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14. ABSTRACT EGFR and HER2 are overexpressed in 20% and 30% of invasive breast cancer, respectively, and are associated with aggressive tumor subtypes and shortened patient survival. Both receptors are important targets of breast cancer therapy. However, despite the apparent promise of some of these therapies, EGFR- and HER2-based monotherapy and combination regimens have serious limitations and need improvement. The goal of this study is, thus, to gain insights into the biology of EGFR- and HER2-expressing invasive breast cancer in order to provide rationales for more effective EGFR- and HER2-based combination therapy for women with breast cancer. Our proposal is built on novel significant findings made from the initial Idea Award. We discovered that proapoptotic PUMA protein is highly expressed in the breast cancer cell lines and patient tumors that overexpress HER2 and/or EGFR. In addition to co-expression, we found HER2 and EGFR to interact with PUMA constitutively and under the treatment of apoptosis inducers. The HER2-PUMA and EGFR-PUMA interactions are not disrupted when breast cancer cells are treated with the EGFR kinase inhibitors, indicating a kinase-independent interaction. Despite the fact that PUMA has been reported to be primarily located on the mitochondrial membranes and initiate apoptosis upon appropriate stress, our results showed PUMA to be sequestered in the cytoplasm of EGFR-expressing breast cancer cells. Although, the BH3-only proapoptotic proteins can be functionally redundant, we observed PUMA to be essential for apoptotic induction in breast cancer cells. Interestingly, while no reports have investigated PUMA phosphorylation, our preliminary results show that PUMA undergoes tyrosine phosphorylation mediated by HER2 and EGFR. These exiting preliminary observations suggest that EGFR and HER2 may modulate PUMA via two modes of actions: (i) interacting with PUMA to prevent PUMA mitochondrial translocation in a kinase-independent fashion, and (ii) phosphorylating PUMA to affect its functionality in a kinase-dependent phosphorylation. Our hypothesis is that the EGFR-PUMA and HER2-PUMA signaling crosstalks modulate PUMA-mediated apoptotic pathway and cellular functions of EGFR and HER2, together contributing to the aggressive behavior of invasive breast cancer. Based on this, we postulate that restoring intrinsic apoptosis will sensitize breast cancer to EGFR- and HER2-targeted therapy. Specific Aims are (1) Characterize EGFR-PUMA and HER2-PUMA crosstalks in breast cancer overexpressing EGFR and/or HER2. (2) Investigate the biological consequence(s) of the phosphorylation of PUMA by EGFR and HER2 in breast cancer. (3) Determine the extent to which PUMA's apoptotic function is associated with breast cancer response to EGFR- and HER2-targeted therapy. If successful, the proposal could shed light on the malignant phenotype of aggressive breast cancer that overexpress HER2 and/or EGFR which constitutes approximately half of invasive breast cancer and could also provide rationales for new more effective therapy for women with aggressive subtypes of breast cancer.					
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INTRODUCTION

Approximately half of the human invasive breast carcinomas overexpress HER2 and/or EGFR and the overexpression leads to more aggressive tumor behaviors and shortened patient survival. Both receptors are important targets of breast cancer therapy. However, despite the apparent promise of some of these therapies, HER2- and EGFR-based regimens have their limitations and need improvement (1, 2). The **goals** of this Idea Expansion Award are to gain insights into the malignant biology and drug-resistant phenotype of EGFR- and/or HER2-overexpressing breast cancer and to use the acquired knowledge for the development of a sensitization strategy that will improve EGFR- and HER2-targeted therapies. The immediate objective of this project is to define the biological significance and therapeutic implications of the novel HER2-PUMA and EGFR-PUMA crosstalks in breast cancer. Our **hypothesis** is two-fold.

