



Induction of heparanase via IL-10 correlates with a high infiltration of CD163+ M2-type tumor-associated macrophages in inflammatory breast carcinomas



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Abstract

Inflammatory breast cancer (IBC) is the most aggressive and lethal form of breast cancer, characterized by a high infiltration of tumor-associated macrophages and poor prognosis. To identify new biomarkers and to elucidate the molecular mechanisms underlying IBC pathogenesis, we investigated the expression pattern of heparanase (HPSE) and its activator cathepsin L (CTSL). First, we quantitated the *HPSE* and *CTSL* mRNA levels in a cohort of breast cancer patients after curative surgery (20 IBC and 20 non-IBC). We discovered that both *HPSE* and *CTSL* mRNA levels were significantly induced in IBC tissue vis-à-vis non-IBC patients ($p < 0.05$ and $p < 0.001$, respectively). According to the molecular subtypes, *HPSE* mRNA levels were significantly higher in carcinoma tissues of triple negative (TN)-IBC as compared to TN-non-IBC ($p < 0.05$). Mechanistically, we discovered that pharmacological inhibition of HPSE activity resulted in a significant reduction of invasiveness in the IBC SUM149 cell line. Moreover, siRNA-mediated HPSE knockdown significantly downregulated the expression of the metastasis-related gene *MMP2* and the cancer stem cell marker *CD44*. We also found that IBC tumors revealed robust heparanase immune-reactivity and CD163+ M2-type tumor-associated macrophages, with a positive correlation of both markers. Moreover, the secretome of axillary tributaries blood IBC CD14+ monocytes and the cytokine IL-10 significantly upregulated *HPSE* mRNA and protein expression in SUM149 cells. Intriguingly, massively elevated *IL-10* mRNA expression with a trend of positive correlation with *HPSE* mRNA expression was detected in carcinoma tissue of IBC. Our findings highlight a possible role played by CD14+ monocytes and CD163+ M2-type tumor-associated macrophages in regulating *HPSE* expression possibly via IL-10. Overall, we suggest that heparanase, cathepsin L and CD14+ monocytes-derived IL-10 may play an important role in the pathogenesis of IBC and their targeting could have therapeutic implications.

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Introduction

Inflammatory breast cancer (IBC) is an aggressive and a deadly form of breast cancer [1], constituting

approximately 11% of breast cancer cases among women in Egypt [2]. It can be differentiated from other forms of locally advanced breast cancer by the clinical appearance of inflammation with diffuse

erythema and peau d'orange, which is thought to result from the presence of tumor emboli blocking the breast dermal lymphatics [1], leading to rapid distant metastasis [3]. IBC patients thus exhibit lower the 5-year overall survival than other breast cancer forms. However, prognosis of IBC can be improved by trimodality treatment, namely neoadjuvant chemotherapy, surgery and radiotherapy [4].

According to expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2); breast cancer can be classified into triple negative (TN) breast cancer, which is defined as (ER, PR and HER2-negative) and non-TNBC, which include three main subtypes: luminal A (ER and/or PR-positive and HER2-negative with low proliferation marker Ki-67), luminal B (ER and/or PR-positive and either HER2-positive or high Ki-67), HER2-enriched (ER-, PR-negative and HER2-positive) [5]. IBC has the same classification of molecular subtypes and that TN-IBC is considered the most aggressive subtype as it is enriched with cancer stem cells [6], thereby leading to a high risk of relapse and metastasis [6,7]. Moreover, currently no targeted therapy is available for TN-IBC, establishing an urgent need to identify new biomarkers and therapeutic targets for this particularly aggressive subtype of cancer.

Emerging evidence connects enhanced cancer cell migration and metastasis to the interplay between cancer cells and the extracellular matrix (ECM) components [8,9]. ECM is a heterogeneous mixture of proteins and polysaccharides that physically support and organize the cells to maintain tissue homeostasis and regulate cell phenotype, and any dysregulation of that composition has an influence on several pathological conditions, including cancer [9–11]. One of the important components of ECM are heparan sulfate proteoglycans (HSPGs), which bind to a multitude of bioactive molecules, thereby regulating physiological and pathological processes such as morphogenesis, tissue repair, inflammation, vascularization, and cancer metastasis [12,13]. Of note, the function of HSPGs can be modified by the release of HS side chains by the action of Heparanase-1 [13]. A heparanase substrate of particular relevance is the cell surface HSPG Syndecan-1, which we recently identified as a novel biomarker of TN-IBC [6,14]. Moreover, we previously demonstrated that downregulation of Syndecan-1 is associated with an increase in heparanase expression in TNBC cells [15]. As the functional status of Syndecan-1 is highly regulated by heparanase [12], heparanase emerges as a potential biomarker and likely accessible druggable target for IBC. Heparanase-1 is the only functional mammalian endoglucuronidase degrading heparan sulfate chains of the HSPGs present on the cell surface and ECM [16]. This degradation can release the bioactive molecules that are bound by HS, and

facilitate their biological functions [13,16]. Additionally, heparanase can exert non-enzymatic activities through stimulating signal transduction pathways, which have an impact on multiple regulatory processes including endothelial cell migration and invasion, increased blood vessel density and angiogenesis [17–20]. During cancer progression, the cancer cells successfully exploit the physiological functions of heparanase for ECM remodeling and facilitate the invasion and metastasis [9,21]. Indeed, breast cancer cells express high levels of heparanase that is found to be associated with increased lymph node metastasis, increased micro-vessel density, large tumor size, high histological grade, and reduced post-operation survival [22], suggesting that heparanase may emerge as a promising marker for IBC.

Cathepsin L, the ubiquitous lysosomal cysteine proteinase responsible for the processing and activation of latent heparanase, is overexpressed not only in breast cancer cells but also in stromal cells in the tumor microenvironment [23,24]. In chronic colitis and colitis-associated cancer, heparanase activates macrophages, while macrophages upregulate (via TNF- α), and potentiate activation of heparanase (via secretion of cathepsin L) [25,26]. This suggests that tumor-associated macrophages (TAMs) are regulators of epithelial heparanase expression and its activation during chronic inflammatory conditions in a tumor-promoting microenvironment [25].

Collectively, heparanase and cathepsin L have been shown to play a vital role during the crosstalk between the cancer cells and the surrounding microenvironment that regulate cancer metastasis [24,25]. Therefore, we aimed in this study to investigate the expression pattern of heparanase and its activator cathepsin L in carcinoma tissue of IBC vs. non-IBC, and to uncover the possible underlying mechanism regulating their expression.

Results

Clinical and pathological characteristics of patients

A total of 40 breast cancer patients selected for curative surgery were included; their median age was 52 years (range: 27–73). Patients were diagnosed as IBC ($n = 20$) and non-IBC ($n = 20$). Patients with IBC showed significantly higher involvement of ≥ 4 lymph nodes (N2) ($p = 0.032$), lymphovascular invasion ($p = 0.004$), and tumor stage ($p < 0.001$) compared to the non-IBC group. Other parameters (age, tumor size, tumor grade, HR status and HER2) did not show a significant difference between both groups (Table 1).

Table 1. Clinicopathologic characteristics of IBC and non-IBC patients.

