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Inhibition of focal adhesion kinase with her-2 targeted antibody pertuzumab (Omnitarg®, 2C4) in breast cancer cells

Research Article

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Abbreviations: American Type Culture Collection, (ATCC); bovine serum albumin, (BSA); Dulbecco's modified Eagle's medium, (DMEM); epidermal growth factor receptor, (EGFR); Focal adhesion kinase, (FAK); phosphatidylinositol 3-kinase, (PI3K)

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Summary

Pertuzumab (Omnitarg®, 2C4) is a recombinant humanized monoclonal antibody targeted to extracellular region of HER-2. Previous results proved the inhibitory effect of Pertuzumab on the survival of breast cancer cells via MAPK and Akt pathway. Focal adhesion kinase (FAK) regulates multiple cellular processes including growth, differentiation, adhesion, motility and apoptosis. Here, we aimed to investigate the effects of Pertuzumab on ligand activated total FAK expression and phosphorylation in the HER-2 overexpressing BT-474 breast cancer cell line. Heregulin was used for ligand activation. We have found that FAK expression and phosphorylation were inhibited in with Pertuzumab in breast cancer cells.

I. Introduction

The HER-2 (c-erbB-2, neu) gene encodes a 185-kDa transmembrane glycoprotein that is a member of the epidermal growth factor receptor (EGFR or erbB) family of receptor tyrosine kinases. HER-2 mediates signal transduction, resulting in mitogenesis, apoptosis, angiogenesis, and cell differentiation (Ménard et al, 2000). The HER-2 gene is amplified and overexpressed in ~20-30% of invasive breast carcinomas, and is associated with increased metastatic potential and decreased overall survival (Slamon et al, 1987; Ménard et al, 2000).

Pertuzumab (Omnitarg®, 2C4; Genentech) is a humanized monoclonal antibody against the dimerization domain of HER-2. This agent is the first in a new class of targeted therapeutics known as HER-2 ''dimerization inhibitors'' (Franklin et al, 2004). In contrast to Trastuzumab, Pertuzumab sterically blocks HER-2 dimerization with other HER receptors and blocks ligand-activated signaling from HER-2/HER-1 and HER-2/HER-3 heterodimers (Agus et al, 2002). It has been shown that the signaling pathways and cellular processes associated with tumor growth and progression could be inhibited with Pertuzumab both in vitro or in vivo models (Agus et al, 2002). Pertuzumab has undergone phase I trials in patients with advanced solid malignancies (Agus et al, 2005; Albanell et al, 2008) and is currently in phase II clinical trials in NSCLC, metastatic breast, ovarian, and prostate cancers (Friess et al, 2005).

The invasion and metastasis of cancer is the process that includes changes in cell adhesion and motility that tumor cells gain the ability to invade and migrate through the extracellular matrix. FAK is a tyrosine kinase considered to be a central molecule in integrin mediated signaling, and it is involved in cellular motility and protection against apoptosis (Parsons et al, 2000).

The aim of the present study was to assess the effects of Pertuzumab on the expression and tyrosine phosphorylation of FAK in HER-2 overexpressing BT-474 breast cancer cells.

II. Materials and Methods

A. Materials

Pertuzumab (Genentech) was provided in freezed dried powder at 50 mg. Heregulin -α and. Calnexin were purchased from Sigma and the anti-Human FAK antibody (M135) was purchased from TaKaRa laboratories. Anti-phospho FAK (pY^{397}) antibody was purchased from Invitrogen. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were obtained from Sigma (USA).

B. Cell culture

BT-474 breast cancer cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1 mM glutamine, 10U/ml penicillin G and 10ug/ml streptomycin at 37ºC in 5% CO_2-containing atmosphere.

C. Immunohistochemistry

BT-474 cells were plated in 6 well-plates (150.000 cells/well) on coverslips one day before the experiment. After 24 hrs, cells were treated with 10ng/ml Heregulin to induce HER-2/ HER-3 or HER-4 dimerization in the cells. Heregulin concentration was used as previously described (Gregory et al, 2005). One and 10 mg/ml of Pertuzumab was added to wells and incubated for 2 to 24 hours. Each experiment was performed in triplicate and repeated three times. After 2 and 24 hours of incubation with Pertuzumab cells were fixed with 4% paraformaldehyde for 20 mins at room temperature and permeabilized with Sodium citrate for 5 mins at 4°C. Blocking was done with 10% bovine serum albumin (BSA) for 30 mins. And the cells were incubated with FAK (p125) antibody. HRP conjugated streptavidin was used in order to visualize the signal of the protein. The signal was developed with a substrate DAB (DAKO). Finally counterstaining was performed by hematoxylene staining.

