An update of the mechanisms of resistance to EGFR-tyrosine kinase inhibitors in breast cancer: Gefitinib (Iressa™)-induced changes in the expression and nucleo-cytoplasmic trafficking of *HER*-ligands (Review)

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Abstract. Intrinsic resistance to the epidermal growth factor receptor (EGFR; HER1) tyrosine kinase inhibitor (TKI) gefitinib, and more generally to EGFR TKIs, is a common phenomenon in breast cancer. The availability of molecular criteria for predicting sensitivity to EGFR-TKIs is, therefore, the most relevant issue for their correct use and for planning future research. Though it appears that in non-small-cell lung cancer (NSCLC) response to gefitinib is directly related to the occurrence of specific mutations in the EGFR TK domain, breast cancer patients cannot be selected for treatment with gefitinib on the same basis as such EGFR mutations have been reported neither in primary breast carcinomas nor in several breast cancer cell lines. Alternatively, there is a general agreement on the hypothesis that the occurrence of molecular alterations that activate transduction pathways downstream of EGFR (i.e., MEK1/MEK2 → ERK1/2 MAPK and PI-3'K → AKT growth/survival signaling cascades) significantly affect the response to EGFR TKIs in breast carcinomas. However, there are no studies so far addressing a role of EGF-related ligands as intrinsic breast cancer cell modulators of EGFR TKI efficacy. We recently monitored gene expression profiles and sub-cellular localization of *HER*-1/-2/-3/-4 related ligands (i.e., EGF, amphiregulin, transforming growth factor-α, ß-cellulin, epiregulin and neuregulins) prior to and after gefitinib treatment in a panel of human breast cancer cell lines. First, gefitinib-induced changes in the endogenous levels of EGF-related ligands correlated with the natural degree of breast cancer cell sensitivity to gefitinib. While breast cancer cells intrinsically resistant to gefitinib (IC_{50} ≥15 μM) markedly up-regulated (up to 600 times) the expression of genes codifying for *HER*-specific ligands, a significant down-regulation (up to 10^6 times) of *HER* ligand gene transcription was found in breast cancer cells intrinsically sensitive to gefitinib (IC_{50} ≤1 μM). Second, loss of HER1 function differentially regulated the nuclear trafficking of *HER*-related ligands. While gefitinib treatment induced an active import and nuclear accumulation of the *HER* ligand NRG in intrinsically gefitinib-resistant breast cancer cells, an active export and nuclear loss of NRG was observed in intrinsically gefitinib-sensitive breast cancer cells. In summary, through *in vitro* and pharmacodynamic studies we have learned that, besides mutations in the HER1 gene, oncogenic changes downstream of HER1 are the key players regulating gefitinib efficacy in breast cancer cells. It now appears that pharmacological inhibition of HER1 function also leads to striking changes in both the gene expression and the nucleo-cytoplasmic trafficking of *HER*-specific ligands, and that this response correlates with the intrinsic degree of breast cancer sensitivity to the EGFR TKI gefitinib. The relevance of this previously unrecognized intracrine feedback to gefitinib warrants further studies as cancer cells could bypass the antiproliferative effects of HER1-targeted therapeutics without a need for the overexpression and/or activation of other *HER* family members and/or the activation of *HER*-driven downstream signaling cascades.

