Long non-coding RNA signatures in breast cancer: Properties as biomarkers?

JASMIN ASBERGER[1,2], ISABELL GE[1,3], BENJAMIN SCHMIDT[2], MARKUS JÄGER[1,2], DANIELA WEISS[1,2], KAI BERNER[1,2], THALIA ERBES[1,2,4], INGOLF JUHASZ-BÖSS[1,2] and SEBASTIAN MAYER[2,5]

[1]Department of Obstetrics and Gynecology, Medical Center, University of Freiburg, D-79106 Freiburg, Germany; [2]Faculty of Medicine, University of Freiburg, D-79110 Freiburg, Germany; [3]Department of Obstetrics and Gynaecology, University Hospital of Basel, 4056 Basel, Switzerland; [4]Department of Gyneaecology and Obstetrics, Diako Mannheim, D-68163 Mannheim, Germany; [5]Department of Gynaecology and Obstetrics, Hospital Krumbach, D-86381 Krumbach, Germany

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Abstract. Breast cancer represents the most common type of cancer in females worldwide. The survival rates for breast cancer patients have been increasing since 1990. However, in 2023 breast cancer is still the second most common cause of malignancy-associated death in women. One decisive reason is the increase of treatment resistance and low therapy response. Therefore, new therapy targets and predictive markers for the response to treatment are needed. The present study analyzed the potential effects triggered by different breast cancer treatments on the transcriptional expression of 12 pre-selected long non-coding (lnc) RNAs and the proliferation markers Cyclin D1 and Ki-67 in six different breast cancer cell lines (BT-474, MDA-MB-231, BT-20, T-47D, SKBR-3 and MCF-7). The results revealed that lncRNA cytoskeleton regulator RNA may be an appropriate biomarker for the response to treatment with both epirubicin and gemcitabine (P<0.001). NF-κB interacting lnc RNA may be a marker for therapy response (P<0.001), while HOX transcript antisense RNA overexpression suggested resistance to treatment (P<0.001) with epirubicin. The transcriptional expression of lncRNA BC4 increased during treatment with epirubicin and gemcitabine, which indicated therapy response. Overall, the present data suggested that the aforementioned lncRNAs have a promising potential as biomarkers to detect early therapy response or resistance in and therefore should be analyzed in more detail.

Correspondence to: Dr Jasmin Asberger, Department of Obstetrics and Gynecology, Medical Center, University of Freiburg, Hugstetterstr. 55, D-79106 Freiburg, Germany
E-mail: jasmin.asberger@uniklinik-freiburg.de

Abbreviations: BC, breast cancer; cDNA, complementary DNA; lncRNA, long non-coding RNA; RT, reverse transcription

Key words: long non-coding RNA, cytoskeleton regulator RNA, NF-κB interacting long non-coding RNA, HOX transcript antisense RNA, breast cancer

Introduction

Breast cancer (BC) is the most common type of cancer in women worldwide (1). The American Cancer Society estimated that 300,590 new cases of invasive BC in women would be diagnosed in 2023 worldwide (2). Breast tumors are heterogeneous and are pathologically classified by immunohistochemical staining. The key proteins for classification of breast tumors are estrogen receptor (ER) α, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Additionally, factors such as tumor stage, nodal status, tumor grade, molecular subtype and the proliferation marker MIB-1 have prognostic relevance. Therapy is planned for the corresponding patient with BC depending on all these characteristics. Molecular subtyping has been implemented in clinical practice as an important tool for risk-adapted therapy in patients with BC (3-5). Treatment options include surgery, radiotherapy, endocrine therapy, chemotherapy and targeted therapies, which vary depending on factors such as TNM status and molecular biology (6,7). Despite improvements in systemic treatment concepts since 1990, BC was projected to be the second leading cause of malignancy-associated deaths in women in 2023 (2). One of the major challenges is the increase in treatment resistance. Current research is focused on exploring the mechanisms by which tumor cells become resistant to endocrine therapy and chemotherapy (8-10). The development of predictive biomarkers for treatment response is crucial for clinicians to detect resistance early and adjust management accordingly.

Chemotherapy represents a well-established and occasionally life-saving treatment option, despite its known severe side effects (6). Overall survival is markedly increased after the application of chemotherapy in BC (11,12). Combined epirubicin and paclitaxel are the cornerstones of early BC therapy (8,11,13,14). Epirubicin is an anthracycline, and the mode of action of anthracyclines is diverse, although most importantly, they intercalate into DNA molecules, thus inhibiting topoisomerase II (a key enzyme in cell division) and generating radicals that lead to DNA degradation (15). Paclitaxel is a taxol-based chemotherapeutic and, as such, acts as a microtubule-stabilizing agent, impacts depolymerization

and triggers apoptosis (11,16). Gemcitabine is a nucleosid analog that plays an important role in the treatment of metastatic BC (14). After being phosphorylated, gemcitabine exhibits cytotoxic activity by inhibiting the enzyme ribonucleotide reductase, which itself catalyzes sufficient and correct DNA synthesis (14,17).

Another treatment option for BC is endocrine therapy. The selective ER degrader fulvestrant is a therapeutic agent with antiestrogenic properties. Compared with the most common antihormonal therapeutics such as tamoxifen, fulvestrant does not exhibit estrogen agonistic effects, including increased risk of endometrial carcinoma or thromboembolic disease (18,19). Therefore, it has become of great interest in BC therapy.

As well as chemotherapy and antihormonal therapy, bone-targeted therapeutics are of outstanding importance for some patients, especially those receiving antihormonal therapies. Not only in advanced but also in early BC therapy, bisphosphonates and denosumab have shown to crucially affect bone health, quality of life, overall and disease-free survival (20), and skeletal-related events such as bone and joint pain, fractures and malignant hypercalcemia can be prevented by these agents (21,22). Among all bisphosphonates, zoledronic acid has been shown to be the most efficient. Its mode of action is to inhibit the proliferation of osteoclasts, thus leading to their cell death (20).

Long non-coding RNAs (lncRNAs) are non-coding (nc) RNA sequences of >200 nucleotides in length. The number of lncRNA genes in the human genome is ~3-fold that of protein-coding genes (23). Accumulating evidence suggests that lncRNAs are involved in a wide range of cellular processes affecting protein, DNA, RNA expression and interactions (24). A previous study showed evidence for regulatory roles of lncRNA in facilitating carcinogenesis, invasion-metastasis and chemoresistance in multiple cancer types (25). Several lncRNAs have been implicated in BC. For example, HOX transcript antisense RNA (HOTAIR) is known to be correlated with tamoxifen resistance (26). The lncRNA cytoskeleton regulator RNA (CYTOR) is highly expressed in triple-negative BC (27). However, the expression of lncRNAs and their role as possible biomarkers for therapy resistance or cancer screening remain to be elucidated.

Cancerogenesis is a very complex process in which a number of factors can play a role. Mutations and copy number variations have a high relevance. However, a number of studies have also shown that ncRNAs play an important role in the field of oncology (25-27). The group of ncRNA contain a wide variety of regulatory RNAs, included are for example piwiinteracting RNAs (piRNAs), microRNAs (miRNAs), small cajal body-specific RNAs (scaRNAs), small nucleolar RNAs (snoRNAs) and lncRNAs, to name but a few (28). For lncRNAs, previous studies have shown evidence of their regulatory role in facilitating carcinogenesis, invasion, metastasis and chemoresistance in different type of cancers, as reviewed by Majidinia and Yousefi (25). In addition, a number of studies have already demonstrated the role of lncRNAs in breast cancer (26,27,29-31).