Characteristic	IBC (N = 20)	Non-IBC (N = 20)	p value
Age (years)			
Range	35–70	27–73	0.695 ^a
Mean ± SD	52.75 ± 10.27	51.3 ± 12.79	
Tumor size (cm)			
≤ 4	7 (35)	10 (50)	0.318
> 4	11 (55)	7 (35)	
NA	2 (10)	3 (15)	
Lymph node status, n (%)			
< 4	4 (20)	11 (55)	0.032*
≥ 4	12 (60)	5 (25)	
NA	4 (20)	4 (20)	
Tumor grade, n (%)			
Grade I	0	1 (5)	1
Grade II	16 (80)	16 (80)	
Grade III	2 (10)	1 (5)	
NA	2 (10)	2 (10)	
Tumor stage, n (%)			
Stage I	0	2 (10)	<0.001*
Stage II	0	7 (35)	
Stage III	20 (100)	6 (30)	
NA	0	5 (25)	
Lymphovascular invasion, n (%)			
Negative	3 (15)	13 (65)	0.004*
Positive	12 (60)	4 (20)	
NA	5 (25)	3 (15)	
ER, n (%)			
Negative	10 (50)	10 (50)	1
Positive	10 (50)	10 (50)	
PR, n (%)			
Negative	11 (55)	13 (65)	0.748
Positive	9 (45)	7 (35)	
HER-2, n (%)			
Negative	16 (80)	18 (90)	0.661
Positive	4 (20)	2 (10)	

Data are expressed as mean ± SD.

NA Data not available.

* Significant p value calculated by ^aStudent's t-test or Pearson Chi-Square.

Heparanase mRNA expression is upregulated in carcinoma tissues of IBC

Heparanase has a critical role in cancer progression of different tumor entities including breast cancer [22], however, its expression pattern in IBC is still unclear. Therefore, we analyzed heparanase mRNA expression in tissues of IBC vs. non-IBC patients by qPCR. Relative to non-IBC carcinoma tissues, heparanase transcript levels in IBC tissues were significantly increased by 6.7-fold ($p < 0.05$, Fig. 1A). According to the molecular subtypes, we found a 5.03-fold overexpression of heparanase mRNA in TN-IBC as compared to TN-non-IBC patients ($p < 0.05$, Fig. 1A). In addition, heparanase mRNA expression was significantly upregulated in TN- compared to non-TN-non-IBC patients by 8.6-fold ($p < 0.05$, Fig. 1B) as

well as in the carcinoma tissue of TN-relative to non-TN-IBC by 8.9-fold ($p < 0.05$, Fig. 1B).

IBC tumors show a strong positive immunostaining of heparanase

Next, immunohistochemical staining of tumor tissue samples of IBC revealed higher levels of heparanase than in non-IBC ($p < 0.05$, Fig. 2A, B). Notably, heparanase positive staining was significantly higher in TN-IBC as compared to TN-non-IBC tissues ($p < 0.05$, Fig. 2A, B). In addition, TN-carcinoma tissue of non-IBC and IBC displayed significantly elevated heparanase protein expression as compared to their non-TN-counterparts ($p < 0.05$, Fig. 2C).

Heparanase inhibition significantly reduces invasiveness of SUM149 IBC cells and its siRNA knockdown is associated with reduced expression of the cancer stem cell marker CD44 and matrix metalloproteinase MMP2

To assess the functional impact of altered heparanase expression in IBC in vitro, we employed the pharmacological heparanase inhibitor OGT2115 [27] and siRNA-mediated knockdown of heparanase expression in SUM149 IBC cells. OGT 2115 significantly reduced the invasiveness of SUM149 cells in matrigel invasion chamber assays ($p < 0.05$, Fig. 3A), suggesting a contribution of heparanase to invasive growth as a prerequisite for metastasis in IBC. siRNA knockdown of heparanase expression in SUM149 cells resulted in a significant and substantial down-regulation of the expression of the hyaluronan receptor and cancer stem cell marker CD44, and of the proinvasive matrix metalloproteinase MMP2, as determined by qPCR ($p < 0.001$, Fig. 3B). These data are in line with the observed effect of heparanase inhibition on invasive behavior. The mRNA expression of additional factors linked to HSPG and heparanase function in IBC (Sdc1, Notch1, Notch2, ZEB1, COX2, MMP9, MMP14, CCL20, PAI1, EGFR, TGFbeta, IL-1beta) was not significantly affected by heparanase siRNA depletion in SUM149 cells (results not shown). Moreover, cell viability was not altered in heparanase-silenced SUM149 cells (Fig. 3C).

Cathepsin L mRNA levels are elevated in carcinoma tissues of IBC

Cathepsin L is considered the only proteolytic enzyme that processes the inactive form of heparanase into the active form. As cathepsin L has a role in breast cancer invasion and metastasis [28], we assessed the mRNA expression level of cathepsin L in carcinoma tissues of IBC and non-IBC patients using qPCR. Consistent with our heparanase mRNA expression data, cathepsin L mRNA expression was significantly

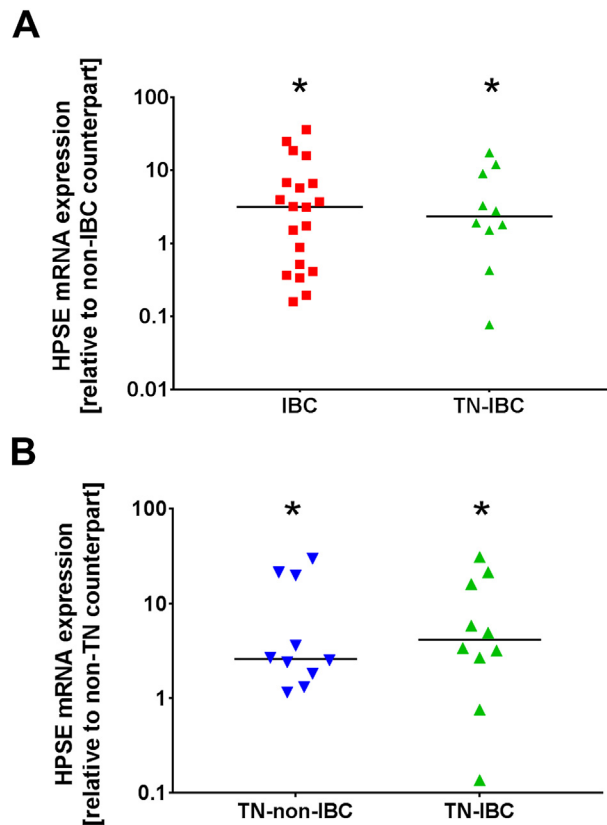


Fig. 1. Heparanase (*HPSE*) mRNA expression in carcinoma tissues of IBC and non-IBC. Heparanase mRNA expression in breast carcinoma tissues was assessed by qPCR. (A) Heparanase mRNA levels in carcinoma tissues of IBC ($n = 20$) and TN-IBC ($n = 10$) relative to their non-IBC counterparts (values are set at 1). (B) Heparanase mRNA expression in carcinoma tissues of TN-non-IBC ($n = 10$) and TN-IBC ($n = 10$) relative to their non-TN-counterparts (values are set at 1). * $p < 0.05$ as determined by Student's *t*-test.

upregulated in IBC tissues by 7.13-fold relative to non-IBC tissues ($p < 0.001$, Fig. 4A). In addition, cathepsin L mRNA expression was significantly increased by 20.07-fold in carcinoma tissues of non-TN-IBC compared to those of non-TN-non-IBC ($p < 0.01$, Fig. 4A). To characterize the source of cathepsin L expression in IBC, we assessed expression of heparanase and cathepsin L mRNA levels in CD14⁺ monocytes obtained from patient axillary tributaries using qPCR. Surprisingly, heparanase mRNA expression in IBC patient-derived monocytes was significantly downregulated by 76% compared to monocytes of non-IBC patients ($p < 0.001$). However, there was no significant difference in cathepsin L mRNA expression in CD14⁺ monocytes of IBC compared to that in non-IBC patients ($p = 0.55$, Fig. 4B).

