Gene Expression Profile in Response to Doxorubicin–Rapamycin Combined Treatment of HER-2–Overexpressing Human Mammary Epithelial Cell Lines

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Abstract
HER-2–positive breast cancers frequently sustain elevated AKT/mTOR signaling, which has been associated with resistance to doxorubicin treatment. Here, we investigated whether rapamycin, an mTOR inhibitor, increased the sensitivity to doxorubicin therapy in two HER-2-overexpressing cell lines: C5.2, which was derived from the parental HB4a by transfection with HER-2 and SKBR3, which exhibits HER-2 amplification. The epithelial mammary cell line HB4a was also analyzed. The combined treatment using 20 nmol/L of rapamycin and 30 nmol/L of doxorubicin arrested HB4a and C5.2 cells in S to G2–M, whereas SKBR3 cells showed an increase in the G0–G1 phase. Rapamycin increased the sensitivity to doxorubicin in HER-2–overexpressing cells by approximately 2-fold, suggesting that the combination displayed a more effective antiproliferative action. Gene expression profiling showed that these results might reflect alterations in genes involved in canonical pathways related to purine metabolism, oxidative phosphorylation, protein ubiquitination, and mitochondrial dysfunction. A set of 122 genes modulated by the combined treatment and specifically related to HER-2 overexpression was determined by finding genes commonly regulated in both C5.2 and SKBR3 that were not affected in HB4a cells. Network analysis of this particular set showed a smaller subgroup of genes in which coexpression pattern in HB4a cells was disrupted in C5.2 and SKBR3. Altogether, our data showed a subset of genes that might be more robust than individual markers in predicting the response of HER-2–overexpressing breast cancers to doxorubicin and rapamycin combination.

Introduction
HER-2/neu, a member of the ErbB receptors, is amplified and/or overexpressed in about 25% to 30% of human breast cancers and generally correlates with poor prognosis (1). Although it has been used in the clinical practice as a parameter to indicate those patients who would better benefit from trastuzumab treatment, almost all HER-2–positive tumors develop resistance to this monotherapy (2). Controversial data arise out of the predictor role of this receptor in response to conventional chemotherapy, because it has been described as conferring either sensitivity (3–5), relative resistance (6–8), or exerting no influences (9) on anthracycline-based regimes, including doxorubicin.

Doxorubicin intercalates in the DNA double strand and forms a stable complex with the enzyme topoisomerase II (TOP2A), the direct target of anthracyclines. The fact that HER-2 and TOP2A genes are frequently coamplified in breast cancers led some researchers to speculate the predictive value of these simultaneous alterations (10–12). One mechanism of resistance to doxorubicin-induced apoptosis in HER-2–positive tumors seems to be the ability of this receptor to activate several cell survival pathways, such as the phosphoinositide 3-kinase (PI3K)/Akt/mTOR signaling, which can induce cell proliferation, transformation, and increased cell motility (13). Moreover, microarray analysis of breast tumor biopsies showed significant correlation between TOP2A and genes involved in mTOR pathway (14).

Considering the role of PI3K/AKT/mTOR signaling in the acquisition of resistance to the antitumoral effects of chemotherapy, it seems reasonable to investigate whether blockade of this pathway could increase the response of breast cancer cells to the antitumoral effects of doxorubicin. Previously published data obtained from different cancer models have shown optimized results after the combination of several chemotherapeutic agents with
PI3K/AKT/mTOR inhibitors, such as rapamycin (14–15). A more recent study showed clinical benefits from metastatic breast cancer patients treated with trastuzumab associated with everolimus, a rapamycin analog (16).

Rapamycin inhibits specifically the mTOR protein, leading to dephosphorylation of its main downstream targets, p70S6K and 4EBP1, which results in the suppression of translation of mRNAs related to cell cycle and proliferation (17). Despite preclinical studies have described favorable effects of rapamycin on different breast cancer models, clinical trials showed only a modest antitumoral activity in patients, which may be attributed to the fact that this drug is essentially cytostatic and not cytotoxic as chemotherapeutic agents (18, 19).

