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Short Communication

Protein array technology to detect HER2 (*erb*B-2)-induced 'cytokine signature' in breast cancer

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ABSTRACT

Identification of genes/proteins that are differentially expressed in HER2 (*erb*B-2) oncogene-dependent breast carcinomas is essential in elucidating the mechanistic basis of their increased metastatic potential and resistance to several anti-cancer therapies. We here applied human cytokine antibody arrays with the goal of identifying a unique HER2-induced 'cytokine signature' in breast cancer. Human Cytokine Array III (RayBiotech, Inc.), which simultaneously detects 42 cytokines and growth factors on one membrane, was used to determine the profile of cytokines in conditioned media obtained from MCF-7/Her2-18 cells, a MCF-7-derived clone engineered to stably express the full-length human HER2 cDNA controlled by a SV40 viral promoter, and from the MCF-7/*neo* control sub-line. We identified two inflammatory and pro-angiogenic CXC chemokines with at least a 10-fold increased expression in HER2-overexpressing MCF-7/Her2-18 transfectants when compared to matched control MCF-7/*neo* cells: CXCL8 (IL-8; Interleukin-8) and CXCL1 and (GRO; Growth-related oncogene). HER2-induced differential overexpression of IL-8 and GRO was validated by ELISA and further confirmed by switching off the HER2 signalling. Treatment with the tyrosine kinase inhibitor gefitinib (Iressa™) returned the expression levels of IL-8 and GRO back to the baseline observed in MCF-7 breast cancer cells, which express physiological levels of HER2. To evaluate the diagnostic utility of these findings, cytokine-specific antibody arrays were incubated with sera retrospectively collected from metastatic breast cancer patients. This approach revealed a high similarity between the 'cytokine signature' observed in serum samples and that obtained in media conditioned by breast cancer-derived cell lines. Thus, IL-8 and GRO circulating levels were significantly higher in HER2-positive breast cancer patients compared with HER2-negative patients. These findings reveal for the first time that: a) Enhanced synthesis and secretion of members of the IL-8/GRO chemokine family, which have recently been linked to oestrogen receptor (ER) inaction, increased cell invasion and angiogenesis, may represent a new pathway involved in the metastatic progression and endocrine resistance of HER2-overexpressing breast carcinomas, and b) Circulating levels of IL-8 and GRO cytokines may represent novel biomarkers monitoring breast cancer responses to endocrine treatments and/or HER2-targeted therapies.

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1. Introduction

The HER2 oncogene (also called *neu* and *erb*B-2) codes for the transmembrane tyrosine kinase orphan receptor $p185^{Her2/neu}$ and, at present, represents one of the most important oncogenes in breast cancer.[1,2] Expression of high levels of HER2 is sufficient to induce neoplastic transformation of some cell lines, suggesting a role for HER2 in the aetiology of some breast carcinomas.[3,4] Accordingly, HER2 is overexpressed and/or hyperactivated not only in invasive breast cancer but also in pre-neoplastic breast lesions such as atypical duct proliferations and in ductal carcinoma *in situ* of the breast.[5,6] Moreover, HER2 overexpression enhances the invasive and metastatic phenotype of breast cancer cells, and clinically accumulated evidence has shown that HER2 gene amplification and/or $p185^{Her2/neu}$ protein overexpression, which occur in about 20–30% of all human breast cancers, associate with a more aggressive breast cancer phenotype and an unfavourable clinical outcome.[7–10] Although there are controversies regarding HER2 and response to chemotherapy and hormonal therapy both in clinical and laboratory studies, HER2 overexpression appears to significantly affect the sensitivity of cancer cells to various treatments, such as cytokine treatment, radiation therapy, chemotherapy, and hormone therapy.[11–13]

Aberrant expression of HER2 triggers the activation of multiple downstream signal transduction pathways, including the phosphatidylinositol 3′-kinase (PI3′-K)/AKT/PTEN pathway and the Ras/Raf/Mitogen-activated protein kinase (MAPK) pathway, which are essential in inducing increased cell proliferation and differentiation, decreasing apoptosis, and enhancing tumour cell motility and angiogenesis. Whereas these signalling pathways emanating from HER2 have been extendedly characterised, much less is known about the specific genes/proteins regulated by HER2 that contribute to its tumourigenic effects. Transcriptome analyses revealed that a large number of differentially HER2 regulated genes were involved in cell-matrix interactions, cell proliferation, and transformation.[14–17] However, we must consider that almost all cell functions are executed by proteins, which cannot be assessed by evaluation of DNA or RNA alone. In addition, there is evidence that indicating that mRNA levels may not necessarily predict the translated protein levels. Indeed, experimental analyses have demonstrated a clear disparity between the relative expression levels of mRNA and their corresponding proteins. In this regard, a few studies on HER2-induced changes in protein expression have been reported using tumour-derived human breast cancer cell lines or breast cancer specimens.[18–20] Thus, the proteins that regulate the 'output' of the HER2 oncogene are not well characterised.