Ki-67 is an established prognostic biomarker in BC (32), and it has been shown that Ki67 dynamics can indicate therapy response (33). Cyclin D1 is a cell cycle regulator reported to be overactive in cancer tissue (34). Notably, it has been shown to be involved in lncRNA-mediated tamoxifen resistance in BC (35).

The present study analyzed the potential effects triggered by different BC treatments on the transcriptional expression of 12 pre-selected lncRNAs and the proliferation markers Cyclin D1 and Ki-67 in six different cell lines. Intracellular analysis of these lncRNAs and their correlation with Ki-67 and Cyclin D1 is expected to indicate a potential biomarker function, since, hypothetically, altered lncRNA expression after treatment that correlates with a simultaneous alteration of Ki-67 and cyclin D1 may highlight the predictive potential of lncRNAs An association with BC has already been described for some of the aforementioned pre-selected lncRNAs, while others have so far only been described in other tumor types (Table I).

Materials and methods

Cell culture conditions and treatments. Established BC cell lines with a varying range of hormone receptor states (Table II) were incubated in a humidified atmosphere at 37˚C and 5% CO_2. MCF-7 cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc.), while i) BT-474, ii) T-47D, SK-BR-3, BT-20 and MDA-MB-231 were cultured in DMEM-F12 (Thermo Fisher Scientific, Inc.) in the presence of i) insulin (2.5 μl/ml) alone or, ii) insulin (2.5 μl/ml) and estrogen (1 μ/ml) supplemented with 1% HEPES buffer (MilliporeSigma), 1% 100 U/ml penicillin/streptomycin (MilliporeSigma) and 5% FBS (Gibco; Thermo Fisher Scientific, Inc. Table II).

Cells were plated in 6-well culture plates and incubated to 70-80% confluence. Pharmaceutical compounds dissolved in DMSO (MilliporeSigma) were applied to *in vitro* models in parallel monotherapy. Control cells received equivalent DMSO volumes. *In vitro* compound concentrations geared to expected serum levels in patients in the clinical setting are listed in Table III. Following application, cells were incubated for 18 h at 37˚C and 5% CO_2.

Treatment response was assessed visually. Therefore, cell cultures were carefully analyzed, and the cells were quantified before and after compound treatment. In case of 50% reduction of cells in a cell culture flask, treatment response was assessed positive. Additionally, Ki67 reduction was used to determine a positive treatment effect. All experiments were performed in triplicate (Table III).

Total RNA isolation. Total RNA of cultured cells was obtained after cell lysis with QIAzol Lysis Reagent® (Qiagen GmbH) using the EURx Total RNA Purification Kit (Roboklon, GmbH) according to the manufacturer's protocol. Assessment of RNA quantity was performed by ultraviolet-spectrometry (NanoDrop ND1000; NanoDrop Technologies; Thermo Fisher Scientific, Inc.). RNA samples were stored at -80˚C until further processing.

Reverse transcription-quantitative (RT-q)PCR. The RT reaction mixture consisted of 5 μl Maxima RT-buffer (Thermo Scientific, Inc.), 1 μl 5 μM random hexamer (IDT DNA, Leuven, Belgium), 1 μl 5 mM dNTPs (Roboklon GmbH), 0.25 μl Maxima reverse transcriptase (Thermo

Table I. Reported function of pre-selected lncRNAs investigated.

A, Tumor-promoting properties in BC

Pre-selected lncRNA	Reported function	Affected genes/proteins/pathways	(Refs.)
CYTOR/linc00152	Promotes cell proliferation, tumorigenesis, invasion and metastasis	EZH2	(34,44)
HOTAIR	Promotes cell proliferation, invasion and metastasis. Prognostic marker for metastasis Positively correlated with tamoxifen resistance	EZH2; estrogen receptor protein	(33,45)
MALAT-1/NEAT2	Promotes cell proliferation, invasion and migration	Phosphatidylinositid-3-Kinase-AKT pathway	(65)
CCAT2	Promotes cell proliferation, tumorigenesis and inhibits apoptosis Positively correlated with tamoxifen resistance	ERK/MAPK signaling pathway TGF-β signaling pathway	(63,64)
BCAR4	Promotes cell proliferation, metastasis and tumor aggressiveness Endocrine resistance	ERBB2/ERBB3 signaling pathway	(61)
TERC	Promotes cell proliferation		(66)
WSPAR/lncTCF7	No data for BC, but Wnt signaling dysfunction mediates progression of triple-negative BC Promotes cell proliferation, invasion and metastasis in colorectal cancer	Wnt/β-catenin pathway	(71,72)

B, Tumor-suppressing properties in BC

Pre-selected lncRNA	Reported function	Affected genes/proteins/pathways	(Refs.)
BC4	Downregulated in BC	Undefined	(67,73)
FTX	Tumor suppressor	Wnt/β-catenin pathway	(73)
JPX	Downregulated in BC	XIST (X inactivate-specific transcript) → AKT Phosphorylation	(62,68)
linc00312	Downregulated in BC Suppression of proliferation, colony forming ability, migration and invasiveness of BC cell lines	Suppression of Cyclin B1 Increase of cadherin 1 (CDH1) Decrease of vimentin	(74,75)
NKILA (NF-κB interacting long non-coding RNA)	- Tumor suppressor	NF-κB-pathway	(59,60)

BC, breast cancer; CYTOR, cytoskeleton regulator RNA; HOTAIR, HOX transcript antisense RNA; MALAT-1, metastasis associated lung adenocarcinoma transcript 1; NEAT2, nuclear enriched autosomal transcript 2; CCAT2, colon cancer associated transcript 2 RNA; BCAR4, breast cancer antiestrogen resistance 4; BCAR4, breast cancer antiestrogen resistance 4; TERC, telomerase RNA component; WSPAR, WNT signaling pathway activating non-coding RNA; NKILA, NF-κB interacting long non-coding RNA.

Scientific, Inc.), 0.25 μl SUPERase in RNase inhibitor (Thermo Fischer Scientific, Inc.) and 2 μg RNA in a volume of 25 μl. The reaction was carried out at 65˚C for 1 min, followed by 25˚C for 10 min, 50˚C for 30 min and 85˚C for 5 min and in a Nexus Thermal Cycler (Eppendorf SE). Processed cDNA was stored at -20˚C until further analysis.

The relative expression levels of specific mRNAs and lncRNAs were assessed by qPCR by using a SYBR Green assay in duplicate. cDNA (1 μl) was mixed with 9 μl Master Mix containing 6.45 μl nuclease-free water, 1 μl 10X qPCR buffer, 0.5 μl 5 mM dNTPs (Jena Bioscience), 0.5 μl specific qPCR primer (Biomers), 0.5 μl SYBR Green 1 μM (Jena Bioscience) and 0.25 U Hot Start Taq-Polymerase (Jena Bioscience). Primers consisted of a specific primer pair for each mRNA, lncRNA type and for the endogenous control genes (Tables IV and V). A negative control (10 μl mastermix; no cDNA) and a minus-RT control (no RNA for Reverse Transcription; 1 μl unspecific cDNA; 9 μl mastermix) were added in order to evaluate if specific or unspecific products were amplified. qPCR was performed on a LightCycler96 (Roche Diagnostics GmbH) at 95˚C for 120 sec, followed by 40 cycles at 95˚C for 10 sec, 60˚C for 20 sec and 72˚C for 10 sec.

Table II. Molecular classification of breast cancer cell lines investigated.