The positive staining of the Heregulin treated HER-2 overexpressing BT-474 cells served as the positive internal control of the experiment. FAK-positive cells were counted in each slide and the ratio of the positive cells to the whole cell count was calculated as percentage. Minimum ten areas were counted from each triplicate slides and the standard deviations were calculated from the mean of the counts. If more than 20% of carcinoma cells were stained more intensely than that of untreated cells the sample was classified as strong FAK overexpression (3+) (Langer et al, 2004; Zinner et al, 2004; Krug et al, 2005). When the FAK immunostaining was equal compared to that of control, the sample was classified as intermediate expression (2+). When the FAK immunostaining was weaker than that of internal control, the cells were classified as low FAK expression (+1). The lack of FAK immunostaining was classified as negative (0).

D. Immunoblotting

BT-474 cells were treated with 10 ng/ml Heregulin and at the same time 1 and 10 μg/ml pertuzumab was added onto the cells. Cells were harvested using lysis buffer (10mM Tris-Cl, pH 7.6, 5mM EDTA, 50mM NaCl, 30mM Na-pyrophosphate, 50mM NaF, 100μM Na-ortovanadate, 1% Triton X and 1mM PMSF). Equal amounts of protein extracts were loaded in 8% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membrane. Total FAK (p125) and Phospho-FAK protein (pY^{397}) were detected by rabbit anti-human FAK (TaKaRa) and anti-human phospho-FAK (Invitrogen) polyclonal antibodies and were used at a 1:1000 dilution in 4% BSA in PBS-Tween-20. Horseradish peroxidase-conjugated anti-rabbit antibody was obtained from Sigma (USA) and was detected using enhanced chemiluminescence (Amersham-Pharmacia Biotech, Piscataway, NJ).

III. Results

A. Immunohistochemical analysis of FAK

Figure 1A shows the basal FAK phosphorylation without ligand activation in Her-2 overexpressed BT-474 cell line (+2). HER-2 dimerization with HER-3 or HER-4 was induced with 10 ng/ml Heregulin for 2 hours in BT-474 cell line which is used as an internal positive control of the experiment (**Figure 1B**). FAK immunostaining revealed strong membranous and cytoplasmic staining with 10 ng/ml of Heregulin treatment for 2 hours in BT-474 cells compared to control cells (+3). **Figures 1C** and 1D show FAK expression in BT-474 cells with 1μg/ml and 10μg/ml Pertuzumab in the presence of 10 ng/ml Heregulin for 2 hours, respectively. The FAK expression was gradually decreased with the increasing amount of Pertuzumab at a concentration of 1 μg/ml and 10 μg/ml after 2 hours (+1). **Figure 2A** shows the basal FAK phosphorylation in BT-474 cells without Heregulin for 24 hours. Her-2 dimerization was activated with 10ng/ml Heregulin for 24 hours in BT-474 cell line (**Figure 2B**).**Figures 2C** and **2D** show decrement of FAK expression with either 1μg/ml or 10μg/ml Pertuzumab in the presence of 10 ng/ml Heregulin for 24 hours in BT-474 cell line. The cells in each condition were seeded triplicate and counted from minumum 10 areas of the slides and the ratio of positive cells to whole cell count of three experiments was calculated as percentage. **Figure 3** shows the mean value of three experiments ± SD. The decrease of FAK expression was not significant in the cells treated with 1μg/ml and 10 μg/ml Pertuzumab in the presence of 10ng/ml Heregulin for 2 hours when compared to the ones treated only with Heregulin ($p>0.05$). However, a significant decrease was present in the FAK expression in the cells treated with 1μg/ml and 10μg/ml Pertuzumab for 24 hours compared to treatment with 10 ng/ml Heregulin alone for 24 hours ($p=0.04$ and $p=0.005$, respectively).

