



Icb-1 expression inhibits growth and fulvestrant response of breast cancer cells and affects survival of breast cancer patients

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Received: 15 June 2020 / Accepted: 17 November 2020 / Published online: 3 January 2021
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Abstract

Purpose Human gene *icb-1* recently has been reported to be part of a gene expression score predicting response to antiestrogen fulvestrant in breast cancer patients. In the present study, we examined to what extent *icb-1* expression would affect the response of breast cancer cells to this antiestrogen in vitro and investigated underlying molecular mechanisms. Using open access mRNA data, we elucidated the significance of *icb-1* expression for survival of breast cancer patients.

Methods *Icb-1* gene expression was knocked down by RNAi. Breast cancer cell growth after treatment with fulvestrant was assessed using the Cell Titer Blue assay. Gene expression was analyzed by Western blot analysis or RT-qPCR. Survival analyses were performed using bioinformatical online tools and data.

Results Knockdown of *icb-1* in T-47D breast cancer cells significantly increased growth of this cell line and also elevated the growth-stimulatory effect of E2 ($p < 0.001$). After treatment with different concentrations of fulvestrant, *icb-1* knockdown cells exhibited a significantly enhanced response to this drug ($p < 0.01$). On the molecular level, *icb-1* knockdown led to elevated expression of ESR1 and its target gene TFF1 (pS2) and enhanced E2-triggered up-regulation of proliferation genes. Finally, bioinformatical meta-analysis of gene expression data of 3951 breast cancer patients revealed that high *icb-1* expression increases their relapse-free survival (HR = 0.87, $p < 0.05$).

Conclusion The presented data further support a tumor-suppressive role of *icb-1* in breast cancer and suggest an inhibitory effect of this gene on fulvestrant action, which both are suggested to be mediated by suppression of cellular E2 response.

Keywords THEMIS2 · Antiestrogen · ICI 182,780 · siRNA · Tumor suppressor

Background

Human gene *icb-1* (THEMIS2) has been originally cloned and described by our group to be involved in differentiation processes of gynecological cancer cells and leukocytes [1–3]. Later, it has been identified as member of a new meta-zoan gene family called THEMIS coding for cytosolic proteins binding to the Grb2 adaptor protein involved in receptor tyrosine kinase signaling [4]. Whereas leukocytes exhibit the highest expression levels of *icb-1*, this gene is also expressed in all human tissues and in a variety of cancer types and cell lines including breast cancer cells. A recent proteomic study

detected ICB-1 protein in the membrane of breast cancer tissue [5]. We were able to show that *icb-1* is a component of signaling pathway-mediating differentiating effects of vitamin D3 and ATRA on breast cancer cells [2]. Other studies demonstrating that *icb-1* is an interferon- γ responsive gene which in turn inhibits the effects of this cytokine on tumor cells showed that the view on *icb-1* function had to be broadened [6]. This was confirmed by identification of an estrogen response element (ERE) in the promoter region of this gene regulating it in an ER α -dependent manner [7]. In turn, *icb-1* gene was reported to inhibit estrogen responsiveness of ovarian and breast cancer cells [8]. The proposed role of *icb-1* in breast cancer was further corroborated by results, suggesting that single-nucleotide polymorphisms in this gene affect breast cancer susceptibility [9]. RNAi studies revealed a growth-inhibitory effect of *icb-1* on ovarian cancer cells [10] and MCF-7 breast cancer cells [8]. A recent study also demonstrated *icb-1* to mediate apoptotic effects on MCF-7 cells, and transcriptome analyses revealed that

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siRNA-triggered *icb-1* knockdown led to down-regulation of a network of apoptosis genes, but to up-regulation of an oncogenic network of genes associated with poor prognosis, invasion, and metastasis [11]. From these in vitro data, *icb-1* was suggested to exert tumor-suppressing functions in breast cancer cells.

Icb-1 has recently been reported to be part of a gene expression score predicting response to the selective estrogen receptor degrader (SERD) fulvestrant (ICI 182,780, Faslodex[®]) in breast cancer patients [12]. In the present study, we examined the role of *icb-1* expression in the response of breast cancer cells to this antiestrogen in vitro and investigated underlying molecular mechanisms. Furthermore, we intended to elucidate the significance of *icb-1* expression for survival of breast cancer patients by means of bioinformatical analyses.

Methods

Materials

Phenol red-free DMEM culture medium was obtained from Invitrogen (Karlsruhe, Germany), and FCS was purchased from PAA (Pasching, Austria). T-47D breast cancer cells were obtained from American Type Culture Collection (ATCC[®], HTB-133[™], Manassas, USA). RNeasy Mini Kit, RNase Free DNase Set and Quantitect SYBR Green PCR Kit were obtained from Qiagen (Hilden, Germany). PCR primers were synthesized at Metabion (Planegg-Martinsried, Germany). Transfectin reagent was obtained from Bio-Rad (Hercules, USA). Platinum Pfx Polymerase and OptiMEM medium were purchased at Invitrogen (Karlsruhe, Germany). Cell Titer Blue (CTB) Assay was from Promega (Madison, USA).

