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IL-8 secreted by tumor associated macrophages contribute to lapatinib resistance in HER2-positive locally advanced breast cancer via activation of Src/STAT3/ERK1/2-mediated EGFR signaling

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ABSTRACT

Locally advanced breast cancer (LABC) is an aggressive disease characterized by late clinical presentation, large tumor size, treatment resistance and low survival rate. Expression of EGFR/HER2 and activation of intracellular tyrosine kinase domains in LABC are associated with poor prognosis. Thus, target therapies such as the anti-receptor tyrosine kinases lapatinib drug have been more developed in the past decade. The response to lapatinib involves the inhibition of RTKs and subsequently signaling molecules such as Src/STAT3/Erk1/2 known also to be activated by the cytokines in the tumor microenvironment (TME). The aim of the present study is to identify the major cytokine that might contribute to lapatinib resistance in EGFR+/HER2+ LABC patients. Indeed, tumor associated macrophages (TAMs) are the main source of cytokines in the TME. Herein, we isolated TAMs from LABC during modified radical mastectomy (MRM). Cytokine profile of TAMs revealed that IL-8 is the most prominent highly secreted cytokine by TAMs of LABC patients. Using in-vitro cell culture model we showed that recombinant IL-8 (50 and 100 ng/mL) at different time intervals interfere with lapatinib action via activation of Src/EGFR and signaling molecules known to be inhibited during treatment. We proposed that to improve LABC patients' response to lapatinib treatment it is preferred to use combined therapy that neutralize or block the action of IL-8.

1. Introduction

Locally advanced breast cancer (LABC) is one of the most advanced breast cancers is an aggressive disease due to the high rate of metastasis, locoregional and failure of systematic therapy including chemotherapy, hormonal and targeted therapies [18]. The incidence of LABC is still high in socially disadvantaged and minority women in USA. Similarly, the incidence of LABC is increasing in the developed countries with disproportionately high mortality rate [4]. Genomic studies revealed that co-expression of epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) are detected in LABC and metastatic breast cancer [48]. EGFR and HER2 comprises receptors that are transmembrane glycoproteins containing an extracellular ligand binding domain and intracellular receptor tyrosine kinase domain [57]. EGFR and HER2 co-expression found to be phosphorylated by membrane associated Src kinases [2,55].

The EGFR and HER2 activate different signaling pathways in normal and malignant breast cancer, as they have shown an elevated stability complex formed between two receptors and ligands, when compared to monomeric receptors [28]. Thus, LABC patients with HER2+ mostly

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receive neoadjuvant chemotherapy such as paclitaxel and EGFR tyrosine kinase targeted therapy including monoclonal antibodies and *tyrosine kinase* inhibitors (TKIs) as treatment protocol [33]. Lapatinib (4-anilinoquinazoline kinase) is a tyrosine kinase inhibitor for EGFR and HER2, it reduces motility and invasion of esophageal squamous cell carcinomas (ESCCs) and esophageal adenocarcinomas (EACs) via inhibition of Akt, signal transducer and activator of transcription (STAT) and extra signal regulated kinases (ERK1/2) [16]. Lapatinib action found to be via inhibiting Src phosphorylation sites on EGFR (Tyr845) and HER2 (Tyr877) [21]. Paradoxically, lapatinib resistance may be mediated via the activation of compensatory pathways such as Src family of non-receptor tyrosine kinase [58]. Src has been involved in the trans-activation of EGFR and can activate EGFR "in the absence of EGFR" ligand [29], leading to the phosphorylation of EGFR and activation of downstream signaling pathways [10].

A study compared the treatment response of LABC-HER2$^+$ to three target therapy the afatinib versus trastuzumab versus lapatinib revealed that the tyrosine kinase afatinib demonstrated the best clinical response compared to trastuzumab and lapatinib [53]. It is suggested that some patients developed resistance to lapatinib treatment, which occurs via various mechanisms; such as HER2 alterations, mutation of the target kinase, thus disrupting the drug binding site [37], activation of alternate factors including chemokine receptors [66] or through simultaneous activation of alternate pathways such as Src activation that modulates the activity of intracellular effectors such as PI3K/AKT and STAT3 consequently regulating the cancer cell migration and invasion [17,50]. Subsequently activation of Src/STAT3 and downstream signaling molecules has been described as a determinant of resistance to anti-EGFR drugs [17]. Despite the great promise of targeted therapies for the treatment of patients with breast cancer, de novo or acquired resistance remains major obstacles [15]. Interestingly, EGFR and HER2 found to be activated both directly through a specific ligand binding or indirectly through cross communication between the EGFR and HER2 utilized by G-protein coupled receptors (GPCR's) or inflammatory cytokines such as interleukin-8 (IL-8) [60]. IL-8 have been extensively found to be abundantly present, in breast cancer patients' serum and cancer tissues, proposing a remarkable "cytokine signature" [19], where the upregulation of IL-8 further activates the CXCR1/2 signaling via Src dependent pathway, Src is oncogenic signaling molecule overly expressed in multiple cancer types leading to the activation of STAT3 and ERK1/2 [57,60]. Indeed, overexpression of IL-8 in the tumor microenvironment (TME) augment tumor growth, metastasis rendering the tumor cells resistant to anti-EGFR drugs [23,30]. IL-8 induce invasive and metastatic properties in hormonal positive and hormonal negative breast cancer patients [14]. IL-8 plays a crucial role in breast cancer poor prognosis by inducing resistance of lung cancer cells to erlotinib a drug that inhibit activation of tyrosine kinase EGFR domain [15]. Previous studies showed that IL-8 was up-regulated in the EGFR-TKI gefitinib-resistant lung adenocarcinoma cell lines (PC9/gef) [36]. It should be noted that the action of gefitinib is similar to lapatinib it inhibits auto-phosphorylation of RTKs [24].

Tumor associated macrophages (TAMs) are the main source of IL-8 in the TME. Previously we showed that TAMs isolated from the TME of the aggressive phenotype inflammatory breast cancer (IBC) is characterized by over-expression and secretion of IL-8 that induce motility and invasion of breast cancer cell lines [43]. Using life cell imaging proteolytic assay, we recently showed that IL-8 induce the proteolytic activity and the expression of the cysteine proteases cathepsin B via activation of Src and Erk1/2 signaling molecules [40]. Furthermore, activation of Src and its signaling molecules found to be associated with treatment resistance to the anti-HER2 drug lapatinib in the HER2-positive BT-474 human breast cancer xenografts athymic mice [49]. In prostate cancer IL-8 suggested to induce carcinogenesis via the activation of Src and FAK [32]. Cellular Src (c-Src) found to regulate the activation of EGFR and its downstream "oncogenic signals" in different types of cancer [5].

Since TAM is the main source of IL-8 in the TME in the present study first we assessed the incidence of different macrophages subtypes in the TME of EGFR/HER2$^+$ LABC patients. Then we isolated CD14+ the most prominent TAMs from the TME of LABC patients during modified radical mastectomy, secretions of the isolated TAM were subjected to cytokine profiling using cytokine antibody array as we previously described [13,43]. IL-8 found to be the most prominent cytokine secreted by TAM. Using in-vitro cell culture models we elucidated the role of IL-8 in the indirect activation of EGFR/HER2 tyrosine kinases and associated signaling molecules. Our present results suggest that IL-8 indirectly activate Src, afterwards Src phosphorylates EGFR on tyrosine 845, in agreement with results published before [20,54]. Then p-EGFR stimulate the signaling molecules ERK1/2 and STAT3 in breast cancer cell lines treated with lapatinib. This was accomplished by measuring the phosphorylation of EGFR2/HER2/Src/Erk1/2 and STAT3 by the SKBR3 breast cancer cell line upon stimulation with different concentration of IL-8 at different time intervals in absence and presence of lapatinib. Additionally, we assessed the capabilities of IL-8 to induce the spheroid structures formation and invasion potential of SKBR3 cells in the absence and presence of lapatinib.