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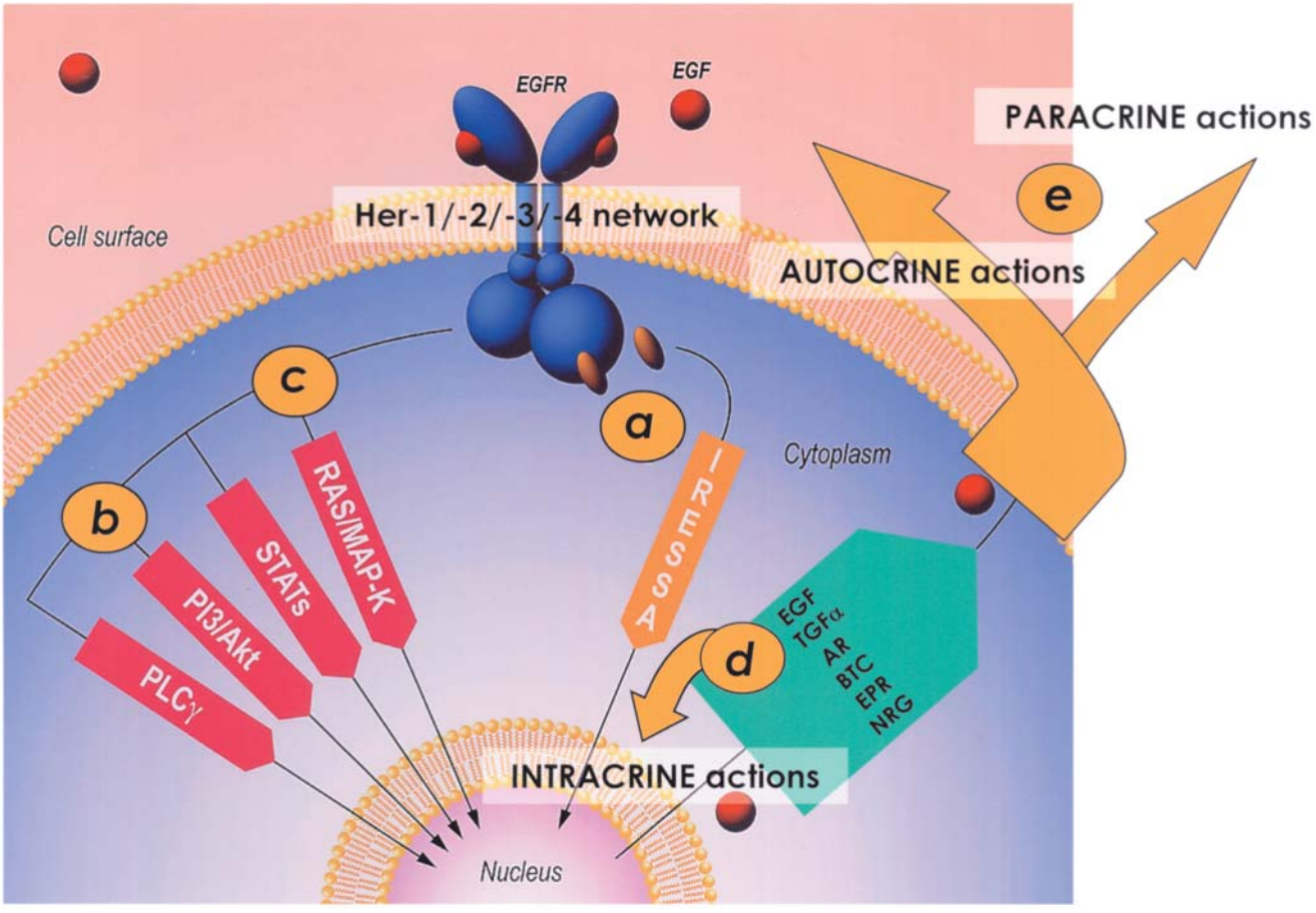

Figure 1. Mechanisms of cancer resistance to Gefitinib (Iressa). (a) HER1 mutations. (b) The 'AKT hypothesis' for breast cancer resistance to gefitinib. (c) The 'MEK/MAPK hypothesis' for breast cancer resistance to gefitinib. (d and e) Up-regulation of EGF-related ligands: a novel mode for breast cancer resistance to gefitinib. Our current findings reveal that gefitinib-induced changes in the expression pattern of genes codifying for EGF-related ligands strikingly correlate with the intrinsic degree of sensitivity of breast cancer cells to the antiproliferative effects of gefitinib; i.e., gefitinib-resistant and gefitinib-sensitive breast cancer cells markedly up-regulate and down-regulate, respectively, the expression of genes codifying for EGF-related ligands. Therefore, activation/deactivation of HER-ligand gene clusters appears to balance loss of HER1-receptor function in breast cancer cells. Moreover, pharmacological blockade of HER1 TK activity differentially regulates an active nuclear import/export of EGF-related ligands in gefitinib-sensitive and gefitinib-resistant breast cancer cells. Since EGF-related ligands may function through intracrine, paracrine and/or autocrine pathways to control cell proliferation and survival in breast cancer cells, altered expression and/or cellular localization of HER-specific ligands may represent a previously unrecognized mechanism determining intrinsic breast cancer resistance/sensitivity to the EGFR TKI gefitinib.