Alteration of cytokine levels is associated with cancer progression, response to chemotherapy and metastatic status, and they are emerging as potential factors that could contribute to key autocrine or paracrine loops in breast cancer aetiology and metastatic phenotype.[21–23] Because of the limitation of technology, however, previous studies only measured single or few cytokines at once. In our current study, and with the goal of identifying those cytokines playing key roles in HER2-driven human breast cancer progression, we took advantage of the recently developed RayBio™ Human Cytokine Array III capable to simultaneously detect 42 cytokines and growth factors on one membrane. Using conditioned media from MCF-7 breast cancer cells, which endogenously express low levels of HER2, before and after re-expression of HER2 (*i.e.* MCF-7/*neo* and MCF-7/Her2-18 cells, respectively) as well as sera obtained retrospectively from metastatic breast cancer patients, we present data to suggest that enhanced protein synthesis and secretion of CXCL8 (IL-8) and CXCL1 (GRO), two members of the CXC (two conserved cysteine residues separated by an additional amino acid residue) chemokine family, may represent a new pathway involved in the metastatic progression of HER2-overexpressing breast carcinomas as they have recently been linked to oestrogen receptor (ER) inaction, increased cell invasion and angiogenesis.[24–31]

2. Materials and methods

2.1. Materials

RayBio™ Human Cytokine Array III (Catalog No: H0108009C) was purchased from RayBiotech, Inc. (Norcross, GA, USA). Gefitinib (Iressa™) was gently provided by AstraZeneca (Macclesfield, United Kingdom).

2.2. Cell lines and culture conditions

MCF-7 breast cancer cells stably overexpressing HER2 oncogene (MCF-7/Her2-18 clone) and the matched control MCF-7/*neo* cells were kindly provided by Prof. Mien-Chie Hung (The University of Texas, M. D. Anderson Cancer Centre, Houston, TX, USA). Cells were routinely grown in DMEM containing 10% (*v*/*v*) heat-inactivated foetal bovine serum (FBS) and 2 mM L-glutamine. Cells were maintained at 37 °C in a humidified atmosphere of 95% air/5% CO_2. Cells were screened periodically for *Mycoplasma* contamination.

2.3. Conditioned medium

To prepare conditioned media, cells were plated in 100-mm tissue culture dishes and cultured in DMEM with 10% FBS until they reached 75–80% confluence. The cells were washed twice with serum-free DMEM, and incubated overnight in serum-free DMEM. Cells were then cultured for 48 h in low-serum (0.1% *v*/*v*FBS) DMEM in the presence or absence of increasing concentrations of gefitinib, as specified. The supernatants were collected, centrifuged at 1000 × g, aliquoted, and stored at −80 °C until testing.

2.4. Cytokine antibody arrays

Assay for cytokine antibody arrays was carried out as per manufacturer's instructions. Briefly, cytokine array membranes were blocked with 5% BSA/TBS (0.01 M Tris HCl pH 7.6/0.15 M NaCl) for 1 h. Membranes were then incubated with about 2 ml of conditioned media prepared from different cell lines or 1 ml of patient's sera after normalisation with equal

amounts of protein. After extensive washing with TBS/0.1% *v*/*v* Tween 20 (3 times, 5 min each) and TBS (2 times, 5 min each) to remove unbound materials, the membranes were then incubated with a cocktail of biotin-labelled antibodies against different individual cytokines. The membranes were then washed and incubated with HRP-conjugated streptavidin (2.5 pg/ml) for 1 h at room temperature. Unbound HRP-streptavidin was washed out with TBS/0.1% Tween 20 and TBS. Finally the signals were detected by ECL system. Densitometric values of spots were quantified using Scion Imaging Software (Scion Corp., Frederick,MD, USA).

