Targeting of HER2 overexpressing breast cancer cells-biosimilars and the impact of multifunctional nanoparticles

Introductions:

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Trastuzumab resistance in HER2-positive breast cancer

Using mostly preclinical models, several different mechanisms have been proposed for conferring resistance to trastuzumab.

Her2 overexpress Herceptin and resistance.

Mechanisms of Resistances:

Resistance against Trastuzumab in the breast cancer cells can be categorized broadly into five categories:

a) Mechanisms that prevent Trastuzumab and HER2 interaction,

b) Mechanisms that prevent Trastuzumab induced inactivation of HER2,

c) Aberrant downstream signaling pathways,

d) Alternate signaling pathways

e) Failure to acquired immune response.

These five mechanisms may, in turn, be executed in a variety of ways as described below.

Mechanisms that prevent Trastuzumab and HER2 interaction.

The interaction of Trastuzumab with HER2 may be obliterated or obstructed by masking off the cognate epitope, or mutations in the binding domain of HER2 (truncated HER2) or a perturbation in the expression levels of HER2 and/or HER3.

Epitope masking:

It has been observed that a membrane-associated glycosylated protein *Mucin4 (MUC4)*, can mask the epitope of HER2 thereby preventing its interaction with the antibody. It was first

reported by Nagy *et a*l in JIMT-1, a cell line derived from Trastuzumab (Herceptin) resistance breast cancer patients with Erb-2 amplification (Nagy *et al*., 2005). The authors observed that the receptor internalization following Trastuzumab exposure and the expression profile of HER-2 were similar in the cell lines resistant or sensitive to Trastuzumab. However, the mean Trastuzumab binding sites number was 1/5th of the HER-2 receptor molecules expressed in the JIMT-1 cell line.

It was observed that this resistant cell line expressed a higher amount of MUC4 and the expression levels of MUC4 were inversely correlated to the HER2 binding capability of the cells. Further, the RNAi mediated knockdown of MUC4 restored the binding of Trastuzumab to the HER2 receptor. It was thus concluded that MUC4 partially masked the HER2 receptor thereby preventing it from interaction with the normal partners.

The same group further reported that the high expression of the hyaluronan binding receptor CD44 also correlated with the downregulation of HER2 and knockdown of CD44 by siRNA causes decreased Trastuzumab internalization (Pályi-Krekk *et al*., 2007). When the JIMT-1 cells were treated with 4-methylumbelliferon (4-MU), an inhibitor of Hyaluronan synthesis, the binding of Trastuzumab to HER2 receptor enhanced. It also increased the inhibitory effect of Trastuzumab on the xenografts of JIMT-1.

Table 1. Expression levels of erbB proteins and Herceptin-induced internalization and down-regulation of erbB2

	JIMT-1	SKBR-3	BT-474	MDA-453
Expression level ($\times 10^3$)				
erbB1	160	190	16	60
erbB2	620	1,100	1,450	620
erbB3	10	42	20	18
erbB4	0	0	0	2
IGF-1R	5	4	9	5
Herceptin internalization (%)	33 ± 4	25 ± 1	22 ± 2	16 ± 2
Herceptin-induced erbB2 down-regulation (%)	40 ± 5	31 ± 2	27 ± 2	38 ± 3

NOTE: The expression levels of proteins were determined by flow cytometry. The following antibodies were used: Ab1-Clone 528 (erbB1), Ab3/OP15 (erbB2), Ab4-Clone H3.90.6 (erbB3), Ab1-Clone H4.77.16 (erbB4), and Ab1-Clone 24-31 (IGF1R). For the determination of Herceptin-induced effects, cells were treated with 10 μg/mL Herceptin. Herceptin internalization and Herceptin-induced erbB2 down-regulation were determined at 3 and 48 hours of Herceptin treatment, respectively. Herceptin internalization is expressed as the percentage of the initial level of bound Herceptin. ErbB2 down-regulation values represent the percentage by which erbB2 expression was reduced after 48 hours of Herceptin treatment, compared with untreated samples.

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Mutated HER2 (truncated HER2):

An amino terminally truncated form of HER2, called p95HER2 is constitutively active with higher kinase activity but is devoid of the extracellular domain. Hence, it cannot bind to the monoclonal antibody Herceptin. A high expression of p95HER2 occurring due to mutation in the HER2 gene over a course of time followed by a selection of such cells may lead to resistance to Trastuzumab in breast cancer patients. Breast cancer cells that express p95HER2 but not the full-length HER2 are sensitive to inhibitors such as lapatinib but not to Trastuzumab. In this context, samples from 46 breast cancer patients treated with Trastuzumab were retrospectively analysed (Scaltriti *et al*., 2007). Patients that express a truncated form of HER2 were resistant to Trastuzumab (1 out of 11; 11% response rate), while those that express full-length form of HER2 showed complete or partial response (19 out of 37; 54.4% response rate)

