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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 translocalization 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.

15. SUBJECT TERMS

EGFR, HER2, PUMA, signal crosstalks

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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 aforementioned 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.

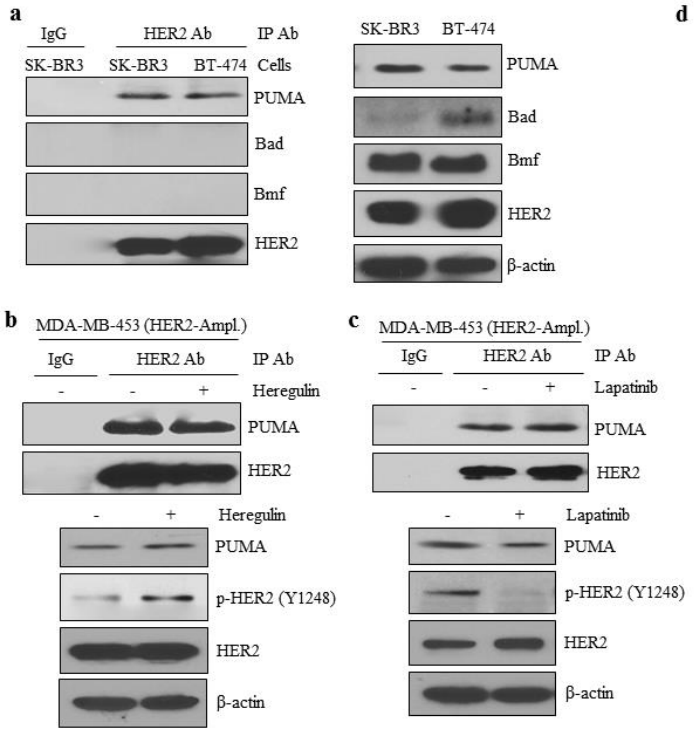
Successful accomplishment of these aims could lead to a greater understanding of the malignant biology and the drug-resistant phenotype of nearly half of the invasive breast carcinomas with HER2 and/or EGFR overexpression which makes them more aggressive. The outcome could also provide a rationale to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

BODY

HER2 physically associates with PUMA (Aim 1).

To investigate whether HER2 has any interplay with PUMA, we first assessed whether HER2 can physically interact with PUMA using immunoprecipitation/western blotting (IP/WB). We used SK-BR3 and BT-474 breast cancer cells as they overexpress HER2 due to HER2 gene amplification. We immunoprecipitated HER2 from these cells and found that PUMA could be detected with HER2 pull down in both cell lines (Figure 1a). This indicated a novel finding that HER2 physically interacts with PUMA. It is worth noting that we did not detect an interaction between HER2 and Bad or Bmf, other BH3-only proteins, suggesting the interaction with HER2 is specific to PUMA (**Figure 1a**). We next assessed whether HER2 kinase activity was required for the interaction with PUMA. For this, cells were treated with or without heregulin as a means of activating HER2 kinase activity. Of note, HER2 does not have obvious ligands and relies on binding to heregulin-bound HER3 for activation; HER3 does not have kinase activity (6). As shown in **Figure 1b**, HER2 was activated by heregulin but this did not significantly change the interaction of HER2 with PUMA. Cells were then treated with or without lapatinib, which inhibits HER2 activation (7). Lapatinib decreased HER2 activation but also did not significantly affect the interaction of HER2 and PUMA (**Figure 1c**). Collectively, these data indicate that HER2 can physically interact with PUMA and this interaction is not dependent on kinase activity of HER2.

PUMA primarily localizes to the mitochondria as it contains a mitochondrial localization signal (4, 8) but PUMA has also been observed to promote apoptosis without mitochondrial localization (9). In addition, HER2 is primarily localized to the plasma membrane but has recently been found to localize to the mitochondria where it influences cellular metabolism and promotes resistance of trastuzumab (10). Therefore, we next determined where PUMA and HER2 physically interact. Mitochondrial (ME) and non-mitochondrial extracts (NME) were isolated from BT-474 cells and immunoblotting for α-tubulin and COX IV confirmed there was effective isolation of mitochondrial and non-mitochondrial fractions (**Figure 1d**). Figure 1d shows that PUMA and HER2 were detected in both the ME and the NME confirming previous observations (10). Despite loading of equal amounts of protein (60 µg), there appeared to be greater PUMA and HER2 levels in the ME than the NME. However, this apparent imbalance is due to the fact that 60 µg is 80% of the ME harvested but only 2% of the NME harvested. To determine interaction between HER2 and PUMA, HER2 was immunoprecipitated from equal amounts of the ME and NME followed by immunoblotting. We observed interaction between HER2 and PUMA in both the ME and the NME (**Figure 1d**). These are the first data indicating HER2 physically interacts with PUMA and that this interaction occurs in and out of the mitochondrial compartment.