Altogether, these evidences highlight the relevance of combining rapamycin with doxorubicin to improve the response of HER-2-positive tumors to both drugs. Nevertheless, the molecular mechanisms involved in this treatment need to be elucidated to provide insights about the biological processes modulated by the combination. Therefore, we aimed to investigate whether rapamycin could increase the sensitivity of HER-2-expressing breast epithelial cell lines to doxorubicin. Gene expression profile generated from this approach might lead to a better understanding about the molecular mechanisms associated with the response of HER-2-overexpressing tumors to the combined treatment.

**Materials and Methods**

**Reagents**

Rapamycin (Calbiochem) and doxorubicin hydrochloride (Sigma-Aldrich) were solubilized in dimethyl sulfoxide (DMSO) and stored at −20°C. Propidium iodide (PI) was purchased from Sigma-Aldrich and Cell Titer Glo was purchased from Promega. Chemical structures of rapamycin and doxorubicin are presented in Supplementary Fig. S1.

**Cell culture**

The HB4a and C5.2 cell lines were kindly provided in 2006 by Dr. Michael O’Hare from Ludwig Institute for Cancer Research, London, United Kingdom. The SKBR3 cells from the American Type Culture Collection were kindly provided by Dr. A.A. Camargo from Ludwig Institute for Cancer Research, Sào Paulo Branch, Brazil. The C5.2 cells were established by transfection of parental mammary luminal epithelial HB4a cells with cDNA full-length corresponding to HER-2 gene (20). HB4a expresses basal levels of HER-2 receptor, and C5.2 expresses high levels of this receptor similarly to SKBR3. The cells were cultured as described elsewhere (21), tested periodically for *Mycoplasma* contamination, and authenticated by quantitative real-time reverse transcriptase (RT)-PCR to evaluate HER-2 overexpression in C5.2 and SKBR3 compared with HB4a cells (data not shown). The 3 cell lines express EGFR (21) and are negative for estrogen receptor and progesterone receptor (data not shown).

**Treatments**

Cells were seeded in 25 cm² flasks or 96-well culture plates at a density of 5 × 10⁴ cells/cm² to 15 × 10⁴ cells/cm². Flow cytometry and luminescence assays were conducted after 24 and 72 hours of treatment, respectively. Cells were treated with DMSO (0.04% v/v), 20 nmol/L of rapamycin, and 30 to 1,000 nmol/L of doxorubicin. The combined treatment was carried out by adding a fixed dose of rapamycin (20 nmol/L) to each doxorubicin dose. Microarray experiments and Western blotting were carried out after 24 hours of treatment with 30 nmol/L of doxorubicin and 20 nmol/L of rapamycin, doses clinically achieved in plasma of patients undergoing these therapies (22, 23).

**Flow cytometry and cell viability assays**

Cell-cycle distribution was determined by a FACS-Calibur flow cytometer (Beckton Dickinson) after PI incorporation. Cell viability was measured with Cell Titer Glo Reagent. GraphPad (version 5.0) software was used for statistical analyses, carried out by ANOVA and Tukey post test. Dose–response curves were constructed by nonlinear regression analyses (P < 0.05).

**Western blotting**

Proteins were extracted in lysis buffer (50 mmol/L Tris-HCl, pH 7.4; 150 mmol/L NaCl; 1% NP-40; 0.5% sodium deoxycholate; 0.1% SDS; and protease inhibitors) and the concentration was determined using the Bradford Protein Assay Reagent. Proteins (40 μg) were boiled for 5 minutes in Laemli buffer, separated by 12% SDS-PAGE, and transferred to nitrocellulose membranes, which were blocked for 1 hour in 5% skimmed milk in TBS-T (0.05% Tween-20) and incubated with monoclonal antibodies (1:500) against human p-AKT (Thr308), p-p70S6K (Thr389), p-4EBP1 (Thr37/46) and total proteins Akt, p70S6K, and 4EBP1 (Cell Signaling Technology) for 16 hours at 4°C. Following incubation with antirabbit immunoglobulin (1:3,000), the results were detected with ECL Western blotting (GE Healthcare) and quantitative densitometric analysis of the bands was conducted by Scion Image Software.