B. Evaluation of expression and phosphorylation of the FAK with immunoblotting

We further confirmed our immunostaining results with immunoblotting. FAK expression was gradually decreased with the increasing amount of Pertuzumab at a concentration of 1μg/ml and 10μg/ml either in 2 or in 24 hours (**Figure 4**). We also investigated whether Pertuzumab could modulate protein phosphorylation of the signal transduction molecule- the FAK. FAK phosphorylation was strikingly inhibited with Pertuzumab in dose-dependent manner after 24 hours. **Figure 5** shows the decrement of FAK phosphorylation in response to Pertuzumab in BT-474 breast cancer cells.

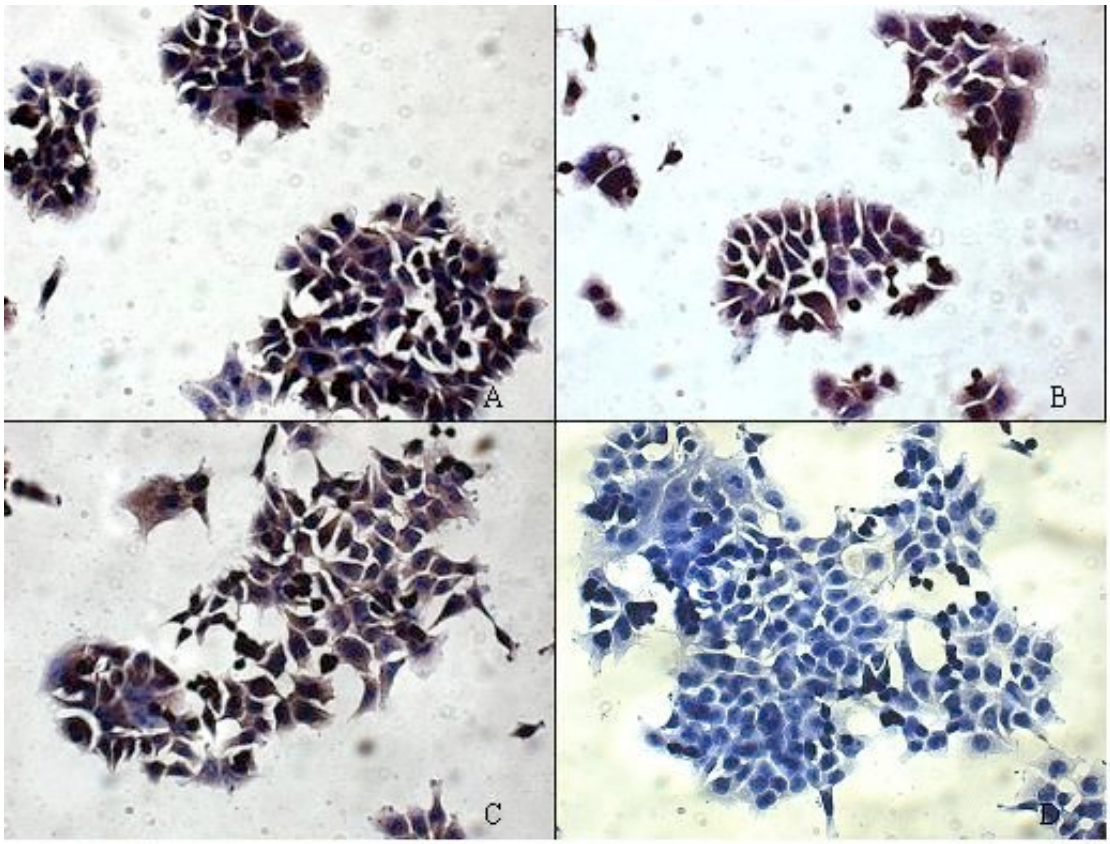

Figure 1. Expression of FAK in BT-474 cells for 2 hours. (**A**) Basal FAK expressionwithout HER-2 dimerization (**B**) with 10ng/ml of Heregulin (**C**) with 1μg/ml Pertuzumab+ 10ng/ml Heregulin (**D**) with 10μg/ml Pertuzumab +10ng/ml Heregulin.

