

Effects of siRNA-mediated Silencing of *ERBB2*, *IGF-1R*, and *ITGB1* in HER2-positive Breast Cancer Cells

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Abstract. *Background/Aim:* One of the hallmarks of cancer is deregulation of multiple signaling pathways, which can lead to uncontrolled proliferation and migration of cells. Over-expression and mutations in human epidermal growth factor receptor 2 (HER2) can lead to overactivation of these pathways, potentially developing cancer in different tissues, including breast tissue. IGF-1R and ITGB-1 are two receptors that have been linked to cancer development. Therefore, the aim of this study was to investigate the effects of silencing of the corresponding genes using specific siRNAs. *Materials and Methods:* Transient silencing of HER2, ITGB-1, and IGF-1R was conducted using siRNAs and expression was quantified by reverse transcription-quantitative polymerase chain reaction. Viability in human breast cancer cells SKBR3, MCF-7, and HCC1954 and cytotoxicity in HeLa cells were tested using WST-1 assay. *Results:* The use of anti-HER2 siRNAs in a breast cancer cell line over-expressing HER2 (SKBR3) led to a decrease in cell viability. However,

silencing of ITGB-1 and IGF-1R in the same cell line had no significant effects. Silencing of any of the genes encoding any of the three receptors in MCF-7, HCC1954, and HeLa had no significant effects. *Conclusion:* Our results provide evidence towards using siRNAs against HER2-positive breast cancer. Silencing of ITGB-1 and IGF-1R did not significantly inhibit the growth of SKBR3 cells. Therefore, there is need for testing the effect of silencing ITGB-1 and IGF-1R in other cancer cell lines over-expressing these biomarkers and explore their potential use in cancer therapy.

Breast cancer is a heterogeneous disease presenting different biological markers, which may have strong effects on therapeutic treatments (1-3). Human epidermal growth factor receptor 2 (HER2) is one of the most relevant biomarkers (4), which is over-expressed in 15-30% of breast cancer cases (5). HER2/ERBB2 is an oncogenic receptor without a natural ligand; its over-expression in malignant breast cells is associated with rapid growth and aggressiveness compared to the other breast cancer subtypes not over-expressing this marker. As a result of these effects, HER2-positive carcinomas tend to proliferate faster, have higher relapse rates, and show higher resistance to drug treatments (6, 7). This rapid progression can also be attributed to the involvement of other over-expressed receptors that can be found in this type of breast cancer. Particularly, the deregulation of insulin-like growth factor receptor (IGF-1R) and integrin $\beta 1$ (ITGB-1) has been observed to accompany HER-2 over-expression in different breast cancer subtypes, including the HER2-positive one, which has also been associated with increasing aggressiveness and growth (8-10).

IGF-1R is a transmembrane protein that can be found over-expressed in 10-20% of HER2-positive breast cancers. Its over-expression is associated with the loss of p53, BRCA, and VHL suppressing activities. When in conjunction with HER2, the activation of IGF-1R promotes the independent phosphorylation of HER2, activating the PI3K/AKT

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Table I. Oligonucleotides and siRNAs sequences.

	Specific primers		siRNAs sequences
<i>HER2</i>	Forward: CTGCTACCAGGACACGATTT Reverse: CCCTTACACATCGGAGAACAGGTAAT	ERBB2 (HSS103333)	GGUCACCUACAACACAGACACGUUU AAACGUGUCUGUGUUGUAGGUGACC
<i>IGF-1R</i>	Forward: CGCAGACACCTACAACATCA Reverse: GTGAAAGGCCGAAGGTTAGA	IGF-1R (HSS105254)	CCUGUGAAAGUGACGUCCUGCAUUU AAAUGCAGGACGUCACUUUCACAGG
<i>ITGB-1</i>	Forward: ATCCCATTGACCTCTACTACCT Reverse: GTCCGAACCTCCTCATT	ITGB-1 (HSS105560)	UGCGAGUGUGGUGUCUGUAAAGUGUA UACACUUACAGACACCACACUCGCA

pathway, inducing transactivation of HER1 and IGF-R1, and promoting resistance to drugs such as gefitinib and trastuzumab (10-13).