First, we **hypothesize** that **the HER2-PUMA and EGFR-PUMA signaling crosstalks modulate PUMA-mediated apoptotic pathway and regulate cellular functions of HER2 and EGFR, together contributing to the aggressive behavior of HER2- and EGFR-overexpressing breast cancer.** The rationales are: **(i)** A paradox was uncovered that the majority (83%) of the invasive breast carcinomas with overexpressed HER2 and/or EGFR also expresses the potent apoptosis inducer PUMA, p53-upregulated modulator of apoptosis and a member of the Bcl-2 family of proteins (3). **(ii)** Both HER2 and EGFR interact with PUMA constitutively and under the treatments with kinase inhibitors in breast cancer cells. **(iii)** Although PUMA has been reported to primarily localize on mitochondrial membranes (4, 5), we found that PUMA is sequestered in the cytoplasm of EGFR-overexpressing breast cancer cells where it is not functional. **(iv)** Subsequent to the interactions, PUMA is tyrosine-phosphorylated by HER2 and EGFR. These results point to the possibility that HER2 and EGFR may modulate PUMA via two modes of actions: interacting with PUMA to prevent PUMA mitochondrial translocalization in a kinase-independent fashion, and phosphorylating PUMA to modulate its functionality in a kinase-dependent manner. **(v)** Since protein-protein interactions can cause reciprocal effects on both proteins, we postulate that the HER2-PUMA and EGFR-PUMA interactions modulate cellular functions of both receptors. We will test this hypothesis by studies proposed in Specific Aims 1 and 2.

Second, we **postulate** that **PUMA's apoptotic function is associated with breast cancer response to HER2- and EGFR-targeted therapies and that restoring PUMA-mediated intrinsic apoptosis will sensitize breast cancer to the therapies.** This is founded on the following rationales: **(i)** PUMA's apoptotic function is compromised by HER2 and EGFR. **(ii)** Ectopic PUMA expression increases apoptotic response in breast cancer cells. **(iii)** The BH3 mimetic ABT-263 that mimics PUMA's apoptotic activity sensitizes breast cancer cells to the Iressa and lapatinib. We will test this hypothesis by the studies proposed in Specific Aim 3.

To test the afore-mentioned hypothesis, we will conduct three Specific Aims:

- 1)** Characterize the HER2-PUMA and EGFR-PUMA crosstalks in breast cancer cells.
- 2)** Investigate the biological consequence(s) of the phosphorylation of PUMA by HER2 and EGFR in breast cancer.
- 3)** Determine the extent to which PUMA's apoptotic function is associated with breast cancer response to HER2- and EGFR-targeted therapies.

Successful accomplishment of these aims could lead to a greater understanding of the malignant biology and the drug-resistant phenotype of nearly half of the invasive breast carcinomas with HER2 and/or EGFR overexpression which makes them more aggressive. The outcome could also provide a rationale to restore PUMA's apoptotic function as a novel strategy that sensitizes aggressive breast cancer to HER2- and EGFR-targeted therapies.

BODY

PUMA is primarily localized in the cytoplasm of HER2- and EGFR-overexpressing breast cancer cells, where PUMA is dysfunctional (Task 1-a). To help determine the extent to which HER2 and EGFR modulate PUMA subcellular locations in breast cancer cells, we first analyzed a panel of human breast cancer cell lines for expression levels of all three proteins. As shown in Figure 1, the majority of breast cancer cell lines analyzed expressed PUMA and some of them co-expressed PUMA and HER2/EGFR. Next, we selected a HER2-overexpressing and an EGFR-overexpressing cell lines, fractionated the cells into mitochondrial and non-mitochondrial fractions, extracted lysates from each fraction, and determined PUMA expression levels using western blot analysis. As shown in Figure 2, in both cell lines PUMA was primarily localized in the non-mitochondrial extracts (NME), but to a lesser degree in the mitochondrial extracts (ME). Mitochondrial fractionation was effective as indicated by the lack of COX IV expression in the NME and the absence of β -actin expression in the ME. These results indicated that PUMA is primarily localized in the cytoplasm of HER2- and EGFR-overexpressing breast cancer cells, where PUMA is not functional.

