Cooperative oncogenic effect and cell signaling crosstalk of co-occurring HER2 and mutant PIK3CA in mammary epithelial cells

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Abstract. Though incidence of *PI3K* oncogenic mutation is prominent in breast cancer (20-30%), pharmacological targeting of this signaling pathway alone has failed to provide meaningful clinical benefit. To better understand and address this problem, we conducted genome-wide analysis to study the association of mutant *PI3K* with other gene amplification events. One of the most significant copy number gain events associated with *PIK3CA* mutation was the region within chromosome 17 containing *HER2*. To investigate the oncogenic effect and cell signaling regulation of co-occurring *PIK3CA*-H1047R and or *HER2* gene, we generated cell models ectopically expressing mutant *PIK3CA*, *HER2* or both genetic alterations. We observed that cells with both genetic alterations demonstrate increased aggressiveness and invasive capabilities than cells with either genetic change alone. Furthermore, we found that the combination of the *HER2* inhibitor (CP-724714) and pan *PI3K* inhibitor (LY294002) is more potent than either inhibitor alone in terms of inhibition of cell proliferation and colony formation. Significantly, four cell signaling pathways were found in common for cells with *HER2*, mutant *PIK3CA* and cells with both genetic alterations through an Affymetric microarray analysis. Moreover, the cells with both genetic alterations acquired more significant replication stress as shown by enriched signaling pathways of cell cycle checkpoint control and DNA damage response signaling. Our study suggests co-occurrence of oncogenic *HER2* and mutant *PIK3CA* cooperatively drives breast cancer progression. The cells with both genetic alterations obtain additional features of replication stress which could open new opportunity for cancer diagnostics and treatment.

Introduction

The PI3K/PTEN/AKT signaling pathway has well-established roles in multiple cellular activities, including cell proliferation, survival, metabolism, cytoskeleton reorganization and membrane trafficking (1-3). The abnormal activation of this signaling pathway leads to various diseases such as diabetes, autoimmunity and cancer. About ten years ago, our group, along with several other research groups, simultaneously found that the somatic mutation frequency of the *PIK3CA* gene in breast cancer is 20-30% (4-7). Our research demonstrated that somatic mutation, rather than gain of *PIK3CA* copy number, is one of the most frequent genetic alterations contributing to human breast cancer progression (7). In another study, we comprehensively analyzed and compared the oncogenic properties of nine different *PIK3CA* somatic mutations, which localized in different domains of the *PIK3CA* gene and with different frequencies in human breast cancer (8). The results of our study are consistent with several other groups, using different research systems, and strongly indicate that different *PI3KCA* mutants exhibit different abilities in contributing to cell proliferation, EGF independent growth, cell morphogenesis, transformation, invasion and

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Abbreviations: PI3K, phosphatidylinositol 3-kinase; ErbB2, v-erb-b2 erythroblastic leukemia viral oncogene homolog; aCGH, array comparative genomic hybridization; CNV, copy number variation; EGF, epidermal growth factor; SFIHE, serum-free medium with insulin, hydrocortisone, and epidermal growth factor; SFHE, serum-free medium with hydrocortisone and EGF; SFIH, serum-free medium with insulin and hydrocortisone; SFH, serum-free medium with hydrocortisone; AhR, aryl hydrocarbon receptor; IPA, ingenuity pathway analysis; PAH, poly aromatic hydrocarbons; AMPK, AMP-activated protein kinase

Key words: PIK3CA, HER2, breast cancer, gene amplification

signaling (9-12). These findings collectively provide fundamental biological evidence to support the critical role of the PI3K/AKT signalling pathway in breast cancer progression. However, to date, there is insufficient clinical data to support that PI3K or AKT inhibitors can be powerful single agents for breast cancer patients (13,14).

HER2 (ErbB2), a member of the HER family of tyrosine kinase receptors (HER1-4), is a major driver of tumor growth in 20% of breast cancers. Due to the well-studied nature of the *HER2* gene in breast cancer and the availability of the monoclonal targeting antibody trastuzumab, targeting HER2 has been the most successful targeted treatment for breast cancer patients (15,16). However, targeting HER2 alone was less effective for breast cancer patients with PIK3CA mutations in clinical studies (17,18). In line with these observations, several groups reported that *HER2* amplification and mutation of *PIK3CA* genes could be co-occurring in certain breast cancer population (6,19-22). However, the cooperative effect of these two genetic alterations in comparison with either single genetic change on cell oncogenic properties has not been well investigated.

In this study, we performed a genome-wide analysis for amplification regions and corresponding genes that correlate to mutant *PIK3CA* in 51 human breast cancer cell lines. We also specifically examined the oncogenic properties driven by expressing both mutant *PIK3CA* and *HER2* and compare the effects to cells with either genetic alteration alone. Additionally, we tested the drug treatment response in cells with ectopic expression of mutant *PIK3CA* and *HER2* amplification. Finally, we investigated the downstream target genes and cell signalling pathways regulated by *HER2*, mutant *PIK3CA* and both of these genetic alterations.

Materials and methods

Bioinformatics analysis for amplification of regions that are correlated with mutant PIK3CA. A published database was used for bioinformatic analysis. This database contains gene expression and copy number information for 51 breast cancer cell lines (23) (http://caarraydb.nci.nih.gov/caarray/publicExperimentDetailAction.do?expId=1015897590151581 at http://cancer.lbl.gov/breastcancer/data.php). Among these 51 cell lines, 13 cell lines contain *PIK3CA* mutations. The other 38 are considered *PIK3CA*-wild type. We then used the strategy as follows to identify amplified gene that could synergize with mutant *PIK3CA* in breast cancer. a) Threshold aCGH and gene expression data: copy number variation (CNV) amplification based on a cut-off ≥0.2. Gene overexpression based on a cut-off >143.767 (3-fold of the median of all samples). b) CNV markers and genes with highly increased amplification/overexpression frequency based on the following criteria: i) frequency difference between cell line w/mutations and w/o ≥0.25 or ii) Fisher exact test P-value of the difference <0.05. c) Pairs of amplified CNV markers and overexpressed genes which are close to each other (distance ≤2 Mb). d) Pairs of amplified CNV markers and overexpressed genes which are close to each other (distance ≤2 Mb) and positively correlated.

