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GPCRs profiling and identification of GPR110 as a potential new target in HER2+ breast cancer

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Abstract

Purpose: G protein-coupled receptors (GPCRs) represent the largest family of druggable targets in human genome. Although several GPCRs can cross-talk with the human epidermal growth factor receptors (HERs), the expression and function of most GPCRs remain unknown in HER2+ breast cancer (BC). In this study, we aimed to evaluate gene expression of GPCRs in tumorigenic or anti-HER2 drug-resistant cells and to understand the potential role of candidate GPCRs in HER2+ BC.

Methods: Gene expression of 352 GPCRs was profiled in Aldeflur+ tumorigenic versus Aldeflur- population and anti-HER2 therapy-resistant derivatives versus parental cells of HER2+ BT474 cells. The GPCR candidates were confirmed in 7 additional HER2+ BC cell line models and publicly available patient dataset. Anchorage-dependent and -independent cell growth, mammosphere formation, and migration/invasion was evaluated upon GPR110 knockdown by siRNA in BT474 and SKBR3 parental and lapatinib+trastuzumab-resistant (LTR) cells.

Results: Adhesion and class A GPCRs were overexpressed in Aldeflur+ and anti-HER2 therapy-resistant population of BT474 cells, respectively. GPR110 was the only GPCR overexpressed in Aldeflur+ and anti-HER2 therapy-resistant population in BT474, SKBR3, HCC1569, MDA-MB-361, AU565, and/or HCC202 cells and in HER2+ BC subtype in patient tumors. Using BT474 and SKBR3 parental and LTR cells, we found that GPR110 knockdown significantly

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Conflict of Interest

The authors declare that they have no conflict of interest.

reduced anchorage-dependent/independent cell growth as well as migration/invasion of parental and LTR cells and mammosphere formation in LTR derivatives and not in parental cells.

Conclusion: Our data suggests a potential role of GPR110 in tumorigenicity and in tumor cell dissemination in HER2+ BC.

Keywords

GPR110; HER2; breast cancer; drug resistance; drug targets; tumorigenesis

Introduction

HER2 plays a key role in cell proliferation, survival, and differentiation [1] and is a well-characterized oncogene [2]. HER2 is amplified and/or overexpressed in ~15–20% of breast cancers (BC), where it is a major driver of tumor growth and survival. HER2-overexpressing (HER2+) tumors are characterized by aggressive phenotypes and poor prognosis [3]. Despite improved clinical outcomes with several Food and Drug Administration (FDA)-approved anti-HER2 drugs [4], *de novo* and acquired drug resistance occur in many patients triggering recurrence. Therefore, identification of new drug targets to improve efficacy of anti-HER2 drugs and/or overcome drug resistance is warranted in HER2+ BC.

G-protein coupled receptor (GPCR) is one of the largest superfamily of cell surface receptors that play an important role in various cellular processes in physiology [5]. GPCRs are considered excellent drug targets due to their plasma membrane localization and unique ligand-binding pockets that facilitate selective and specific drug design. Indeed, 30–50% of FDA-approved drugs target GPCR pathways and are often used to treat chronic diseases due to their excellent safety profile [6, 7]. Although several recent studies suggest that GPCRs may have a crucial role in cancer initiation, progression, and metastases [6], their expression and function largely remain unknown in HER2+ BC. Since GPCRs are known to cross-talk with the HER superfamily [8–14], it is possible that some GPCRs may signal to modulate the HER2 pathway and anti-HER2 drug sensitivity. The goal of this study was to profile and identify potential GPCR targets in HER2+ BC, especially in the context of tumorigenesis and anti-HER2 therapy resistance.

Material and Methods

Cell lines and reagents

The BT474 cell line was obtained from AstraZeneca (Cheshire, UK) [15]. SKBR3, MDA-MB-361, HCC1954, and HCC202 cell lines were from Dr. Joe Gray's lab (Berkeley Lab, Berkeley, CA, USA) [16, 17]. AU565, UACC812, and HCC-1569 cell lines were from ATCC. BT474, UACC-812, and MDA-MB-361 cell lines were maintained in Dulbecco's modified Eagle medium (DMEM); SKBR3 cells were grown in McCoy's 5A; and AU-565, HCC-1569, HCC-1954, and HCC-202 were cultured in RPMI 1640 modified with 1X L-glutamine with all media supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin-glutamine [17, 18].

Aldeflur assay and FACS sorting

ALDH+ tumorigenic cells were isolated from various HER2+ BC cell lines using ALDEFLUR™ kit (Catalog# 01700, Stem Cell Technologies) [19]. Aldeflur+ cells were sorted using FACSAria sorter II (BD Biosciences).

Development of anti-HER2 resistant derivatives

Cell lines resistant (R) to HER-targeted therapy were generated by long-term culture of the cells in their original media with increasing concentrations of trastuzumab (1 to 50 μg/ml), lapatinib (0.1 to 1 μM), or both as described before [17, 18, 20]. Parental cell lines and their resistant derivatives were authenticated twice a year while being used for these experiments.

Drugs

Trastuzumab (Herceptin®, manufactured by Genentech, San Francisco, CA, USA) was purchased from McKesson and was dissolved in sterile distilled water provided with the drug. Lapatinib was obtained from LC Laboratories (MA, USA) and was dissolved in sterile dimethyl sulfoxide (DMSO). Drug dilutions were made in appropriate media such that the final DMSO concentration was less than 0.1%.

TaqMan GPCR gene expression Array and data analysis

cDNA samples were analyzed on pre-designed 384-well Human GPCR TaqMan Array cards (Catalog# 4367785, Life Technologies) using ViiA™ 7 real-time PCR instrument. These arrays contained validated primer/probe sets for 352 GPCRs, which represented 50 different subfamilies excluding the sensory GPCRs. The results of the assay were analyzed using DataAssist™ v3.0, Life Technologies. Threshold cycle (C_T) values of GPCRs were normalized to the C_T values of the housekeeping gene (PPIA) to obtain ΔC_T. A $\Delta\Delta C_T$ approach was utilized to assess relative levels of mRNA in comparison to a calibrator sample. Relative expression level was calculated as $2^{-\Delta\Delta CT}$. GPCR expression was considered high, moderate, low, or very low if the Ct values were <25.0, 25.0 – 29.9, 30.0 – 39.9, and ≥ 40, respectively.

Real Time q-PCR

Real time quantitative (q)-PCR was conducted using Taqman gene expression assays (Life Technologies). The level of the different mRNAs was normalized to the level of the housekeeping gene (PPIA or GAPDH). A comparative C_T analysis was utilized to determine fold change in RNA expression based on the $\Delta\Delta C_T$ approach as described above.

siRNA Knockdown

GPR110-targeting silencer select siRNAs (Catalog# 4392420, id: S49024 (referred to as si-GPR110(1)) and S49026 (referred to as si- GPR110(2)) and non-targeting control siRNAs (referred to as (si-control) (Catalog# 4390843) were purchased from Ambion (Waltham, MA). Reverse transfection was carried out using 20 nM siRNAs, lipofectamine (Catalog# 13778150, Life technologies), and OptiMEM reduced serum medium (Catalog# 31985–088, Life technologies) incubated for 15–20 minutes to which cells 100,000–500,000 were added in 6 or 24-well plates. After 24 hours, the cells were either used for subsequent experiments

immediately or the transfection media was replaced with fresh complete media. Effective knockdown of GPR110 was confirmed by real time q-PCR in all experiments.

MTT assay to assess anchorage-dependent cell growth

Cell growth and viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Catalog# 30–1010K™, ATCC®). Briefly, the cells were seeded at 10,000–15,000 cells/well in a 96-well tissue culture plate. After the overnight attachment, the cells treated with various concentrations (1 nM to 10 μM) of lapatinib for 72 hours, after which the kit protocol was followed. The data was normalized to vehicle (100%) and was plotted using the lapatinib concentration ([lapatinib]), represented in log M. For determination of IC_{50} of lapatinib, the data was fitted using non-linear regression analysis and 3-parameter logistic equation: Y=Bottom + (Top-Bottom)/(1+10^((X-LogIC50))) using GraphPad Prism version 5.0c.