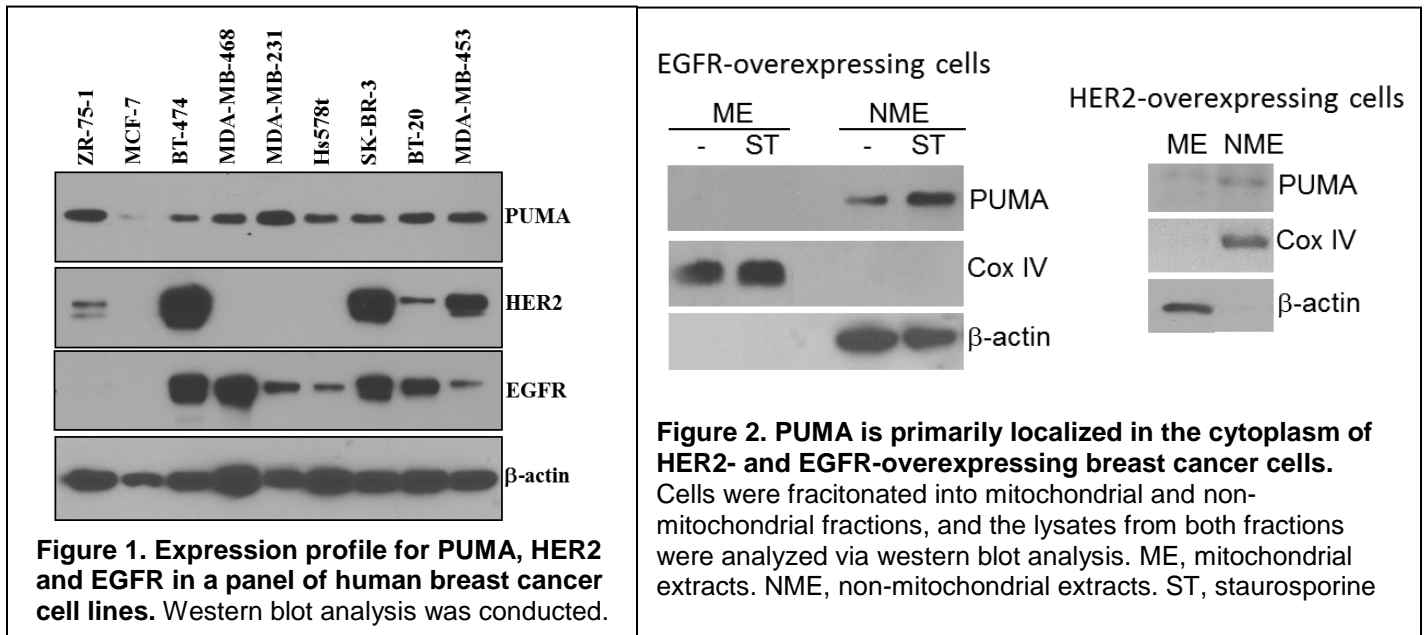


Figure 2. PUMA is primarily localized in the cytoplasm of HER2- and EGFR-overexpressing breast cancer cells. Cells were fractionated into mitochondrial and non-mitochondrial fractions, and the lysates from both fractions were analyzed via western blot analysis. ME, mitochondrial extracts. NME, non-mitochondrial extracts. ST, staurosporine

PUMA knockdown increased EGFR expression (Task 1-b). To examine the effects of PUMA on EGFR, we knocked down PUMA expression using siRNA, treated the cells with and without EGF for 20 minutes, and then determined EGFR levels and activation status using western blot analysis. As shown in Figure 3, we found the PUMA siRNA to be effective in reducing PUMA expression while the non-specific (NS) siRNA served as negative controls. Interestingly, our results showed that PUMA downregulation led to increased expression of EGFR, independent of EGF stimulation. Consistent with the increase in EGFR, we observed a higher level of activated EGFR (p-EGFR) in EGF-treated cells with PUMA siRNA compared to those with NS siRNA. This potentially important observation will be further validated using additional breast cancer cell lines with EGFR and HER2 overexpression.