Cell culture. MCF 10A and HCC1954 cells were obtained from the American Tissue Culture Collection. MCF10A cell lines expressing LacZ (negative control), *PI3KCA*-H1047R, *HER2*, and both *PI3KCA*-H1074R and *HER2* genes were created in our laboratory at the Barbara Ann Karmanos Cancer Institute (KCI). Briefly, full-length *PIK3CA*-H1047R and *HER2* were subcloned into a pENTR vector and recombinated into the pLenti-6/V5-DEST vector. The lentiviruses for the full-length genes were generated using the pLentivirus-expression system (Invitrogen). The generated virus was used to infect targeted model cells. Stable cells were generated after being selected with blasticidin (10 μg/ml, Invivogen) and were grown at 37˚C in an incubator with 5% CO_2. Most of the cell lines used were originally grown in standard SFIHE medium (serum-free medium with insulin, hydrocortisone, and epidermal growth factor) made by Ham's F12 media supplemented with 0.1% bovine serum albumin, 0.5 μg/ml fungizone, 5 μg/ml gentamycin, 5 mM ethanolamine, 10 mM HEPES, 5 μg/ml transferrin, 10 μM T3, 50 μM selenium, 1 μg/ml hydrocortisone, 10 ng/ml EGF, and 5 μg/ml insulin. MCF10A cells expressing HER2 were grown in SFHE medium (serum-free medium with hydrocortisone and EGF). For experimentation, each individual cell line with its own different overexpressed gene was first placed in SFIHE or SFHE medium for a few days, and then placed in either SFIHE medium, SFIH (lacking EGF or epidermal growth factor), SFHE (lacking insulin), or SFH (lacking both EGF and insulin) medium in order to observe cell growth and colony formation. For the invasion assay, cells were first plated in each well insert with SFIH medium, and then SFIHE with 5% FBS was placed outside in the well. Tests on inhibition were done in SFIHE medium.

Cell proliferation/MTT assay. MCF10A/*HER2*, MCF10A/*PIK3CA*-H1047R, MCF10A/*HER2*/*PIK3CA*-H1047R and MCF10A/LacZ were seeded in triplicate at a density of $2x10^3$ cells per well in 96-well plates on day 0. Cells were grown in SFIHE (growth medium with insulin, hydrocortisone and EGF), SFIH (growth medium lacking EGF) or SFHE (growth medium lacking insulin) or SFH (lacking both EGF and insulin) medium. Cell proliferation was measured with an MTT assay kit (Fisher Scientific, Pittsburgh, PA, USA), trypsinized and counted with a particle counter (Beckman Coulter) with an absorbance value of 540 nm on days 1, 3, 5, and 7 to make the growth curve. Media was changed at each of these time points.

Colony formation. Each cell line was seeded in two wells of a 6-well plate with a density of $2x10^3$ cells per well for 1-2 weeks in SFIHE, SFIH, SFHE, or SFH culture mediums. As soon as distinct clones were visible with a microscope and were almost starting to merge, all plates were stained with crystal violet on day 12 of growth for approximately 5 min and then exposed to water gently. The numbers of individual clones were then counted and recorded, and the plates were scanned for clear pictures of clones.

Migration and invasion assay. A cell migration and cell invasion assay was performed in BD control chamber and BioCoat Matrigel Invasion chambers (BD Bioscience, Corning, NY, USA) according to the manufacturer's instructions. Cells ($5x10^4$) were plated in the upper chamber in 500 μl of SFIH

growth medium without FBS, and the lower chamber was filled with 750 μl of SFIHE growth medium with 5% FBS. After 48-h incubation, the cells that had invaded to the underside of the membrane were fixed and stained with the HEMA 3 kit (Fisher Scientific). The migration and invasive cells were then determined by counting the stained cells by microscopy of x100 and images were taken.

Inhibition of cell proliferation or colony formation by inhibitors. LY294002 [pan PI3K inhibitor (24,25)], and CP-724714 [selective HER2 inhibitor (26,27)] were diluted to the concentrations of 3 and 10 μM and 0.1 and 1 μM in SFIHE medium, respectively, according to previous data. SFIHE medium with either concentration of the PI3K inhibitor, the HER2 inhibitor, or both inhibitors at both concentrations were added to only the MCF10A cell line expressing both the mutant *PIK3CA*-H1047R and the *HER2* gene in a 96-well plate for cell growth, and in 6-well plates for colony formation with $2.5x10^4$ cells/well. Medium with fresh inhibitors was replaced every other day. For the 96-well plate, cells were trypsinized and counted with a particle counter (Beckman Coulter) with an absorbance value of 540 nm on the 9th day of growth. Clones in the 6-well plates were stained with crystal violet on the 12th day of experimentation, and clones were counted. The plates were also scanned for pictures of clones.

Affymetrix microarray. Gene expression levels for the four different cell lines were measured with Human Gene 2.1 ST Array Strip at the Genomics Core of University of Michigan. Microarray data reported herein have been deposited at the NCBI Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE99512) with the accession number GSE99512. Microarray analyses and pathway analysis were performed by the Biostatistics Core of the KCI. The raw intensity probe-level values were background corrected, log2 transformed, quantile normalized, and summarized to probe set level expression values. The 'rma' function from R package 'oligo', based on robust multi-array average (RMA) method (28), was used for all above pre-processions. Control type of probe sets, such as 'Background probes', 'Poly-A controls' and 'Hybridization controls', were excluded from further analyses. Probe sets with log2 expression values less than 4 for any two cell lines compared were excluded from the analyses of differential expression (DE) probe sets. The k-mean clustering method was used for identifying DE probe sets based on each of three comparisons *PIK3CA* vs. MCF10A_Control, *HER2* vs. MCF10A_Control, and *PIK3CA_HER2* vs. MCF10A_Control. The identified up-/down-regulated probe sets were subjected to ingenuity pathway analysis (IPA). IPA canonical pathway analysis identified the enriched pathways that were most significant to the data set. The significance of the association between the data set and the IPA canonical pathway was measured by the Fisher's exact test. The -Log (P-value) ≥3 was used as the cut-off for identifying the common or unique pathways across three comparisons.