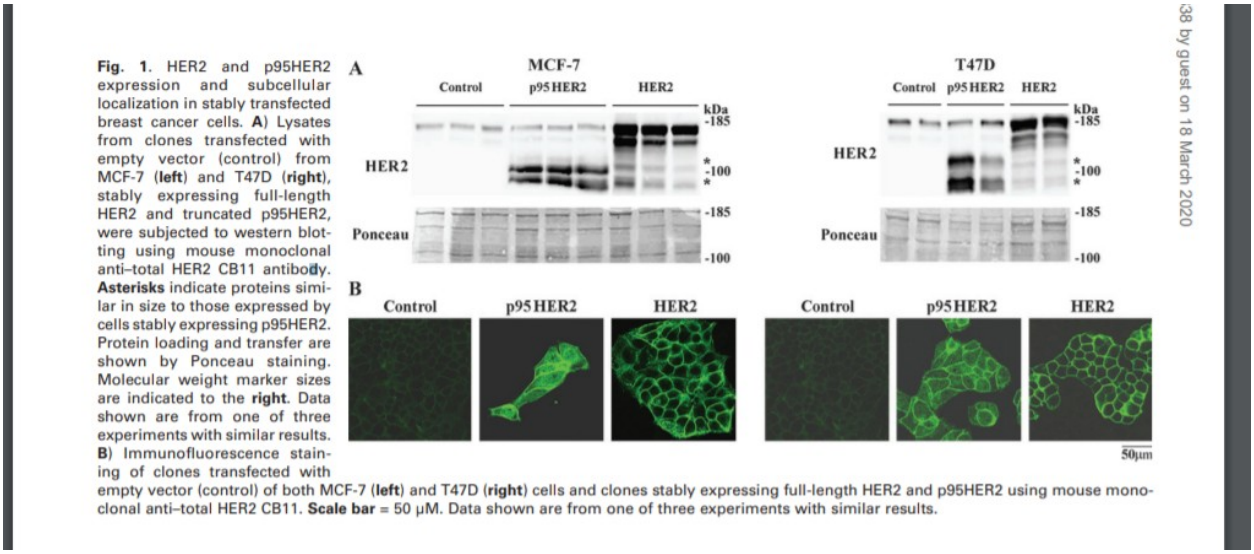

Fig. 1. HER2 and p95HER2 expression and subcellular localization in stably transfected breast cancer cells. **A**) Lysates from clones transfected with empty vector (control) from MCF-7 (**left**) and T47D (**right**), stably expressing full-length HER2 and truncated p95HER2, were subjected to western blotting using mouse monoclonal anti–total HER2 CB11 antibody. **Asterisks** indicate proteins similar in size to those expressed by cells stably expressing p95HER2. Protein loading and transfer are shown by Ponceau staining. Molecular weight marker sizes are indicated to the **right**. Data shown are from one of three experiments with similar results. **B**) Immunofluorescence staining of clones transfected with empty vector (control) of both MCF-7 (**left**) and T47D (**right**) cells and clones stably expressing full-length HER2 and p95HER2 using mouse monoclonal anti–total HER2 CB11. **Scale bar** = 50 μM. Data shown are from one of three experiments with similar results.

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Perturbation of the Expression/Activation of HER2 and/or HER3/EGFR receptors:

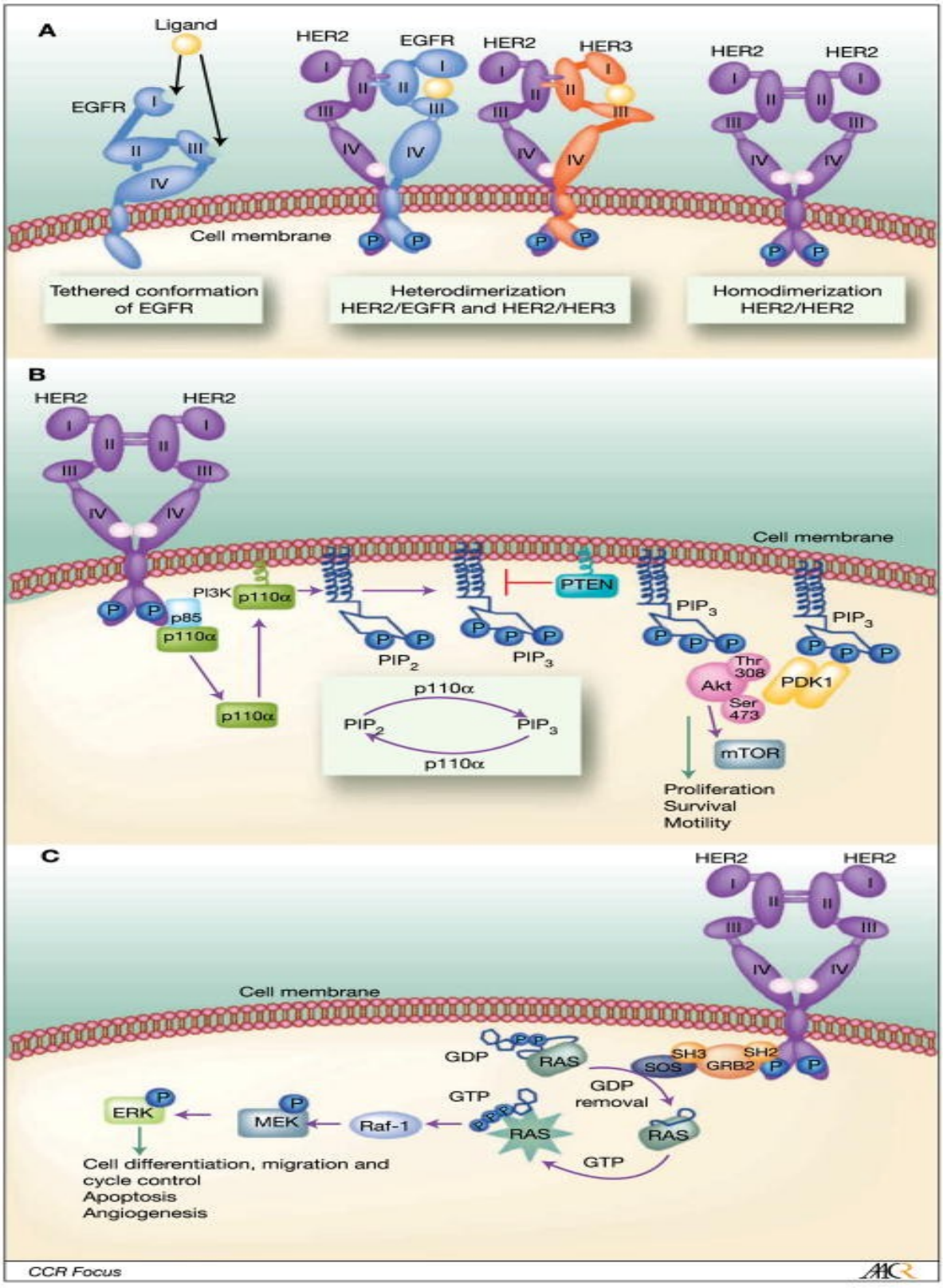
Cancer is a heterogeneous population of cells exhibiting different phenotypes. The effectiveness of Trastuzumab is thus only limited to the cells overexpressing the HER2 receptor. When the patients are treated with the monoclonal antibody the cell population of the breast cancer cells in the patient might shift from a HER2+ve to a HER2-ve state thus making Trastuzumab ineffective. A retrospective study conducted on the specimens of patients that had received neoadjuvant therapy with anthracycline, taxane, and Trastuzumab, it was observed that 1/3rd of the patients (8 out of 32) developed HER -ve phenotype (Mittendorf *et al*., 2009). In the patients

that retained HER2+ve status the response, the free survival rate was significantly better (87%) as compared to the patients that lost HER2 (50%).