Integrins are heterodimeric membrane proteins implicated in signaling between the cytoskeleton and extracellular matrix. ITGB-1 over-expression has been observed in approximately 30% to 50% of breast cancers. Because ITGB-1 regulates the FAK and Src kinases, the activity of HER2 induces over-activation of RAS/ERKPK, resulting in greater proliferation of cancer cells. Therefore, inhibition of ITGB-1 in HER2-positive breast cancer cells has attracted attention for targeted therapy (9, 14, 15).

Current treatment schemes for HER2-positive breast cancer include, besides classic chemotherapy, different targeting approaches inhibiting HER2 downstream activity. Monoclonal antibodies (mAbs) and tyrosine kinase inhibitors (TKIs) target the exposed receptor and the phosphorylation cascade induced upon its activation, respectively. Trastuzumab is the main mAb used against HER2-positive breast cancer and lapatinib one of the most used TKIs. While showing greater targeting and therapeutic effects, minimizing adverse effects such as myelosuppression observed with typical chemotherapy drugs, these therapeutics have also adverse effects. For mAbs targeting the extracellular domain of HER2, the truncated form p95HER-2 where the external domain is absent nullifies binding with HER2. In the case of TKIs, compensatory signaling pathways, such as EGF, can neutralize the effects of TKIs and mAbs alike, whereas mutations in the tyrosine domain of HER2 reduce binding with TKIs (16). These obstacles have prompted the search for other strategies to more effectively treat these diseases, for example, with antisense therapy.

One way to silence a gene and its mRNA translation into a protein is through the activity of miRNAs and siRNAs. miRNAs are small RNA molecules that mediate the down-regulation of target genes by different processes leading to the recruitment of the RNA-induced silencing complex (RISC) that either blocks translation or breaks down its complementary mRNA. siRNAs carry out the same function; the biggest difference between them is that a single siRNA silences one specific mRNA whereas an miRNA can target multiple mRNAs. Perfect base pairing between the

dsRNA and its mRNA target elicits cleavage by RISC and subsequent further degradation by exonucleases (17, 18). The usage of siRNAs in the clinic has already provided very promising results with the ability to target specific genes, silencing genes encoding proliferation factors (19).

This study aimed to evaluate the activity of three different siRNAs targeting HER2, IGF-R, and ITGB-1 in different cancer cell lines over-expressing these biomarkers to assess their effects on gene expression and cell viability alone or in synergy.

Materials and Methods

Cell culture. HER2-positive breast carcinoma cell lines SKBR3 (ATCC®: HTB-30™) and HCC1954 (ATCC®: CRL-2338™), HER2-low breast cancer cell line MCF-7 (ATCC®: HTB-22™), and HeLa (ATCC®: CCL-13™) were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured at 37°C in a 5% CO₂ atmosphere. McCoy's 5A medium was used for SKBR3 culture, RPMI-1640 for HCC1954 and EMEM (Sigma-Aldrich, St. Louis, MO, USA) for MCF-7 and HeLa. All were supplemented with 10% fetal bovine serum (FBS), and 1X penicillin-streptomycin (10,000 U/ml) solution supplied by Thermo Fisher Scientific (Waltham, MA, USA). To assess gene-silencing, cells were seeded at 1×10⁶ cells/well in a 6-well plate and for the antiproliferative test, cells were seeded at 5000 cells/well in a 96-well plate, both were cultured for 24 h at 37°C in a 5% CO₂ atmosphere before siRNAs treatments.

siRNA-liposome complex preparation. siRNA-liposome complexes were obtained using Lipofectamine 2000 according to manufacturer's protocol, siRNAs ERBB2 (HSS103333), IGF-1R (HSS105254), ITGB-1 (HSS105560) and Mix (ErbB2, IGF-1R and ITGB-1) (Thermo Fisher Scientific) were prepared at a final concentration of 20 nM. siRNAs sequences are presented in Table I.