2.5. *Immunoblotting analyses*

For assaying levels of protein expression and phosphorylation status of HER2, p42/p44-MAPK and AKT, cells were cultured as described above in the absence or presence of gefitinib. Cells were then washed with cold-PBS, placed on ice and lysed in a non-denaturing 1X lysis buffer (Cell Signalling; Beverly, MA) containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% *v*/*v* Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin and 1 mM PMSF. Cells were scraped, added to eppendorf tubes and incubated on ice for 20 min before debris were removed by a 15 min spin at 14,000 rpm at 4 °C. A BCA protein reagent kit (Pierce, Rockford, IL, USA) was used to determine levels of total protein. Equal amounts of protein were heated in SDS sample buffer (Laemli) for 10 min at 70 °C, subjected to electrophoresis on either 3–8% Tris-Acetate NuPAGE (HER2, →–HER2) or 10% SDS-PAGE (MAPK, →–MAPK, AKT, and →–AKT), and transferred to nitrocellulose membranes. For immunoblotting analyses of HER2/ →–HER2, nonspecific binding on the nitrocellulose filter paper was minimised by blocking for 1 h at room temperature (RT) with TBS-T [25 mM Tris-HCl, 150 mM NaCl (pH 7.5), and 0.05% Tween 20] containing 5% (*w*/*v*) nonfat dry milk. The treated filters were washed in TBS-T and then incubated with primary antibodies for 2 h at RT in TBS-T containing 1% (*w*/*v*) nonfat dry milk. The membranes were washed in TBS-T, horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno Research, West Grove, PA, USA) in TBS-T were added for 1 h, and immunoreactive bands were visualised with ECL detection reagent (Pierce, Rockford, IL, USA). For immunoblotting analyses of MAPK, →–MAPK, AKT, and →–AKT, membranes were blocked as described above and incubated overnight at 4 °C with primary antibody in TBS-T/5% bovine serum albumin (BSA). The membranes were washed in TBS-T, horseradish peroxidase-conjugated secondary antibodies in TBS-T containing 5% (*w*/*v*) nonfat dry milk were added for 1 h, and primary antibody binding was detected with ECL detection reagent (Pierce, Rockford, IL, USA). Blots were re-probed with an antibody for β-actin to control for protein loading and transfer. Densitometric values of protein bands were quantified using Scion imaging software (Scion Corp., Frederick, MD, USA).

The following primary antibodies were used at the concentrations indicated: HER2 (Ab-3; Oncogene Research Products, Cambridge, MA, USA; 2.5 μg/ml), Phosphor-HER2 (Ab-18; Lab Vision Corp., Fremont, CA, USA; 2.5 μg/ml), p44/p42-MAPK (Cell Signalling, Beverly, MA, USA; 1:500), Phosphor-p44/p42-MAPK (Cell Signalling, Beverly, MA, USA; 1:500), AKT (Cell Signalling; 1:500), and Phosphor-AKT (Cell Signalling; 1: 500). To verify equivalent protein loadings, β-actin (Sigma-Chemicals) was included at a concentration of 0.2 μg/ml. HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (Cell Signaling) were used at a concentration of 1:2,000.

2.6. *ELISA*

IL-8 and GRO levels were measured using quantitative immunometric sandwich enzyme immunoassays (ELISA), following the manufacturer's recommended procedures (R & D Systems, Minneapolis, MN, USA). Triplicate cultures of cells were tested for each experimental condition.

2.7. *Statistical analysis*

Statistical analysis of mean values was performed using the non-parametric Mann–Whitney test. Differences were considered significant at $P < 0.05$ and $P < 0.005$.

3. Results

3.1. *In vitro identification of IL-8 and GRO as HER2-related breast cancer cytokines using chemokine antibody array technology*

We first applied cytokine antibody array system to identify the 'key cytokine(s)' associated with HER2-driven breast cancer progression (Fig. 1*a*). By using a RayBio™ Human Cytokine Array III, we simultaneously screened the expression of 42 cytokines when breast cancer cells naturally expressing physiological levels of HER2 (i.e. MCF-7 breast cancer cells) were engineered to overexpress HER2 gene (i.e. MCF-7/Her2-18 transfectants). MCF-7/Her2-18 cells are known to express ~45 times the level of HER2 than parental MCF-7 cells or the MCF-7/*neo* control subline. MCF-7/*neo* cells express a neomycine phosphotransferase gene (*neo*), and stable transfectants MCF-7/Her2-18 express full-length HER2 cDNA under SV40 promoter control. Fig. 1*b* illustrates the location of cytokine antibodies spotted onto the RayBio™ Human Cytokine Array III.