Similarly, an ineffective blockade of HER2 heterodimerization might also contribute to Trastuzumab resistance. In Herceptin resistant (HR) cells that have been cultured in the continued presence of Trastuzumab, a higher amount of heregulin mRNA was observed (Ritter *et al*., 2007). It was accompanied by a higher amount of ligand-activated EGFR (phosphorylated EGFR) and consequent EGFR/HER2 heterodimers. Such HR cells were sensitive to EGFR inhibitors such as erlotinib and gefitinib and EGFR/HER2 inhibitor lapatinib.

Mechanisms that prevent Trastuzumab induced inactivation of HER2

In this above context, the role of ADAM10 (metalloprotease and disintegrin) and ADAM17 cell surface protease , they have been involved in ectodomain shedding of many ligands of HER family receptors (Duffy, Crown and Mullooly, 2014). ADAM17 has been reported to be causing the release of HER family ligands of EGFR, HER3, and HER4 via a PKB (protein kinase B) negative feedback loop, resulting in a hetero-dimerization of HER2 with these receptors (Gijsen *et al*., 2016). That eventually maintains a phosphorylation level of HER2. In other words, due to ADAM17, Trastuzumab cannot execute its effect despite binding to HER2, because HER2 is still activated. Similarly, the expression of ADAM10 was also observed to be enhanced in breast cancer cells following Trastuzumab exposure and it correlated well with the concurrent decrease in PKB phosphorylation (Feldinger *et al*., 2014). Knockdown or inhibition of ADAM10 enhances the sensitivity of breast cancer cells to Trastuzumab even if they are resistant to it. Moreover, if breast cancer patients have higher ADAM10 levels before treatment with Trastuzumab, then there is a high probability of them showing poor clinical response. The higher the level of ADAM10 the poorer relapse-free survival rates in patients treated with Trastuzumab.



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Aberrant downstream signaling pathways:

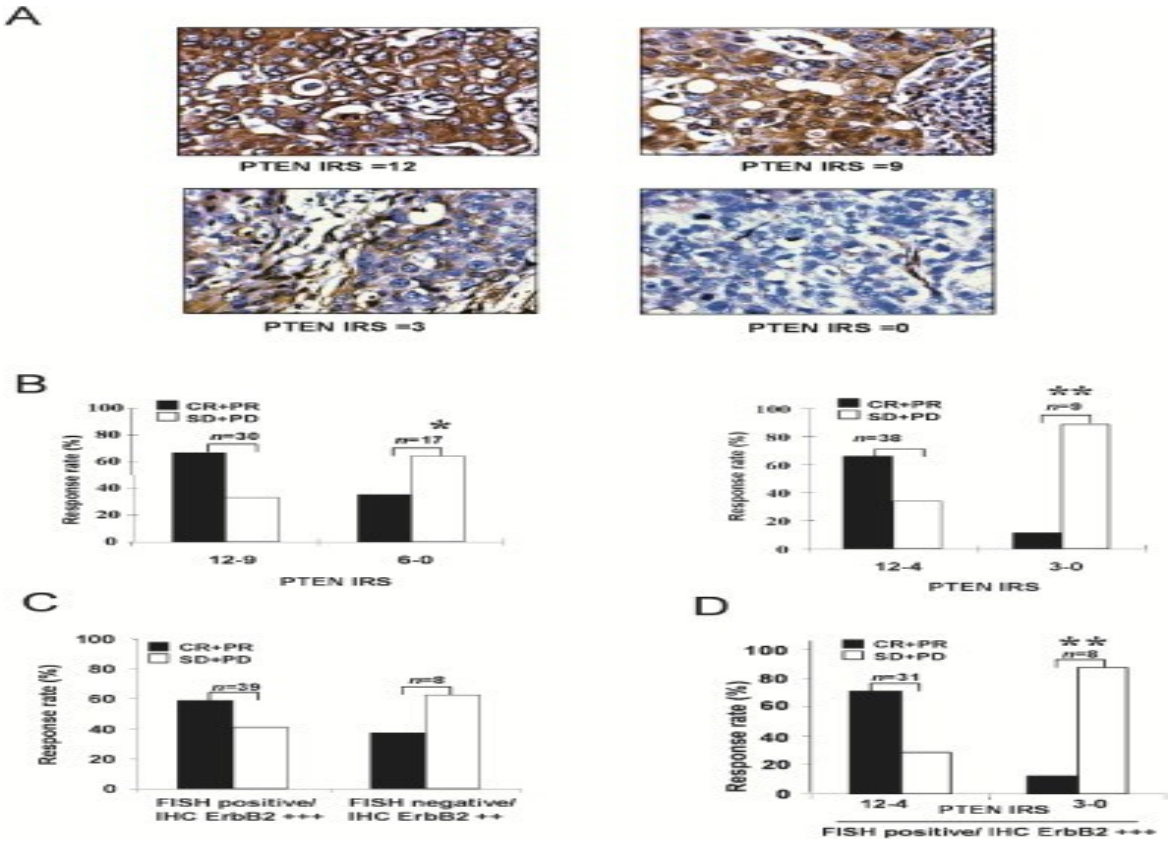
Upon activation, HER2 may hetero-dimerize with other receptors such as HER3, auto-phosphorylate and activate a plethora of downstream signaling pathways. Prominent among them are the Ras-Raf-MAPK pathway and PI3K-PDK-AKT pathway. Aberrant signaling events in these pathways may, in turn, lead to unexpected results following Trastuzumab treatment despite an apparent normal HER2 binding.