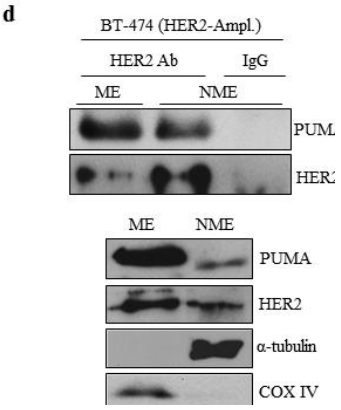


Figure 1. HER2 Directly Interacts with PUMA. a) SKBR3 and BT-474 cells were lysed and total protein subjected to immunoprecipitation with either control IgG or HER2 antibodies followed by immunoblotting with indicated antibodies. Whole cell lysates were also subjected to immunoblotting with indicated antibodies. **b)** MDA-MB-453 cells were incubated in serum-free medium for 16 hrs followed by treatment with heregulin (100 ng/mL) for 30 minutes. Cells were then lysed and total protein was subjected to immunoprecipitation with either control IgG or HER2 antibodies followed by immunoblotting with indicated antibodies. Whole cell lysates were also subjected to immunoblotting with indicated antibodies. **c)** MDA-MB-453 cells were incubated with lapatinib (10 µM) for two hrs. Cells were then lysed and total protein was subjected to immunoprecipitation with either control IgG or HER2 antibodies followed by immunoblotting with indicated antibodies. Whole cell lysates were also subjected to immunoblotting with indicated antibodies. **d)** The mitochondrial (ME) and non-mitochondrial extract (NME) were isolated from BT-474 cells and both extracts were subjected to immunoprecipitation and immunoblotting.

HER2 directly phosphorylates PUMA (Aim 2).

Following detection of a direct interaction between HER2 and PUMA, we next determined whether PUMA could be phosphorylated by HER2. To the best of our knowledge, PUMA tyrosine phosphorylation has not been previously reported. To first assess whether PUMA can be tyrosine phosphorylated intracellularly, we starved HER2-overexpressing cells for 16 hrs and then treated the cells with or without heregulin to activate HER2. We subjected the cell lysates to IP/WB using a PUMA antibody for IP and immunoblotted with anti-phospho-tyrosine antibodies. As shown in **Figure 2a**, tyrosine-phosphorylated PUMA was readily detected in heregulin-stimulated cells which expressed activated phosphorylated HER2 (p-HER2). However, MCF-7 cells, which express low levels of HER2, did not respond to heregulin and did not show significant PUMA tyrosine phosphorylation (**Figure 2b**). In contrast, MCF-7 cells with stable, forced HER2 overexpression (MCF-7/HER2 cells) shows PUMA tyrosine phosphorylation in response to heregulin (**Figure 2c**). These results are the first to show that PUMA can be phosphorylated on tyrosine residues and this occurred with HER2 stimulation by heregulin.

We next wanted to determine whether HER2 could directly phosphorylate PUMA. To this end, we used commercially available purified recombinant PUMA and HER2 proteins to perform a cell-free kinase assay. As shown in **Figure 2d**, PUMA was strongly phosphorylated at tyrosine residues in the presence of HER2. As expected, HER2 underwent auto-phosphorylation. In the presence of lapatinib, HER2 phosphorylation was lost, and consequently, there was no tyrosine phosphorylation of PUMA. We also observed a dose-response increase in tyrosine phosphorylation of PUMA with increasing levels of PUMA protein in the presence of HER2 (**Figure 2e**). Using IP-WB, we further show that pulldown of recombinant HER2 also results in pulldown of purified PUMA (**Figure 2f**) confirming HER2 directly associates with PUMA in the context of the cell-free kinase assay. These results show for the first time that PUMA can be phosphorylated at tyrosine residues directly by HER2.