CD163⁺ M2-type TAM infiltration is increased and shows a positive correlation with heparanase expression in IBC

It has been recently reported that IBC tumors secrete factors that drive the recruitment and differentiation of monocytes into tumor-promoting, immune-suppressing

M2-like macrophages [29]. Therefore, we assessed the positive staining of CD163⁺ M2-type TAMs in carcinoma tissues of IBC and non-IBC patients using immunohistochemistry. Relative to non-IBC, CD163⁺ M2-type TAMs were more highly immunostained in IBC tumor ($p < 0.05$, Fig. 5A, B). Accordingly, we further verified whether CD163⁺ M2-type TAM infiltration would associate with heparanase expression. Indeed, as depicted in Fig. 5C, D we found a significant positive Pearson's correlation between heparanase and CD163⁺ protein expression ($n = 10$, $r = 0.81$, $p = 0.004$) in IBC, but not in non-IBC carcinoma tissues ($n = 10$, $r = 0.461$, $p = 0.18$).

The secretome of axillary tributaries CD14⁺ monocytes of IBC and rh IL-10 augment the expression of heparanase mRNA and protein in IBC SUM149 cells

To understand the potential role that may be played by TAMs in regulating expression of heparanase mRNA in IBC and non-IBC, we stimulated MDA-MB-231 and SUM149 cell lines with the secretome of CD14⁺ monocytes isolated from

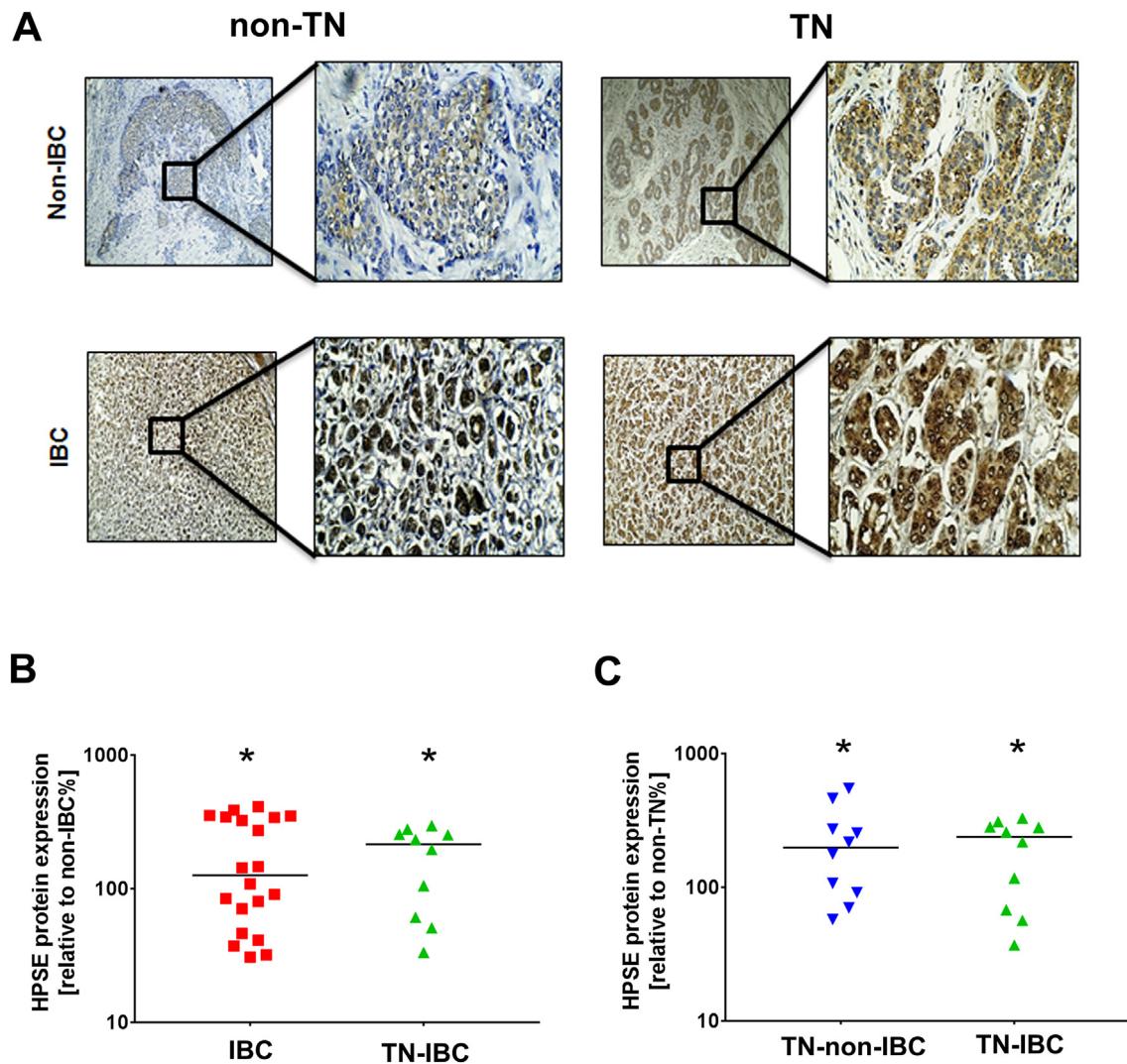


Fig. 2. Immunohistochemical detection of heparanase (HPSE) protein expression in carcinoma tissues of non-IBC and IBC. (A) Representative fields of immunostaining (brown color) of heparanase protein in the TN- and non-TN-subgroups of both non-IBC and IBC. The original magnifications on the left and right panels are 100 \times and 400 \times , respectively. (B) Quantitative analysis of the area fraction of positive heparanase immunostaining in carcinoma tissues of IBC ($n = 20$) and TN-IBC ($n = 10$) relative their non-IBC counterparts (values are set at 100%) as assessed by NIH Image J. (C) Quantitative analysis of the area fraction of positive heparanase immunostaining in carcinoma tissues of TN-non-IBC ($n = 10$) and TN-IBC ($n = 10$) relative to their non-TN-counterparts (values are set at 100%) as assessed by NIH Image J. * $p < 0.05$ as determined by Student's t -test.

non-IBC and IBC patients, respectively, and subsequently assessed the expression of heparanase transcript levels using qPCR. Our data revealed that basal heparanase mRNA expression was significantly downregulated in SUM149 as compared to MDA-MB-231 cell lines ($p < 0.01$, Fig. 6A). Interestingly, SUM149 heparanase mRNA expression was significantly increased by 1.78-fold upon stimulation with the secretome of IBC CD14 $^{+}$ cells compared to unstimulated control cells ($p < 0.05$, Fig. 6B).

However, this effect was not observed in MDA-MB-231 cells when stimulated with secretome of non-IBC CD14 $^{+}$ cells.

We then analyzed the contributing factors by which secretome of IBC CD14 $^{+}$ monocytes may enhance heparanase expression. A previous study has reported that relative to non-IBC, CD14 $^{+}$ monocytes isolated from blood drained from axillary vein tributaries of IBC secrete significantly higher levels of chemotactic cytokines, including