2. Materials and methods

2.1. Patients and samples

The present study was approved by the institutional Review Board (IRB) of Faculty of Medicine, Ain Shams University, Egypt and all participants signed informed consent that agree with publication of their anonymous data. Fifteen patients were diagnosed with LABC as described before [19,59] and as EGFR/HER2$^+$. HER2$^+$ diagnosed depended on the HER2 immunostaining [22] and Chromogenic In Situ Hybridization (CISH) analysis [27] (Fig. S1). While EGFR+ detected by quantitative real time polymerase chain reaction (RT-PCR) as described before [31].

2.2. Immunohistochemistry

To assess the infiltration of tumor associate macrophages (TAMs) in the carcinoma tissues of LABC we used immunohistochemistry (IHC). The staining was performed after chemical dewaxing of 4 μm thick formalin-fixed paraffin-embedded (FFPE) tissue sections as we described before [44,46] using CD14 antibody (1:50) (Chemicon, CA, USA), CD163 (1:500) (Abcam, Cambridge, UK) and CD68 (1:50) (M0814) from Dako (Agilent, CA, USA). The stained area fractions (brown color) were calculated using image J software (National Institutes of Health, Bethesda, MD, USA) as we described before [38,39].

2.3. Isolation of tumor associated monocytes/macrophages (TAMs) CD14$^+$ cells

During axillary dissection of modified radical mastectomy (MRM) operations, the surgeon (M.ES) withdraw 10–15 mL blood in a heparinized syringe with angular needle from the identified axillary tributaries [13]. After plasma separation, mononuclear cells were separated from the precipitated blood content by Ficoll-Hypaque density gradient centrifugation (Lonza, ME, USA) at 1500 rpm for 30 min and CD14$^+$ were purified from the mononuclear cells using "EasySep™ Human Monocyte Enrichment Kit without CD16 Depletion" (StemCell Technologies, VAN, Canada) as we described before [30]. Isolated TME CD14$^+$ monocytes/macrophages were seeded overnight at concentration of (1×10^6 cells/mL) in RPMI media with 1% of penicillin/streptomycin antibiotic mixture in 3% fetal bovine serum (FBS) and incubated at 37 °C in 5% CO_2 for 24 h. CD14$^+$ monocytes/macrophages secretome was collected and concentrated 1:100 using Vivaspin™ protein concentrator column (Sartorius, NI, Germany) with 10,000 molecular weight cutoff value (MWCO value) and its protein content was determined via Bradford assay (Biorad Laboratories, CA, USA) using

Infinite®200 PRO NanoQuant (Tecan, ZH, Switzerland).

2.4. Human cytokine antibody Array

Cytokines, chemokines and growth factors profile secreted by $CD14^+$ cells isolated from LABC-TME were identified quantitatively using RayBio™ human cytokine antibody array-3 (RayBiotech Life, GA, USA). Methods were conducted as described in the kit guidelines and as we described before [41]. Array membranes were exposed to X-ray film for different time intervals and the presence of each cytokine, chemokines and growth factors were represented as dots with different intensities and diameters. Calculation of relative density value of each cytokine was achieved by quantification of the different cytokines was achieved by densitometric methods using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.5. Treatment of SKBR3 cancer cells by recombinant IL-8 in the absence and presence of lapatinib

SKBR3 cell line is a human breast cancer expressing EGFR and HER2 receptors (gift from Prof. Bonnie F. Sloane, Pharmacology Department, Wayne State University, Detroit, 48,102, MI, USA). SKBR3 cells were cultured in DMEM medium with 10% fetal bovine serum and 1% of penicillin/streptomycin antibiotic mixture at 37 °C in 5% CO_2. SKBR3 cells (35×10^4 cells/well) were plated and at 80% confluence of SKBR3 cell line cultured media was removed, cells were washed twice with phosphate buffered saline (PBS) (Serva, HDB, Germany). Then cells were starved for 24 h in serum free culture media. After that, cells were seeded in media contained two different concentrations (50 and 100 ng/mL) of recombinant IL-8 (R&D Systems, MN, USA) for 15 and 30 min. After that the media was removed and cells were rinsed with ice cold PBS and scraped with lysis buffer (RIPA buffer with addition of protease and phosphatase inhibitors) (Serva, HDB, Germany). For studying the effect, the recombinant IL-8 on SKBR3 cells pretreated with lapatinib (gift from Dr. Julie Boerner, Department of Oncology and Pharmacology Wayne State University, Detroit, 48,201, MI, USA). SKBR3 cells were pretreated with 1 μL/mL lapatinib for 2 h as recommended before [60] followed by adding 50 and 100 ng/mL of recombinant IL-8 in the presence of lapatinib according to the methods mentioned above.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Immunoblotting

Protein concentration of cell lysates were determined by Bradford assay (Biorad Laboratories, Hercules, CA, USA) using Infinite®200 PRO NanoQuant (Tecan, ZH, Switzerland). Equal concentrations of protein (30 μg/μL) were loaded for each sample, then separated by 7.5% SDS-PAGE, followed by immunoblotting on Polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA.) as described before [40]. 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° C with 1:1000 diluted antibodies against p-EGFR $^{(Tyr845)}$, p-Src $^{(Y527)}$, p-MAPK p-44 (ERK1/2) $^{(T202/Y204)}$, p-STAT3 $^{(Y705)}$, and from (Cell Signaling, MA, USA), AB Rabbit and pAB to ErbB2 $^{(Y1248)}$ from (Abcam, CAM, UK) then washed and incubated for 1 h with 1:1000 diluted peroxidase-labeled goat anti-rabbit secondary antibody. After washing, the bands were visualized and detected proteins bands were analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA) that measures the density of each band using β-actin as a loading control as described before [40,43].

2.7. Effect of IL-8 on spheroid formation of SKBR3 pretreated with lapatinib

To assess spheroid formation, as an indicator of stem cell properties and a structure known to resist cancer treatment [51], the SKBR3 cells were grown on 3D culture, sterile glass coverslips were coated with 50 μL Cultrex® Basement Membrane Extract (BME) (Trevigen, MD, USA) and incubated in humidified atmosphere at 37 °C with 5% CO_2 for 15 min to solidify. SKBR3 cells were used at density of (1×10^4 per coverslip) and mixed with 2% BME before overlaying onto each coated coverslip and incubated for 40 min at 37 °C with 5% CO_2 to allow cell attachment and then culture media (DMEM with 10% FBS) was added and cells were incubated at 37 °C with 5% CO_2 for 24 h [42]. After that, culture media was replaced with media contained 100 ng/mL of recombinant IL-8 in the absence and presence of 1 μL/mL of lapatinib respectively to determine its effect. Cells incubated for 8 days and culture media was changed every 3 days. Control cells are those grown in complete culture media without lapatinib (control) or with lapatinib (control + lapatinib). Controls are subjected to the same conditions and grown in parallel to cells grown on media conditioned with IL-8 in the absence and presence of lapatinib. Coverslips were examined by phase contrast and average number of spheroids (>50 μm) were recorded and counted by Zeiss Axiovert microscope (Carl Zeiss, AG, Germany) [24].