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1. Gefitinib and breast cancer: A disappointing history

The epidermal growth factor receptor (EGFR; HER1) and its cognate ligands have been shown to actively contribute to the pathogenesis and progression of breast cancer (1-4). This evidence led to the development of different anti-EGFR agents, and several have undergone clinical trials, including the small molecule tyrosine kinase inhibitor (TKI) gefitinib (Iressa; AstraZeneca, Macclesfield, UK). Gefitinib is a low molecular weight inhibitor with highly selective and reversible TK inhibition properties directed at the EGFR, being a competitive inhibitor of adenosine triphosphate binding to this receptor (5-6).

Initial phase II studies have suggested that gefitinib does not have a high efficacy in a heavily pre-treated population of patients with metastatic breast cancer, particularly post chemotherapy (7). With the exception of a recent neoadjuvant trial in estrogen receptor (ER)-positive breast cancer patients (8), clinical studies of the EGFR TKI gefitinib in breast cancer resulted in few clinical responses and in a disease control rate of approximately 10% (7,9). These observations clearly indicate that intrinsic resistance to gefitinib, and more generally to EGFR TKIs, is a common phenomenon in breast cancer (7).

2. How breast cancer cells escape the anti-tumor effects of gefitinib

Unraveling the ultimate molecular mechanisms determining the resistance/sensitivity of breast cancer cells to gefitinib is a major challenge that is only beginning to be addressed. It has been recently demonstrated that in non-small-cell lung cancer (NSCLC) response to gefitinib is directly related to the occurrence of specific mutations in the EGFR TK domain (10-13; Fig. 1a). Unfortunately, breast cancer patients cannot

be selected for treatment with gefitinib on the same basis as such EGFR mutations have been reported neither in primary breast carcinomas nor in several breast cancer cell lines (12,14,15). NSCLCs carrying mutations of the HER1 tyrosine kinase domain are highly sensitive to gefitinib (reviewed in ref. 13). Although the rate of mutations in breast cancer patients remains unclear, Lynch *et al* did not find any mutations of HER1 exons 19 and 21 in any of 15 primary breast cancer samples analyzed nor in the breast cancer-derived cell line MDA-MB-468 (12). The absence of HER1-activating mutations in breast carcinomas has also been confirmed by Bhargava *et al* (14). Normanno *et al* recently investigated the status of the HER1 gene in SK-Br3 and MDA-MB-361 cells that have not been previously screened for HER1 mutations. Sequence analysis of the whole HER1 TK domain revealed no mutations as compared with the wild-type HER1 gene sequence (15). Alternative mechanisms should be involved in regulating breast cancer cell responses to gefitinib.

There is a general agreement on the hypothesis that the occurrence of molecular alterations that activate signal transduction pathways downstream of EGFR might affect the response to EGFR TKIs in breast carcinomas. In this regard, it has been hypothesized that the PI-3'K/AKT pathway is the main signaling pathway involved in the resistance to gefitinib (16-18; Fig. 1b). Gefitinib treatment has been found to produce a significant reduction of AKT activation in gefitinib-sensitive cell lines. On the contrary, inhibition of MAPK1/2 activation upon gefitinib treatment occurred in cell lines with either high or low sensitivity to this drug. The main model in which the role of the PI-3'K/AKT pathway in the resistance to gefitinib has been explored is the MDA-MB-468 breast cancer cell line. These cells carry a deletion of the PTEN tumor suppressor protein leading to constitutive activation of the PI-3'K/AKT pathway. Moreover, the reconstitution of PTEN function or inhibition of PI-3'K activity with specific inhibitors can restore gefitinib sensitivity in MDA-MB-468 cells. This hypothesis has been confirmed by a pharmacodynamic study of Baselga *et al* (20). These authors found that treatment of advanced breast cancer patients for 28 days with gefitinib produced a significant inhibition of both HER1 phosphorylation and MAPK activation but not of AKT activation in tumor tissue, and this phenomenon was associated with no significant effects of gefitinib on tumor cell proliferation as measured by Ki67 immunostaining. Evidence also suggests that the MEK/MAPK pathway might be involved in the spontaneous and acquired resistance of breast cancer cells to EGFR TKIs (15,19; Fig. 1c). Normanno *et al* recently demonstrated the direct involvement of the MAPK signaling cascade in the resistance of breast cancer cells to gefitinib (15). In their study, overexpression of a constitutively activated form of MAPK in MCF10A non-transformed human mammary epithelial cells resulted in a 2- to 3-fold increase in the IC_{50} value to gefitinib. In agreement with this observation, combined treatment with gefitinib and the MEK inhibitor PD98059 produced a synergistic anti-tumor effect and also produced a significant increase in the levels of apoptosis in breast cancer cells with natural resistance to gefitinib (e.g., MDA-MB-468 cells) as compared with treatment with a single agent (15). This hypothesis has been confirmed by Magne *et al* (19), who showed an association between intrinsic activation of MAPK signaling and resistance to gefitinib in head and neck cancer cells. Pre-operative treatment of ER-positive/HER1-positive breast cancer patients with gefitinib alone or gefitinib plus anastrozole, the only clinical trial so far demonstrating significant benefits for gefitinib-containing regimens in breast cancer, produced significant reduction of MAPK activation and Ki67 labeling index (8). These hypotheses have been confirmed by pharmacodynamic studies (20).