**Microarray procedures**

Total RNA was isolated with TRIzol reagent (Invitrogen) and purified by the RNeasy Mini Kit (QIA-GEN). The RNA quality and purity were analyzed by NanoDrop ND-1000 (Thermo Scientific) and Agilent 2100 Bioanalyzer (Agilent). Only total RNA samples with 260/280 ratios between 1.9 and 2.0 and RNA integrity number more than 7 were further processed. Total RNA (250 ng) from samples were amplified by the GeneChip 3’IVT Expression Kit Assay and hybridized to Human Genome U133 Plus 2.0 GeneChips. After hybridization, genechips were scanned with a GeneChip Scanner 3000 7G and data were recovered using Expression Console Software (Affymetrix). All experiments presented the indexes of quality control as.

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specified in the Affymetrix guidelines for assessing sample and array quality.

**Gene expression analysis**

The data were normalized with RMA Express Release 1.0 software. Statistical analyses were conducted by Multi-Experiment Viewer v.4.5 (Mev) software. Differentially expressed genes were identified after comparing each treatment to the control cells by SAM method using criteria based on FDR less than 5%. The genes were assigned to canonical pathways and functions by Ingenuity Pathway Analysis software ($P < 0.05$).

**Network construction**

Network analysis was carried out for the 122 genes related to HER-2-mediated response to the combined treatment, based on the interactome database (human protein data base, HPRD; ref. 24). For each cell line (HB4a, C5.2, and SKBR3), we selected the shortest pathways between 2 nodes and the first neighbors. Next, the R software (25) was used to calculate Pearson correlation coefficient (PCC) among normalized expression values of genes in each sample. PCC values of both C5.2 and SKBR3 cells were compared with HB4a cells and we selected for further investigation only those gene pairs that displayed differences greater than 1.5. Data analysis and visualization were conducted by Cytoscape software (version 2.8.0; www.cytoscape.org).

**Quantitative real-time RT-PCR**

Validation experiments were carried out for 11 out of 122 genes related to HER-2-mediated response to the combined treatment. Samples of C5.2 and SKBR3 cells were reverse transcribed with 2 μg of total RNA, random primers (Promega Corporation), and Superscript III Reverse Transcriptase (Invitrogen Corporation). Primers were designed with Primer-Blast software (http://www.ncbi.nlm.nih.gov/tools/prime-blaster/) and synthesized by IDT (Integrated DNA Technologies). Real-time RT PCR reactions were carried out in duplicate using SYBR Green PCR Power MasterMix in the 7900HT Fast Real Time System (Applied Biosystems) with the following thermal cycling: 95°C (10 minutes) and 40 cycles of 95°C (15 seconds) and 60°C (1 minute). β-Actin (ACTB) gene expression was used as endogenous control, and a pool of C5.2 and SKBR3 cells was used as calibrator for calculating relative expression of target genes with $2^{-ΔΔCt}$ method. Statistical analyses were carried out with Student’s t test.

**Results**

**Effects of doxorubicin and rapamycin on cell-cycle distribution**

The effects of doxorubicin and rapamycin on cell-cycle distribution were determined by flow cytometry. The HB4a cells showed a remarkable 50% increase in cells situated in S to G2–M ($P < 0.001$) by doxorubicin, because the percentage of cells in this phase shifted from 26.8% in control cells to 74.6% after exposure to a 30 nmol/L dose. Increased doses of doxorubicin showed no significant alterations in this cell-cycle distribution up to 250 nmol/L dose ($P > 0.05$). Nevertheless, higher concentrations (500–1,000 nmol/L) resulted in depletion of cells in S to G2–M, which could be explained by an increase in the percentage of cells in G0–G1 and in sub-G1 ($P < 0.05$; Fig. 1A). The C5.2 cells seemed to be more resistant to the effects of doxorubicin on S to G2–M arrest, because these cells displayed 50% increase in S to G2–M only after exposure to 120 nmol/L dose ($P < 0.01$). Although these results were maintained up to 500 nmol/L of doxorubicin, the 1,000 nmol/L dose reduced the percentage of cells in the S to G2–M phase, with a concomitant increase in G1 phase ($P < 0.01$; Fig. 2A). The ability of doxorubicin to induce S to G2–M arrest was less prominent in SKBR3 cells compared with the others. The maximum increase in the percentage of cells in S to G2–M phase consisted in 30% and occurred after treating cells with a 120 nmol/L dose ($P < 0.001$; Fig. 3A). We observed no substantial cell death as shown by absence of peaks in sub-G1 phase for C5.2 and SKBR3, even at higher doses ($P > 0.05$).