Soft agar assay to assess anchorage-independent cell growth

Soft agar assay was performed as described before [21]. Number of colonies (at least 50 μm in size) were counted on day 10 with Gelcount™ (Oxford Optromix, Germany).

Mammosphere Assay

Mammocult Human medium kit (Catalog# 05620) from Stem cell Technologies was used for the culturing of the mammospheres as previously described [22]. The mammospheres were counted on Day 7 and 14 by Gelcount™ (Oxford Optromix, Germany). For second generation passaging, the spheres were disaggregated using pre-warmed trypsin-EDTA for 3–5 minutes after which 1,000 cells per well were plated again, and the spheres were counted as described above.

Migration and invasion assay

Migration and invasion assays were performed without or with matrigel on inserts, respectively, as described before [12, 23]. The images were taken using Zeiss Axioskop 40 microscope and the migrated and invaded cells were quantified using ImageJ (National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2016).

Statistical Analyses

All cell-based studies were run at least in triplicate and repeated at least three times. All data analysis was done using the GraphPad Prism version 5.0c. Values were presented as mean ± SEM of biological replicates unless otherwise specified. Statistical differences between the groups were analyzed by student's t-test, One-way ANOVA or Repeated Measures ANOVA, both followed by Dunnett's multiple comparison post-hoc test. A P value of < 0.05 was considered statistically significant.

Results

GPCR gene expression array in Aldeflur+ vs. Aldeflur- BT474 cells

Relative expression of a panel of 352 non-sensory GPCR genes in Aldeflur+ versus Aldeflur- BT474 cells was determined in 4 independent experiments. The average Ct values for individual GPCRs are summarized in Figure 1A. None of the GPCRs had high expression in Aldeflur+ or Aldeflur- BT474 cells. A total of 19 GPCRs had moderate expression in Aldeflur+ and 21 GPCRs in Aldeflur- BT474 cells (Figure 1A). Out of these, 18 GPCRs (*BDKRB1, EBI2, FZD3, FZD4, FZD6, GPR160, GPR21, GPR126, GPR153, GPR143, GPR81, GPR22, LGR4, NPY1R, OPN3, OR2A4, P2RY2* and *TM7SF1*) were common to both Aldeflur+ and Aldeflur- cells. Three GPCRs (*GABBR1, VN1R1*, and *VN1R5*) had the moderate expression in only Aldeflur+ and 1 GPCR (*LPHN2*) had moderate expression in only Aldeflur- BT474 cells. Most GPCRs had either low (212 in Aldeflur+ and 206 in Aldeflur- cells) or very low (121 in Aldeflur+ and 125 in Aldeflur- cells) expression in BT474 cells.

A total of 11 GPCRs were at least two fold up-regulated in Aldeflur+ vs. Aldeflur- BT474 cells. These were *ADORA1, BAI3, EDG2, EDNR1, EMR2, GCGR, GPR110, GPR116, GPR124, GPR87*, and *MTNR1A*. The International Union of Basic and Clinical Pharmacology (IUPHAR) class and family of these 11 GPCR as well as the endogenous ligands and average fold-upregulation from all 4 experiments are tabulated in Table 1. Among the 11 GPCRs upregulated in Aldeflur+ cells, 5 belonged to class A (*ADORA1, EDG2, EDNRA*, *GPR87, and MTNR1A*); 1 to class B (*GCGR*); and 5 to Adhesion class (*BAI3, EMR2, GPR110, GPR116, and GPR124*). Considering that there are 284 GPCRs in class A and 33 in Adhesion class [24], there was significantly more representation from the Adhesion family of GPCRs upregulated in Aldeflur+ vs. Aldeflur- BT474 cells.

GPCR gene expression array in anti-HER2 therapy-resistant vs. parental BT474 cells

We determined the relative expression of a panel of 352 GPCR genes between parental vs. LR, TR and LTR in 2 independent experiments. The average Ct values for individual GPCRs are summarized in Figure 1B. High expression was found for 3 GPCRs (*GPR142*, *GPR160*, and *HTR7*) in parental and TR BT474 cells, for 2 GPCRs (*HTR7* and *GPR160*) in LR BT474 cells, and for 1 GPCR (*GPR142*) in LTR BT474 cells. Moderate expression (Ct value 25.0–29.9) was found for 39 GPCRs in parental, 40 GPCRs in TR, 31 GPCRs in LR, and 42 GPCRs in LTR. Most GPCRs had either low or very low expression in BT474 cell model of anti-HER2 treatment-resistance.

Ten GPCRs were at least two-fold up-regulated in LTR as well as LR and/or TR compared to parental cells in 2 independent replicates. These were *CCBP2, CCR9, EBI2, F2RL1, GALR2, GPR1, GPR24, GPR110, LGR4* and *OXER1*. Among these GPCRs, 9 belonged to Class A (*CCBP2, CCR9, EBI2, F2RL1, GALR2, GPR1, GPR24, LGR4* and *OXER1*) and only one (*GPR110*) belonged to Adhesion class. The IUPHAR class and family of GPCR as well as endogenous ligands and level of upregulation are tabulated in Table 2.

Candidate GPCR selection and validation of upregulation

Among the 11 GPCRs upregulated in Aldeflur+ vs. Aldeflur- and 10 GPCRs upregulated LTR and LR and/or TR, only GPR110 was common to both and hence was selected as the first candidate for further analysis (Figure 2).

To validate the upregulation of GPR110 in BT474 cell line model of tumorigenicity and in anti-HER2 therapy resistance and determine if the upregulation of GPR110 is common in other HER2+ BC models, an independent real time q-PCR was conducted using a panel of BC cell lines. We found that GPR110 gene expression was seven-fold higher in Aldeflur+ BT474 cells compared to Aldeflur- BT474 cells ($P < 0.05$, student's t-test, N= 3) (Figure 3A). Also, GPR110 was five- and nine-fold upregulated in LR and LTR, respectively, but not in TR of BT474 model of anti-HER2 resistance ($P < 0.05$, One-way ANOVA followed by Dunnett's test, N= 3) (Figure 4A). These results were in agreement with our GPCR gene array data for GPR110. In addition, GPR110 expression was significantly higher in Aldeflur + compared to Aldeflur- populations of SKBR3, HCC1569, and MDA-MB-361 cell lines ($P < 0.05$, student's t-test, N= 3) (Figure 3B-D). GPR110 expression was also significantly elevated in the TR, LR, and LTR derivatives of SKBR3 and UACC812 and also in TR and LR derivatives of AU565 and HCC202 cells compared to parental cells ($P < 0.05$, One-way ANOVA followed by Dunnett's test, N= 3) (Figure 4B-E). GPR110 expression was unchanged in Aldeflur+ compared to Aldeflur- population of AU565 and HCC1954 cells (Figures 3E-F) and in anti-HER2 resistant derivatives compared to parental cells of HCC1954 cells (Figure 4F).

GPR110 expression in publicly available datasets with different BC subtypes

Publicly available TCGA datasets were examined to determine the expression of GPR110 in different subtypes of BC [25]. Box and Whisker plot was employed for presenting differential GPR110 expression in basal, HER2+, luminal A, and luminal B subtypes of BC in patients. The gene expression of GPR110 was significantly higher in HER2+ and basal compared to luminal A and B subtypes ($P < 1E-13$, t-test on log-transformed data) (Figure 5). There was no significant difference in GPR110 expression between HER2+ and basal BC ($P > 0.05$).

Effects of GPR110 knockdown with GPR110-targeting siRNAs on anchorage-dependent cell growth and efficacy of lapatinib in HER2+ BC models

Next, we measured the effects of GPR110 knockdown by siRNAs on the growth of BT474 and SKBR3 parental and LTR cells in absence or presence of lapatinib using MTT assay. GPR110 siRNAs resulted in at least 70% knockdown of GRP110 expression by RT-PCR (data not shown). In absence of lapatinib, GPR110 knockdown using 2 independent siRNAs significantly inhibited the growth of parental and LTR cells of BT474 (Figure 6A) and SKBR3 (Figure 6B) models ($P < 0.05$, One-way ANOVA followed by Dunnett's test, N= 3). Lapatinib, in a concentration-dependent manner, inhibited growth of BT474 and SKBR3 parental cells (Figure 6C and6D). But, knockdown of GPR110 did not significantly alter the efficacy (bottom of the curve) or potency (IC50) of lapatinib in either BT474 or SKBR3 cells (Figure 6C and6D; Table 3). These results suggested that GPR110 might regulate cell

growth of HER2+ BC cells regardless of anti-HER2 drug sensitivity without affecting the efficacy or potency of lapatinib.

