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14. ABSTRACT

Identification of drug targets with novel mechanism of actions as well as excellent safety profile is needed to improve the "chemotherapy-sparring" regimen of anti-HER2 drug combination (lapatinib (L) + trastuzumab (T) (L+T)), which is effective in a larger group of patients. Drugs targeting G protein-coupled receptors (GPCRs) have low toxicity because of their high specificity and target-selectivity. In a GPCR expression profiling study and subsequent *in vitro* studies, we have identified GPR110 as a potential candidate in HER2+ breast cancer. Our overall goals of the proposed research are to investigate the role of GPR110 in tumorigenicity and anti-HER2 drug resistance in HER2+ BC. In the first year of the funded study, we have shown that GPR110 overexpression occurs commonly in various anti-HER2 drug resistant cells and in tumorigenic population using a broad panel of cell line models. We have successfully generated inducible lentiviral plasmids with GPR110 cDNA and cell lines that inducibly overexpress GPR110. Generation of cell lines with lentiviral plasmids containing GPR110 shRNA is ongoing. Using GPR110-overexpression and siRNA-mediated knockdown strategies, we have also demonstrated that GPR110 may contribute to tumorigenicity in HER2+ breast cancer. Further, we have uncovered previously unanticipated role of GPR110 in cell adhesion, invasion, and migration, which may facilitate anti-HER2 drug resistance. In vitro and in vivo experiments proposed in the coming years will further establish a role of GPR110 in HER2+ breast cancer.

15. SUBJECT TERMS

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1. Introduction

HER2+ breast cancer is treated using the combination of chemotherapy and anti-HER2 drugs. However, chemotherapy is associated with life-threatening toxicities without significantly improving long-term survival. Recently, "chemotherapy-sparring" regimen consisting of only anti-HER2 drug combination (lapatinib (L) + trastuzumab (T) (L+T)) has shown efficacy, albeit in a small group of HER2+ breast cancer patients. Therefore, identification of drug targets with novel mechanism of actions as well as excellent safety profile is needed to further improve the "chemotherapy-sparring" regimen that is effective in a larger group of patients. Drugs targeting G protein-coupled receptors (GPCRs) have low toxicity because of their high specificity and target-selectivity. Indeed, 30-50% FDA-approved drugs target GPCRs or their pathways. In a GPCR expression profiling study and subsequent *in vitro* studies, we have identified GPR110 as a potential candidate in HER2+ breast cancer. In this research, we proposed to investigate the role of GPR110 in tumorigenicity and anti-HER2 drug resistance in HER2+ breast cancer. In this 1st annual report, we summarize our accomplishments to date and describe challenges and potential solutions to address these challenges.

2. Keywords

HER2+ Breast Cancer, Lapatinib, Trastuzumab, drug resistance, G protein-coupled receptors, GPR110, tumorigenicity

3. Accomplishments

What were the major goals of the project?

In the proposed research, we hypothesized that GPR110 overexpression activates HER signaling and other survival pathways, resulting in resistance to anti-HER therapy, and GPR110 knockdown can improve efficacy and delay resistance to anti-HER therapy. We also hypothesized that GPR110 is induced in patient tumors that do not respond to anti-HER therapy and that its expression correlates with a lack of response to anti-HER therapy in HER2+ breast cancer patients. Our specific aims were to (1) determine GPR110 expression and its effects on anti-HER therapy efficacy and resistance in preclinical HER2+ breast cancer models; (2) investigate HER-dependent and –independent signaling mechanisms by which GPR110 mediates tumorigenicity and anti-HER therapy resistance; and (3) investigate the predictive value of tumoral GPR110 expression in HER2+ breast cancer patients treated with neoadjuvant L+T regimen.

What was accomplished under these goals?

In the first 12 months we proposed accomplishments of the following major aims and tasks. A detailed summary of what was accomplished is also given below under each task.

<u>Aim 1: Determine GPR110 expression and its effects on anti-HER therapy efficacy and resistance in preclinical HER2+ breast cancer models</u>

<u>Major task 1.1</u>: Measure the expression of GPR110 in parental and anti-HER therapy resistant derivatives of 12 cell line models. 1-6 months

In our preliminary data, we had demonstrated that GPR110 was overexpressed in anti-HER therapy resistant derivatives of 2 HER2+ breast cancer cell line models. To understand whether GPR110 overexpression is a common phenomenon in anti-HER2 therapy resistance, we first interrogated the RNAseq data obtained in various anti-HER2 resistant derivatives in collaboration with Dr. Joe Gray at the Oregon Health and Science University (OHSU). These results showed that GPR110 expression was higher in at least one of the resistant models

(LR, TR, or LTR) in 4 out of 9 models of BT474, SKBR3, HCC1954, and HCC202 models (Figure 1). To confirm these results, we determined the mRNA expression of GPR110 using the Taqman RT-PCR assay in selected resistant models, prioritized based on the RNAseq data. We have found that GPR110 mRNA levels were significantly higher in LR, TR, and LTR derivatives of 3 models (BT474, SKBR3, and UACC812) compared to the parental cells (Figure 2A-C). In the AU565 and HCC202 models, GPR110 mRNA levels were significantly higher only in the LR and TR derivatives, but not in the LTR derivatives (Figure 2D-E). Whereas, in HCC1954, the mRNA expression of GPR110 was not significantly different in the LR, TR, or LTR derivatives compared to the parental cells (Figure 2F). We are in the process of validating these results at the protein level by performing immunohistochemistry using these anti-HER2 resistant models.

