

ZNF217 Gene Copy Number as a Marker of Response to Standard Therapy Drugs According to ER α Status in Breast Cancer

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Purpose: The therapeutic decision for the management of breast cancer (BC) patients is based on the evaluation of prognostic factors alongside clinical and pathological parameters. Despite the use of standard biomarkers, response and resistance to therapy represent a challenge for clinicians. Among the new potential biomarkers for BC the *ZNF217* gene have gained importance in recent years. However, while associations between *ZNF217* gene copy number and clinicopathological characteristics have been established, its correlation with treatment response remains unclear.

Patients and Methods: This study aimed to evaluate the *ZNF217* gene copy number and establish its associations with treatment response in estrogen receptor positive (ER α +) and ER α negative (ER α -) BC cell lines. In addition, a validation of the relationship between *ZNF217* gene copy number and its prognostic value was performed using datasets of BC patients retrieved from the cBioPortal public database.

Results: Our data show that in ER α + cells, *ZNF217* gene copy number increase (amplification), while cell proliferation decreases in response to standard drug treatments. In contrast, both *ZNF217* gene copy number (gain) and cell proliferation increases in response to standard drug treatments in ER α - cells. The results obtained align with findings from the cBioPortal database analysis, demonstrating that ER α + /HER2- low proliferation patients, exhibiting *ZNF217* gene amplification or gain, have a significantly higher survival probability after treatment, compared to ER α - /HER2- and HER2+ patients.

Conclusion: Our results suggest that in ER α + BC cells, *ZNF217* gene amplification could be indicative of a favorable response, while in ER α - BC cells, *ZNF217* gene gain could be postulated as a potential predictor of treatment resistance. A broader understanding of the role of *ZNF217* gene in treatment response, together with prospective studies in BC patients, could contribute to confirming our data, as well as optimizing existing treatments and exploring novel approaches to improve overall cancer treatment outcomes.

Keywords: gene amplification, gene gain, hormone therapy, chemotherapy, treatment resistance, FISH

Introduction

Breast Cancer (BC) is the most common type of cancer in women worldwide with around 2.261.419 of new cases and 684.996 deaths annually.¹ The molecular classification of this disease is based on the expression of four biomarkers already standardized at the clinical level: Estrogen receptor alpha (ER α), Progesterone receptor (PR), Human epidermal growth factor receptor type 2 (HER2) and the cell proliferation marker Ki67. These biomarkers allow classifying this neoplasm into at least four subgroups according to their presence or absence.^{2,3} Although this has been a fundamental pillar in directing treatments in recent years, the increase in resistance has contributed to significant difficulties in the success of therapeutic approaches. Consequently, for every 100 women who have received some treatment for their disease at an early stage, 30 relapses.^{4,5} Therefore, establishing phenotypes of sensitivity to treatments based on the use of predictive biomarkers could be an additional useful tool to help physicians in decision-making regarding response to treatment in BC patients.⁶ Among the many genes that have gained importance in recent years, for its possible role in the response to treatment in BC is the *ZNF217* gene.⁷ *ZNF217* is a candidate oncogene located on chromosome 20. The

chromosomal region containing *ZNF217* gene (20q13), is amplified in up to 29% of BC and has been observed mainly in ER α -/PR negative (PR-)^{8,9} and in luminal A, node-negative BC patients.⁷ *ZNF217* gene amplification has been associated with aggressive phenotypes, poor clinical prognosis,¹⁰ higher clinical stages,¹¹ high cancer risk,^{12–14} and shorter patient survival in breast⁷ and in ovarian cancers.¹⁵ In addition, *ZNF217* was shown to be more highly expressed in breast cancer cell lines carrying the 20q13 amplification than in cells with normal 20q13 copy number.¹⁰

ZNF217 gene encodes for a krüppel-like zinc finger protein that localizes to the nucleus,¹⁶ and interacts with co-repressors and histone modifying proteins,^{17–19} suggesting that *ZNF217* may be part of a transcriptional repressor complex. Furthermore, the product of the *ZNF217* gene has been shown to interfere with apoptotic pathways at early and later tumor progression stages, which may be associated with resistance to chemotherapy.^{20,21} Indeed, it has been indicated that ectopic expression of *ZNF217* conferred resistance to doxorubicin and paclitaxel, but *ZNF217* silencing increased the sensitivity of the BC cells to these drugs.^{20,21} However, while a specific association between *ZNF217* mRNA expression with treatment response has been established in BC, studies that report associations between *ZNF217* gene copy number with treatment response are scarce or absent.

Since amplification seems to be the predominant mechanism leading to overexpression,^{16,22} the value of assessing the relationship of *ZNF217* gene copy number with cell proliferation (a marker of response to standard treatments), independently of the *ZNF217* gene-expression levels, appears worthwhile. Additionally, it has been shown that high *ZNF217* mRNA expression levels are not necessarily correlated to *ZNF217* gene amplification in BC cells.^{7,16,22,23} The hypothesis established around this behavior is that some tumors overexpress *ZNF217* as an early antiapoptotic and anti-stress mechanism that can later disappear when other mechanisms take over.⁷ This highlights the value of assessing *ZNF217* gene copy number as a possible predictive biomarker of response to therapy.

Considering the above, this study aimed to evaluate the *ZNF217* gene copy number and to establish its associations with cell proliferation, as an indicator of response to standard treatments, in BC cell lines representative of the Luminal [ER α + /HER2- and ER α + /HER2+], HER2-enriched [ER α - /HER2+], and Triple Negative (TNBC - ER α - /HER2-) tumor subtypes. In addition, a validation of the relationship between *ZNF217* gene copy number and its prognostic value, was performed using datasets of BC patients retrieved from the cBioPortal public database.

Materials and Methods

Cell Lines

The human BC cell lines MCF7 (RRID: CVCL_0031; ER α + /HER2-), MDA-MB468 (RRID: CVCL_0419; ER α - /HER2-), and BT474 (RRID: CVCL_0179; ER α + /HER2+), were obtained from the American Type Culture Collection (ATCC). KPL4 (RRID: CVCL_5310; ER α - /HER2+) cells were a kindly gift from Professor Anna Sapino, from the University of Turin, Italy. Cell lines were expanded and stored at -80°C for short-term use. Cells from these stocks were thawed and used for the experiments. MCF7, MDA-MB468 and KPL4 were cultured in RPMI-1640 medium (Sigma), whereas BT474 was cultured in DMEM medium (Sigma). Culture media were supplemented with antibiotic-antimycotic solution (1X) (Sigma), 10% fetal bovine serum (FBS) (Sigma) and L-glutamine (2 mM) (Invitrogen GmbH). Cells were cultured in 75 cm² flasks using general conditions (37°C and 5% CO₂). PCR assays were performed to confirm the absence of contamination with mycoplasma. The research was approved by the Ethics Committee of Universidad Pedagógica y Tecnológica de Colombia.