Activation of the PI3K-AKT pathway

PI3K is an oncogene that codes for an enzyme that consists of a regulatory subunit p85 and a catalytic subunit p110α. Activating mutations in this gene may lead to an activation of this pathway leading to enhanced cell proliferation and reduced apoptosis in breast cancer cells. PTEN (Phosphatase and tensin homolog) on the other hand is a tumour suppressor that inactivates the PI3K pathway and a homozygous deletion or downregulation of this confers a survival advantage in cancer cells.

Activation of PTEN is associated with Trastuzumab antitumor activity and a reduction in PTEN expression by antisense oligonucleotide imparts resistance to Trastuzumab both *in vitro* and *in vivo* by activating PI3K pathway (Nagata *et al*., 2004). Together, the inhibition of the PI3K pathway rescues the resistance phenotype due to the loss of PTEN.

Further, in an RNAi screen to identify genes conferring Trastuzumab resistance in breast cancer cells, both *PTEN* and *PIK3CA* genes were identified as the key modulators (Berns *et al*., 2007). Further, in a cohort of 55 patients of breast cancer, either the low expression of PTEN or activating mutation in PIK3CA were observed to be associated with poor prognosis following Trastuzumab therapy.



Patients with PTEN-deficient breast tumors have a poor response to trastuzumab-based therapy

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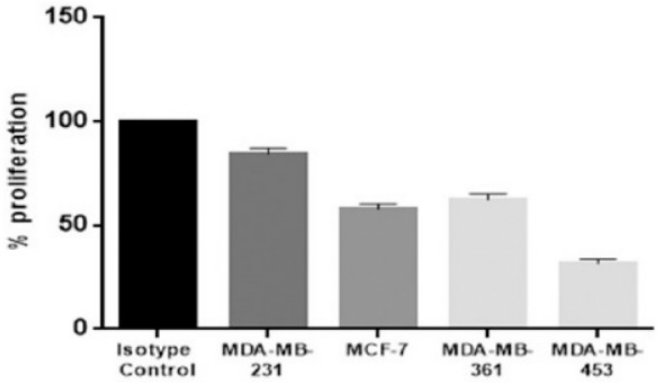
AKT is a transcription factor that is one of the culminating points of the PI3K pathway. Activating mutations in AKT1 has been reported in 1-8% of breast carcinoma. In a study involving 78 samples of invasive breast carcinoma, it was observed that only 3 (3.8%) samples contained AKT1 E17K mutations and authors concluded that they occur early during the breast tumor formation (Dunlap *et al*., 2009). Chan *et al*, isolated a Trastuzumab resistant sub-clone BT/Her^{R} from breast cancer cell line BT474 by continuous selection in Trastuzumab containing medium for 5 months. These resistant sub-clones had higher levels of both total and phosphorylated Akt protein levels (Chan, Metz, and Kane, 2005). These Trastuzumab resistant sub-clones had higher sensitivity to PI3K inhibitor LY294002, but the original BT cells had lost sensitivity to this inhibitor in the presence of Trastuzumab. This indicated that one of the mechanisms of the monoclonal antibody against HER2 may be a down-regulation of the PI3K-AKT pathway and the activating mutations of this pathway add to the resistance to this therapy.

In accordance with this, it was hypothesized that concurrent use of AKT inhibitors might synergistically potentiate the response to Trastuzumab in breast cancer cells. Thus a PDK-1 inhibitor, 2-amino-*N*-[4-[5-(2-phenanthrene)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl] phenyl]-acetamide (OSU-03012), potentiated the anti-tumor effect of Trastuzumab in a breast cancer cell line SKBR3/IGF-IR (Tseng *et al*., 2006). The effect of the same inhibitor was mild in the other three cell lines BT474, MDA-MB-231, and SKBR3 suggesting that the higher expression of Insulin-like growth factor-1 receptor (IGF-1R) might contribute to the effect.

The PI3K-AKT pathway is also important in the light of the finding that, in the cells that escape and survive tyrosine kinase inhibition by inhibitors of EGFR/HER2, an activation of HER3 is observed (Sergina *et al*., 2007). The expression of HER3 is higher along with its trafficking to the plasma membrane and the shifting of the phosphorylation/dephosphorylation equilibrium such that more amount of phosphorylated HER3 is present. This eventually leads to AKT activation and cell survival. Thus, when HER2 is inhibited then HER3 is activated. It had been observed that for HER2, the HER3 is a preferred hetero-dimerization partner, however, Trastuzumab only effectively blocks EGFR/HER2 dimerization ((Wehrman *et al*., 2006)). Trastuzumab does not block HER2/HER3 dimerization. Hence, in the scenario of continuous Trastuzumab therapy cells that have higher activated HER2 and consequent AKT activation, might be selected over a period of time. Definitive evidence of whether this mechanism is also involved in Trastuzumab resistance is yet to be explored.

Activation of the Ras-Raf-MAPK pathway:

Ras-Raf-MAPK is an important pathway downstream of EGFR. Since Trastuzumab effectively blocks HER2/EGFR dimerization a logical question arises if mutations in the Ras-Raf-MAPK pathway provide any proliferation or survival advantage to Trastuzumab resistant cells? Considering this logic, a cell culture study was conducted in which different cell lines of breast cancer were treated with Trastuzumab at a dose of 7µg/ml (Patra *et al*., 2017). It was observed that the cell line MDA-MB-231 that contained activating mutations in BRAF and KRAS was least sensitive to the optimum dose of Trastuzumab. On the other hand, the cell lines such as MDA-MB-631 and MCF-7 that lack this mutation but have mutations in PIK3CA were more sensitive to Trastuzumab. These observations support the hypothesis that activating mutations in the Ras-Raf-MAPK pathway might be a key modulator of Trastuzumab resistance.



Response of Different Breast Cancer Cell Lines to 7 µg Trastzumab Treatment

The isotope control had 100% proliferation after being treated with trastuzumab.

T-test was applied with a p value of < 0.05 considered to be significant.