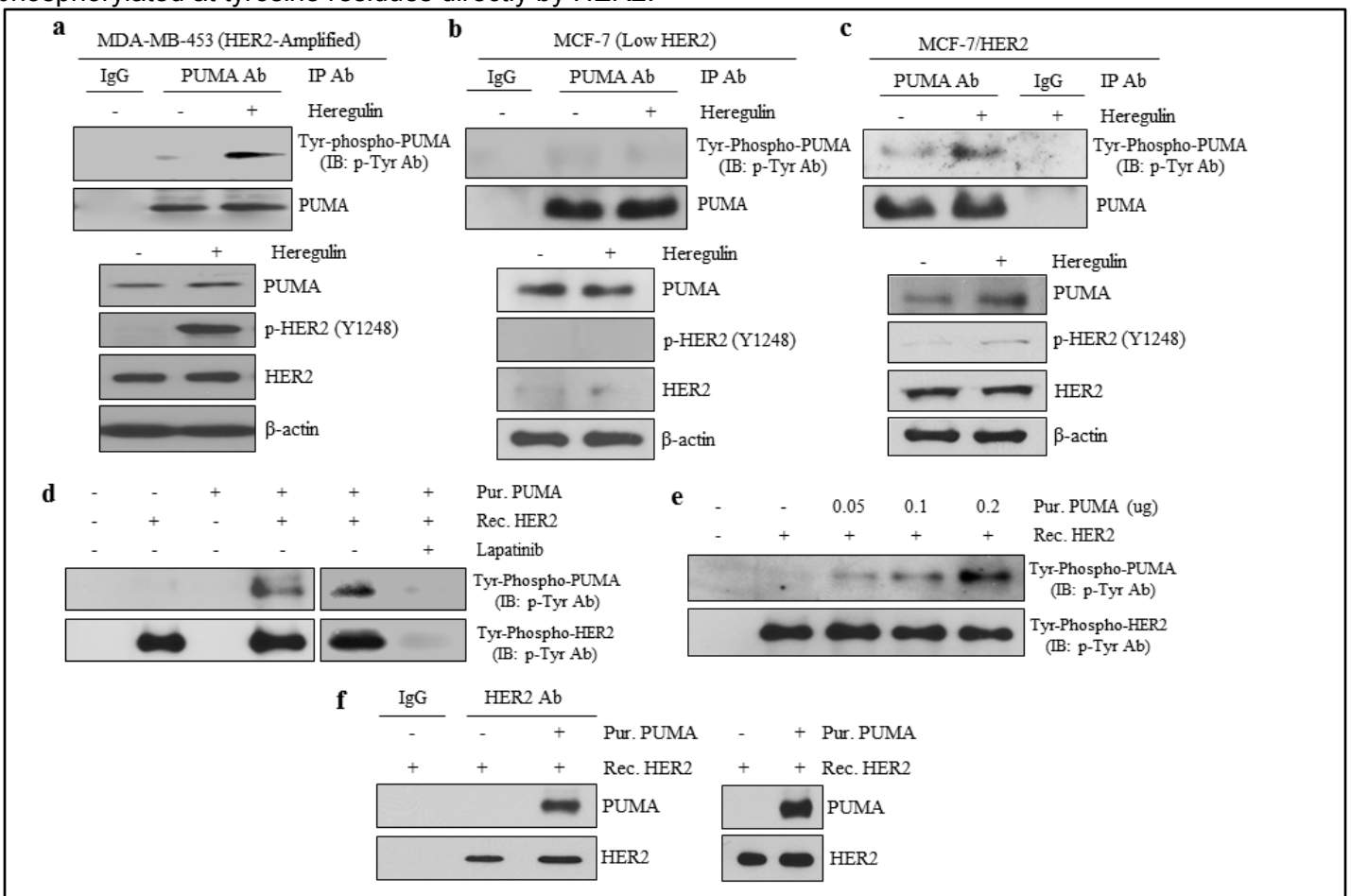


Figure 2. HER2 Directly Phosphorylates PUMA. MDA-MB-453 **(a),** or MCF-7 **(b),** or MCF-7/HER2 **(c)** cells were incubated in se free medium for 16 hrs followed by treatment with heregulin (100 ng/mL) for 30 min. Cells were then lysed and total protein subjected to immunoprecipitation with either control IgG or PUMA antibodies followed by immunoblotting with indicated antibo Whole cell lysates were also subjected to immunoblotting with indicated antibodies. Tyrosine-phosphorylated PUMA was detected 4G10 phospho-tyrosine antibodies. Recombinant PUMA protein was subjected to the HER2 kinase assay in the presence or absen lapatinib **(d)** or with increasing levels of recombinant PUMA protein **(e). f)** Recombinant HER2 was immunoprecipitated in the pres or absence of purified PUMA followed by immunoblotting with indicated antibodies.

HER2 phosphorylates PUMA at three tyrosine residues (Aim 2).

A search of the human PUMA protein sequence revealed the presence of three tyrosine residues, namely Y58, Y152, and Y172 (**Figure 3a**). All three tyrosine residues in PUMA were found to be conserved across multiple mammalian species (**Figure 3a**), indicating these residues are potentially functionally important. To determine which specific PUMA tyrosine residue(s) that HER2 phosphorylates, we conducted site-directed mutagenesis to mutate each tyrosine (Tyr; Y) to phenylalanine (Phe; F) using an expression vector carrying HA-tagged PUMA as the template. Phenylalanine has the same R group as tyrosine without the oxygen to bind phosphate and, thus, cannot be phosphorylated. These PUMA mutants (Y58F-, Y152F-, Y172F-PUMA), along with wild-type PUMA (WT-PUMA), were expressed in cells, immunoprecipitated using an HA-tag antibody, and subjected to the HER2 kinase assay. As shown in **Figure 3b**, WT-PUMA was strongly phosphorylated by recombinant HER2 while all of the mutants showed a low level of phosphorylation, indicating that all three tyrosines can be phosphorylated. To fully understand the biological consequences of PUMA tyrosine phosphorylation we created an additional PUMA mutant, a triple mutant PUMA (TM-PUMA), in which all three tyrosines (Y58, Y152, and Y172) were mutated to phenylalanine. Using the cell-free HER2 kinase assay (**Figure 3b**), WT-PUMA showed phospho-tyrosine bands whereas none were detected with TM-PUMA, indicating the TM-PUMA is not phosphorylated by HER2. To rule out the possibility that TM-PUMA cannot be tyrosine-phosphorylated due to its inability to interact with HER2, we next determined whether TM-PUMA can physically interact with HER2. IP/WB with a HER2 antibody (**Figure 3c**) demonstrated that HER2 interacted with both WT-PUMA and TM-PUMA equally indicating the lack of TM-PUMA phosphorylation by HER2 is not due to decreased interaction between the two proteins. Taken together, the results in Figures 2 and 3 are the first evidence showing that PUMA undergoes tyrosine phosphorylation and that HER2 can directly phosphorylate PUMA.