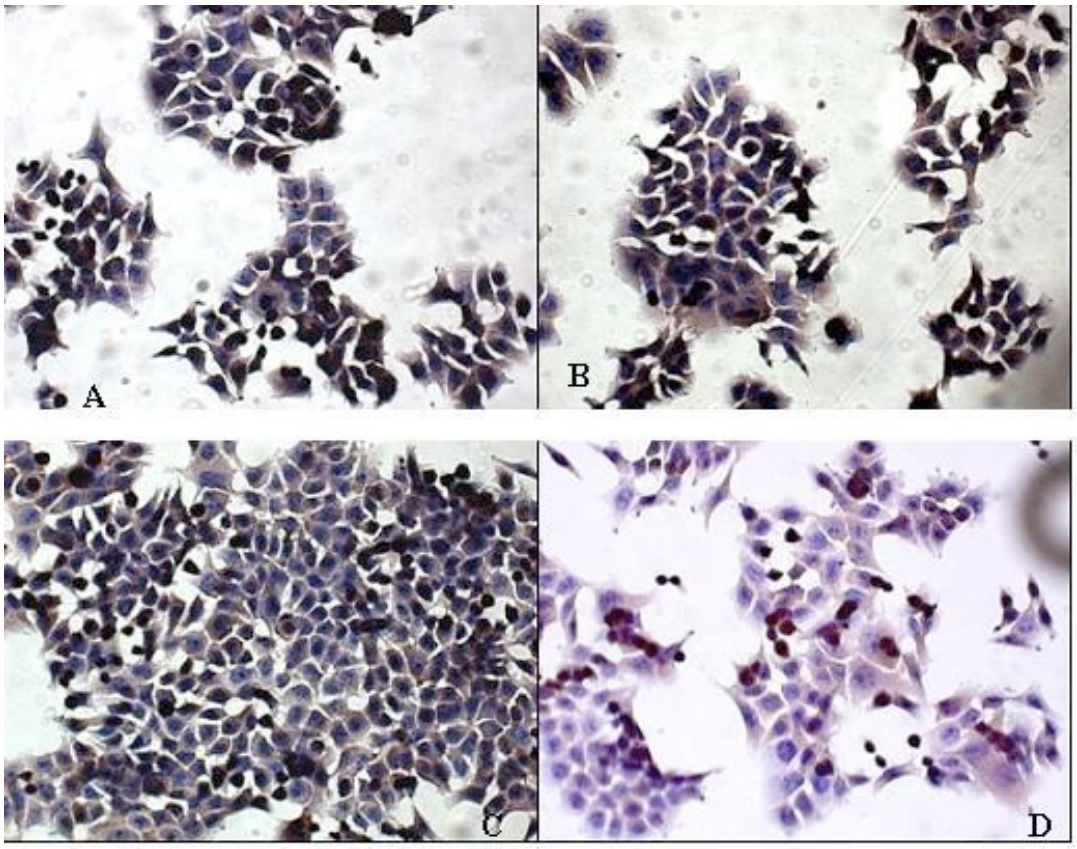

Figure 2. Expression of FAK in BT-474 cells for 24 hours. (**A**) Basal FAK expression without HER-2 dimerization (**B**) with 10ng/ml of Heregulin (**C**) with 1μg/ml Pertuzumab + 10ng/ml Heregulin (**D**) with 10μg/ml Pertuzumab + 10ng/ml Heregulin.

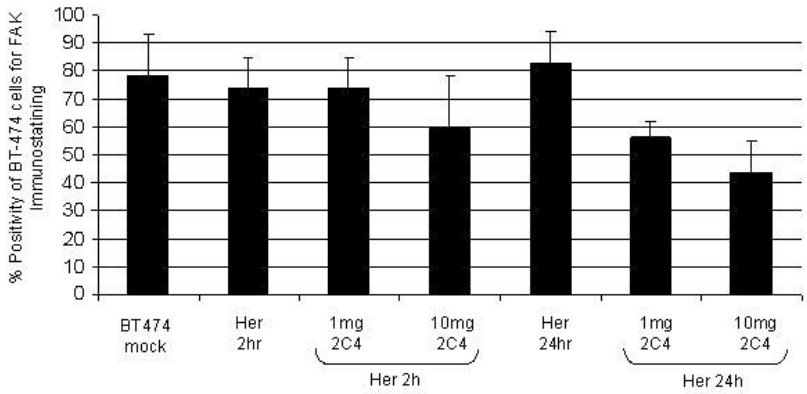

Figure-3. Pertuzumab inhibits FAK expression with immunostaing. Each immunostaining experiment was done in triplicates. The cells were counted and the ratio of positive cells to whole cell count was calculated as percentage. Minimum 10 areas from each slide were counted. The standart deviations were calculated according to the whole cell count. The decrease of the FAK expression is significant in the cells treated with 10 μg/ml Pertuzumab for 24 hours when compared to the ones treated with 10ng/ml Heregulin for 24 hours (p=0.005). Likewise a significant decrease is present in the FAK expression in the cells treated with 1 μg/ml Pertuzumab for 24 hours (p=0.04). Decrease in FAK expression was not significant in the cells treated with 1 μg/ml and 10 μg/ml 2C4 in the presence of 10ng/ml heregulin for 2 hours.