2.8. Invasion assay

The SKBR3 cells (3×10^3 per well) were grown in the upper chamber of BD Bio-Coat Matrigel™ Invasion Chambers (Becton Dickinson Labware, NJ, USA), while the recombinant cytokines in absence and presence of lapatinib were added to the lower chamber. The concentrations used were as follows; 100 ng/mL of IL-8 and 1 μL of lapatinib for each mL. The cells were cultured in DMEM and 10% FBS for 24 h and cells were fixed and stained according to studies by [42]. Where the non-invasive cells attached to the upper chamber were removed with cotton swabs and the cells remaining on the lower side of the membrane were stained and counted using light microscopy. The mean number of invasive cells passed through the BD Matrigel was quantified by counting in five randomly selected microscopic fields using light microscopy. Data represent the mean number of cells that had invaded in response to IL-8 divided by the mean number of cells that had invaded in response to culture media (control) and multiplied by 100 as described before [3].

2.9. Statistical analysis

The Statistical Package of the Social Sciences software (SPSS, Chicago, IL, USA), version 22.0 was used for data analysis. The data were presented as the mean ± standard deviation (SD). In addition, Differences among two groups of variables were evaluated using Student's t and the Chi square tests. The statistical difference between more than two groups was evaluated using one-way ANOVA followed by Tukey's HSD Post Hoc tests. The level of significance was set at $P < 0.05$.

3. Results

3.1. Clinical and pathological characterization of $HER2^+$ LABC patients

Clinical and pathological data of $HER2^+$ LABC patients showed that all patients have tumor stage T3N1M0 and average tumor size 4.9 ± 1.6 cm. In addition, 80% of patients were grade 2 and 20% grade 3, 73.6% of patients have metastatic lymph nodes and 40% with positive lymphovascular invasion (Table 1).

3.2. High infiltration of $CD14^+$ monocytes/macrophages in the carcinoma tissues of $EGFR/HER2^+$ LABC patients

Previously we found high infiltration of $CD14^+$ monocytes in IBC carcinoma tissues compared to non-IBC [39]. Herein, IHC analysis of LABC patients' cancer tissues was used to determine the infiltration of $CD14^+$ monocytes having strong potential for differentiation into M1 and M2 macrophages [67] in the carcinoma tissues of $HER2^+$ LABC patients. Analysis of microscopic images analysis showed high

Table 1
Clinical and pathological characterization of HER2$^+$ LABC patients.

Characteristic	HER2$^+$ LABC ($N = 15$)
Age [year]	
Range	34–70
Mean ± SD	49.8 ± 7.03
Tumor size [cm]	
Mean ± SD	4.9 ± 1.6
Tumor grade	
G1	0 (0%)
G2	12 (80%)
G3	3 (20%)
Axillary lymph node metastasis	
Negative	4 (26.7%)
Positive	11 (73.3%)
Lymphovascular invasion	
Negative	9 (60%)
Positive	6 (40%)

Data are reported as means ±SD.

infiltration of CD14$^+$ monocytes (Fig. 1A). To determine the incidence of differentiated M1 and M2 macrophages in the tumor microenvironment of HER2$^+$ LABC tissues, IHC staining of CD68 as marker of M1 macrophages and CD163 as marker for M2 macrophages. The obtained results showed no significant difference in the prevalence of M1 and M2 macrophages in the tumor microenvironment of HER2$^+$ LABC tissues (Fig. 1B-D).

3.3. Cytokines profile of TAMs-CD14+ characterized by high secretions of IL-8

Cytokine array for the secretions of TAMs (CD14$^+$) isolated from EGFR/HER2$^+$ LABC patients showed that the highest expressed cytokine is IL-8. In addition, other cytokines such as regulated upon Activation, Normal T Cell Expressed and Presumably Secreted (RANTES) and Epidermal Growth Factor (EGF) cytokines are highly expressed but less than IL-8. On the other hand, Granulocyte colony-stimulating factor (GCSF) cytokine showed very low expression level (Fig. 1E and F).

3.4. IL-8 activates Src/EGFR/Erk1/2 and STAT3 signaling in SKBR3 breast cancer cell line

Study by Singh and colleagues have demonstrated that IL-8 signaling is mediated by Src and EGFR/HER2-dependent pathway [60]. Thus, we studied whether different concentrations of recombinant IL-8 (50 and 100 ng/mL) can modulate the phosphorylation level of Src, EGFR/HER2 and signaling molecules ERK1/2 and STAT3 by SKBR3 breast cancer cell line at different time intervals (15 and 30 min) using immunoblot assay (Fig. 2A). IL-8 (50 ng/mL) significantly ($P < 0.05$) induced the expression of p-EGFR$^{(Tyr\text{-}845)}$ after 30 mins. While, SKRB3 cells seeded in high concentration of IL-8 (100 ng/mL) showed a significant ($P < 0.05$) increase in the expression of the receptor tyrosine kinase p-EGFR$^{(Tyr\text{-}845)}$ after 15 and 30 mins (Fig. 2B and G). We did not detect any significant difference in the expression of p-EGFR2 (HER2$^{(Tyr\text{-}Y1248)}$) in SKRB3 cells seeded in different IL-8 concentrations (Fig. 2C and H) compared to control cells. Moreover, IL-8 (50 and 100 ng/mL) significantly ($P < 0.05$) induced the expression of p-Src $^{(Y527)}$, ERK1/2$^{(202/204)}$ and p-STAT3$^{(705)}$, respectively (Fig. 2D-F) and (Fig. 2I-K) at different time intervals compared to control cells.

3.5. IL-8 interferes with the inhibitory effect of lapatinib by activating Src, EGFR, EGFR2 (HER2), Erk1/2 and STAT3 in SKBR3 breast cancer cell line at different time intervals

To corroborate with the above functional results, we investigated the role of different IL-8 concentrations (50 and 100 ng/mL) in activating Src, EGFR, EGFR2 and the signaling molecules Erk1/2 and STAT3 (Fig. 3A), since it has been reported to aid in lapatinib resistance [60]. Statistical analysis showed that IL-8 (50 and 100 ng/mL) in presence of lapatinib significantly ($P < 0.05$) induced the expression of p-EGFR$^{(Tyr\text{-}845)}$ and p-EGFR2 $^{(Tyr\text{-}Y1248)}$ compared to SKBR3 in presence of lapatinib only (Fig. 3B and C) and (Fig. 3G and H), respectively at different time intervals. IL-8 (50 and 100 ng/mL) abolish the inhibitory effect of lapatinib by activating p-EGFR$^{(Tyr\text{-}845)}$ and p-EGFR2 $^{(Tyr\text{-}Y1248)}$, p-Src$^{(Y527)}$, ERK1/2$^{(202/204)}$ and p-STAT3$^{(705)}$ and adding IL-8 to cells pretreated with lapatinib significantly ($P < 0.01$) induced the expression of p-Src $^{(Y527)}$, p-ERK1/2$^{(202/204)}$ and p-STAT3$^{(705)}$ respectively compared to SKBR3 in presence of lapatinib (Fig. 3D and F) and (Fig. 3I and K), respectively at different time intervals compared to SKBR3 in the presence of lapatinib only.

3.6. IL-8 increase SKBR3 proliferation and size of spheroids in the presence of lapatinib

Using 3D cell culture models, we studied the effect of IL-8 (100 ng/mL) on spheroids formation capability by SKBR3 in the absence and presence of lapatinib (1 μL/mL) (Fig. 4A-D). Microscopic results showed that IL-8 significantly increased the number of the formed spheroids by 23.52% after normalization to control cells ($P < 0.01$) (Fig. 4E). On the other hand, lapatinib significantly reduced the size and number of spheroids formed to 38.2% after normalization to control cells ($P < 0.001$) (Fig. 4E). However, the number of spheroids for the cells grown in media contained IL-8 (100 ng/mL) in the presence of lapatinib increased to 105.8% after normalization to control cells ($P < 0.001$) demolishing the effect of lapatinib (Fig. 4F).