First author/s, year	Cell line	Receptor status	(Refs.)
Brooks, 1973	MCF7	ER+, PR+, HER2neu -	(76)
Judge, 1983	T-47D	ER+, PR+, HER2neu -	(77)
Lasfargues, 1978	BT-474	ER+, PR+, HER2neu +	(78)
Trempe, 1976	SK-BR-3	ER-, PR-, HER2neu +	(58)
Lasfargues, 1958	BT-20	ER-, PR-, HER2neu -	(57)
Brinkley, 1980	MDA-MB-231	ER-, PR-, HER2neu -	(79)

ER, estrogen receptor; PR, progesterone receptor; HER2neu., HER2neu receptor.

Table III. Concentration of the medications used.

Medication	Concentration	Supplier
Epirubicin	5 μg/ml	Onkovis
Fulvestrant	16 ng/ml	CC-Pharma GmbH
Gemcitabine	20 μg/ml	Abmol Bioscience
Paclitaxel	5 nM	LC Laboratories
Zoledronic acid	5 ng/ml	Novartis International AG

All results from RT-qPCR were normalized against the geometric mean of Alas1 and TOP2α using the DD-CT_q method (36) Each statistically significant lncRNA alteration was compared to Ki67-alterations. Only in case of simultaneous expression alteration, altered lncRNAs expression was considered relevant in terms of biomarker properties (Tables IV and V).

Statistical analysis. The influence of treatment on lncRNA expression levels in different BC cell lines was investigated using a linear regression t-tests with factors treatment (epirubicin, fulvestrant, gemcitabine, paclitaxel or zoledronic acid) compared to control conditions and cell line as factorial predictors, including an interaction term as coefficients. For calculation the Statistic program R was used [R Core Team (Release year 2017; http://www.R-project.org/)]. This scenario inherently suggested complex associations where both the type of cell line and the treatment could affect the expression levels. A linear model with interaction terms allows for the examination of these effects in a nuanced manner, including how the effect of treatment may vary across different cell lines, where simpler tests cannot accommodate. The use of regression analysis with interaction terms provides a comprehensive, efficient, and statistically rigorous method for investigating complex relationships in biological data. It is well-suited to address the specific research questions posed in the study, offering insights into the nuanced effects of treatments across different BC cell lines that simpler statistical tests could not provide (37). lncRNA expression levels were graphically visualized as boxplots, showing the medians, quartiles and interquartile range (IQR) in the cell lines BT-474, MCF-7, T-47D, SK-BR-3, BT-20 and MDA-MB-231 under control conditions. The estimated coefficients represented the average effects on the lncRNA expression levels under treatment were visualized in a heatmap that represented the fold change.

Results

In the present study, the potential regulatory effects of epirubicin, gemcitabine, paclitaxel, fulvestrant and zoledronic acid on the expression levels of pre-selected lncRNAs in six BC cell lines were analyzed (Table IV). The characteristics of the six investigated cell lines (MCF-7, T-47D, BT-474, SK-BR-3, BT-20 and MDA-MB-231) are summarized in Table II. Regression analysis was based on the target-control value of BT-20 and was selected as intercept (Figs. 1-4) The parameter estimates from the regression model with their 95% confidence intervals are presented in Table SI).

Basal lncRNA and Ki-67/Cyclin D1 expression levels. The expression level of HOTAIR was lower in the BT-474 and MDA-MB-231 cell lines (P<0.001; Fig. 1A) compared to the intercept. TERC was highly expressed in BT-20 cells (P<0.001; Fig. 1C). metastasis associated lung adenocarcinoma transcript 1 (MALAT-1) expression levels under standard culture conditions revealed elevated expression in the ER-positive cell lines MCF-7 (P<0.001) and T-47D (P=0.001; Fig. 1D). Under standard control conditions, CYTOR was overexpressed in BT-20 and MDA-MB-231 cells (P<0.001), and downregulated in MCF-7, T-47D and BT-474 cells (Fig. 1B). The expression level of lncRNA BC4 was increased in BT-20 cells (P<0.001), and reduced in MCF-7, T-47D and BT-474 cells (P<0.001; Fig. 2B). Higher BCAR4 and FTX RNA expression levels were observed in MCF-7 cells (P<0.001; Fig. 2A and C). The expression level of JPX was higher in BT-20 and MCF-7 cells (P<0.001; Fig. 2D). The mRNA expression level of the proliferation marker Ki-67 was elevated in BT-20 (P<0.001) and SK-BR-3 (P=0.001) cells. Downregulation of Ki-67 was detectable in T-47D (P<0.001) and MDA-MB-231 (P=0.01) cells (Fig. 3A). Under standard culture conditions, Cyclin D1 was overexpressed in MCF-7 and BT-474 cells (P<0.001; Fig. 3B).

lncRNA and Ki-67/Cyclin D1 expression levels following treatment with epirubicin, gemcitabine, paclitaxel, fulvestrant and zoledronic acid. Treatment with epirubicin triggered an increase in HOTAIR expression in BT-20, SK-BR-3 and T-47D cells (P<0.001) and an increase in CYTOR expression in

Table IV. Specific lncRNA primer sequences for reverse transcription-quantitative PCR.

Primer for lncRNA		Sequence
BC4	Sense	5'-CCTTCCTTCGCACCACTAAA-3'
	Antisense	5'-CGAGGAGGCATGGGTAAATATG-3'
BCAR 4	Sense	5'-CGAGGCTAAGAGTAGGAGTGATA-3'
	Antisense	5'-GCGAGGTGCTAGCGATTATT-3'
CCAT2	Sense	5'-TCTCAACTGCCCAGGTAATATG-3'
	Antisense	5'-GTTGGGACTTGCTGGTAGAA-3'
CYTOR	Sense	5'-GATGGCTTGAACATTTGGTCTTC-3'
	Antisense	5'-TCCTGTTTCATCTCCCAGTTATTC-3'
FTX	Sense	5'-CCAGTTTGCCTCCCTCTTT-3'
	Antisense	5'-CAGCACCTCATTCAACCTAGT-3'
HOTAIR	Sense	5'-GTGTAGACCCAGCCCAATTTA-3'
	Antisense	5'-GGCTGGACCTTTGCTTCTAT-3'
JPX	Sense	5'-GAGTCCACCACCACCATAATC-3'
	Antisense	5'-GCATGTCTTCCAGCACCATA-3'
linc312	Sense	5'-GACGCTGTTGAAGGAAGAAATG-3'
	Antisense	5'-CCAAAGGAATCAGACCAGGAG-3'
MALAT-1	Sense	5'-GATTTGAGCGGAAGAACGAATG-3'
	Antisense	5'-TGCCATGTGCCTGGAATTA-3'
NKILA	Sense	5'-GAATTGCTTTGGAAGGAGCATAG-3'
	Antisense	5'-CTGAACTGGGTGTCCTGTATTT-3'
TERC	Sense	5'-CGAGGTTCAGGCCTTTCAG-3'
	Antisense	5'-CATGTGTGAGCCGAGTCC-3'
WSPAR	Sense	5'-GTCCTTGGACCTGAGCTAAC-3'
	Antisense	5'-GGCTGGCATATAACCAACAATG-3'

BCAR4, breast cancer antiestrogen resistance 4; CCAT2, colon cancer associated transcript 2 RNA; CYTOR, cytoskeleton regulator RNA; HOTAIR, HOX transcript antisense RNA; lincRNA, long intergenic non-coding RNA; MALAT-1, metastasis associated lung adenocarcinoma transcript 1; NKILA, NF-κB interacting long non-coding RNA; TERC, telomerase RNA component; WSPAR, WNT signaling pathway activating non-coding RNA.

Table V. Specific mRNA primer sequences for reverse transcription-quantitative PCR.