Effects of GPR110 knockdown with GPR110-targeting siRNAs on anchorage-independent cell growth of HER2+ BC models

The effect of GPR110 knockdown on anchorage-independent cell growth of the parental cells and LTR derivatives of BT474 and SKBR3 cell lines was determined using soft agar assay. GPR110 knockdown resulted in a significant decrease in number of colonies in parental as well as LTR derivatives of BT474 (Figure 7A) and SKBR3 (Figure 7B) cell line models ($P < 0.05$, Repeated Measures ANOVA followed by Dunnett's test, N= 3–4).

Effects of GPR110 knockdown with GPR110-targeting siRNAs on mammosphere formation capacity of BT474 cell line model

GPR110 knockdown also resulted in a significant inhibition of mammosphere formation in BT474 LTR derivatives ($P < 0.05$, Repeated Measures ANOVA followed by Dunnett's test, N=3), but not in parental cells (Figure 8A). Similarly, the second generation passaging of the spheres from GPR110-targeting siRNAs formed significantly fewer spheres compared to those from non-targeting siRNAs in BT474 LTR cells ($P < 0.05$, Repeated Measures ANOVA followed by Dunnett's test, N=3) but not in parental cells (Figure 8B). These results suggest that GPR110 may have a role in tumorigenicity of HER2+ BC cells primarily in the setting of anti-HER2 drug resistance. The SKBR3 parental and LTR cells did not form mammospheres and hence could not be assessed for the effects of GPR110 knockdown on this phenomenon.

Effects of GPR110 knockdown with GPR110-targeting siRNAs on migration and invasion of SKBR3 cell line model

Because adhesion GPCRs have been implicated in cancer metastases, we investigated whether GPR110 knockdown has an effect on migration and invasion of parental and LTR derivatives of various cell line models. Both migration and invasion of parental SKBR3 cells was significantly higher than that of their LTR derivatives ($P < 0.05$, Student's t-test, N= 3) (Figure 9). GPR110 knockdown resulted in a significant inhibition of migration and invasion of SKBR3 parental cells as well as LTR cell lines ($P < 0.05$, Repeated Measures ANOVA followed by Dunnett's test, N=3). These results suggest that GPR110 may have a role in migration and invasion of HER2+ BC cells regardless of anti-HER2 therapy resistance. A similar inhibition of invasion/migration upon GPR110 knockdown was also seen in BT474 parental cells (data not shown), but the LTR derivatives of BT474 could not be tested as they did not migrate or invade.

DISCUSSION

In this study, our primary objective was to profile and identify potential GPCR targets in HER2+ BC, especially in the context of tumorigenesis and anti-HER2 therapy resistance. We found that the adhesion GPCR family is well represented at least at the transcriptomic level in the Aldeflur+ tumorigenic cell population of BT474 cells. This family of GPCRs has been implicated in various cancer cell processes such as tumorigenicity and metastases that

promote disease progression. On the other hand, the GPCRs that were highly expressed in the anti-HER2 drug-resistant population of BT474 cells belonged mostly to class A GPCRs, which are best represented within FDA-approved drugs and in development pipeline. The distinction between the classes of GPCRs implicated in tumorigenesis versus drug resistance may have implications for identifying potential endogenous GPCR ligands important in HER2+ BC biology in future studies [26, 27]. We have further identified GPR110 as a candidate GPCR target of interest based on its overexpression in the tumorigenic population as well as anti-HER2 resistant population of various HER2+ BC cell line models. Knockdown of GPR110 caused inhibition of cell growth of BT474 and SKBR3 parental and LTR cells without affecting the short-term potency of lapatinib in both parental cells. GPR110 gene knockdown led to a significant decrease in mammosphere formation in only LTR derivatives and not in parental cells. Whereas, inhibition in anchorage-independent cell growth as well as cell migration and invasion by GPR110 knockdown was regardless of anti-HER2 drug sensitivity status. Our experimental findings suggest that GPR110 may have a role in tumorigenicity, especially in the setting of anti-HER2 drug resistance, and also in metastatic progression regardless of anti-HER2 drug sensitivity.

GPR110 is an orphan GPCR and is thought to be a proto-oncogene based on its high expression in lung cancer, prostate cancer, high-risk acute lymphocytic lymphoma, and glioma [28–32]. GPR110 belongs to the subfamily of adhesion GPCRs [33, 34]. Recently, adhesion GPCRs have shown to play an important role in cell motility, cancer metastases and progression [35–38]. However, the signaling and function of GPR110 remains unknown except the observation that the cleavage of N-terminal extracellular domain may be important for activation of GPR110 via exposure of a cryptic tethered agonist [39]. These findings along with the data presented here provide a rationale for further investigating GPR110 as a potential drug target in HER2+ BC.

In our studies, we have primarily utilized *in vitro* gene knockdown by an siRNA approach to investigate the role of GPR110 using multiple cell line models of HER2+ BC. Our observation that GPR110 overexpression is not seen in all HER2+ BC models emphasizes the heterogeneity within HER2+ BC as well as mechanisms of resistance. Furthermore, targeting GPR110 may be important even when cancer cells are sensitive to anti-HER2 drugs. This is evidenced by our observation that GPR110 inhibits colony formation in the soft agar assay as well as migration and invasion of the treatment-sensitive parental cells. However, targeting GPR110 may be more pronounced in the setting of anti-HER2 resistance, which may be a clinically significant, and should be investigated in the future. Finally, various adhesion GPCRs have been shown to have an important role in cancer metastasis [36, 38, 40–43]. Since we also observed the effects of GPR110 knockdown on migration and invasion of cells, the future studies should also focus on understanding downstream signaling of GPR110 as well as its role in metastasis using animal models.

In summary, we have taken the first step to profile the expression of a subset of GPCRs in HER2+ BCs. We have identified and used a proof-of-concept *in vitro* approach to demonstrate that GPR110 influences the growth of HER2+ BC cells and potentially drug resistance in these cells. Our findings raise exciting future prospects (i) to understand GPR110 crosstalk with the HER family to promote tumor growth and dissemination, (ii) to

evaluate if GPR110 modulation is an effective approach using HER2+ BC xenograft models, and (iii) to determine if GPR110 is amenable to pharmacological modulation. Finally, our findings provide rationale for an in-depth analysis of other GPCRs in breast tumors, which may lead to better therapeutics for HER2+ BC.