3.7. High concentration of IL-8 overcomes the action of lapatinib and increase SKBR3 cells invasion

In vitro studies showed IL-8 signaling is important for breast cancer cell invasion and that both migration and invasion of SKBR3 acquired lapatinib-resistant cells were inhibited when *IL-8* expression was silenced [1]. In fact, another study on SKBR3 and other breast cancer cell lines such as MDA-MB-231, MDA-MB-468, HS578t, BT474, BT549, MCF-7 and T47, showed that cell invasion is directly proportional to IL-8 expression level. Moreover, they found that upon IL-8 neutralization invasion of breast cancer cells was significantly inhibited [35]. Herein, we aimed to validate the chemotactic properties of IL-8 overexpression on invasion potential of EGFR/HER2$^+$ breast cancer cell lines SKBR3, in absence and presence of lapatinib (Fig. 5A-D). The results revealed that IL-8 at concentration of 100 ng/mL significantly increased the invasive potential of SKBR3 to be 110.9% normalized to the control which is set as 100% (Fig. 5E). On the other hand, lapatinib drug was able to significantly reduce the invasive potential of SKBR3 cells to be 41.47% normalized to the control which is set as 100%. In the presence of lapatinib recombinant IL-8 reduced the effect of lapatinib drug by inducing the invasion of SKBR3 by 63.41% normalized to the control which is set as 100% (Fig. 5F).

4. Discussion

Members of EGFRs family are considered as an important target for the development of anticancer therapeutic drugs, since they are aberrantly activated by over expression or mutation in many common human cancer types such as breast and lung cancer [23]. Therapeutic targets such as trastuzumab were developed to target the HER2$^+$ breast

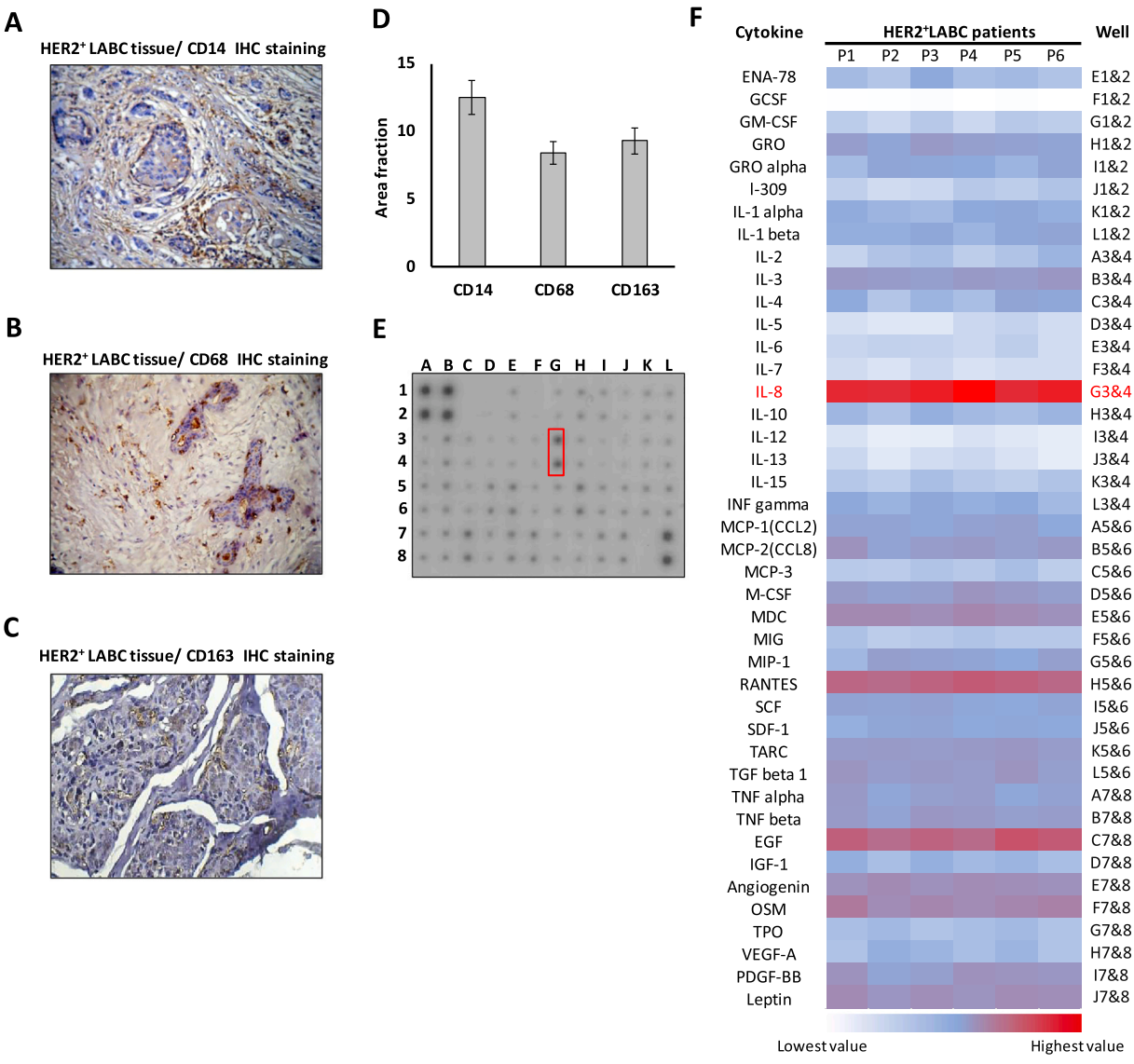

Fig. 1. CD14$^+$ monocytes highly infiltrate tumor microenvironment of LABC patients and characterized by high secretions of IL-8 cytokine. (A-C) Microscopic imaging showing positive CD14$^+$, CD68$^+$ and CD163$^+$ staining (brown color) in breast carcinoma tissues of HER2$^+$ LABC patients ($n = 15$) (magnification 40×). (D) bars showed no significance differences between the prevalence of M1 and M2 macrophages in HER2$^+$ LABC tissues (E) Cytokine profiling membrane of the secretome of CD14$^+$ monocytes. Spots represents the signal intensity of each cytokine calculated by using ImageJ software (National Institutes of Health, MD, USA) and normalized according to algorithm equation provided with kit instruction manual. (F) Heat map showing the different expression level of 42 cytokines in the secretome of isolated TME CD14$^+$ monocytes ($n = 6$). The colors range of the heat map from (white - light blue - deep red), where the white rectangles represent the Lowest expression values, light blue rectangles represent the moderate expression values and dark red rectangles represent the highest expression values. Data represent the mean of ± SD.

cancer patients however, up to 70% of patients developed acquired resistance within 1–2 years [21]. Resistance to trastuzumab encouraged scientists and pharmaceutical companies to introduce lapatinib that inhibits the receptor tyrosine kinases (RTKs) of EGFR and HER2 through binding to their ATP binding sites preventing the activation associated intracellular signaling pathways such as Src, ERK1/2 and MAPK [34,65].