Primer for mRNA		Sequences
ALAS 1	Sense	5'-AGCGCAACGTCAAACTCAT-3'
	Antisense	5'-TTTTAGCAGCATCTGCAACC-3'
Cyclin D1	Sense	5'-GGGTTGTGCTACAGATGATAGAG-3'
	Antisense	5'-AGACGCCTCCTTTGTGTTAAT-3'
Ki-67	Sense	5'-GACCTCCAAACTGGCTCCTAATC-3'
	Antisense	5'-GCTGCCAGATAGAGTCAGAAAG-3'
TOP2α	Sense	5'-GACGCTTCGTTATGGGAAGATA-3'
	Antisense	5'-GGGCCAGTTGTGATGGATAA-3'

BT-20, MDA-MB-231 and BT-474 cells (P<0.001). In SK-BR-3 cells, the CYTOR-increasing drug-dependent effect did not reach statistical significance but demonstrated a pronounced trend. The expression levels of lncRNA BC4 increased in BT-20 and SK-BR-3 cells (P<0.001), and the expression of NF-κB interacting long non-coding RNA (NKILA) increased in MCF-7 cells (P<0.001). A slight upregulation of BCAR4 was observed in T-47D cells (P=0.05). For JPX, downregulation was observed in BT-474, SK-BR-3, BT-20 and MCF-7 cells. However, the decrease was only significant in BT-474 cells. In response to epirubicin, the expression of Ki-67 decreased in MCF-7 and BT-474 cells (P<0.001; data not shown). The same trend was observed in MDA-MB-231 cells, albeit it was not statistically significant (P=0.05). In BT-474 cells, epirubicin-dependent downregulation was observed for Cyclin D1 (P=0.01; Fig. 4).

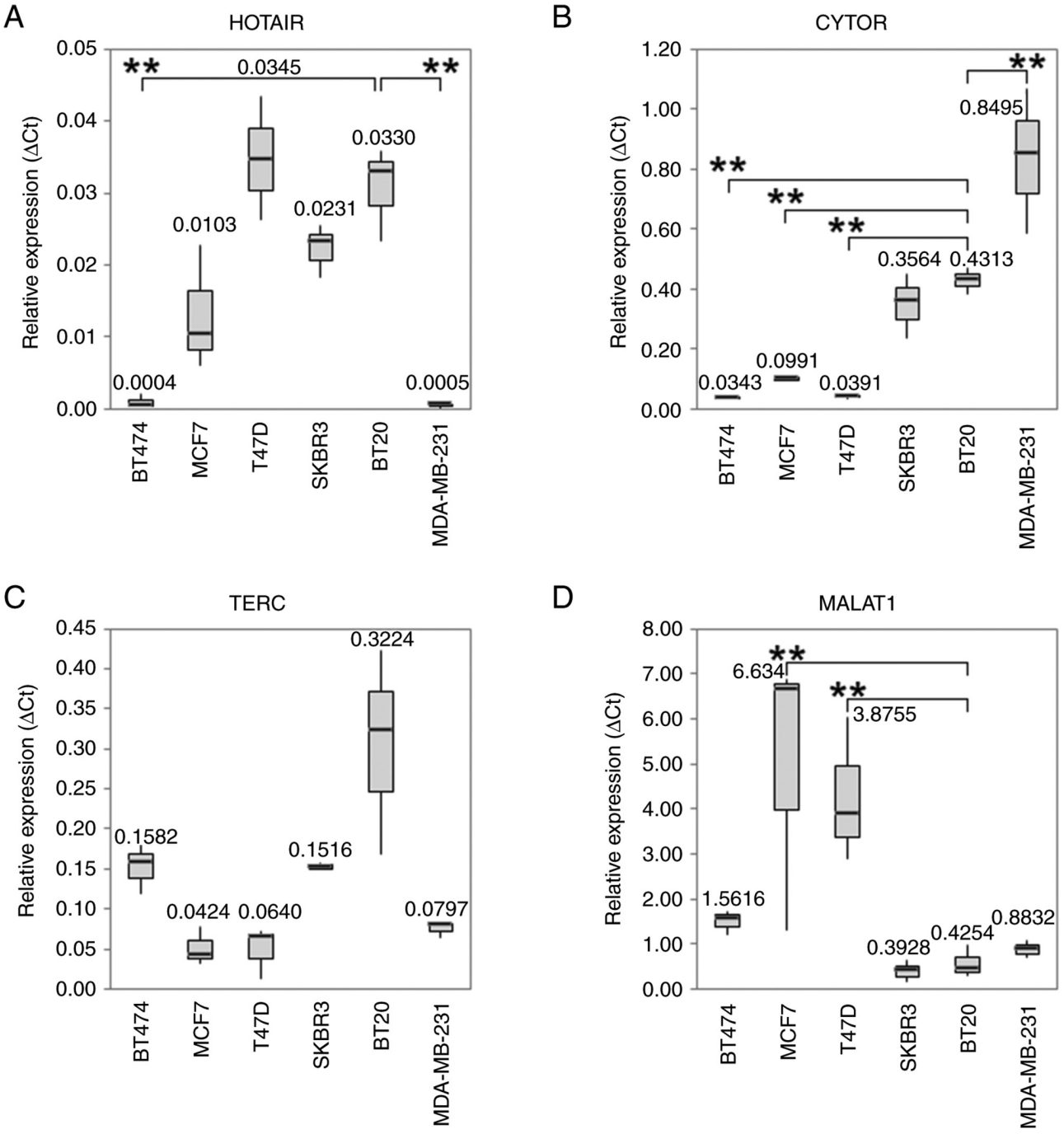

Figure 1. Boxplots with median values of mRNA expression levels. The median values of mRNA expression levels of (A) HOTAIR, (B) CYTOR, (C) TERC and (D) MALAT-1 under control conditions in different BC cell lines. The significance level refers to the calculation of the linear regression model. The untreated BT20 cells served as an intercept. (A) The expression level of HOTAIR was lower in BT-474 and MDA-MB-231 (P<0.001). The IQR for PCR is from left to right: 0.0008, 0.0083, 0.0086, 0.0036, 0.0063 and 0.0003. (B) Under standard control conditions CYTOR was overexpressed in MDA-MB-231 cells (P<0.001). In the cell lines MCF7, T-47D and BT-474 CYTOR was downregulated (P<0.001). The IQR for PCR is from left to right: 0.0068, 0.0070, 0.0050, 0.1069, 0.0429 and 0.2405. (C) TERC shows no significant differences in the expression of the different cell lines. The IQR for PCR is from left to right: 0.0307, 0.0230, 0.0299, 0.0046, 0.1266 and 0.0089. (D) MALAT-1 expression levels under standard culture conditions revealed elevated expression in the ER-positive cell lines MCF-7 (P<0.001) and T-47D (P=0.001). The IQR for PCR is from left to right: 0.2615, 2.7902, 1.5664, 0.2396, 0.3329 and 0.1853. All Box plots demonstrate median (thick black line), lower and upper quantile range (box lines), and minimum and maximum values. Based on triplicate experiments, reverse transcription-quantitative PCR. **P<0.001 vs. the intercept. IQR, interquartile range; HOTAIR, HOX transcript antisense RNA; CYTOR, cytoskeleton regulator RNA; TERC, telomerase RNA component; MALAT-1. metastasis associated lung adenocarcinoma transcript 1.

