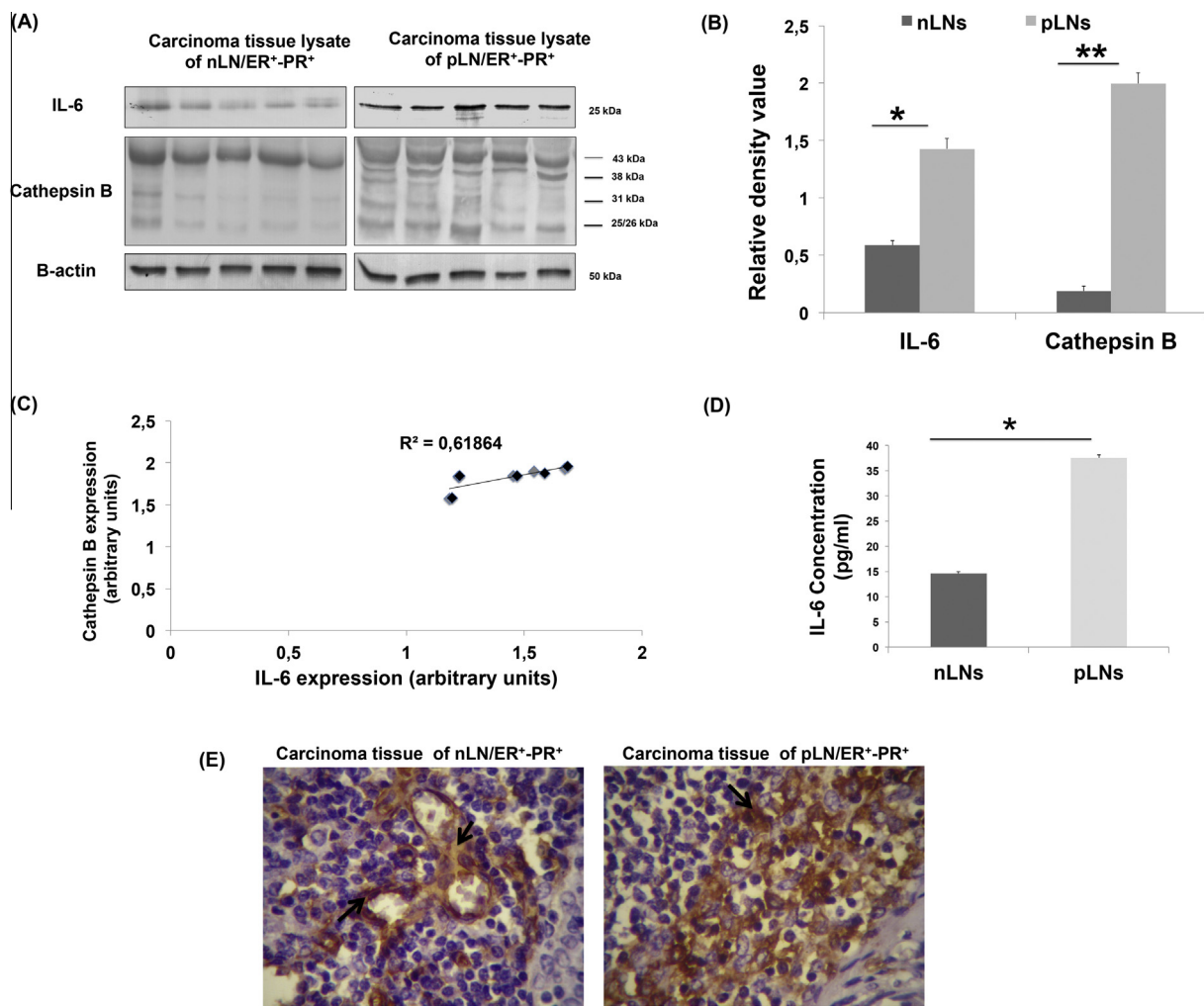


Fig. 3 Up-regulation of IL-6 and CSTB expression in carcinoma tissues of HRP-breast cancer with pLNs as compared to those with nLNs patients. (A) Representative Western blot of IL-6 and CTSB expression in 5 patients. (B) Densitometric analysis of Western blot shows a significant upregulation of expression levels of IL-6 and CTSB in carcinoma tissues of HRP with pLNs ($n = 10$) as compared to those with nLNs ($n = 10$). (C) A positive correlation between IL-6 and CTSB expression in HRP-breast cancer patients with pLN ($n = 10$) as analyzed by a Pearson's correlation. (D) Carcinoma tissues of HRP with pLNs ($n = 10$) breast cancer patients exhibit higher IL-6 protein levels than those of nLNs ($n = 14$), as quantified by ELISA. * represents $P < 0.05$ and ** represents $P < 0.01$ as determined by Student's *t*-test. (E) Representative immunohistochemical staining of higher CTSB expression in carcinoma tissue of HRP-breast cancer with pLNs ($n = 14$) as compared to those with nLNs ($n = 14$). Scale bar = 100 μm , arrow points to CTSB staining.

sion of MCF-7 breast cancer cells [32]. Moreover, it is also documented that both IL-6 and its receptors (IL-6R and sIL-6R) are overexpressed in breast carcinoma tissues [35]. Therapeutic targeting via monoclonal antibodies either against IL-6 or IL-6R has been reported to be used in phase I/II clinical trials in several tumor entities as reported before [31].