Response to EGFR TKIs might also correlate with the total levels of expression of the different *HER* receptors (HER1/-2/-3/-4). In fact, low levels of EGFR might be sufficient to trans-activate other *HER* receptors through the formation of *HER* heterodimers and through lateral signaling (21,22). In this respect, breast cancer cell lines with low levels of expression of EGFR are extremely sensitive to the anti-tumor effects of gefitinib, if they co-express high levels of HER2 (16,23-25). This effect has been demonstrated to be mediated, at least in part, by gefitinib reduction of HER1/HER2 heterodimer phosphorylation (16,23-25).

3. Ligands of the EGFR network: Forgotten protagonists in gefitinib resistance

The ligands that bind to and activate the *HER* family of TKs are known as EGF-related peptide growth factors. They exhibit differential receptor binding specificities and are grouped into three classes: one group of ligands including EGF, amphiregulin (AR) and transforming growth factor-α (TGFα) bind specifically to HER1; a second group composed of ß-cellulin (BTC) and epiregulin (EPR) bind to HER1 and HER4; and a third group composed of neuregulins (NRG) bind to HER3 and HER4 (22,27,28). HER2 remains an orphan receptor, with no soluble ligand identified to date. However, this receptor occupies a pivotal role in the TK function of the EGFR network, being the preferred and most potent heterodimerization partner for the other *HER* receptors (29).

Because the ligands activate different *HER* receptors and most tumors of epithelial origin express multiple *HER* receptors and coexpress one or more of the EGF-related ligands, multiple *HER* receptor combinations might be active in a tumor. This characteristic should influence tumor response to an *HER*-targeted therapeutic and, therefore, the efficacy of anticancer drugs that block a single *HER* receptor may be comprised by the presence of EGF-related ligands. In this regard, Motoyama *et al* demonstrated that the growth-inhibitory effects of monoclonal antibodies and TKIs targeting HER1 and HER2 are significantly attenuated in the presence of exogenous ligands (26). However, there are no studies so far addressing the effects of EGFR TKIs on the endogenous expression of EFG-related ligands. Using real-time RT-PCR, we recently characterized MCF-7, MDA-MB-231, MDA-MB-468 and SK-Br3 breast cancer cell lines according to the mRNA expression of ligands from the EGF network prior to and after gefitinib treatment (Table I). When expression data were normalized relative to ribosomal RNA (18S RNA) as endogenous control gene and related to the gefitinib 50% inhibitory concentration (IC_{50}) in each cell line, we made the following conclusions.

Low HER1-expressing MCF-7 cells, as expected, were insensitive to gefitinib (IC_{50} >20 μM). Interestingly, a subset

Table I. Gene expression profiles for ligands of the EGF network following gefitinib treatment.

		HER receptor status				Gefitinib-induced changes in *HER*-specific ligands					
						HER1 ligands			HER1/4 ligands		HER3/4 ligand
	Gefitinib IC_{50}	HER1	HER2	HER3	HER4	EGF	AR	TGFα	BTC	EPR	NRG
MCF-7	21±2 μM	-/+	+	+++	+++	+12	n.d.	+7	n.d.	+600	+18
MDA-MB-231	18±2 μM	+++	-/+	-/+	-/+	n.d.	-2	-3	+6	n.d.	-2
MDA-MB-468	15±1 μM	+++	-/+	++	-/+	+21	+9	n.d.	+12	n.d.	+8
SK-Br3	1±0.2 μM	++	+++	++	-/+	n.d.	n.d.	-10^4	n.d.	n.d.	-10^6

