Title: Patient derived-driver mutations in the interaction domain of HER2 changes the dynamics of HER2 interaction, thereby changing the targeted therapy response in breast cancer

- Introduction

Breast cancer is the currently the most common cancer in the world and, also, the most frequent cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012 (30% of all cancers) (1). It is also the second most common cause of cancer related death in women worldwide. The scenario of India is much worse, with incidence as high as 25.8 per 100,000 women and mortality 12.7 per 100,000 women, which makes it the most prevalent cause of cancer deaths. While only a small portion of all newly diagnosed breast cancer patients are presented with metastatic disease at the time of initial diagnosis, up to one third of patients with early stage disease will subsequently develop resistance and metastasis.

The underpinning signalling of breast cancer, and the major pathways involved in tumor progression, resistance and metastases, are still partly understood. Breast cancer conventionally has been classified into three different subtypes based on the presence or absence of three receptors found on these cancer cells. Hormone receptor (HR) positive breast cancers express estrogen and/or progesterone receptors (ER/PR), and constitute approximately 60% of all breast cancer cases. The oncogene human epidermal growth factor receptor 2 (HER-2/neu) is over-expressed in approximately 25-30% of all breast cancer cases; while approximately 10% of breast cancer cases are negative for the expression of ER, PR, and HER-2/neu, also known as triple negative breast cancer (TNBC).

The HER2 receptor, a tyrosine kinase, can drive the cancer via over expression and/or mutations. There is a plethora of HER2 targeted cancer drugs available for treatment of patients with HER2 positive breast cancer such as Trastuzumab, Lapatinib, Neratinib and Pertuzumab. Unfortunately, many of the patients stop responding to these drugs while undergoing treatment. In this context, investigation on the dynamics of HER2 receptor homo-dimerization or hetero-dimerization with Epidermal Growth Factor Receptor (EGFR), HER3 or c-MET are considered important for this thesis study. Interactions and cross signalling from the HER2 receptor to other growth factor receptors may potentially contribute to such therapeutic resistance. Understanding co-operative interaction amongst these primary target receptor kinases keeping Her2 as the central target gene may actually help to control various disease condition and treatment response to important small molecule kinase inhibitors applied in clinical practice.

Reports suggest that some of the mutations that occur in Her2 and Her3 are oncogenic (5-7). However, therapeutically the focus has been primarily on the tyrosine kinase domain. The mutations occurring in the domain required for interaction with other receptor tyrosine kinases has never been studied in context of resistance. Additionally, activation/incomplete inhibition of a receptor in receptor dimer complex have been shown to promote drug-resistance (2, 3). Thus, mutations occurring in dimerization domain of Her2 and Her3 which might change the binding partner of these receptors can result in incomplete inhibition of the signalling thereby, promoting resistance. The aim of this study would be to understand the changes that accompany these mutations providing novel insights about the molecular interaction dynamics occurring in Her2 with its family members and other RTKs due to mutations. The understanding of the underpinning mechanism as well as the visualization of receptor switching as a result of mutations could help us in better targeting of the cancer in these patients and prevent metastasis and relapse in future.

The mutations of Her2 serving as a driving factor in breast cancer is well known (6). However, the change in interaction dynamics of Her2 with that of other receptor tyrosine kinases that are responsible for chemo-resistance is comparatively new topic in breast cancer research and less documented (8). Further, with respect to the mutations, the tyrosine kinase domain has been

the prime focus in research with very little importance given to the dimerization domain. After ligand binding, the dimerization of the receptor occurs via domain II (major role in dimerization) and domain IV (stabilization of the receptor complex). This event is followed by the phosphorylation of the receptors and transduction of signals which promote the proliferation and prevent the apoptosis of cancer cells. It is known that HER2:HER2 homodimer results in the activation of MAPK signalling and HER2:HER3 heterodimer results in the activation of primarily PI3K-AKT signalling (9). Therefore, in this study we want to build a focus on disease relevant mutants in the dimerization domain, which mediates the interaction of Her2 with other RTK receptor molecules and cause receptor switching. As per the reports available so far, the receptor switching phenomena subsequently promotes chemo-resistance. In line with this thought, based on available breast cancer database mining, interaction domain mutants will be identified. Further, exploring the structural changes that occur due to these mutations in the dimerization domain and the biological outcome of these changes on the dynamics of Her2 receptor interaction would provide us with useful and novel insights into the mechanism of receptor biology in the context of promoting chemo-resistance.

- Objectives

We asked two key questions:

- **Whether disease relevant mutations that occur in dimerization domain is changing the interaction dynamics of HER2 with other receptor tyrosine kinases?**

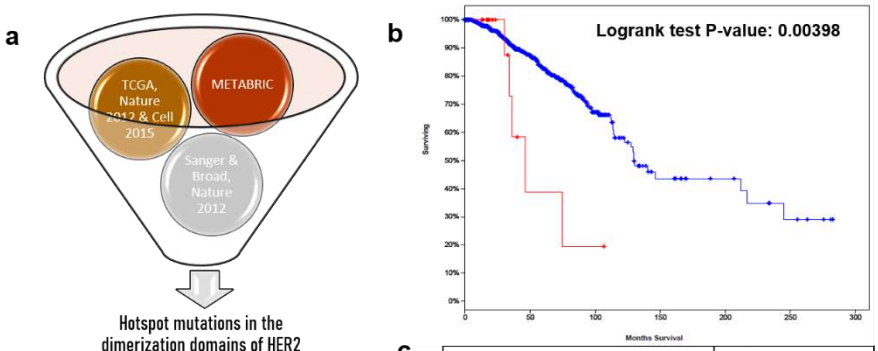
- **Whether such changes are linked to development of resistance against HER2 targeted medicine in real?**

To address the key questions, we have framed three objectives for the study:

1. Identification and in-silico validation of the structural changes occurring due to mutations in HER2 and HER3 dimerization domains (domain II and domain IV).
2. Characterization of interaction dynamics due to the mutations occurring in the dimerization domains of HER2 and HER3.
3. Establishment of functional relevance of mutations to the targeted drug response.

- Results

1. **Identification and in-silico validation of the structural changes occurring due to mutations in HER2 and HER3 dimerization domains (domain II and domain IV).**



c

	Median months survival
Cases with HER2 dimerization domain mutations	46.35
Cases without HER2 dimerization domain mutations	129.6

d

HER2 Dimerization domain mutants			
Mutations	**Panther**	**Provean**	**Mupro**
S305C	Probably benign (176)	Neutral (-0.122)	Decrease stability (-1.07)
G309A	Probably damaging (797)	Neutral (-2.406)	Decrease stability (-0.6)
S310F	Probably damaging (455)	Deleterious (-4.738)	Decrease stability (-0.13)
S310Y	Probably damaging (455)	Deleterious (-4.768)	Decrease stability (-0.29)
P523S	Probably damaging (797)	Deleterious (-6.736)	Decrease stability (-0.765)