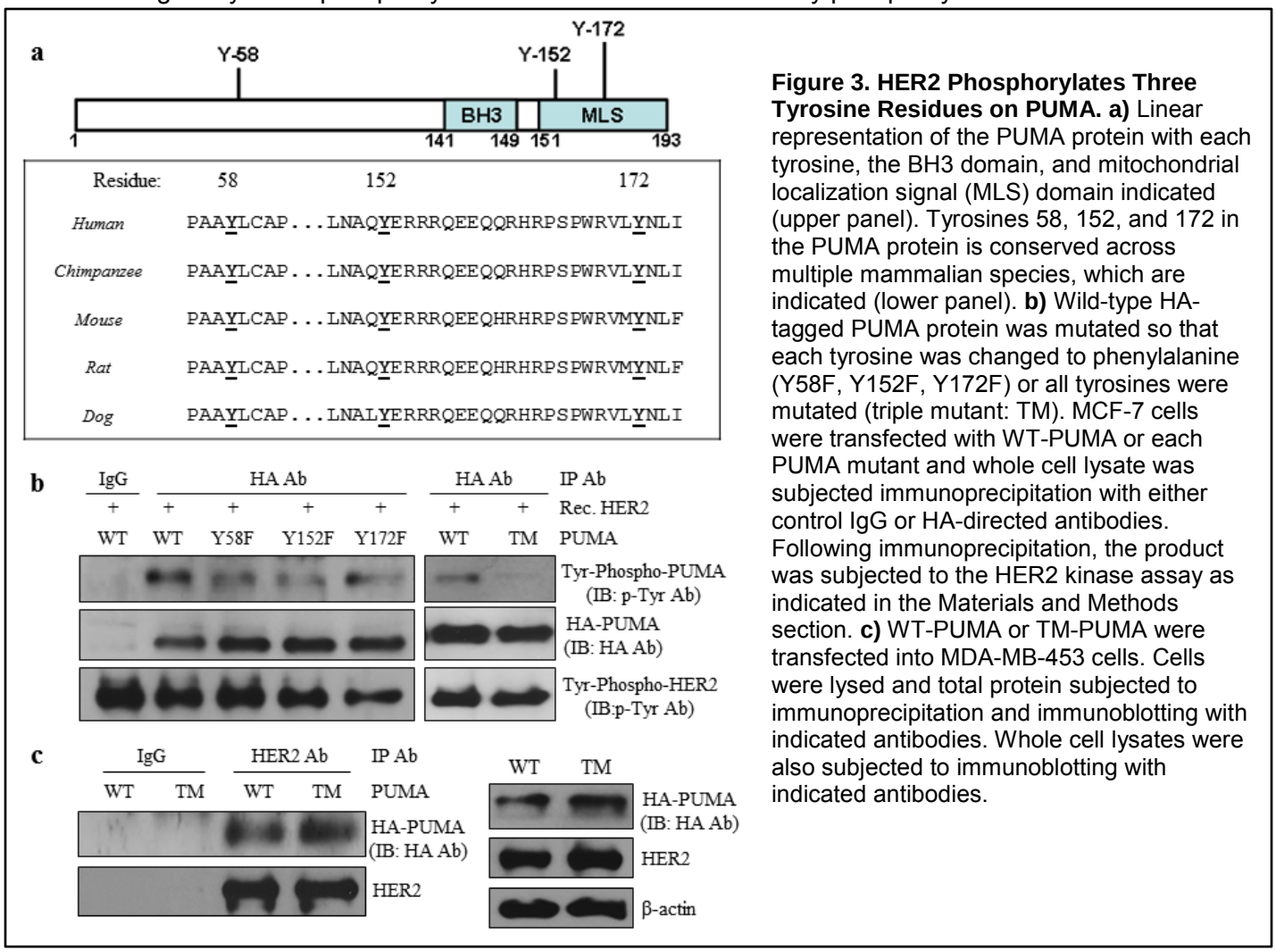


Figure 3. HER2 Phosphorylates Three Tyrosine Residues on PUMA. a) Linear representation of the PUMA protein with each tyrosine, the BH3 domain, and mitochondrial localization signal (MLS) domain indicated (upper panel). Tyrosines 58, 152, and 172 in the PUMA protein is conserved across multiple mammalian species, which are indicated (lower panel). **b)** Wild-type HA-tagged PUMA protein was mutated so that each tyrosine was changed to phenylalanine (Y58F, Y152F, Y172F) or all tyrosines were mutated (triple mutant: TM). MCF-7 cells were transfected with WT-PUMA or each PUMA mutant and whole cell lysate was subjected immunoprecipitation with either control IgG or HA-directed antibodies. Following immunoprecipitation, the product was subjected to the HER2 kinase assay as indicated in the Materials and Methods section. **c)** WT-PUMA or TM-PUMA were transfected into MDA-MB-453 cells. Cells were lysed and total protein subjected to immunoprecipitation and immunoblotting with indicated antibodies. Whole cell lysates were also subjected to immunoblotting with indicated antibodies.

TM-PUMA has a longer half-life than WT-PUMA (Aim 2).