Treatments

BC cell lines were treated with tamoxifen (TAM) (T5648; Sigma), docetaxel (DOC) (sc-201436; Santa Cruz Biotechnology), doxorubicin (DOX) (sc-200923; Santa Cruz Biotechnology), Herceptin[®] (HT) and combined treatments (TAM+DOC, TAM+DOX, HT+TAM, HT+DOC and HT+DOX). TAM, DOC, DOX and HT were dissolved in absolute ethanol and diluted in medium at 1 μ M, 10 nM, 0.05 μ M and 50 μ g/mL, respectively. Subsequently, these doses were added to the culture media for 24h, 48h, and 96h, both for individual and combined treatments. The above concentrations were selected considering that they were the highest and best doses in which a change in cytoskeleton architecture and cell death was observed in BC cell lines.^{24–27} TAM, DOC and DOX as single treatments, as well as TAM+DOC, and TAM+DOX as combined treatments, were added to all cell lines. While HT as single treatment and, HT+TAM, HT

+DOC, and HT+DOX, as combined treatments, were added only to HER2+ cells (BT474 and KPL4). Control cells were treated with ethanol (vehicle control) at appropriate concentrations and incubated together with the drug treated cell lines.

Proliferation Assay

Cells were seeded at a density of $2.5\text{--}5 \times 10^3$ cells in 100 μL of phenol red-free medium. After 24h, cells were treated with single and combined treatments for 24h, 48h, and 96h. The BrdU Cell Proliferation ELISA Kit (Roche Diagnostics Deutschland GmbH) was used to evaluate the cell proliferation after each treatment. The absorbance was measured using a Tecan Infinite M200 reader (Tecan Trading AG, Switzerland) against a background control as a blank. Each treatment was performed in 24 replicates (3 biological replicates with 8 technical replicates each one).

Nuclei Spreads

Nuclei from control and treated BC cell lines were obtained through standardized harvesting protocols. Briefly, 2.5h before cell harvesting colcemid solution (0.03 $\mu\text{g}/\text{mL}$) (Sigma) was added to cultures. Then, cells were detached from flasks by Trypsin-EDTA solution 1X (Sigma) and treated with hypotonic solution. Subsequently, cells were fixed three times with Carnoy's fixative (3:1 methanol to acetic acid), and spread on glass. Nuclei spreads were analyzed using Fluorescence in situ Hybridization (FISH).

ZNF217 Gene Copy Number Evaluation

To evaluate the *ZNF217* gene copy number, molecular cytogenetic analysis on control and treated BC cell lines were performed. The *ZNF217* gene copy number was evaluated by FISH, on the nuclei spreads obtained previously, using the *ZNF217* amplification probe (Cytocell, Cambridge) and standard procedures. The above is a 197kb red probe spanning the *ZNF217* gene and neighboring regions. The 20p13 probe in green, acts as a control for chromosome 20. *ZNF217* gene copy number was assessed in a minimum of 100 intact and non-overlapping nuclei. The analysis of the *ZNF217* dual probe was performed by counting the number of red and green signals on the images taken in an Olympus microscope and transferred to the Cytovision software version 7.4. A ratio of *ZNF217* gene signals (red signals) divided by the number of control signals (green signals) (*ZNF217*/20p13) was calculated for all cell lines analyzed, both control and treated. Cell lines were considered as amplified when *ZNF217*/20p13 ratio was greater than 2 (≥ 2) in $\geq 10\%$ of analyzed cells [28–30]. Whilst, cells lines were considered to carry a *ZNF217* gene gain (increase in *ZNF217* copy number) when the *ZNF217* gene copy number was ≥ 3 and the *ZNF217*/20p13 ratio was less than 2 (≥ 2) in $\geq 10\%$ of analyzed cells.^{8,28–30} In order to understand the reason for the use of the *ZNF217*/20p13 ratio, it is important to clarify the difference between gene amplification and gene gain (polysomy). Although both, can give rise to an increase in gene copy number in tumor cells, gene amplification is a copy number increase for a specific gene (or group of genes) on a given chromosome arm, that does not change the copy number of genes located in other regions of the chromosome.³¹ However, gene gain increases the copy number for a given gene as a result of the presence of extra copies of the entire chromosome. Thus, the *ZNF217*/20p13 ratio, makes it possible to differentiate an increased copy number of the *ZNF217* gene attributable to gene amplification, from that resulting from extra copies of the chromosome (polysomy/gene gain).

cBioPortal Database Analysis

In order to evaluate the prognostic value of *ZNF217* gene copy number and its potential role as predictor of response to treatments, we analyze breast tumor data published in the cBioPortal database. Specifically, cBioPortal is a publicly available web program for exploring and analyzing genomic, transcriptomic, proteomic and clinical data from cancer patients and cell lines (<http://cbioportal.org/>).^{32,33} First, we analyzed *ZNF217* gene amplification and gain events in BC cell lines (MCF7, MDA-MB468, BT474 and KPL4) in 3 different datasets included in the cBioPortal database (data not shown). Then, we analyze data from BC patients included in the METABRIC studies,^{34,35} which have data of primary tumors from 2509 BC patients with long-term clinical follow-up. Using the cBioPortal database, we created a virtual study where the main parameter selected for the analysis was information of somatic copy number aberrations/alterations (CNAs). A total of 2173 out of 2509 BC patients have data for CNAs. In these cases, the *ZNF217* gene was queried to differentiate patients having amplification from those with gains or without amplification or gains (Diploids/Deletions). After creating the virtual

study, *ZNF217* CNAs, clinicopathological characteristics, type of treatment [hormone therapy (HoT) or chemotherapy (CT)], tumor subtypes and survival data (OS and RFS) of BC patients were downloaded from the cBioPortal website and examined with classical statistical packages in order to establish the prognostic value of the *ZNF217* gene copy number.

As tumor subtypes we use those defined by the 3 gene classifier,² a subtype classification model which group the major and clinically relevant molecular subtypes [ER α -/HER2-, ER α +/HER2- High Proliferation (HP), ER α +/HER2- Low Proliferation (LP), and HER2+], by quantitative measurements of three key BC genes: *ESR1* (ER α), *HER2*, and aurora kinase A (*AURKA*).