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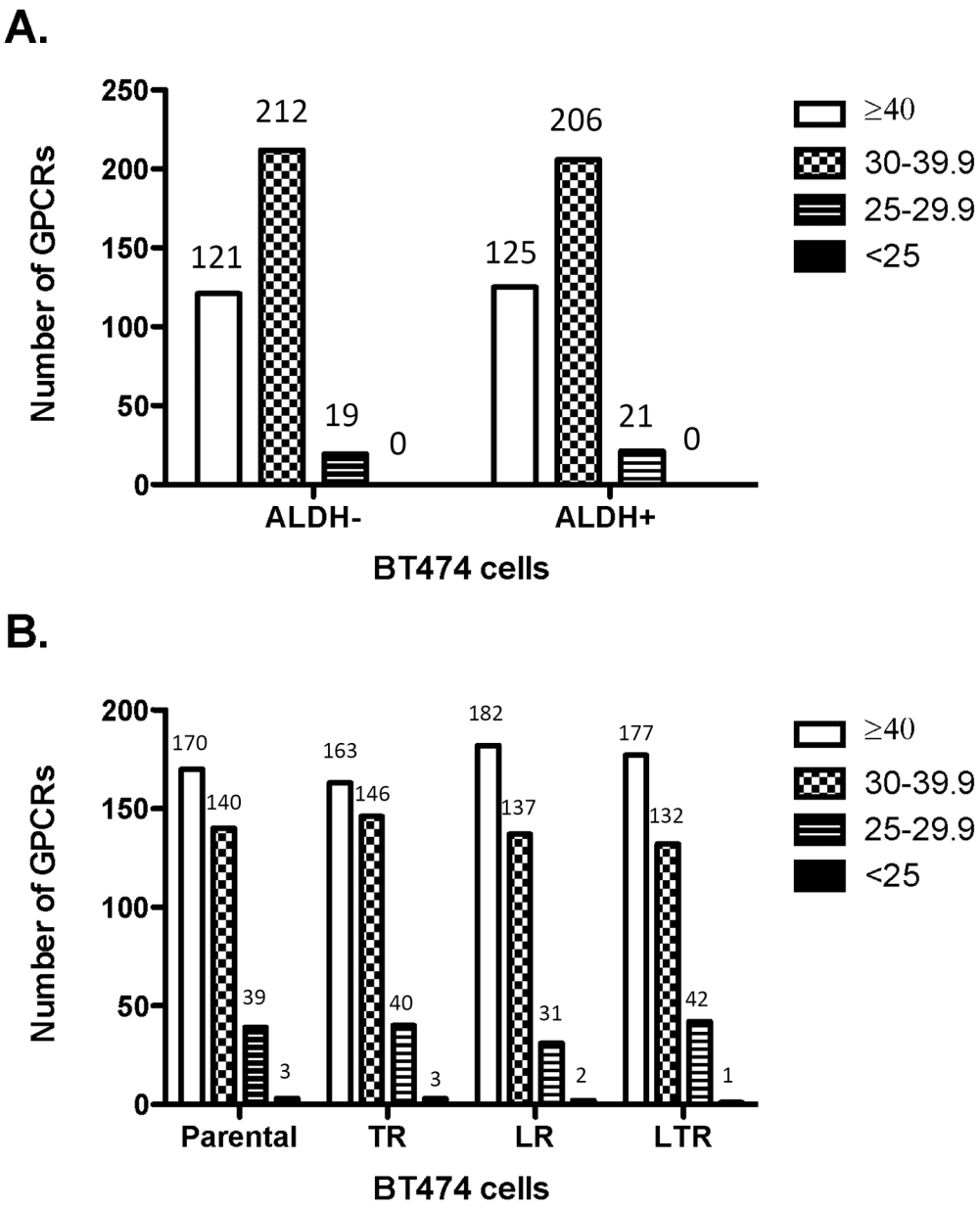

Figure 1. Distribution of 352 GPCRs with high, intermediate, low, and very low expression in Aldeflur+ tumorigenic versus Aldeflur- population and in parental cells and their anti-HER2 drug resistant derivatives of BT474 cells.

GPCR gene expression was determined using the 384-well Human GPCR TaqMan Array cards in (**A**) Aldeflur+ cell versus Aldeflur- cell population and (**B**) anti-HER2 drug resistant derivatives compared to parental cells of BT474 cells (N=2–4). Aldeflur+ cells were separated using Aldeflur assay followed by FACS sorting. Trastuzumab-resistant (TR), lapatinib-resistant (LR), and lapatinib + trastuzumab-resistant (LTR) cells were generated by long-term culture of the parental cells in their original media with increasing concentrations of respective drugs. GPCR expression was considered high, moderate, low, or very low if the Ct values were <25.0, 25.0 – 29.9, 30.0 – 39.9, and ≥ 40, respectively. Although most

GPCRs had very low or low expression, a few GPCRs had moderate or high expression in various BT474 cell line model of tumorigenic cells and anti-HER2 drug resistance.

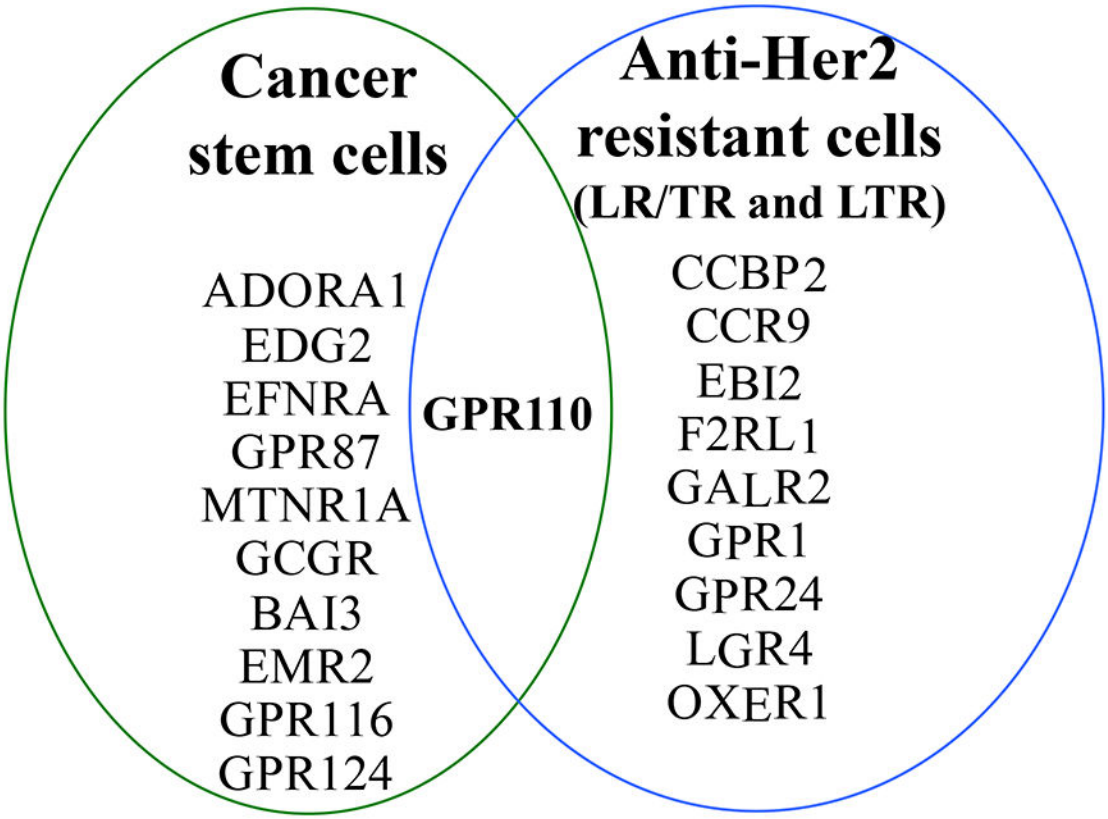

Figure 2. Candidate GPCR selection in Aldeflur+ vs. Aldeflur- cells and anti-HER2 resistant cells:

Eleven GPCRs (ADORA1, EDG2, EFNRA, GPR87, MTNR1A, GCCR, BAI3, EMR2, GPR116, GPR124, GPR110) were upregulated in Aldeflur+ tumorigenic cells compared to Aldeflur- cell population of BT474 cells. In addition, 10 GPCRs (CCBP2, CCR9, EBI2, F2RL1, GALR2, GPR1, GPR24, LGR4, OXER1 AND GPR110) were over-expressed in the LTR and LR or TR derivatives compared to the parental BT474 cells. GPR110 was the only GPCR overexpressed in both tumorigenic as well as resistant derivatives of BT474 cells, and hence was selected as a candidate GPCR for further investigation.