Results

Bioinformatics analysis reveals the co-occurrence of mutant PIK3CA and HER2 amplification in breast cancer cells. Six distinct chromosomal regions were identified within chromosomes 3, 7, 12, 14, 16, and 17 as having increased frequency of amplification in *PIK3CA*-mutant versus *PIK3CA*-WT cell lines. The regions with the most amplification include Chromosome 7, 14, 16, and 17 ($P<0.05$, Table I). In particular, the *ERBB2*/*HER2* region was one of the most correlated regions. The percent difference of amplification of the *HER2* region between the cell lines with and without the *PIK3CA* mutation was 37%, which indicated that there may be a synergistic relationship between the *PIK3CA* mutation and *HER2* gene amplification. In addition to the chromosome 17 region, which contains *HER2* and two other genes, multiple other chromosomal regions were also found to be correlated with *PIK3CA* mutation. Chromosome 7 region contains three genes. Chromosome 12 region contains ten genes. Chromosome 14 region contains seven genes and chromosome 16 contains 11 genes (Table II).

Table I. Chromosome regions show different CGH frequency between cell lines with different status of PIK3CA.

Chromosome	Difference in CGH frequency (%)	Fisher P-value
3	33	0.07
7	39.40	0.04
12	30	0.09
14	36.60	0.04
16	46.10	0.002
17	38.40	0.04

Co-occuring HER2 and mutant PIK3CA-H1047R synergistically enhance cell proliferation, colony formation, and cell invasion in MCF10A cells. To specifically study the cooperative effect of mutant *PIK3CA* and *HER2* genes in breast cancer cell growth, we chose to create MCF10A cell models expressing mutant *PIK3CA*-H1047R (MCF10A/*PIK3CA*-H1047R), *HER2* (MCF10A/*HER2*) or both mutant *PIK3CA*-H1047R and *HER2* genes (MCF10A/*HER2*/*PIK3CA*-H1047R). A MCF10A cell line expressing LacZ was used as a control (MCF10A/LacZ) (Fig. 1A). A cell proliferation assay was performed in SFIHE, SFIH, SFHE, and SFH media in order to address proliferation under different growth stimulating conditions. In SFIHE medium, all the cell models demonstrate similar proliferation rate (Fig. 1B). However, in media deprived of the growth factor EGF and insulin, the MCF10A/*HER2*/*PIK3CA*-H1047R cells exhibit the greatest growth advantage over MCF10A/*PIK3CA*-H1047R, MCF10A/*HER2*, and MCF10A/LacZ (negative control) (Fig. 1B). Epidermal growth factor (EGF) is an essential factor in the acceleration of cell growth in cancer cells (29-31). Insulin allows cells to use glucose for energy and maintains the glucose level in blood and is tightly associated with cancer cell growth and proliferation (32-34). This finding suggests a cooperative effect of *HER2* and mutant *PIK3CA* that is insulin- and EGF-independent. A colony formation assay showed similar synergistic effect of the two genetic alterations on the promotion of colony formation (Fig. 1C and D).

