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Inhibition of Increased Invasiveness of Breast Cancer Cells With Acquired Tamoxifen Resistance by Suppression of CYR61

GERD BAUERSCHMITZ, SILKE HÜCHEL, JULIA GALLWAS and CARSTEN GRÜNDKER

Department of Gynecology and Obstetrics, University Medical Center Göttingen, Göttingen, Germany

Abstract. *Background/Aim: Hormone sensitivity-targeted therapy with selective estrogen receptor modulators (SERMs), such as 4-hydroxytamoxifen (4-OHT), is the mainstay of treatment for breast cancers (BCs) that express estrogen receptor α (ERα). However, development of resistance limits this therapy approach. The question arises whether changes associated with 4-OHT resistance could be exploited therapeutically. Materials and Methods: First, 4-OHT-resistant sublines of ERα-positive breast carcinoma cell lines MCF-7 and T47D were generated. Viability was assessed by the Alamar Blue assay. Cell invasion was quantified in modified Boyden chambers with Matrigel. Changes in expression of CYR61, S100A4, and ERα were examined by RT-qPCR. Expression of CYR61 was suppressed by transient gene silencing using siRNA. Successful suppression was verified by western blot. Efficacy of 4-OHT treatment was analyzed by quantification of viability using Alamar Blue assay. Correlation of CYR61 levels in patients with luminal A BC to distant metastases-free survival was determined by Kaplan-Meier analysis. Results: ERα-positive MCF-7 and T47D BC cells exhibit an extremely weak invasion rate. Acquired tamoxifen resistance significantly increased the invasive behavior of both tamoxifen-resistant MCF-7-TR and T47D-TR sublines. In addition, expression of CYR61 and S100A4 showed significantly increased levels, whereas expression of ERα was decreased. Suppression of CYR61 expression resulted in a significant decreased invasion rate. In addition, expression of S100A4 was reduced, whereas expression of ERα was increased. Furthermore, suppression of CYR61 resulted in re-sensitization to 4-OHT. High CYR61 levels in patients with luminal A BC resulted in reduced distant metastases-free survival. Conclusion: The prometastatic factor CYR61 appears to play an important role in the increased invasiveness of tamoxifen-resistant ERα-positive BC cells. Its suppression leads to a lower invasion rate. Given the few therapeutic options available for tamoxifen-resistant BC, therapy that reduces CYR61 may improve its treatability in future.*

Tamoxifen works on estrogen receptors (ERs) as a selective estrogen receptor modulator (SERM) and is used in breast cancer (BC) cells of luminal A and luminal B subtypes, as these express estrogen receptor α (ERα). Tamoxifen aims for specific antagonist effects on ERα in most tissues and thus exhibits an antiproliferative effect. Following intake, tamoxifen is converted in the liver into active and significantly more powerful metabolites by a variety of cytochrome p (CYP) enzymes. This also applies to 4-hydroxytamoxifen (4-OHT) (1). It binds to ER in a competitive manner but does so in a way that causes a confirmation shift, resulting in ER antagonism. A complex is formed with coactivators, leading to an inhibitory effect on estrogen response elements (EREs) and suppressing ER-dependent transcription. Thus, tamoxifen functions at the transcriptional level as a selective estrogen receptor modulator (SERM). Seventy-five percent of BCs are hormone-sensitive and can therefore be treated with SERMs, such as tamoxifen (2). Along with potential side effects, development of tamoxifen resistance is a significant issue in clinical practice. Tamoxifen resistance either primary or secondary affects more than 50% of patients (3). We still do not fully understand the precise mechanisms that lead to development of tamoxifen resistance. For these patients, it is crucial to develop new, innovative therapy choices.

Cysteine-rich angiogenic inducer 61 (CYR61) and S100 calcium binding protein A4 (S100A4) have been found to be highly expressed in tissue samples of breast hyperplasia, carcinoma in situ, and malignant BC. Both prometastatic

Correspondence to: Prof. Dr. Carsten Gründker, Department of Gynecology and Obstetrics, University Medical Center Göttingen, Robert-Koch-Street 40, 37075 Göttingen, Germany. Tel: +49 (0)5513969810, e-mail: grundker@med.uni-goettingen.de

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factors play important roles in epithelial-mesenchymal transition (EMT), invasion, and metastasis by promoting tumor cell motility. Aggressive, metastatic BCs cells express high levels of CYR61 and S100A4. Suppression of these factors results in a significantly decreased cell invasion. Therefore, they are considered potential therapeutic targets (4). The ERα positive BC cells lines MCF-7 and T47D show little invasive behavior and low expression of the prometastatic factors CYR61 and S100A4 (5). After mesenchymal transition of MCF-7 cells, there is an increase in expression of these prometastatic factors as well as a significantly increased invasion (5). Reduction in the CYR61 expression resulted with in reduced invasion (5, 6).