Fig. 1*b* shows the raw images of cytokine antibody array data from MCF-7/*neo* (*left panels*) and MCF-7/Her2-18 cells (*right panels*). The relative expression levels of 42 cytokines were then determined by densitometry. Interestingly, we found that solely four different cytokines were significantly up-regulated in MCF-7 cells engineered to overexpress HER2. Thus, HER2 overexpression induced a dramatic >10-fold increase in the expression of IL-8 (interleukin-8) and GRO (growth-related oncogene), while inducing a highly significant 5 to 10-fold increase in the expression of GROα (growth-related oncogene alpha), one of the 3 GRO isotypes (i.e. GROα, GROβ and GROγ). A noteworthy up-regulation of vascular endothelial growth factor (VEGF), a well-characterised pro-angiogenic effect driven by HER2 overexpression,[8–10] was also observed in our experimental system.

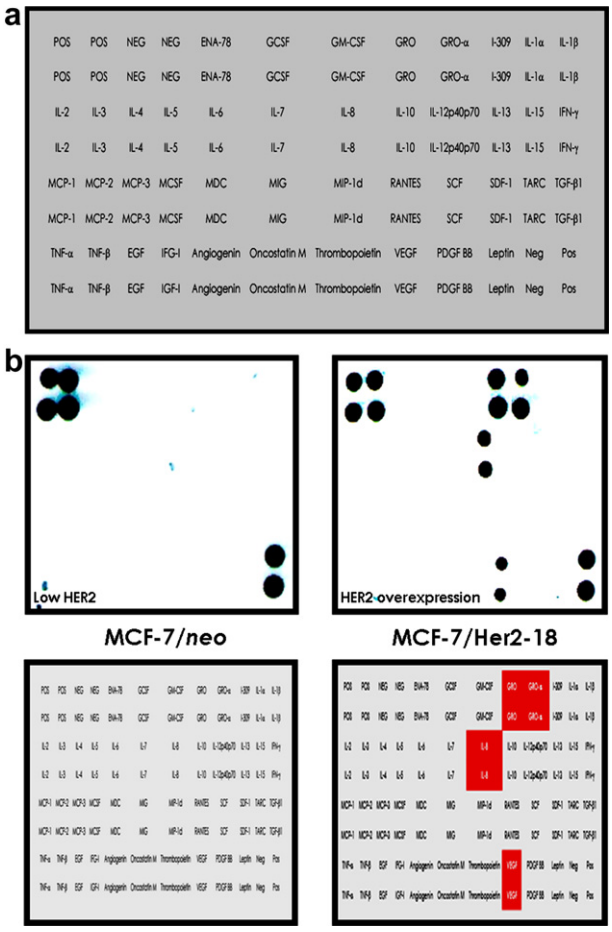

Fig. 1 – (a) Template showing the location of cytokine antibodies spotted onto the RayBio™ Human Cytokine Array III. (b) Detection and modulation of cytokines secreted from MCF-7 cells by overexpression of HER2. Top panels. Forty-eight hour CM prepared from an equal number of MCF-7 cells stably expressing HER2 oncogene (i.e.,MCF-8/Her2-18 cells, *right*) and MCF-7/*neo* matched control cells (*left*) was assayed for cytokine content by the protein array methodology described in the Materials and methods section. *Bottom panels*. Densitometric data were arbitrarily expressed as red for extremely high (> 10-fold increase) when compared to those found in MCF-7/*neo* matched control cells. Figure shows representative sample results ($n = 3$) revealing marked increase of GRO, GROα, IL-8 and VEGF secreted from MCF-7/Her2-18 cells when compared to MCF-7/*neo* control cells.

3.2. *Pharmacological inhibition of HER2-driven cellular signalling knock-down IL-8 and GRO expression*

When the expression levels of IL-8 and GRO were confirmed by ELISA, there was a clear correlation between the HER2-induced fold-changes in cytokines expression as determined by both array technology and ELISA. Thus, conditioned supernatants from MCF-7 and MCF-7/Her2-18 cultures contained 353 ± 7 and 4240 ± 48 pg IL-8/mg protein respectively (Fig. 2*a*). Overexpression of HER2 also resulted in a dramatic increase in secreted GRO protein, from 14 ± 5 pg GRO/mg protein in MCF-7 cells to 2302 ± 300 pg GRO/mg protein in MCF-7/Her2-18 transfectants (Fig. 2a).