Following treatment with gemcitabine, the expression level of CYTOR significantly increased in BT-20 and BT-474 cells. The increase of CYTOR expression in SK-BR-3 cells followed a statistical trend. The RNA expression level of BC4 significantly increased in BT-20 and in SK-BR-3 cells, although without statistical significance. In MCF-7 cells, the expression of NKILA increased (P=0.05), whereas the expression of JPX decreased (P=0.05). For Ki-67, gemcitabine-dependent down-regulation was observed in the cell lines SK-BR-3 (P<0.001), BT-474 (P=0.001), BT-20 (P=0.01) and MDA-MB-231

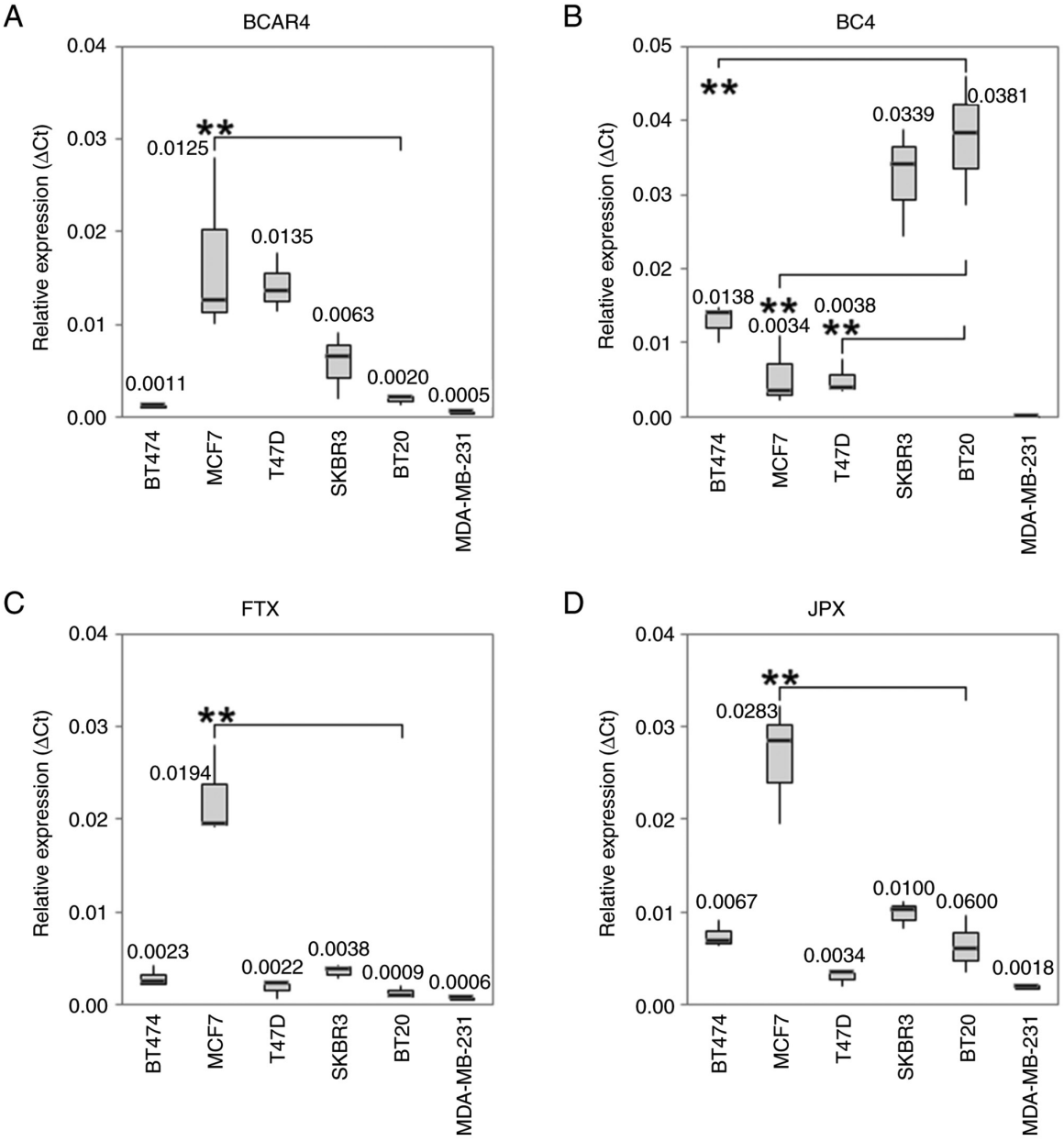

Figure 2. Boxplots with median values of mRNA expression levels. The median values of mRNA expression levels of (A) BCAR4, (B) BC4, (C) FTX and (D) JPX under control conditions in different BC cell lines. The significance level refers to the calculation of the linear regression model. The untreated BT20 cells served as an intercept. (A) In MCF-7 cells a statistically significant higher BCAR4 expression level was observed. The IQR for PCR is from left to right: 0.0002, 0.0089, 0.0031, 0.0035, 0.0005 and 0.0001. (B) The RNA expression level of BC 4 was significant reduced in MCF7, T-47D and BT-474 cells (P<0.001). The IQR for PCR is from left to right: 0.0024, 0.0043, 0.0021, 0.0072, 0.0086 and 0. (C) MCF-7 cells expressed a significant higher RNA level of FTX. The IQR for PCR is from left to right: 0.0010, 0.0044, 0.0008, 0.0008, 0.0006 and 0.0001. (D) The expression level of JPX was statistically significant higher in MCF-7 cells. The IQR for PCR is from left to right: 0.0014, 0.0063, 0.0007, 0.0014, 0.0030 and 0.0002. All Box plots demonstrate median (thick black line), lower and upper quantile range (box lines), and minimum and maximum values. Based on triplicate experiments, reverse transcription-quantitative PCR. **P<0.001 vs. the intercept. IQR, interquartile range; BCAR4, breast cancer antiestrogen resistance 4.

(P=0.01). The mRNA expression level of Cyclin-D1 decreased in BT-474 cells (Fig. 4).

Paclitaxel led to an increased expression level of lncRNA FTX in MCF-7 cells (P=0.001). The other pre-selected lncRNAs showed no alterations of their expression levels after treatment with paclitaxel (Fig. 4). Following treatment with fulvestrant, the expression of JPX decreased in MCF-7 cells (P=0.01), while other alterations did not reach statistical significance (Fig. 4). No zoledronic acid-driven alterations in the expression levels of the pre-selected lncRNAs were observed in any of the cell lines investigated (Fig. 4).

Discussion

The present study analyzed the potential effects triggered by different BC treatments on the transcriptional expression of pre-selected lncRNAs and the proliferation markers Cyclin D1 and Ki-67 in six different BC cell lines. Following treatment with epirubicin and gemcitabine, the levels of the proliferation marker Ki-67 decreased in all six cell lines, indicating a decreased proliferation after chemotherapy and thus treatment response, while Cyclin D1 levels only decreased in the cell line BT-474. Therefore, in the present study, Ki-67 was considered