Acquired tamoxifen resistance in ERα-positive BC likely leads to increased aggressiveness associated with increased invasion and metastasis. The question arose whether suppression of CYR61 can reduce the increased invasiveness of BC cells with acquired tamoxifen resistance.

Materials and Methods

Cell lines and culture conditions. The human BC cell lines MCF-7 and T47D were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). 4-hydroxytamoxifen (4-OHT) resistant sublines MCF-7-TR and T47D-TR were developed as previously described (7) using a concentration of 4-OHT (Sigma, Deisenhofen, Germany) of 1.25 μM in culture medium. The cells were cultured as previously described (7).

Small interfering RNA transfection. MCF-7-TR (10^6 cells/ml) and T47D-TR (1×10^5 cells/ml) BC cell lines were grown in 25 cm^2 cell culture flasks with 2 ml of MEM [w/o penicillin/streptomycin (P/S)] supplemented with 10% FBS. The cells were treated with siRNA specific to CYR61 (sc-39331; Santa Cruz Biotechnology, Dallas, TX, USA) in OPTI-MEM I medium (Gibco, Carlsbad, CA, USA) with siRNA transfection reagent (sc-29528; Santa Cruz Biotechnology). Non-targeting siRNA was used as control (sc-37007; Santa Cruz Biotechnology). After 6 h, MEM supplemented with 20 % FBS and 20% P/S was added.

Viability assay. The BC cells were seeded in 96 well plates (1.25×10^3) in DMEM w/o phenol-red supplemented with 10% cs-FBS. Relative AlamarBlue (BioRad, Hercules, CA, USA) reduction was quantified after 120 h, as previously described (8).

Co-culture transwell vertical invasion assay. The co-culture transwell vertical invasion assay (9) was performed as described earlier (8, 10) using 1×10^4 BC cells seeded into the upper wells and 2.5×10^4 MG63 osteosarcoma cells seeded (2.5×10^4) into the lower wells. After 24 h, cells were co-cultured for 96 h.

Real-time quantitative PCR analysis. Real-time quantitative PCR analysis was performed as previously described (6). Primers were, for CYR61 5'-CTC CCT GTT TTT GGA ATG GA-3' (forward) 5'-TGG TCT TGC TGC ATT TCT TG-3' (reverse), for S100A4 5'-GTA CTC GGG CAA AGA GGG TG-3' (forward) 5'-TTG TCC CTG TTG CTG TCC AA-3' (reverse), for ERα 5'-TGG GCT TAC TGA CCA ACC TG-3' (forward) 5'-CCT GAT CAT GGA GGG TCA AA -3' (reverse), and for GAPDH 5'-GAA GGT CGG AGT CAA CGG AT-3' (forward) 5'-TGG AAT TTG CCA TGG GTG GA -3' (reverse). PCR conditions were: denaturing once at 95˚C (2 min), 95˚C (5 sec), and 60˚C (15 sec) for 40 cycles.

Western blot analysis. Western blot analysis was performed as previously described (11). Primary antibodies against CYR61 1:250 (HPA029853; Sigma, St. Louis, MO, USA) and GAPDH 1:2000 (MAB379; Chemicon International, Temecula, CA, USA) were used.

Kaplan-Meyer analysis. To analyze the prognostic value of CYR61 on distant metastases-free survival, a Kaplan-Meyer plotter analysis (12) was performed as previously described (6) using 241 patient samples.

Statistical analysis. All experiments were performed on at least three biological and technical replicates. Data were analyzed using the GraphPad Prism Software version 8.41 (GraphPad Software Inc., La Jolla, CA, USA) using unpaired, two-tailed, parametric *t*-tests comparing two groups (treatment to respective control) by assuming both populations had the same standard derivation or with an ANOVA one-way analysis when more than two groups were compared. F-values were recorded, and a Dunnett's or a Tukey's multiple comparison test with no matching or pairing between groups was calculated. $p<0.05$ was considered statistically significant.