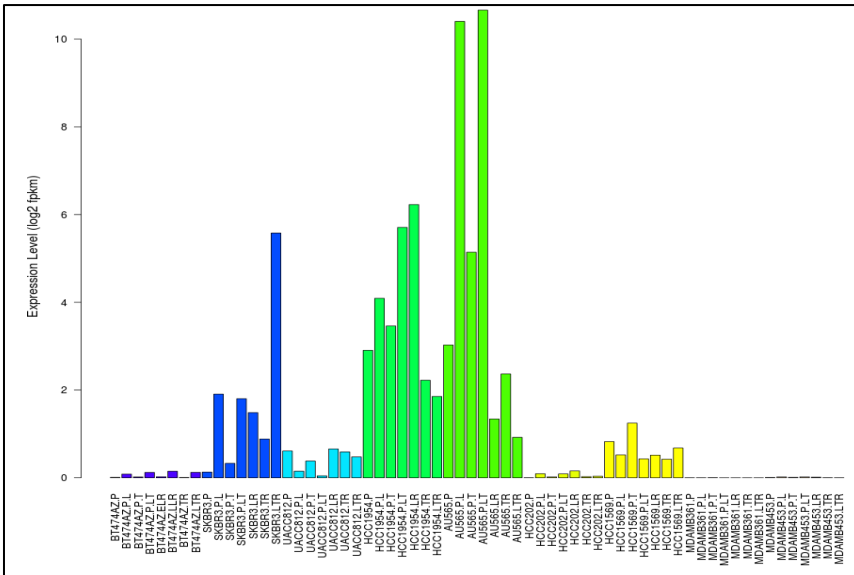

Figure 1. GPR110 mRNA expression analysis in anti-HER2 resistant derivatives vs. parental cells by RNAseq. A total of 9 anti-HER2 resistant models that included lapatinib (L)-resistant (LR), trastuzumab (T)-resistant (TR), and L+T-resistant (LTR) derivatives as well as the parental cells with short-term treatment with L (PL), T (PT), and L+T (PLT).

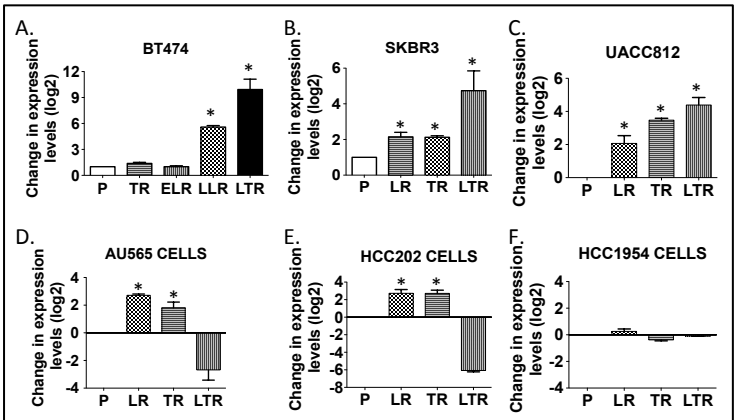

Figure 2. GPR110 mRNA expression analysis in anti-HER2 resistant derivatives vs. parental cells by quantitative RT-PCR using Taqman assay. qRT-PCR was conducted using the RNA isolated from parental, lapatinib (L)-resistant (LR) [early (ELR) or late (LLR) resistant if applicable], trastuzumab (T)-resistant (TR), L+T-resistant (LTR) derivatives of BT474 (A), SKBR3 (B), UACC812 (C), AU565 (D), HCC202 (E), and HCC1954 cell line models. * indicates $p<0.05$ (N=3-6).

Overall, our results suggest that GPR110 is overexpressed in multiple anti-HER resistant derivatives of various HER2+ breast cancer cell line models, confirming that it is a common phenomenon. These results are also in agreement with overexpression of GPR110 in various types of cancers and its possible role as a proto-oncogene (Lum et al., BMC Cancer, 2010; Harvey, Blood, 2010; Espinal-Enriquez et al., BMC Genomics, 2015; Hasan et al., Bioinformation, 2015). Based on the GPR110 expression, 2 cell line models (BT474 and SKBR3) have been selected for generation of stable cell lines capable of inducing GPR110 overexpression and knockdown as described below.

<u>Major task 1.2</u>: Generate stable parental and anti-HER therapy resistant cell lines capable of inducing GPR110 overexpression and knockdown. 3-12 months

Since GPR110 is an orphan GPCR, pharmacological tools to study its role are not available. Furthermore, the cellular function of GPR110 is still unknown. In order to understand the

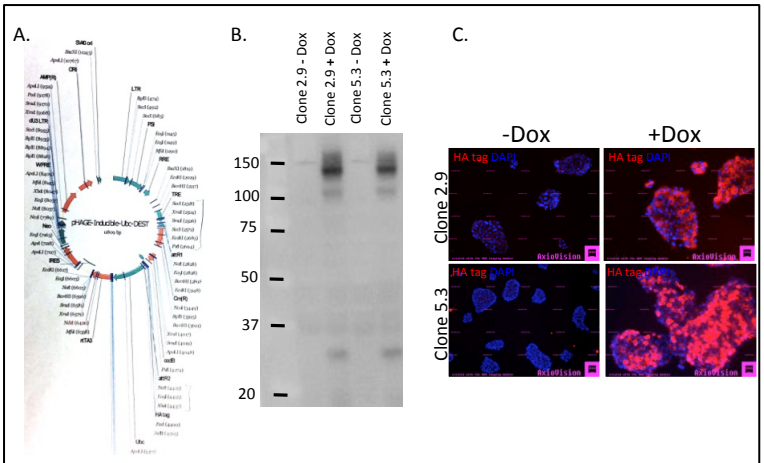

Figure 3. GPR110-overexpression using pHAGE lentiviral mediated infection of BT474 cells. A. Map of pHAGE lentiviral plasmid. B. Western blot analysis for the expression of GPR110 using anti-HA antibodies in clones 2.9 and 5.3 in in absence (-) or presence (+) of doxycycline (dox). C. Immunofluorescence staining for the expression of GPR110 using anti-HA antibodies in clones 2.9 and 5.3 in absence (-) or presence (+) of doxycycline (dox).

role of GPR110 in HER2+ breast cancer, we proposed to generate stable cell line models of inducible GPR110 overexpression and knockdown. We have been successful in generation of the lentiviral plasmids containing GPR110 cDNA using the pHAGE system, which includes the HA tag, under the control of inducible Tet-on promoter. The map of pHAGE lentiviral plasmid is shown in Figure 3A. Using this, the BT474 and SKBR3 parental cells were stably infected with the lentiviral plasmid and single cell cloning was performed. A single major band corresponding to the appropriate molecular weight (101kDA + 42kDA for HA tag = 143 kDA) was observed on the western blot. Two clones (clone 2.9 and clone 5.3) were selected for subsequent experiments based on the intermediate level of GPR110 overexpression in presence of doxycycline (+dox) by western blotting (Figure 3B). We have also validated the GPR110 overexpression in both these clones in +dox condition by immunofluorescence (IF) using anti-HA antibodies, which showed a strong cell surface signal (Figure 3C), corresponding with the plasma membrane expression of GPR110. No signal was observed in the dox-untreated (-dox) cells. GPR110-overexpressing SKBR3 cells have also been developed, and we are in the process of amplifying the single cell colonies.