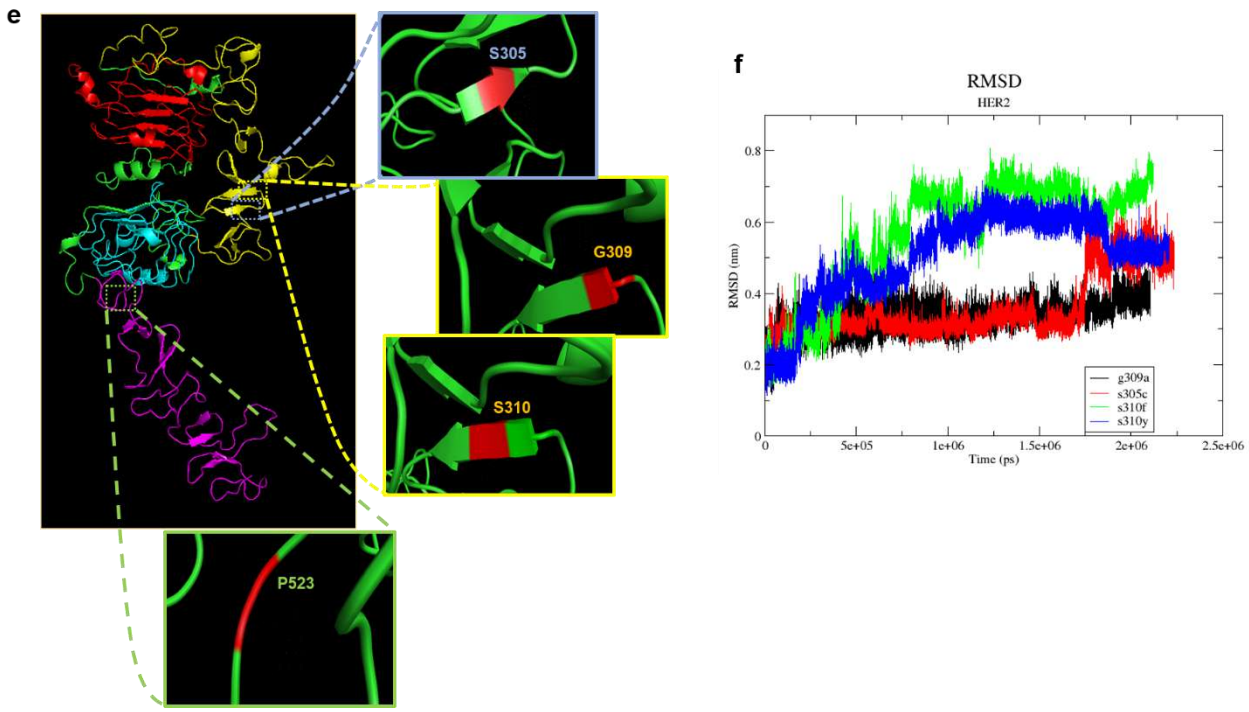


Fig:1 *(A) TCGA-analysis for identification of hotspot HER2 dimerization domain mutants in HER2 positive breast cancer. (B and C) Kaplein-Meier survival analysis depicts significantly lower survival in HER2 dimerization domain mutation harbouring patient. (D) 2-D structural changes analysis due to mutations in HER2 domain II and domain IV. (E) X-ray crystal structure of HER2 extracellular region highlighting the position of mutations. (F) 2μs molecular dynamics simulation of HER2 extracellular region harbouring mutations to get energy minimized 3-D structures.*

The prevalent mutations of dimerization domain of HER2 and HER3 are identified by analysing tumor tissue databases enlisted above (Fig. 1A). For HER2 five mutations was identified including S305C, G309A, S310F, S310Y and P523S (Fig. 1D). The patients harbouring these mutations have poorer survival as compared to the wildtype HER2 harbouring patients (OS: 46.35 months vs 129.6 months; P=0.00398) (Fig. 1B and C). To get an idea about the 2-dimensional structural changes occurring due to these mutations, 3 tools i.e Panther, Provean and Mupro were employed. Every dimerization domain mutant was somewhat detrimental to the stability of the structure, hinting that they might affect the interaction of HER2 (Fig. 1D). To gain more insights into the structural changes occurring at the 3-dimensional level, MDS (Gromacs) was employed. For molecular dynamic simulations (MDS), the structures of the proteins were modelled using SWISS-MODEL keeping the already reported X-ray structure of HER2 as the template (3N85), further, it was verified using PROCHECK (data not shown). The structures (HER2-WT and its Mutants) were simulated for 2 μs to generate energy minimized structures (Fig. 1F), that was further docked with itself to form the homo-dimers. These HER2-WT and its mutant homo-dimers were further simulated for additional 500ns to check the stability of those structures to access whether there is any change in the interaction between HER2:HER2 due to dimerization domain mutants.

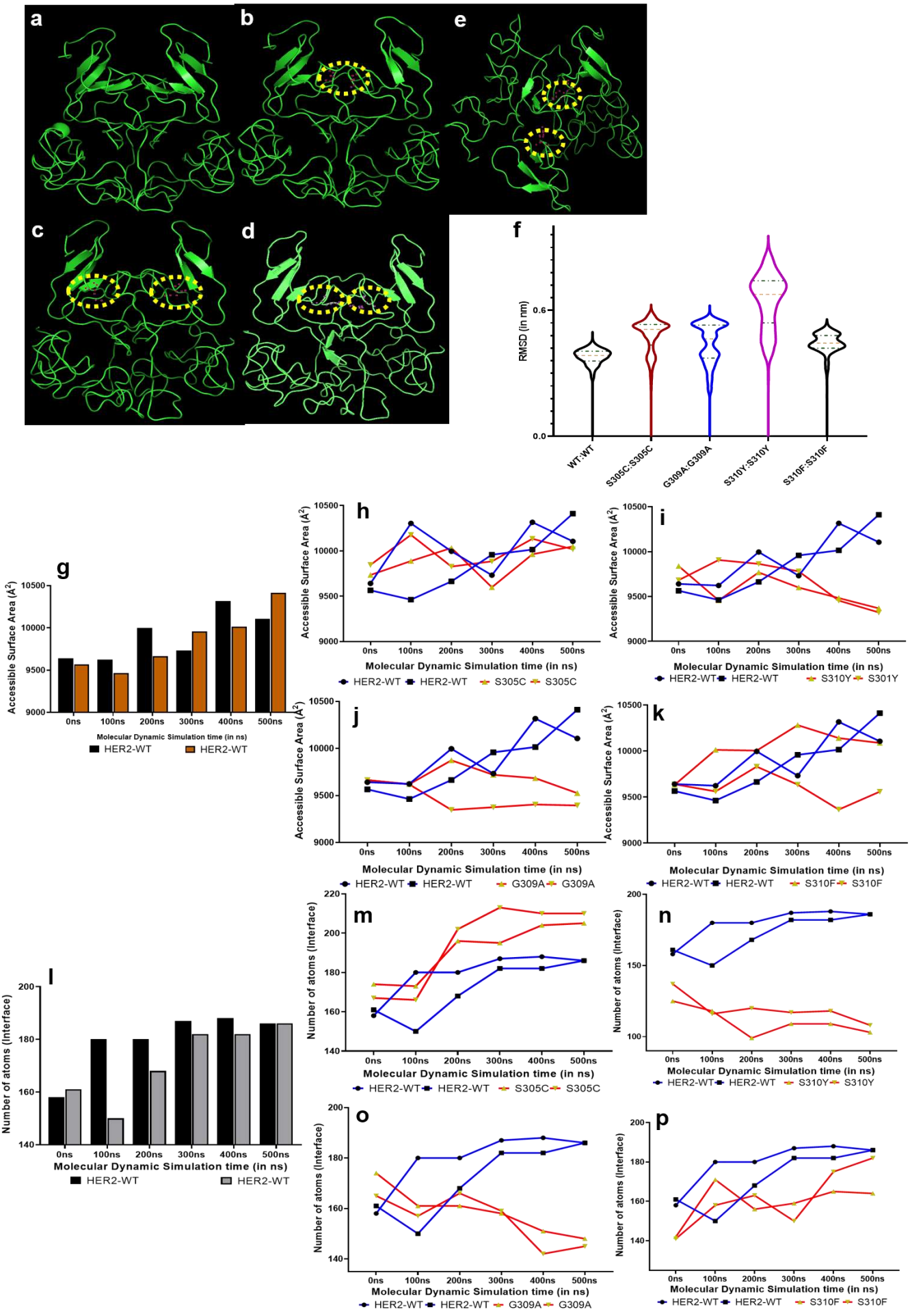

a
b
e
c
d
f
RMSD (in nm)
0.6
0.0
WT:WT
S305C:S305C
G309A:G309A
S310Y:S310Y
S310F:S310F
g
Accessible Surface Area (Å²)
10500
10000
9500
9000
0ns
100ns
200ns
300ns
400ns
500ns
Molecular Dynamic Simulation time (in ns)
HER2-WT
HER2-WT
h
HER2-WT HER2-WT S305C S305C
i
HER2-WT HER2-WT S310Y S301Y
j
HER2-WT HER2-WT G309A G309A
k
HER2-WT HER2-WT S310F S310F
l
Number of atoms (Interface)
200
180
160
140
m
HER2-WT HER2-WT S305C S305C
n
HER2-WT HER2-WT S310Y S310Y
o
HER2-WT HER2-WT G309A G309A
p
HER2-WT HER2-WT S310F S310F