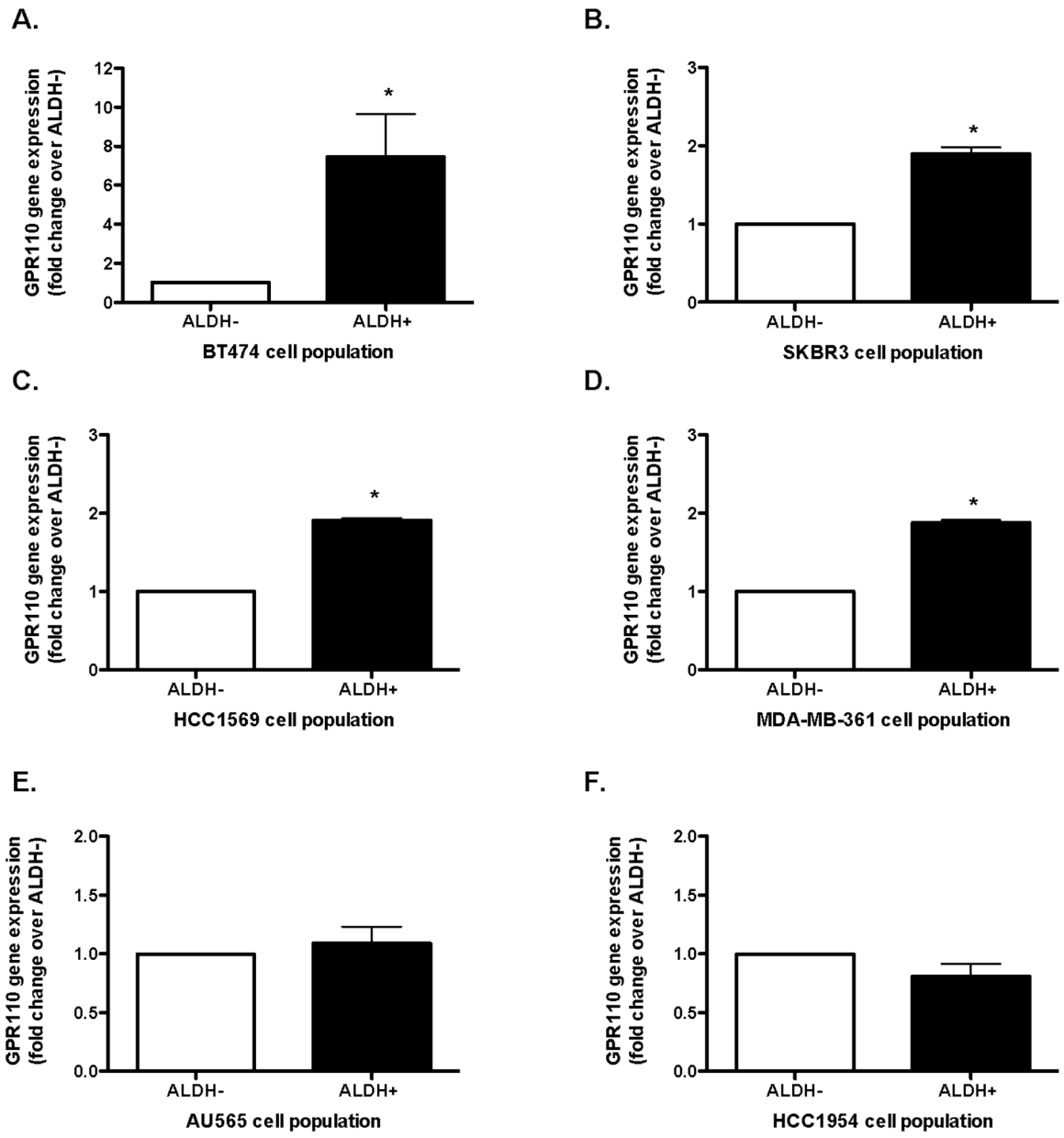

Figure 3. Relative gene expression of GPR110 in Aldeflur+ tumorigenic cells versus Aldeflur- cells population of various HER2+ BC cell line models.

Aldeflur+ cells were separated using Aldeflur assay followed by FACS sorting. GPR110 gene expression was determined using real time quantitative PCR using Taqman gene expression assays (Life Technologies). The level of the different mRNAs was normalized to the level of the housekeeping genes (PPIA). A comparative C_T analysis was utilized to determine fold change in RNA expression based on the $\Delta\Delta C_T$ approach (N=3). GPR110 expression was significantly higher in Aldeflur+ compared to Aldeflur- populations of (**A**) BT474, (**B**) SKBR3, (**C**) HCC1569, and (**D**) MDA-MB-361 cell lines, but not in (**E**) AU565 and (**F**) HCC1954 cells. * indicates $P < 0.05$ (student's t-test).

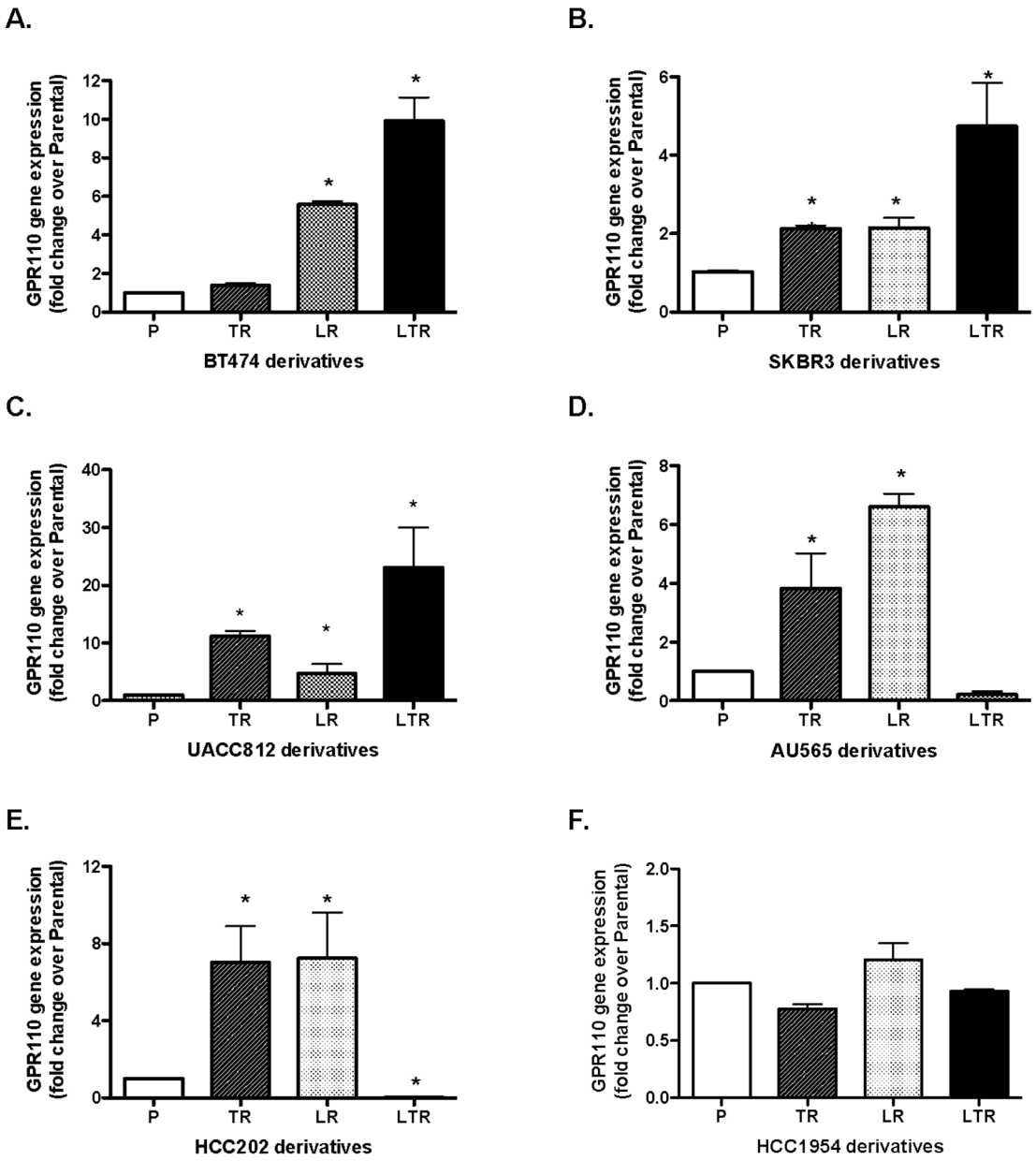

Figure 4. Relative gene expression of GPR110 in anti-HER2 drug-resistant derivatives versus parental cells of various HER2+ BC cell line models.

Trastuzumab-resistant (TR), lapatinib-resistant (LR), and lapatinib + trastuzumab-resistant (LTR) cells were generated by long-term culture of the parental cells in their original media with increasing concentrations of respective drugs. GPR110 gene expression was determined using real time quantitative PCR using Taqman gene expression assays (Life Technologies). The level of the different mRNAs was normalized to the level of the housekeeping gene (PPIA). A comparative C_T analysis was utilized to determine fold change in RNA expression based on the $\Delta\Delta C_T$ approach (N=3). GPR110 expression was significantly elevated in the TR, LR, and LTR derivatives compared to parental cells of (**A**) BT474, (**B**) SKBR3, and (**C**) UACC812; and in TR and LR derivatives of (**D**) AU565 and (**E**) HCC202

cells, but not in (**F**) HC1954 cells. * indicates $P < 0.05$ (One-way ANOVA followed by Dunnett's posthoc test).