Gene-silencing assay using reverse transcription-quantitative polymerase chain reaction. Two hundred and fifty µl of siRNA-liposome complexes was added to each well and incubated for 6 h at 37°C in a 5% CO₂ atmosphere. After exposure, complexes were removed and replaced with fresh medium. Total RNA was isolated after 48 h using TRIzol reagent and cDNA synthesis was carried out using the SuperScript™ IV First-Strand Synthesis System (Thermo Fisher Scientific) according to the manufacturer's instructions. The cDNA was diluted to the final concentration ~5 ng/µl and reacted with ErbB2, IGF-1R, and ITGB-1 specific primers (shown in Table I) and PowerUp™ SYBRTM Green Master Mix. Glyceraldehyde-3-

phosphate dehydrogenase (*GAPDH*) gene was used as an endogenous control and amplified employing the following primers: 5'-AGA AGG TGG TGA AGC AGG-3' and 5'-GTC AAA GGT GGA GGA GTG G-3'. PCR was performed using Applied Biosystems StepOne™ Thermal Cycler according to the manufacturer's conditions. All equipment and reagents were obtained from Thermo Fisher Scientific. Samples were run in triplicate and relative gene expression was calculated using the comparative C_T ($\Delta\Delta C_T$) method as described by Livak and Schmittgen (20).

Antiproliferative assays. An aliquot of 50 μ l of siRNA-liposome complex was added to each well and incubated for 6 h at 37°C in a 5% CO₂ atmosphere. Then, complexes were removed and replaced with fresh medium. Plates were incubated for 24, 48, 72, and 96 h, followed by WST-1 assay (Sigma-Aldrich). Cell viability was quantified spectrophotometrically at 450 nm.

Statistical analysis. Statistical analysis was performed on data from three independent experiments. Significant differences relative to the control and between the time sets were tested using ANOVA and Tukey's multiple comparison test. $p < 0.05$ was considered to indicate significant differences.

Results

Effect of siRNA treatment on targeted gene expression. The effect of siRNA treatments on the targeted genes expression in SKBR3 cells was evaluated. After siRNA treatment, no expression of *ITGB-1* was detected, which appeared to be completely silenced. *IGF-1R* expression did not show a notable reduction following treatment with the corresponding siRNA. However, *HER-2* gene expression was not completely silenced; it was strongly down-regulated (Figure 1).

siRNA's antiproliferative activity in cancer cells. Antiproliferative activity results derived from siRNA treatments are shown in cancer cell lines SKBR3, HCC1954, MCF-7, and HeLa (Figure 2). *IGF-1R* and *ITGB-1* siRNA treatments had no significant effect on the viability of any cell line. However, treatment with ErbB2 and Mix siRNAs resulted in a significant reduction in the viability of the HER2-positive breast cancer cells SKBR3. Viability decreased to 67% after ErbB2 siRNA treatment for 72 h (Figure 2A). However, the same effect was not observed in the HER2-positive breast cancer cells HCC1954 (Figure 2B). Interestingly, the effect of siRNA Mix treatment (ErbB2, *IGF-1R* and *ITGB-1*) in SKBR3 was comparable to that of ErbB2 but not better (Figure 2A). Nonspecific cytotoxicity tests were carried out in MCF-7 and HeLa cell lines. None of the siRNA treatments had significant cytotoxic effects on MCF-7 (Figure 2C) or HeLa cells (Figure 2D).

Discussion

The gene silencing through siRNA interference at a transcriptional level continues to attract more and more attention as a novel approach in the treatment of various

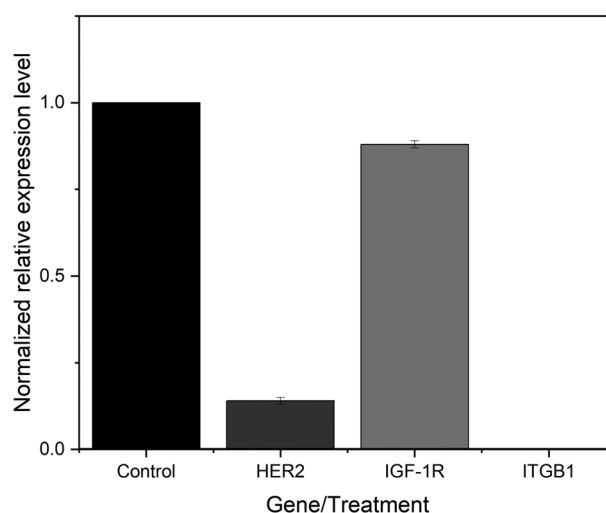


Figure 1. Columns represent means $\pm$ SEM of siRNAs treatment effect on the expression of *HER2*, *IGF-1R*, and *ITGB-1* genes in SKBR3 cells treated with *HER2*, *IGF-1R* or *ITGB-1* siRNAs or left untreated. Expression of each gene was normalized to that of *GAPDH* used as endogenous control.