To determine a causative role of HER2 in the overproduction of IL-8 and GRO cytokines, MCF-7/Her2-18 cells were exposed to graded concentrations of the small molecule tyrosine kinase inhibitor (TKIs) gefitinib (Iressa™; AstraZeneca, Macclesfield, United Kingdom). As expected, HER2 overexpression resulted in phosphorylation and activation of both the HER2 receptor and the signalling molecules AKT and ERK1/2 mitogen-activated protein kinase (MAPK) (Fig. 2*b*, *left panels*). Treatment with gefitinib inhibited HER2 activation, while down-stream reducing both AKT and p42/p44-MAPK phosphorylation in MCF-7/Her2-18 cells (Fig. 2*b*, *right panels*). Importantly, treatment of these cells with graded concentrations of gefitinib (0.1, 1 and 10 μM) decreased, in a dose-dependent manner, IL-8 and GRO secretion to levels similar to those secreted from HER2-negative MCF-7 parental cells (Fig. 2*a*). Equivalent results were found when gefitinib was substituted by trastuzumab (Herceptin®), a humanised monoclonal antibody directed against the extracellular domain of HER2 (data not shown).

3.3. *HER2-positive breast cancer patients exhibit high levels of circulating IL-8 and GRO cytokines*

To evaluate the diagnostic utility of the above findings, cytokine-specific antibody arrays were incubated with sera retrospectively collected from breast cancer patients. Fig. 3 shows the raw image of cytokine antibody array data from two representative metastatic breast cancer (MBC) patients' sera with HER2 extracellular domain (ECD) serum concentrations of 13.7 and 484 ng/ml, respectively, and from one representative healthy donor serum (HER2 ECD = 5.5 ng/ml). This approach revealed a high similarity between the 'cytokine signature' observed in MBC patient's sera and that obtained in conditioned media from human breast cancer-derived cell lines. Thus, 'cytokine signature' differed significantly in patients with elevated baseline HER2 ECD concentrations (⩾15 ng/ml) and those with normal concentrations (<15 ng/ml). HER2-positive MBC patients analysed consistently exhibited high levels of circulating IL-3, IL-6, IL-8, IL-13, IL-15, GRO and VEGF (e.g. with a cytokine profile similar to that found in Sample#121 – Fig. 3), whereas HER2-negative MBC patients mostly exhibited high levels of circulating EGF and angiogenin (e.g. with a cytokine profile similar to that found in Sample#212 – Fig. 3).

4. Discussion

4.1. *CXCL8 (IL-8) and CXCL8 (GRO): HER2-induced 'cytokine signature' in breast cancer cells*

The concept of autocrine or paracrine loops in breast cancer aetiology and metastatic phenotype has been proposed by several studies. Cytokines, in particular, are emerging as potential factors that could contribute to the progression of breast cancer.[21–23] Because HER2-overexpressing cancer cells display distinct phenotypes to those observed in HER2-negative cancer cells, we here hypothesised that such phenotypes may be a result of differential expression of inflammatory and pro-angiogenic chemokines, which are known to play a role