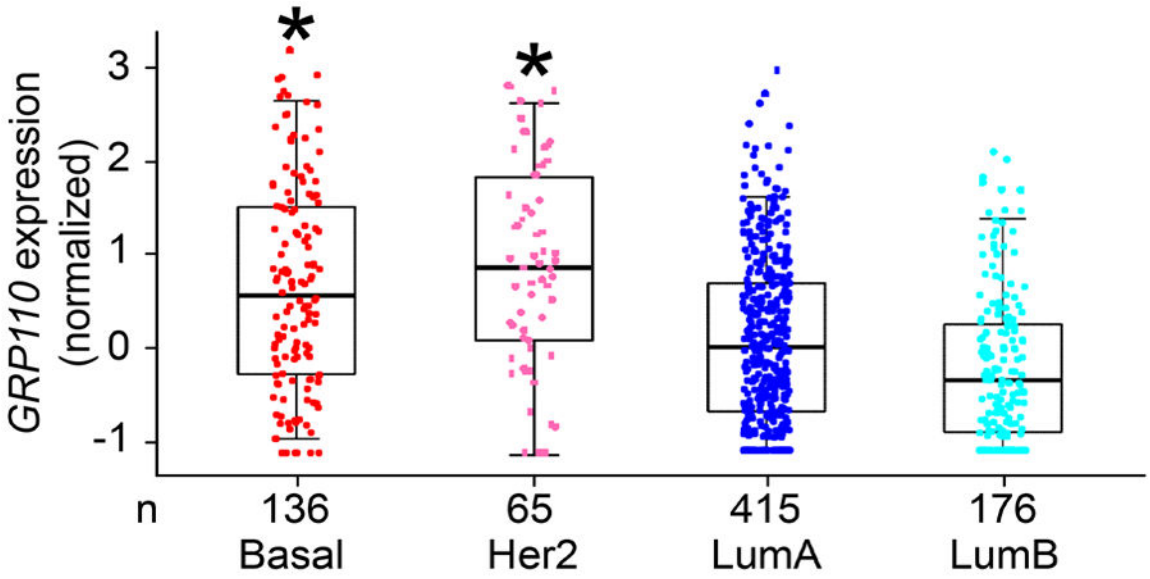

Figure 5. GPR110 expression in publicly available datasets with different BC subtypes.

Publicly available TCGA dataset [25] was used to determine the expression of GPR110 mRNA in different subtypes of BC. Box and Whisker plot was used for showing differential GPR110 expression in basal, HER2+, luminal A, and luminal B subtypes of BC in human patients. As indicated, gene expression of GPR110 was significantly higher in HER2+ and basal like BC compared to luminal A and B subtypes. * indicates $P < 0.05$, t-test on log-transformed data. Box plots represent 5%, 25%, 50%, 75%, and 95%. Log2 FPKM expression values are normalized to standard deviations from the median.

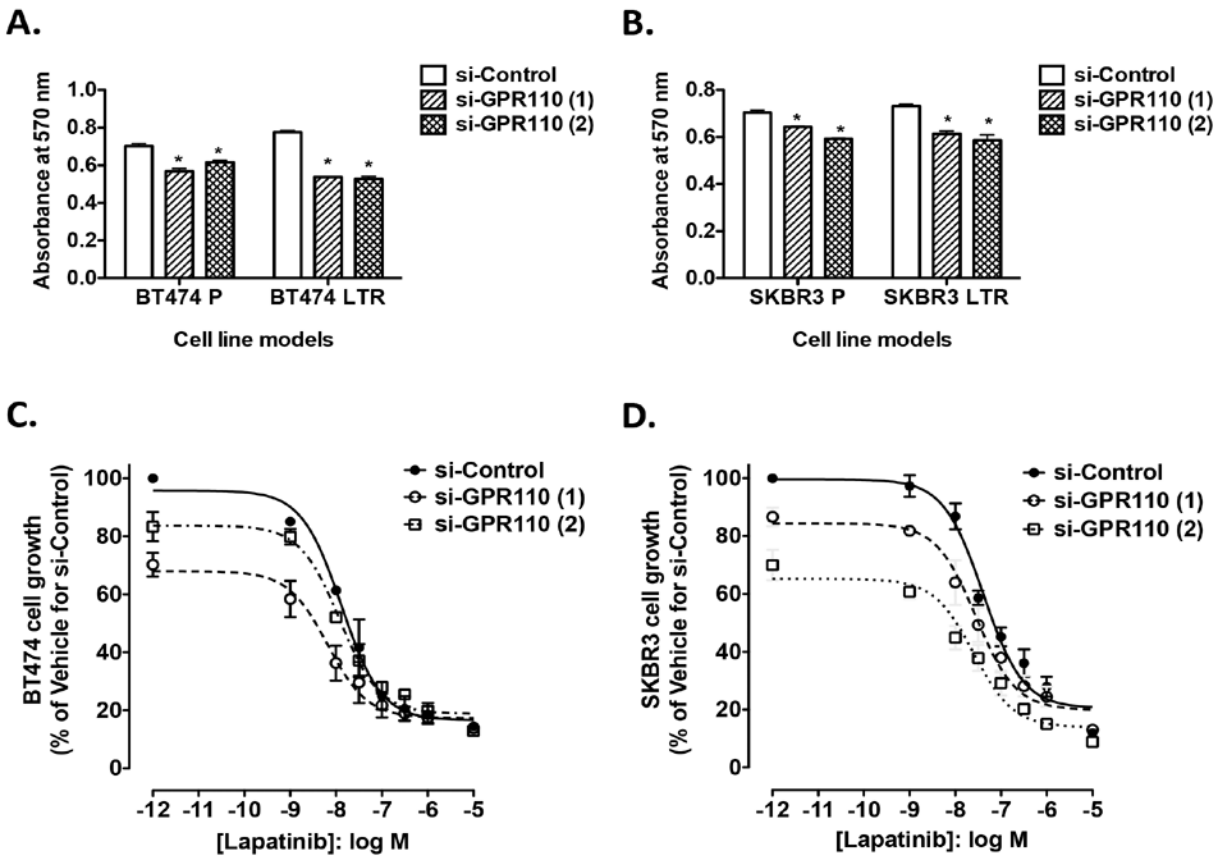

Figure 6. Concentration-response curve of lapatinib in affecting anchorage-dependent cell growth of HER2+ BC cells in presence or absence of GPR110 knockdown by siRNA.

Parental and LTR derivatives of (**A**) BT474 and (**B**) SKBR3 cells were reverse transfected with non-targeting (si-control, solid line) or with 2 independent GPR110-targeting (si-GPR110, dotted lines) siRNAs. Parental (**C**) BT474 and (**D**) SKBR3 cells were also treated with vehicle or various concentrations of lapatinib (1 nM to 10 μM) for 72 hours. MTT assay was conducted using the kit from ATCC to assess cell growth and viability (N=3). The data was normalized to vehicle (100%) and was plotted using the lapatinib concentration ([lapatinib]) in log M for concentration curve. The data was fitted using the 3-parameter logistic equation: Y = Bottom + (Top-Bottom)/(1+10^((X-LogIC50))). The growth of parental and LTR derivatives of BT474 and SKBR3 cells was significantly inhibited by GPR110-targeting siRNAs. But, knockdown of GPR110 did not affect potency of lapatinib in BT474 and SKBR3 parental cells. * indicates $P < 0.05$, Repeated Measures ANOVA followed by Dunnett's post-hoc test.