Fig. 2. *(A-E) HADDOCK structures of HER2 dimerization domain mutants. The yellow circle highlights the position of the mutation (A: HER2-WT, B: S305C, C: G309A, D: S310F E: S310Y) (F) Box and violin plot of RMSD fluctuations analysed after 500ns of simulations. (G-K) Accessible surface area available for binding in HER2-WT and its mutant condition. (L-P) Number of atoms involved in the interaction graphs showing increasing trend for HER2-WT, S305C and S310F and decreasing trend for S310Y and P523S.*

HADDOCK restraint docking of HER2-WT and its mutants was performed and showed that G309A (Fig. 2C) and S310Y (Fig. 2E) mutation results in distorted homo-dimer formation, however, S305C (Fig. 2B) and S310F (Fig. 2D) resembled the homo-dimer of HER2-WT. These homo-dimers were simulated for 500ns and analysed for stability changes during the course of MDS. Box and violin plot of RMSD changes revealed that HER2-WT homo-dimers don't fluctuate during the simulations depicting stable interaction. S305C and S310F rmsd graphs also denotes stable interaction (Fig 2F). However, G309A and S310Y showed un-stable interaction, depicted by the elongated tail of the box and violin plot (Fig 2F). We also found that the accessible surface area for binding increased over the course of simulations for HER2-WT (Fig. 2G), S305C (Fig. 2H) and S310F (Fig. 2K) homo-dimers, suggesting increase in the interaction or stronger interaction between the monomers during the simulation. A decreasing trend in the accessible area for binding was observed for G309A (Fig. 2J) and S310Y (Fig. 2I) mutants, hinting that due to these mutations the homo-dimer of HER2 are not stable and patient harbouring these mutations might not behave in the conventional or wildtype HER2 manner. In addition, we also found that the number of atoms for binding increased over the course of simulations for HER2-WT (Fig. 2I), S305C (Fig. 2M) and S310F (Fig. 2P) homo-dimers, suggesting stronger interaction. A decreasing trend in the accessible area for binding was observed for G309A (Fig. 2O) and S310Y (Fig. 2N) mutants, hinting that due to these mutations the homo-dimer of HER2 are not stable.

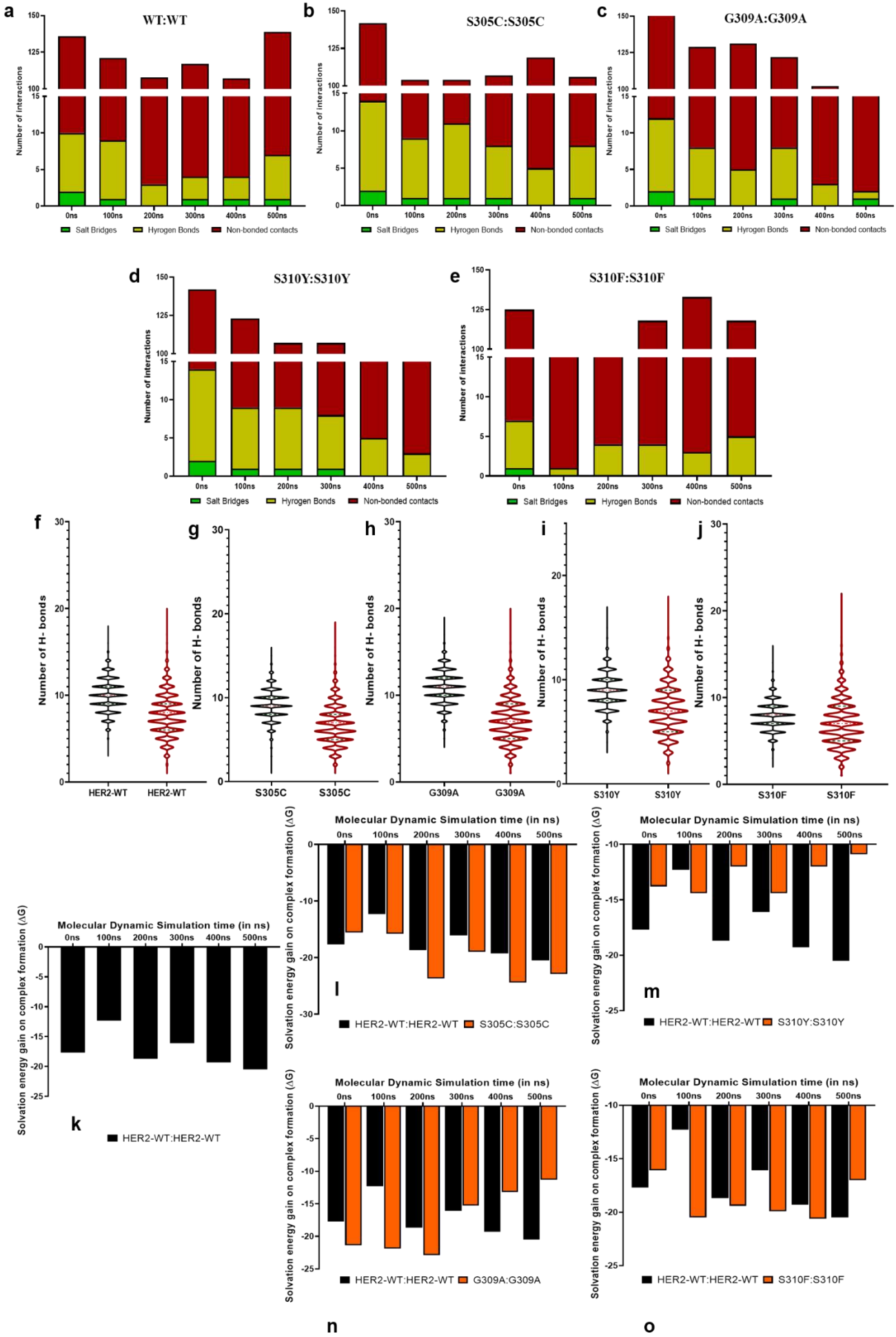

a
WT:WT
b
S305C:S305C
c
G309A:G309A
Number of interactions
0ns
100ns
200ns
300ns
400ns
500ns
Salt Bridges
Hyrogen Bonds
Non-bonded contacts
d
S310Y:S310Y
e
S310F:S310F
f
g
h
i
j
Number of H- bonds
HER2-WT
HER2-WT
S305C
S305C
G309A
G309A
S310Y
S310Y
S310F
S310F
Molecular Dynamic Simulation time (in ns)
Solvation energy gain on complex formation (ΔG)
k
HER2-WT:HER2-WT
l
HER2-WT:HER2-WT
S305C:S305C
m
HER2-WT:HER2-WT
S310Y:S310Y
n
HER2-WT:HER2-WT
G309A:G309A
o
HER2-WT:HER2-WT
S310F:S310F