Statistical Analysis

Results are expressed as the mean \pm standard deviation (SD). Two-way analysis of variance (ANOVA) with the Dunnett's multiple comparisons test were used to evaluate the potential differences for both cell proliferation and gene copy number (*ZNF217/20p13* ratio) between groups (control vs treated cells). For data downloaded from the cBioPortal website, Pearson's chi-square test was preliminarily performed to compare categorical variables and to evaluate the potential differences in the variable distribution among the groups. Survival distribution curves were plotted using the Kaplan–Meier method. Statistical comparisons were performed using the Log rank test. The progression/relapse-free survival (RFS) indicates whether patient's disease has recurred/progressed, and at what time the disease recurred, or the patient was last seen. Overall survival (OS) indicates time of diagnosis to time of death for BC or last follow-up. Cox regression analysis were carried out on the RFS and OS to calculate the Hazard Ratio (HR) with 95% confidence intervals (CIs) for the different study groups. All statistical tests were two sided. *p* values < 0.05 were considered significant. Statistical analyzes were performed using Prism v.7.0. (GraphPad) statistical software and R: Language and Environment for Statistical Computing.

Results

MCF7 Shows *ZNF217* Gene Amplification and Decreased Cell Proliferation After Treatments

In MCF7 cells (ER α +/HER2-), a reduction in cell proliferation was observed in response to all treatments, both individual and combined, at all evaluated times (Figure 1A). However, these effects were significant at 48 and 96h (*p* \leq 0.04), suggesting that in early times (24h) therapies seems not to be yet effective enough (Supplementary Table 1). To note, combined treatments (both TAM [1 μ M] + DOC [10 nM], and TAM [1 μ M] + DOX [0,05 μ M]) showed a greater cell proliferation inhibition.

Regarding to the *ZNF217* gene copy number, the control cells (cell without treatment), showed *ZNF217* gene gain (*ZNF217* gene copies \geq 3 and *ZNF217/20p13* ratio < 2) (Figures 1B and 2). After all treatments (individual and combined), and at 48 and 96h, MCF7 cells showed a significant *ZNF217* gene amplification (*ZNF217/20p13* ratio \geq 2), indicating that in these cells, there is a biologically relevant induction of *ZNF217* gene copy number in response to treatments (Figure 1B and Supplementary Table 2). It is noteworthy, that the behavior mentioned was more evident when the cells were individually treated with TAM [1 μ M] and DOC [10 nM] (Figure 1B and Supplementary Table 2). These results suggest that in MCF7 cells (ER α +/HER2), *ZNF217* gene amplification could serve as an indicator of a positive response to both, individual and combined drug treatments.

BT474 Exhibits the Highest Increase in *ZNF217* Gene Copy Number Following Treatments

In ER α +/HER2+ cells, TAM [1 μ M], DOX [0,05 μ M] and HT [50 μ g/mL] single treatments, significantly reduced cell proliferation at all evaluated times (*p* \leq 0.002); while, DOC [10 nM] treatment induced a significant increase (*p* \leq 0.01) but only at 96h (Figure 1C and Supplementary Table 1). Among the combined treatments, TAM [1 μ M] + DOC [10 nM] and HT [50 μ g/mL] + DOC [10 nM], induced a significant reduction in cell proliferation in two of three evaluated times (*p* \leq 0.04). The other combined treatments do not seem to have significant effects on the proliferation of this cell type (Figure 1C and Supplementary Table 1).