The aim of this study was to explore the effect of IL-6 on non-invasive phenotype of HPR-breast cancer cell line MCF-7. Data of this study revealed that IL-6 induced invasion of breast carcinoma cells *in vitro* in a dose-dependent manner. Furthermore, loss of cell-cell contact with acquisition of fibroblastic phenotype via loss of E-cadherin expression and upregulation by vimentin was prominent in MCF-7 cells upon stimulation with IL-6. These findings are in agreement with two reports: In a study using the orthotopic xenograft MCF-7^{IL-6} breast cancer model, IL-6 was found to induce EMT phenotype via over-expression of vimentin and repression of E-

cadherin. In addition, mice bearing MCF-7^{IL-6} xenograft tumors possessed higher invasive and metastatic properties as compared to control MCF-7 xenograft mice models [36]. A recent study [37] showed that over-expression of IL-6 induced invasion and lymph node metastasis in gallbladder cancer via stimulation of EMT molecular mechanism.

CTSB, a lysosomal cysteine protease, is considered as a "multifunctional enzyme in cancer" and essentially contributes to the proteolytic cascades associated with tumor progression including growth, invasion and metastasis [7]. Mechanistically, CTB initiates proteolytic cascades on the surface of cancer cells which ultimately result in the activation of downstream proteases such as uPA, pro-matrix metalloproteinases (MMP)-2 and -9 that degrade ECM components and adhesion molecules (E-cadherin) [7,38–40]. A study showed that IL-6 had no impact on the expression of CTB mRNA and protein levels in A-549 human lung epithelial cells [41]. However, other

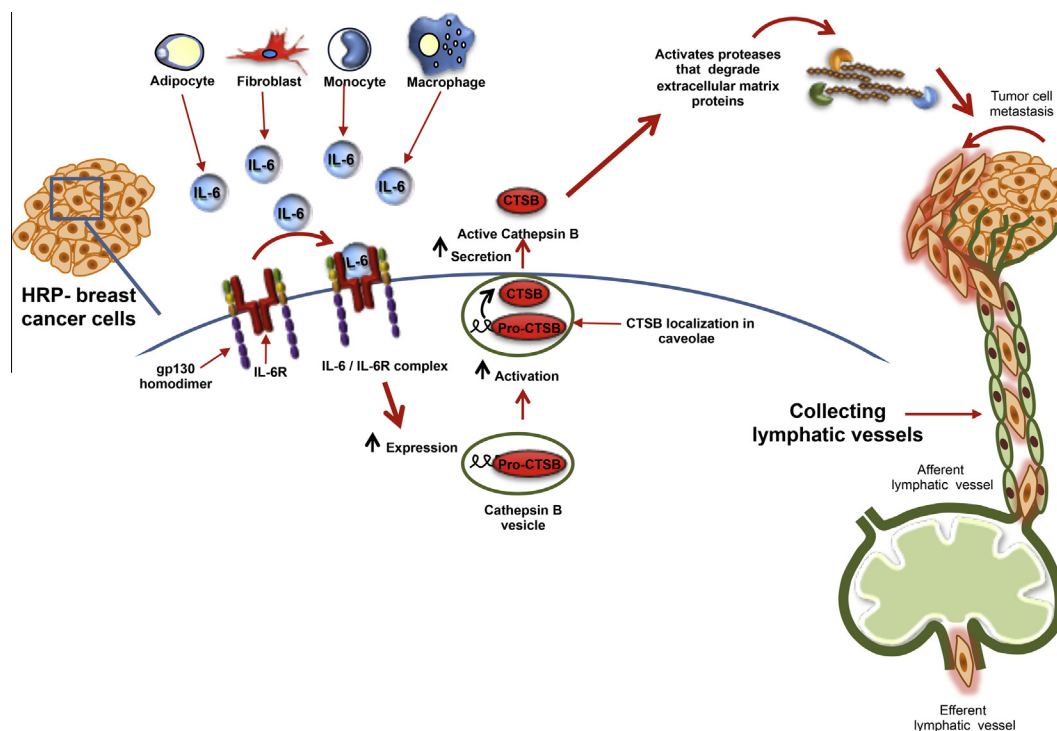


Fig. 4 Schematic representation demonstrating the role of IL-6 in regulating expression and activity of CTSB, and in lymph node metastasis in HRP-breast cancer cells. Exogenous IL-6 or IL-6 secreted from tumor-associated cells (e.g. adipocytes, fibroblasts and monocytes/macrophages) binds to its receptor complex IL-6R/gp-130, which consequently increases expression and conversion of pro-CTSB into active CTSB via its binding into lipid raft of cell membrane. The active form of CTSB secreted into extracellular space enhances proteolytic activities cascades and degradation of ECM constituents. Cancer cells lose cell-to-cell contact, acquire invasive phenotype and metastasize via lymphatic vessels into the regional lymph nodes.