Results

Generation of tamoxifen-resistant BC cells. First, sublines of ERα-positive breast carcinoma cell lines MCF-7 and T47D with secondary resistance against 4-hydroxytamoxifen (4-OHT) were generated according Günthert *et al*. (7). Treatment of parental MCF-7 cells with 5 μM 4-OHT resulted in a reduction of viability to 51.57±7.13 % ($p<0.01$ *vs*. control=100%, n=3; Figure 1A), while viability of tamoxifen-resistant MCF-7-TR cells remained unchanged (99.87±1.54 %, n=3; Figure 1A). Viability of parental T47D cells treated with 5 μM 4-OHT was reduced to 44.78±7.03 % ($p<0.01$ *vs*. control=100%, n=3; Figure 1B). Tamoxifen-resistant T47D-TR cells treated with 5 μM 4-OHT showed no changes in viability (92.49±3.71 %, n=3; Figure 1B).

Increased invasiveness of tamoxifen-resistant BC. The ERα-positive BC cells lines MCF-7 and T47D showed very low invasiveness (Figure 2A). In contrast, their tamoxifen-resistant sublines MCF-7-TR and T47D-TR showed significantly increased invasion. The invasiveness of MCF-7-TR cells was increased 3.5-fold to 363.71±56.77 % ($p<0.0001$ *vs*. MCF-7=100±14,89%, n=22). The T47D-TR cells showed a 3-fold increase in invasion to 310.05±62.28 % ($p<0.01$ *vs*. T47D=100±25.35%, n=22).

Increased expression of CYR61 and S100A4 and decreased expression of ERα in tamoxifen-resistant BC. mRNA expression of CYR61, S100A4, and ERα was detectable in

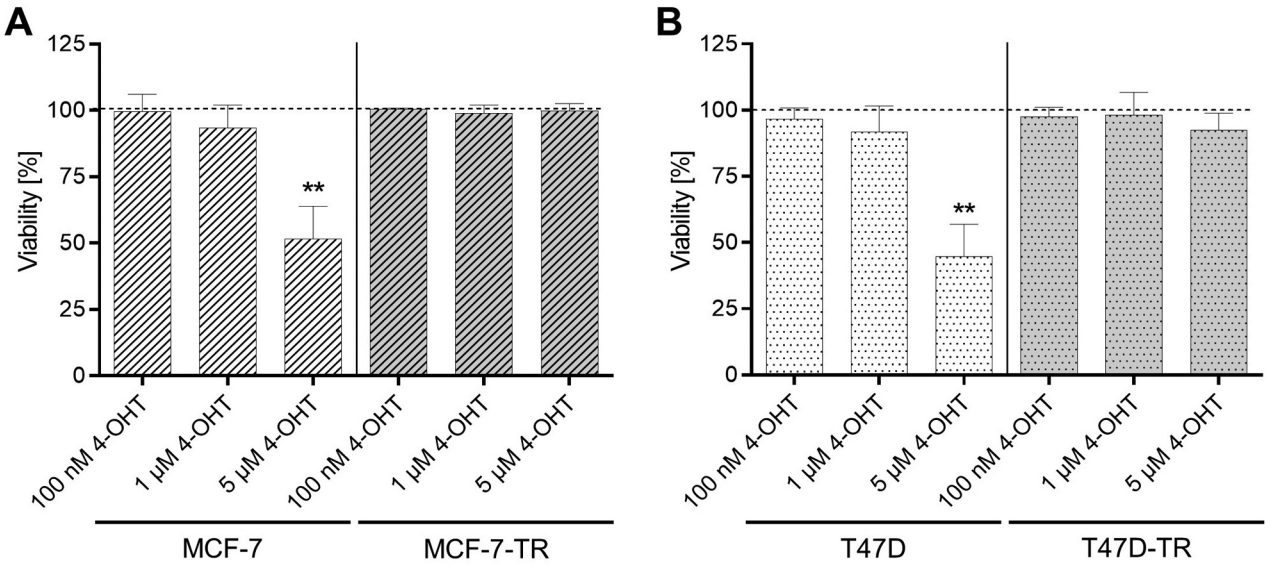

Figure 1. *Generation of tamoxifen-resistant BC cells. ERα-positive BC cell lines MCF-7 (A) and T47D (B) with secondary resistance against 4-OHT were generated according Günthert et al. (7). Viability of parental MCF-7 (A) and T47D (B) BC cells and tamoxifen-resistant MCF-7-TR (A) and T47D-TR (B) BC cells after treatment with 4-OHT. Mean±SEM; n=3; unpaired two-tailed t-test; **p<0.01. 4-OHT: 4-hydroxytamoxifen; BC: breast cancer; ERα, estrogen receptor α.*