-/+, negative/weak; ++, moderate; +++, strong; n.d., not determined. Using real-time RT-PCR, MCF-7, MDA-MB-231, MDA-MB-468 and SK-Br3 breast cancer cell lines were characterized according to the mRNA expression of ligands from the EGF network prior and after gefitinib treatment (3 days). Data indicate x-fold changes in the expression of genes codifying for EGF-related ligands by comparing with control (DMSO-treated) cells. The metabolic status of gefitinib-treated cells was first evaluated using an MTT-based cell viability assay and constructing dose-response curves. Gefitinib concentrations required to produce the IC_{50} value (the drug concentration needed to reduce cell viability by 50% relative to untreated control cells) were calculated by interpolation. HER receptor status was characterized by immunoblotting and ELISA procedures and yielded equivalent results to those previously reported by Moasser *et al* (16).

of ligands of the EGF network significantly increased following gefitinib treatment. Specifically, the mRNA expression of EPR and NRG, two ligands of the HER3 and HER4 receptors, which are overexpressed in MCF-7 cells, showed up to 600-fold increased concentration upon gefitinib exposure.

In agreement with Moasser *et al* (16), HER1-overexpressing MDA-MB-231 cells were rather insensitive to the antiproliferative effects of gefitinib (IC_{50} = 18±2 μM), as compared with the vulvar carcinoma cell line A431, which expresses ~$2x10^6$ HER1 receptors/cell and is classically used as a positive control for anti-HER1 therapies (IC_{50} = 0.8±2 μM; data not shown). Of note, gefitinib-treated MDA-MB-231 cells significantly up-regulated by 6-fold mRNA expression of BTC, a potent survival factor even in the absence of its HER1 receptor (30).

MDA-MB-468 cells (IC_{50} = 15±1 μM), the main 'HER1-positive' breast cancer model of gefitinib resistance via constitutive activation of the intracellular signaling downstream of the HER1 receptor (i.e., PI-3'K/AKT and MEK/MAPK pathways) (15-18), provided an interesting comparison. Upon gefitinib treatment, HER1- and HER3-overexpressing MDA-MB-468 cells significantly up-regulated (up to 21 times) the HER1 ligands EGF, AR and BTC as well as the HER3 ligand NRG.

HER2-overexpressing SK-Br3 breast cancer cells, which also express HER1 and HER3, were exquisitely sensitive to gefitinib (IC_{50} <1 μM). Searching for potential mechanisms explaining the high sensitivity of SK-Br3 cells to gefitinib, Anido *et al* observed that gefitinib promotes the formation of inactive (unphosphorylated) HER1/HER2 and HER1/HER3 heterodimers (31). Moreover, they found that gefitinib-treated SK-Br3 cells are no longer stimulated by the non-HER1 ligand NRG, suggesting that gefitinib also exerts its antitumor effects by sequestration of HER2 and HER3, a favorite heterodimerization partner of HER2, into inactive HER1-based heterodimers (31). Remarkably, we here found that the HER1 ligand TGFα was profoundly down-regulated (up to 104 times) following gefitinib treatment. Moreover, the expression of the HER3 ligand NRG was completely abolished (up to 106 times down-regulation) in the presence of gefitinib (Table I).

We have partially validated our findings using immunoblotting analyses, which likewise demonstrated a significant modulation of the protein levels of *HER*-specific ligands upon gefitinib treatment (data not shown). Collectively, these findings reveal for the first time the following: a) gefitinib-induced changes in the expression pattern of genes codifying for EGF-related ligands strikingly correlate with the intrinsic degree of sensitivity of breast cancer cells to the antiproliferative effects of gefitinib; i.e., gefitinib-resistant and gefitinib-sensitive breast cancer cells markedly up-regulate and down-regulate, respectively, the expression of genes codifying for EGF-related ligands; and b) gefitinib exposure appears to regulate *HER* ligand gene clusters in an *HER* receptor-dependent manner; i.e., gefitinib-treated breast cancer cells specifically modulate the gene expression of EGF-related ligands with high affinity to the set of *HER* receptors present in the cells.

These findings suggest that activation/deactivation of *HER* ligand gene clusters might balance loss of *HER* receptor function and, therefore, may represent a previously unrecognized mechanism determining intrinsic breast cancer resistance/sensitivity to the EGFR TKI gefitinib.