studies showed that IL-6 stimulated the expression of proteases such as MMP-2 and MMP-9 and intracellular protease CTSB and CTSL in C2C12 myotubes as a model of the muscle [42], non-Hodgkin's lymphomas [43], squamous cell carcinoma [44] and in breast tumor-associated monocytes [27]. Although many reports showed the potential role of IL-6 in regulating CTSB expression, the present study is the first report addressed this regulation in breast cancer. In this regard, results of this study indicate that 25 ng/mL and 50 ng/mL IL-6 augmented the expression and activity of CTSB in MCF-7 cells, suggesting that CTSB may play a crucial role in IL-6-mediated enhanced invasiveness phenotype of MCF-7 cells.

IL-6 and CTSB have been assigned as markers whose expressions are associated with poor prognosis of breast cancer patients [34,45]. A high level of IL-6 is associated with increased tumor stage, lymph node infiltration, recurrence, and treatment resistance in breast cancer patients [34,46,47]. In addition, local intratumoral IL-6 was found to regulate breast cancer cell growth, metastasis, and self-renewal of cancer stem cells [48]. IL-6 is significantly upregulated in the plasma of ER⁺ metastasized cancers, and essentially can be utilized to distinguish between women with breast cancer and healthy controls [30]. Nouh et al. [9] have previously shown that CTSB is a potential prognostic marker and its over-expression induces lymph-node metastasis in IBC patients. In addition, inhibition of CTSB activity attenuates ECM degradation and IBC cell invasion [8]. Patients suffering from lung tissue damage (cystic fibrosis) show an increased

level of sputum IL-6 correlating with CTSB [49]. In this study, Western blotting and ELISA data showed that IL-6 was over-expressed in HRP-breast carcinoma tissues of pLNs as compared to those of nLNs patients. In addition, an over-expression of CTSB in HRP-breast carcinoma tissues of pLNs when compared to nLNs patients was detected. Of particular importance, the present findings indicate an existence of a positive correlation ($r = 0.78$, $P < 0.01$) between IL-6 and CTSB in pLNs patients. Mechanistically, IL-6 induces CTSB activation via p38 MAPK and NF κ B in human osteoblasts [25]. Of note, NF κ B regulates expression of CTSB via binding to the promoter region of CTSB gene in Hela cells [50], suggesting a mechanism may be exerted by IL-6 in stimulating CTSB expression by HRP-breast carcinoma cells. Indeed, CTSB secreted by carcinoma cells plays crucial role in the activation of proteolytic pathways, degradation of ECM, motility and invasion of carcinoma cells, which may ultimately lead to lymph node metastasis (Fig. 4).

Conclusions

The present study demonstrated that IL-6 promotes invasive properties of the low metastatic potential MCF-7 cells via increasing expression and activity of CTSB. In addition, this study validated the role of IL-6 in stimulating CTSB expression in human HRP-breast carcinoma tissues. Results showed that IL-6 is upregulated and correlates with the expression of CTSB levels in the carcinoma tissues of HRP-breast cancer

with pLNs when compared to nLN patients. Given the therapeutic modality of the monoclonal antibody against IL-6 in clinical trials of different tumor entities [51,52], thus the present results provided evidence that IL-6 as single agent or in combination with CTSB are relevant therapeutic targets for HRP-breast cancer with pLNs patients (Fig. 4). Further studies are warranted to fully identify signaling pathway(s) regulating IL-6/CTSB axis in different molecular subtypes of breast cancer.

Study limitation

One limitation of this study is only MCF-7 cells tested; however, previous work performed by authors showed a modest increased migratory phenotype of the triple negative breast cancer cell line MDA-MB-231 upon incubation with IL-6 [53], suggesting a similar mechanism may be at least in part relevant for other breast cancer cell lines. Nevertheless, it is documented that hormonal negative breast cell lines produced autocrine IL-6, which prone the cells less or unresponsive to exogenous IL-6 [54].

Conflict of Interest

The authors have declared no conflict of interest.

Acknowledgments

Special thanks to Dr. Bonnie Sloane's laboratory Wayne State University, Detroit, MI, for the support in the experiments. We thank Dr. Nader S. Metwalli, PhD Biomedical and Metabolic Imaging, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Branch, National Institutes of Health (NIH) Clinical Center, Bethesda, MD, USA, for critical review of the English language. This work was supported by Avon Foundation-USA (MMM), Cairo University Scientific Research Sector (SAI, HH and MMM).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jare.2016.06.007>.

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