For the construction of the shRNA GPR110 plasmid, the 4 independent shRNAs we purchased did not effectively knockdown GPR110 as assessed by qRT-PCR (Figure 4A) when transiently transfected in the SKBR3 LTR cells, which has a high GPR110 expression. We are using a new strategy for designing the GPR110 shRNA using the GPR110 siRNA sequences. These siRNAs have been validated by RT-PCR in significantly knocking down the expression of GPR110 in SKBR3 model (Figure 4B, 4C). We are in the process of constructing these inducible GPR110 shRNA plasmids, and will then carry out the infection to generate stable cell lines.

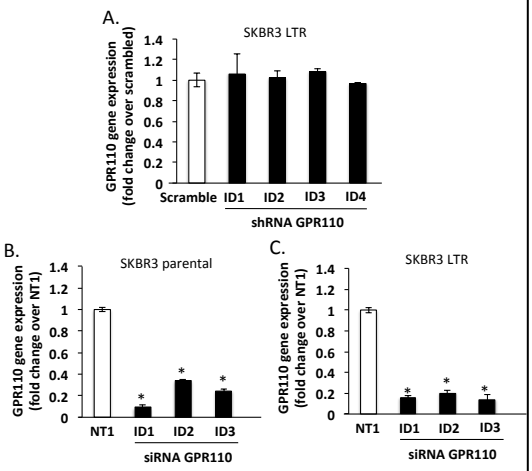

Figure 4. Level of GPR110 knockdown by shRNA or siRNA in SKBR3 parental and LTR cells. GPR110 mRNA expression by qRT-PCR after transient transfection with shRNA (A) or siRNA (B,C) targeting GPR110 in SKBR3 parental (B) or LTR derivatives (A,C). * indicates $p<0.05$ (N=3).

In this major task, we have generated stable cell lines with inducible GPR110 overexpression. The development of cell lines capable of inducible GPR110 knockdown is in progress using a revised strategy to ensure adequate GPR110 knockdown. The generation of these cell lines is critical in understanding the role of GPR110 in HER2+ breast cancer.

<u>Major task 1.3</u>: Determine the *in vitro* cell growth in response to L, T, and L+T when GPR110 gene is overexpressed and/or knocked down in parental and anti-HER therapy resistant derivatives of 2 cell line models. 9 to 18 months

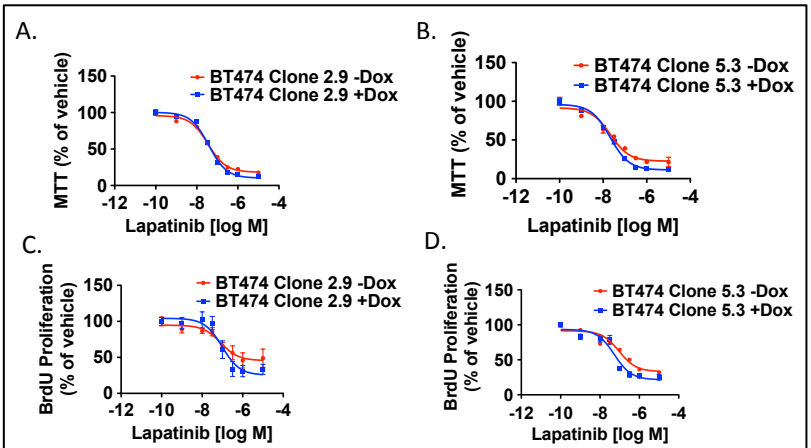

Figure 5. Effects of GPR110 overexpression on cell growth and proliferation. **A-B**. MTT assay and **C-D**. BrDU proliferation assay for clones 2.9 and 5.3 in absence (-) or presence (+) of doxycycline (dox) in presence of various lapatinib concentrations (N=3).

In order to understand the effect of GPR110 on the *in vitro* cell growth in response to various anti-HER drugs, we have initiated the testing in BT474 cell line with inducible GPR110 overexpression. In assessing the cell growth in 2-dimension (2D), we found that GPR110 overexpression in BT474 with +dox did not affect cell growth measured by MTT assay (Figure 5A-B) and cell proliferation as detected by BrDU incorporation assay (Figure 5C-D) at baseline or in presence of various lapatinib concentrations when compared to –dox. These data is consistent with our preliminary data using cells with stable overexpression of GPR110, suggesting that GPR110 may not have a role in 2-dimension (2D) cell growth or proliferation in HER2+ breast cancer.

To determine the effect of GPR110 overexpression on mammosphere-forming capability, BT474-GPR110 overexpressing cells were used for mammosphere assay. We found that both of the stably transfected clones had a significantly lower numbers of mammospheres on day 7 (Figure 6A,C) and after the first passage of the mammospheres (data not shown), but an increase in mammosphere diameter (Figure 6B,D). Additional analysis on day 3 has revealed that this may likely occur from aggregation of mammospheres, which was selectively present in GPR110-overexpressing cells with +dox, but not in –dox conditions (Figure 6). Because GPR110 belongs to an adhesion GPCR family, the role of GPR110 in cell-cell adhesion may be possible, which is currently under investigation. Interestingly, knockdown of GPR110 using 3 independent siRNAs selectively inhibited colony formation in the soft agar assay preferentially in the SKBR3 LTR