Nonetheless, studies have reported that only 20% to 35% of patients with HER2$^+$ metastatic breast cancer respond to lapatinib [17,60]. Concluding that, lapatinib resistance by breast cancer patients is a vital issue [11,65], which can result from various mechanisms such as; HER2 alterations, aberrant activation of escape pathways mediated by other RTK's or intra- signaling effectors and changes in apoptosis or cell cycle regulation [56]. The resistance to lapatinib was also revealed to be due hypoxia that promotes lapatinib resistance in HER2+ breast cancer cells through activation of Src, AKT and ERK1/2 pathways [26]. It should be noted that EGFR was associated with HER2 over-expression, lower hormone receptor levels, higher proliferation and genomic instability in breast cancer patients [52]. IL-8 strongly correlates with poor prognostic tumor stage, lymph node metastasis, and HER2 antigen expression in breast cancer patients [38]. IL-8 serum levels were significantly higher in HER2$^+$ compared to HER2$^-$ breast cancer patients, also IL-8 found to be the most prominent chemokine with at least a 10-fold increased expression in HER2-overexpressing transfectant cell lines when compared with control [64]. The majority of clinical studies confirmed that overexpression of IL-8 in the most advanced stages of breast cancer such as LABC. IL-8 interact with ER and HER2 activating cellular pathways that contribute to breast cancer poor prognosis and therapeutic resistance [56]. TAMs secrete high levels of IL-8 in breast TME and the signaling pathways stimulated by IL-8 assumed to be potential therapeutic target in breast cancer [61]. Indeed, monocyte/macrophage infiltration within the TME is associated with poor prognostic breast

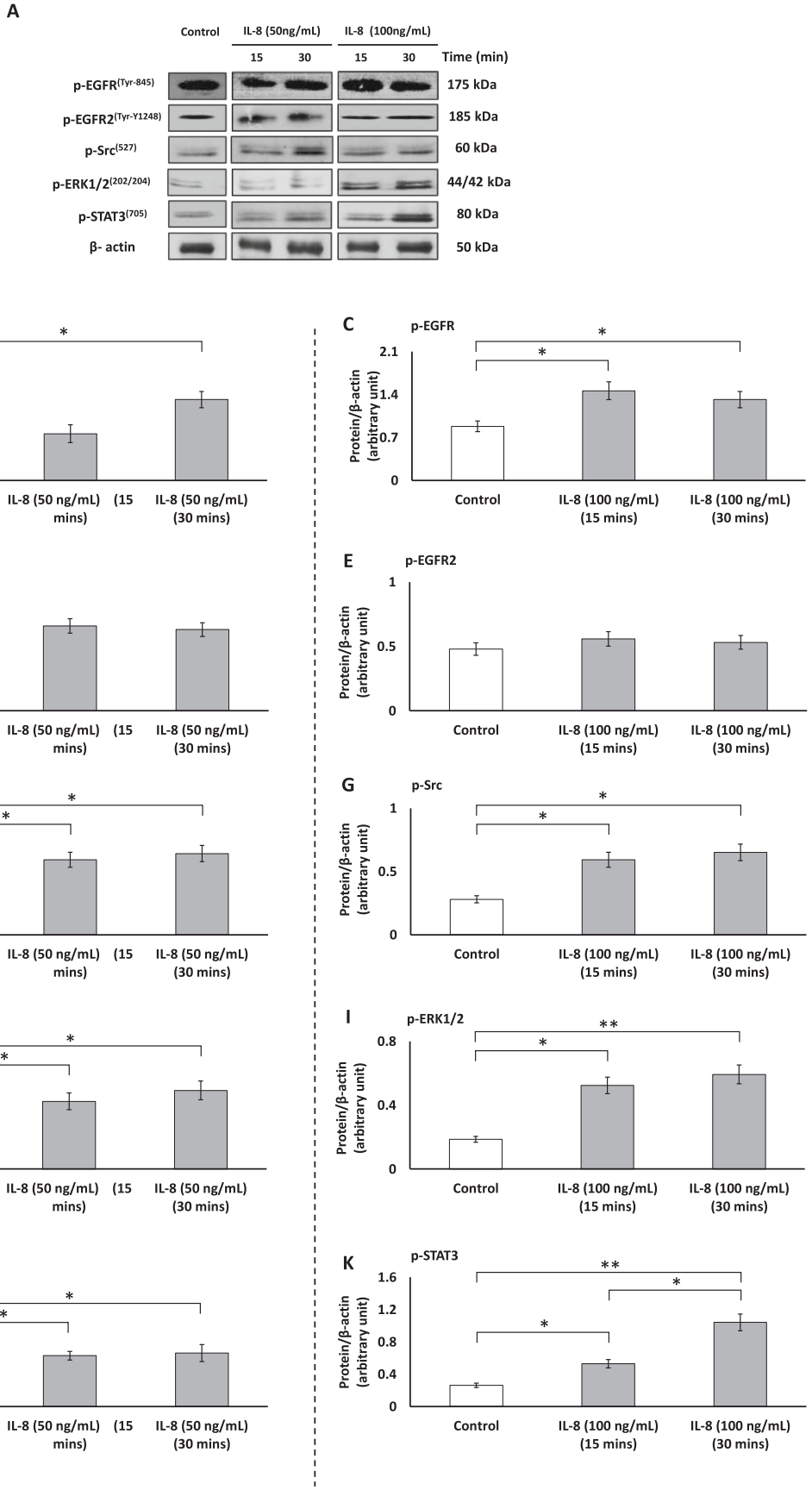

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Fig. 2. IL-8 induces the expression of EGFR, HER2 and downstream signaling pathways Src, ERK1/2 and STAT3 of SKBR3. (A) Immunoblot representatives of protein expression of p-EGFR$^{(Tyr\text{-}845)}$ and p-HER2$^{(Tyr\text{-}Y1248)}$ tyrosine kinases and downstream signaling pathways (p-Src$^{(527)}$, ERK1/2$^{(202/204)}$ and STAT3$^{(705)}$ in SKBR3 cell lysates after stimulation with IL-8 (50 and 100 ng/mL) at different time intervals (15 and 30 mins). (B - E) Bars represent the phosphorylation level of the of p-EGFR$^{(Tyr\text{-}845)}$ and p-HER2$^{(Tyr\text{-}Y1248)}$ after stimulation with IL-8 (50 and 100 ng/mL, respectively) compared to control cells (stimulated for 15 and 30 mins). (F-K) Bars represent the phosphorylation of the downstream signaling pathways p-Src$^{(527)}$, STAT3$^{(705)}$ and ERK1/2$^{(202/204)}$, respectively IL-8 (50 and 100 ng/mL, respectively) compared to control cells. Data represented as mean ± SD, * significant differences at a P value <0.05 and ** significant differences at a P value <0.01 as determined by one-way ANOVA test followed by Tukey's HSD post-hoc test. Results are representatives of at least 3 independent experiments.

cancer characteristics; larger tumor size, higher tumor grade, lymph node metastasis, vascular invasion, hormone receptor negativity, HER2 overexpression [47,63]. Previously we showed that monocytes contribute to the aggressive behavior of inflammatory breast cancer (IBC), we showed that human monocyte secrete IL-8 that induce expression of mesenchymal marker fibronectin; a mechanism modulated by PI3k/AkT signaling pathway [41]. In addition, we also demonstrated that TAM drained from breast TME through axillary tributaries secrete cytokines, IL-8 is one of the most prominent secreted cytokines, that induce motility and invasion of IBC cells in-vitro [43].