The effects of the combined treatment on cell-cycle distribution were similar to those generated by doxorubicin alone in HB4a and C5.2 cells, because the increase in S to G2–M also occurred after exposure to 30 nmol/L doxorubicin and was only 10% smaller after the combined treatment than doxorubicin alone (Figs. 1B and 2B). These effects were attenuated after increasing the doses of the chemotherapeutic agent. In SKBR3 cells, the response to the combined treatment was different from that with doxorubicin alone. Rapamycin seemed to cause an increase of 10% of cells in G0–G1 phase with a concomitant decrease in S to G2–M after its addition to 30 nmol/L of doxorubicin ($P < 0.05$). Increased doxorubicin doses resulted in a cell-cycle distribution similar to that of control cells ($P > 0.05$; Fig. 3B).

Although 20 nmol/L of rapamycin alone caused no cell-cycle alterations in HB4a cells compared with the control ($P > 0.05$), C5.2 and SKBR3 cells displayed 12% and 15% increase, respectively, in the percentage of cells in G0–G1 ($P < 0.01$; Figs. 1C, 2C, and 3C). These increases were accompanied by a depletion of cells accumulated in S to G2–M. Increased doses of rapamycin caused no changes in cell-cycle distribution for all cell lines studied (data not shown).

**Effects of doxorubicin and rapamycin on cell growth**

Doxorubicin inhibited cell growth of the 3 cell lines in a dose-dependent manner (Fig. 4). The results showed that 30 nmol/L doxorubicin caused a 35% inhibition in cell growth of HB4a and C5.2 cells, reaching 90% to 100% after exposure to doses ranging from 250 nmol/L to 1,000 nmol/L ($P < 0.01$). According to the flow cytometric results, SKBR3 cells seemed to be more resistant to the antiproliferative effects of doxorubicin, which consisted
in 23% of growth inhibition at a 30 nmol/L dose ($P < 0.01$). Escalating doses of doxorubicin caused 77% of cell growth inhibition, reaching up to 90% at 1,000 nmol/L. Rapamycin caused 35% growth inhibition in HB4a cells, 47% in C5.2 cells, and 40% in SKBR3 cells ($P < 0.001$; data not shown).

The addition of rapamycin was 20% more effective in inhibiting cell growth than doxorubicin alone, particularly after adding rapamycin to 30 to 120 nmol/L doxorubicin for HB4a ($P < 0.05$) and C5.2 cells ($P < 0.001$) and to 30 nmol/L for SKBR3 cells ($P < 0.01$). Higher concentrations of doxorubicin had similar effects to those exerted by this agent alone ($P > 0.05$; Fig. 4). Nonlinear regression analyses showed that rapamycin was able to reduce about 2-fold the IC$_{50}$ of doxorubicin ($P < 0.0001$), which changed from 54.2, 56.3, and 88.2 nmol/L to 23.4, 22.9, and 47.1 nmol/L for HB4a, C5.2, and SKBR3, respectively (Supplementary Fig. S2). Moreover, the higher IC$_{50}$ values of SKBR3 cells suggested that these cells were more resistant to doxorubicin than the other cell lines.

**Impact of rapamycin, doxorubicin, and the combination of these two drugs on the phosphorylation level of AKT, p70S6K, and 4EBP1**

Determination of the impact of rapamycin, doxorubicin, and their combination on activation of the mTOR pathway was carried out in HB4a, C5.2, and SKBR3 cells. We analyzed the effects of each treatment on phosphorylation levels of AKT protein (upstream regulator), p70S6K, and 4EBP1 (downstream targets of mTOR; Supplementary Fig. S3). Constitutive levels of phosphorylated proteins were observed for control samples of all cell lines and, compared with the others, SKBR3 cells showed about 2-fold increased levels for p-AKT and p-4EBP1 proteins. After 24 hours of treatment, Western blotting analysis detected no changes in the levels of p-AKT by each drug individually or the combined therapy in HB4a and SKBR3 cells. On the contrary, C5.2 cells showed increased levels of p-AKT only in response to the combined treatment. The levels of p-p70S6K were significantly decreased by all treatments in the 3 cells, mainly after treatment with rapamycin.
and its association with doxorubicin. Only SKBR3 cells displayed decreased levels of p-4EBP1 by rapamycin and combination therapies.