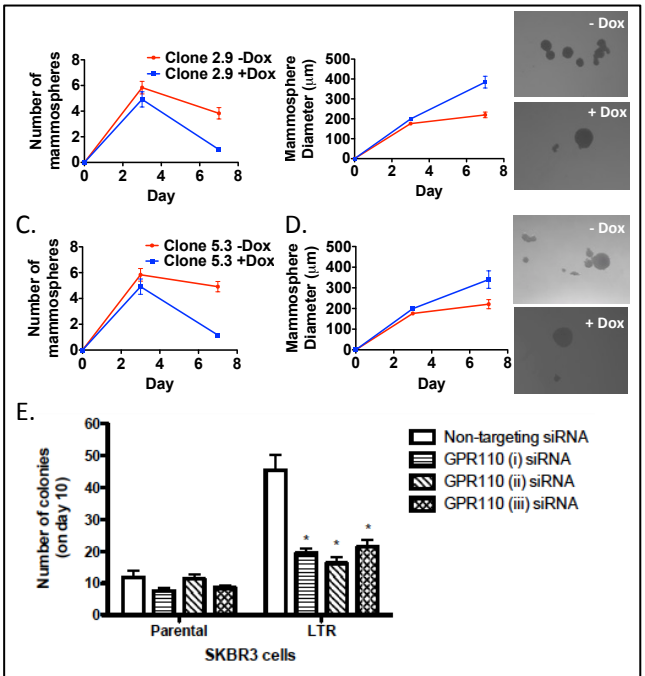

Figure 6. Effects of GPR110 on mammosphere formation. **A-D.** Mammosphere count (A,C) and diameter (B,D) of clones 2.9 (A,B) and 5.3 (C,D) in absence (-) or presence (+) of doxycycline (dox) (N=2). Representative figures are on the right. E. Number of colonies in the soft agar assay in SKBR3 parental and LTR cells transfected with non-targeting or GPR110 siRNAs (N=3).

derivatives but not in SKBR3 parental cells (Figure 6E), suggesting that GPR110 may indeed have a role in 3-dimension cell growth of HER2+ breast cancer cells in the setting of anti-HER resistance. The soft agar experiments using GPR110-overexpressing cells are ongoing.

Ongoing and future experiments include confirmation of these findings in SKBR3 cell line with inducible GPR110 overexpression and also to determine the effects of GPR110 knockdown using the GPR110-shRNA expressing cell lines. In addition, the effects of GPR110 overexpression or knockdown on cell growth in the context of drug resistance will also be determined to understand the possible role of GPR110 in anti-HER2 drug resistance setting.

Major task 1.4: **Determine the *in vivo* tumor growth in response to L, T, and L+T when GPR110 gene is overexpressed and/or knocked down in parental and anti-HER therapy resistant xenografts. 12-33 months**

In order to understand the effect of *in vivo* tumor growth in response to GPR110 overexpression, we have already obtained the approval of IACUC at Baylor College of Medicine (BCM), where all the proposed animal experiments will be conducted (approval date: June 10, 2015). Necessary documents were also submitted to the USAMRMC Animal Care and Use Review Office (ACURO) on July 10, 2015. We expect to hear from ACURO by the end of October regarding the approval status in order to begin the animal experiment proposed in the project. Since all the proposed animal work will be done at BCM, obtaining IACUC approval at University of Houston (UH) is not necessary.

Aim 2: Investigate HER-dependent and –independent signaling mechanisms by which GPR110 mediates tumorigenicity and anti-HER therapy resistance.

Major task 2.1: **Measure the expression of GPR110 in ALDH+ vs. ALDH- population of 12 cell line models. 1-6 months**

In our preliminary data, we had demonstrated that GPR110 was overexpressed in tumorigenic ALDH+ vs. ALDH- population of 2 HER2+ breast cancer cell line models. To understand whether GPR110 overexpression is a common phenomenon in tumorigenesis, we have measured GPR110 expression in the ALDH+ vs. the ALDH- population of various HER2+ breast cancer cells. For this, we first carried out the flow cytometry analysis to quantify the ALDH positivity in various cell line models. As shown in figures 7A-I, the aldeflur+ population varied (1.1% to 58.4%) in various cell line models, as described before. Based on the aldeflur positivity, only 6 models were chosen for FACS sorting and subsequent RNA extraction for measuring GPR110 mRNA using RT-PCR and protein using IHC. We observed that GPR110 mRNA levels were significantly up-regulated in the aldeflur+ population of cells of BT474, SKBR3, MDA-MB-361 and HCC1569 models (Figure 8A-D). There was no difference in the

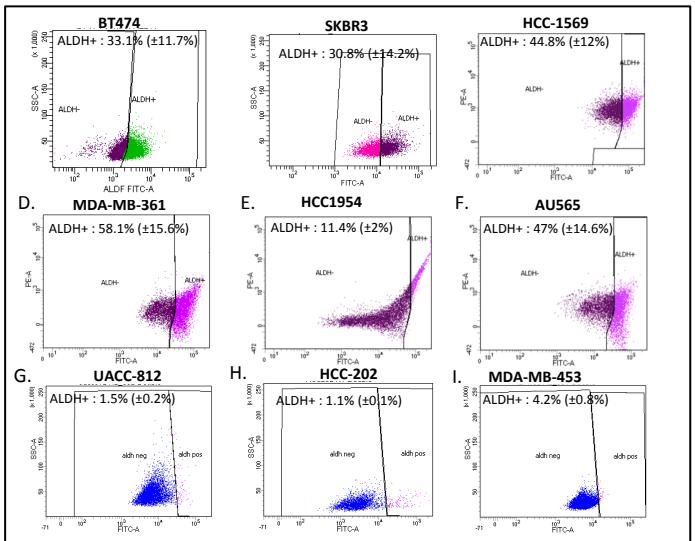

Figure 7. ALDH expression analysis in various HER2+ breast cancer cell line models. Aldeflur assay was conducted in various HER2+ breast cancer cell lines. ALDH+ population is the mean of 2-6 independent experiments.

GPR110 mRNA levels observed in the Aldeflur+ versus Aldeflur- cells of the AU565 and HCC1954 models (Figure 8E-F). IHC analysis for GPR110 protein levels in the aldeflur+ vs. the aldeflur- population of various cell models is ongoing. Overall, our results suggest that GPR110 is overexpressed ALDH+ population of multiple HER2+ breast cancer cell line models confirming that it is a common phenomenon.