luminal A BC cell lines MCF-7 and T47D (Figure 2B-D). Their tamoxifen-resistant sublines MCF-7-TR and T47D-TR showed significantly increased relative mRNA expression of CYR61 [MCF-7-TR: 2.23±0.17 of MCF-7 (=1), $p<0.0001$; T47D-TR: 3.04±0.62 of T47D (=1), $p<0.01$; Figure 2B] and of S100A4 [MCF-7-TR: 1.84±0.27 of MCF-7 (=1), $p<0.05$; T47D-TR: 1.47±0.16 of T47D (=1), $p<0.05$; Figure 2C], whereas relative ERα mRNA expression (Figure 2D) was significantly decreased to 0.36±0.09 [$p<0.001$ *vs.* MCF7 (=1)] and to 0.42±0.13 [$p<0.01$ *vs.* T47D (=1)].

Reduced invasiveness after suppression of CYR61 in tamoxifen-resistant BC. First, we checked whether CYR61 expression was effectively reduced by siRNA. Significant suppression of CYR61 expression in MCF-7-TR cells (Figure 3A) was detectable as early as 24 h after siRNA transfection (35.89±7.49%; $p<0.0001$ *vs.* control=100%) and CYR61 expression remained significantly reduced until 96 h (46.42±7.55%; $p<0.0001$ *vs.* control=100%). CYR61 suppression was significant in T47D-TR cells 24 h after transfection (29.23±9.88; $p<0.01$ *vs.* control; not shown) and CYR61 expression remained significantly reduced until 48 h (46.58±16.74; $p<0.05$ *vs.* control; not shown). Suppression of CYR61 in T47D-TR cells remained reduced up to 96 h but was no longer significant (60.50±17.53%; not shown).

Suppression of CYR61 expression resulted in significantly reduced invasion (Figure 3B). The number of invaded MCF-7-TR cells was reduced to 18.10±3.79% of siRNA control (=100%; $p<0.0001$). The number of invaded T47D-TR cells was reduced to 44.49±8.27 % of control (=100%; $p<0.01$).

Reduced expression of S100A4 and increased expression of ERα after suppression of CYR61 in tamoxifen-resistant BC. Suppression of CYR61 expression (Figure 3A) resulted in significantly reduced expression of S100A4 (Figure 3C) and significantly increased expression of ERα (Figure 3D). After CYR61 suppression, relative mRNA expression of S100A4 in MCF-7-TR cells was reduced to 0.64±0.05 of siRNA control (=1; $p<0.001$) and S100A4 relative mRNA expression in T47D-TR cells was reduced to 0.78±0.08 of siRNA control (=1; $p<0.05$). Relative mRNA expression of ERα was increased to 1.68±0.17 of siRNA control (=1; $p<0.01$) in MCF-7-TR cells and to 1.41±1.45 of siRNA control (=1; $p<0.05$) in T47D-TR cells after suppression of CYR61.

Re-sensitization to 4-OHT after suppression of CYR61 in tamoxifen-resistant BC. Since CYR61 is increased in tamoxifen-resistant MCF-7-TR and T47D-TR cells, it was of interest to examine whether 4-OHT might be effective in the MCF-7-TR and T47D-TR after suppression of CYR61 expression. Suppression of CYR61 expression using siRNA resulted in re-sensitization to 4-OHT treatment (Figure 4A and B). While 4-OHT had no effect on the viability of both cell lines treated with control siRNA (Figure 4A, B), 4-OHT treatment resulted in a significant decrease of viability of both