We next wanted to determine whether PUMA phosphorylation by HER2 altered PUMA stability. To this end, we assessed protein half-life using cycloheximide, which inhibits protein synthesis allowing detection of when proteins are degraded. Cycloheximide is a common method to determine protein stability as several relevant papers have used this method in recent years (11-14). Thus, HER2-overexpressing MDA-MB-453 cells were treated with cycloheximide for up to 16 hrs in the presence or absence of heregulin to activate HER2. As shown in **Figure 4a**, heregulin induced activation of HER2 in these cells and also led to enhanced PUMA protein degradation. To further examine the stability of PUMA, we assessed PUMA half-life using MCF-7 cells, which have low HER2 expression, or MCF-7/HER2 cells, which have stable overexpression of HER2. **Figure 4b** shows that PUMA is degraded faster in MCF-7/HER2 cells compared to MCF-7 cells indicating HER2 overexpression reduces PUMA stability. We next determined whether the half-life of TM-PUMA, which cannot be tyrosine phosphorylated, differs from that of WT-PUMA. Cells were transfected with either WT-PUMA or TM-PUMA followed by cycloheximide treatment. As shown in **Figure 4c**, WT-PUMA levels significantly decreased at 16 hrs whereas TM-PUMA levels did not substantially decline. Following quantification of PUMA band signals and plotting them over time, we found that the half-life for WT-PUMA was approximately 7 hrs whereas that of TM-PUMA was longer than 16 hrs. It has been previously shown that PUMA can be targeted to the proteasome for degradation (11). To determine if WT-PUMA or TM-PUMA is regulated by the proteasome, we performed the half-life experiment in the presence of the proteasome inhibitor MG132. As shown in **Figure 4d**, we observed that WT-PUMA half-life could be extended with inhibition of the proteasome confirming previous results (11). These results suggest HER2-mediated phosphorylation reduces the half-life of PUMA.

We next asked whether TM-PUMA retains the ability to undergo translocalization to the mitochondria where PUMA promotes apoptosis. Thus, WT-PUMA or TM-PUMA were transfected into cells followed by isolation of the ME and NME with subsequent immunoblotting. As **Figure 4e** indicates, TM-PUMA retained the ability to undergo mitochondrial localization. Furthermore, we observed greater levels of TM-PUMA compared to WT-PUMA in the ME, which was confirmed by calculation of the mtPUMA Index resulting in 3.3 times more TM-PUMA in the mitochondria than WT-PUMA. A greater TM-PUMA level in the mitochondria is likely the result of enhanced protein stability of TM-PUMA protein in the presence of HER2. Together, these data show that PUMA protein stability is decreased with HER2 activation and blocking PUMA tyrosine phosphorylation enhances PUMA stability and results in greater mitochondrial levels of PUMA.

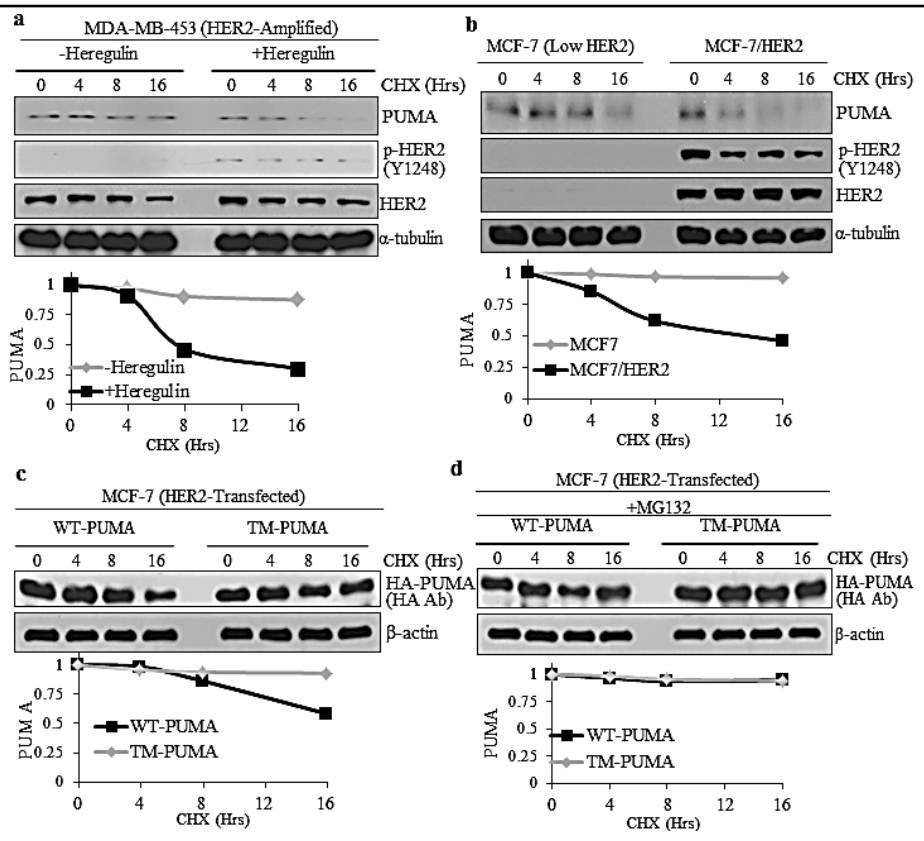
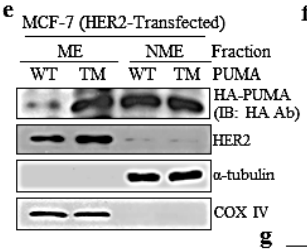


Figure 4. HER2 phosphorylation regulates PUMA half-Life and mitochondrial Levels. **a)** MDA-MB-453 cells were incubated in serum-free medium for 16 hrs followed by treatment with heregulin (100 ng/mL) and cycloheximide (10 ug/mL). Whole cell lysate was subjected to immunoblotting with indicated antibodies. **b)** MCF-7 and MCF-7/HER2 cells were incubated in serum-free medium for 16 hrs followed by treatment with heregulin (100 ng/mL) and cycloheximide (10 ug/mL). Whole cell lysate was subjected to immunoblotting with indicated antibodies. **c,d)** MCF-7 cells were transfected with HER2 and either WT-PUMA or TM-PUMA. Cells were incubated in serum-free medium for 16 hrs followed by treatment with heregulin (100 ng/mL) and cycloheximide (10 ug/mL) without **(c)** or with MG132 (10 μM) co-treatment **(d).** Whole cell lysate was subjected to immunoblotting with indicated antibodies. **e)** MCF-7 cells were transfected with WT-PUMA or TM-PUMA and mitochondria were isolated. ME and NME were subjected to immunoblotting with indicated antibodies. **f)** Chi-square tables for analysis of clinical cancer samples. Med-High HER2=2-3+. Low HER2=0-1+. g) Sample IHC images of clinical cancer samples for High HER2 + Low PUMA (case 1) and Low HER2 + High PUMA (case 2).