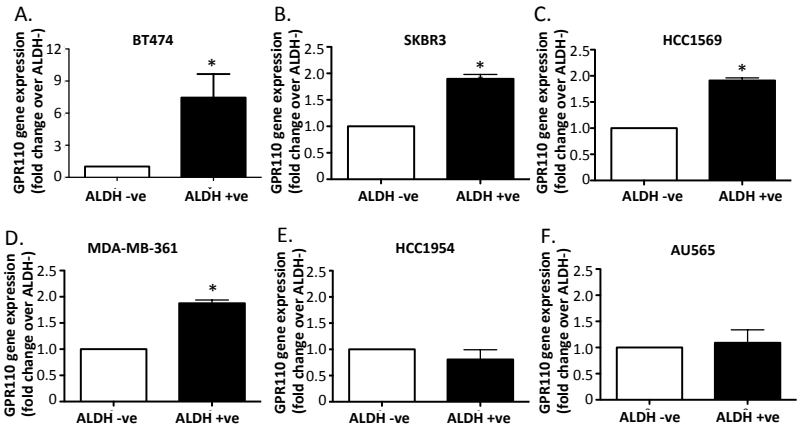

Figure 8. GPR110 mRNA expression analysis in ALDH+ vs. ALDH- population of various HER2+ breast cancer cell line models. A-F. qRT-PCR analysis for GPR110 mRNA expression in Aldeflur+ (ALDH+) vs. Aldeflur- (ALDH-) populations. * indicates p<0.05 (N=3-5).

Major task 2.2: **Measure the effects of GPR110 gene-overexpression and -knockdown on tumorigenic potential of HER2+ breast cancer cells and their anti-HER therapy resistant derivatives. Also, determine their HER pathway dependence. 12-36 months**
To determine the tumorigenic potential of GPR110, the proposed experiments to test aldeflur-positive population are ongoing.

Aim 3: Investigate the predictive value of tumoral GPR110 expression in HER2+ breast cancer patients treated with neoadjuvant L+T regimen.

Major task 3.1: Evaluate GPR110 expression in tumor biopsies at baseline and in residual disease after neoadjuvant L+T treatment. 12-30 months
The IRB protocol was approved at BCM on November 21, 2014 and renewed without any modification on 8/24/2015. The US Army Medical Research and Materiel Command, Office of Research Protections, Human Research Protection Office (HRPO) has also approved the BCM protocol on January 13, 2015.

The UH IRB has approved the protocol on December 22, 2014. The HRPO has also approved the UH protocol on January 9, 2015. The proposed studies in the human samples will begin in year 2 of the project.

Additional experiments demonstrating a role of GPR110 in cancer invasion and migration: Because GPR110 belongs to adhesion GPCR family, members of which have shown a role in cancer metastasis, we also have evaluated whether GPR110 has a role in metastasis. We conducted a preliminary experiment to determine the effect of GPR110 knockdown using

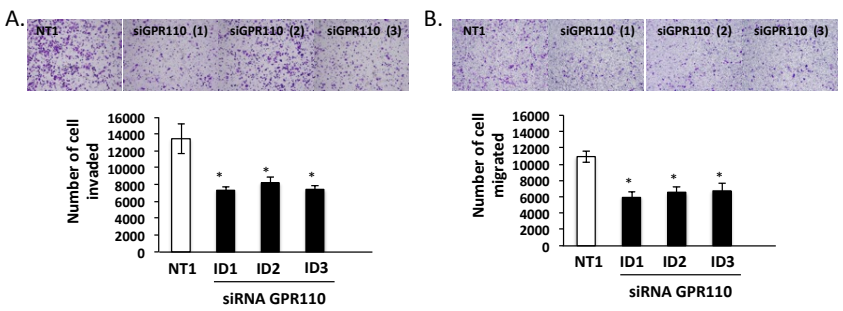

Figure 9. Effects of GPR110 knockdown on cell invasion and migration in SKBR3 cells. Invasion and migration assay using the transwell inserts with (A) or without (B) matrigel for SKBR3 cells transfected with non-targeting (NT1) or 3 independent GPR110 siRNAs. * indicates p<0.05 (N=3).

siRNAs on invasion and migration of SKBR3 cells. We found that knockdown of GPR110 using 3 independent siRNAs in SKBR3 parental cells resulted in a significant reduction in the cell invasion (Figure 9A) and migration (Figure 9B), suggesting a role of GPR110 in tumor cell dissemination. These experiments using the GPR110 overexpressing cell lines are ongoing.

What opportunities for training and professional development has the project provided?
A highly qualified postdoctoral fellow, Dr. Debashish Sahay, was recruited in February 2015 for the project, who completed his PhD from INSERM UMR 1033 at Universite Claude Bernard Lyon in France in January 2015. Because his PhD thesis focused on the study of a GPCR (lysophosphatidic acid receptor) in mediating bone metastasis of triple-negative breast cancer, he was considered to be the most qualified candidate to carry out the proposed project. Dr. Sahay's involvement in this research has provided him opportunities to present the findings at two conferences. The details are provided below under the Products section of this report. Furthermore, the funding from DOD has also facilitated Dr. Trivedi's career independence and advancement.

How were the results disseminated to communities of interest?
Findings from these studies have been presented at 2 meetings. The details are given below under Products section.

What do you plan to do during the next reporting period to accomplish the goals?
Based on the confirmation of our initial results, we will inject GPR110-overexpressing cells in mice to determine the effect on tumor formation as proposed in the SOW. Furthermore, we will evaluate the role of GPR110 in cell adhesion, invasion, and migration as suggested by our analysis described above. RPPA and/or phospho-proteomic studies will also be undertaken in order to uncover the downstream signaling pathways for GPR110 and its potential involvement in resistance to anti-HER2 drug therapy and tumorigenicity. We will generate stable cell line with the GPR110 shRNAs and carry out functional assays to better understand the role of GPR110 in anti-HER2 resistance and tumorigenesis as proposed in the SOW. We will use these genetically engineered cells to test the effects of GPR110 gene-overexpression and/or -knockdown in the absence or presence of the HER pathway inhibition on tumorigenic potential as well as evaluate the effects on HER and the alternate survival pathways in these preclinical models using *in vitro* and *in vivo* studies as proposed in the SOW. Additional GPCR candidates from our initial screen will also be evaluated for their potential role in tumorigenesis as well as anti-HER2 resistance.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?
This project has allowed the investigation of GPR110 as a potential novel target in improving efficacy and delaying resistance for anti-HER2 therapy. We have shown that GPR110 overexpression occurs commonly in anti-HER2 drug resistant cells and in tumorigenic population using a broad panel of cell line models. In addition, we have demonstrated the role of GPR110 in tumorigenicity, cell adhesion, invasion, and migration, which may facilitate anti-HER2 drug resistance. We have successfully generated inducible lentiviral plasmids with GPR110 cDNA and cell lines that inducibly overexpress GPR110. Generation of cell lines with GPR110 knockdown is ongoing. These tools provide the necessary resources to investigate the role of GPR110 in HER2+ breast cancer as proposed in this research.