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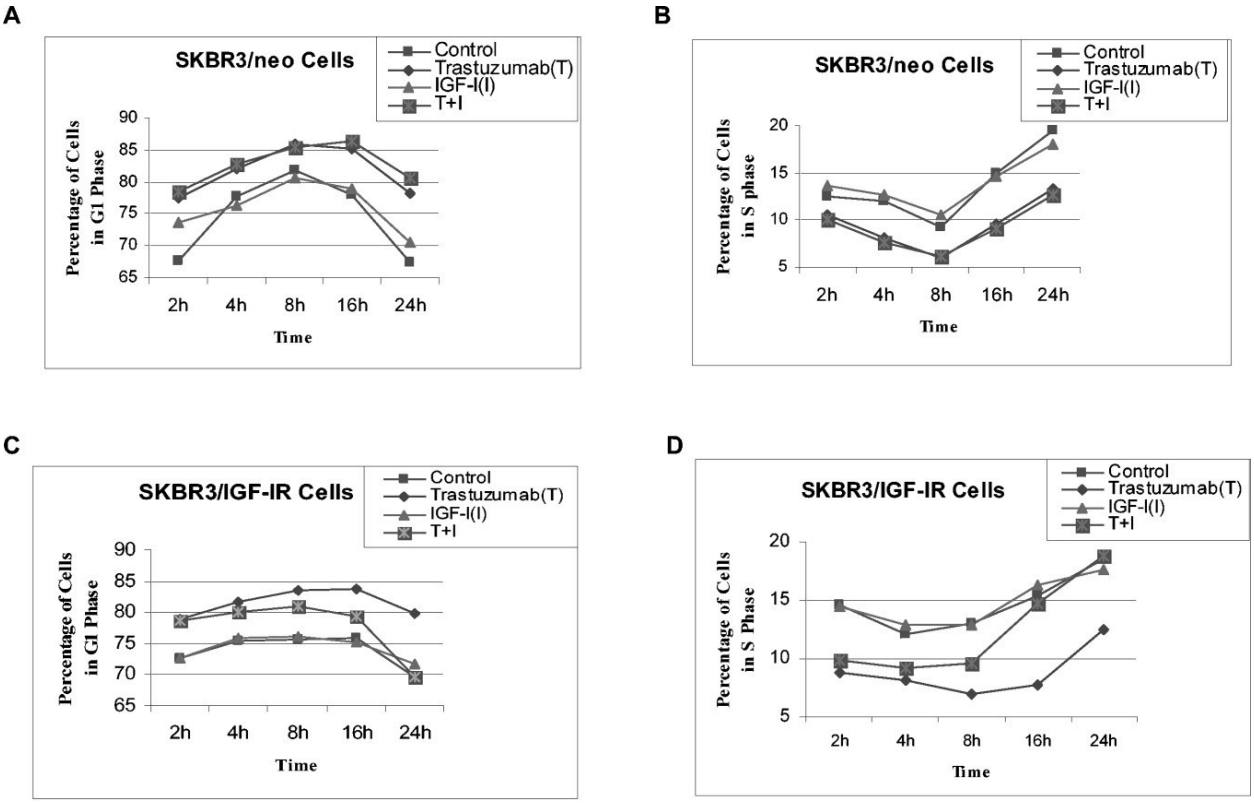
Alternate signaling pathways:

The sea of the mammalian cell is flooded with intricacies involving a "network" of cellular signaling pathways. Thus perturbation of one signaling pathway may lead to observable phenotype in the other pathways that at first sight appear to be remote.

In the case of Trastuzumab resistance in breast cancer cells, the involvement of signaling molecules such as Insulin Growth Factor 1 Receptor, p27 tumor suppressor, c-met, integrins, and even some miRNAs have been observed to be involved.

IGF-1R

In an initial study with three breast cancer cell lines grown in Trastuzumab (10µg/ml), it was observed the cells grown either in 1% FBS or the absence of IGF were sensitive, but they were insensitive under 10% FBS or upon IGF(40ng/ml) treatment (Lu *et al*., 2001). Also, the SKBR3 cells that express higher HER2/neu but less IGF-1R were sensitive to Trastuzumab, regardless of IGF treatment. That indicates that IGF-IR expression might contribute to Trastuzumab resistance in breast cancer cells.