**Gene expression profiling**

Gene expression profiles were obtained for HB4a, C5.2, and SKBR3 cells treated with doxorubicin, rapamycin, and the association of both drugs. Figure 5 is a Venn diagram showing that doxorubicin consistently upregulated global gene expression and this response was more remarkable in HB4a cells (n = 1,453) than C5.2 (n = 468) and SKBR3 (n = 174). Repressed genes by doxorubicin treatment were also observed predominantly in HB4a cells (n = 231) compared with C5.2 (n = 12) and SKBR3 (n = 11).

Unlike doxorubicin, rapamycin substantially downregulated global gene expression and these effects occurred predominantly in C5.2 (n = 1,589) and SKBR3 (n = 867) compared with HB4a cells (n = 167). The induction of gene expression by rapamycin occurred prevalently in SKBR3 cells (n = 518 genes) compared with HB4a (n = 190) and C5.2 (n = 188).

Genes deregulated by the combined treatment were predominantly upregulated in HB4a cells (1,439 up vs. 821 down), downregulated in C5.2 cells (509 up vs. 1,203 down), and altered in both directions in SKBR3 (926 up vs. 1,109 down). Altogether, up- and downregulated genes showed a high percentage (40%–50%) of genes exclusively regulated by the combined treatment of HB4a cells (1,129 of 2,260), C5.2 cells (656 of 1,712), and SKBR3 (1,023 of 2,035). Overlapping genes between each drug alone and the combined treatment showed that the contribution of rapamycin and doxorubicin on gene expression consisted mainly in decrease by the former and upregulation by the latter.

**Canonical pathways modulated by the combined treatment**

To better understand the molecular mechanisms involved in the response to the association between the
2 drugs, differentially expressed genes by the combined treatment were classified in canonical pathways. These annotations included up- and downregulated genes representing 3 gene sets: (i) overlapped with doxorubicin alone, (ii) overlapped with rapamycin alone, and (iii) exclusively regulated by the combined treatment. The complete list of genes referred to the canonical pathways found in each cell line is available in Supplementary Tables S1A, S1B, S2A, S2B, S3A, and S3B.

Ingenuity Pathway Analysis of the transcripts differentially regulated in C5.2 cells by the combined treatment revealed that downregulated genes were involved in pathways such as aminoacyl-tRNA biosynthesis, glycolysis/gluconeogenesis, pyrimidine and purine metabolism, cell-cycle control of chromosomal replication, mitochondrial dysfunction, and oxidative phosphorylation. Genes upregulated fell into the following most significant pathways: PI3K signaling in B lymphocytes, p53 and ATM signaling, G2–M DNA damage checkpoint regulation, antigen presentation, and NF-κB signaling.

Some pathways in C5.2 cells were also regulated by the combination in the parental HB4a, such as glycolysis. Nevertheless, we decided to focus on canonical pathways commonly regulated in both C5.2 and SKBR3 and not evidenced in HB4a cells to identify those related to the HER-2–mediated response to the combined treatment. Among these pathways, we found PI3K signaling in B lymphocytes, mitochondrial dysfunction, protein ubiquitination, oxidative phosphorylation, cyclins and cell-cycle regulation, ubiquinone biosynthesis, and citrate cycle, which, therefore, might underlie some molecular mechanisms of this response (Supplementary Fig. S4A).
Genes exclusively regulated by the combined treatment might play relevant roles in the HER-2–mediated response as they were altered irrespective of the action of each drug alone (Supplementary Fig. S4B). Accordingly, a significant percentage of genes in the canonical pathways related to HER-2 overexpression belonged to this category, mainly in SKBR3 cells, which displayed 60% to 80% of genes represented in citrate cycle, mitochondrial dysfunction, and oxidative phosphorylation as exclusively regulated by the combination therapy.