Considering all of the above results introduced by different studies and our previous studies herein we assumed that resistance to lapatinib treatment in LABC patients may be due to cytokines secreted by TAMs that activate RTKs and associated signaling molecules used to be inhibited by lapatinib treatment. To test role of cytokines secreted by TAMs on lapatinib treatment we used innovative surgical approach to isolate TAMs (CD14+) from the TME of LABC-HER2+ patients. The isolated TAMs were subjected to cytokine profile our results revealed that IL-8 is considered as the major cytokine secreted by TAM of LABC patients. This agree with a previous study showed that serum IL-8 is highly detected in LABC patients and high level of IL-8 correlate with distant metastasis and poor survival rate [6]. IL-8 found to interact with EGFR/HER2 and activate signaling pathways associated with stem cell properties, motility and invasion of breast cancer [60]. Studies demonstrated that IL-8 induced transient phosphorylation of the EGFR and HER2 tyrosine kinases suggesting that HER2 can be transactivated by IL-8/CXCR1/2 by a mechanism independent from the ligand activation of EGFR [8,60]. Of note, the EGFR phosphorylation at the transphosphorylation site Y845, is an indicator for the involvement of Src kinase in the activation of EGFR. The phosphorylated Y845 act as a "docking site" that attract and induce phosphorylation of STAT 3 and/or STAT5 [62]. In the same manner, the present results suggest that IL-8 activate Src kinase that induce the activation of p-EGFR$^{(Tyr\text{-}845)}$ and associated signaling molecules STAT3 and Erk1/2.

Similarly, IL-8 secreted by human pancreatic cancer cells induce muscle atrophy via activation of Src, STAT3 and ERK1/2 signaling molecules [9]. Herein, our results showed that after different time intervals (15 and 30 min) different concentrations of recombinant IL-8 (50 and 100 ng/mL) induced the activation of p-EGFR$^{(Tyr\text{-}845)}$ receptor tyrosine kinase, non-receptor tyrosine kinase p-Src$^{(Y527)}$ and signaling molecules ERK1/2$^{(202/204)}$ and STAT3$^{(705)}$ in SKBR3 breast cancer cell line. In addition, the inhibitory action of lapatinib drug on EGFR /HER2 receptor tyrosine kinases and the signaling molecules as p-Src$^{(Y527)}$, STAT3$^{(705)}$ and ERK1/2$^{(202/204)}$ was abolished in the presence of 50 and 100 ng/mL IL-8 after 15 & 30 min. Similarly, studies revealed that inhibition of EGFR/ HER2 induced by lapatinib solely did not show appropriate biological effect whereas, when they used a combination of lapatinib and Saracatinib drug the proliferation and invasion rate of cells decreased which in turn seem to depend on Src activation [17,45].

The present result agrees with a study suggested that HER2 activation can be mediated via intracellular mechanisms using non-receptor Src tyrosine kinase that transduces the intracellular signals of the cytokine receptor and interacts with the EGFR and HER2 in a dependent or independent manner modulating the activity or intracellular effectors such as the ERK1/2 [60]. It should be noted that we also detected an increase in the phosphorylation level ERK1/2 the Src and STAT3 signaling molecule upon stimulation with IL-8. The present results confirm previous results published by Singh and his colleagues [19] finding that IL-8 markedly increased the phosphorylation of AKT and ERK1/2 within 10 min. Herein, IL-8 abolished the inhibitory effect of lapatinib via inducing ERK1/2 phosphorylation by SKBR3 cells treated with IL-8 (50 and 100 ng).

Cells grown in 3D culture recapitulate the actual tumor microenvironment [12] and cellular physiological responses in 3D culture are more similar to their in vivo behavior [41]. We conducted an in vitro 3D culture to test the effect IL-8 on the proliferation rate of SKBR3 cell and the formation of tumor spheroids in the absence and presence of lapatinib. We found that lapatinib significantly reduced the size and number of the 3D spheroids as compared to control cells. On the other hand, IL-8 induced the size and number of 3D spheroids as compared to control cells. In the presence of lapatinib IL-8 was able to demolish its effect and increase the size and the number of the tumor spheroids.

Since, accumulating evidence suggests that the inflammatory cytokine IL-8 aid in facilitating the tumor invasion. Therefore, we investigated both the chemotactic properties and its effect on the invasion potential of the SKBR3 minimizing the mode of action of lapatinib by indirect activation of the EGFR/HER2 and associated signaling molecules contributing to breast cancer progression an in-vitro. We found that lapatinib drug was able to decrease the invasion potential of cells compared to the control. However, recombinant IL-8 was able to decrease the action of lapatinib by inducing the invasive properties of SKBR3. Blocking of IL-8 was found to reduce invasive properties of melanoma cells [25]. Recent phase one clinical trial using specific IL-8 monoclonal antibody HuMax-IL8 (BMS-986253) showed that blockade and inhibition of IL-8 reduce mesenchymal transition associated with metastasis of solid cancers such as ovarian, prostate, esophageal and papillary thyroid [7]. Our present study encourages using IL-8 monoclonal antibody HuMax-IL8 (BMS-986253) during lapatinib treatment in LABC patients.

5. Conclusion

The obtained results suggested that the ineffective action of lapatinib might be due to the secretion of IL-8 by TAMs infiltrating TME of LABC/HER2+ breast cancer patients. IL-8 interfere with lapatinib drug action via activating target EGFR/HER2 and associated signaling molecules that contribute to cancer poor prognosis and treatment resistance. In this regard, neutralization or down-regulation of IL-8 in the TME of LABC patients seems a promising therapeutic. We recommend that combined therapy targeting the inflammatory cytokines IL-8 which is over expressed in patient's tumor microenvironment should be taken in consideration during the treatment with lapatinib drug.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamcr.2021.118995.

CRediT authorship contribution statement

Shaza Ahmed: Methodology, Writing – original draft. **Hossam Taha Mohamed:** Methodology, Validation, Formal analysis, Writing - original draft. **Noura El-Husseiny:** Methodology. **Manal M. El Mahdy:** Methodology. **Gehan Safwat:** Writing – review & editing, Supervision. **Ayman A. Diab:** Writing – review & editing, Supervision. **Ahmed A. El-Sherif:** Writing – review & editing, Supervision. **Mohamed El-Shinawi:** Resources, Writing – review & editing, Supervision. **Mona Mostafa Mohamed:** Validation, Writing – original draft, Supervision.