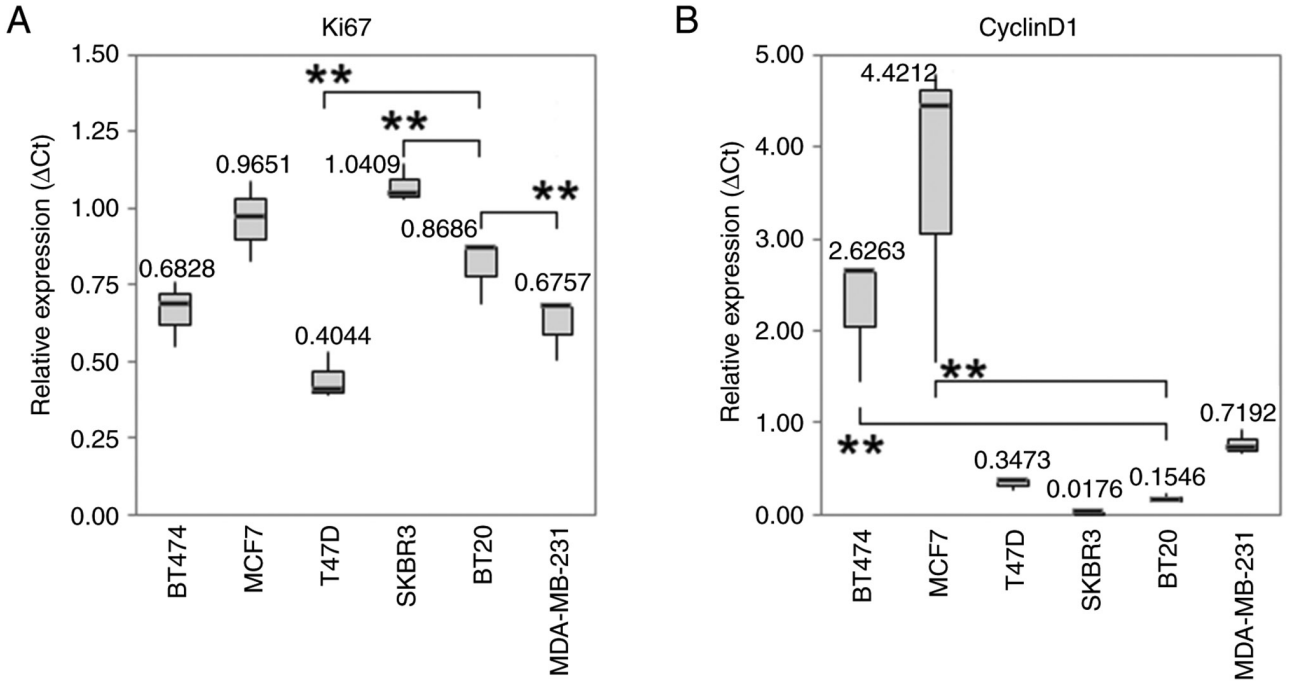

Figure 3. Boxplots with median values of mRNA expression levels. The median values of mRNA expression levels of (A) Ki67 and (B) cyclin D1 under control conditions in different BC cell. The significance level refers to the calculation of the linear regression model. The untreated BT20 cells served as an intercept. (A) The RNA expression level of the proliferation marker Ki-67 was overexpressed in SK-BR-3 (P=0.001). In T-47D cells (P<0.001) and MDA-MB-231 cells (P=0.01) were a downregulation of Ki-67 detectable. The IQR for PCR is from left to right: 0.1051, 0.1304, 0.0700, 0.0589, 0.0943 and 0.0860. (B) Under standard culture conditions cyclin D1 was statistically significant higher expressed in MCF-7 and BT-474 cells. The IQR for PCR is from left to right: 0.5895, 1.5604, 0.0607, 0.0077, 0.0430 and 0.1269. All Box plots demonstrate median (thick black line), lower and upper quantile range (box lines), and minimum and maximum values. Based on triplicate experiments, reverse transcription-quantitative PCR. **P<0.001 vs. the intercept. IQR, interquartile range.

to serve as a predictive marker for the six cell lines analyzed following treatment with epirubicin and gemcitabine, while Cyclin D1 could only be used for interpretation in the BT-474 cell line.

LncRNA CYTOR has emerged as an important player in cancer progression across various malignancies, underscoring its potential both as a biomarker and a therapeutic target. Recent research agrees with the present observations that CYTOR expression is dysregulated in multiple cancer types, which is further altered following treatment with chemotherapy agents such as epirubicin and gemcitabine (38). Specifically, the current study noted an upregulation of CYTOR in selected cell lines under chemotherapy, coinciding with a concurrent downregulation of Ki67, indicating a potential therapeutic response. This observation is supported by a body of evidence suggesting the involvement of CYTOR in cancer cell proliferation, migration and invasion. For instance, a previous study demonstrated the role of CYTOR in modulating key signaling pathways implicated in cancer progression, such as the PI3K/Akt signaling pathway, thus highlighting its contribution to enhanced cancer cell survival and proliferation (39). Furthermore, dysregulation of CYTOR has been associated with chemoresistance in certain cancer types, similar to the present findings. Elevated CYTOR expression has been linked to resistance against gemcitabine-based chemotherapy in pancreatic cancer (40) and to tamoxifen therapy resistance in BC (41).

Additionally, studies have begun to unravel the molecular mechanisms underlying the function of CYTOR, elucidating its important role in cancer biology. For example, previous research has highlighted its involvement in regulating apoptosis and cell cycle progression, thereby contributing to cancer therapy resistance (38-42). Such evidence suggests that targeting CYTOR could offer a new avenue for combating treatment resistance and improving patient outcomes. With the role of CYTOR in cancer biology becoming increasingly clear, it can be concluded that CYTOR may serve not only as a marker of disease progression but also as a potential target for therapeutic intervention, especially in the context of chemoresistance (42,43).

The alignment of the present study with these findings underscores the importance of further investigating CYTOR in cancer research, particularly its response under chemotherapy, to develop more effective treatments. In the current study, CYTOR was consistently expressed in every cell line and underwent a marked treatment-induced regulation. Under control conditions, CYTOR expression was high in the triple-negative BC cell lines BT-20 and MDA-MB-231 (P<0.001). A lower expression level was observed in the hormone-receptor positive cell lines MCF-7 and T-47D (P<0.001). The increased expression levels of CYTOR in triple-negative BC cell lines support the hypothesis that CYTOR is associated with higher aggressiveness and worse overall survival in different tumor types, as demonstrated by Liang *et al* (44).

Following treatment with epirubicin and gemcitabine, the levels of the proliferation marker Ki-67 decreased in all cell lines, although to a different extent. The RNA expression of CYTOR increased following therapy with epirubicin and gemcitabine. After epirubicin treatment, this increase was

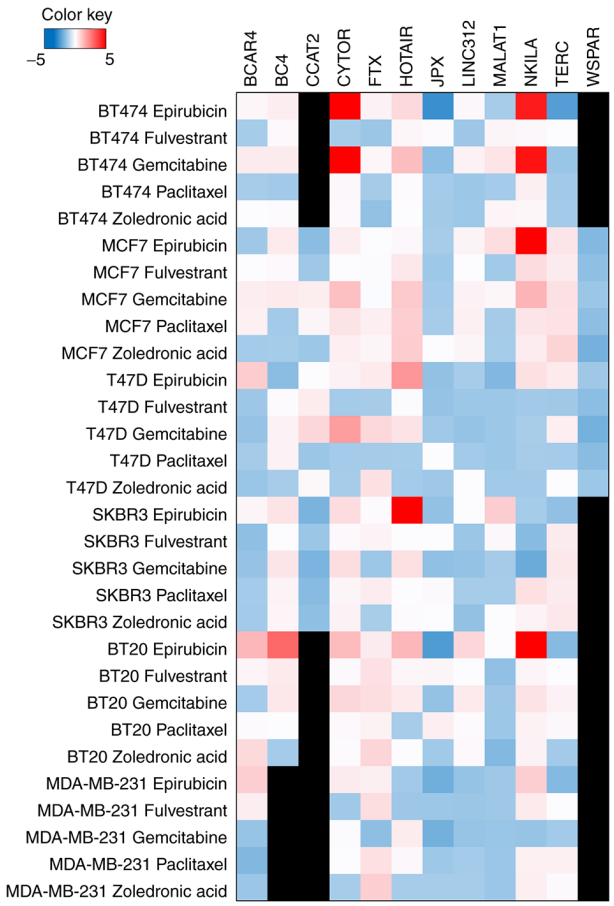

Figure 4. The influence of treatment on lncRNA expression levels of the different BC cell lines. Expression profiles of selected lnc-RNAs in BT-474, MCF-7, T-47D, SKBR-3, BT-20 and MDA-MB-231cells under control conditions and after different breast cancer treatments. Lnc-RNA-expressions were determined in triplicates by real-time quantitative PCR and calculated using ΔCt method based on reference value (geometric mean of housekeeping genes Alas1 and TOP2α) to determine expression of single lncRNAs. After calculating the expression level. The data were visualized in a heatmap according to the color key. It represents the fold change of the ΔΔCt values compared to the same cell line untreated. lncRNA, long non-coding RNA; CCAT2, colon cancer associated transcript 2 RNA; CYTOR, cytoskeleton regulator RNA; HOTAIR, HOX transcript antisense RNA; MALAT-1, metastasis associated lung adenocarcinoma transcript 1; NKILA, NF-κB interacting long non-coding RNA TERC, telomerase RNA component; WSPAR, WNT signaling pathway activating non-coding RNA.