Genes commonly regulated in HER2-overexpressing cell lines

Considering that our results showed the relevance of HER-2 in the modulation of canonical pathways by the combined treatment, we decided to determine a group of genes related to this response. Firstly, we compared the gene expression profiles generated from C52 and HB4a cells, which showed that 27% (139 of 509) of the up-regulated and 65% (780 of 1,203) of the downregulated genes by the combined treatment were found only in C52 compared with HB4a cells. Our next step was to identify which of these genes were also regulated in SKBR3, which resulted in a set containing 122 genes (16 up- and 106 downregulated; Supplementary Table S4). Although most of these genes have seemed not to exert known roles in canonical pathways, they were functionally annotated according to Gene Ontology database (Supplementary Table S5).

Experimental validation by real-time RT PCR

Among the 122 genes corresponding to HER-2–mediated response to the combined treatment, we selected 11 genes to be validated by real-time RT-PCR (4 up- and 7 downregulated; Supplementary Table S6). We observed validation of 6 genes (ALDOC, ELOVL6, PGHDH, TIPIN, ...

Figure 4. HB4a, C5.2, and SKBR3 cell lines were treated with varying doses of doxorubicin alone (30–1,000 nmol/L) and associated with a fixed dose of rapamycin (20 nmol/L) for 72 hours and growth inhibition was determined by luminescence assays. Results are expressed as percentage of the control. Experiments were conducted in 3 different days. Error bars represent the values of SEM. Dox, doxorubicin; Dox + Rapa, combined treatment.

Figure 5. Venn diagrams of the number of differentially expressed genes generated from HB4a, C5.2, and SKBR3 cell lines after exposure to doxorubicin alone (D), rapamycin alone (R) and the combination of the 2 drugs (D + R).
combined treatment showed a preponderance of doxorubicin-mediated S to G2–M arrest of HB4a and C5.2 cells, whereas SKBR3 cells displayed prominent effects of rapamycin over doxorubicin as shown by G0–G1 arrest of these cells.

According to previous works (29, 15), our cell growth assays showed that inhibition of mTOR caused a decrease of doxorubicin IC50 for all cell lines. Because HB4a cells lack HER-2 overexpression, we expected they were more resistant to the effects of the combined treatment compared with the HER-2-overexpressing cell lines. The ability of rapamycin to increase the chemosensitivity of cell lines depends on constitutive activation of the AKT pathway (30). Indeed, our Western blotting results showed that the HB4a cells, like C5.2 and SKBR3, had constitutive levels of phosphorylation of AKT and downstream targets of mTOR (p70S6K and 4EBP1). We suppose that the high levels of EGFR receptor described for HB4a cells lead to the formation of EGFR–HER3 heterodimers, which might trigger an alternative signaling cascade that activates AKT/mTOR pathway and increases the response of HB4a cells to the growth inhibitory effects of the combined treatment (21).

Our Western blotting analyses also showed a significant decrease of p70S6K activation after rapamycin and its association with doxorubicin for all cell lines whereas AKT remained activated irrespective of the treatment. These results might be explained, in part, by the fact that doxorubicin has been shown to activate AKT pathway (31). In addition, mTOR leads to a feedback inhibition of PI3K/AKT through activation of p70S6K so that its inhibition by rapamycin decreases p70S6K activity and, therefore, abolishes this regulatory loop, resulting in AKT activation (32). Decreased phosphorylation levels of 4EBP1 were observed only for SKBR3 cells undergoing rapamycin and combined treatment. These data do not necessarily indicate resistance to treatment because p-4EBP1 levels might not correlate with rapamycin sensitivity (33).

Gene expression profiling was carried out to better understand the processes involved in HER-2–mediated response to the combined treatment. For this purpose, we identified canonical pathways commonly regulated in C5.2 and SKBR3 and not evidenced in HB4a cells. Downregulated genes were categorized in purine metabolism, cyclins and cell-cycle regulation, protein ubiquitination, lysine degradation, oxidative phosphorylation, mitochondrial dysfunction, ubiquinone biosynthesis, and citrate cycle. Considering that our recent report showed enrichment of oxidative phosphorylation for genes upregulated by HER-2 overexpression (34), these data suggest that the pathways identified by our approach were, in fact, specifically related to HER-2 overexpression.