4. Gefitinib and the HER network: From the cell surface to the nucleus

Both intrinsic and acquired resistance to EGFR inhibitors have been identified as potential treatment obstacles for patients receiving *HER* receptor-directed therapeutics. *In vitro* and pharmacodynamic studies support a role for the signaling pathways downstream of the EGFR (i.e., PI-3'K/AKT and MEK/MAPK) in the intrinsic resistance of breast cancer cells to gefitinib (5-20). We here report the selective increase in the expression of genes codifying for growth factors of the EGF

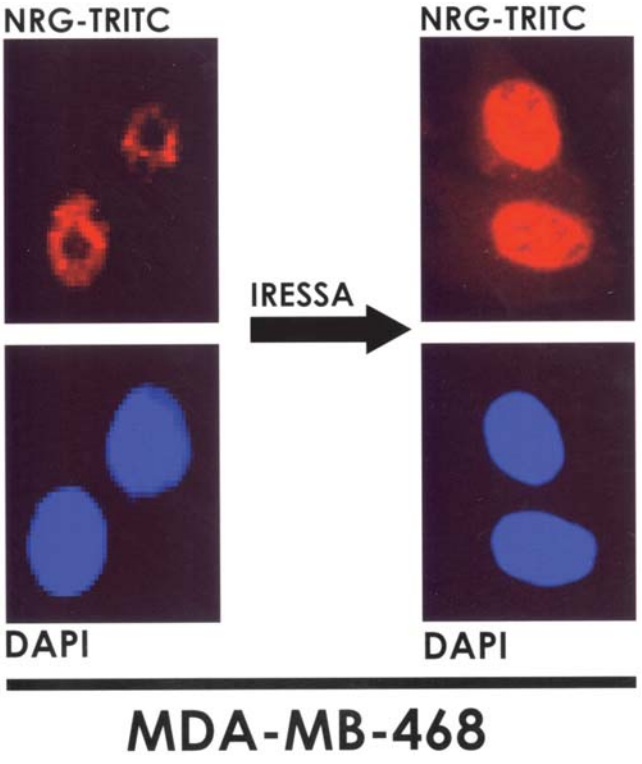

Figure 2. Gefitinib treatment modifies nuclear accumulation of the *HER*-specific ligand NRG. Gefitinib (Iressa)-resistant MDA-MB-468 breast cancer cells grown on cover-slides in the absence or presence of 10 μM gefitinib (48 h) were fixed for 15 min in PBS containing 2% paraformaldehyde and then permeabilized in PBS containing 0.2% Triton X-100 for 5 min, washed with PBS, and post-fixed for 20 min in 100% methanol at -20˚C. After two washes with PBS, cells were blocked in PBS containing 5 mg/ml BSA and 20 mM Glycine for 30 min. Cells were stained with an anti-NRG antibody (1:100 dilution, 1 h, room temperature; C-20, sc-348, Santa Cruz Biotech., Santa Cruz, California, USA) followed by TRITC-conjugated anti-rabbit IgG antibody (45 min, room temperature), both of which were diluted with PBS containing 5 mg/ml BSA. DNA was stained with diamidino-2-phenylindole (DAPI). Stained cells were observed and analyzed using a Nikon Eclipse E800 microscope equipped for epifluorescence. A representative immunofluorescence is shown (n=3).

network as a previously unrecognized molecular response of breast cancer cells naturally resistant to gefitinib. Interestingly, breast cancer cells with acquired resistance to the anti-HER2 monoclonal antibody trastuzumab (Herceptin®) express higher levels of EGF-related ligands as compared with parental, trastuzumab-sensitive cells (32). Therefore, the intrinsic or acquired ability of breast cancer cells to specifically regulate *HER* ligand gene clusters may represent a common molecular mechanism determining the efficacy of anticancer drugs blocking a single *HER* receptor.

An intriguing question, however, arises from the above hypothesis. If an overproduction of EGF-related growth factors actively rescues the antiproliferative effects of gefitinib, we should expect ligand-induced activation of other *HER* family members and reactivation of downstream signaling pathways upon gefitinib exposure, which does not occur. How can we reconcile these conflicting findings? Firstly, mitogenic growth factors including EGF-related ligands are generally considered cell surface-associated or secreted proteins, which produce effects by binding to cell surface receptor TKs. However, evidence is mounting that several members of the *HER* receptor ligand family can translocate to the nucleus and, more importantly for this study, they play a role in the etiology, progression and chemosensitivity of breast cancer by mechanisms independent of *HER* receptor activation (33-37). Secondly, like other receptor TKs located in the cell membrane, the members of the EGFR TK family are conventionally considered as integral membrane molecules transferring signals through the cell membrane. However, members of the *HER* receptor family have also an important role directly within the nucleus. For instance, it now appears that a direct mode of EGFR signaling in which EGF shuttles its HER1 receptor into the nucleus, results in transcriptional activation of target genes (38,39). Recent studies have also revealed the presence of HER2 in the nucleus of breast cancer cells and identified the HER2 nuclear function as a transcriptional regulator (40,41).