What was the impact on other disciplines?

GPR110 belongs to the adhesion GPCR family. The biologic function of this family of receptors is largely unknown, but a few studies indicate their role in promotion of cancer metastasis. To date, there are no reports of the function of GPR110 in physiology or disease. This DOD-funded project has facilitated generation of novel cell lines that overexpress GPR110 or GPR110 shRNA for its knockdown to investigate pharmacology of GPR110, for which we plan to acquire additional funding.

What was the impact on technology transfer?
Nothing to report

What was the impact on society beyond science and technology?
Nothing to report

5. Changes/Problems:

Changes in approach and reasons for change
In addition to our proposed studies, we will investigate the role of GPR110 on cell adhesion, invasion, and metastasis as suggested by our analysis described above. We will also evaluate potential role of additional GPCR candidates in improving the efficacy and delaying resistance to anti-HER2 therapy with the goal of identifying targets or drugs with excellent safety profile.

Actual or anticipated problems or delays and actions or plans to resolve them
For the construction of the shRNA GPR110 plasmids, the 4 independent shRNAs we purchased did not effectively knockdown GPR110 as assessed by qRT-PCR when transiently transfected in the SKBR3 LTR cells, which has a high GPR110 expression (Figure 4A). We are using a new strategy for designing the GPR110 shRNA using the GPR110 siRNA sequence. These siRNAs have been validated by RT-PCR in significantly knocking down the expression of GPR110 in SKBR3 model (Figure 4B,C). We are in the process of constructing the inducible plasmid with these GPR110 shRNA, and will then carry out the infection to generate stable cell lines.

Changes that had a significant impact on expenditure
Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report

Significant changes in use or care of human subjects
Nothing to report

Significant changes in use or care of vertebrate animals.
Nothing to report

Significant changes in use of biohazards and/or select agents
Nothing to report

6. Products

Publications, conference papers, and presentations

4th Annual Conference on Recent Advances in the Development of Combinatorial Therapies for Cancer

Debashish Sahay, Raksha R. Bhat, Puja Yadav, Ahmed Al-rawi A, Vikas Yadav, Hosu Kim, Pavel Christiny, Sarmistha Nanda, Mario Giuliano, Chad Creighton, C. Kent Osborne, Vihang A. Narkar, Rachel Schiff, Meghana V. Trivedi. Identification of GPR110 as a Novel Target in Her2+ Breast Cancer. June 2015 in Rice University, Houston, USA. *(Poster Presentation)*

2nd University of Houston Department of Pharmacological and Pharmaceutical Sciences annual symposium

Debashish Sahay, Raksha R.Bhat, Puja Yadav, Ahmed Al-rawi A, Vikas Yadav, Hosu Kim, Pavel Christiny, Sarmistha Nanda, Mario Giuliano, Chad Creighton, C. Kent Osborne, Vihang A.Narkar, Rachel Schiff, Meghana V. Trivedi. Identification of GPR110 as a Novel Target in HER2+ Breast Cancer. August 2015 at University of Houston, Houston, USA. *(Oral Presentation)*

Website(s) or other internet site(s)
Nothing to report

Technologies or techniques
Nothing to report

Inventions, patent applications, and/or licenses
Nothing to report

Other products
Nothing to report

7. Participants & Other Collaborating Organizations:

What individuals have worked on the project?

Name	Rachel Schiff
Project Role	Partnering principal investigator
Nearest person month worked	1
Contribution to project	Dr. Schiff has contributed to planning of experiments, interpretation of data, supervision of Dr. Sahay and Ms. Rajendran, and overall supervision of the project. Specifically, she has provided supervision for the generation of inducible lentiviral plasmids for GPR110 overexpression and knockdown and infection of cell lines with these lentivirus. She is also the PI of IACUC and IRB protocol approved at BCM.

Name	Mahitha Rajendran
Project Role	Research assistant
Nearest person month worked	1
Contribution to project	Ms. Rajendran has assisted with maintenance of various HER2+ breast cancer cell line models of anti-HER2 drug resistance for the GPR110 expression analysis.

Name	Lanfang Qin
Project Role	Research associate
Nearest person month worked	1
Contribution to project	Dr. Qin has provided conceptual guidance for the generation of lentiviral plasmids.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report

What other organizations were involved as partners?
Organization name: University of Houston
Location of organization: Houston, Texas
Partner's contribution to the project: as an initiating PI
Financial support: None
In-Kind support: None
Facilities: Cell-based assays, molecular analysis
Collaborations: Meghana Trivedi
Personnel exchanges: None

8. Special reporting requirements

Collaborative awards
Nothing to report

Quad charts
Nothing to report

9. Appendices
Abstracts of conferences, poster

Identification of GPR110 as a Novel Target in HER2+ Breast Cancer.

Debashish Sahay[1], Raksha R.Bhat[1], Puja Yadav[1], Ahmed Al-rawi A[1], Vikas Yadav[2], Hosu Kim[1], Pavel Christiny[1], Sarmistha Nanda[3], Mario Giuliano[3], Chad Creighton[3], C. Kent Osborne[3], Vihang A.Narkar[2], Rachel Schiff[3], Meghana V. Trivedi[1,3].

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[3]Lester and Sue Smith Breast Center, Dan L. Duncan Cancer Center, Baylor College of Medicine, Houston, TX.