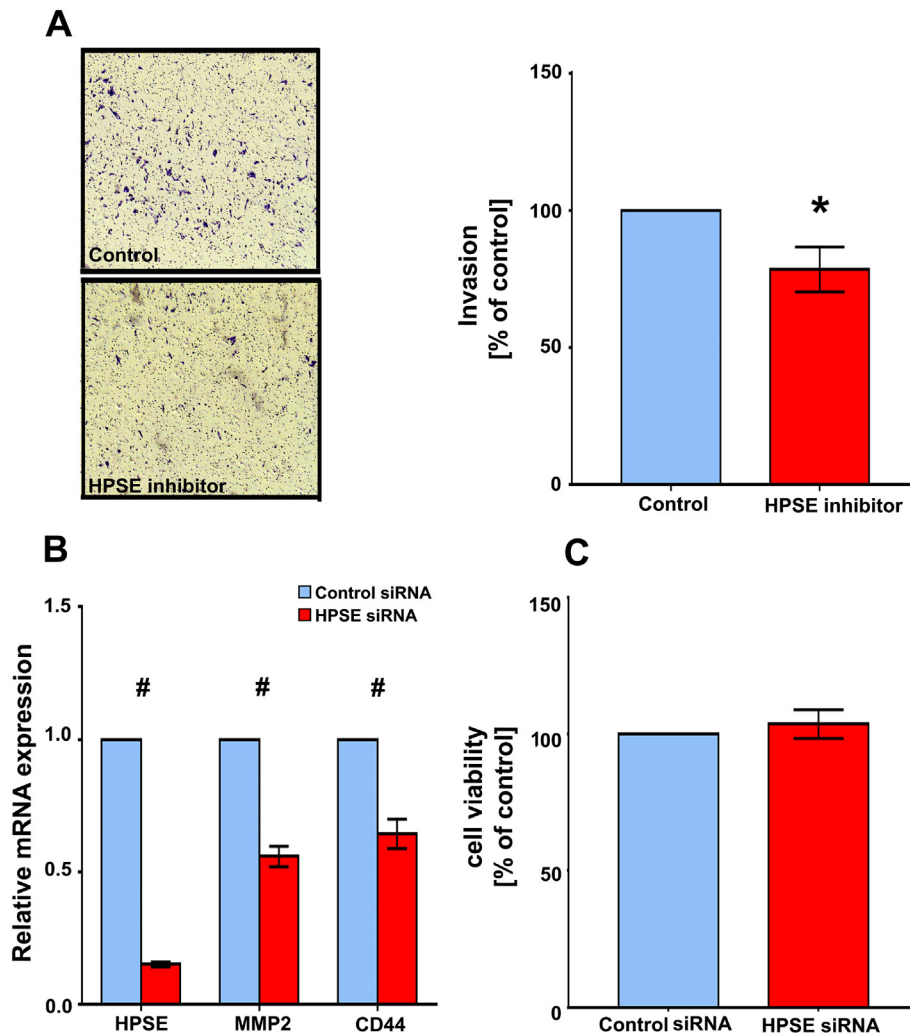


Fig. 3. Influence of pharmacological and siRNA-mediated heparanase (HPSE) inhibition on SUM149 cell behavior. (A) Left panel: representative images of stained matrigel coated-invasion inserts; right panel: quantitative analysis of invading control and OGT 2115-treated SUM149 cells. Treatment of SUM149 cells with 1 μ M OGT 2115 for 24 h significantly inhibits matrigel invasiveness. Data represent the mean \pm SEM, $n = 3$. * $p < .05$ as determined by Student's t -test. (B) HPSE expression knockdown results in a significant downregulation of CD44 and MMP-2 mRNA expression levels relative to control cells. Data represent the mean \pm SEM, $n = 3$. # $p < 0.001$, * $p < 0.05$ determined by Student's t -test. (C) Heparanase expression silencing has no effect on SUM149 cell viability as determined by MTT assay. Data represent the mean \pm SEM, $n = 3$.

interleukin-10 (IL-10) and tumor necrosis factor- α (TNF- α) [30]. Data of the same study revealed that IL-10 was the most potent candidate cytokine that significantly enhanced SUM149 cell invasion and motility [30]. Therefore, we assessed heparanase mRNA expression in SUM149 cells stimulated with rh IL-10 compared to controls, using qPCR. We found that IL-10 caused a mild but significantly increase in heparanase mRNA expression by 1.2-fold as compared to unstimulated SUM149 cells ($p < 0.05$, Fig. 6C). Accordingly, we verified these data at the protein level by Western blot. Our data uncovered that heparanase protein expression was increased by 1.4-fold in IL-10-treated SUM149 cells as compared to unstimulated control cells (Fig. 6D).

IL-10 mRNA expression is increased, and shows a positive correlation with heparanase expression in carcinoma tissues of IBC

To validate the relevance of our findings in a clinical setting, we analyzed the mRNA expression level of *IL-10* and *TNF- α* in IBC tissue specimen compared to non-IBC patients by qPCR. Relative to non-IBC, the mRNA expression level of *IL-10* and *TNF- α* was upregulated by 30.53- and 2.69-fold in carcinoma tissues of IBC, respectively (both $p < 0.05$, Fig. 7A). Intriguingly, as depicted in Fig. 7B, C there was a trend for a positive Pearson's correlation between *IL-10* and heparanase transcripts in IBC ($n = 14$, $r = 0.494$, $p = 0.072$), but not in non-IBC

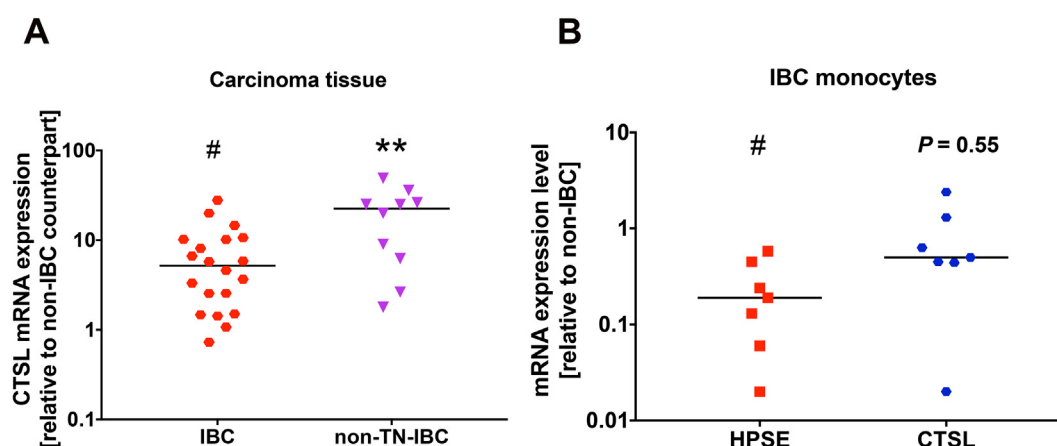


Fig. 4. The transcript levels of the heparanase (HPSE)-processing protease cathepsin L (*CTSL*) are upregulated in carcinoma tissues, but not in monocytes obtained from the axillary tributaries of IBC relative to non-IBC patients. Cathepsin L mRNA expression was quantified by qPCR. (A) Cathepsin L mRNA expression is higher in carcinoma tissues of IBC ($n = 20$) and non-TN-IBC ($n = 10$) relative to their non-IBC counterparts (Values are set at 1). # $p < 0.001$ and ** $p < 0.01$ as determined by Student's *t*-test. (B) mRNA expression levels of heparanase and cathepsin L in monocytes obtained from axillary tributaries of IBC- relative to non-IBC-patients. Heparanase mRNA expression is significantly downregulated in monocytes of IBC ($n = 7$) vs. non-IBC patients ($n = 7$; values are set at 1). # $p < 0.001$ as determined by Student's *t*-test.

carcinoma tissues ($n = 14$, $r = -0.063$, $p = 0.831$). However, we found no a significant correlation between *TNF- α* and heparanase mRNA levels in both groups (Data not shown).

Discussion

As heparanase represents an important link between inflammation and cancer in different experimental models and clinical settings, such as chronic gastritis to intestinal-type gastric carcinoma [31], chronic hepatitis C to hepatocellular carcinoma [32], pancreatitis to pancreatic adenocarcinoma [33] and colitis to colorectal cancer [26], it may emerge as a biomarker for IBC. The current study demonstrates for the first time a higher transcript level and more positive immunostaining of heparanase in carcinoma tissues of IBC vs. non-IBC patients. This result is in agreement with previous studies demonstrating that heparanase is upregulated in several human tumors, including breast carcinoma [22,25]. In addition, augmented heparanase expression is associated with poor prognostic markers, such as increased lymph node metastasis, larger tumor size, higher histological grade, and poor survival [22].