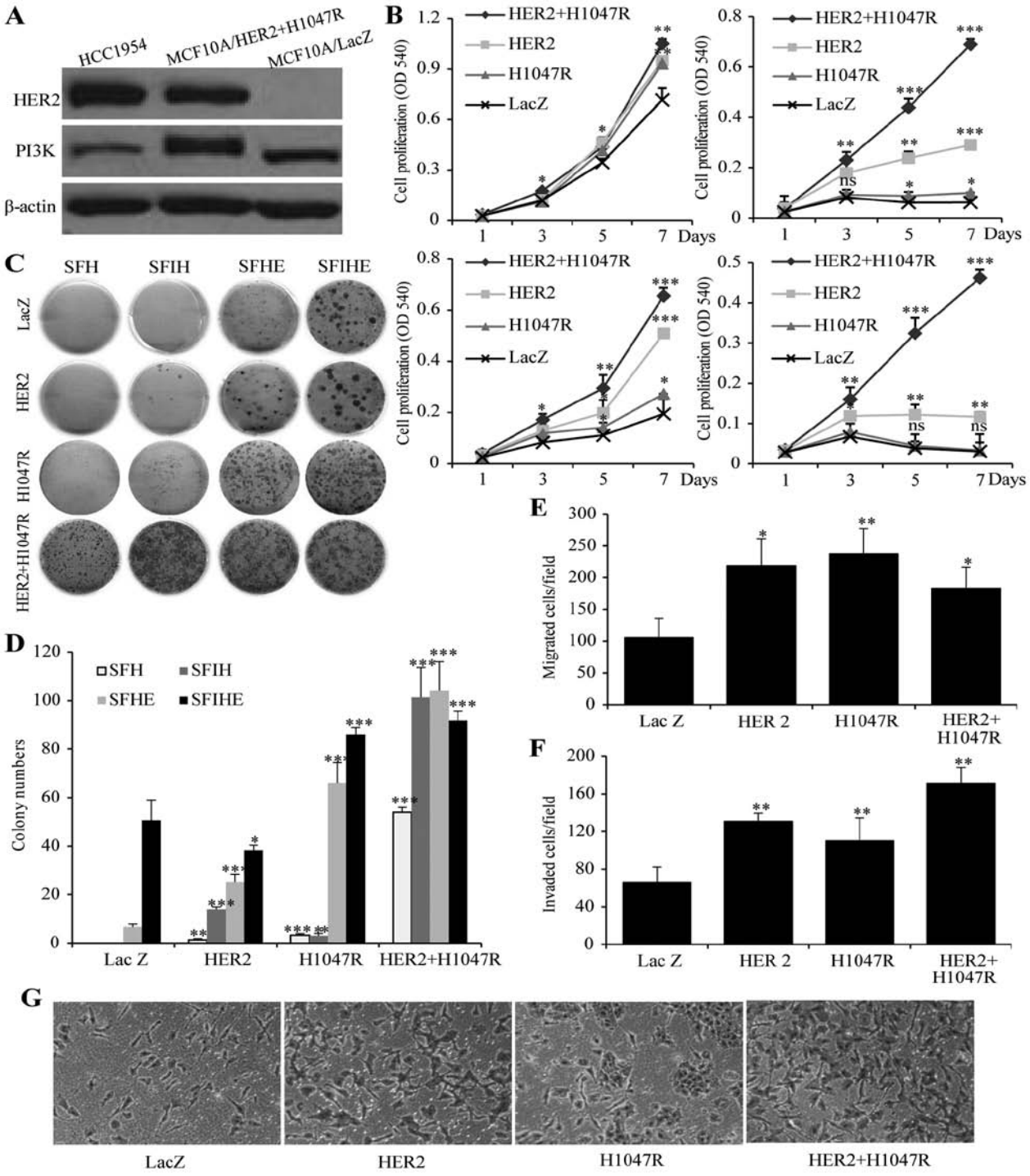

Figure 1. The effect of *HER2* or/and *PIK3CA*-H1047R on cell proliferation, invasiveness and colony formation. (A) Expression of *PIK3CA* and *HER2* gene in MCF10A/*HER2*/*PIK3CA*-H1047 cells and HCC1954 cells. MCF10A/LacZ was used as negative control. (B) Cell proliferations of MCF10A/*HER2*/*PIK3CA*-H1047R, MCF10A/*HER2*, MCF10A/*PIK3CA*-H1047R and MCF10A/LacZ cells when cultured in SFIHE (top left), SFIH (top right), SFHE (bottom left), and SFH (bottom right) media. (C) Different capability in colony formation of MCF10A/*HER2*/*PIK3CA*-H1047R, MCF10A/*HER2*, MCF10A/*PIK3CA*-H1047R and MCF10A/LacZ cells when cultured in SFH, SFHE, SFIH and SFIHE media. (D) Summary of the colony formation assay under different cell culture conditions. Data are mean ± SD. (E) MCF10A/*HER2*/*PIK3CA*-H1047R, MCF10A/*HER2* and MCF10A/*PIK3CA*-H1047R exhibited higher migration ability than MCF10A/Lac Z (negative control). (F) The effects of MCF10A/*HER2*/*PIK3CA*-H1047R, MCF10A/*HER2*, MCF10A/*PIK3CA*-H1047R and MCF10A/LacZ on cell invasion. Columns mean of three independent experiments. Bars mean SD. The MCF10A/LacZ was used as a control (100%). The invasion ability (%) was measured by the invading cell number of each cell line comparing to the invading cell number of MCF10A/LacZ cell line. Student's t-test was used for statistical analysis. $^{***}P<0.001$, $^{**}0.01>P>0.001$; $^{*}0.05>P>0.01$; ns, not significant. (G) Representative images showing invading cells in Matrigel invasion chambers for four cell models. Original magnification, x100.

We further investigated the effect of *HER2*, the mutant *PIK3CA*-H1047R, or both genetic alterations on the migration and invasion using a chamber assay. In the migration assay, all the oncogene expressing cells showed higher migration efficiency than control cell MCF10A/LacZ. MCF10A/*HER2* and MCF10A/*PIK3CA*-H1047R showed a similar migration

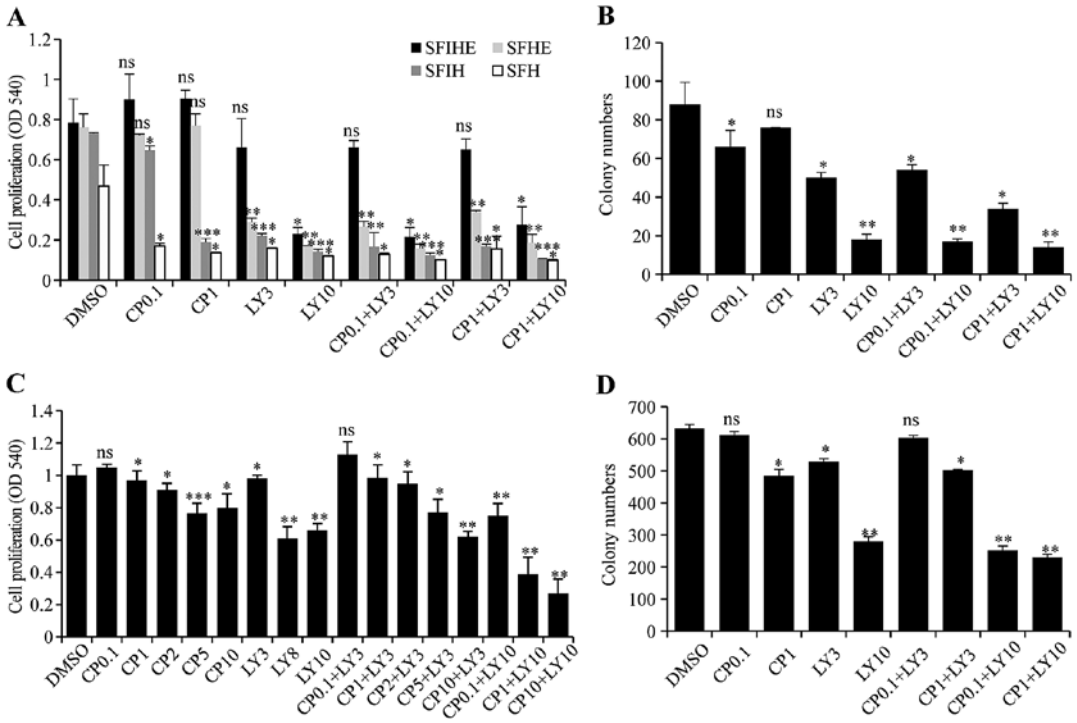

Figure 2. Inhibitory effects on cell proliferation and colony formation when the established MCF10A models and breast cancer cell were treated with PI3K and HER2 inhibitor individually and in combination. (A) Cell proliferations were detected with treatment of LY294002 (LY) and/or CP724714 (CP) in four different media. Combination of both inhibitors played drastic role in decreasing cell viabilities of MCF10A/*HER2*/*PIK3CA*-H1047R cells. (B) The effect of LY294002 (LY) and/or CP724714 (CP) treatment on colony formations of MCF10A/*HER2*/*PIK3CA*-H1047R cell model. Evaluation of inhibitory effect of LY294002 (LY) or/and CP724714 (CP) on cell proliferation (C) and colony formation (D) of HCC1954 cells. For all the experiments in this figure, drug dose (μM) is shown next to the drug name. Data represent the mean ± SD of three independent experiments. Student t-test was used for statistical analysis. *0.05>P>0.01, **0.01>P>0.001, ***P<0.001; ns, not significant.

cell numbers, while MCF10A/*HER2*/*PIK3CA*-H1047R did not demonstrate higher migration capability than either oncogene alone (Fig. 1E). In the invasion assay, all the oncogene-expressing cells showed higher invasive efficiency than control cell MCF10A/LacZ. Specifically, MCF10A/*HER2*/*PIK3CA*-H1047R demonstrated a stronger invasion capability than MCF10A/*HER2* and MCF10A/*PIK3CA*-H1047R cells (Fig. 1F and G).