Increasing evidence has shown that protein-based network analysis might provide a more effective means to identify molecular biomarkers involved in response to treatments (35), because it allows to study gene–gene interactions related to a set of biological processes instead looking at a specific pathway (Fig. 6). Using more stringent criteria to understand the effects of HER-2 on the
response to the combined treatment, we selected only the genes exclusively regulated in HER-2–overexpressing cell lines for network construction. Among the 122 genes analyzed, we found a subgroup of genes in which interconnections in HB4a were inverted in C5.2 and SKBR3 cells and, therefore, might represent a putative set of markers for HER-2–mediated response to the combined treatment.

Our network analyses showed the TCF19 (SC1) gene as a central player (hub) of the interaction network, because it presented disruption of interactions with several genes in response of HER-2–overexpressing cells to the combined treatment. These data are consistent with the role of this gene as a relevant mediator of cell growth and transcription of targets required for G_{1}–S transition and entry to S to G_{2}–M (36). Among the genes interacting with TCF19, we found PHGDH, which encodes for 3-phosphoglycerate dehydrogenase, an important mediator of production of biosynthetic precursors required for cell proliferation. The diversion of glycolysis into

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Figure 6. Network generated from genes related to HER-2–mediated response to the combined treatment and based on the human interactome. PCC calculated among normalized expression values of the genes is represented by the lines connecting genes and colors vary according to these values: the red lines correspond to PCC values close to −1 and the green lines represent PCC values close to 1. Only gene pairs related to significant differences (>1.5) of PCC values in HB4a compared with C5.2 and SKBR3 cells were selected for network construction.
serine biosynthetic pathway by this enzyme leads to accumulation of serine and α-ketoglutarate essential for the synthesis of proteins, nucleotides, carbohydrates, and lipids that occurs through citrate cycle in mitochondria (37).

In addition to its role in these biosynthetic processes, mitochondria also supplies 40% to 75% of ATP requirements in cancer cells, which indicates that therapeutic interventions targeting this organelle might contribute to mitochondrial dysfunction and could lead to loss of cell viability. Indeed, other genes represented in the network encode for mitochondrial components, such as mitochondrial ribosomal protein (MRPS7) and translocases (TIMM50, TIMM10, and TOMM40L), which recognize and mediate the import of nucleus-encoded mitochondrial precursor proteins (38).

Altogether, these data suggest that the combined treatment might affect the mitochondria, which is supported by previous reports unraveling the coordinated relationship between mTOR and mitochondrial metabolism. Inhibition of mTOR by rapamycin has been described to decrease mitochondrial membrane potential, oxygen consumption, and ATP synthetic capacity (39, 40).

Other genes represented in the network involved upstream regulators of AKT/mTOR (IMPA2, CORO1A, and TXNIP). The CORO1A (41) and IMPA2 (42) genes have been described as involved in the phosphatidylinositol signaling, which triggers a signaling cascade that activates the AKT protein. The gene TXNIP was related to the stimulation of PTEN protein, a negative regulator of this pathway, which indicates a relevant role of this gene in the attenuation of AKT/mTOR effects on cell growth (43).

Other genes in which interactions with TCF19 were disrupted play functions related to DNA metabolism (ORC1L, GINS2, and DTYMK). The DTYMK gene encodes for thymidylate kinase, a key enzyme for dTTPs supply in DNA synthesis of cancer cells. Knockdown of GINS2 was reported to lead to growth inhibition and polyploidy by the suppression of M-phase progression in human breast cancer cells, showing that besides its roles in DNA replication initiation, this gene might also facilitate cell division and chromosome segregation required for cell growth (44).

We also noticed genes encoding for enzymes involved in energy homeostasis, such as CKB (creatine kinase) and FKBP11 (peptidyl prolyl isomerase), with the latter described as inhibited by rapamycin (45).

Conclusions

Our results suggest that HER-2-mediated response to the combined treatment might lead to alterations of pathways related to mitochondrial metabolism. A protein network analysis of a smaller set of genes corresponding to this response showed a loss of coordinated coexpression of genes related to oxidative phosphorylation, citrate cycle, mitochondrial proteins, and DNA metabolism, which might contribute to the impairment of processes relevant for increased cell survival mediated by HER-2.

We suppose that, compared with strategies based on individual gene markers analyses, our network might be more robust in predicting response of HER-2-overexpressing breast carcinomas to the combined treatment with doxorubicin and rapamycin.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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