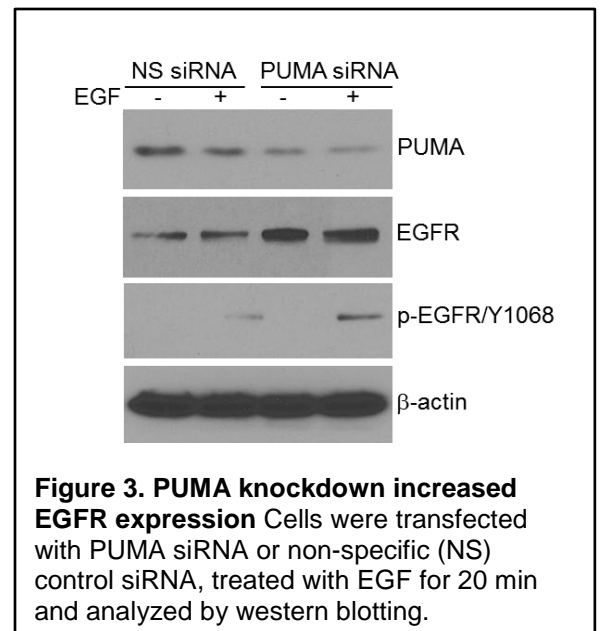
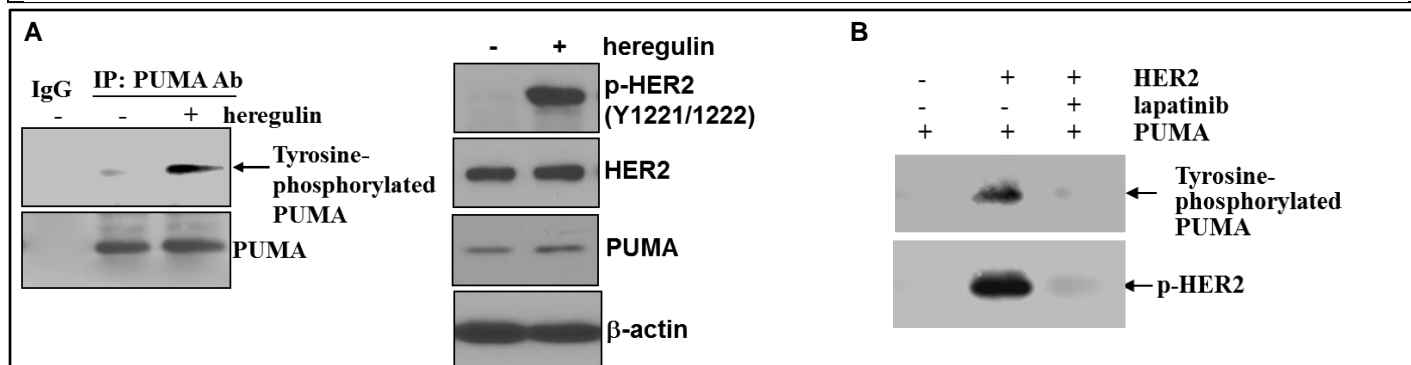
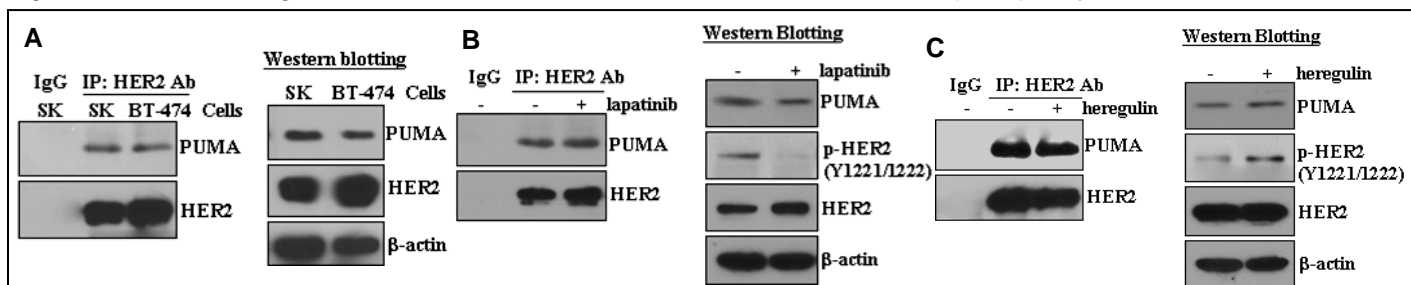


Figure 3. PUMA knockdown increased EGFR expression Cells were transfected with PUMA siRNA or non-specific (NS) control siRNA, treated with EGF for 20 min and analyzed by western blotting.