Pharmacological inhibition of HER2 and PIK3CA-H1047R in MCF10A/HER2/PIK3CA-H1047R and HCC1954 cells. To test whether the cell model (MCF10A/*HER2*/*PIK3CA*-H1047R) with both genetic alterations can be synergistically inhibited by simultaneous targeting of both genes, LY294002, a pan-PI3K inhibitor, was used for targeting PIK3CA-H1047R and CP-724714, a selective HER2 inhibitor, was used for targeting active HER2. The results showed that the cells treated with CP-724714 had little to no change in cellular proliferation. Low dose LY294002 (3 μM) alone also had a minimal efficacy as an inhibitor of cell growth. However, cells exposed to a combination of both inhibitors with 10 μM LY294002 had a drastic decrease in cell proliferation. The combined inhibition effects were more significant in SFIH, SFHE, and SFH medium than in SFIHE medium (Fig. 2A and B).

To validate the results obtained from the established MCF10A/*HER2*/*PIK3CA*-H1047R cell model, we repeated drug treatment using the HCC1954 cell line, which contains both endogenous *PIK3CA* mutation and *HER2* amplification. The treatment with different doses of CP724714 (0.1, 1, 2, 5, and 10 μM) marginally changed cell growth. The treatment of HCC1954 cells with LY294002 only significantly inhibited cell proliferation at high doses (8 and 10 μM). The combination of these two drugs evinced a dose-dependent inhibition in cell proliferation. With a low dose of LY294002 (3 μM) treatment, increasing the dose of CP724714 from 0.1 to 10 μM resulted in a cell proliferation inhibition effect similar to the high dose of LY294002 (10 μM). However, when a high dose of LY294002 (10 μM) was used, further treatment with CP724714 at 0.1, 1, 10 μM obtained an even more significant reduction in cell growth (Fig. 2C). A similar result was obtained in colony formation assay when HCC1954 cells were treated with different combinations (Fig. 2D).

Common and distinct downstream genes and signaling pathways controlled by HER2 and/or mutant PIK3CA. The study of the biological function of three different cell models showed that the MCF10A/*HER2*/*PIK3CA*-H1047R cells harbor unexpected characteristics in comparison to MCF10A/*HER2* and MCF10A/*PIK3CA*-H1047R including EGF and insulin-independent growth, stronger invasion capability, and sensitive only to double inhibition. These results suggest that the co-occuring oncogenic *HER2* and mutant *PIK3CA* could lead to activation of novel cell signaling pathways which drive unique oncogenic properties in cancer cells. To investigate the underlying mechanism of the functional differences between cells with mutant *PIK3CA*, *HER2* or both mutant *PIK3CA* and *HER2*, we performed an Affymetrix microarray analysis. After normalization with MCF10A/LacZ control cells,

Table II. Differential amplified chromosome regions and genes.

Clone	Chrom	HUGO	Description
CTD-2172D17	3	EIF4G1	Eukaryotic translation initiation factor 4 γ, 1
CTD-2172D17	3	ABCC5	ATP-binding cassette, sub-family C (CFTR/MRP), member 5
CTD-2172D17	3	EIF2B5	Eukaryotic translation initiation factor 2B, subunit 5 (ε, 82 kD)
CTC-329F6	7	FTSJ2	FtsJ homolog 2 *(E. coli)*
GS1-165K6	7	AHR	Aryl hydrocarbon receptor
GS1-165K6	7	AGR2	Anterior gradient 2 homolog *(Xenepus laevis)*
RP11-270J9	12	U5-100K	prp28, U5 snRNP 100 kd protein
RP11-112M10	12	GALNT6	GalNAc-T6
RP11-101H10	12	TARBP2	TAR (HIV) RNA binding protein 2
RP11-101H10	12	MGC11308	Hypothetical protein MGC11308
RP11-101H10	12	AAAS	Achalasia, adrenocortical insufficiency, alacrimia
RP11-132H4	12	HOXC10	Homeo box C10
VYS12P2692	12	SAS	Sarcoma amplified sequence
RP11-234C19	12	PXN	Paxillin
RP11-234C19	12	MGC5139	Hypothetical protein MGC5139
RP11-234C19	12	GCN1L1	GCN1 general control of amino-acid synthesis 1-like 1 (yeast)
RP11-70F9	14	THTP	Thiamine triphosphate
RP11-189N14	14	PCK2	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)
RP11-189N14	14	TM9SF1	Transmembrane 9 superfamily member 1
RP11-189N14	14	FLJ23338	Importin 4
RP11-189N14	14	LOC51016	CGI-112 protein
RP11-189N14	14	TINF2	TERF1 (TRF1)-interacting nuclear factor 2
RP11-111F22	14	RNB6	RNB6
CTD-2271I17	16	CES2	Carboxylesterase 2 (intestine, liver)
CTD-2271I17	16	ELMO3	Engulfment and cell motility 3 (ced-12 homolog, *C. elegans*)
RP11-354N7	16	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)
RP11-354N7	16	VPS4A	Vacuolar protein sorting factor 4A
RMC16P004	16	LOC64146	Peptide deformylase-like protein
RP11-253O10	16	RAP1	TRF2-interacting telomeric RAP1 protein
RP11-110L8	16	MBTPS1	Membrane-bound transcription factor protease, site 1
RP11-162I18	16	USP10	Ubiquitin specific protease 10
RP11-118F19	16	LOC51659	HSPC037 protein
RP11-122P17	16	KIAA0182	KIAA0182 protein
RMC16P026	16	GALNS	Galactosamine (N-acetyl)-6-sulfate sulfatase
RMC17P077	17	GRB7	Growth factor receptor-bound protein 7
RMC17P077	17	ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
RMC17P077	17	MGC9753	Hypothetical gene MGC9753

Table III. Four common pathways in HER2, mutant PIK3CA and mutant PIK3CA+HER2 overexpression models.