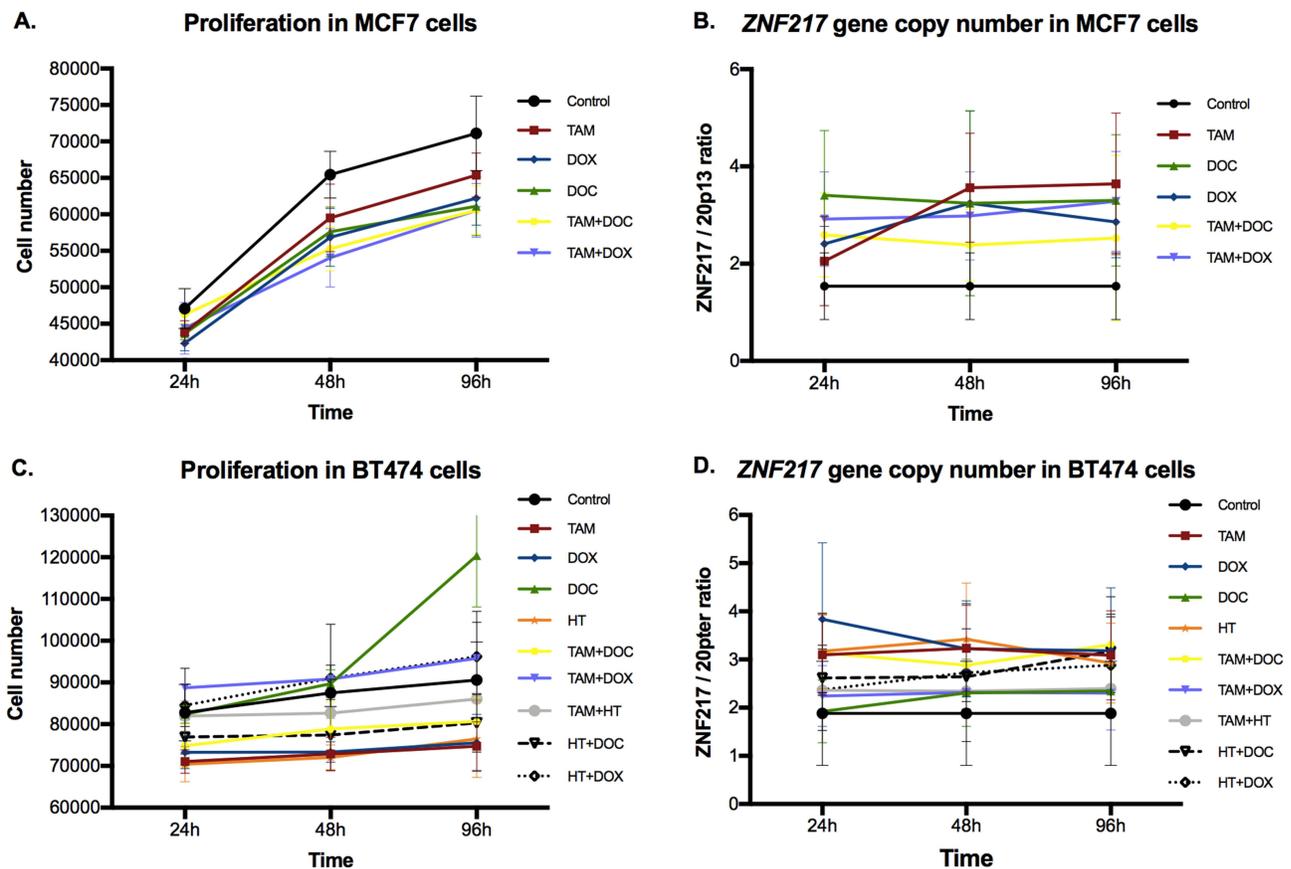


Figure 1 Effects of single and combined treatments on cell proliferation, and on *ZNF217* gene copy number in ER α + cells after 24h, 48h and 96h. (A and B) MCF7 cells. (C and D) BT474 cells. Error bars represents mean standard deviation of 24 replicates. Differences between control and treatments were evaluated with Dunnett's multiple comparisons test (see [Supplementary Tables 1](#) and [2](#)).

Regarding the *ZNF217* gene copy number, control cells were characterized by having *ZNF217* gene gain (*ZNF217* gene copies ≥ 3 and *ZNF217/20p13* ratio < 2). For most individual and combined treatments, there was a significant induction of *ZNF217* gene amplification (*ZNF217/20p13* ratio ≥ 2) at all evaluated times (Figures 1D and 2, and [Supplementary Table 2](#)). Only DOC at 24h, did not show a significant change. It should be noted that, the amplification of this gene was higher in cells exposed to individual (TAM, DOX and HT) and combined (TAM+DOC and HT+DOC) treatments, which also showed a significant reduction in cell proliferation. In addition, BT474 was the cell line that presented the greatest increase in the number of copies of the *ZNF217* gene after the treatments, with increases ranging from 9 copies in the control, up to a maximum of 19 copies after the treatments ([Supplementary Table 2](#)). These findings suggest that *ZNF217* gene amplification in ER α + / HER2+ BC cells, holds promise as a favorable predictor of treatment response.

MDA-MB468 Shows a Significantly Higher Increase in *ZNF217* Gene Gain and Increased Cell Proliferation After Treatments

In the ER α - / HER2- cell line, most treatments induced a significant increase in cell proliferation at all evaluated times. Although an increase in cell number was observed, TAM [1 μ M] as individual treatment, was the only treatment showing no meaningful effects ($p \geq 0.05$) (Figure 3A and [Supplementary Table 3](#)).

About the *ZNF217* gene copy number, two copies were observed in the control cells (Figures 2 and 3B). However, MDA-MB468 cells showed a significantly higher increase in the *ZNF217* gene copy number (gene gain) (*ZNF217* gene copies ≥ 3 and *ZNF217/20p13* ratio < 2) ($p \leq 0.0001$), after DOC, DOX and TAM+DOC treatments, at all evaluated times ([Supplementary Table 4](#)). The above was not evident at any time for TAM and TAM+DOX treatments. Our findings suggest a potential correlation between *ZNF217* gene gain and treatment resistance in triple-negative cells (MDA-MB468).

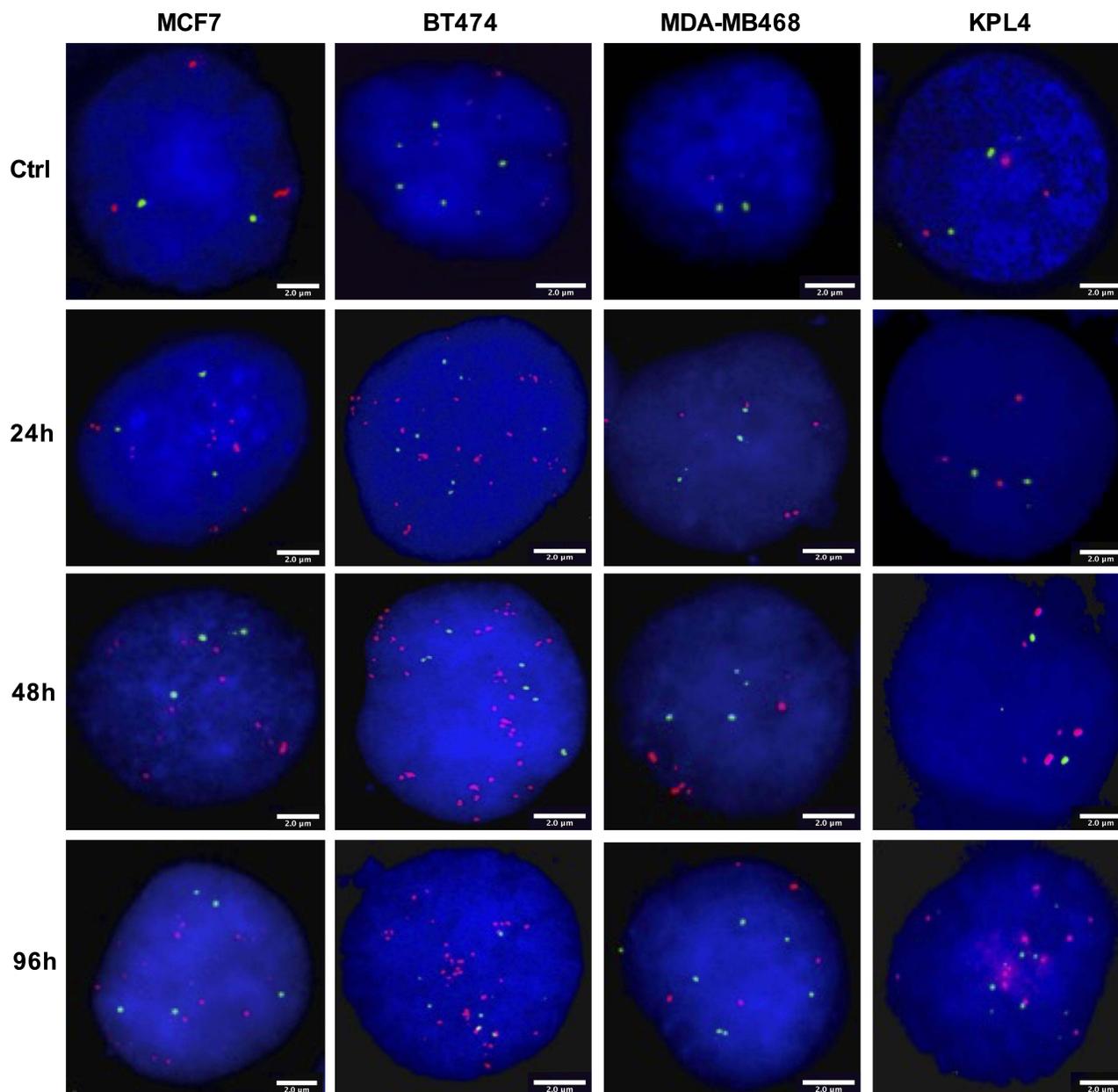


Figure 2 Representative FISH images of BC cells after TAM treatment for 24h, 48h and 96h. FISH was performed on nuclei spreads for *ZNF217* gene and for neighboring regions (20p13) as control, using a dual color probe labeled with different spectrum colors: spectrum orange for *ZNF217* gene and spectrum green for 20p13 region. Interphase nuclei at each treatment time point are indicated. The scale bars at the bottom right of each FISH image are indicative of the surface area of the nucleus. Ctrl: Control, untreated cells.