HER2 interacts with and phosphorylates PUMA in breast cancer cells (Task 2-a).

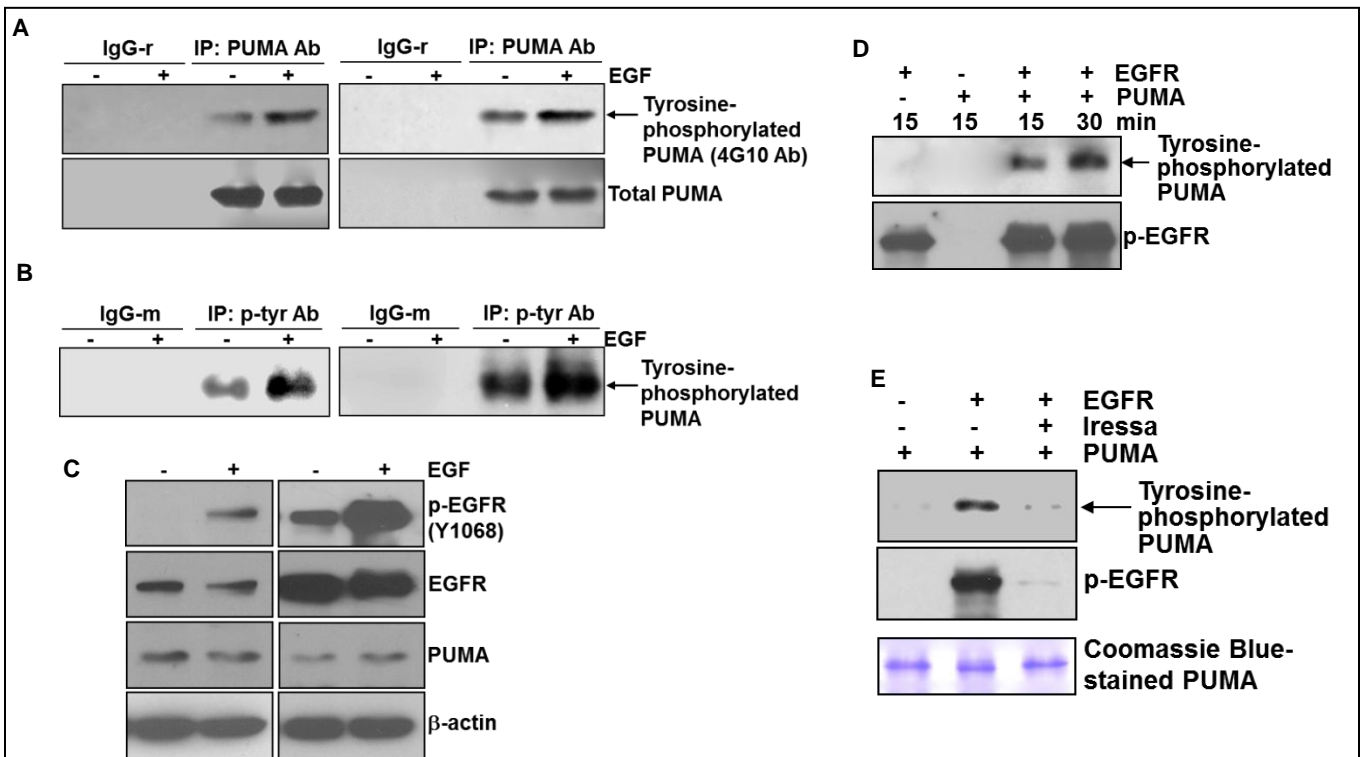
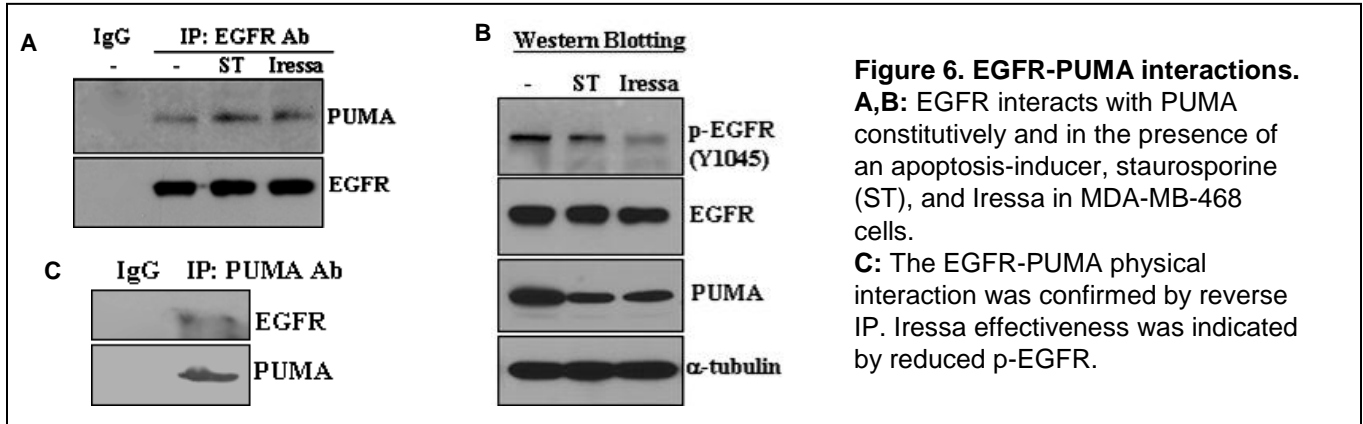
Using immunoprecipitation/western blotting (IP/WB) and HER2-overexpressing breast cancer cells, we found HER2 to interact with PUMA constitutively (Figure 4A). The HER2-PUMA interaction was sustained when breast cancer cells were treated with lapatinib, a dual HER2/EGFR kinase inhibitor that has effectively inhibited HER2 phosphorylation (Figure 4B). In line with the results of lapatinib, the HER2-PUMA interaction is independent of heregulin-induced receptor activation (Figure 4C). These results indicate that HER2 interacts with PUMA constitutively in a kinase-independent fashion.