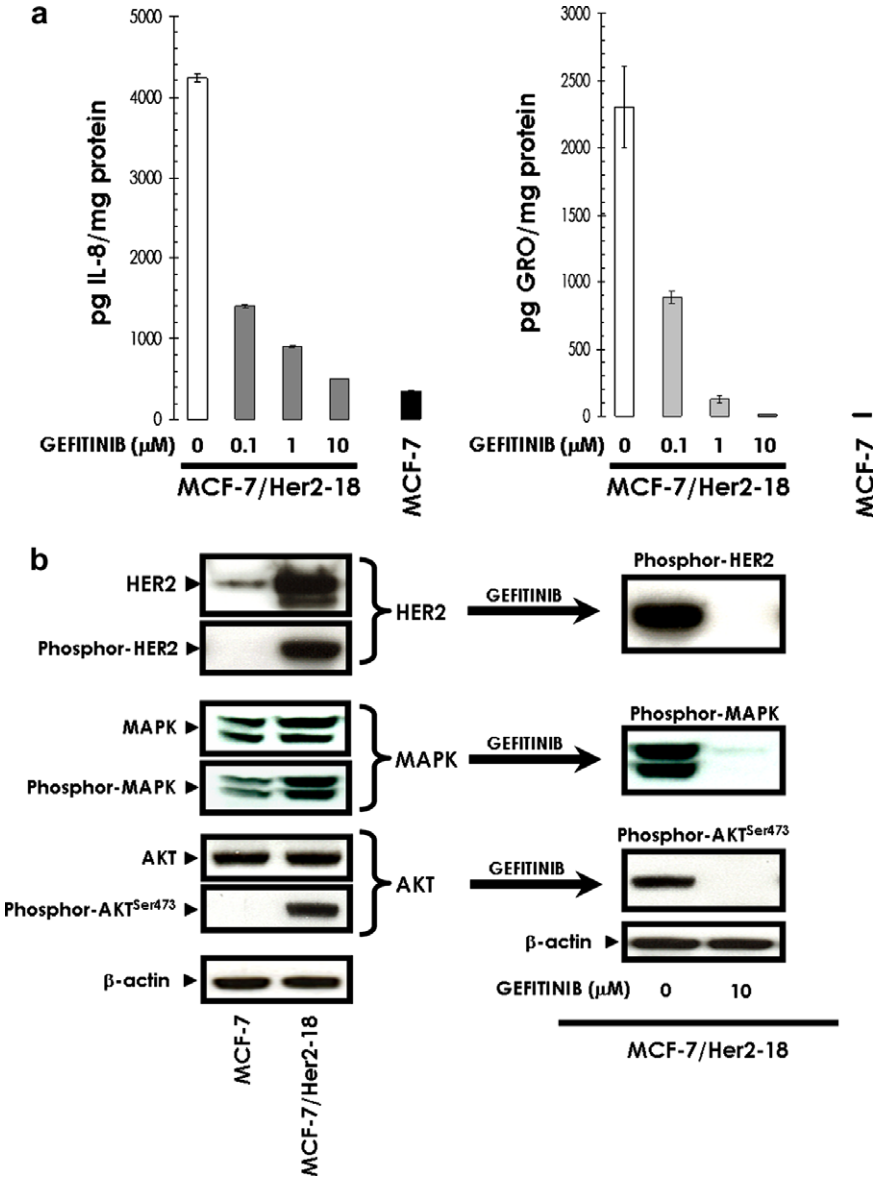

Fig. 2 – Regulation by HER2 of IL-8 and GRO secretion from breast cancer cells. (a) IL-8 (*left*) and GRO (*right*) concentrations in CM of gefitinib-treated MCF-7/Her2-18 cultures as well as untreated CM of MCF-7 parental cell cultures were assessed by ELISA as per manufacturer's instructions. Values represent means ± SD of results from three independent experiments. (*b*) Overnight serum-starved MCF-7 and MCF-7/Her2-18 cells were cultured for 48 h in low-serum (0.1% FBS) DMEM in the presence or absence of gefitinib (10 μM). The activation status of HER2, MAPK and AKT was tested using immunoblotting procedures as described in the Material and methods section. Figure shows a representative immunoblotting analysis. Equivalent results were obtained in at least three independent experiments.

in cell migration, invasion and/or metastasis.[21–32] Using human cytokine antibody arrays, a recently developed proteomic technique capable of simultaneously detecting expression levels of multiple cytokines, we found that HER2 re-expression in breast cancer cells endogenously expressing low levels of HER2 (i.e. MCF-7 → MCF-7/ Her2-18 transition) leads to the specific up-regulation of solely CXCL8 (IL-8) and CXCL1 (GRO), two members of the CXC family of chemokines. Given that gefitinib (Iressa™)-induced pharmacological blockade of HER2 autophosphorylation and HER2-activated transduction cascades PI-3′K → AKT and MEK1/2 → ERK1/2 MAPK returned IL-8 and GRO expression levels back to the baseline observed in parental HER2-negative MCF-7 breast cancer cells, it is reasonable to suggest that hypersecretion of IL-8 and GRO chemokines is a previously unrecognised molecular feature that specifically accompanies HER2-dependent metastatic phenotype in breast cancer disease (Fig. 4).

4.2. *HER2-induced 'cytokine signature' mimics 'cytokine signature' in highly invasive oestrogen receptor (ER)-negative breast cancer cells*

Young and colleagues were pioneers observing that breast carcinoma cells, especially ER-positive MCF-7 cells, can