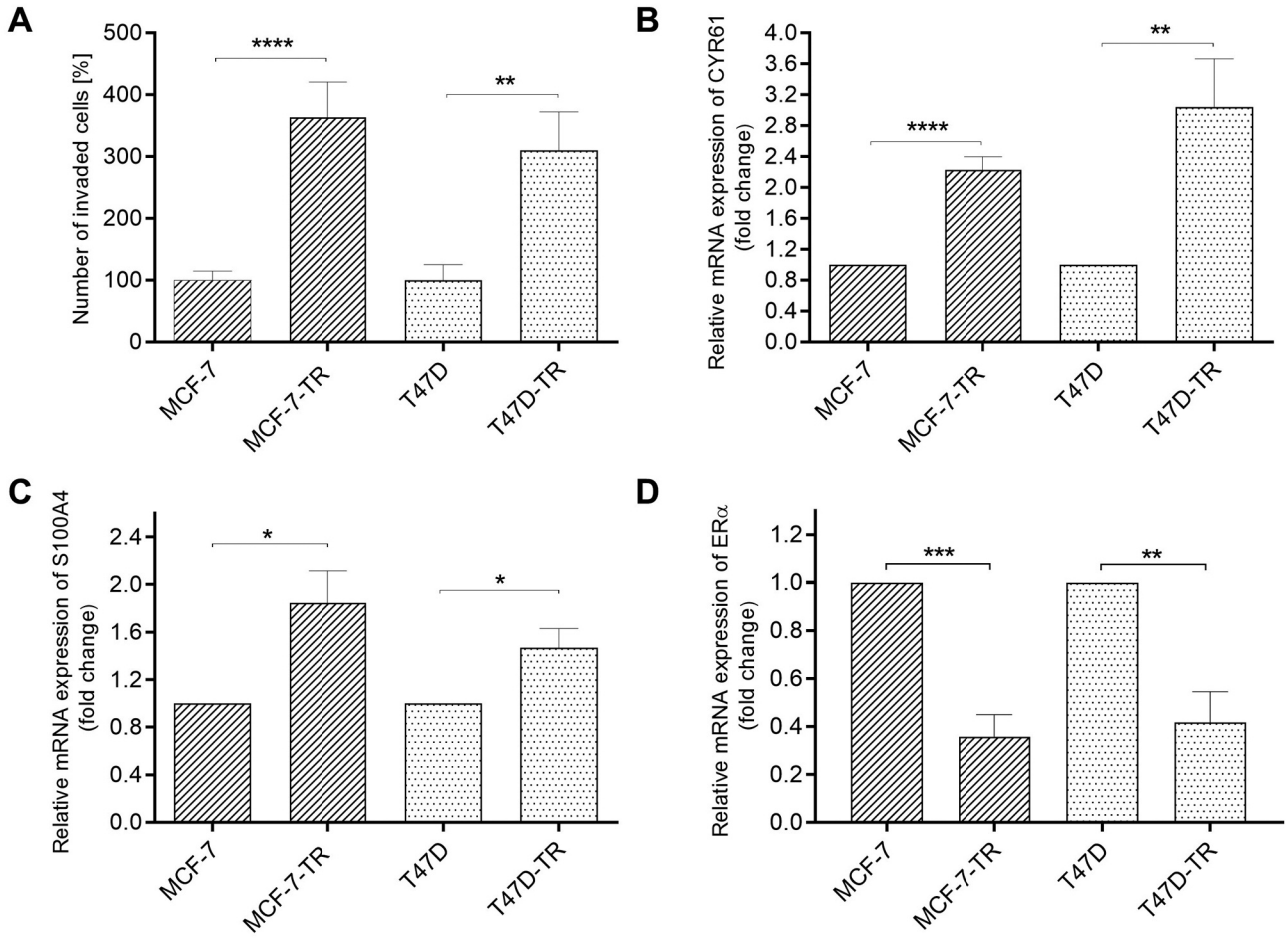

Figure 2. *Effects of 4-OHT treatment on invasion and expression of CYR61, S100A4, and ERα in tamoxifen-resistant BC cells and their tamoxifen-sensible parental cells. Invasion of tamoxifen-resistant MCF-7-TR and T47D-TR BC cells and their tamoxifen-sensible parental MCF-7 and T47D BC cells (A). Relative mRNA expression of CYR61 (B), S100A4 (C), and ERα (D) in tamoxifen-resistant MCF-7-TR and T47D-TR BC cells their tamoxifen-sensible parental MCF-7 and T47D BC cells. Mean±SEM; n=22 (A), n=6 (B), n=5 (C), n=4 (D); unpaired two-tailed t-test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. 4-OHT: 4-hydroxytamoxifen; BC: breast cancer; ERα, estrogen receptor α; S100, calcium binding protein A4.*

cell lines after suppression of CYR61 expression (MCF-7-TR: 56.33±9.42%, $p<0.001$ *vs.* siRNA control; Figure 4A; T47D-TR: 50.83±7.50%, $p<0.0001$ *vs.* siRNA control; Figure 4B).

Correlation of CYR61 levels in patients with luminal A BC to distant metastases-free survival. Expression of CYR61 had an impact on the survival of patients. The 10-year distant metastases-free survival (DMFS) of luminal A BC patients with high expression of CYR61 was significantly reduced as compared with luminal A BC patients with low expression of CYR61 ($CYR61_{low}$, n=122; $CYR61_{high}$, n=119; $p=0.00076$; Figure 4C). We also found significant results for 10-year regression-free survival (RFS). RFS of luminal A BC patients with high expression of CYR61 was significantly reduced as compared with luminal A BC patients with low expression of CYR61 ($CYR61_{low}$, n=305; $CYR61_{high}$, n=303; $p=0.0014$; not shown).