The well-established traditional function of the *HER* signaling pathway is known to transmit extra-cellular mitogenic signals from EGF-related ligands through activating a number of downstream signaling cascades. In cancer cells, the common outcomes following the activation of the *HER* network are altered gene activities, leading to uncontrolled tumor proliferation and apoptosis. As described above, emerging evidence suggests the existence of a novel 'direct mode' involving cellular transport of *HER* receptors or *HER* ligands to the cell nucleus, their association with gene promoters, and transcriptional regulation of target genes (36,42-44). Auspiciously suggested by Lo *et al* (43,44), a better understanding of this new 'nuclear mode' of the *HER* network will facilitate the identification of patients that are likely to respond to anti-*HER* agents as well as future development of more effective therapeutic interventions aimed to block the *HER* pathway. An active role of nuclear *HER* receptors in the response of breast cancer to chemotherapy- and radiotherapy-induced cell damage has already been established (40,41). However, little is known about the role of nuclear EGF-related ligands on the response of breast cancer cells to *HER*-targeted therapies. Nevertheless, the relevance of this mechanism warrants further studies as breast cancer cells could bypass the antiproliferative effects of a HER1-directed inhibitor without a need for the overexpression and/or activation of *HER* family members and/or activation of *HER*-driven downstream signaling cascades.

In a first attempt to reveal gefitinib-induced changes in the nuclear mode of the *HER* network, we recently assessed the cellular accumulation and compartmentalization of the EGF-related ligand NRG prior to and after gefitinib treatment using immunofluorescence microscopy. NRG was shown recently to localize to the nuclei of human breast cancer cells in a receptor-independent manner, supporting the notion that secretion and subsequent cell surface receptor binding of some *HER*-ligands are not a prerequisite for nuclear localization and that nonsecreted *HER*-ligands may have highly specific functions in defined nuclear compartments (35,36). Indeed, indirect immunofluorescent studies with an anti-NRG antibody revealed a prevalent nuclear localization of endogenous NRG in MDA-MB-468 and SK-Br3 breast cancer cell lines (Fig. 2). Remarkably, this pattern of sub-nuclear dot formation of endogenous NRG was significantly affected upon gefitinib treatment. Gefitinib-resistant MDA-MB-468 cells exhibited a striking nuclear accumulation of NRG following gefitinib

exposure (Fig. 2). Conversely, gefitinib treatment significantly reduced dot-like structures containing NRG in the nuclei of gefitinib-sensitive SK-Br3 breast cancer cells (data not shown). Considering that nuclear accumulation of endogenous NRG is not a passive diffusion process (36), the above findings are extremely relevant as they clearly implicate that loss of HER1 function differentially regulates an active nucleo-cytoplasmic trafficking of NRG in gefitinib-resistant and -sensitive breast cancer cells, respectively.

At present, it is unclear what all the NRG-regulated nuclear functions are, and which of them could play a role in the response of breast cancer to gefitinib-induced cell damage. Interestingly, nuclear NRG has been shown to specifically interact with several proteins implicated in transcriptional regulation, including the transcriptional repressor histone deactylase 2 (HDAC2) (36). Using cDNA microarrays, Chinnaiyan *et al* recently identified HDAC inhibitors as having strong potential to enhance the effects of anti-*HER* agents (45). Moreover, when used cooperatively, HDAC inhibitors and gefitinib treatment has been suggested a valuable pharmacological strategy for overcoming resistance to HER1 inhibitors in patients with lung cancer (46). Further investigations should determine whether a causal connection exists between an enhanced or reduced presence of 'nuclear *HER* ligands' such as NRG, their interaction with target genes and/or protein such as HDAC, and breast cancer cell responses to gefitinib. Nevertheless, this is the first report showing that pharmacological inhibition of HER1 function leads to significant changes in both the expression and the nucleo-cytoplasmic trafficking of EGF-related ligands, and that these changes correlate with the intrinsic degree of breast cancer sensitivity to gefitinib.