Fig.3 *(A-E) Graphs showing the number of non-covalent interaction stabilizing the HER2:HER2 homodimer. (F-J) Graph showing total number of h-bonds fluctuating between the monomers during the course of simulation. (K-O) Solvation free energy gain on complex formation graph showing that HER2-WT, S305C and S310F form stable homo-dimers and S310Y and P523S forms unstable homo-dimers.*

In-depth structural analysis revealed that the number of interactions between the monomers increased over the course of simulations for HER2-WT (Fig. 3A) and S310F (Fig. 3E) homo-dimers, suggesting stronger interaction. A decreasing trend in the number of interactions including salt bridges and hydrogen bonding was observed for G309A (Fig. 3C) and S310Y (Fig. 3D) mutants, hinting that due to these mutations the homo-dimer of HER2 don't form long-term interaction, thereby are not stable. Further, analysing the number of hydrogen bonds only during the simulations suggested that HER2-WT and S305C might not have the highest number of h-bonds but the number of stronger salt bridges are higher in these conditions only, thus they form stronger interactions (Fig. 2A and B; 3F-J). Solvation free energy gain upon complex formation that describes whether the structure forming is feasible or not, suggested that HER2-WT, S305C and S310F has more stable dimer structures as the delta G is more negative (Fig, 3K, Land O). However, G309A and S310Y structures have less negative delta G values, thereby are less likely to form stable homo-dimer interaction (Fig. 3M and N).

2. **Characterization of interaction dynamics due to the mutations occurring in the dimerization domains of HER2 and HER3**

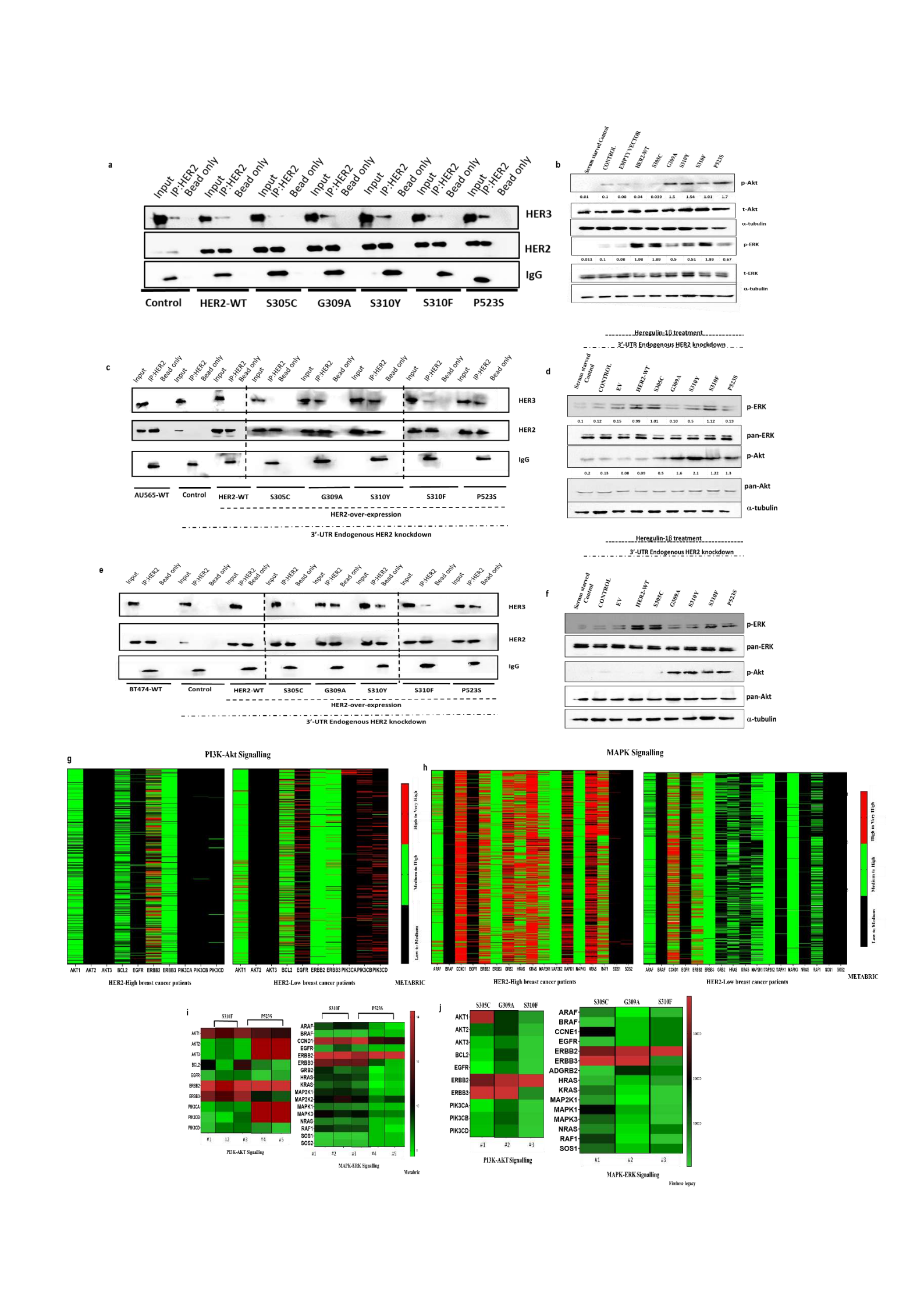

a
Input
IP:HER2
Bead only
HER3
HER2
IgG
Control
HER2-WT
S305C
G309A
S310Y
S310F
P523S
b
Serum starved Control
CONTROL
EMPTY VECTOR
HER2-WT
S305C
G309A
S310Y
S310F
P523S
p-Akt
t-Akt
α-tubulin
p-ERK
t-ERK
c
AU565-WT
HER2-over-expression
3'-UTR Endogenous HER2 knockdown
d
Heregulin-1β treatment
3'-UTR Endogenous HER2 knockdown
pan-ERK
pan-Akt
e
BT474-WT
f
g
PI3K-Akt Signalling
High to Very High
Medium to High
Low to Medium
HER2-High breast cancer patients
HER2-Low breast cancer patients
METABRIC
h
MAPK Signalling
i
PI3K-AKT Signalling
MAPK-ERK Signalling
Metabric
j
Firehose legacy

Fig. 4 *(A-F) Co-IP studies highlighting the switching in the signalling in HER2 dimerization domain mutants due to interaction with HER3 in various HER2 low (A-B) and HER2 high (C-F) breast cancer cells. (G and H) Transcriptome analysis in HER2 high and HER2 low patients highlights the activation of either MAPK or AKT signalling. (I and J) Transcriptomic analysis of HER2 dimerization domain mutation harbouring patients revealed the switching in the signalling arm. α- tubulin was used as a loading control for the western blots.*