Discussion

The ERα-positive MCF-7 and T47D breast cancer (BC) cells of the luminal A type are characterized by a very weak invasion rate. However, when MCF-7 and T47D BC cells were made resistant to tamoxifen, this resulted in a significantly increased invasive behavior. At the same time, both tamoxifen-resistant MCF-7-TR and T47-TR BC sublines showed significantly increased expression of CYR61 and subsequently of S100A4. CYR61, a member of the matricellular family, binds to integrin receptors (αVβ3, αVβ3, α6β1, αMβ2), syndecan 4 (SDC4), and heparan sulfate proteoglycans (HSPGs) to carry out its

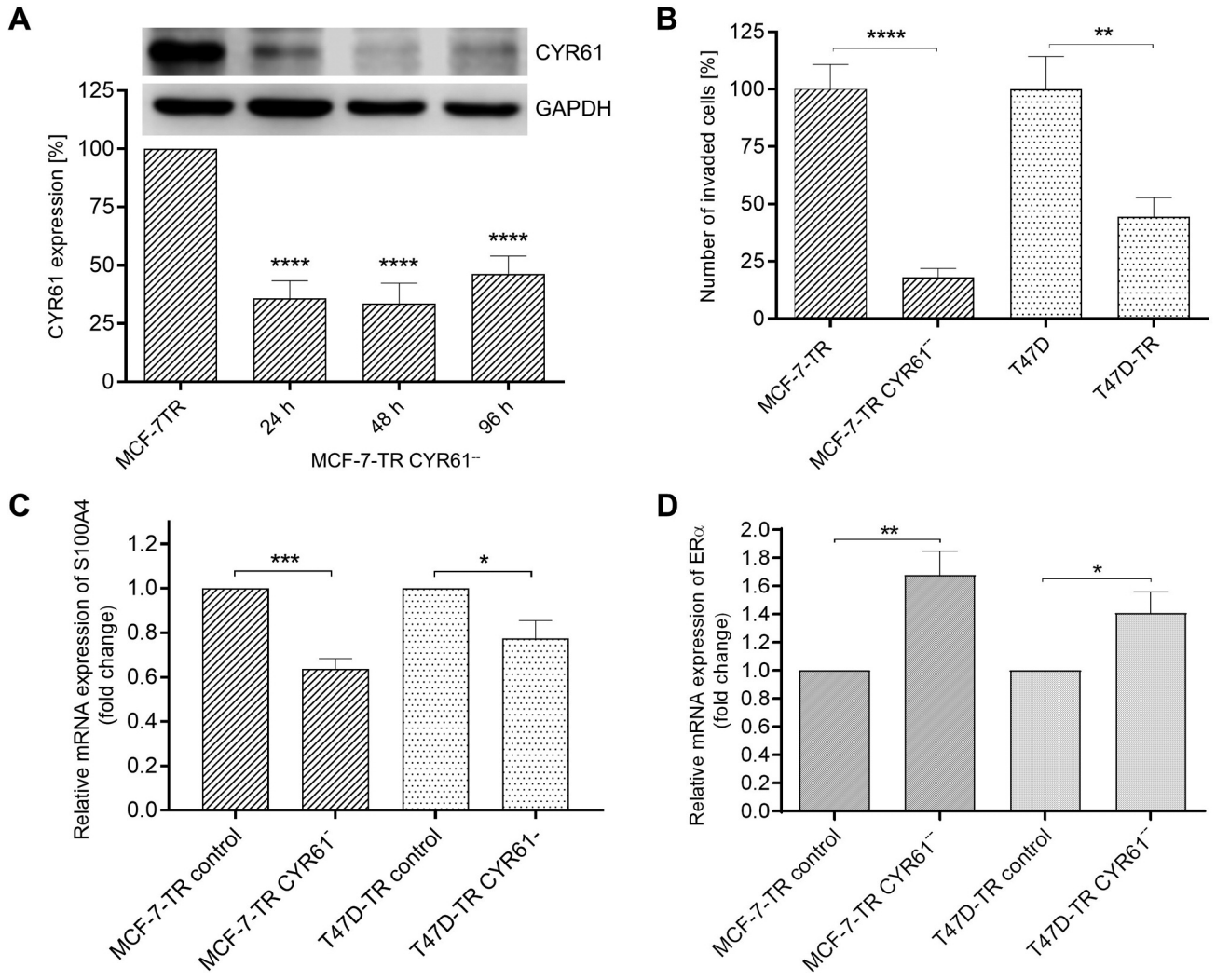

Figure 3. *Effects of CYR61 suppression on invasion and expression of S100A4 and ERα in tamoxifen-resistant BC cells. Time-dependent CYR61 protein expression after suppression of CYR61 mRNA expression by siRNA (A). Mean±SEM; n=6; ANOVA; ****p<0.0001. Invasion of tamoxifen-resistant MCF-7-TR and T47D-TR BC cells without and with CYR61 suppression (B). Relative mRNA expression of S100A4 (C) and ERα (D) in tamoxifen-resistant MCF-7-TR and T47D-TR BC cells without and with CYR61 suppression. Mean±SEM; n=18 (B), n=4 (C), n=5 (D); unpaired two-tailed t-test; *p<0.05, **p<0.01, ***p<0.001. BC: Breast cancer; CYR61, cysteine-rich angiogenic inducer 61; ERα, estrogen receptor α; S100, calcium binding protein A4.*