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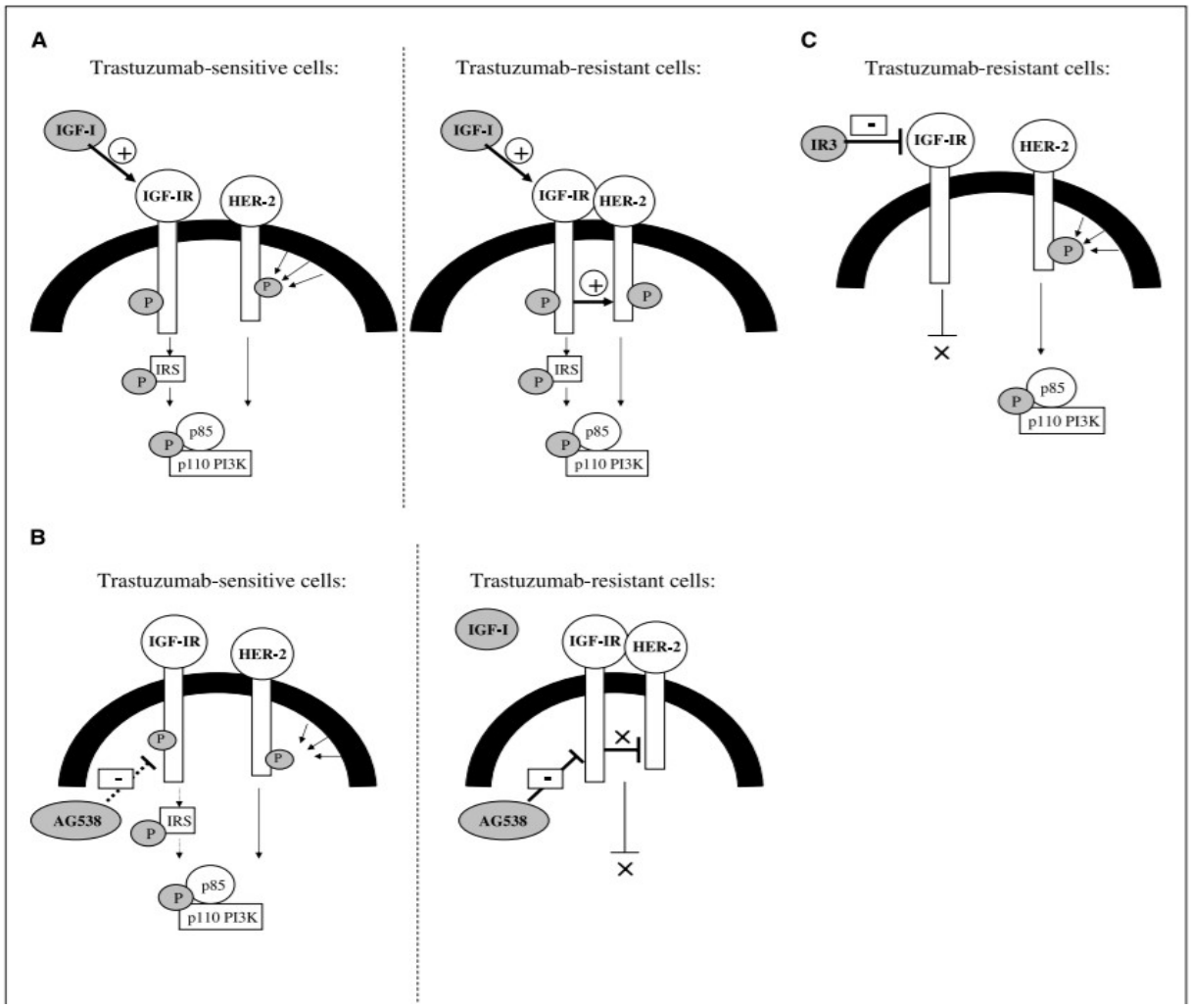
IGF-I rescues SKBR3/IGF-IR cells from trastuzumab-induced G_1 arrest. After SKBR3/neo and SKBR3/IGF-IR cells were synchronized in SFM for 24 hr and treated or not with trastuzumab (10 μg/ml) for a further 24 hr, 40 ng/ml IGF-I were added and the cells collected at the times indicated thereafter for flow-cytometric analysis. (*a*) Percentage of SKBR3/neo cells in G_1 phase. *(b)* Percentage of SKBR3/neo cells in S phase. *(c)* Percentage of SKBR3/IGF-IR cells in G_1 phase. *(d)* Percentage of SKBR3/IGF-IR cells in S phase. Experiments were repeated in triplicate.

Further studies on cells expressing both HER2/neu and IGF-1R (SKBR3/IGF-1R), it was revealed that IGF signaling prevented the expression of $p27^{KIP1}$ induced by Trastuzumab treatment (Lu, Zi, and Pollak, 2003). This results in decreased interaction of $p27^{KIP1}$ with CDK2 thereby restoration of CDK2 and removal of G1 cell cycle arrest induced by Trastuzumab.

It has been observed that HER2 doesn't interact with IGF-1R in the Trastuzumab sensitive Her2+ breast cancer cells. However, in the cells that acquire resistance to Trastuzumab, HER2 interacts with IGF-1R in a unique way (Nahta *et al*., 2005). Further, the activation of IGF-1R in these cells leads to an activation of HER2. The sensitivity of these Trastuzumab resistant cells got restored when they were treated with I-OMe-AG538, an inhibitor of IGF-1R. Together these three studies prove that IGF-1R signaling is an important modulator of Trastuzumab resistance in breast cancer cells.



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Proposed model for IGF-IR signaling and cross talk in trastuzumab resistance. A, in trastuzumab-sensitive cells, IGF-I stimulates IGF-IR signaling, resulting in phosphorylation of IGF-IR, IRS, and Akt. HER-2 is phosphorylated by other membrane receptor tyrosine kinases such as EGFR (arrows) and by autophosphorylation. In trastuzumab-resistant cells, IGF-I stimulates IGF-IR signaling with increased phosphorylation of IGF-IR, IRS, and Akt. Interaction between IGF-IR and HER-2 facilitates IGF-IR signaling to HER-2, resulting in increased HER-2 phosphorylation. B, in trastuzumab-sensitive cells, IGF-IR tyrosine kinase inhibition partially blocks phosphorylation and activation of IGF-IR signaling. HER-2 phosphorylation is still regulated by other membrane receptor tyrosine kinases and by autophosphorylation. Trastuzumab-resistant cells are more sensitive to IGF-IR tyrosine kinase inhibition, which results in a dramatic decrease in IGF-IR downstream signaling as well as significantly decreased cross-signaling to HER-2 even in the presence of IGF-I. This inhibition of IGF-IR activity results in increased trastuzumab sensitivity. C, antibody-mediated blockade of IGF-IR disrupts receptor interaction and diminishes IGF-IR signaling in trastuzumab-resistant cells. HER-2 is maintained in a phosphorylated and active state perhaps due to activity of other membrane receptor tyrosine kinases and autophosphorylation (Nahta and Esteva, unpublished results). IGF-IR blockade results in a dramatic restoration of trastuzumab sensitivity, supporting the concept that IGF-IR is a promising molecular target in this subset of breast cancers.

P^{27} tumor suppressor:

$P27^{KIP1}$ is a tumor suppressor gene the deletion of which might lead to cancer. As we have observed that enhancement of p27KIP1 interaction with CDK2 upon Trastuzumab treatment leads to G1 cell cycle arrest, this protein becomes an important candidate for resistance acquisition. In agreement with this Nahata et al, observed downregulation of P27KIP1 and upregulation of CDK2 in two pools of Trastuzumab resistant SKBR3 breast cancer cells (Nahta, Takahashi, Ueno, Hung & Esteva, 2004). Increasing the expression of $p27^{KIP1}$ by transfection or increasing its half-life by treating with proteasome inhibitors, restored the sensitivity of these cells to Trastuzumab.