most pronounced in the triple-negative BC cell lines BT-20 and MDA-MB-231 (P<0.001). The same effect was observed in BT-474 cells. Under the influence of gemcitabine, the expression level of CYTOR increased in BT-20 and BT-474 cells. Wu *et al* (27) described a proliferation-stimulation effect of CYTOR *in vitro* and *in vivo*. It appears that cell lines with a high proliferation activity and responsiveness to chemotherapy (epirubicin and gemcitabine) cause reactive CYTOR overexpression. This confirms the results reported by Van Grembergen *et al* (45), who showed that CYTOR was essential for the proliferation in MDA-MB-231 BC cells. Knockdown of CYTOR leads to cell accumulation at the end of the S phase and in the G_2/M phase. Since epirubicin and gemcitabine have their maximum toxic effect in the S/G_2 phase, the increased expression of CYTOR observed during treatment can be interpreted as an adaptation of the cancer cells to try to maintain proliferation. Based on the present results, it may be hypothesized that CYTOR will be overexpressed if the treatment is effective. In the current study, treatment with paclitaxel, fulvestrant or zoledronic showed no significant alteration in the expression levels of Ki-67 or CYTOR.

The lncRNA HOTAIR is observed to be upregulated in various cancer types (46). In the present study, HOTAIR was consistently expressed in the selected cell lines. Following treatment with epirubicin, the RNA expression level of HOTAIR increased. This is in agreement with previous findings, such as those by Teschendorff *et al* (47), who found a correlation between overexpression of HOTAIR and carboplatin resistance in ovarian cancer. The epirubicin-dependent overexpression of HOTAIR may also be a sign of resistance. A statistically significant increase in HOTAIR expression was observed in BT-20, SK-BR-3 and T-47D cells. None of the cell lines analyzed in the present study showed a simultaneous significant decrease in the proliferation marker Ki-67. This contrasts with cell lines lacking HOTAIR overexpression, which displayed an epirubicin-dependent decline in Ki-67 levels. According to the current findings and recent research, HOTAIR is overexpressed under therapies such as paclitaxel, cisplatin, paclitaxel and doxorubicin, temozolomide and sunitinib (48-52). Of note, the present and other studies have shown that HOTAIR knockout can lead to increased chemotherapy efficacy, suggesting that HOTAIR may indicate resistance (48,53). Additionally, the current study contributed to the understanding of the role of HOTAIR in treatment response, particularly in the context of epirubicin therapy. Furthermore, there is a relevant study on NSCLC and EGFR-TKI therapy that shows conflicting results (54), where HOTAIR reduction was demonstrated post-therapy, contrasting with the present findings. Wang *et al* (55) were able to show, in a pancreatic cancer cell line, that treatment with gemcitabine led to overexpression of HOTAIR, which promoted cell proliferation and migration and reduced the apoptosis rate. After suppression of HOTAIR with small interfering RNA, the sensitivity to gemcitabine increased. This process could be reversed by lentivirus-induced expression of HOTAIR, so that chemotherapy resistance can be assumed by induction of HOTAIR after gemcitabine administration (55). Thus, HOTAIR may be a target for new therapy against chemotherapy resistance. In the present study on BC cell lines, overexpression of HOTAIR was only significantly detectable after epirubicin treatment, while the alterations observed after gemcitabine treatment were not statistically significant. In addition, a study has shown overexpression of HOTAIR in association with endocrine resistance, more precisely tamoxifen resistance (26). The fulvestrant-dependent alteration of the expression level of HOTAIR was examined in the present study. However, no significant alteration in HOTAIR was measurable after fulvestrant treatment. The results of the current study are congruent with those of Milevskiy *et al* (56), who hypothesized that the association between estrogen and HOTAIR may play a role in endocrine resistance. The authors used well-documented tamoxifen-resistant, fulvestrant-resistant and estrogen-independent sublines of MCF7 cells (56). HOTAIR expression increased in both low-estrogen MCF7X and tamoxifen-resistant TAMR cells, which was consistent with a repressive

role of the estrogen/ER pathway. After fulvestrant treatment, HOTAIR expression did not increase significant.

In contrast to the established body of research, the findings of the present study on the expression of 10 lncRNAs NF-κB interacting long non-coding RNA (NKILA), breast cancer antiestrogen resistance 4 (BCAR4), colon cancer associated transcript 2 RNA (CCAT2), metastasis associated lung adenocarcinoma transcript 1 (MALAT1), telomerase RNA component (TERC), WNT signaling pathway activating non-coding RNA (WSPAR), BC4, FTX, JPX and long intergenic non-coding RNA (lincRNA) 00312 do not present a consistent pattern of regulation. This deviation underscores a complex interaction that may be influenced by the specific conditions of the present experimental setup, including cell line selection, drug dosage, and treatment duration, which are crucial factors as outlined in the referenced publications. For instance, Lasfargues and Ozzello (57) as well as Trempe (58) have emphasized the heterogeneity of breast cancer cell lines, which might explain the sporadic regulation patterns observed in the present study, contrasting with the uniform responses reported in previous studies (4,5).

The effect of different chemotherapeutic agents on lncRNA expression, particularly epirubicin and gemcitabine, also reveals a disparity when compared to literature. Khasraw *et al* (13) and Seidman (14) discuss the general mechanisms and effects of these drugs in breast cancer treatment but do not delve into their specific impacts on lncRNA regulation. The present findings that NKILA, BCAR4 and others exhibit varied expression under these treatments suggested a more complex interaction than previously understood, further diverging from studies that did not observe such effects (10,17).

Focusing on the body of literature on the single lncRNAs, especially NKILA, BCAR 4 and JPX should be discussed in more detail. Previous studies have shown NKILA to be involved in suppressing breast cancer metastasis through inhibition of NF-κB signaling (59,60). The present observation of NKILA upregulation in MCF-7 cells under epirubicin and gemcitabine treatment could suggest a stress response mechanism. However, the lack of a consistent response across cell lines and treatments indicates a potential context-dependent regulation.

Highlighted in the work of Godinho *et al* (61), BCAR4 is known to drive endocrine resistance in breast cancer. The present findings of BCAR4 upregulation in MCF-7 untreated cells and T-47D cells under epirubicin treatment could reflect its role in resistance mechanisms, yet the absence of this trend across other treatments and cell lines underscores the variability in its expression and potential function.

Involved in X chromosome inactivation and regulation, JPX shows downregulation under epirubicin treatment in BT-474 and gemcitabine in MCF-7 (62). This suggests a potential involvement in stress response or treatment resistance mechanisms, albeit in a cell line-specific manner.

In addition, CCAT2, associated with breast cancer growth and metastasis, showed no significant changes, suggesting that its effect may lie in metastatic processes rather than direct treatment response (63,64). MALAT1, linked to poor prognosis and tumor growth, was more highly expressed in ER-positive cell lines, hinting at a fundamental role in tumorigenesis (65). TERC, crucial for telomere maintenance, exhibited cell line-specific upregulation in BT-20, indicating a complex interplay with treatment conditions (66). Lesser-studied lncRNAs such as WSPAR and BC4 showed minimal or specific regulation, underscoring the need for further research to clarify their functions in breast cancer biology and treatment responses.