Compared to other breast cancer subtypes, TNBC is considered the most lethal subtype of breast cancer [34] because of its aggressiveness and high metastatic capacity [35]. In this study, qPCR and immunohistochemistry results showed that heparanase was overexpressed in both TN-IBC and TN-non-IBC as compared to their non-TN-counterparts. Importantly, we observed a higher heparanase protein expression in carcinoma tissues of TN-IBC

compared to TN-non-IBC. Given the positive association between expression of heparanase and mammary tumor growth and metastasis [36], and that heparanase overexpressing TNBC cells possess the unique clinical characteristic of forming clusters that mediate their metastatic potential [37], this may suggest the aggressiveness of TN-IBC is attributed to the elevated heparanase expression. This supposition is corroborated by our *in vitro* mechanistic experiments. Our data indicate that SUM149 cell invasion was significantly reduced upon treatment with the heparanase inhibitor OGT2115, implying that heparanase activity at least in part regulates invasive growth of IBC cells, consistent with the tumor promoting role of heparanase in different tumor entities [38–40]. In an attempt to decipher the molecular clues underlying heparanase mechanism of action, our qPCR data indicate that heparanase silencing was associated with downregulation of the ECM degrading enzyme MMP2 and the cancer stem marker CD44 [41] in SUM149 cells. This is the first report underscoring the effect of heparanase expression on CD44, which is overexpressed in IBC carcinoma tissues [6]. This suggests that heparanase may act as a modulator of cancer stem function along with Syndecan-1 in IBC [6], which is consistent with the role for heparanase in promoting cancer stemness and *in vivo* tumorigenesis in myeloma [41]. Moreover, another report showed that the concurrent expression of heparanase and CD44v3 may be mechanistically linked to cancer invasion and metastasis in colon cancer patients [42]. Another important molecule that mediates ECM remodeling and breast cancer cell invasion and metastasis is MMP-2 [35,43]. Our

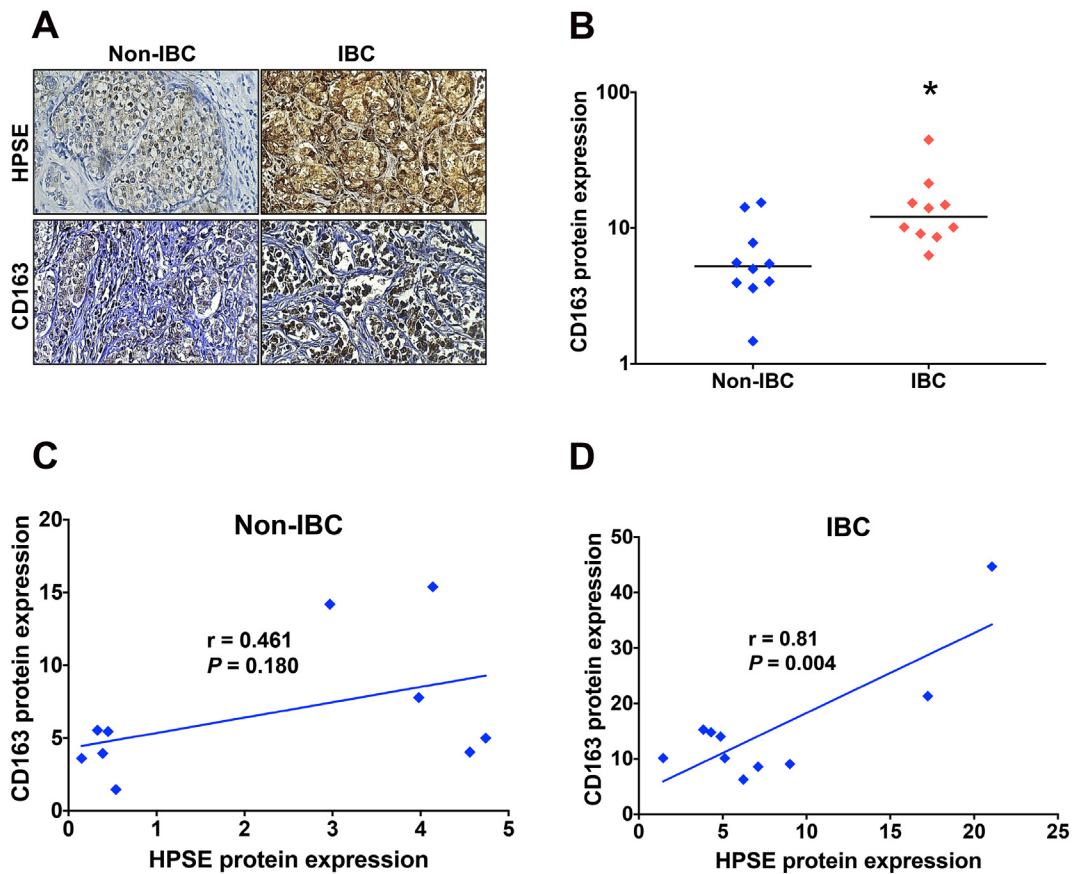


Fig. 5. Heparanase (HPSE) protein expression is positively correlated with CD163+ M2-type-TAM infiltration. (A) Representative fields of immunostaining (brown color) of heparanase and CD163+ M2-type TAM in carcinoma tissues of non-IBC and IBC. The original magnification is 400x. (B) Quantitative analysis of the area fraction of positive CD163 immunostaining in carcinoma tissues of IBC vs. non-IBC. * $p < 0.05$ as determined by Student's *t*-test. Pearson's correlation between heparanase and CD163+ protein expression in carcinoma tissues of non-IBC (C) and in those of IBC (D).

finding that MMP-2 expression is reduced upon heparanase depletion in SUM149 cells contradicts the notion of a marked decrease in MMP-2 expression in heparanase overexpressing MDA-MB-231 cells [44], suggesting heparanase may have a cell-type and context-dependent effect in IBC (SUM149) vs. non-IBC (MDA-MB-231) cells.

As the lysosomal cysteine protease cathepsin L is the only mammalian activating enzyme for heparanase [28], we assessed the mRNA expression level of cathepsin L in breast cancer tissues. We found a significantly elevated expression of cathepsin L mRNA level in IBC carcinoma tissues in comparison with those with non-IBC. It has previously demonstrated that cathepsin L mRNA levels were upregulated in carcinoma tissue specimens of TN-compared to non-TN-non-IBC patients [45]. Analogously, we assessed the mRNA expression of cathepsin L in carcinoma tissue of non-TN- and TN-IBC patients. Surprisingly, our qPCR data

revealed that cathepsin L expression was not significantly different; suggesting that expression of cathepsin L mRNA is high in carcinoma tissues of IBC irrespective of its molecular subtype. This is in accordance with the notion that immunohistochemical staining of cathepsin L expression in breast cancer tissue specimens is associated with advanced tumor stages [46], a clinical feature of IBC. We and others have shown that IBC tissues are characterized by enrichment with TAMs [30], which secrete high levels of the pro-inflammatory cytokine IL-6 [47]. Notably, IL-6 has a critical role in enhancing cathepsin L promoter activity and synthesis in lung epithelial cells [48]. Moreover, TAMs release augmented levels of cathepsin L into the tumor microenvironment during heparanase-induced inflammation [25]. Our qPCR data showed no significant difference for cathepsin L mRNA expression level in CD14+ monocytes isolated from both non-IBC and IBC, suggesting that the observed