Co-immunoprecipitation of HER2 mutants in HER2 low, MCF7 breast cancer cells highlighted that G309A, S310Y and P523S causes the change in the interaction dynamics of HER2. These mutants preferred HER3 as their binding partner rather than HER2, which is favoured by HER2-WT and its S305C and S310F mutant (Fig. 4A). The shift in the interaction dynamics of mutants were also validated by shift in the activation of signalling arm from MAPK in HER2-WT and its S305C mutant to PI3K-AKT in G309A, S310Y and P523S mutants (Fig. 4B). The shift in the interaction dynamics of HER2 mutants from HER2 homo-dimer in HER2-WT and its S305C mutant to HER2:HER3 hetero-dimer in G309A, S310Y and P523S mutants was validated in HER2 high breast cancer cell lines such as AU-565 (Fig. 4C) and BT-474 (Fig. 4E) post 3'-UTR knockdowns of endogenous HER2 and subsequently expressing exogenous HER2-WT and its Dimerization domain mutants. As reported earlier, interaction of G309A, S310Y and P523S mutants to HER3 resulted in shift in signalling to PI3K-Akt from MAPK-ERK observed in HER2-WT and S305C mutant in both AU-565 (Fig. 4D) and BT-474 (Fig. 4F). Surprisingly, S310F mutant interacted with both HER2 as well as HER3, however the major interaction was with HER2 (Fig. 4A, C and E). This was also highlighted by the activation of both MAPK as well PI3K-AKT signalling in this mutant (Fig. 4B, D and F).
To gain more confidence on the switching of the signalling due to dimerization domain mutants of HER2, we analysed the transcriptome data of breast cancer patients from the METABRIC and Firehose legacy cohorts. As reported earlier, we found that patient expressing higher levels of HER2 have elevated MAPK signalling, in contrast to HER2 low patients that have high PI3K-Akt signalling (Fig. 4G and H). Surprisingly, as reported by us patient harbouring S305C mutation had higher MAPK signalling, but S310F, G309A and P523S mutation harbouring patient had elevated levels of PI3k-Akt signalling, suggesting that these mutations have a preferable binding partner i.e. HER3 leading to the activation of PI3K-Akt signalling (Fig.4I and J).

3. Establishment of functional relevance of mutations to the targeted drug response

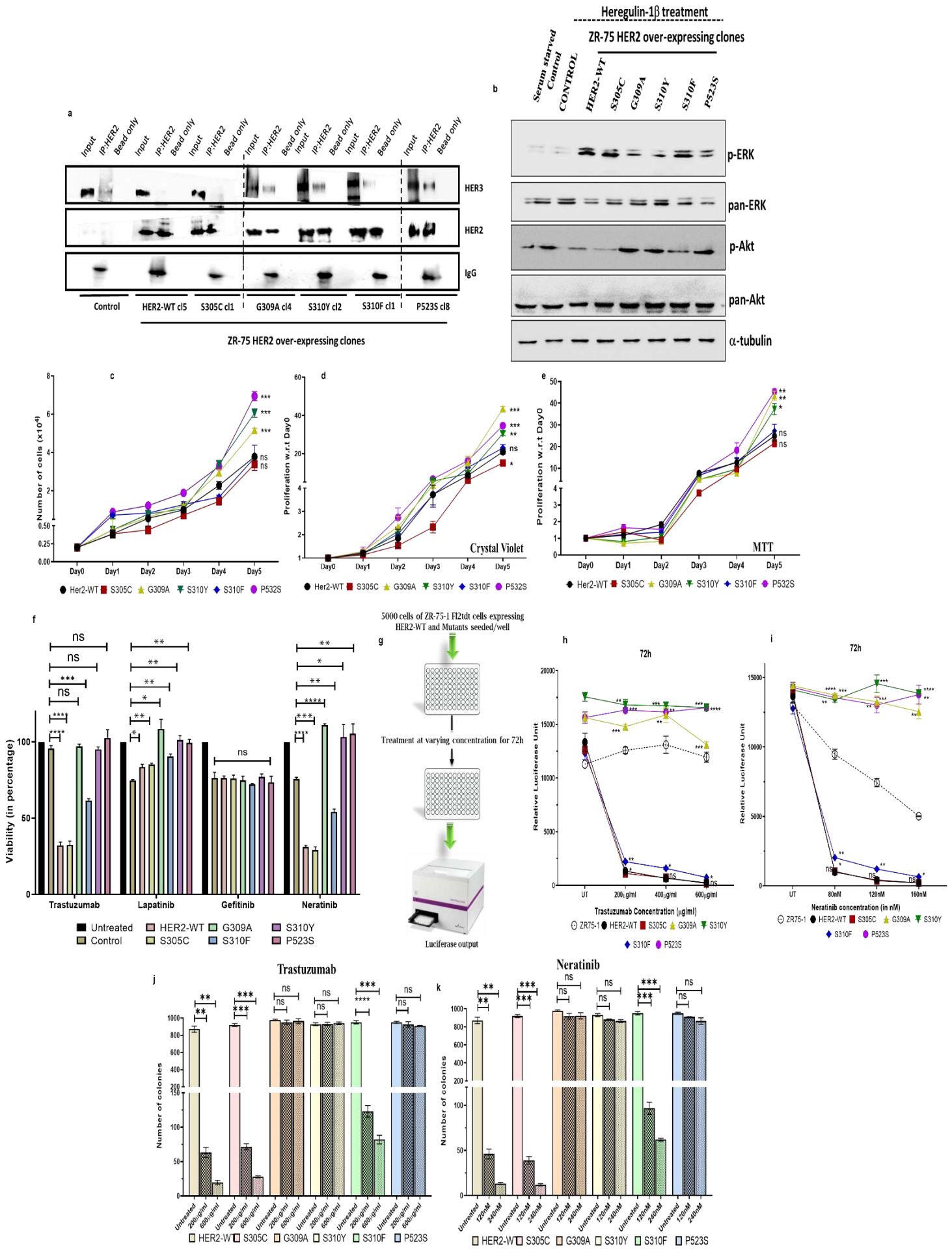

a
Input
IP:HER2
Bead only
HER3
HER2
IgG
Control
HER2-WT cl5
S305C cl1
G309A cl4
S310Y cl2
S310F cl1
P523S cl8
ZR-75 HER2 over-expressing clones
b
Heregulin-1β treatment
ZR-75 HER2 over-expressing clones
Serum starved Control
CONTROL
HER2-WT
S305C
G309A
S310Y
S310F
P523S
p-ERK
pan-ERK
p-Akt
pan-Akt
α-tubulin
c
Number of cells (x10⁴)
Day0
Day1
Day2
Day3
Day4
Day5
Her2-WT S305C G309A S310Y S310F P532S
d
Proliferation w.r.t Day0
Crystal Violet
e
Proliferation w.r.t Day0
MTT
f
Viability (in percentage)
Trastuzumab
Lapatinib
Gefitinib
Neratinib
Untreated
Control
HER2-WT
S305C
G309A
S310F
S310Y
P523S
g
5000 cells of ZR-75-1 Fl2tdt cells expressing HER2-WT and Mutants seeded/well
Treatment at varying concentration for 72h
Luciferase output
h
72h
Relative Luciferase Unit
UT
200 g/ml
400 g/ml
600 g/ml
Trastuzumab Concentration (μg/ml)
ZR75-1 HER2-WT S305C G309A S310Y S310F P523S
i
72h
Relative Luciferase Unit
UT
80nM
120nM
160nM
Neratinib concentration (in nM)
ZR75-1 HER2-WT S305C G309A S310Y S310F P523S
j
Trastuzumab
Number of colonies
Untreated
200 g/ml
600 g/ml
HER2-WT S305C G309A S310Y S310F P523S
k
Neratinib
Number of colonies
Untreated
120nM
240nM
HER2-WT S305C G309A S310Y S310F P523S