C-Met receptor

It has been observed that the c-Met receptor is frequently overexpressed in HER2(+) breast cancer cells and exposure of these HER2 positive cells to Trastuzumab further enhances the expression of c-Met (Shattuck, Miller, Carraway & Sweeney, 2008). Higher expression of c-Met eventually abrogates the p27KIP1 mediated signaling and thus the cells become resistant to Trastuzumab. RNAi mediated inhibition c-Met in these cells leads to enhanced sensitization to Trastuzumab induced cell cycle arrest.

Integrins;

It has been hypothesized that the integrin $\alpha_6\beta_1$and $\alpha_6\beta_4$ might be a crucial mediator of resistance in HER2+ cells because they are required for cancer progression in breast cancer cases (Huang, Gee, Nicholson, Osborne & Schiff, 2008). A subclone of HER2+ breast cancer cell line BT474, obtained by serial passage in Trastuzumab containing media for over 1year, exhibited higher nuclear CXCR4 expression ((Tripathy, et al., 2007)). Inhibition of this CXCR4 by the inhibitor AMD3100 leads to the partial rescuing of the resistance to Trastuzumab. Furthermore, a signal transduction/cell cycle pathway. CXCR4 is upregulated and can be targeted to reverse resistance CXCR4 antagonists may be useful in preventing or reversing trastuzumab resistance. This observation was presented in a meeting, however, till now **no peer-reviewed** research article has been published to corroborate these findings.

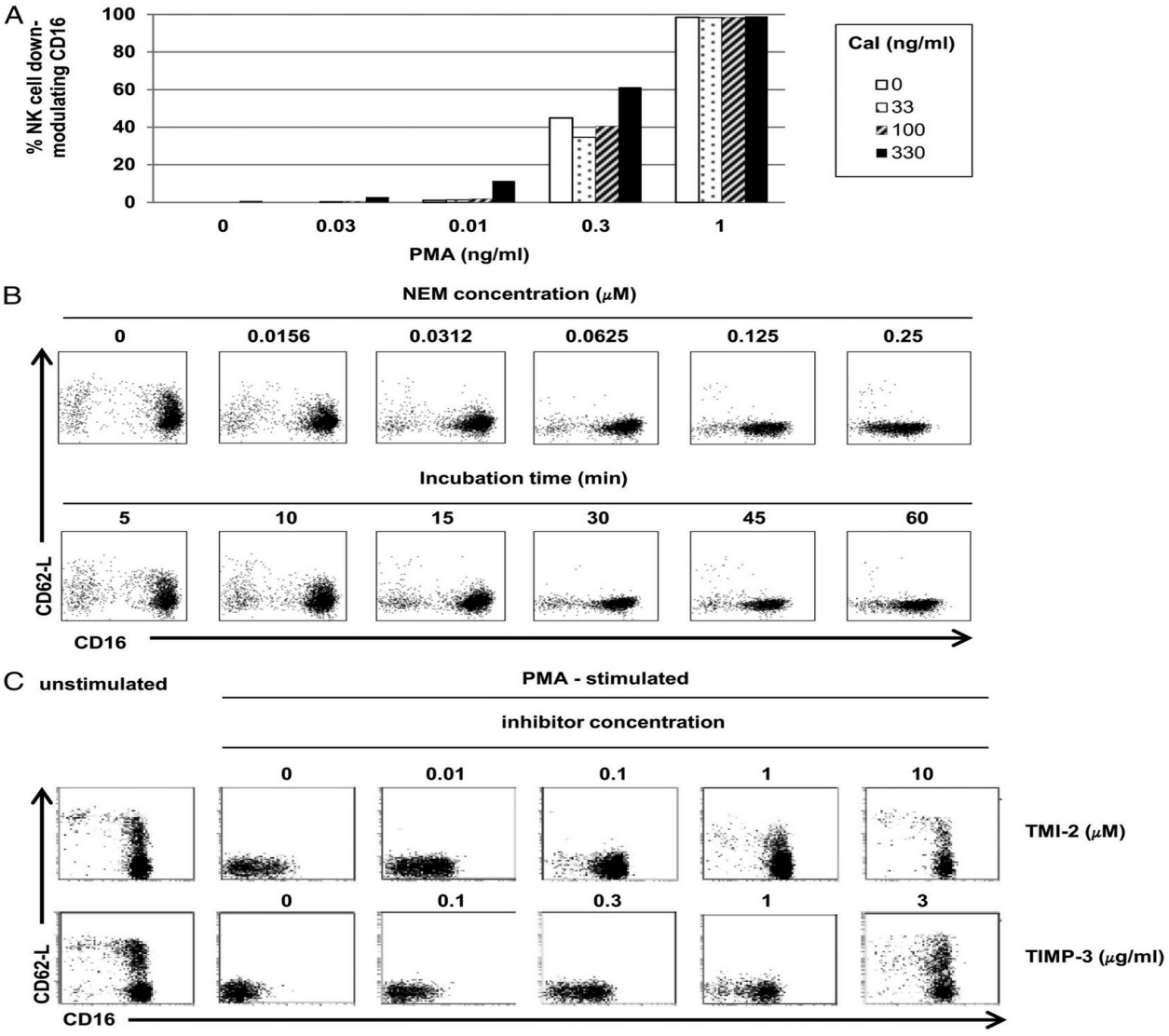
Failure to acquired immune response:

Trastuzumab is a monoclonal antibody comprising the kappa chain of IgG against the HER2 receptor. Upon binding to the epitope present in the ectodomain of this receptor it may elicit an immune response directed by its Fc portion independently of the Fab portion. A failure of breast

cancer HER2+ cells to elicit an immune response of optimum magnitude might confer resistance to Trastuzumab. This may happen in many ways.