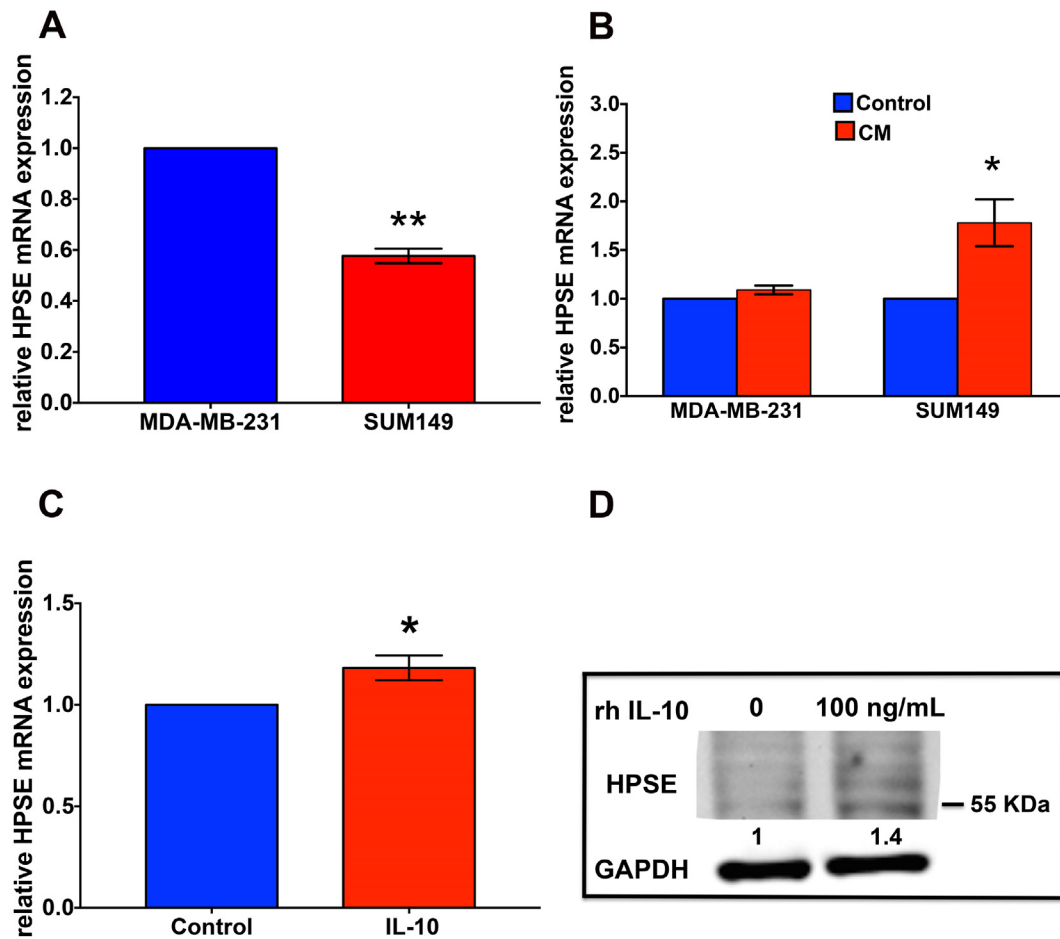


Fig. 6. Effect of cytokines secreted by axillary tributary-derived CD14⁺ monocytes isolated from IBC and non-IBC, and effect of IL-10 on expression of heparanase (HPSE) in SUM149 and MDA-MB-231 breast cancer cell lines. (A) Basal mRNA expression of heparanase in SUM149 vs. MDA-MB-231 cell lines. Data represent the mean \pm SEM, $n \geq 3$, ** $p < 0.01$ as determined by Student's *t*-test. (B) The secretome of CD14⁺ monocytes isolated from patients with IBC upregulated heparanase mRNA level in SUM149 cells relative to unstimulated control cells. Data represent mean \pm SEM, $n \geq 3$, * $p < 0.05$ as determined by Student's *t*-test. (C) IL-10 enhanced the expression of heparanase mRNA as assessed by qPCR in SUM149 cells relative to unstimulated control. Data represent mean \pm SEM, $n \geq 3$, * $p < 0.05$ as determined by Student's *t*-test. (D) Heparanase protein expression was upregulated post 48 h upon stimulation with 100 ng/mL rh IL-10 in SUM149 cells. Data shown are a single experiment representative of two independent experiments.

upregulation of cathepsin L mRNA in carcinoma tissues of IBC may be confined to cancer cells. However, we cannot rule out the possibility of altered expression of cathepsin L mRNA in CD14⁺ monocytes when they infiltrate and interact with tumor cells. Overall, cathepsin L may represent an additional marker for IBC patients.

In contrast to the *in vivo* findings, our qPCR data revealed the basal mRNA level of heparanase was significantly elevated in non-IBC MDA-MB-231 cells compared to IBC SUM149 cells. Moreover, IBC CD14⁺ monocytes displayed a significantly reduced level of heparanase mRNA in comparison with non-IBC CD14⁺ monocytes. One of the most frequent players in the tumor microenvironment are TAMs that have the ability to stimulate heparanase expression during

cancer-induced inflammation [26]. Interestingly, our immunohistochemical analysis showed that carcinoma tissues of IBC displayed a significantly higher infiltration of CD163⁺ M2-type TAMs than those of non-IBC. This is in agreement with recent data indicate that CD163⁺ M2 macrophages were higher infiltrated in IBC tumors compared to normal breast tissue controls [29]. Additionally, the same study showed that IBC have elevated levels of many monocyte recruitment and macrophage polarization factors that attract and augment differentiation of monocytes into immune-suppressing M2-like macrophages [29]. Although heparanase facilitates M1 polarization in U937 and renal macrophages induced by ischemia/reperfusion injured tubular cells [49], we detected a significant positive correlation between heparanase protein