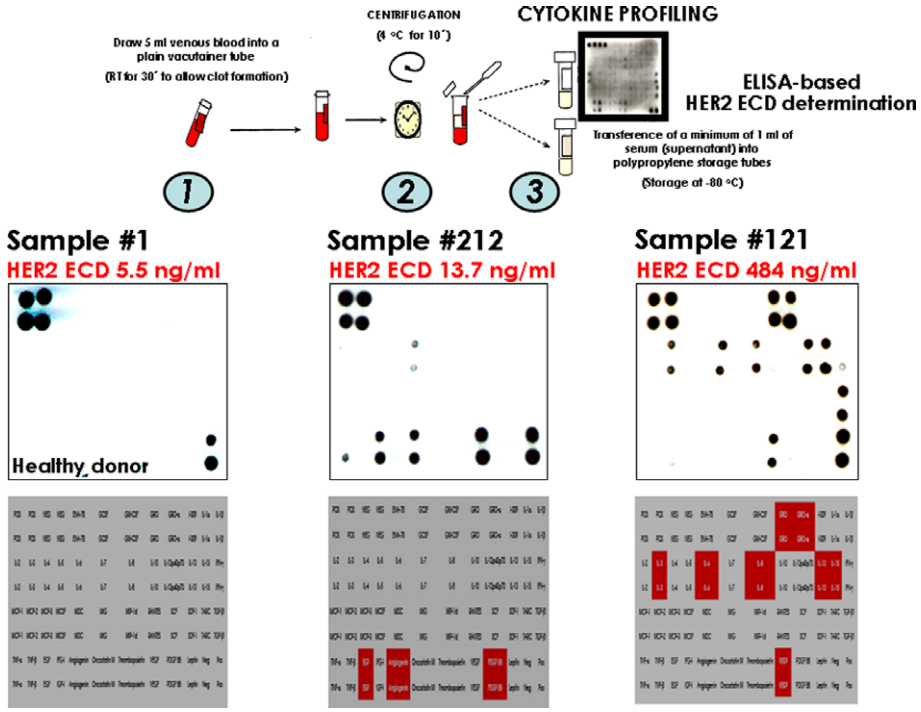

Fig. 3 – Detection of cytokine expression from metastatic breast cancer (MBC) patient's sera. *Top*. Preparation of sera from venous blood of MBC patients ($n = 12$; $n = 6$ for HER2 ECD > 15 ng/ml and $n = 6$ for HER2 ECD < 15 ng/ml) and healthy donors ($n = 6$). *Bottom*. Undiluted MBC patient's sera (1 ml/each) were incubated with cytokine array membranes, and the signals were detected as described under Materials and methods. Densitometric data were arbitrarily expressed as red for extremely high (>10-fold increase) when compared to those found in healthy donor's sera. HER2 ECD values were determined as previously described.[39,40]

respond to chemokines such as IL-8 and GRO, and suggested a potential role for these molecules in the process of tumour cell migration, invasion and metastasis.[32] By using human cytokine antibody array technology, Lin and colleagues recently identified IL-8 as a key factor involved in breast cancer invasion and angiogenesis, but not in breast cancer proliferation.[28] Interestingly, IL-8 overexpression in invasive breast cancer cells has been found to inversely correlate with ER status. Thus, ER-positive breast cancer cells express low levels of IL-8, IL-8 overexpression naturally occurs in ER-negative breast cancer cells, and exogenous expression of ER in ER-negative cells decreases IL-8 levels.[27–30] Li and Sidell, also using protein array technology, determined that highly invasive and ER-negative MDA-MB-231 breast cancer cells secrete a number of cytokines known to regulate cellular growth and motility.[31] One such cytokine, GRO, which was previously not described in breast cancer, actively mediated the invasive potential of MDA-MB-231 cells. Similarly to IL-8, GRO was not involved in the proliferation rate of metastatic breast cancer cells.[31] Here, human antibody array technology revealed that IL-8 and GRO cytokines are significantly up-regulated upon HER2 overexpression in MCF-7 breast cancer cells. Thus, HER2-induced 'cytokine signature' in ER-positive MCF-7/Her2-18 cells appears to be molecularly equivalent to that observed in ER-negative breast cancer cells. Signal transduction mediated by HER2 can partially overcome the oestrogen dependence of 'ER-positive' breast cancer cells for growth and that HER2 overexpression confers a selective advantage to such cell in the absence of oestrogen.[33,34] Indeed, increased ER-HER2 cross-talk has been recognised as a main molecular mechanism underlying tamoxifen resistance in patients receiving adjuvant tamoxifen whose tumours express high levels of both HER2 and ER, while treatments such as gefitinib blocking receptor cross-talk efficiently restore tamoxifen's antitumour effects.[35,36] Future research should elucidate whether or not IL-8 and GRO chemokines actively contribute to the process of HER2-promoted oestrogen-independence and anti-oestrogen resistance in breast cancer disease.