To assess whether this relationship is maintained *in vivo*, we performed immuno-histochemistry on a set of clinical cancer samples (n=93) to detect HER2 and PUMA. After scoring, we divided the samples into low HER2 (0-1+ intensity) or medium to high HER2 (2-3+ intensity). PUMA was divided into high PUMA (either ≥150 H-Score or ≥100 H-Score) or low PUMA (either <150 H-Score or <100 H-Score). We then performed a chi-square analysis to determine the relationship between HER2 and PUMA expression (**Figure 4f**). The chi-square analysis using either PUMA barrier (150 H-Score or 100 H-Score) resulted in statistical significance (p=0.045 and p=0.027, respectively). These data suggest the tissues with high HER2 expression tend to have lower PUMA expression *in vivo* (**Figure 4g**) supporting our data from cell lines that HER2 can downregulate PUMA expression.



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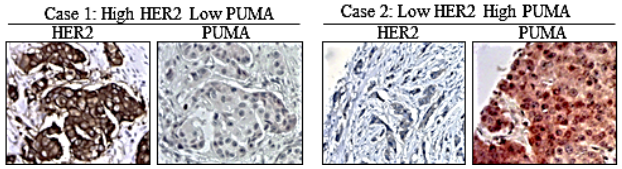
High PUMA ≥ 150 H-Score
Low PUMA < 150 H-Score

	Med-High HER2	Low HER2
High PUMA	20*	7
Low PUMA	34	32
n=93	$\chi 2$=4.00	p=0.045

High PUMA ≥ 100 H-Score
Low PUMA < 100 H-Score

	Med-High HER2	Low HER2
High PUMA	26	10
Low PUMA	28	29
n=93	$\chi 2$=4.84	p=0.027

* Number of tumors



TM-PUMA has a stronger effect than WT-PUMA on suppressing clonogenic growth (Aim 2).

Figure 4 indicated that TM-PUMA had greater protein stability and greater protein levels in the mitochondria, which may indicate that TM-PUMA has an enhanced ability to promote apoptosis. To examine the effect of TM-PUMA on cell viability, we expressed an empty vector, WT-PUMA or TM-PUMA in two different HER2-overexpressing breast cancer cell lines, namely BT-474 (**Figure 5a,c,e**) and MDA-MB-453 (**Figure 5b,d,f**) cells, and monitored the ability of these cells to form colonies. As shown by the anchorage-dependent colony assay (**Figures 5a and 5b**), TM-PUMA significantly decreased colony formation compared to WT-PUMA, indicating that TM-PUMA had a stronger growth suppression than WT-PUMA. As expected, compared to the empty vector, WT-PUMA had a stronger propensity to decrease the colony formation ability of both cell lines. Both of these cells are aggressive and will grow independent of attachment. Therefore, a similar experiment was also performed with the same cell lines but using an anchorage-independent soft agarose colony assay. TM-PUMA significantly reduced soft agarose colony formation compared to WT-PUMA (**Figures 5c and 5d**). WT-PUMA also reduced colony formation compared to vector in both cell lines. Together, these data demonstrate that TM-PUMA has a greater effect than WT-PUMA on decreasing clonogenic growth of breast cancer cells and suggests tyrosine phosphorylation of PUMA decreases the ability of PUMA to suppress cell growth.