KPL4 Shows Increased Cell Proliferation, with No Alterations in the *ZNF217* Gene Copy Number Following Treatments

In ER α -/HER2+ cells, all individual treatments showed an increase in cell proliferation, this increase being greater and significant, after treatments with DOX [0,05 μ M] (at all times evaluated), DOC [10 nM] (at 24h) and HT [50 μ g/mL] (at 24 and 48h) ($p \leq 0.006$). Concerning combined treatments, the effects were variable, since TAM [1 μ M] + DOC [10 nM] and TAM [1 μ M] + HT [50 μ g/mL] showed a statistically significant reduction in cell proliferation in two of the three evaluated times ($p \leq 0.02$) (Figure 3C). While HT [50 μ g/mL] + DOC [10 nM] (at 24 and 48h) and HT [50 μ g/mL] + DOX [0,05 μ M] (at all times) induced a significant increase in cell proliferation ($p \leq 0.0001$) (Figure 3C and [Supplementary Table 3](#)).

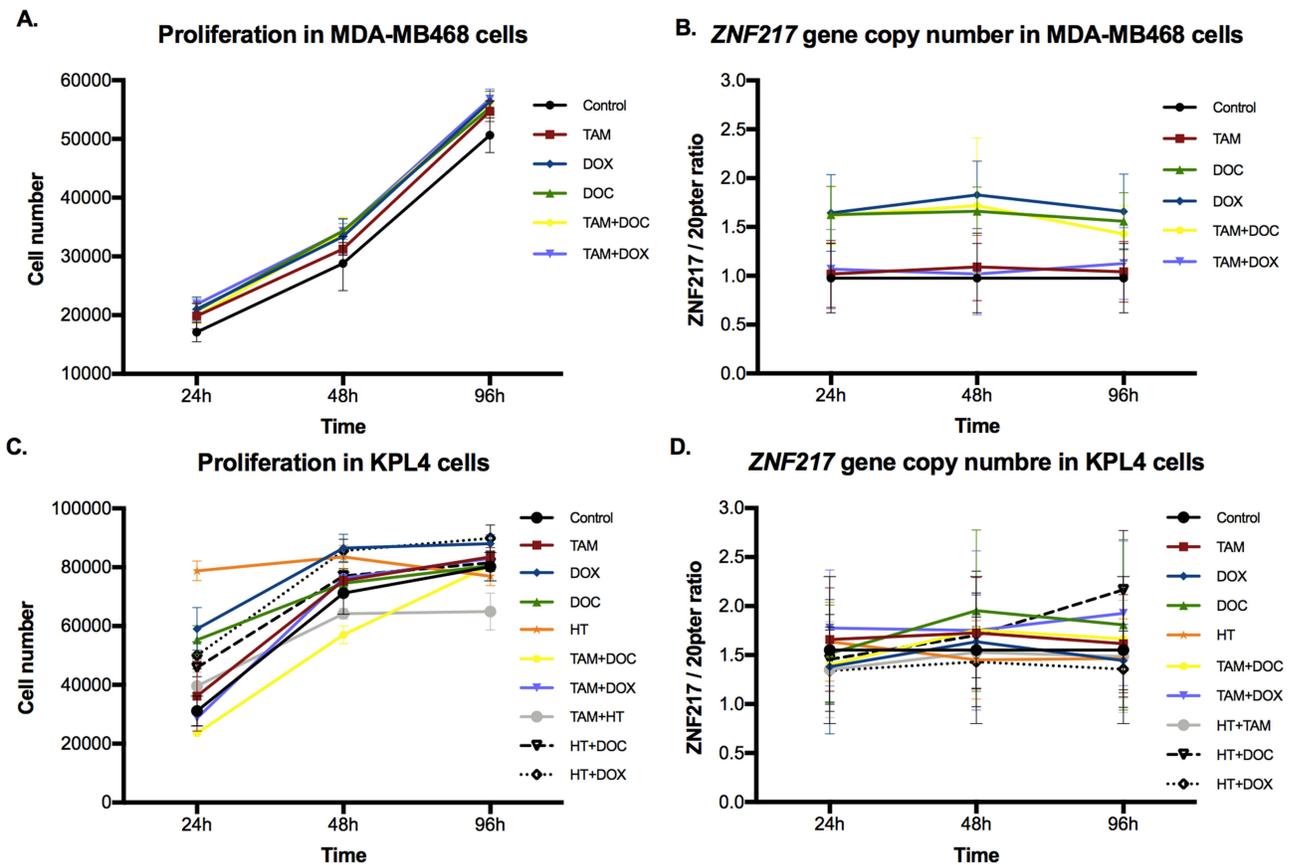


Figure 3 Effects of single and combined treatments on cell proliferation, and on *ZNF217* gene copy number in ER α - cells after 24h, 48h and 96h. (**A** and **B**) MDA-MB468 cells. (**C** and **D**) KPL4 cells. Error bars represent mean standard deviation of 24 replicates. Differences between control and treatments were evaluated with Dunnett's multiple comparisons test (see [Supplementary Tables 3 and 4](#)).