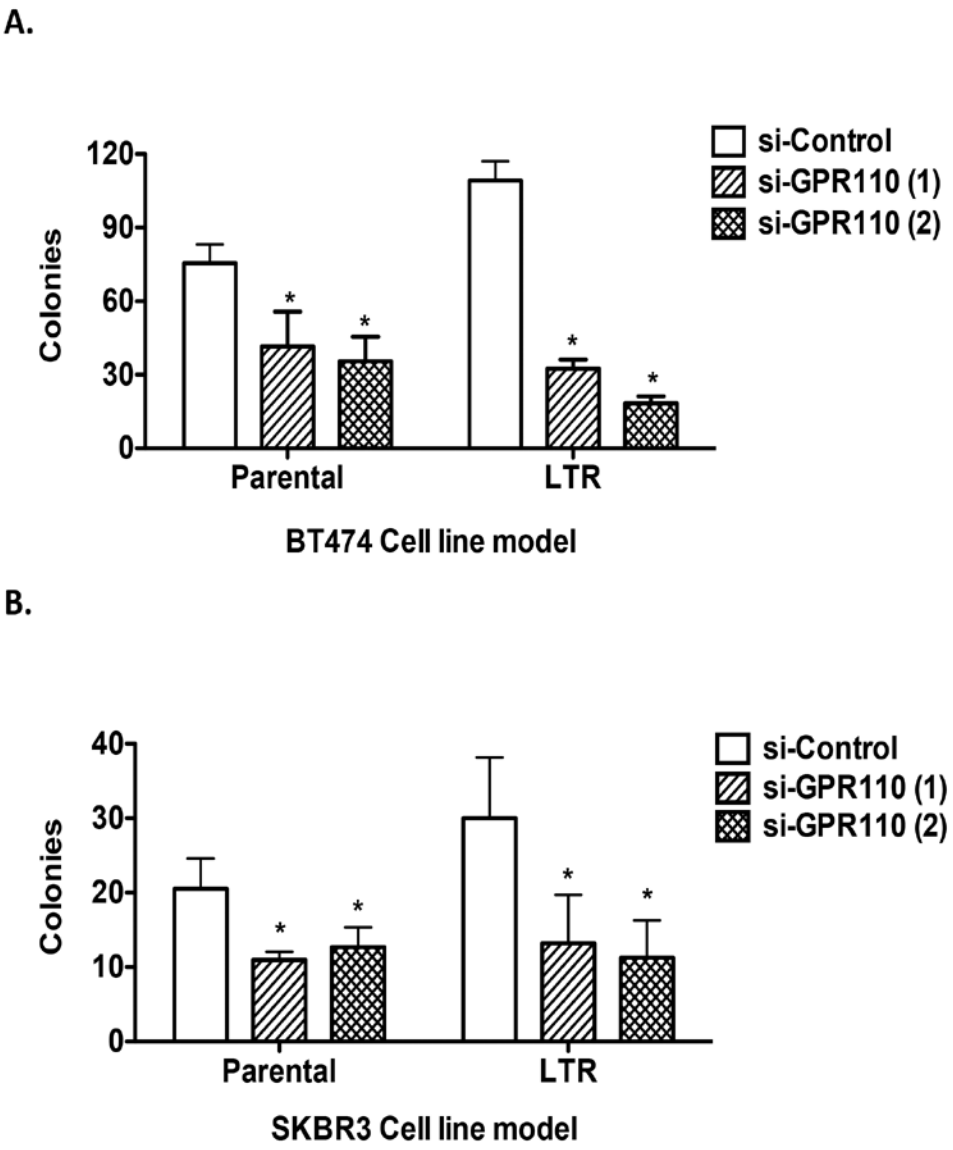

Figure 7. GPR110 knockdown inhibits anchorage-independent cell growth as measured by soft agar assay better in LTR derivatives compared to parental cells in HER2+ BC cell lines.

Parental and LTR derivatives of (**A**) BT474 and (**B**) SKBR3 cells were reverse transfected with non-targeting (si-control, white bar) or with 2 independent GPR110-targeting (si-GPR110, lined bars) siRNAs. After 24 hours, the cells were subjected to soft agar assay to assess anchorage-independent growth (N=3–4). Number of colonies (at least 50 μm in size) was counted on day 10 with Gelcount™ (Oxford Optromix, Germany). Knocking down GPR110 by two independent siRNAs resulted in a significant decrease in the number of colonies in both parental and LTR derivatives of BT474 and SKBR3 models. However, the decrease was more pronounced in the LTR cells compared to parental BT474 and SKBR3 cells. * indicates $P < 0.05$, Repeated Measures ANOVA followed by Dunnett's post-hoc test.

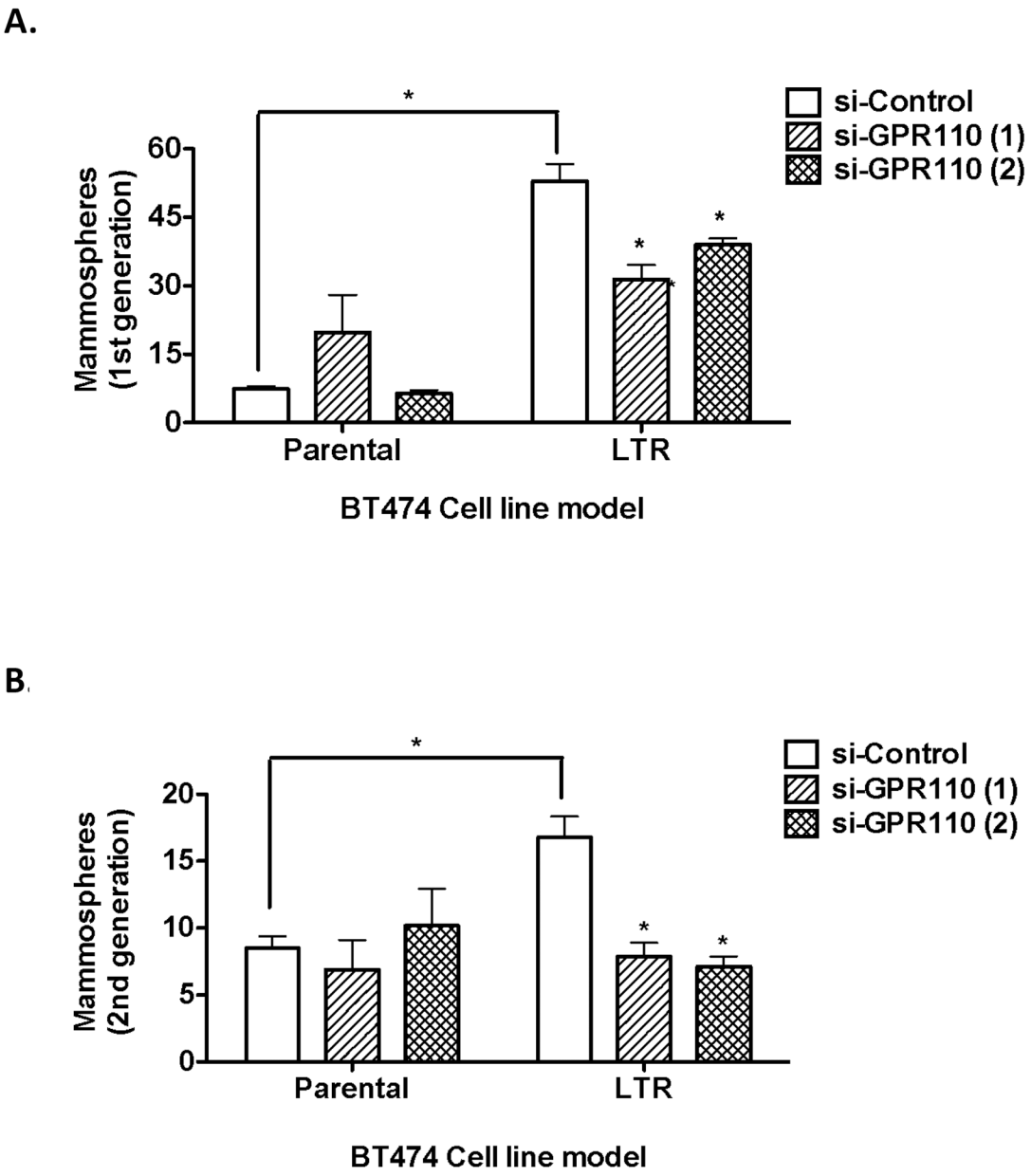

Figure 8. GPR110 knockdown inhibits mammosphere formation in LTR derivatives but not in parental BT474 cells.