Fig 5. *(A and B) Change in the interaction of HER2 dimerization domain mutant and its downstream signalling activation in ZR-75-1 cells stably expressing HER2-WT and mutants. (C-E) Proliferation assay of HER2 mutants expressing cells. (F-I) Short-term cell viability assay showing resistivity of HER2 mutant expressing cells interaction with HER3 against Trastuzumab and Neratinib. (I and J) Graph showing anchorage-independent growth of HER2-WT and mutants expressing cells with and without treatment with Trastuzumab and Neratinib. Error bars represent ± s.e.m; *P<0.05; **P<0.01; ***P<0.001. α- tubulin was used as a loading control for the western blots.*

To further gain insights into the phenotypic changes as well as the changes in the targeted drug response due to HER2 dimerization domain mutants, HER2-WT and their mutant stable cell lines were established in HER2 low ZR-75-1 breast cancer cells. The similar change in interaction dynamics was reported in G309A, S310F, S310Y and P523S mutant expressing cells (Fig.5A). Interaction of G309A, S310Y and P523S with HER3 results in activation of Akt, whereas HER2-WT and S305C causes activation of ERK (Fig 5B). S310F mutation expressing cells had both the activation of ERK and AKT (Fig 5B). In addition, proliferation assay of ZR-75-1 HER2 mutant expressing cells revealed that G309A (P<0.01), S310Y (P<0.01) and P523S (P<0.001) had higher proliferation than HER2-WT, S305C and S310F expressing breast cancer cells suggesting that these mutant harbouring breast cancer cells might be more aggressive in nature (Fig. 5C, D and E).

Further, short term cell survival assay using MTT revealed that G309A, S310Y and P523S expressing cells were not sensitive to Trastuzumab or Neratinib. However, HER2-WT expressing cells were highly sensitive to Trastuzumab (P<0.0001) and Neratinib (P<0.0001). S305C expressing mutant also responds to Trastuzumab (P<0.001) and Neratinib (P<0.001). S310F expressing cells were slightly more resistant to HER2 targeting drugs like Trastuzumab (P< 0.001) and Neratinib (P<0.01) (Fig. 5F). Mutants interacting with HER3 lost their sensitivity to Lapatinib but no change in the sensitivity towards Gefitinib was observed (Fig. 5F). We also performed Luciferase assay of Firefly luciferase labelled HER2-WT and its Mutants expressing ZR-75-1 breast cancer cells. It showed that HER2-WT and S305C cells responded to Trastuzumab and Neratinib, resulting in significant decrease in the Relative Luciferase activity (RLU) of the cells. Additionally, G309A (P<0.001; P<0.001), S310Y (P<0.001; P<0.001)) and P523S (P<0.001; P<0.001)) were resistant to Trastuzumab and Neratinib respectively when compared to HER2-WT expressing breast cancer cells, thereby no significant drop in the RLU was observed. S310F expressing cells were less sensitive to Trastuzumab (P<0.05) and Neratinib (P<0.05) as compared to HER2-WT expressing cells, thus a drop in RLU was also observed (Fig. 5H and I). 3-D soft agar assay of HER2-mutant expressing cells portrayed that HER2-WT and S305C expressing cells were highly sensitive to Trastuzumab (P<0.01; P<0.001 respectively) and Neratinib (P<0.01; P<0.001 respectively) (Fig. 5J). However, G309A, S310Y and P523S were resistant to Trastuzumab and Neratinib. S310F expressing cells were less sensitive to Trastuzumab (P<0.001) and Neratinib (P<0.001) as compared to HER2-WT cells (Fig. 5K).

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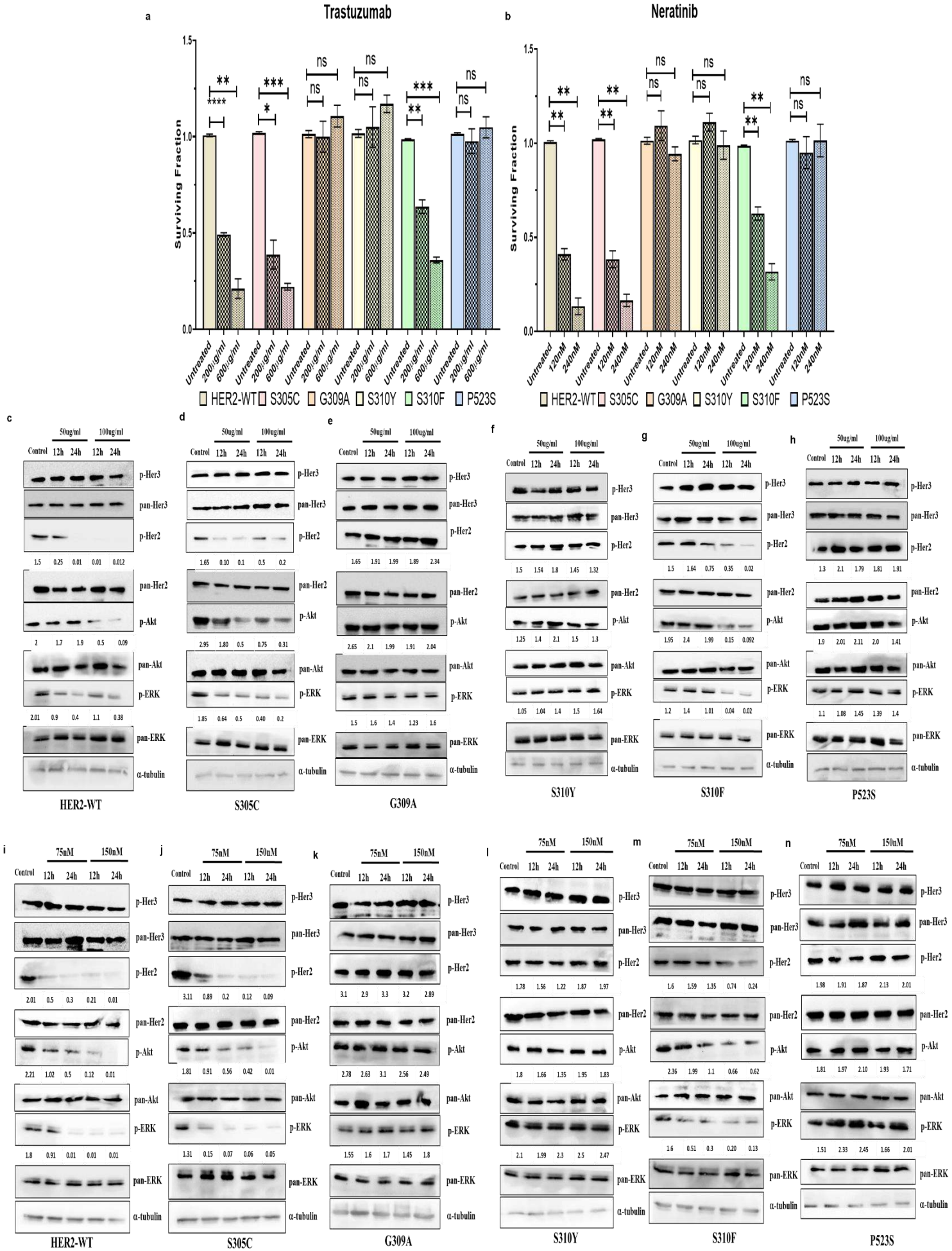
a
Trastuzumab
b
Neratinib
Surviving Fraction
1.5
1.0
0.5
0.0
Untreated
200μg/ml
600μg/ml
120nM
240nM
HER2-WT
S305C
G309A
S310Y
S310F
P523S
c
d
e
f
g
h
50ug/ml
100ug/ml
Control
12h
24h
p-Her3
pan-Her3
p-Her2
pan-Her2
p-Akt
pan-Akt
p-ERK
pan-ERK
α-tubulin
HER2-WT
S305C
G309A
S310Y
S310F
P523S
i
j
k
l
m
n
75nM
150nM