Regarding the *ZNF217* gene copy number, control cells showed *ZNF217* gene gain (*ZNF217* gene copies ≥ 3 and *ZNF217*/20p13 ratio < 2). Nevertheless, after individual and combined treatments, little and variable changes in the *ZNF217* gene copy number were identified, most of them without statistical meaning. In fact, only DOC (at 48 and 96h), TAM+DOX (at 24 and 96h) and HT+DOX (at 96h) treatments, showed a significant increase respect to control. However, despite these changes, the status of the *ZNF217* gene (*ZNF217*/20p13 ratio < 2) was not altered with respect to the control ([Figures 2 and 3D](#), and [Supplementary Table 4](#)). Overall, our findings suggest that ER α -/HER2+ cells exhibit resistance to both chemotherapeutic and HER2 targeted treatments, with no discernible alterations in the *ZNF217* gene copy number.

cBioPortal Database Analysis

The characteristics of the 2173 BC patients having data for copy number alterations (CNAs) and stratified by *ZNF217* gene copy number are reported in [Supplementary Table 5](#). Of the 2173 cases, 178 (8.2%) had amplification and 423 (19.5%) gain of the *ZNF217* gene. Patients with amplification or gain, carried larger tumors with higher grading, frequently with more positive lymph nodes and most of them negative for PR status ($p \leq 0.04$). Univariate cox regression analysis showed that *ZNF217* gene amplification is a significant marker of poor survival [HR = 1.26 for overall survival (OS), and HR = 1.57 for relapse-free survival (RFS), both $p \leq 0.02$], associated with high grades, tumor size ≥ 15 mm, nodal involvement ≥ 1 , and related with more aggressive molecular subtypes ([Supplementary Table 6](#)). Multivariate analysis, adjusted by age, showed that the prognostic value of *ZNF217* is maintained only for gene amplification in RFS ([Supplementary Table 7](#)). Furthermore, in BC patients with *ZNF217* gene amplification or gene gain, a lower survival probability was observed for both, OS and RFS ($p \leq 0.024$) ([Figures 4A and B](#)). According to the BC molecular subtypes, our data suggest a possible association between *ZNF217* gene amplification

(Figure 4C) and/or gene gain (Figure 4D) with reduced RFS probability in patients classified as ER α -/HER2-, HER2+ and ER α + /HER2- HP compared to those ER α + /HER2- LP. In this context no associations were observed between *ZNF217* CNAs and OS.

Also, we decided to analyze whether the presence of *ZNF217* gene amplification or gene gain, may be a prognostic predictor of response to CT or HoT, according to the BC molecular subtypes (3 gene classifier model). Cases with *ZNF217* gene amplification, treated with HoT and classified as ER α -/HER2- and HER2+, were associated with reduced OS, compared to ER α + /HER2- BC patients ($p \leq 0.0018$) (Figure 5A). No associations between survival and cases with *ZNF217* gene amplification treated with CT were observed among subtypes (data not shown). With regard to *ZNF217* gene gain, HER2+ and ER α + /HER2- HP BC patients treated with CT presented a significantly reduced OS ($p \leq 0.042$), compared to ER α + /HER2- LP BC patients (Figure 5B). Like above, BC patients with *ZNF217* gene gain classified as HER2+, ER α + /HER2- HP and ER α - /HER2- but treated with HoT (Figure 5C), presented a significantly reduced RFS, compared to those ER α + /HER2- LP BC patients ($p \leq 0.015$).

Discussion

In this study, we provide evidence suggesting that *ZNF217* gene copy number might be a predictive biomarker of response to standard drug treatment (individual or combined) in BC cells according to the ER α status. Our results allowed us to identify (before treatments), two copies of the *ZNF217* gene in triple negative cells (MDA-MB468), and an increase in the gene copy number in ER α + cells (MCF7 and BT474). Analysis of BC cell lines using the cBioPortal database, showed that at least one of the cBioPortal datasets was concordant with our results. KPL4 cell line was the only cell line not studied in any of the 3 data sets included in the tool. The slight differences with some of the datasets can be explained by the methods used to establish CNAs, since while we use FISH, the cBioPortal dataset analysis reports CNAs data obtained by Next Generation Sequencing.

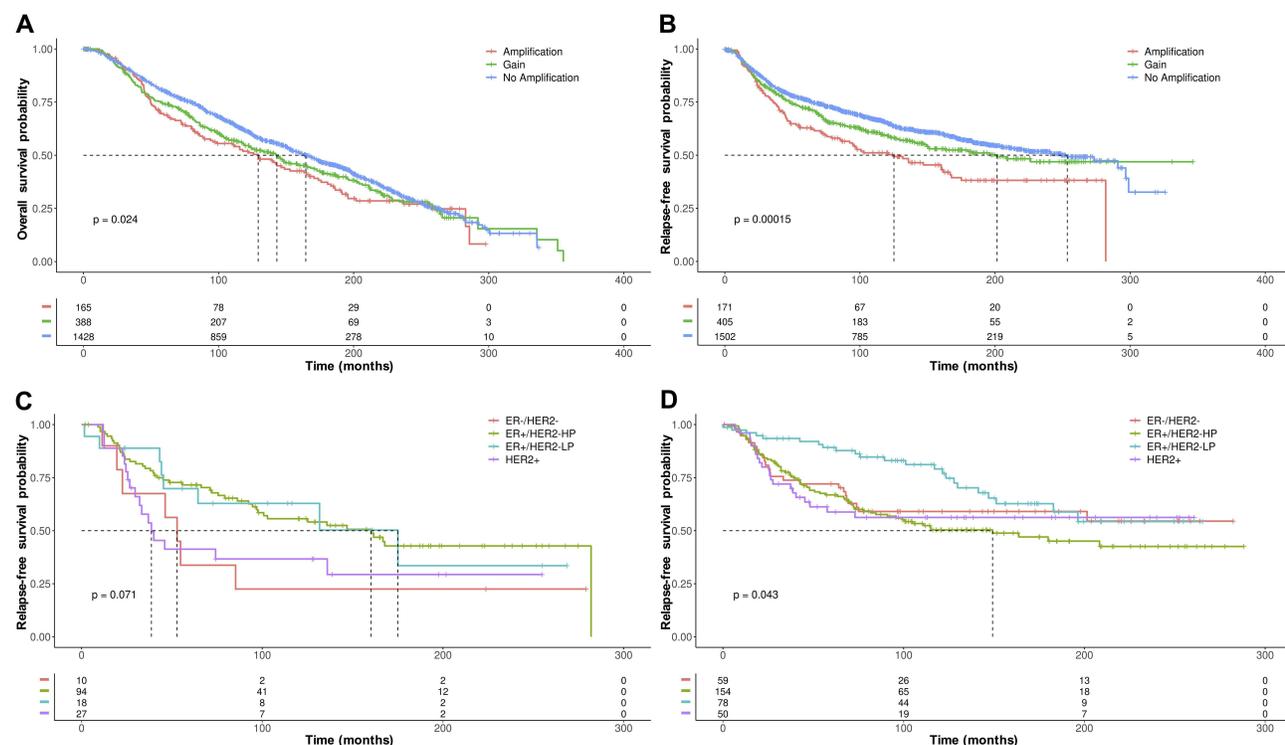


Figure 4 Kaplan-Meier plots of (A) OS and (B) RFS in BC patients with *ZNF217* gene amplification or gene gain, versus BC patients without *ZNF217* gene amplification or gene gain. (C) Kaplan-Meier plots of RFS in BC patients with *ZNF217* gene amplification or (D) *ZNF217* gene gain according to molecular subtypes defined by 3 gene classifier model. p value was determined using the Log rank test.