WSPAR has been described in association with colorectal and hepatocellular cancer. WSPAR overexpression correlates with cell proliferation, invasion and metastatic growth. However, no function of WSPAR has been reported in BC thus far. In the present study, only the hormone receptor-positive cell lines MCF-7 and T-47D showed WSPAR expression. However, the expression levels showed no significant drug-induced alterations.

Ding *et al* (67) described the downregulation of BC4 in BC, which was confirmed in the present study, since each BC cell line showed low BC4 expression. After treatment with epirubicin and gemcitabine, the expression level of BC4 increased in BT-20 and SK-BR-3 cells, while Ki-67 expression levels decreased in both cell lines at the same time after treatment with gemcitabine only. Epirubicin did not induce a significant alteration in Ki-67 expression in BT-20 or SK-BR-3 cells. Due to the divergent expression-alteration of Ki-67, it is difficult to interpret the meaning of the increase in BC4 expression during therapy. Thus, further studies are needed to elucidate the meaning of BC4 overexpression for therapy efficiency monitoring.

The lncRNAs TERC, MALAT-1 and linc312 were consistently expressed in each cell line, but showed no significant treatment-dependent alterations. In addition, the expression of CCAT2 showed no significant therapy modification. Notably, CCAT2 was consistently expressed in every cell line with the exception of BT-474.

BCAR4, a lncRNA associated with endocrine resistance (61), showed limited therapy-dependent alterations in the present study. The expression level of BCAR4 decreased ($P=0.01$) after epirubicin treatment only in T-47D cells, without any alterations in Ki-67 or Cyclin D1 expression.

Wu *et al* (59) reported a negative correlation between the expression level of NKILA and tumor/metastasis proliferation. Liu *et al* (60) have shown NKILA to be involved in suppressing breast cancer metastasis through inhibition of NF-κB signaling. In the present study, the effect of epirubicin in NKILA expression was observed only in the MCF-7 cell line.

The expression level of the lncRNA JPX was higher in MCF-7 and BT-20 cells and lower in MDA-MB-231 cells. Following epirubicin treatment, a decrease in JPX expression was detectable in BT-20, BT-474, MCF-7 and SKBR-3 cells. However, a reduction in the expression level of Ki-67 was observed at the same time only in BT-474 and MCF-7 cells. Fulvestrant also led to a decrease in JPX expression level in MCF-7. The interpretation of the role of downregulation of JPX is therefore challenging. Huang *et al* (68) reported that suppression of JPX via reduced expression of the tumor suppressor Xist led to reduced inactivation of AKT phosphorylation and thus to increased cell viability. Overexpression of XIST markedly inhibited BC cells proliferation, migration and invasion via sponging to microRNA-155 in BC (69).

The present study has certain limitations. The current study investigated whether there were any connections between

selected BC drugs and pre-selected RNAs; future studies should focus on the identification of the signaling pathways involved.

Although paclitaxel is an established, potent drug in the treatment of BC, overall, it rarely appeared to have effects on the different cell lines analyzed in the present study. The concentration of the drugs, including paclitaxel, was based on the maximum serum levels during *in vivo* therapy under clinical conditions. The duration of drug application was based on the half-value period of the drug and findings from our preliminary experiments. Li *et al* (70) observed a clear proliferation-inhibiting effect of paclitaxel with prolonged exposure (48 instead of 18 h) and double concentration (10 instead of 5 nM). However, with an incubation time of 48 h at 10 nM, the drug dose of such study is within a range that is not reached clinically in BC therapy. The reaction of cells *in vitro* cannot, however, represent the exact behavior of an organism with metabolism and redistribution. The conditions may suggest that paclitaxel, in the present study, was applied in an excessively low concentration for finding effects in proliferation and lncRNA expression. Considering the interpretation of these results, it is not certain whether lncRNAs hold predictive value for paclitaxel in reality. Potential effects could have been missed in the current study due to the aforementioned lower dosage and exposure of the drug. Therefore, further explorations with adjusted experimental conditions should be pursued in the future. As potential avenues for future research, a step-wise procedure could be envisioned. Firstly, *in vitro* experiments should be performed with increased dosages and longer exposure of paclitaxel. Secondly, *in vivo* models could be introduced to mimic the clinical metabolic conditions in a more realistic manner. Lastly, *in vitro* and *in vivo* results could be validated in clinical trials by analyzing the breast tissue or liquid samples of patients under paclitaxel treatment. Another limitation is that the expression of the lncRNAs and the proliferation markers are regulated by a number of factors. Further studies must confirm whether the discovered correlations can really be causally linked. An additional limitation was that the response to the treatment was assessed visually. At a 50% reduction of cells in a cell culture flask, the response to treatment was assessed as positive. A viability assay would be useful for further projects.

In summary, the strength and innovative findings of this study relate to the significant correlation of lncRNA alterations to established disease biomarkers such as Ki67. CYTOR and HOTAIR showed significant expression alterations under different chemotherapeutic treatments (especially under anthracyclines) simultaneously to Ki67 downregulation and thus suggesting biomarker properties for treatment response. In clinical practice, CYTOR and HOTAIR could potentially be used to determine treatment effects under anthracyclines specifically in tissue and liquid biopsies. Based on the findings of the present study, the upregulation of HOTAIR and CYTOR potentially indicate treatment response whereas their downregulation or expression level maintenance might indicate treatment failure. As the proper therapy response is crucial for overall and progression free survival, these findings could be game-changing for the management of chemotherapy patients. Thus, further studies must be undertaken to analyze HOTAIR and CYTOR with regards to therapy response. In the present study, the other 10 lncRNAs did not show significant expression alterations under the aforementioned treatments. This could mean that they are not suitable for an early detection of therapy response under the given therapeutics. Furthermore, it might also mean that the treatment doses in the present study were not adequate.

In conclusion, the present study analyzed *in vitro* the potential effects of various drugs on the transcriptional expression of pre-selected lncRNAs to indicate therapy resistance and response in BC. The results revealed that lncRNA CYTOR may be an appropriate biomarker for the response to treatment with both epirubicin and gemcitabine, while NKILA may be a marker for treatment response. Furthermore, HOTAIR overexpression may suggest therapy resistance to epirubicin, while BC4 transcriptional overexpression may indicate therapy response to gemcitabine and epirubicin. Overall, treatment-dependent alterations were not observed in every cell line investigated and the extent of the alteration differed, which may reflect the heterogeneity of BC disease even *in vitro*. However, the therapy-dependent alterations shown in the current study suggest that lncRNAs may hold potential to serve as biomarkers for treatment response or resistance. In addition, lncRNAs may be explored as a prognostic and diagnostic molecule in patients with metastatic BC. However, the expression of lncRNAs and their role as potential biomarkers remain to be fully clarified. Further research should focus on the elucidation of the pathways involved in lncRNAs regulatory mechanisms and the analysis of lncRNAs for monitoring of BC treatment *in vivo*.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JA, BS, KB, MJ, IJB, TE and SM designed the present study. MJ, DW and BS performed experiments (cell culture, RNA isolation and RT-qPCR). JA and SM confirm the authenticity of all the raw data. BS performed statistical analysis. JA, KB, IG, BS, TE, IJB and SM analyzed and interpreted the data. JA, TE, KB and BS wrote the manuscript in consultation with and under critical revision of DW, MJ, IG, SM and IJB. JA and SM supervised the overall experimental design. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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