functions in matrix signaling in a cell type and tissue specific manner (13-17). In addition to acting as an oncogene in BC, ovarian cancer, stomach cancer, pancreatic cancer, and glioblastoma (18, 19), CYR61 also serves as a tumor suppressor in human hepatocellular carcinoma and non-small cell lung cancer (20-24). CYR61 physically contributing to cardiovascular development throughout embryogenesis (13). Proliferation, survival, and angiogenesis are induced by its binding to integrin αvβ3 (13, 25, 26). Additionally, it has been hypothesized that CYR61 stimulates angiogenesis by increasing the production of vascular endothelial growth factors (27, 28). Previous research demonstrated that CYR61 may influence chemotherapy resistance and estrogen resistance, as well as accelerate BC tumor development, progression, and metastasis, which has been linked to a poor prognosis (20, 29-32). Additionally, a poor prognosis and elevated CYR61 expression are linked to BC growth *in vivo* (30). In triple-negative BC (TNBC) cells, decreased CYR61 expression reduced invasion and transendothelial migration, and reduced lung metastases (33-35). In addition, it has been proposed that BC cells undergoing EMT had enhanced CYR61 expression, metastasis, and tumor cell invasion (5). *In vitro* and *in vivo* studies have revealed that neutralizing CYR61 antibodies reduces BC cell invasion and metastasis (5, 36). Aggressive