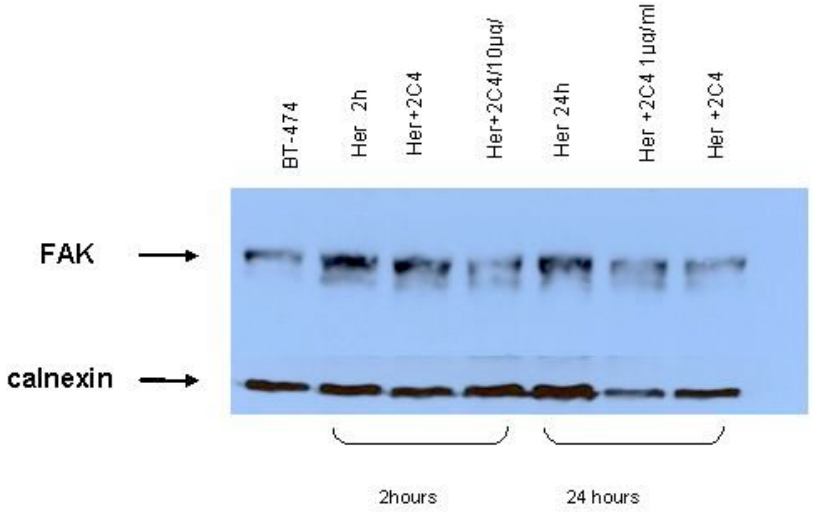

Figure 4. Pertuzumab decreases the expression of FAK with immunoblotting. The BT-474 cells were treated with 1 μg/ml and 10 μg/ml of Pertuzumab in the presence of 10ng/ml Heregulin for 2 and 24 hours. The inhibitory effect of Pertuzumab on FAK expression was observed in the cells treated with 10 μg/ml of Pertuzumab for 24 hours. The equal loading was adjusted with calnexin according to the Comassie blue staining.

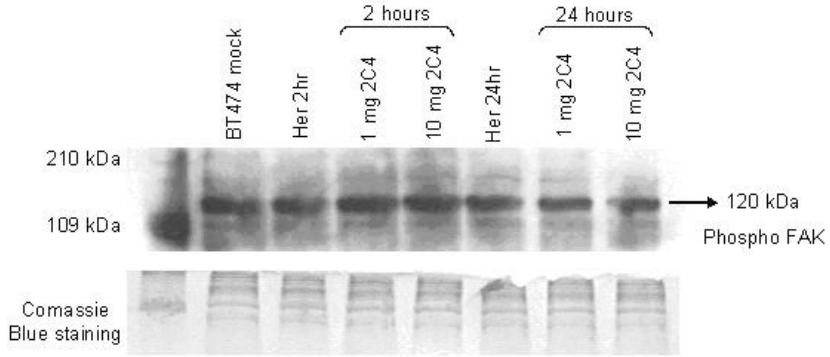

Figure 5. Pertuzumab decreases the phosphorylation of FAK. The BT-474 cells were treated with 10ng/ml of Heregulin for 2 and 24 hours. Meanwhile they were treated with 1 μg/ml and 10 μg/ml of Pertuzumab. The inhibitory effect of Pertuzumab on phosphorylated-FAK was observed in the cells treated with 10 μg/ml of Pertuzumab for 24 hours. The equal loading was adjusted with calnexin according to the Comassie blue staining.

IV. Discussion

In this report, for the first time we have shown that Heregulin activated total FAK expression and FAK phosphorylation were inhibited with Pertuzumab in BT-474 HER-2 overexpressing breast cancer cell line.