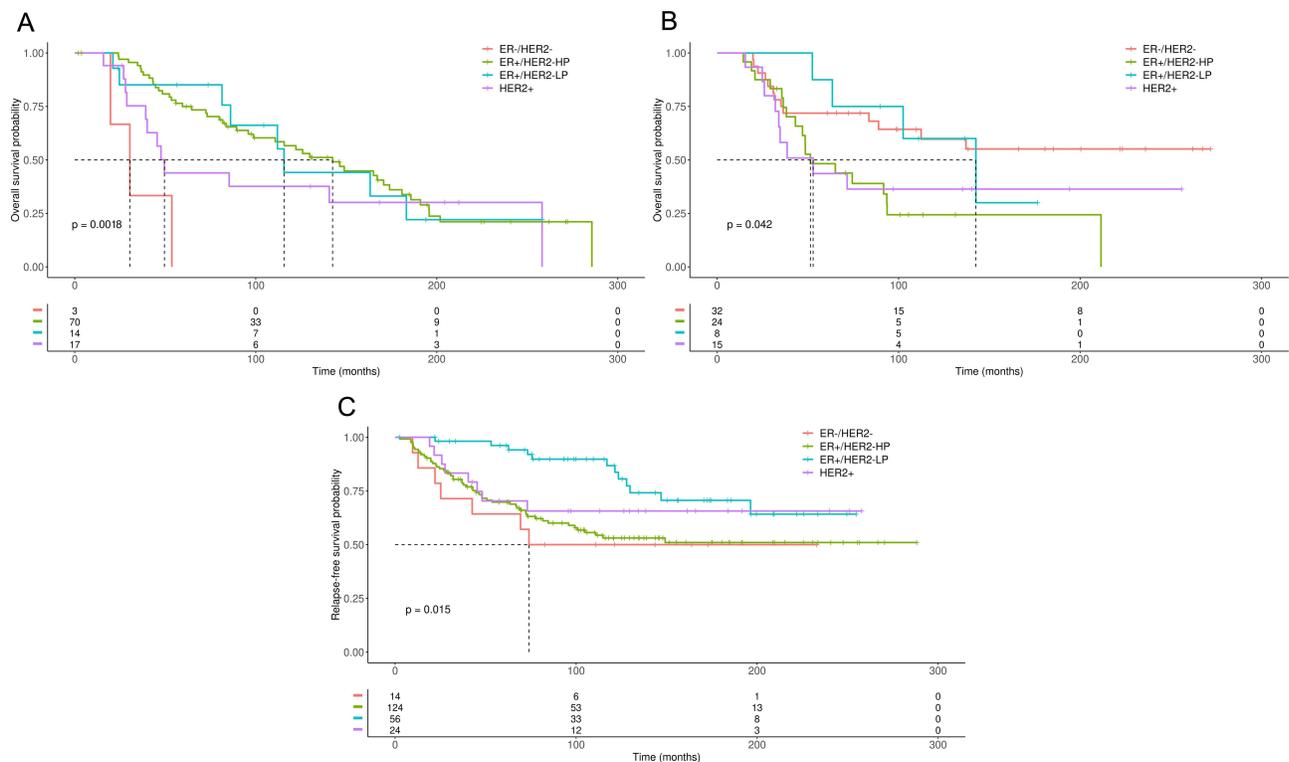


Figure 5 Kaplan–Meier plots of OS or RFS in BC patients with *ZNF217* gene amplification or gain according to molecular subtypes defined by 3 gene classifier model and treated with CT or HoT. **(A)** OS in BC patients with *ZNF217* gene amplification treated with HoT. **(B)** OS in BC patients with *ZNF217* gene gain treated with CT. **(C)** RFS in BC patients with *ZNF217* gene gain, treated with HoT. *p* value was determined using the Log rank test.

In MCF7 cells, a relevant induction of *ZNF217* gene amplification over time (48h and 96h), together with a decrease in cell proliferation, was evidenced in response to all treatments, mainly to TAM. A similar behavior was observed in BT474 cells, where most treatments (TAM, DOX, HT, TAM+DOC and HT+DOC) induced a significant reduction in cell proliferation that coincided with a significant induction of *ZNF217* gene amplification (Figure 1). These results align with analysis from cBioPortal database, where it was observed that, ER α +HER2- LP BC patients, having *ZNF217* gene amplification or gain, and treated with HoT, have higher survival probability (Figure 5). Our results are in line with previous studies indicating that TAM reduce proliferation of ER α + cells. These studies further indicate that the effects of TAM can be explained mainly by downregulation of ER α target pathways (eg, JAK/STAT, MAPK) and by mitochondria-mediated apoptosis.^{36,37}

ZNF217 gene amplification observed in ER α + cells, may be related to genome integrity loss of many human cancers which is indicative of chromosomal instability (CIN) of tumor cells.¹⁹ In fact, it has been indicated that CIN plays an important role in molding BC genomes and is useful for therapeutic purposes.^{31,38–40} Indeed, previous studies have indicated that ER α + cells, having increased CIN, are sensitive to different standard treatments.⁴¹ However, paradoxical associations between extreme CIN and improved outcome have also been reported.^{40–43} Thus, although most studies have assigned oncogenic properties to the *ZNF217* gene expression in ER α + BC,⁴⁴ based on our data and those of others who have not found clear associations with gene expression or amplification,^{7,9,44–46} we cannot rule out that the increased CNAs observed for the *ZNF217* gene in MCF7 and BT474 cells, as well as in ER α + BC patients (representative of luminal subtypes), could be a potential indicator of CIN related to improved outcomes for some types of treatments.