Furthermore, we found that PUMA was tyrosine-phosphorylated in heregulin-stimulated HER2-overexpressing MDA-MB-453 cells (Figure 5A). We further confirmed this results using cell-free kinase assays in which the reactions contained HER2 (recombinant C-terminal HER2 expressed in Sf9 insect cells; Promega) and pre-dephosphorylated PUMA (from HEK293 cells infected with a PUMA viral vector; OriGene). Reactions were subjected to WB for tyrosine-phosphorylated PUMA using an anti-phosphotyrosine Ab (4G10; Upstate). Importantly, results of the kinase assay (Figure 5B) show that HER2 phosphorylated PUMA at the tyrosine residue(s) and the phosphorylation was inhibited by lapatinib. Together, results in Figures 4 and 5 indicate that HER2 interacts with and phosphorylates PUMA.



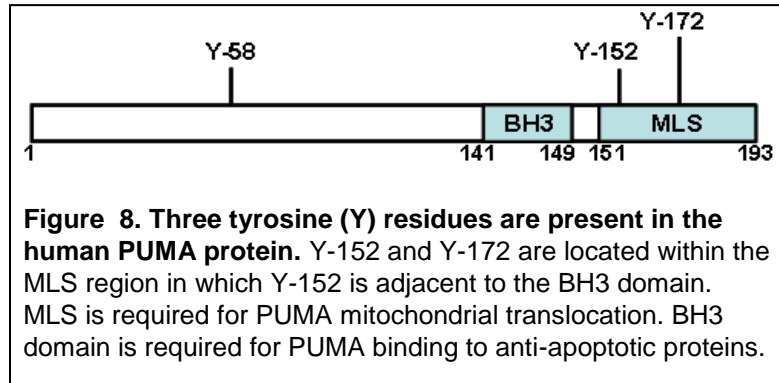
EGFR interacts with and phosphorylates PUMA in breast cancer cells (Task 2-a).