Objectives: Human epidermal growth factor receptor-2-overexpressing breast cancer (HER2+ BC) is an aggressive tumor with high rates of anti-HER2 drug resistance. Identification of novel drug targets in HER2+ breast cancer is an unmet clinical need. In this context, G-protein coupled receptors (GPCRs) may be excellent drug targets because they cross-talk with the HER family members. However, the expression and function of the majority of GPCRs are unknown in HER2+ breast cancer. Our goal was to identify novel G-protein coupled receptor (GPCR) targets to improve efficacy and overcome anti-HER2 drug resistance. In preliminary studies, we identified GPR110 as a potential drug target in HER2+ BC. Here, we investigated the role of GPR110 in modulating HER2 signaling and anti-HER2 drug efficacy and resistance in HER2+ BC. Method: The publically available TCGA dataset was interrogated to determine differential mRNA expression of GPR110 in HER2+ and other subtypes of breast cancer. Stable GPR110 overexpression was obtained by lentiviral-mediated delivery of GPR110 cDNA. GPR110 gene knockdown was achieved using target-specific siRNAs. Tumorigenic potential was determined by calculating the % of ALDH+ cells using Aldefluor assay and by evaluating the anchorage-independent cell growth using soft agar assay over 14 days. The influence of GPR110 overexpression on HER signaling pathway was investigated by measuring the levels of phosphorylated (active) and total protein levels of HER1 and HER2 using immunoblotting. Results: In TCGA dataset, GPR110 expression was significantly higher in HER2+ and basal subtypes of breast cancer compared to ER+ luminal A and B subtypes. Anchorage-independent cell growth was 5-fold higher in GPR110-overexpressing cells compared to control cells. In addition, GPR110-overexpressing cells had a significantly higher % of ALDH+ population compared to control cells. Conversely, GPR110 knockdown reduced tumorigenic potential selectively in drug resistant SKBR3 cells but not in parental cells. Phosphorylated (but not total) HER1 and HER2 protein levels were significantly higher in GPR110-overexpressing cells compared to control cells. Conclusions: We show for the first time a pro-tumorigenic role of GPR110 in HER2+ BC. GPR110 is a novel pharmacological target, inhibition of which may potentially improve anti-HER2 drug efficacy and delay anti-HER2 drug resistance.

This study has been supported by grants from AACP and DoD and faculty start-up funds.

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ABSTRACT

Objectives: Human epidermal growth factor receptor-2-overexpressing breast cancer (HER2+ BC) is an aggressive tumor with high rates of anti-HER2 drug resistance. Identification of novel drug targets in HER2+ breast cancer is an unmet clinical need. In this context, G-protein coupled receptors (GPCRs) may be excellent drug targets because they cross-talk with the HER family members. However, the expression and function of the majority of GPCRs are unknown in HER2+ breast cancer. Our goal was to identify novel G-protein coupled receptor (GPCR) targets to improve efficacy and overcome anti-HER2 drug resistance. In preliminary studies, we identified GPR110 as a potential drug target in HER2+ BC. Here, we investigated the role of GPR110 in modulating HER2 signaling and anti-HER2 drug efficacy and resistance in HER2+ BC.

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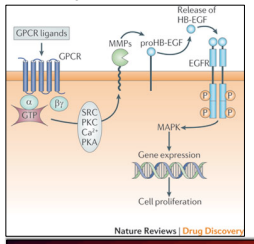
Conclusions: We show for the first time a pro-tumorigenic role of GPR110 in HER2+ BC. GPR110 is a novel pharmacological target, inhibition of which may potentially improve anti-HER2 drug efficacy and delay anti-HER2 drug resistance.

BACKGROUND

G protein-coupled receptors (GPCRs)

- Are excellent drug targets due to their plasma membrane localization, unique ligand-binding pocket, & availability of assays for drug screening.
- Are known to cross-talk with the HER superfamily.
- It is possible that some GPCRs may signal to modulate the HER2 pathway.
- The expression and function of the majority of GPCRs are largely unknown in HER2+ BC.



GPCRs interacting with HER2 (and HER1) pathways in breast cancer
β2 adrenoceptor
Chemokine receptor CXCR1/2
Chemokine receptor CXCR4/5
Endothelin receptor ET(A)R
Estrogen G protein-coupled receptor GPER
Lysophosphalipid receptor S1P3/4
Melatonin receptor MT1
Tachykinin receptor NK1R
Vasoactive intestinal peptide receptor VPAC

METHODS

BT474 & SKBR3 (HER2+ breast cancer cell line) → Aldefluor assay → ALDH$^+$ versus ALDH$^-$ cell population

BT474 & SKBR3 (HER2+ breast cancer cell line) → Anti-HER therapy resistance by long-term exposure to trastuzumab (T), lapatinib (L), and L+T[3]. → Parental and TR, LR, and LTR derivatives

METHODS

Human GPCR Taqman real-time PCR array:

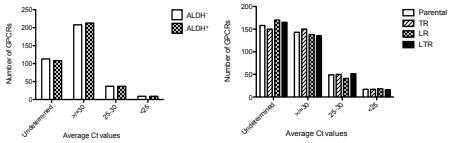
RNA was isolated from ALDH$^+$ and ALDH$^-$ cells as well as parental, TR, LR, and LTR derivatives of BT474 and SKBR3 cell line models using RNeasy Mini kit (Qiagen ,Valencia, CA). The cDNA was prepared from 1.5 µg using high capacity RNA to cDNA master mix (Invitrogen, Carlsbad, CA). Each port of the TaqMan real-time PCR GPCR 384 microarray card (Invitrogen) was loaded with cDNA (from 1.5 µg of RNA) and the TaqMan Gene Expression Master mix (Invitrogen). The array card was analyzed on the ViiA™ 7 System and the results were analyzed using data assist 3.01 software (Invitrogen). Candidate genes were validated using the cDNA (from 0.5 µg of RNA) in 96-well PCR plates.

Aldefluor assay and sorting of Aldefluor+ population by FACS:

The Aldefluor kit (stem cell technologies) was used to isolate the population with a high ALDH ezymatic activity. 10^6 (10^7 for sorting) cells were harvested and suspended in Aldefluor assay Buffer. Next steps were followed as per manufacturer's protocol. Cells were counterstained with Sytox red to discriminate viable cells from dead cells in FACS flow cytometer (Cell sorter: BDFACS Aria; Analyser: LSR Fortessa, BD Biosciences)

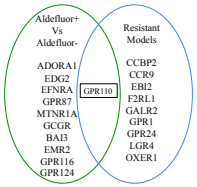
RESULTS

Figure 1. Number of GPCRs with different Ct values in (A) ALDH$^-$ and ALDH$^+$ BT474 cells and in (B) parental, TR, LR, and LTR derivatives of BT474 cells.



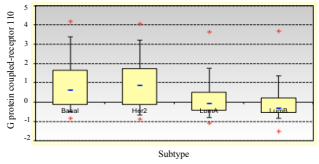
Average Ct values of GPCRs from 2-4 independent experiments were computed and is represented. Only a few GPCR genes are expressed in HER2+ BC.