Fig.6 *(A and B) Long-term clonogenic assay showing the resistivity of HER2 dimerization domain mutants against Trastuzumab and Neratinib. (C-H) Immunoblot analysis showing the effect of Trastuzumab of HER2-WT and its mutants expressing ZR-75-1 breast cancer cells. (I-N) Immunoblot analysis showing the effect of Neratinib of HER2-WT and its mutants expressing ZR-75-1 breast cancer cells. Error bars represent ± s.e.m; *P<0.05; **P<0.01; ***P<0.001. α- tubulin was used as a loading control for the western blots.*

Long-term clonogenic assay revealed that G309A, S310Y and P523S expressing breast cancer cells does not respond to Trastuzumab, which is the first line of treatment. These mutants were also resistant to Neratinib, the last line of HER2 targeted therapy. However, HER2-WT expressing cells were highly sensitive to trastuzumab ($P<0.01$) as well as Neratinib ($P<0.001$). Similarly, S305C were also highly responsive to Trastuzumab ($P<0.001$) and Neratinib ($P<0.01$). S310F expressing cells were slightly less sensitive to Trastuzumab ($P<0.01$) and Neratinib ($P<0.01$) when compared to HER2-WT and S305C expressing cells (Fig. 6A).

To shed light on the underlying mechanism of either enhanced sensitivity or resistivity, we performed immunoblot analysis after treating ZR-75-1 cells expressing HER2-WT and mutants with Trastuzumab and Neratinib. Treatment of HER2-WT and mutants expressing cells with trastuzumab for 12h and 24h, showed dramatic decrease in the levels of pHER2, pERK and pAKT in HER2-WT and S305C expressing cells (Fig. 6C and D). Significant albeit slower decrease in the levels of pHER2, pERK and pAKT was reported in S310F expressing cells (Fig. 6G). No change in the levels of pHER2, pERK and pAKT was observed in G309A, S310Y and P523S expressing ZR-75-1 cells (Fig. 6E, F and H). In addition, treatment of HER2-WT and mutants expressing cells with pan-HER family inhibitor i.e. Neratinib for 12h and 24h, showed dramatic decrease in the levels of pHER2, pERK and pAKT only in HER2-WT and S305C expressing cells (Fig. 6I and J). Significant albeit slower decrease in the levels of pHER2, pERK and pAKT was reported in S310F expressing cells (Fig. 6M). No change in the levels of pHER2, pHER3, pERK and pAKT was observed in G309A, S310Y and P523S expressing ZR-75-1 cells (Fig. 6K, L and N). We did not find any changes in the levels of pHER3 after treatment suggesting that the activation of signalling in HER2-WT and S305C expressing cells is mediated by HER2:HER2 homodimer (Fig. 6C, D, G, I, J and M). The inability of Trastuzumab and Neratinib to inhibit HER3 mediated signalling was highlighted in G309A, S310Y and P523S expressing cells as we reported no change in the levels of pHER3 (Fig.6E, F, H, K, L and N).

- Statistical Analysis

All data are expressed as mean ± s.e. Statistical significance was analysed by Student's t-test using GraphPad Prism 8 software (GraphPad Software, La Jolla, CA). P values of ≤ 0.05 were considered statistically significant and the confidence interval (CI) was set at 95%. Statistical analysis was done for a minimum of two independent biological repeats.

- Discussion

Targeted therapy against breast cancer has proven to be a success story, improving the clinical outcome of patients. In that regard, Tamoxifen and Fulvestrant is prescribed as the first line of therapy to treat Estrogen positive breast cancer, for HER2 positive breast cancer Trastuzumab was FDA approved as a first line therapy in 1998. The underpinning 'oncogene addiction' of cancer cells is implicated for the efficacy of these targeted therapeutics. For HER2 positive breast cancer, signalling through HER2 drives the breast cancer proliferation and growth. Inhibition of this HER2 signalling either via monoclonal antibody (trastuzumab) or small

molecule inhibitor (neratinib) results in death of breast cancer cells expressing HER2. However, patients develop resistance against these targ
eted therapeutics either due to activation of compensatory pathway activation or mutations that abrogates the binding of small molecule inhibitors to the tyrosine kinase domain. In this work, we have highlighted for the first time that mutations in the interaction domain of HER2 changes the dynamics of its interaction as well dictates the therapeutic response of breast cancer cells.
We scrutinized the TCGA to identify the hotspot dimerization domain mutations, and found 5 mutations i.e. S305C, G309A, S310F, S310Y and P523S predominantly occurring as well as affecting the overall survival of HER2 positive breast cancer patients. Rudimentary 2-D analysis revealed that all the identified mutations abrogate the structure of HER2, hinting that they might affect the interaction of HER2. We also found that all the mutations were present in the β-Sheet, suggesting that amino acid change at this position might alter the 3-D structure. To gain more insights into the structural changes, we performed a total simulation of 12 μs and further docked the structure. Subsequently, we performed a total of 2μs MDS of the dimers to access whether the mutation is changing the interaction dynamics of HER2. Analysing the HER2-WT and its mutants dimer structures during the simulations revealed that HER2-WT homo-dimers don't fluctuate during the simulations depicting stable interaction. S305C and S310F rmsd graphs also revealed stable interaction. Surprisingly, G309A and S310Y showed un-stable interaction, as depicted by the box and violin plot. Further analysis revealed that the accessible surface area for binding as well as the atoms involved in the interaction increased over the course of simulations for HER2-WT, S305C and S310F homo-dimers, suggesting stronger interaction between the monomers. A decreasing trend in the accessible area for binding and atoms in interactions were observed for G309A and S310Y mutants, hinting that due to these mutations the homo-dimer of HER2 are not stable. Interestingly, we found that although the S310F mutant form homo-dimers but the stability is lower than that of HER2-WT and S305C condition. Solvation free energy gain on complex formation analysis during the simulation suggested that HER2-WT, S305C and S310F has more stable dimer structures as the delta G is more negative. However, G309A and S310Y structures have less negative delta G values, thereby are less likely to form stable homo-dimer interaction.
To validate whether the decreased stability of HER2 dimerization domain mutants cause any change in the interaction we performed co-immunoprecipitation of HER2 mutants in MCF7 breast cancer cells. Intriguingly, we reported that G309A, S310F and P523S causes the change in the interaction dynamics of HER2. These mutants preferred HER3 as their binding partner rather than HER2, which is favoured by HER2-WT and its S305C and S310F mutant. The shift in the interaction dynamics of mutants were also validated by shift in the activation of signalling arm from MAPK in HER2-WT and its S305C mutant to PI3K-AKT in G309A, S310Y and P523S mutants. S310F mutant interacted with both HER2 as well as HER3, however the major interaction was with HER2. This was also highlighted by the activation of both MAPK as well PI3K-AKT signalling in this mutant. In line with our in-vitro observation, we reported that patient harbouring S305C mutation had higher MAPK signalling which is the major cell survival signalling arm activated in HER2 high patients, but G309A and P523S mutation harbouring patient had elevated levels of PI3k-Akt signalling, which is known to get activated when HER2 interacts with HER3, suggesting that these mutations have a preferable binding partner i.e. HER3. This interaction as well as activation of different signalling arm in G309A, S310Y and P523S mutants might alter the targeted therapy response in breast cancer cells.
HER2-WT and their mutant stable cell lines were established in ZR-75-1 breast cancer cells, a similar switching of the interaction from HER2:HER2 homo-dimer in HER2-WT, S305C and S301F to HER2:HER3 heterodimer in G309A, S310Y and P523S. We also found that the proliferation of ZR-75-1- G309A, S310Y and P523S mutants expressing breast cancer cells