4.3. *HER2-induced 'cytokine signature': Molecular and clinical implications*

Our current findings expand the concept that inflammation and inflammatory cytokines are critical components of aggressive breast cancer progression and may explain, at least in part, HER2-promoted breast cancer cell dissemination. Remarkably, IL-8 and GRO circulating levels were significantly higher in HER2-positive metastatic breast cancer patients when compared to those found in HER2-negative patients. Despite the small sample size of patients' sera profiled using cytokine-specific antibody arrays, the high similarity between the secretory status of the CXC chemokines IL-8 and GRO observed in serum samples and that obtained in media conditioned by breast cancer-derived cell lines deserves to be confirmed in larger studies. If enhanced synthesis and secretion of members of the IL-8/GRO chemokines family – which have recently been linked to ER inaction, increased cell invasion and angiogenesis[24–31] – in fact represent

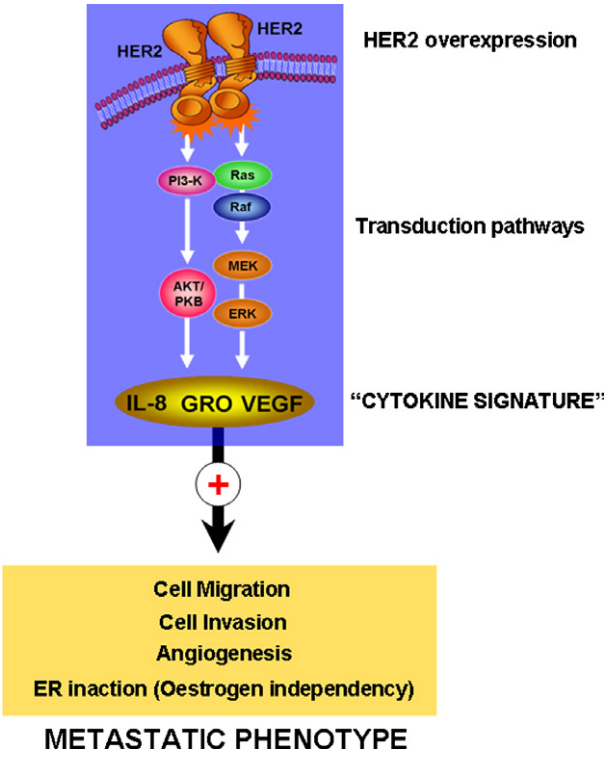

Fig. 4 – HER2-regulated 'cytokine signature' and breast cancer metastasis. We here describe that HER2 overexpression leads to the specific hypersecretion of IL-8, GRO and VEGF chemokines in human breast cancer cells. Considering the ability of IL-8, GRO and VEGF to positively modulate cell migration, cell invasion, and angiogenesis as well as the relationship between high levels of IL-8 and ER inaction, it is reasonable to suggest that HER-regulated 'cytokine signature' actively contributes to the acquisition, maintenance and/or enhancement of the metastatic phenotype in human breast cancer disease.

a new pathway involved in the metastatic progression and endocrine resistance of HER2-overexpressing breast carcinomas, circulating levels of IL-8/GRO cytokines may represent novel biomarkers monitoring breast cancer responses to endocrine treatments and/or HER2-targeted therapies. Moreover, considering that neutralisation of IL-8 or GRO by functional antibodies has been found to specifically block cell migration and invasion of metastatic breast cancer cell lines *in vitro*,[28,30,31] development of therapeutic molecules targeting IL-8/GRO chemokines and/or human IL-8/GRO receptors may represent a novel avenue in the management of breast carcinomas that overexpress HER2.

Because of the limitation of technology previous studies only measured single or few cytokines at once when assessing the role of chemokines in breast cancer progression. Human cytokine antibody arrays, which can simultaneously detect expression levels of multiple cytokines, combine advantages of the specificity of ELISA, sensitivity of ECL, and high-throughput of microspot.[37] Our study supports the notion that, by using this innovative, simple, flexible and cost-effective protein array technology, comparative cytokine mapping of conditioned media from tumour-derived breast cancer cell lines and sera from breast cancer patients should represent a valuable discovery tool to identify potential targets involved in breast cancer progression.

Note: While preparing this manuscript, When and colleagues[38] reported that re-expression of HER2 in MCF-7 and T-47D breast cancer cells likewise resulted in elevated expression of VEGF and IL-8.

Conflict of interest statement

None declared.

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