types of cancers (21, 22). In this study, ErbB2 siRNA showed the greatest inhibition of SKBR3 cell growth and viability amongst the siRNAs tested, as observed using the WST-1 assay, showing 67% viability at 72 h with a dose of 20 nM siRNA. Comparable results in SKBR3 have been obtained in other studies. For example, Choudhury *et al.* reported 60-61% viability after siRNA transfection targeting ErbB2 with three different siRNA pairs and evaluation 72 h after transfection (23). Furthermore, Faltus *et al.* evaluated cell viability with four different anti-HER2 siRNAs, obtaining 66.8%, 56%, 82.9%, and 70.1% viability 96 h after transfection (24).

When comparing expression of targeted genes, it must be noted that while *HER-2* was strongly silenced, *ITGB-1* was completely silenced by its siRNA; however, no noticeable antiproliferative activity was detected in SKBR3. According to our results and literature, ErbB2 up-regulation appears to be necessary for SKBR3 proliferation (25), whereas *ITGB-1* is not over-expressed in this cell line (9). It has also been observed that SKBR3 cells appear to have constitutively activated AKT and ERK, diminishing the effects of down-regulating *IGF-1R* (26).

Although HCC1954 cells also over-express *HER2*, no noticeable antiproliferative effects were observed in the WST-1 assays, indicating no dependence on *HER-2* pathways for its viability (25). Western blot assays have shown that HCC1954 cells express c-Met growth factor receptor, encoded by the proto-oncogene *MET*, which could be reducing the impact of silencing *HER2* on cell viability (27). Another study that evaluated the effect of anti-HER2 siRNA-