had higher proliferation than HER2-WT, S305C and S310F expressing breast cancer cells suggesting that these mutants might be more aggressive in nature. Short term cell survival assay highlighted the resistivity of G309A, S310Y and P523S expressing cells against Trastuzumab (first line) or Neratinib (last line) treatment. Expectedly, HER2-WT and S305C expressing cells were highly sensitive to Trastuzumab and Neratinib. Interestingly, S310F expressing cells were slightly more resistant to HER2 targeting drugs like Trastuzumab and Neratinib as compared to HER2-WT and S305C expressing cells. In addition, we also found, mutants interacting with HER3 lost their sensitivity to Lapatinib but no change in the sensitivity towards Gefitinib was observed suggesting that over-expression of HER2 in these HER2 low breast cancer cells did not alter the levels of EGFR. Trastuzumab and Neratinib abrogated the anchorage-independent growth of HER2-WT and S305C expressing cells. However, G309A, S310Y and P523S were resistant to Trastuzumab and Neratinib effect on their anchorage-independent growth. As observed earlier, S310F expressing cells were less sensitive to Trastuzumab and Neratinib as compared to HER2-WT and S305C cells in 3D Soft agar assay. Clonogenic assay revealed that G309A, S310Y and P523S expressing breast cancer cells does not respond to Trastuzumab as well as Neratinib. However, HER2-WT and S305C expressing cells were highly sensitive to trastuzumab and Neratinib. S310F expressing cells were slightly resistant to Trastuzumab and Neratinib when compared to HER2-WT and S305C expressing cells. To gain insights into the effect of HER2 targeted therapy on the downstream signalling, ZR-75-1 cells expressing HER2-WT and its mutants were treated with trastuzumab and neratinib for 12h and 24h, A significant decrease in the levels of pHER2, pERK and pAKT in HER2-WT and S305C expressing cells was observed when treated with Trastuzumab and Neratinib in a dose-dependent and time-dependent manner. Significant albeit slower decrease in the levels of pHER2, pERK and pAKT was reported in S310F expressing cells after treatment with Trastuzumab and Neratinib. Interestingly, no change in the levels of pHER2, pERK and pAKT was observed in G309A, S310Y and P523S expressing ZR-75-1 cells after treating with Trastuzumab and Neratinib.

- Impact of the research in the advancement of knowledge or benefit to mankind

Targeting the cancer cells specifically has always been the primary aim of any anti-cancer therapy. Decades of research has led to the advent of HER2 targeted therapy namely Trastuzumab as the first line and Neratinib as the last line of therapeutic. Keeping in line with this thought, targeting HER2 and its downstream signalling has been a success story for breast cancer therapy. However, activation of compensatory pathway like HER3 or mutations in the receptor kinase domain that affects the inhibitor binding is still a conundrum challenging HER2 targeted therapy. Activation of ERK signalling after HER2 homo-dimerization mediated via its interaction domain (domain II and domain IV) drives the HER2 positive breast cancer. However, we report for the first time that mutations in the dimerization domains causes switching in the interaction from HER2:HER2 homo-dimer to HER2:HER3 hetero-dimer. This receptor switching also causes a change in the signalling activation from MAPK to AKT signalling. We also highlighted that patient harbouring these inherent HER2 mutations have altered signalling activation. Further, we reported that mutations interacting with HER3 were aggressive as well as resistant to Trastuzumab and Neratinib. In the current clinical practice, the therapy is decided on the basis of IHC scoring, however we report that patients harbouring HER2 dimerization domain mutation would not form HER2:HER2 homo-dimer that dictates the therapy response of a patient. Patients harbouring G309A, S310Y and P523S mutation might not respond to the Trastuzumab (first line) or Neratinib (last line) treatment as both of these drugs in in-effective in inhibiting the HER3 signalling.

- Material and Methods
Materials

We used HER3 monoclonal antibody (Cell Signaling Technology, 12708S, 1:1000), , phospho-HER2 monoclonal antibody (Cell Signaling Technology, 2247, 1:1000), HER2 monoclonal antibody, (Invitrogen, MA5-13675, 1:1000), ERK monoclonal antibody (Cell Signaling Technology, 46951:1000), phosphor-ERK (Cell Signaling Technology, 4370, 1:1000), AKT monoclonal antibody (Cell Signaling Technology, 4691, 1:1000), phosphor-AKT monoclonal antibody (Cell Signaling Technology, 9271, 1:1000), anti-mouse HRP secondary antibody (Abcam, ab6728), goat anti-rabbit HRP antibody (Thermo Scientific, 31460)
The drugs were bought from Selleckchem. Neratinib (S2150), Trastuzumab was a kind gift from Dr. Sudeep Gupta (ACTREC, TMC).

Molecular Dynamic Simulation
Validation of the HER2 model was done using Ramachandran plot. All the 3-D structures were subjected to molecular dynamics simulation studies using by GROMACS 2018.1 with implementation of OPLS all atom force-field. Solvation of the system was done usingTIP3P water model in a cubic box with periodic boundary conditions. Required counter-ions were added to neutralize the system before starting the run. The three systems were initially energy minimized using steepest descent algorithm with a tolerance of 1000kJ/mol/nm. The electrostatic interactions were computed using Particle Mesh Ewald (PME) summation with 1 nm cut-offs. Further, Van der Waal and Columbic interactions were calculated with a distance cut-off of 1.4 nm. System was subsequently equilibrized by employing positional restraints on the structure using NVT and NPT ensemble for 100 ps each. Temperature of 300K was coupled by Berendsen thermostat with pressure of one bar using the SHAKE algorithm. The three equilibrized systems were then subjected to 50ns of production run with time-step integrationof2fs. Trajectories of the simulation were saved at every 2ps and was analyzed using Gromacs2018.1. The root mean square fluctuations (RMSF), root mean square deviation (RMSD), hydrogen bonds, radius of gyration (Rg), solvent accessible surface area (SASA), and dictionary of secondary structure of protein (DSSP) were analyzed.

Docking Study
Molecular docking studies of HER2 wt and its mutants were performed using HADDOCK server. The structure of the HER2 (3N85) was downloaded from the protein data bank and used as a template for structure building. Chain assignment of both proteins was edited by PDB editor. Prior to docking, the energy minimized structure of HER2 was taken from the GROMACS simulation and all co-factors atoms were removed from the structure. Best cluster showing lowest HADDOCK score was selected. These docked structures were then subjected to PDBsum analysis as well as further GROMACS simulation to gain information about the residues involved in interactions and changes in the stability.

Immunoblotting

After treatment with Trastuzumab and Neratinib at desired concentration for designated time-points cells were collected and lysed using RIPA buffer containing protease inhibitor cocktail. Equal amounts of protein from control and treated cells were separated by 7.5% SDS-PAGE and transferred onto a nitrocellulose membrane by using a wet transfer blotting apparatus. After blocking with 5% non-fat dry milk, membranes were probed with antibodies against various

cell survival regulating proteins. The blots were then probed with HRP-conjugated secondary antibodies and developed using a Chemidoc system.

Cell survival assays

For MTT, 3000 cells were seeded for the cell viability assay. 16h after seeding, the ZR-75-1 cells were treated with Trastuzumab (10ug/ml), Neratinib (50nM), Gefitinib (50mM) and Lapatinib (75mM) for 48h. After 48h, the treated and control cells were processed for MTT and clonogenic assay. Similarly, for clonogenic assay and soft agar assay, the cells were treated with the mentioned concentrations and allowed to form colonies. The colonies were stained with 0.05% crystal violet for visualization and subsequently, counted.

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The above written work is done by me and is novel to the best of my knowledge.

Mr. Pranay Dey