Ingenuity canonical pathways	Common genes
Aryl hydrocarbon receptor signaling	RELA, NFIX, MYC, CTSD, ALDH7A1, TP53, MED1, NQO1, BAX, CYP1B1, CDKN1A, ALDH3B1, NRIP1, HSPB1
AMPK signaling	SMARCD2, MAPK13, ELAVL1, CRTC2, TSC2, PIK3R2, AKT1S1, CPT1A, GRB2, STK11, PPP2R1A, CDKN1A
Protein ubiquitination pathway	USP5, HSPA1A/HSPA1B, SACS, BAG1, USP36, USP19, PSMD3, SKP2, USP31, PSMD11, BAP1, HSPB1, USP21, PSMB3, HLA-A
Hereditary breast cancer signaling	SMARCD2, POLR2C, PIK3R2, TP53, GRB2, POLR2E, CDKN1A, SMARCC1, HLTF, RFC3

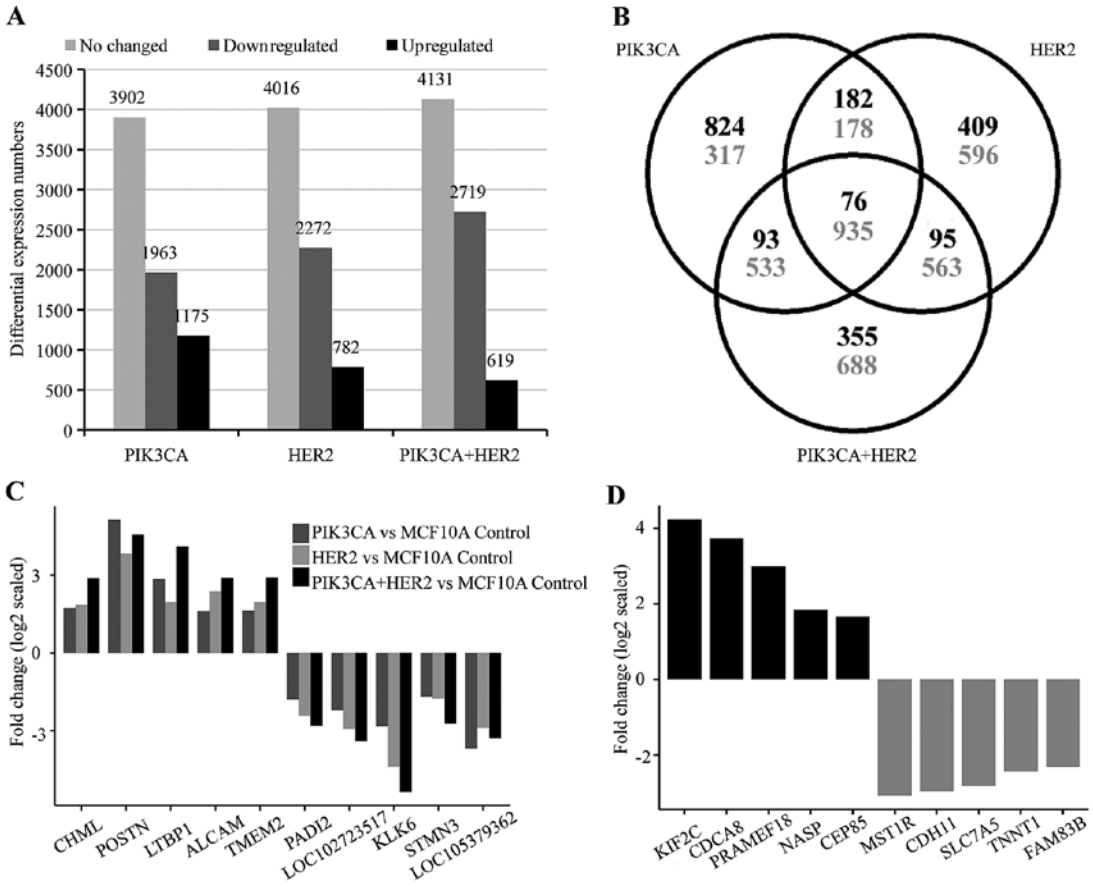

Figure 3. Downstream target genes regulated by *HER2*, mutant *PIK3CA* and both of *HER2* and mutant *PIK3CA*. (A) The resulted number of probe sets that upregulated, downregulated, or no change in MCF10A/*HER2*, MCF10A/*PIK3CA*-H1047R and MCF10A/*HER2*/*PIK3CA*-H1047R models after compared to control cell model MCF10A/LacZ. (B) Venn diagram shows common and differential expressed probe sets among three cell models. Black: upregulated; gray: downregulated. (C) The top 5 upregulated and top 5 downregulated common downstream gene targets in three comparisons of MCF10A/*HER2* vs. MCF10A/LacZ; MCF10A/*PIK3CA*-H1047R vs. MCF10A/LacZ, and MCF10A/*HER2*/*PIK3CA*-H1047R vs. MCF10A/LacZ control. (D) Specific deregulated genes were identified in MCF10A/*HER2*/*PIK3CA*-H1047R cells, but not in either MCF10A/*HER2* or MCF10A/*PIK3CA*-H1047R cells, after compared to MCF10A/LacZ control cells. Top 10 up-/down-regulated genes are shown here. Probe sets with log2 expression values <4 for any two cell lines compared were excluded.

MCF10A/*PIK3CA*-H1047R cells showed 1,175 upregulated, 1,963 downregulated and 3,902 no changed genes; MCF10A/*HER2* cells showed 762 upregulated, 2,271 downregulated and 4,016 no changed genes. The MCF10A/mutant *PIK3CA*/-*HER2* showed 619 upregulated, 2,719 downregulated and 4,131 no changed genes (Fig. 3A). Although there are 76 upregulated and 935 downregulated common genes shared in all three cell models, each cell model was shown to contain hundreds of unique genes (Fig. 3B-D).