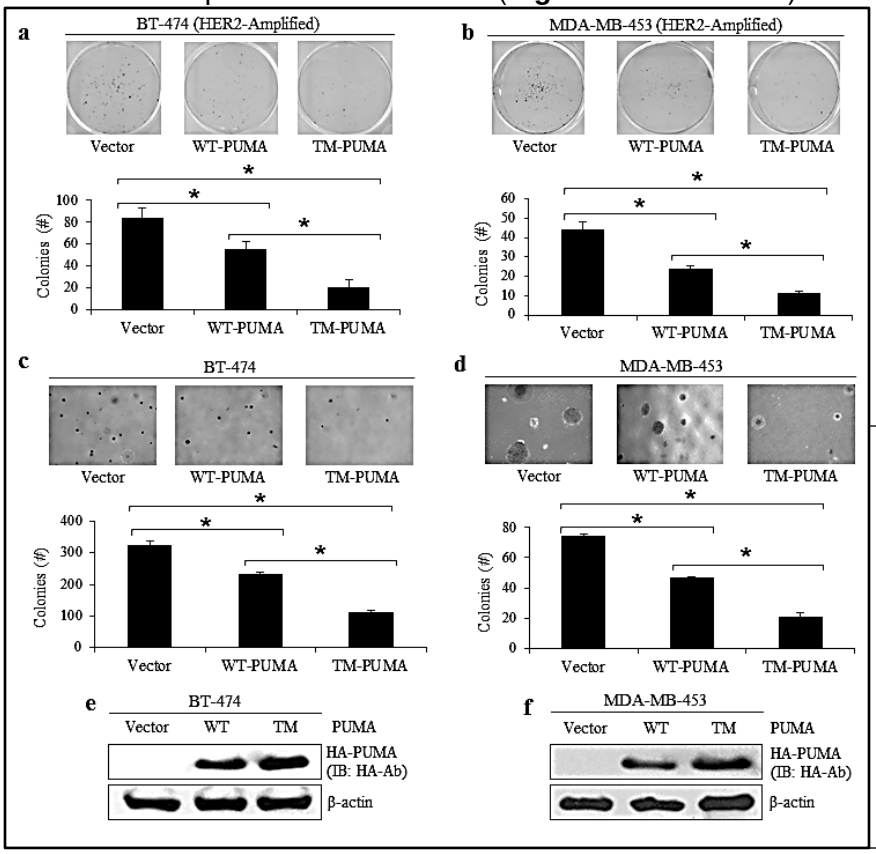


Figure 5. TM-PUMA decreases clonogenic Growth in HER2 overexpressing Cells. BT-474 cells **(a, c, e)** and MDA-MB-453 cells **(b, d, f)** were transfected with an empty vector, WT-PUMA, or TM-PUMA for 48 hrs (e, f). Transfected cells were then seeded into 6-well plates either without (a and b) or with soft agarose (c and d) and incubated at 37°C for 14-21 days. Colonies were then counted, stained with crystal violet blue, and images were taken.

TM-PUMA induces apoptosis to a greater degree than WT-PUMA (Aim 2).

We observed that TM-PUMA has a greater effect on cell growth than WT-PUMA in the context of HER2 overexpressing cells. However, whether this decrease in cell growth with TM-PUMA was due to enhanced apoptosis cannot be determined from analysis of the colony assays. To determine the effect of TM-PUMA on apoptosis, BT-474 cells were transfected with an empty vector, WT-PUMA, or TM-PUMA followed by treatment with heregulin to ensure HER2 activation. We then assessed the extent of apoptosis in the treated cells by the Annexin V binding assay using flow cytometry. **Figures 6a and 6c** show that TM-PUMA induced the greatest levels of apoptosis compared to WT-PUMA or empty vector. PUMA has been shown previously to sensitize cancer cells to treatment with apoptosis-inducing chemotherapeutic agents (15). Therefore, we next assessed whether TM-PUMA could further enhance apoptosis in the presence of a low dose of anisomycin, an apoptosis inducer (16). To this end, cancer cells were transfected with vector, WT-PUMA, or TM-PUMA followed by exposure to heregulin and anisomycin with subsequent assessment of Annexin V binding. As shown in **Figures 6b and 6c**, TM-PUMA expression significantly promoted apoptosis in the presence of anisomycin compared to vector and WT-PUMA. As expected, we observed modest increases in apoptosis in anisomycin-treated cells expressing vector or WT-PUMA compared to untreated cells.

To confirm the effects of WT-PUMA and TM-PUMA on apoptosis, cell lysates were analyzed by WB for the presence of PARP-1 cleavage. Consistent with the results of the Annexin V staining, the results revealed that TM-PUMA induced the greatest levels of cleaved PARP-1 (**Figure 6d**). Together, results presented in Figure 6 indicate TM-PUMA as a stronger apoptosis inducer than WT-PUMA and that tyrosine phosphorylation of PUMA reduces the ability of PUMA to promote apoptosis.