Figure 2. Two fold or more up-regulated GPCRs in ALDH+ vs. ALDH- cells AND in at least one resistant line vs. parental cells.



We found 11 GPCRs that were two fold or more up-regulated in ALDH+ vs ALDH- in atleast 2 out of 4 independent experiments. 10 GPCRs were atleast two fold up-regulated in LTR as well as LR and/or TR compared to parental cells in independent replicates. We only found GPR110 as the common GPCR in both the groups and hence it was selected as a candidate for further analysis.

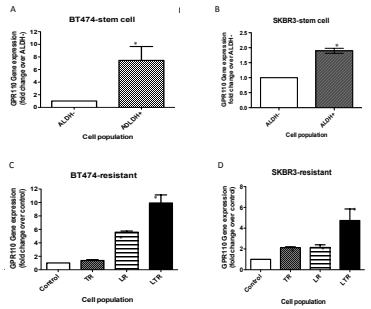
Figure 3. GPR110 mRNA expression in HER2+ and other subtypes of breast cancer using the publicly available TCGA data



Publicly available TCGA dataset was used to determine the GPR110 expression in different subtypes of breast cancer. Box and Whisker plot was used to show the differential GPR110 expression. The GPR110 mRNA expression was significantly higher in HER2+ and basal like breast cancer vs luminal A and B subtypes ($p < 0.05$, One way Anova)

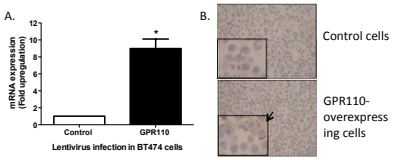
RESULTS

Figure 4. Differential expression of GPR110 in ALDH$^+$ vs. ALDH$^-$ cells and in anti-HER therapy resistant derivatives vs. parental cells of HER2+ BC cells.



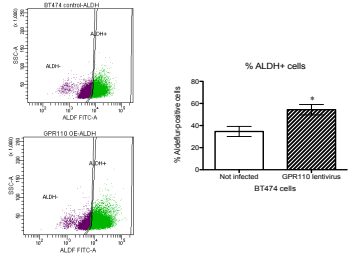
To validate candidate genes from the array, the cDNA (from 0.5 µg of RNA), TaqMan Gene Expression Master mix, the TaqMan gene expression assay for each of the genes of interests, and RNase-free water were added to a 96-well PCR plate (Applied Biosystems, Carlsbad, CA) according to manufacturer's instructions. * indicates $p < 0.05$, Student's t-test (A and B) or One-way ANOVA followed by Tukey post-hoc test (C and D), N=4.

Figure 5. GPR110 expression in BT474 cells infected with lentivirus containing GPR110 construct.



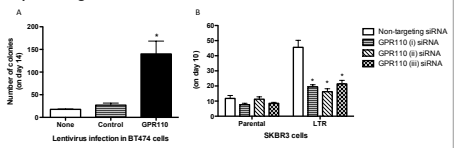
GPR110 mRNA (A) and protein (B) expression was higher in BT474 cells infected with GPR110 containing lentivirus vs. control cells. mRNA expression was measured by q-RT-PCR as described above. Protein expression was evaluated with immunohistochemistry using anti-GPR110 antibodies (Sigma-Aldrich, St. Louis, MO) * indicates $p < 0.05$, Student's t-test, N=6.

Figure 6. Effects of GPR110 overexpression on Aldefluor+ cells in HER2+ BC cells.



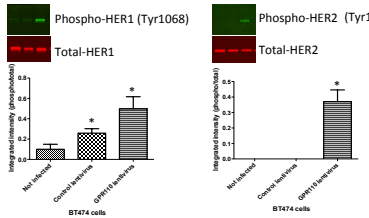
Aldefluor+ cells with high ALDH enzyme activity were determined using ALDEFLUR assay kit (Stem Cell Technologies, Vancouver, BC) using 10^6 (10^7 for sorting) cells (Cell sorter: BD FACS Aria II and analyser: LSR Fortessa, BD Biosciences). * indicates $p < 0.05$, Student's t-test, N=6.

Figure 7. Effects of GPR110 genetic modulation on anchorage-independent growth in HER2+ BC cells.



GPR110 genetic modulation was achieved by lentivirus-mediated stable overexpression using cDNA (GeneCopoeia, Rockville, MD) (A) or by transient knockdown using Silencer® Select siRNAs targeted to GPR110 (Ambion, Carlsbad, CA) (B). Soft agar assay was performed using 5000 cells/well in quadruplicates for each independent experiment. Colonies were counted using GelCount™ (Oxford-optronix, UK) on day 14 (A) or 10 (B). Overexpression of GPR110 in BT474 cells induces anchorage-independent growth. On the other hand, GPR110 gene knockdown in SKBR3 LTR cells, in which GPR110 is overexpressed, significantly reduces anchorage-independent cell growth, but not in SKBR3 parental cells. * indicates $p < 0.05$, One-way ANOVA followed by Tukey post-hoc analysis, N=4.

Figure 8. Effects of GPR110 overexpression on HER signaling in HER2+ BC cells.



GPR110 was stably overexpressed by lentivirus-mediated delivery in BT474 parental cells. Cells were lysed in RIPPA buffer containing protease inhibitor and Phosphatase inhibitor (Roche, Indianapolis, IN, USA). Cell lysates (30-40 µg protein) were separated under denaturing conditions by electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE) and transferred onto nitrocellulose membranes, which were then immunoblotted with the specific primary (Cell Signaling Technologies, Danvers, MA) and secondary antibodies (LI-COR, Lincoln, NE). Significant increase in phospho/total expression of HER1 and HER2 proteins were seen in GPR110-overexpressing BT474 cells compared to not infected cells and the ones infected with empty vector alone.

* indicates $p < 0.05$, One-way ANOVA, Tukey post-hoc analysis, N=3.

CONCLUSIONS

- We demonstrate a previously unknown pro-tumorigenic role of GPR110 in HER2+ BC.
- GPR110 is a novel pharmacological target, inhibition of which may potentially improve anti-HER2 drug efficacy and delay anti-HER2 drug resistance

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