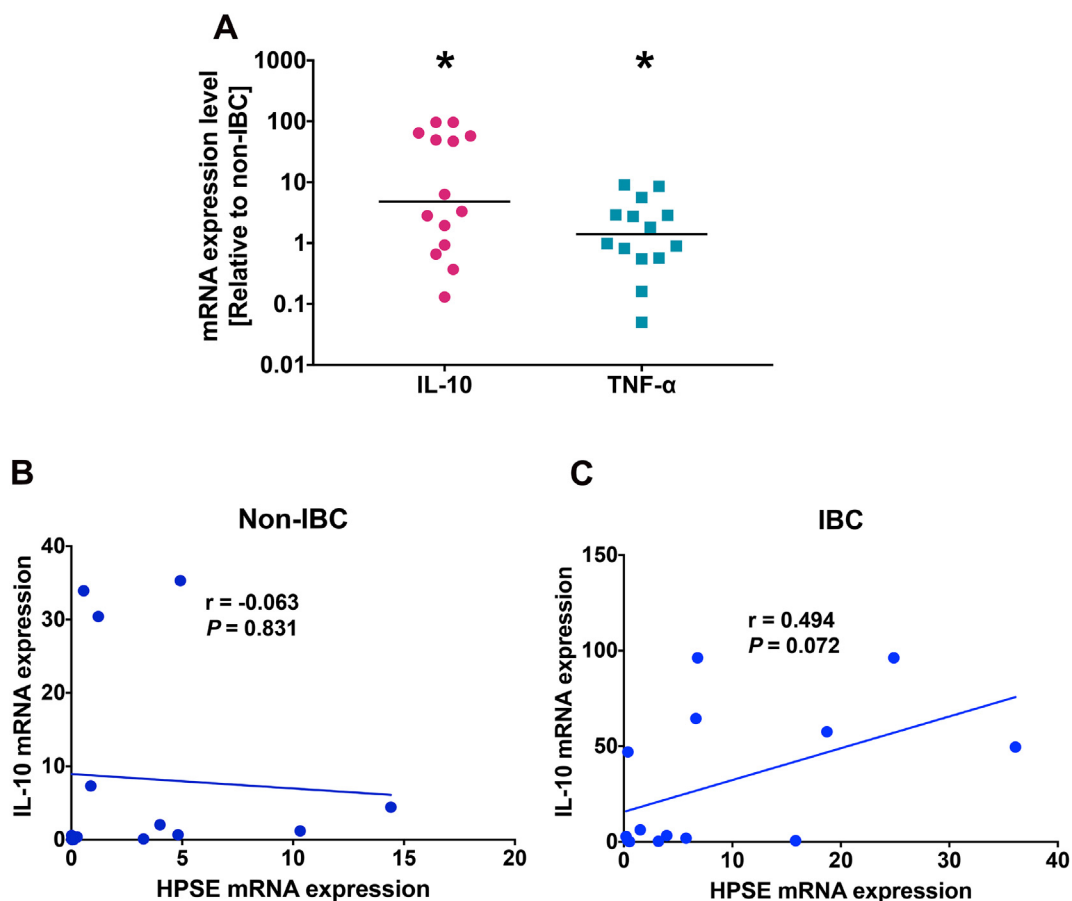


Fig. 7. mRNA expression levels of *IL-10* and *TNF- α* in carcinoma tissues of IBC vs. non-IBC patients. Expression of *IL-10* and *TNF- α* mRNA levels in breast carcinoma tissues was assessed by qPCR. (A) Expression of *IL-10* and *TNF- α* mRNA levels in carcinoma tissues of IBC ($n = 14$) relative to those of non-IBC (values are set to 1). * $p < 0.05$ as determined by Student's *t*-test. Pearson's correlation between heparanase (*HPSE*) and *IL-10* mRNA expression levels in carcinoma tissues of non-IBC (B) and IBC (C).

expression and CD163+ M2-macrophage infiltration in IBC carcinoma tissues. This is in line with a study showing that overexpression of heparanase in experimental and clinical settings of human pancreatic ductal adenocarcinoma results in a high infiltration of TAMs with a pro-cancerous phenotype (i.e. IL-10 secretion) [50]. This suggests that heparanase expression and/or activity may have a differential impact on macrophage polarization in the context of disease type, e.g. acute injury vs. tumor entity. These findings were further confirmed by ex vivo stimulation experiments. Heparanase mRNA levels were elevated in SUM149 cells when stimulated with the TAM secretome of IBC patients compared to unstimulated cells. However, this finding was not observed in MDA-MB-231 cells when stimulated with the TAM secretome of non-IBC patients. Previously, the secretome composition of CD14+ monocytes of IBC vs non-IBC was profiled for its cytokine composition [30]: Relative to non-IBC, blood drained CD14+ cells isolated from axillary vein tributaries of IBC secrete significantly higher levels of TNF- α , monocyte chemoattractant protein-1/CC-che-

mokine ligand 2, IL-8 and IL-10 [30]. Previous studies have reported the relevance of those cytokines/chemokines in regulating heparanase expression and/or activity in different experimental models [50,51]. Consistent with the enhanced invasive phenotype of IL-10-stimulated SUM149 cells [30], IL-10-treated SUM149 cells showed elevated levels of heparanase mRNA and protein expression. Of note, our in vitro results were validated in clinical carcinoma tissue samples. In accordance with our previous findings [52], our qPCR data showed massively elevated levels of IL-10 mRNA with a trend for positive correlation with heparanase mRNA expression in carcinoma tissues of IBC. This correlation was not observed in non-IBC carcinoma tissues. Although TNF- α mRNA was upregulated in carcinoma tissues of IBC relative to those of non-IBC, no correlation was observed with heparanase mRNA. Earlier studies indicated that heparanase augments the release of pro-inflammatory cytokines (e.g. IL-10 and TNF- α) from human peripheral blood mononuclear cells [53]. Overall, these findings suggest a potential role of IL-

10 in regulating heparanase expression and that regulation may be occur in a reciprocal and self-sustained manner between tumor cells and TAMs in IBC patients.

Numerous studies addressed the application of heparanase inhibitors as an anti-cancer therapy [38,54] as well as the potential implications of heparanase in cancer immunotherapy [55]. For example, PG545 (pixatimod) has been used as a potent anti-lymphoma drug [38] and in a phase I clinical trial [54] for advanced solid tumors. In addition, other reports focused on cathepsin L inhibitors such as 2,5-Diaryloxadiazoles [56] and benzoylbenzophenone thiosemicarbazone analogues [57] as anti-cancer agents. This supports the relevance of our findings that may provide potential treatment strategies using heparanase inhibitors or a combination with cathepsin L inhibitors for IBC patients.

In conclusion, this study demonstrates that carcinoma tissues of IBC displayed upregulated expression of heparanase, cathepsin L, and *IL-10* mRNA levels relative to those of non-IBC. Expression of heparanase protein was positively associated with the high infiltration of CD163+ M2-type TAMs and that expression is regulated by IL-10, one of the predominant cytokines secreted by CD14+ monocytes isolated from IBC. While, HSPE and cathepsin L may emerge as tissue biomarkers and new druggable targets along with IL-10 in IBC, more studies are still required to validate our findings in a large cohort of IBC patients.

Methods

Patients' samples

In this study, we enrolled 40 breast cancer patients from the breast clinic of Ain Shams university hospitals; all patients were candidate to curative surgery (whether modified radical or conservative mastectomy). Half of the patients were diagnosed as IBC and the rest as non-IBC. According to breast cancer molecular subtypes, each of the IBC and non-IBC groups were divided into 2 subgroups; TN ($n = 10$) and non-TN patients ($n = 10$). Fresh tumor tissue specimen from every patient was divided into two parts: the first part was fixed in 10% formalin buffered for immunohistochemical staining and the other part was frozen in $-80\text{ }^{\circ}\text{C}$ for subsequent isolation of total RNA.

Reagents and supplies

Unless otherwise stated, all reagents and supplies were purchased from Sigma (Deisenhofen, Germany).

Quantitative real-time PCR

Total RNA was isolated from cultured cells and fresh or frozen tissues using the GeneJET RNA Purification Kit (Thermo Fisher Scientific, USA), and $1\text{ }\mu\text{g}$ RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's instructions. Quantitative real-time PCR and melting curve analysis of heparanase (#QT00009555, Qiagen, Hilden, Germany), heparanase (upstream 5'-GAATGGACGGACTGCTACCAAG-3' and downstream 5'-CTCCTAACCCAGACCTTCTTGCC-3', Biologio BV, Nijmegen, the Netherlands) cathepsin L (#QT01664978, Qiagen, Hilden, Germany), *CD44* (upstream 5'-GCCCATGTTTCATTCTTGCT-3' and downstream 5'-AAACCAGAGGAAGGGTGTGCTC-3', Biologio BV, Nijmegen, the Netherlands) and *MMP2* (upstream 5'-AGCGAGTGGATGCCGCTTTAA-3' and downstream 5'-CATTCCAGGCATCTGCGATGAG-3', Biologio BV, Nijmegen, the Netherlands) gene products were performed using SYBRTM Green PCR Master Mix (Applied Biosystems, Foster City, USA) in a StepOnePlus Real-Time PCR System (Applied Biosystems). Each sample was initially denatured at $95\text{ }^{\circ}\text{C}$ for 10 min, then subjected to 40 cycles of the following: Denaturation at $95\text{ }^{\circ}\text{C}$ for 15 s, annealing at $55\text{ }^{\circ}\text{C}$ for 30 s and extension at $60\text{ }^{\circ}\text{C}$ for 30 min, and subsequently exposed to melting curve cycle step one $95\text{ }^{\circ}\text{C}$ for 15 s, step 2 $60\text{ }^{\circ}\text{C}$ for 1 min and step 3 $95\text{ }^{\circ}\text{C}$ for 15 s, followed by amplification and melting curve generation. After normalization to GAPDH (#QT00079247, Qiagen, Hilden, Germany) for tissue sample and to 18S (upstream 5'-AACCCGTTGAACCCATT-3' and downstream 5'-CCATCCAATCGGTAGTAGCG-3') for cell lines and monocytes, the $2^{-\Delta\Delta\text{Ct}}$ method was used to determine relative gene transcript levels as described before [58].