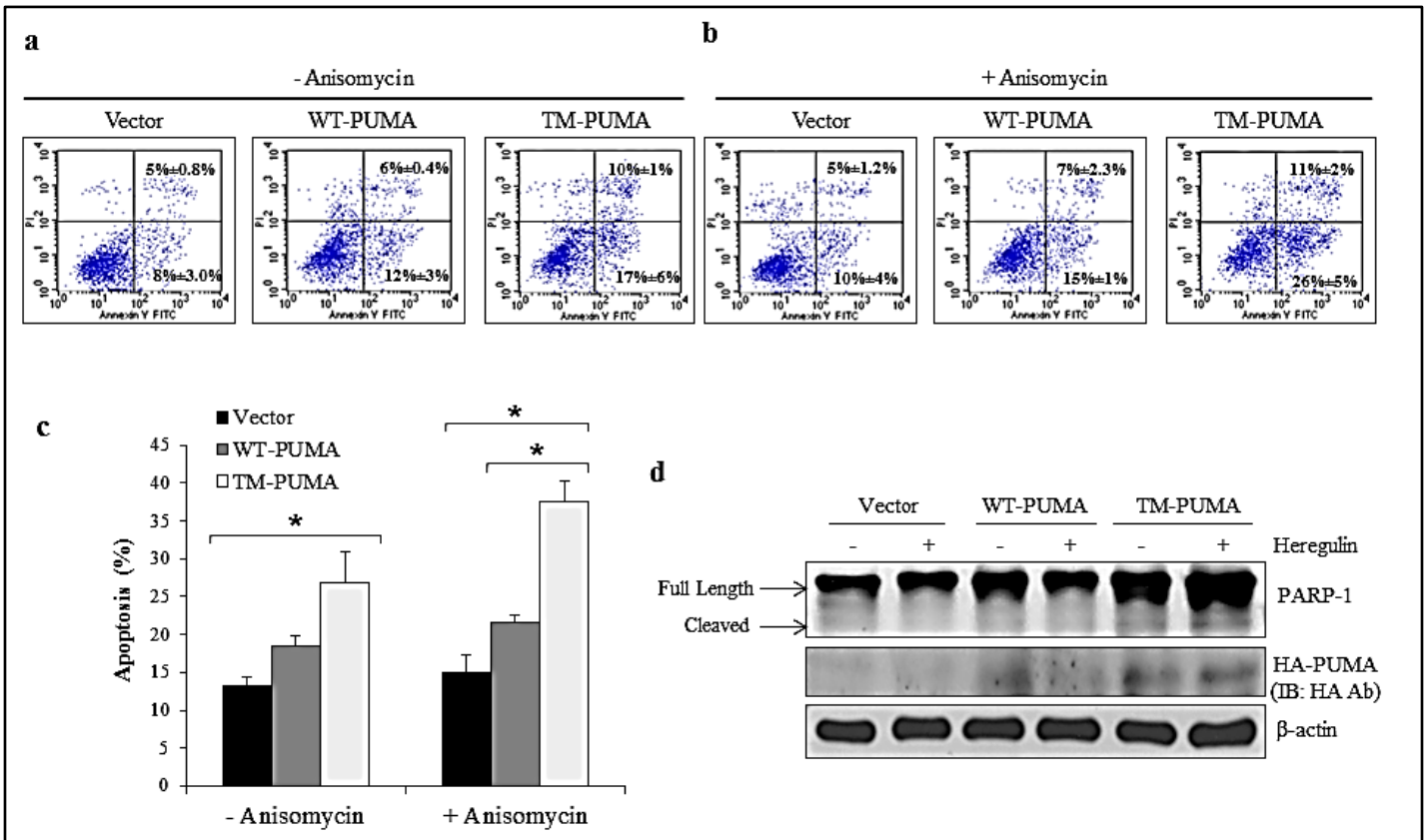


Figure 6. TM-PUMA Induces Apoptosis in HER2 Overexpressing Cells. a) and **b)** BT-474 cells were transfected with an empty vector, WT-PUMA, or TM-PUMA. Cells were treated with heregulin (100 ng/mL) and with or without anisomycin (25 ng/mL) for 16 hrs. Cells were detached and incubated with annexin-V-FITC and PI according to manufacturer's instructions followed by analysis by flow cytometry. **c)** Graph representing measurements of apoptosis from (a and b). **d)** BT-474 cells were transfected with an empty vector, WT-PUMA, or TM-PUMA. Cells were incubated in serum-free medium for 16 hrs followed by treatment with heregulin (100 ng/mL) for 4 hrs. Cells were then lysed and total protein was subjected to immunoblotting with indicated antibodies.

KEY RESEARCH ACCOMPLISHMENTS

- Aim 1:
  (a) PUMA is primarily localized in the cytoplasm of 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.
  (b) PUMA interacts with both HER2 and EGFR in breast cancer cells.
  (c) PUMA knockdown increased EGFR expression.
- AIM 2:
  (a) HER2 and EGFR phosphorylate PUMA in breast cancer cells.
  (b) HER2 phosphorylates PUMA at all three tyrosine residues.
  (c) TM-PUMA has a longer half-life than WT-PUMA.
  (d) TM-PUMA has a stronger effect than WT-PUMA on suppressing clonogenic growth.
  (e) TM-PUMA induces apoptosis to a greater degree than WT-PUMA.
- Aim 3:
  Ongoing

REPORTABLE OUTCOMES

Peer-reviewed publications:

1. 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. (17)

2. 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. PMID:22693070. Published online: 12 JUN 2012 | DOI: 10.1002/mc.21936. (18)

3. Lo, H.-W. Akt destabilizes p57Kip2: Akt at the converging crossroad? Invited News & Views. Cell Cycle 12(6):870-871, 2013. (19)

4. Carpenter, R. L and Lo, H.-W. Regulation of Apoptosis by HER2 in Breast Cancer. Invited review. Journal of Carcinogenesis & Mutagenesis 2013 (In Press). (20)

5. Carpenter, R. L, Han, W., Paw, I. and Lo, H.-W. HER2 phosphorylates and destabilizes proapoptotic PUMA, leading to antagonized apoptosis in cancer cells. PLOS ONE, 2013 (In Press). (21)

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 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. **Second,** 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. HER2 interacted with PUMA in both mitochondrial and non-mitochondrial compartments. **Third,** we found that activation of HER2 decreased PUMA protein half-life. **Fourth,** we generated three PUMA non-phosphorylation mutants each with a single Tyr→Phe substitution and the results indicated that each PUMA single mutant had lost some, but not all phosphorylation by HER2 indicating that HER2 targets all three tyrosines. Consequently, we created an additional PUMA mutant with all three tyrosines mutated (TM-PUMA) that could not be phosphorylated by HER2. Importantly, TM-PUMA was found to have a longer half-life than PUMA. **Fifth,** an inverse association was observed between HER2 and PUMA in 93 invasive breast carcinoma samples. **And finally,** we found that TM-PUMA suppressed

growth of breast cancer cells to a greater degree than PUMA. Also, TM-PUMA had a stronger propensity to induce apoptosis than PUMA. In summary, we have made considerate progress in the past year towards the objectives of this Award. The aforementioned 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 with a special focus to complete studies proposed in Specific Aim 3.

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