As shown by IP/WB in Figure 6, EGFR interacts with PUMA constitutively and under the treatments with an apoptosis-inducer, staurosporine (ST), and the EGFR kinase inhibitor, Iressa, in MDA-MB-468 cells with EGFR gene amplification. In the intracellular analyses, serum-starved breast cancer cells were stimulated with and without EGF for 10 minutes. Total proteins were subjected to IP to pull down PUMA followed by WB to detect tyrosine-phosphorylated PUMA. As shown in Figure 7A-C, PUMA was tyrosine-phosphorylated in two EGFR-overexpressing cancer cells line and the phosphorylation was enhanced by EGF. In Figure 7D-E, we used the cell-free EGFR kinase assay to further show that recombinant PUMA was phosphorylated by EGFR and the phosphorylation was inhibited by the EGFR kinase inhibitor Iressa. Collectively, results in Figures 6 and 7 indicate that PUMA is phosphorylated by EGFR.



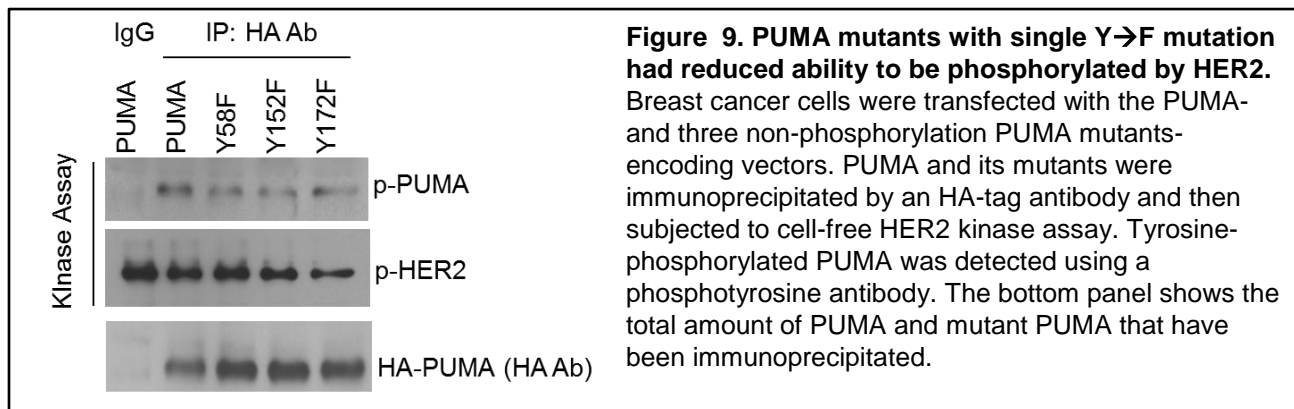
Creation of three non-phosphorylation PUMA mutants with single Y->F mutation (Task 2-b).

There are three tyrosine residues within human PUMA protein, namely, Y-58, Y-152 and Y-172 (Figure 8). Notably, Y-152 and Y-172 are within the mitochondrial localization signal (MLS; required for PUMA mitochondrial entry). Y-152 is adjacent to the BH3 domain (required for PUMA binding to anti-apoptotic proteins). To determine which tyrosine residue(s) is phosphorylated by EGFR and/or HER2, we conducted site-directed mutagenesis to create non-phosphorylation PUMA mutants, PUMA^{Y58F}, PUMA^{Y152F} and PUMA^{Y172F}, each containing a tyrosine (Y) to phenylalanine (F) mutation. Mutant PUMA expression vectors were made by PCR-mutagenesis using the pHA-PUMA vector as the PCR template plasmid and DNA sequences confirmed.



PUMA is phosphorylated by HER2 at all three tyrosine residues (Task 2-b).