ADCC:

Trastuzumab is known to cause Antibody-dependent cell cytotoxicity in the breast cancer cells in a mice xenograft model. When JIMT-1 cancer cells that are intrinsically resistant to Trastuzumab were injected subcutaneously into a nude mouse, the antibody was able to reduce the xenograft tumors in 5-7 weeks (Barok *et al*., 2007). The leucocyte mediated antibody-dependent cell cytotoxicity reaction was equally strong in the Trastuzumab sensitive cells SKBR-3 cells as well. In the antibody-dependent cell cytotoxicity response, many cells such as Natural killer (NK) cells, neutrophils, monocytes and dendritic cells are involved. Several receptors of the FcγR family are involved in passing the signal from the antibody to the ADCC executing leucocytes. FcγRIIIA/CD16A is a low-affinity receptor for the IgG Antibody Fc constant region. Upon activation of the NK cells, this receptor is shed. ADAM17 has been reported to be involved in the shedding of FcγRIIIA/CD16Ain NK cells and since the role of ADAM17 has been reported in Trastuzumab resistance, it would be interesting to know the status of FcγRIIIA/CD16A receptor shedding by ADAM17 following Trastuzumab treatment of breast cancer cells (Lajoie *et al*., 2013).



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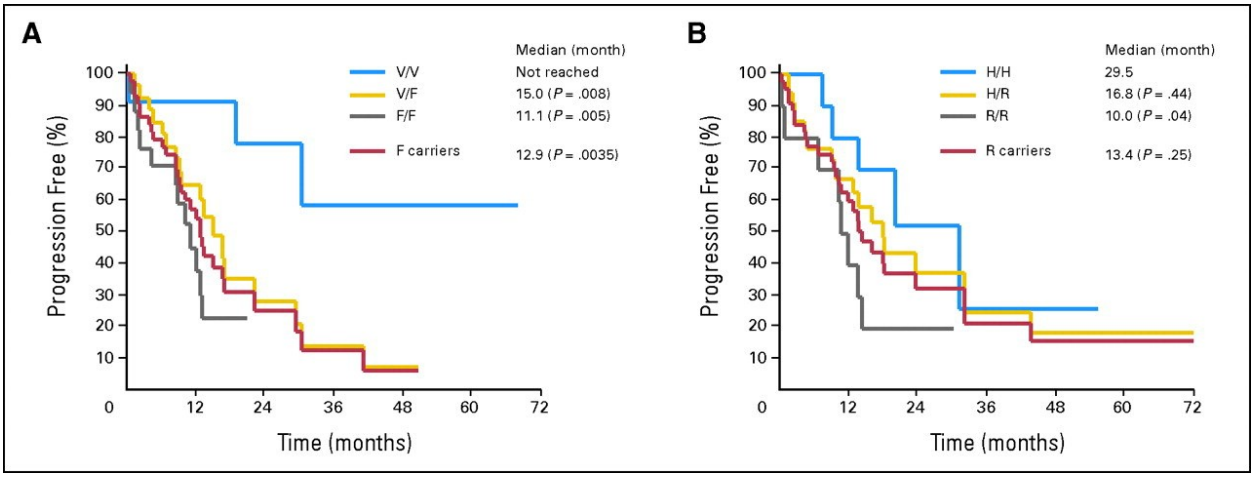
ADAM17 involved in the shedding of FcγRIIIA/CD16A. (**A**) NK cells were stained with anti-CD16 mAb after incubation with indicated concentrations of PMA and/or CaI and analyzed by FCM. Results are expressed as the percentage of NK cells downmodulating CD16. This experiment was repeated four times, and one representative experiment is shown. NK cells were stained with anti-CD16 and anti-CD62L mAbs after incubation for 1 h in the absence or presence of indicated concentrations of NEM (*top panels*) (**B**) or for the indicated times in the presence of 0.125 μM of NEM (*bottom panels*) (**C**) or after initial incubation in the absence or presence of indicated concentrations of specific pharmacological inhibitor (TMI-2) or physiological inhibitor (TIMP-3) of ADAM17 followed by a second incubation in the presence of PMA and analyzed by FCM. Each experiment was repeated at least three times, and one representative experiment is shown.

Base substitution of Phenylalanine (F) in place of Valine (V) at 158th position in the FcγRIIIA or Histidine (H) in place of Arginine (R) at 131st position in FcγRIIA may significantly alter the affinity of antibody Fc chain with this receptor. Consequently, in patients having these polymorphisms the affinity of Trastuzumab to these receptors may be affected. In a study involving 54 breast cancer patients, it was thus observed that FcγRIIIA-158 V/V and FcγRIIIA-131 H/H were the favorable genotypes (Musolino *et al*., 2008).



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Kaplan-Meier estimates of progression-free survival (PFS) to trastuzumab-based therapy by immunoglobulin G (IgG) fragment C receptor (FcγR) polymorphisms. (A) PFS curves were plotted by FcγRIIIa 158 valine (V)/phenylalanine (F) genotype. F carriers represent patients with either 158 V/F or 158 F/F genotype. (B) PFS curves were plotted by FcγRIIa 131 histidine (H)/arginine (R) genotype. R carriers represent patients with either 131 H/R or 131 R/R genotype.

Patients with these receptors exhibited better overall response rate (ORR) and progression-free survival (PFS) indicating higher ADCC. Conversely, we can presume that in breast cancer, the subpopulation of cells having mutations leading to base substitution in FcγIIIA or FcγIIa might be selected such that those cells have minimal ADCC response against Trastuzumab conferring resistance. We have to wait for any such scientific report involving such a study.

In continuation with the above observations, we can also expect that tailor-made antibodies showing better ADCC response may be engineered. In agreement with this Lazar *et al*, used computational algorithm and high throughput screening to produce variants of Trastuzumab

having better affinity to FcγIIIa receptors (Lazar *et al*., 2006). For both the V158 and F158 forms of FcγIIIa receptors, better variants have been synthesized that show enhanced cytotoxicity.

Coming soon😊

Nanotechnology

What is the nanotechnology (nanoparticles carriers) DDS

What Benefit of(Biosimilar & nanoparticles) for DDs

Application

Biosimilar drugs for the her2 Herceptin

Current nanotechnology biosimilar drug carrier's DDS for her2 Herceptin

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