Further ingenuity pathway analysis showed that 24, 20 and 18 cell signaling pathways were enriched in MCF10A/*PIK3CA*-H1047R, MCF10A/*HER2* and MCF10A/*HER2*/*PIK3CA*-H1047R, respectively, with a cut-off of -log (P-value) ≥3 (Fig. 4A-C). Under this condition, four common cell signaling were identified in all three cell models, including aryl hydrocarbon receptor (AhR) signaling, AMP-activated protein kinase (AMPK) signaling, protein ubiquitination pathways and hereditary breast cancer signaling (Fig. 4D and E, and Table III). Additionally, 25% (7 out of 24) signaling pathways in cells expressing *PIK3CA*-H1047R overlapped with 35% (7 out of 20) signaling pathways in *HER2* overexpression cells (Fig. 4D). There is 55% (10 of 18) signaling pathways enriched only in cells with both *PIK3CA*-H1047R and *HER2* and do not overlap with cells expressing either mutant *PIK3CA* or *HER2* alone (Fig. 4F). Importantly, 50% (5 out of 10) of these cell signaling pathways are related to DNA replication stress, including role of BRCA1 in DNA damage response, role of CHK1 in cell cycle checkpoint control, estrogen-mediated S-phase entry, cell cycle control of chromosomal replication and mismatch repair in eukaryotes (Fig. 4F and Table IV).

Discussion

Our current study represents the first large scale genome-wide screening of gene amplifications that correlate with the presence of activating *PIK3CA* mutations in a panel of 51 human breast cancer cell lines. Our results indicate that in the human genome there are multiple other amplification regions besides the *HER2* region localized at different chromosomes that correlate with the *PIK3CA* mutation in human breast cancer. Further study of these novel regions could identify more novel therapeutic targets in the future for breast cancer treatment.

Using the cell models established, we showed that the co-occurrence of *PIK3CA* mutation and *HER2* gene amplification greatly enhances proliferation. In the MTT assay, the MCF10A/*HER2*/*PIK3CA*-H1047R cell model exhibited

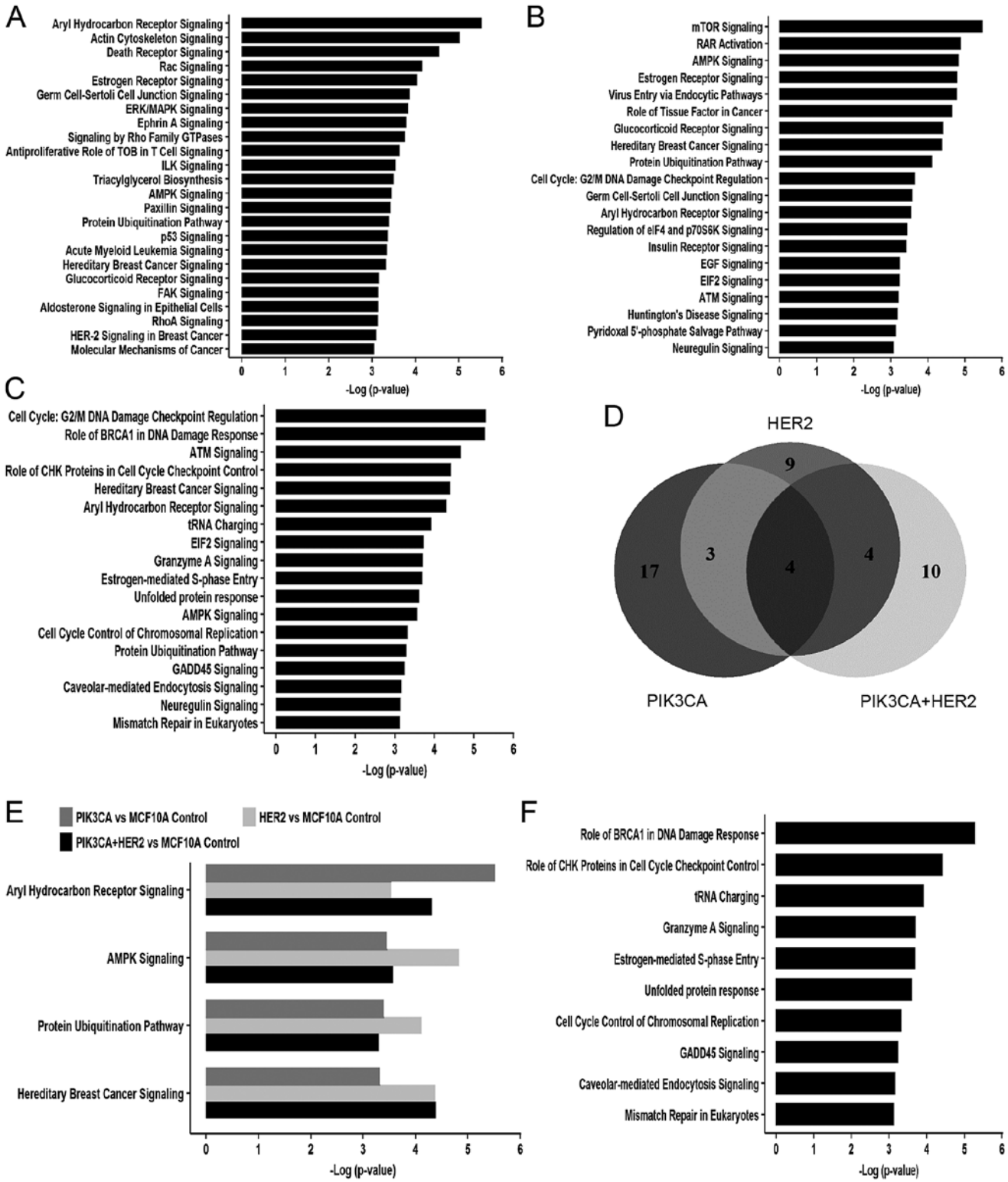

Figure 4. IPA canonical pathway analysis on targeted gene regulated by *HER2*, mutant *PIK3CA* and both of *HER2* and mutant *PIK3CA*. (A-C) The signaling pathway controlled by mutant *PIK3CA* (A), *HER2* (B) and both mutant *PIK3CA* and *HER2* (C) with -log (P-value) ≥3. (D) Venn diagram shows common and differential cell signaling pathways regulated by mutant *PIK3CA*, *HER2* and both mutant *PIK3CA* and *HER2*. (E) Four common signaling pathways with -Log (P-value) ≥3, among mutant *PIK3CA*, *HER2*, and both mutant *PIK3CA* and *HER2* overexpression cells after compared to MCF10A control cell model. (F) Top ten signaling pathways with -Log (P-value) ≥3 in cells with both mutant *PIK3CA* and *HER2* overexpression, but not in cells expressing either *HER2* or mutant *PIK3CA*.