Even though the surgical techniques and the adjuvant therapies have been proven to be useful in the treatment of primary tumors (Entschladen et al, 2004), invasion and metastasis remain a major cause of poor prognosis and death in cancer patients. Trastuzumab monotherapy offers clinical benefit to a subset of HER-2-overexpressing metastatic breast cancers. However, the majority of breast cancers that initially respond to Trastuzumab-containing regimens begin to progress again within 1 year (Albanell and Baselga, 2001). The recombinant humanized HER-2 monoclonal antibody Pertuzumab sterically blocks dimerization of HER-2 with other HER receptors (Agus et al, 2002) known as ''HER dimerization inhibitors''. Reports from phase I and II trials indicate that Pertuzumab plays an important role in the inhibition of the solid tumors progression including breast cancer (Parsons et al, 2000; Friess et al, 2005; Albanell et al, 2008). Beside these reports, which ligand activated HER-2 signaling molecules inhibited by Pertuzumab are not completely detected.

Signaling pathways activated by HER-2 include the phosphatidylinositol 3-kinase (PI3K)/Akt and MAPK cascades (Mendoza et al, 2002; Nahta et al, 2004). The reports of studies on the effects of Pertuzumab show that inhibiting the survival of breast cancer cells via MAPK (Agus et al, 2002) and Akt pathway (Nahta et al, 2004). The combination of Trastuzumab and 2C4 reduced the serine phosphorylation of Akt whereas signaling from the MAPK cascade was not inhibited (Nahta et al, 2004). Previous studies also show that Pertuzumab inhibited Heregulin-activated mitogenic signaling in breast and prostate cancer models in vitro and in vivo because of dissociation of HER-2/HER-3 dimers (Agus et al, 2002; Mendoza et al, 2002).

FAK is a cytoplasmic tyrosine kinase that plays an important role in integrin-mediated signal transduction pathways closely related to cell adhesion, motility, and growth (Parsons et al, 2000; Parsons et al, 2000; Schlaepfer et al, 2004). Upregulation of FAK expression is associated with oncogenesis (Cance et al, 2000) and decrease in FAK is associated with the loss of ability to attach (Mitra et al, 2005), decreased migration (Schlaepfer et al, 2004) and induction of apoptosis (Parsons, 2003). In our study, the FAK expression and phosphorylation were increased in response to HER-2 dimerization induced by Heregulin. Specifically, FAK is phosphorylated at multiple sites in cells stimulated by mitogenic agonists that act via heptahelical GPCRs including bombesin (Zachary et al, 1992; Salazar and Rozengurt, 2001) and lysophosphatidic acid (Seufferlein and Rozengurt, 1995), ligands of tyrosine kinase receptors, including EGF (Leventhal et al, 1997; Ojaniemi and Vuori, 1997), integrin clustering induced by cell adhesion (Owen et al, 1999; Ruest et al, 2000) and activated variants of pp60src (Guan and Shalloway, 1992; Parsons and Parsons, 1997). It is increasingly recognized that FAK functions as a point of convergence and integration in the action of multiple signals (Rozengurt, 1995). In recent studies, Vadlamudi and colleagues utilized human breast cancer cell lines in vitro to establish a novel signaling pathway involving HER-2, phospho-Src Tyr-215 and phospho-FAK Tyr-861 leading to increased cellular motility (Vadlamudi et al, 2002, 2003). The authors showed that heregulin-induced HER-2 activation resulted in phosphorylation of FAK at tyrosine 861. Further support to our study was reported by Schmitz et al. They have reported that HER-2 and FAK associated signaling in tumor tissue of breast cancer patients (Schmitz et al, 2005). A recent study also identified frequent polysomic patterns for chromosome 1, chromosome 8 and chromosome 17 that are indicative for increased tumor malignancy in breast cancer (Nakopoulou et al, 2002). The FAK is located on chromosome 8 and the HER-2 is located on chromosome 17. These polysomic patterns can be lead to the alterations in HER-2 and FAK expression and signaling in breast cancer.

In the present study, a significant downregulation of FAK expression and phosphorylation with Pertuzumab was observed, suggesting that Pertuzumab may serve as a potential important anticancer agent for breast cancer. Increased FAK expression and phosphorylation by ligand activated HER-2 signaling and inhibition with Pertuzumab indicating that FAK also could be an important pharmacologic target site and whether FAK is the upstream molecule of MAPK/Akt pathway of apoptosis and/or metastasis remains to be investigated.

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