BT474 parental cells and their LTR derivatives were reverse transfected with non-targeting (si-control, white bar) or with independent GPR110-targeting (si-GPR110, lined bars) siRNAs. After 24 hours, the cells were subjected to mammosphere assay using the Mammocult media from stem cell technologies in 24-well ultra low attachment plates (N=3). Numbers of (**A**) first- and (**B**) second-generation mammospheres of at least 50 μm in size were counted on day 7 with Gelcount™ (Oxford Optromix, Germany) after plating. BT474 LTR derivatives formed more mammospheres compared to parental cells. GPR110 knockdown by siRNAs resulted in a significant inhibition of first and second generation mammosphere formation in BT474 LTR derivatives, but not in parental cells. * indicates $P < 0.05$, Repeated Measures ANOVA followed by Dunnett's posthoc test.

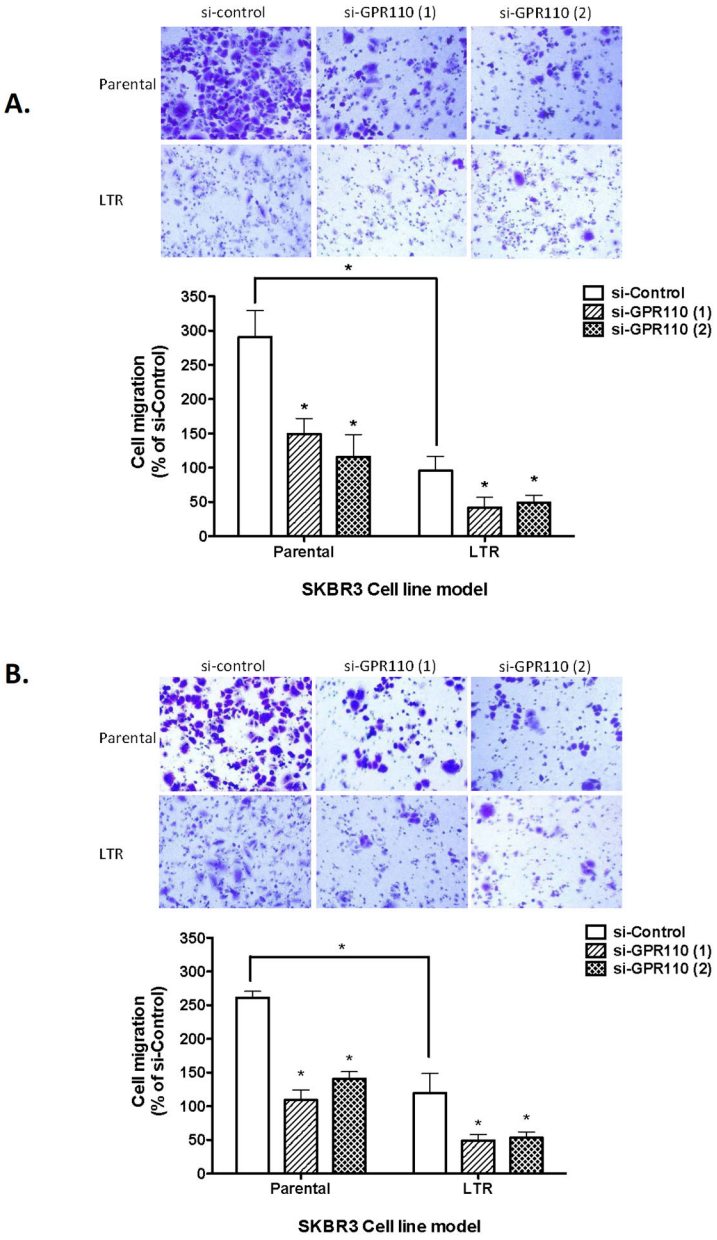

Figure 9. GPR110 knockdown inhibits migration and invasion better in LTR derivatives compared to parental cells of SKBR3 cell line.

SKBR3 parental cells and their LTR derivatives were reverse transfected with non-targeting (si-control, white bar) or with 2 independent GPR110-targeting (si-GPR110, lined bars) siRNAs. After 24 hours, the cells were subjected to migration (**A**) and invasion (**B**) assays using 8μm cell culture inserts (N=3). GPR110 knockdown resulted in significant inhibition of migration and invasion of SKBR3 parental as well as LTR cells. * indicates $P < 0.05$, Repeated Measures ANOVA followed by Dunnett's posthoc test.

Table 1.

Upregulated GPCRs in Aldeflur^{+} tumorigenic cells in BT474 cells (N=4).

Gene name	Class	Family	Endogenous ligands	Fold upregulation (SEM) in Aldeflur+ vs. Aldeflur-
ADORA1	Class A	Adenosine receptors	Adenosine	2.4 (1.7)
BAI3	Adhesion	VII Adhesion GPCR	Glycerol	>15.6 (12.7)
EDG2 (or *LPAR1*)	Class A	LPA receptors	LPA	7.4 (2.8)
EDNRA	Class A	Endothelin receptors	Endothelin-1, 2	8.5 (2.7)
EMR2	Adhesion	Adhesion GPCR	NI	23.4 (10.4)
GCGR	Class B	Glucagon receptor	Glucagon	12.1 (5.3)
GPR110	Adhesion	VI adhesion GPCR	NI	5.3 (0.9)
GPR116	Adhesion	VI adhesion GPCR	NI	28.9 (18.6)
GPR124	Adhesion	VI adhesion GPCR	NI	8.1 (3.3)
GPR87	Class A	Class A orphans	LPA	7.4 (3.4)
MTNR1A	Class A	Melatonin receptors	Melatonin	3.8 (2.3)

Abbreviations: GPCR- G protein coupled receptor, LPA- Lysophospholipid, NI- Not identified, SEM- standard error of mean.

Table 2.

Upregulated GPCRs in anti-HER2-resistant derivatives of BT474 cells (N=2).

Gene	Class	Family	Endogenous ligands	Fold up-regulation vs. Parental (SEM)		
				TR	LR	LTR
CCBP2	Class A	Chemokine receptors	-	2.1 (1.0)	10.7 (5.7)	4.9 (2.2)
CCR9	Class A	Chemokine receptors	*CCL25*	4.0 (0.6)	11.5 (11.4)	23.0 (5.0)
EBI2	Class A	Class A orphans	7α, 25-dihyroxy cholesterol	2.7 (0.7)	4.0 (0.1)	5.9 (3.3)
F2RL1 (*PAR2)*	Class A	Proteinase activated receptors	Serine proteases	1.5 (0.0)	4.2 (0.0)	3.5 (1.2)
GALR2	Class A	Galanin receptors	Galanin; Galanin-like peptide	25.3 (9.3)	2.2 (1.1)	50.1 (2.0)
GPR1	Class A	Class A orphans	Not identified	6.3 (6.1)	104.3 (101.9)	72.6 (65.5)
GPR24	Class A	MCH receptors	MCH	0.9 (0.2)	31.3 (28.1)	113.9 (77.8)
GPR110	Adhesion	Adhesion	Not identified	0.6 (0.3)	7.9 (0.5)	8.7 (0.1)
LGR4	Class A	Class A orphans	R-spondins	1.8 (0.3)	5.4 (0.9)	3.9 (1.0)
OXER1	Class A	Leukotriene receptors	5-oxo-C20; 3, 5-oxo-ETE; 5S-HETE; 5S-HPETE	20.7 (16.6)	30.5 (22.6)	47.7 (25.0)

Abbreviations: 5-oxo-C20:3: (6Z,8Z,11Z)-5-oxoicosa-6,8,11-trienoic acid, 5-oxo- ETE - (6E,8Z,11Z,14Z)-5-oxoicosa-6,8,11,14-tetraenoic acid; 5S-HETE: (6E,8Z,11Z,13E,15S)-15-hydroxy-5-oxoicosa-6,8,11,13-tetraenoic acid; 5S-HPETE: (5S,6E,8Z,11Z,14Z)-5-hydroperoxyicosa-6,8,11,14-tetraenoic acid; MCH- Melanin-concentrating hormone.

Table 3.

Potency (IC_{50}) in nanomolar (nM) of lapatinib in presence of GPR110 siRNAs in various HER2+ BC cell lines.

Cell lines	si-Control	si-GPR110 (1)	si- GPR110 (3)
BT474	13	6.5	11
SKBR3	41	28	25