highest proliferation in all four media, and a synergistic effect was evident in SFH (medium lacking insulin and EGF) and in SFIH (medium lacking EGF). In the colony formation assay, the MCF10A/*HER2*/*PIK3CA*-H1047R cell model had the most colonies than any other cell model in all media. We also determined that the cooperative action of *HER2* and *PIK3CA*-H1047R genes is EGF and insulin-signaling pathway independently, because even in conditions lacking insulin and

Table IV. Top 10 signaling pathways enriched only in mutant PIK3CA and HER2 co-occuring model.

Ingenuity canonical pathways	Genes
Role of BRCA1 in DNA damage response	ARID1A, FANCF, SMARCD2, RBL1, CHEK1, RB1, FANCD2, SMARCB1, RFC2, BRCA1, BRIP1, BLM, TP53, E2F4, PLK1, SMARCD1, RFC5, FANCL, NBN, MSH2, RFC4, CDKN1A, BRCA2, SMARCC1, HLTF, FANCA, RFC3
Role of CHK proteins in cell cycle checkpoint control	TP53, CDC25C, E2F4, PLK1, RFC5, CDK1, CHEK1, NBN, PPP2R1A, PCNA, RFC4, CDKN1A, HUS1, ATMIN, RFC2, CLSPN, TLK2, BRCA1, CDK2, RFC3
tRNA charging	CARS, MARS2, LARS2, MARS, QARS, FARSA, WARS, YARS, FARS2, VARS2, AARS, VARS, SARS, SARS2, IARS
Granzyme A signaling	HIST1H1B, SET, HIST1H1C, NME1, CREBBP, HMGB2, H1F0, APEX1, EP300
Estrogen-mediated S-phase entry	MYC, RB1, CCNA2, E2F4, CCNE2, CDKN1A, RBL1, CCND1, CDK1, CDK2, SKP2
Unfolded protein response	SCAP, MAP2K7, ERN1, INSIG1, HSPH1, HSPA1A/HSPA1B, HSPA9, CEBPD, CANX, OS9, MAP3K5, CEBPB, CEBPG, MBTPS2, SEL1L, MBTPS1, PPP1R15A, AMFR
Cell cycle control of chromosomal replication	MCM5, MCM3, MCM6, PCNA, PRIM1, POLA1, CDC6, CDC7, ORC6, TOP2A, MCM4, DBF4, CDK2, ORC1
GADD45 signaling	TP53, PCNA, CCNE2, CDKN1A, BRCA1, CCND1, CDK1, CDK2, CCNB1
Caveolar-mediated endocytosis signaling	B2M, FLNB, HLA-A, ITGA2, COPE, ABL1, ITGA6, ITGA10, ITGB8, CD55, ITGA3, FLOT2, ACTA2, HLA-C, ITGB4, ITGB6, ACTG1, PTRF, DNM2, ITGB5, EGFR
Mismatch repair in eukaryotes	PCNA, MSH2, RFC4, RFC2, FEN1, RFC5, EXO1, RFC3

EGF, the growth of cells with both genetic alterations is similar to that in regular conditions (SFIHE medium) suggesting a strong self-sufficient capability (Growth factor independent proliferation). Consistent with previous studies (35,36), the treatment with two inhibitors is more potent in inhibiting cell proliferation and colony formation than using either inhibitor alone in cells with both *HER2* and *PIK3CA* mutation.

We demonstrated that AhR signaling, AMPK signaling, protein ubiquitination pathway and hereditary breast cancer signaling are four common signaling pathways in cells expressing *HER2*, mutant *PIK3CA* and *HER2* plus mutant *PIK3CA*. The AhR is a ligand-activated transcription factor that regulates a battery of genes in response to exposure to a broad class of environmental poly aromatic hydrocarbons (PAH). AhR is historically characterized for its role in mediating the toxicity and adaptive responses to these chemicals. However, mounting evidence has established a role for it in ligand-independent physiological processes and pathological conditions, including cancer. The AhR is overexpressed and constitutively activated in advanced breast cancer cases and was shown to drive the progression of breast cancer (37-39). The AMPK is a central regulator of cellular metabolism and energy homeostasis in mammalian tissues. The importance of such regulation of fundamental process poses the AMPK signaling pathway in one of the most attractive therapeutic targets in diabetes and cancer (40-42). Ubiquitination is a protein posttranslational modification reservedly regulated by a series of ubiquitination-associated enzymes. The cellular functions of ubiquitination span a wide spectrum that includes cell death, DNA damage repair, autophagy, proteasomal degradation of proteins and metabolism (43). Thus, dysregulation ubiquitination has broad consequences that may lead to aberration of tumor-promoting pathways and tumor-suppressing pathways (44,45). Consistent with these published results, the finding of our study highlights that these pathways are basic and essential fundamental signaling pathways in *HER2* and *PIK3CA* driven breast cancer.

More importantly, we identified 10 signaling pathways that were specifically enriched in the cells with both mutant *PIK3CA* and *HER2* overexpression. Among these pathways, we found that 50% (5/10) are related to cell cycle control and DNA damage response, including role of BRCA1 in DNA damage response, role of CHK proteins in cell cycle checkpoint control, estrogen-mediated S phase entry, cell cycle control of chromosomal replication and mismatch repair in eukaryotes. Oncogene expression has been shown to drive cell proliferation by interfering with the regulatory pathways of cell cycle progression control, which could further lead to replication stress, a term that is characterized by DNA synthesis slow down and/or replication fork stalling and is the primary cause of genome instability (46-49). Our study for the first time showed that co-occuring oncogenic *HER2* and mutant *PIK3CA* could induce replication stress in mammary epithelial cells. This result highlights the acquisition of unique characteristics arising from co-occurrence of two prominent oncogenes, which will lead to us to re-think the tumor progression mechanism and open up new possibilities for breast cancer diagnostics and treatment.

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