To determine the ability of the PUMA single mutants to undergo HER2/EGFR-dependent phosphorylation, we have transfected breast cancer cells with the PUMA- and non-phosphorylation PUMA mutants-encoding vectors. PUMA and its mutants were immunoprecipitated by an HA-tag antibody and then subjected to the cell-free HER2 kinase assay. As shown in Figure 9, our results indicated that all three single Y→F mutants had reduced phosphorylation compared to wild-type PUMA. The fact that each of the three single mutants retained phosphorylation, albeit at reduced levels, indicated that more than one tyrosine residue are targets of phosphorylation. Based on this result, we have created a PUMA triple mutant in order to determine the effects of HER2 phosphorylation on PUMA properties. We are also analyzing the three single non-phosphorylation PUMA mutants for EGFR phosphorylation.



KEY RESEARCH ACCOMPLISHMENTS

- Task 1-a: PUMA is primarily localized in the cytoplasm of HER2- and EGFR-overexpressing breast cancer cells, suggesting that PUMA is unable to enter the mitochondria to induce apoptosis in breast cancer cells with high levels of EGFR and/or HER2.
- Task 1-b: PUMA knockdown increased EGFR expression.
- Task 2-a: HER2 interacts with and phosphorylates PUMA in breast cancer cells.
- Task 2-a: EGFR interacts with and phosphorylates PUMA in breast cancer cells
- Task 2-b: Creation of three non-phosphorylation PUMA mutants with single Y→F mutation.
- Task 2-b: All three PUMA single mutants had reduced phosphorylation by HER2, indicating PUMA is phosphorylated by HER2 at all three tyrosine residues. We have created a PUMA triple Y→F mutant to determine the effects of tyrosine phosphorylation on PUMA properties.

REPORTABLE OUTCOMES

Peer-reviewed publications:

Carpenter, RL. and Lo, H.-W. Hedgehog Pathway and GLI1 Isoforms in Human Cancer. (invited review) *Discovery Medicine* 13:105-113, 2012. (6)

Han, W. and Lo, H.-W. Landscape of EGFR Signaling Network in Human Cancers: Biology and Therapeutic Response in Relation to Receptor Subcellular Locations. (invited review) *Cancer Letters* 318:124-134, 2012. (7)

Han, W., Carpenter, RL., Cao, X. and Lo, H.-W. STAT1 gene expression is enhanced by nuclear EGFR and HER2 via cooperation with STAT3. *Molecular Carcinogenesis*. Published on-line 12 June 2012. (8)

CONCLUSION

Our research effort in the past award year has resulted in several interesting findings that support the study hypothesis: the EGFR-PUMA and HER2-PUMA signaling crosstalks modulate PUMA-mediated apoptotic pathway and cellular functions of EGFR and HER2, together contributing to the aggressive behavior of invasive breast cancer. First, we observed that PUMA was primarily localized in the cytoplasm of HER2- and EGFR-overexpressing breast cancer cells, suggesting that PUMA is unable to enter the mitochondria to induce apoptosis in breast cancer cells with high levels of EGFR and/or HER2. Second, in the course of understanding the effects of PUMA on EGFR, we found that PUMA knockdown increased EGFR expression. This interesting finding provides a rationale to further determine the impact of PUMA on EGFR and HER2 signaling. Third, we observed that both HER2 and EGFR interact with and phosphorylate PUMA in breast cancer cells. The interaction is constitutive independent of kinase activity while the interaction provides the opportunity for the receptors to phosphorylate PUMA. Finally, to gain insights into the consequences of PUMA tyrosine phosphorylation, we created three non-phosphorylation PUMA mutants, each with single Y→F mutation, and examined their ability to be phosphorylated by HER2. The results indicated that the single mutants had reduced phosphorylation, indicating that all three tyrosine residues within PUMA are targeted by HER2. This interesting finding has directed us to generate a PUMA mutant with triple Y→F mutations in order to determine HER2 effects on PUMA properties. We are also examining the PUMA single mutants for EGFR phosphorylation. In summary, we have made considerable progress in the past year towards the objectives of this Award. The afore-mentioned promising results have built a strong foundation for us to further explore the HER2-PUMA and EGFR-PUMA crosstalks in breast cancer cells in the next award year.

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