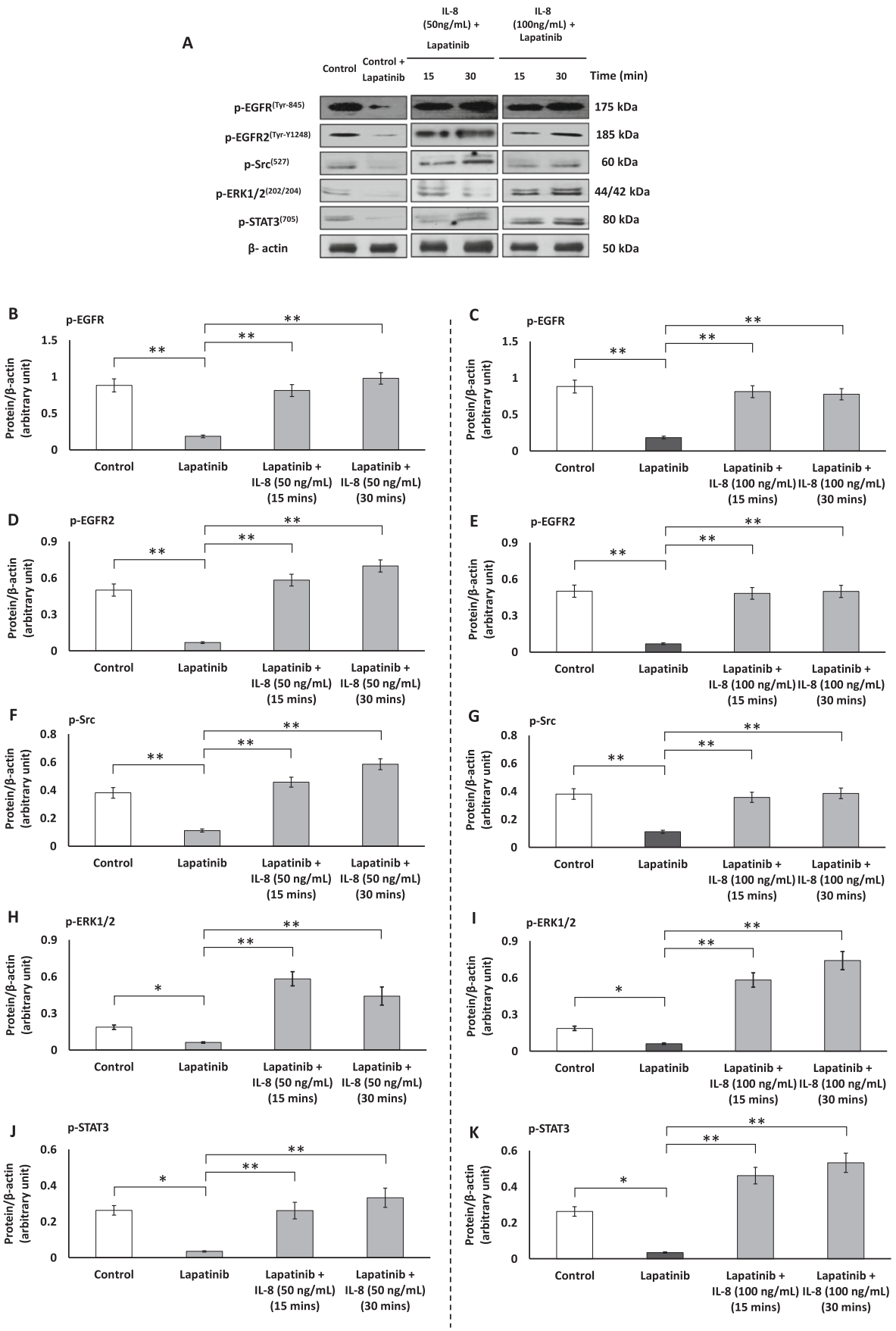

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Fig. 3. IL-8 induces the expression of EGFR, HER2 and downstream signaling pathways Src, ERK1/2 and STAT3 of SKBR3 in presence of lapatinib. (A) Immunoblot representatives of protein expression of p-EGFR$^{(Tyr\text{-}845)}$ and p-HER2$^{(Tyr\text{-}Y1248)}$ tyrosine kinases and downstream signaling pathways (p-Src$^{(527)}$, ERK1/2$^{(202/204)}$ and STAT3$^{(705)}$ in SKBR3 cell lysates after stimulation with IL-8 (50 and 100 ng/mL) in presence of lapatinib after different time intervals (15 & 30 min). (B - E) Bars represent the phosphorylation level of the p-EGFR$^{(Tyr\text{-}845)}$ and p-HER2$^{(Tyr\text{-}Y1248)}$ after stimulation with IL-8 (50 and 100 ng/mL, respectively) in the presence of lapatinib drug (1 μL/mL). (F - K) Bars represent the phosphorylation of the downstream signaling pathways p-Src$^{(527)}$, p-ERK1/2$^{(202/204}$ and p-STAT3$^{(705))}$, respectively after stimulation with IL-8 (50 and 100 ng/mL, respectively) in presence of lapatinib drug (1 μL/mL). data represented as mean ± SD, * significant differences at a *P* value <0.05 and ** significant differences at a *P* value <0.01 as determined by one-way ANOVA test followed by Tukey's HSD post-hoc test. Results are representatives of at least 3 independent experiments.