5. Clinical perspectives

Overall clinical response rates to EGFR TKIs are extremely low in breast cancer, often in the range of 5-20%. Landmark studies indicate that a cohort of lung cancer patients harbor specific EGFR mutations that play a significant role in determining tumor sensitivity/resistance to gefitinib (10-13). However, preliminary findings suggest that such mutations do not occur in breast carcinomas cells (12,14,15). Therefore, it is unlikely that breast cancer patients might be selected for treatment with gefitinib on the basis of EGFR mutations. Indeed, it is unclear at this time how to select a breast cancer patient population most likely to be sensitive to gefitinib.

Although we are lacking retrospective studies evaluating the response to gefitinib treatment in breast carcinomas with known content of both HER1 and EGF-related growth factors, and caution must be applied when extrapolating *in vitro* results into clinical practice, it is reasonable to suggest that a correct molecular pathological analysis of breast carcinomas should incorporate the detection of EGF-related ligands and their intracrine, autocrine and/or paracrine actions as clinical valuable markers in gefitinib-based regimens (Fig. 1d and e). Although we acknowledge that additional experiments are required (e.g., siRNA evaluation of the impact of the up-regulation *HER* ligands on the EGFR inhibitor-resistant breast cancer phenotype), our current findings strongly suggest that gefitinib-resistant breast cancer cells retain the ability to compensate for loss of HER1 receptor function by significantly up-regulating EGF-related ligands. Therefore, dual assays monitoring the expression profile and the sub-cellular localization not only of *HER* receptors but also of *HER*-specific ligands might be more useful than a single assay to identify responders among breast cancer patients receiving *HER* receptor-targeted anticancer therapeutics. In this regard, we recently demonstrated that breast cancer cells naturally or ectopically overexpressing the HER3 ligand NRG and single-copy low levels of HER2 are unexpectedly hypersensitive to chemotherapy when co-treated with the anti-HER2 monoclonal antibody trastuzumab, while this sensitizing effect was mimicked by transfection of an antisense cDNA against the HER3/HER4 ligand NRG (47). These results and our current findings strongly reaffirm the role of ligand-induced signal amplification in mediating *HER*-dependence in breast cancer and, in agreement with a recent editorial by Arteaga, further highlight the need of incorporate *HER* ligands to the molecular profile obtained in tumors/patients enrolled in trials with drugs targeted against HER1, HER2 or HER3 (48).

6. Conclusions

According to Mendelsohn's hypothesis, formulated in the early 1980s, the pharmacological blockade of HER1 activation may be able to inhibit cancer proliferation, and cancer cells may be selectively sensitive to HER1 inhibition as compared to normal cells (49). We have learned that simple measurement of HER1 expression is not predictive of the activity of HER1 inhibitors, including gefitinib (50). Besides mutations in the HER1 gene that make the target sensitive or resistant to gefitinib, studies have revealed that additional oncogenic changes downstream of HER1 could also result in resistance to EGFR TKIs (50). According to the conventional approach, the main strategy to overcome resistance to anti-HER1 drugs should be to combine such agents with inhibitors of the intracellular signaling downstream of HER1. 'Cyclotherapy', an elegant therapeutic approach recently proposed by Blagosklonny and Darzynkiewicz, further suggests that the combination of low doses of gefitinib with cycle-dependent and apoptosis-inducing chemotherapies will be most beneficial to patients who do not respond to monotherapy with HER1 TKIs (51,52). Unfortunately, virtually nothing is known on what the autocrine and/or paracrine actions of HER ligands involved in the efficacy of gefitinib are. Furthermore, the intracrine actions of *HER*-specific ligands, bypassing the antiproliferative effects of gefitinib without a need for the overexpression and/or activation of *HER* receptors and/or the activation of *HER* receptor-driven downstream signaling cascades, has not been considered as a molecular mechanism underlying natural cancer cell resistance to gefitinib. Our current findings provide evidence that gefitinib treatment leads to significant changes in both the gene expression and the nucleo-cytoplasmic trafficking of EGF-related ligands, and that these changes correlate with the intrinsic degree of breast cancer cell sensitivity to gefitinib. We are conscious that this 'direct mode' involving active transport of *HER*-ligands to the cell nucleus, their association with gene promoters, and transcriptional regulation of target genes may add even more complexity to the 'gefitinib scenario', but it should offer a

novel rationale for a better molecular understanding and a more correct use of HER1-targeted therapeutics in breast cancer.

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