Immunohistochemistry

Immunohistochemical staining of heparanase in breast carcinoma tissues was performed as described previously [6] using the EnVisionTM FLEX Target Retrieval Solutions (Dako, Glostrup, Denmark). FFPE tissues were deparaffinized in xylene and rehydrated through a descending series of ethanol to water. Slides were incubated in citrate buffer (pH = 6) as an antigen-retrieval step in a steamer for 30 min. After washing with distilled water, tissue sections were blocked with hydrogen peroxide solution for 10 min then with 1% BSA/TBST for 1 h. Subsequently, slides were incubated overnight in a humid chamber with anti-human heparanase (Abcam, UK) (1:100) or anti-human CD163 (Santa Cruz Biotechnology, Texas, USA)

(1:100) primary antibodies diluted in 1% BSA/TBST. Detection was carried out by incubating tissue sections with HRP-labeled rabbit secondary antibody (DAKO) for 1 h at room temperature and staining was developed with 3,3'-diaminobenzidine (DAB) chromogen (DAKO) for ~3 min. Finally, the slides were counterstained by Mayer's hematoxylin (Merck, Darmstadt, Germany) then dehydrated in ascending series of ethanol, cleared by xylene and mounted with DPX mounting media. Negative control slides were run in parallel where primary antibodies were omitted and replaced by 1% BSA/TBST. The stained slides were analyzed semi-quantitatively using ImageJ software (National Institutes of Health, Bethesda, MD, USA) as we described before [6].

Blood sample collection, TAM isolation and preparation of conditioned media

About 10–15 ml blood was collected from tumor microenvironment of IBC or non-IBC patients, through axillary tributaries during surgery in heparinized tubes. Collected blood was transferred directly to the laboratory for isolation of tumor-associated macrophages (TAMs) using density-gradient centrifugation as we described before [30]. Briefly, mononuclear cells were suspended in RPMI 1640 medium containing 1% heat-inactivated FBS at density of 1×10^6 cells/ml. A total of 2 ml of the cell suspension was added to each plate and incubated in a humidified incubator at 37 °C, 5% CO₂. On the next day, the medium with non-adherent cells was removed and the adherent monocytes were washed with 2 ml PBS. Adherent monocytes/macrophages were cultured overnight RPMI-1640 media containing 1%FBS for 24 h. Media conditioned by TAM secretions were collected and 5-fold concentrated using Amicon Ultracell10K filters (Millipore, Billerica, MA).

Stimulation of cell lines with TAM-conditioned media or rh IL-10

Concentrated conditioned media of TAMs isolated from axillary tributaries of IBC patients ($n = 15$) and non-IBC patients ($n = 15$) were respectively re-diluted with cell culture media specific for each cell line to equal protein content as described before [59]. SUM149 and MDA-MB-231 cells were seeded at 2.5×10^5 cells/ml in complete culture media of HAM's-F12 containing 5% FBS and DMEM containing 10% FBS in a humidified atmosphere of 5% CO₂ at 37 °C, respectively. At 80% confluence, the cells were washed with PBS and seeded in media with 1%FBS and equal protein content of secretion of TAMs isolated from IBC and non-IBC patients. Control cells were seeded in RPMI with 1% FBS and run in parallel at the same conditions for those cells seeded in TAMs-CM. Analogously, the previous steps were

performed for incubation in absence or presence of 100 ng/mL rh IL-10 (Immunotools, Friesoythe, Germany) for 24 h or 48 h and SUM149 cells were lysed for qPCR or Western blot, respectively.

siRNA-mediated knockdown of heparanase expression

siRNA knockdown was performed essentially as described [6] using a negative control siRNA (negative control #1, Ambion, Cambridgeshire, UK) and validated siRNA S21304 (Ambion) to target exon 7 of human heparanase (accession # NM_001098540.2). SUM149 cells were transfected with 20 nM siRNA using Dharmafect reagent (Dharmacon, Lafayette, CO, USA) according to the manufacturer's instructions. Successful knockdown was confirmed by qPCR.

MTT cell proliferation assay

Metabolic activity as a readout of cell viability was determined using 3-(4,5-dimethyl-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT). siRNA-transfected SUM149 cells were seeded in 96-well plates (1×10^4 cells/well) 48 h after transfection, and cultured for an additional 48 h. MTT assay was performed as described previously [15], measuring the optical density at 595 nm in a microplate reader.

Matrigel Invasion chamber assay

Matrigel invasion chamber (BD Biosciences, Heidelberg, Germany) assays were performed essentially as described before [15] in the presence and absence of 1 μM heparanase inhibitor OGT 2115 (2-[4-[[3-(4-Bromophenyl)-1-oxo-2-propenyl]amino]-3-fluorophenyl]-5-benzoxazoleacetic acid; Cat.no. 2710, TOCRIS bioscience, Wiesbaden-Nordenstadt), using an invasion time of 48 h. Relative invasiveness was expressed as percentage of cells on compound-treated compared to control inserts ($n > 3$).

SDS-PAGE and Western blot

Control and stimulated SUM149 cells were lysed in RIPA buffer containing protease inhibitor cocktail as we previously described [58]. Total protein content was determined with BCA method and 50 μg protein /lane was loaded into 10% SDS-PAGE. After the semi-dry transfer step, nitrocellulose membrane was blocked with 5% skimmed milk/TBST solution, washed thrice with TBST, and probed with anti-human heparanase antibody (1:500; ab85543, Abcam, UK) overnight at 4°C. Following 1 h incubation at room temperature with HRP-conjugated secondary antibody, the membrane was subjected to a chemiluminescence reaction and signal quantification was performed with NIH ImageJ software. Subsequently, the

membrane was stripped and incubated with anti-GAPDH (1:1000, Santa Cruz Biotechnology, Texas, USA) as housekeeping controls for normalization.

Statistical analysis

All data were analyzed using IBM SPSS version 18 (Chicago, IL, USA) and GraphPad Prism 4.02 (GraphPad Software, La Jolla, CA). Data were presented as mean \pm SEM or SD as indicated. Fisher's exact test was used to evaluate differences between variables. Unpaired Student's *t*-test was used to evaluate the significance between two groups. Correlations were conducted using Pearson's Rank correlation test. Data were considered significant at $p < 0.05$.

Author contributions

HH, BG, MG, and SAI conceived and designed experiments. MN, HH, MSE, EN, YMI, SAI conducted experiments. MN, HH, MSE, EN, YMI, MA, ME, and SAI analyzed data. YMI analyzed the clinical data. MN, HH, YMI, BG, MG, and SAI drafted/edit the manuscript. MG, BG, and SAI supervised the study. All authors have read and approved the final version of the manuscript.

Ethical approval

This study was approved by Institutional Review Board (IRB#00006379) from research ethics committee of General Surgery Department, Faculty of medicine, Ain Shams University, Cairo, Egypt. All patients enrolled in this study signed written informed consent to participate in this study.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CTSL, cathepsin L; ECM, extracellular matrix; ER, estrogen receptor; FFPE, Formalin-Fixed Paraffin-Embedded; HER-2, human epidermal growth factor receptor-2; HPSE, heparanase; HSPGs, heparan sulfate proteoglycans; IBC, inflammatory breast cancer;; rh IL-10, recombinant human interleukin-10; IRB, Institutional Review Board; MMP2, matrix metalloproteinase2; MTT, 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide; OGT 2115, 2-[4-[[3-(4-Bromophenyl)-1-oxo-2-propenyl]amino]-3-fluorophenyl]-5-benzoxazoleacetic acid; PR, progesterone receptor; qPCR, quantitative real-time PCR; TAMs, tumor-associated macrophages; TN, triple negative; TNF- α , tumor necrosis factor- α .

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