In contrast to what was observed in ER α + cells, our findings suggest a potential correlation between *ZNF217* gene gain and treatment resistance in triple-negative cells (MDA-MB468). Except for TAM, all treatments in MDA-MB468, induced a significant increase in cell proliferation, which agreed with an increase in the *ZNF217* gene copy number. These results, also agree with analysis from cBioPortal database, indicating that ER α -HER2- BC patients treated with HoT and, having *ZNF217* gene amplification or gene gain, had worse OS and RFS, respectively (Figures 5A and 5B). The lack of significant

differences between *ZNF217* gene amplification and survival of TNBC patients treated with CT, may be due to the low number of cases included in the analysis, by dilution of the sample after the cases were grouped according to each tumor subtype. However, it must be noted that in this analysis, ER α +HER2- LP BC patients had a higher survival probability respect to ER α -HER2- BC. Besides, when BC patients were not classified by tumor subtypes, those treated with CT and having *ZNF217* gene amplification or gene gain, had worse survival ([Supplementary Figure 1A](#) and [1B](#)). The resistance phenotype observed is consistent with studies showing that the drugs used here do not always inhibit ER α - cell growth, and evidence the aggressiveness and worse prognosis associated with TNBC.^{47,48} This behavior could be explained by the fact that alterations in chromosome copy number and gene dosage (*ZNF217* gene gain) may induce cellular stresses, including CIN, which has been associated with therapy resistance.^{49,50} In fact, an interesting hypothesis indicates that cancer cells might exploit CIN to survive under conditions of selective pressure. Thus, CIN could provide phenotypic variation which increases heterogeneity within a tumor and drives the ability of cancer cells to adapt to stressful conditions, including chemotherapy.⁴⁹ In addition, it has been reported that progression of TNBC could be due to oncogenic *ZNF217*-driven activation of PTEN/PI3K/AKT/mTOR pathway,^{39,51,52} which is hyperactivated mainly due to loss of *PTEN*, and is not only associated with progression but also with chemoresistance of TNBC.⁵³

Like TNBC cell line, the ER α -/HER2+ cells (KPL4) showed a resistance phenotype (increased cell proliferation) in response to DOC, DOX, HT and some combined treatments (HT+DOC and HT+DOX). However, this behavior does not seem to be related to the *ZNF217* gene copy number, since none of the mentioned treatments induced significant changes in the status of this gene with respect to control cells ([Figure 3](#)). It should be noted that, treatments such as TAM+DOC and TAM+HT, induced a reduction in cell proliferation of KPL4 cells, an interesting behavior, since the addition of TAM as a single treatment, did not induce any notable effect on these ER α - cells. Although these treatments did not modify the *ZNF217* gene copy number, these results confirm that hormonal therapy combined with other therapeutic strategies, may be useful for the treatment of ER α - tumors, as previously reported.^{54,55} Likewise, it cannot be ruled out that the variable effects observed in KPL4 cells could be explained by the increased expression of the HER2 receptor. In fact, on the HER2+ BT474 cells, DOC, TAM+DOX and HT+DOX, unlike to all others treatments, induced a slight increased cell proliferation. However, in both KPL4 and BT474 cells, most treatments induced changes similar to those observed in its partner cells lines MDA-MB468 and MCF7, respectively. The above suggests that responses to treatments seem to be mainly influenced by the ER α status. In general, results in KPL4 cells suggest resistance to chemotherapeutic and HER2 targeted treatments, with no evident changes in the *ZNF217* gene copy number. The above agrees with analysis from the cBioPortal database, since HER2+ BC patients treated with CT and having *ZNF217* gene amplification, not showed differences in OS or RFS with respect to other subtypes. However, HER2+ BC patients treated with CT or HoT, but with *ZNF217* gene gain, were associated with a reduced survival probability ([Figure 5](#)). This variable behavior suggests that, at least in ER α -/HER2+ cells, the *ZNF217* gene copy number, may not be informative enough and that other mechanisms (eg, changes in the mRNA and protein expression levels) must be related to the oncogenic role assigned to *ZNF217* in ER α -/HER2+ subtypes.⁴⁶ In fact, it has been reported that the increased expression of the *ERBB3* gene (a member of the HER family receptors), which is a direct target for the *ZNF217* transcription factor, could favor the *ZNF217*-driven activation of the survival signaling pathways PI3K/AKT and MAPK, both of which are implicated in BC therapy resistance.^{39,51,56} The above suggests that variations in the expression levels of the *ZNF217* gene could be a better indicator of survival related to BC cases that are positive for the HER family receptors.

Our study has limitations related to the in vitro models (cell lines) used for treatment evaluation, which could provide variable results. However, it is important to note that, in order to reduce such variability, the effect of each treatment was evaluated at least in 24 replicates and the gene copy number was studied in at least 100 non-overlapping nuclei for each chromosome. Moreover, is also worth mentioning that due to the retrospective nature of the analysis performed with the cBioPortal dataset, the samples included correspond to tumors from patients before treatments. However, the CNA data from these patients gives us insights into the relationship between *ZNF217* CNAs and the possible prognosis of BC patients after they undergo HoT or CT. To further address these limitations and validate our data, future studies need to be performed with in vivo models, as well as evaluating a large cohort of BC patients with long follow-up in prospective studies, including cases with neoadjuvant regimens for different types of treatment.

Conclusions

In this study, we suggest the value of *ZNF217* gene copy number (gene amplification and gene gain) as a predictive biomarker of response to standard drug treatment (individual or combined), according to the ER α status in BC cells. Our “in vitro” study in BC cell lines, suggests that *ZNF217* gene amplification appears to be a good predictor of favorable drug response in ER α + cells (MCF7 and BT474 cell lines), while in ER α -/HER2- cells (MDA-MB468 cell line), gene gain appears to be a good predictor of resistance to CT. The above results are consistent with analysis from cBioPortal database, where was observed that ER α + /HER2- LP patients, with *ZNF217* gene amplification or gain, have significant higher survival probability after treatments (mainly HoT), in contrast to ER α -/HER2- patients. These data reinforce the insight that *ZNF217* CNAs could be useful to improve the prognostic and predictive utility of classical markers, like ER α and HER2, as well as, the prognostic value of BC subtypes related to these markers. Our results highlight the role of *ZNF217* gene amplification or gain, in predicting favorable response to CT and HoT in ER α + BC cases and resistance to CT for ER α -, mainly for ER α -/HER2- BC patients. A broader understanding of the role of *ZNF217* gene copy number in the treatment response of BC could contribute to the optimization of therapeutic strategies and/or support new ones for improved cancer treatment outcomes.

Ethics Approval and Consent to Participate

The study was approved by the Ethical Committee of Universidad Pedagógica y Tecnológica de Colombia.

Funding

This work was supported by Universidad Pedagógica y Tecnológica de Colombia and by Pontificia Universidad Javeriana through the “Support for the publication of high-quality research articles 2024” grant.

Disclosure

The authors report no conflicts of interest in this work.

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