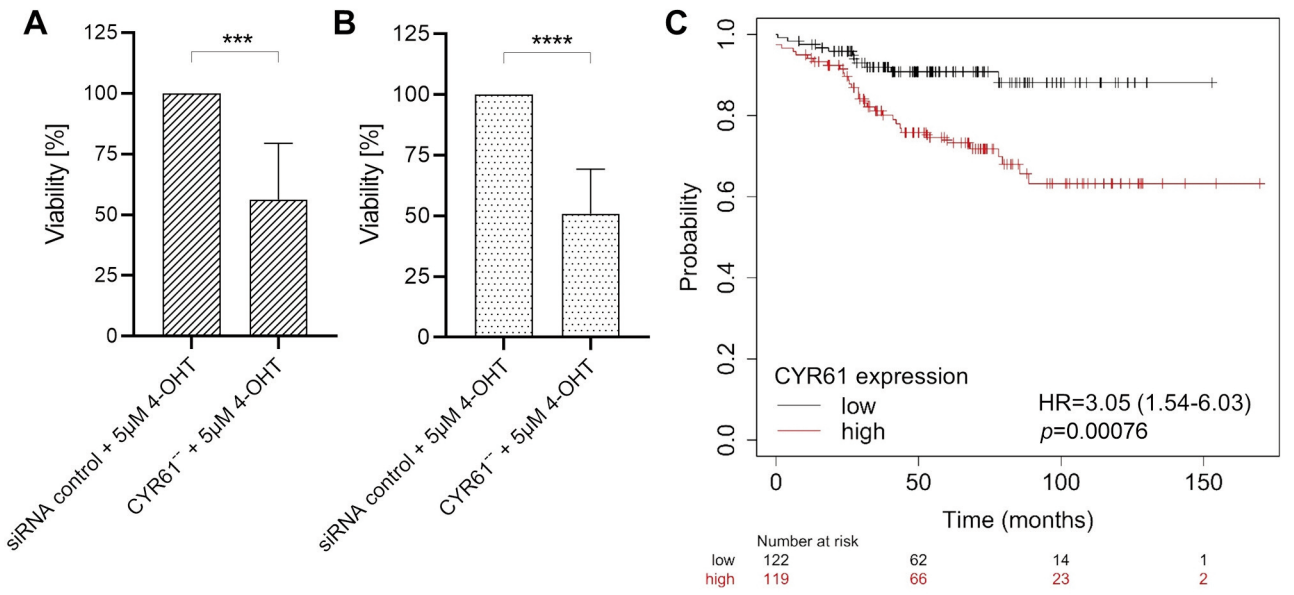

Figure 4. *Re-sensitization to 4-OHT after suppression of CYR61 in tamoxifen-resistant BC and correlation of CYR61 levels in patients with luminal A breast cancer to distant metastases-free survival. Viability of tamoxifen-resistant MCF-7-TR (A) and T47D-TR (B) BC cells without and with CYR61 suppression after treatment with 4-OHT. Mean±SEM, n=6, unpaired two-tailed t-test. ***p<0.001, ****p<0.0001. Ten-year distant metastases-free survival (DMFS) of luminal A breast cancer patients with high and low expression of CYR61 (C). $CYR61_{low}$, n=122; $CYR61_{high}$, n=119; p=0.00076. 4-OHT: 4-hydroxytamoxifen; BC: breast cancer; ERα, estrogen receptor α; CYR61, cysteine-rich angiogenic inducer 61; DMFS, distant metastases-free survival.*

mesenchymal transformed BC cells and TNBC cells show elevated expression of CYR61 and S100A4 (5). There is evidence that S100A4 facilitates BC invasion (37). It was demonstrated that suppression of CYR61 and S100A4 extracellular signaling reduced the ability of BC cells to invade (5). Transient silencing of CYR61 or S100A4 was shown to reduce invasion of mesenchymal transformed and TNBC cells (6). Hellinger *et al*. (6) showed that CYR61 regulates S100A4 expression in mesenchymal transformed BC cells and TNBC cells through regulating ERK1/2 phosphorylation. Reduced expression of S100A4 lead to decreased invasiveness of BC cells (6). Furthermore, they demonstrated a close correlation between CYR61 and S100A4 expression and BC cell invasion as well as metastasis in BC patients (6).

Expression of ERα was significantly decreased in tamoxifen-resistant BC cells in comparison to their parental tamoxifen-sensitive BC cells. It is known that tamoxifen-resistant BCs have partially reduced ERα expression (38, 39). However, although ERα expression may be lost in some patients who develop acquired tamoxifen resistance and may be the mechanism of resistance in these patients, the majority of patients still express ERα (39).

When expression of CYR61 was suppressed in tamoxifen-resistant BC cells, it resulted in a significant reduction in invasion rate. In addition, expression of S100A4 was reduced. The relationship between CYR61 and S100A4 has been previously described (6). Furthermore, suppression of CYR61 resulted in increased expression of ERα. This raised the question whether the tamoxifen-resistant BC cells could thereby become more sensitive to treatment with tamoxifen again. Cyr61 downregulates ERα expression at the transcriptional level by binding to ERα regulatory subunits, resulting in increased tamoxifen resistance (40). Therefore, suppression of CYR61 should reverse this effect. Indeed, we were able to demonstrate that the tumor-inhibitory effect of tamoxifen was restored. Thus, suppression of CYR61 resulted in de-sensitization to tamoxifen. Looking at CYR61 levels in patients with luminal A BC, 10-year distant metastases-free survival was significantly worse in patients with high CYR61 expression compared with patients with low CYR61 expression. This supports the important role of CYR61 in the progression of ERα-positive BC and, in particular, in the treatability with tamoxifen. However, long-term survival analyses with large patient populations are needed to support that Cyr61 serves an important role in the development of endocrine treatment resistance in patients with BC.

Regarding targeted therapy for ERα-positive BC of the luminal A type, CYR61 might be utilized as therapeutic target and prognostic marker. To provide the most effective therapeutic strategy, a thorough histological investigation of

tumor tissues should precisely identify the CYR61 expression. In the future, CYR61 might be used as a possible target to overcome tamoxifen resistance. For this, well-applicable and well-tolerated therapeutic strategies to block CYR61 in patients need to be developed.

Conflicts of Interest

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

Authors' Contributions

Conceptualization, Carsten Gründker; Investigation, Gerd Bauerschmitz and Silke Hüchel; Project administration, Carsten Gründker; Writing original draft, Carsten Gründker; Review & editing, Gerd Bauerschmitz and Julia Gallwas.

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