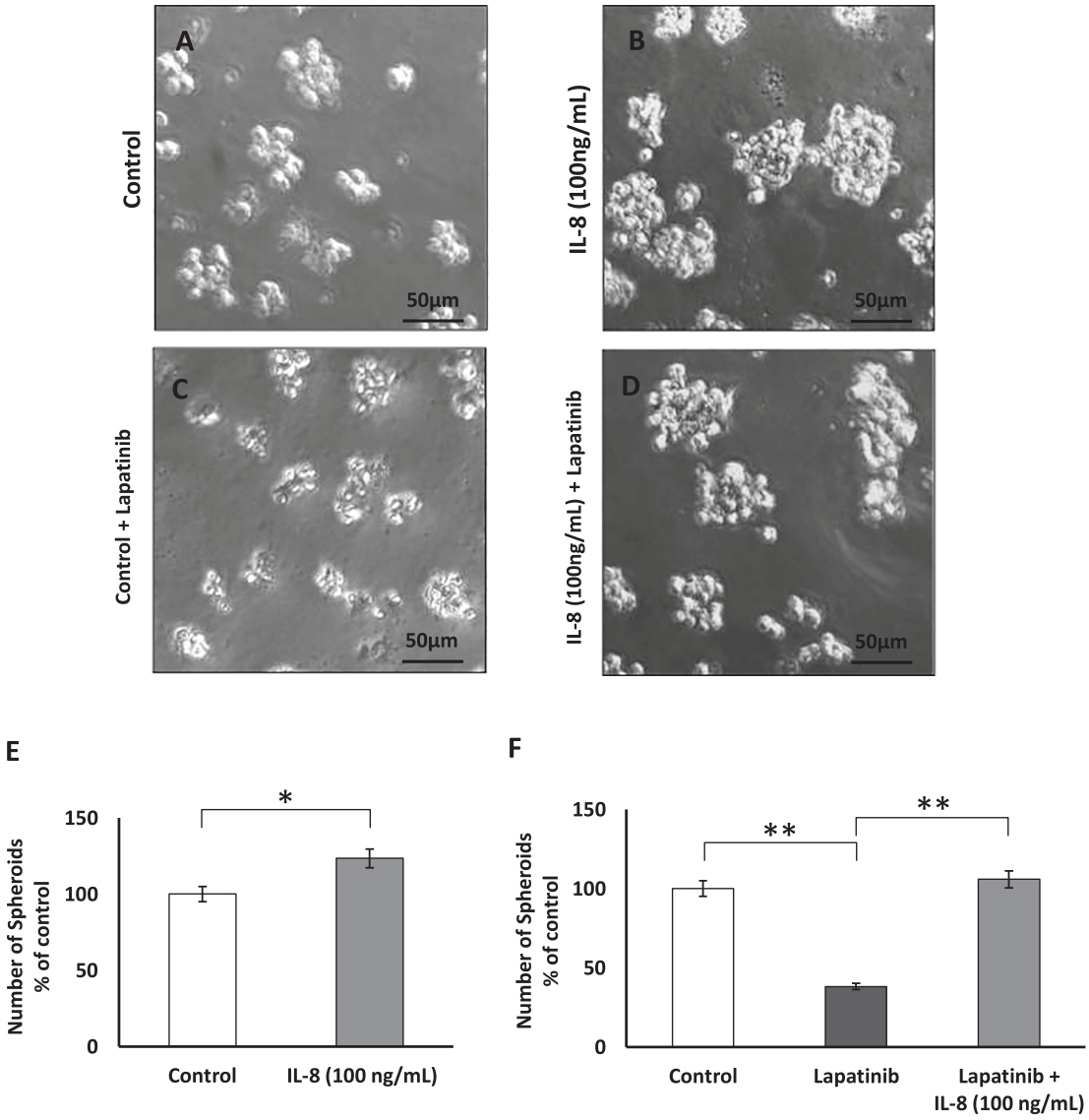

Fig. 4. Spheroid formation of SKBR3 in 3D cultured system upon stimulation with IL-8 (100 ng/mL) in the absence and presence of lapatinib. (A) Microscopic image showed spheroid formation as indictor to stem cell properties and drug resistance by SKBR3 cells grown in complete culture medium. (B) Microscopic images represent one microscopic field showing size and number of spheroids formation in SKBR3 cells in response to recombinant IL-8 (100 ng/mL). (C) Microscopic image showed spheroid formation in SKBR3 cells grown in complete culture medium with addition of lapatinib drug (1 μL/mL). (D) Microscopic images showed spheroid formation in SKBR3 cells pretreated with lapatinib drug (1 μL/mL) in response to recombinant IL-8 (100 ng/mL) (E) bars represent the count of spheroids of SKBR3 cells in response to recombinant IL-8 (100 ng/mL), respectively compared to SKBR3 cells grown in complete culture medium. Data represented as mean ± SD, the different letters (a, b) denote significant differences at a *P* value <0.05 as determined by Student's *t*-test. (F) bars represent the count of spheroids of SKBR3 cells pretreated with lapatinib drug (1 μL/mL) in response to recombinant IL-8 (100 ng/mL), respectively compared to SKBR3 cells grown in complete culture medium with addition of lapatinib drug (1 μL/mL). data represented as mean ± SD, * significant differences at a *P* value <0.05 and ** significant differences at a *P* value <0.01 as determined by one-way ANOVA test followed by Tukey's HSD post-hoc test. Scale bar = 150 μm. Results are representatives of at least 3 independent experiments. Images of spheroids were captured using AxioVision software (Zeiss, Germany) and the measurement of spheroids was performed manually by applying the "Count and Measure" tool of the CellSens software. Scale bars = 50 μm.

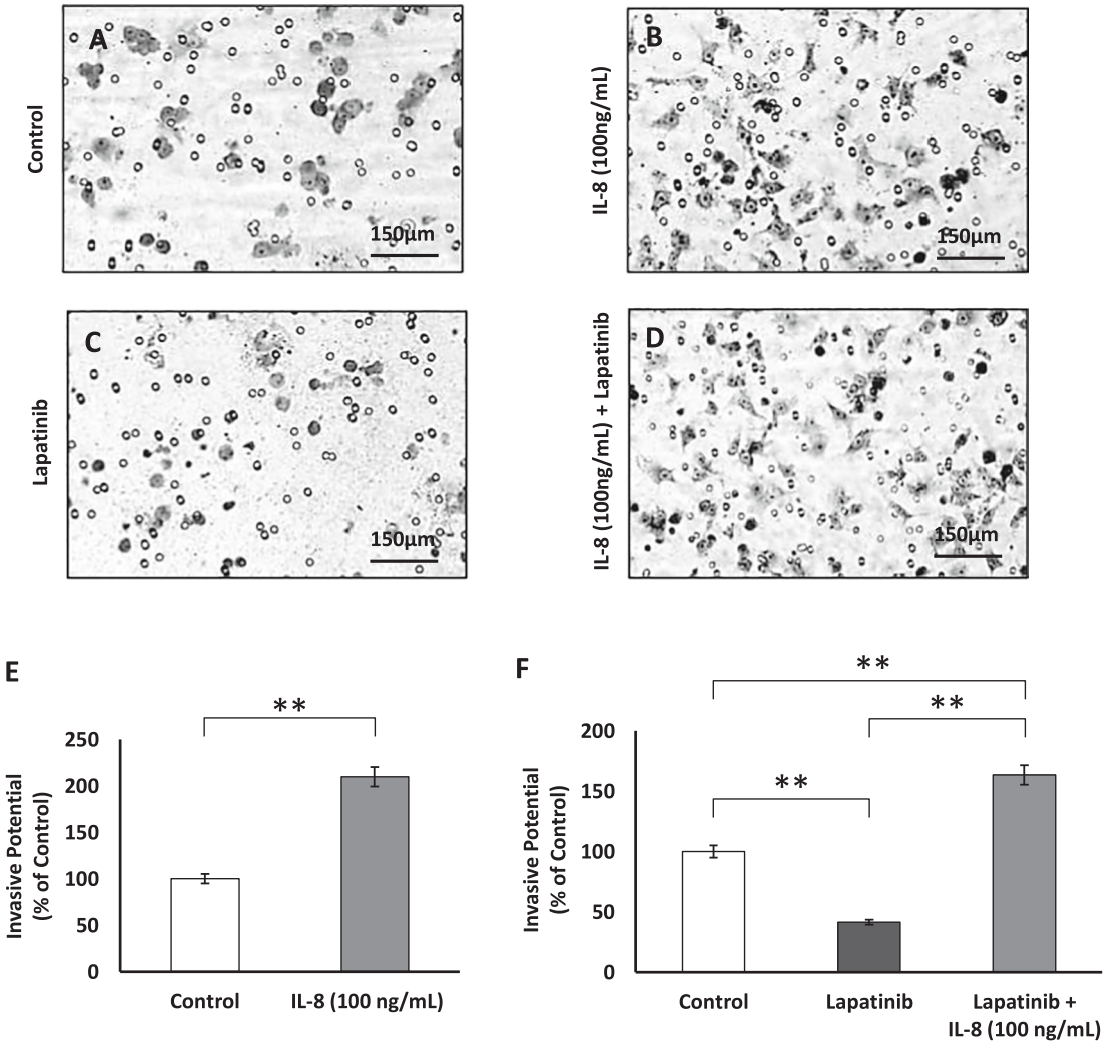

Fig. 5. Effect of IL-8 (100 ng/mL) on in vitro invasion of SKBR3 in the absence and presence of lapatinib drug. The SKBR3 cells (3×10^3 per well) were grown in the upper chamber of BD Bio-Coat Matrigel™ Invasion. IL-8 (100 ng/mL) was added to lower chamber in absence and presence of lapatinib. (A) SKBR3 control cells grown in upper chamber and complete culture media in lower chamber. (B) SKBR3 cells that invaded the BD Bio-Coat Matrigel the upper chamber in response to recombinant IL-8 (100 ng/mL) in the lower chamber. (C) SKBR3 cells grow in presence of lapatinib drug (1 µL/mL). (D) SKBR3 cells pretreated with lapatinib drug (1 µL/mL) that invaded the BD Bio-Coat Matrigel in the presence of recombinant IL-8 (100 ng/mL). (E) bars represent the invasive potential of SKBR3 cells in response to recombinant IL-8 (100 ng/mL) normalized to the control which is set as 100%. data represented as mean ± SD, ** significant differences at a *P* value <0.01 as determined by by Student's t-test. (F) bars represent the invasive potential of SKBR3 cells in response to recombinant IL-8 (100 ng/mL) in the presence of lapatinib drug (1 µL/mL) compared to SKBR3 cells grown in media with of lapatinib drug only (1 µL/mL) normalized to the control which is set as 100%. data represented as mean ± SD, ** significant differences at a *P* value <0.01 as determined by by one-way ANOVA test followed by Tukey's HSD post-hoc test. Scale bar = 150 µm. Results are representatives of at least 3 independent experiments.

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.

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

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Data availability

There are no restrictions on availability of the presented materials, data and associated protocols.

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