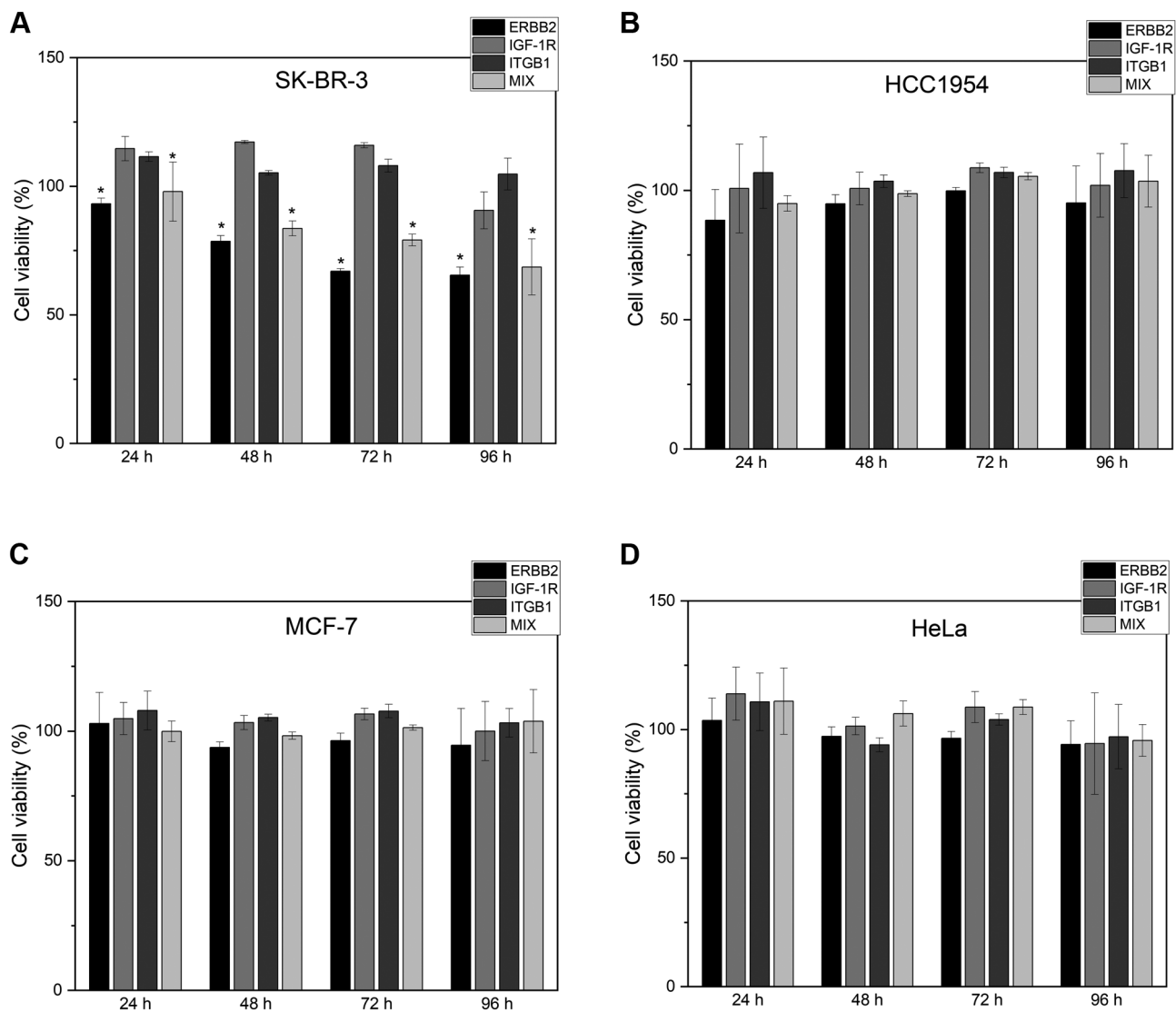


Figure 2. Columns represent means $\pm$ SEM of cell viability data after siRNA treatment for 24-, 48-, 72-, and 96-h post-exposure in SKBR3 cells (A), HCC1954 (B), MCF-7 (C) and HeLa (D). ANOVA analysis was followed by Tukey's multiple comparisons test.

loaded nanoparticles provided similar results, with a viability to ~90% after 48 h of treatment (28). As expected, MCF-7 and HeLa cell lines were unaffected by siRNA treatments since their survival and proliferation do not depend on HER2, IGF-1R, and ITGB-1 expression. Although our results were not as expected for all target genes, they provide evidence that inhibition of cancer-driver genes can be used in cancer treatment. An example of this is the work of Lim *et al.*, whereby silencing of the caspase recruitment domain-containing protein 14 (*CARD14*) gene using siRNAs led to significant anti-proliferative effects in MCF-7 and SKBR3 cells, thus other cancer-driver genes should be studied to explore their potential use in cancer treatment (29).

In summary, these results indicate that targeted ErbB2 suppression can be an effective means to selectively reduce SKBR3 cell proliferation given that growth and proliferation of MCF-7 and HeLa cell lines was not altered. Also, while the expression of ITGB-1 was completely suppressed, cell growth and proliferation were not reduced by the siRNA treatment. Finally, our results indicate that there is no synergistic effect of HER2, ITGB-1, and IGF-1R silencing in SKBR3 cells, prompting the need for additional testing to precisely assess the antiproliferative effects of IGF-1R, HER2, and ITGB-1 suppression in other HER2 positive cancer cell lines and other cancer types.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

J.H.-J: Investigation, Writing – Review & Editing, Formal Analysis, Data Curation; A.O.G.-C: Investigation, Writing – Review & Editing, Formal Analysis, Visualization, Data Curation; R.M.-E: Investigation, Writing – Original Draft, Writing – Review & Editing; M.D.R.-S: Methodology, Formal Analysis; K.R.-E: Methodology; I.B.-R: Methodology, Supervision; E.A.-E: Conceptualization, Project Administration, Funding Acquisition, Writing – Review & Editing. All Authors read and approved the final manuscript.

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