Cell culture, transfection, and proliferation assays

T-47D breast cancer cells were maintained in DMEM/F12 medium supplemented with 10% FCS and 0.2 units/ml insulin. Cells were cultured with 5% CO₂ at 37 °C in a humidified incubator. Cells were transfected in 6-well plates containing 2 × 10⁵ freshly seeded cells using 20 nM of *icb-1* siRNA (Silencer Select siRNA s18156, ThermoFisher) or the same amount negative control siRNA (Silencer[™] Select Negative Control No. 1, Invitrogen[™]) and 7 µl Transfectin in OptiMEM medium. 72 h after transfection, total mRNA was isolated using RNeasy Mini Kit Qiagen (Hilden, Germany). For cell proliferation assays, the transfected cells were serum-starved by changing medium to 1% FCS and 1 × SR2 (serum replacement 2, Sigma) the day after transfection and after 16 h were seeded in phenol red-free DMEM/1xSR2 into 96-well plates in triplicates (2000 cell /

well). For the fulvestrant experiments, culture medium contained 1 nM E2 + vehicle as control or E2 + 3, 10, 30, 100, 300, or 1000 nM fulvestrant. On days 0, 4, 5, and 6, relative numbers of viable cells were measured using the fluorimetric, resazurin-based Cell Titer Blue assay (Promega) according to the manufacturer's instructions at 560Ex/590Em nm in a Victor3 multilabel counter (PerkinElmer, Germany). Cell growth was expressed as percentage of day 0 or in percentage of vehicle (± E2), as indicated.

Western blot analysis

T-47D (*icb-1* KD) and T-47D (control) cells were lysed in RIPA buffer (1% (v/v) Igepal CA-630, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS) in phosphate-buffered solution (PBS) containing aprotinin and sodium orthovanadate 72 h after transfection. Aliquots containing 10 µg of protein were resolved by 10% (w/v) SDS–polyacrylamide gel electrophoresis, followed by electrotransfer to a PVDF hybond (Amersham, UK) membrane. Immunodetection was carried out using anti-ICB-1 antibody (C-term) (AP9910b, Abgent, USA) in a dilution of 1:100 and β-actin antibody (8226, Abcam, Germany) diluted 1:5000 in PBS containing 5% skim milk (w/v) followed by horseradish peroxidase-conjugated secondary antibody (1:20,000) which was detected using chemiluminescence (ECL) system (Amersham, Buckinghamshire, UK). Western blot bands were quantified using ImageJ software [13] and band densities were calculated in relation to β-actin.

Reverse transcription and qPCR

Total RNA from the transfected tumor cells was isolated 72 h after transfection, using RNeasy Mini Kit Qiagen (Hilden, Germany), according to the manufacturer's instructions. From 0.3 µg total RNA, cDNA was synthesized using 100 U M-MLV-P reverse transcriptase (Promega), 2.5 mM dNTP mixture, and 50 pM random primers (Invitrogen). For *real-time* PCR detection of gene expression in an intron-spanning manner (primer sequences in Table 1), 2 µl cDNA were amplified using LightCycler[®]FastStart DNA master mix SYBR Green I and the LightCycler 2.0 PCR device (Roche Diagnostics, Mannheim, Germany). The PCR program was 95 °C for 15 min, followed by 40 PCR cycles (95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s) and a final extension for 5 min at 72 °C, followed by a standard melting curve analysis. In all RT-PCR experiments, a 190 bp β-actin fragment was amplified as reference gene using intron-spanning primers *actin-2573* and *actin-2876*. Data were analyzed using the comparative $\Delta\Delta C_T$ method [14, 15] calculating the difference between the threshold cycle (C_T) values of the target and reference gene of each sample and then comparing the resulting ΔC_T values between different samples. In these experiments, mRNA not

Table 1 Primers used for RT-qPCR analyses

Gene	Primer sequences (5'–3')
ICB1	TCGAGGGCTCCATCTATGA GAAGTAGCCCTGGAAGTTGG
ACTB	CTTCTTTCTGGGCATGGAGT CAGGAGGAGCAATGATCTTGATCTTC
PR	CAACTACCTGAGGCCGGATT CATTGCCCTCTTAAAGAAGACCT
ESR1	CACATGAGTAACAAAGGCATGG ATGAAGTAGAGCCCGAGTG
TFF1	GGCCCAGACAGAGACGTGTA GGGACGTCGATGGTATTAGG
CCND1	CTGGAGGCTGCGAGGAA GGGGATGGTCTCCTTCATCT
CCNA2	CTGCTGCTATGCTGTTAGCC TGTTGGAGCAGCTAAGTCAAAA
MKI67	CAGTCCACAAATCCAACACA CTTTCATTTTCATACCTGAAGGAAC

subjected to reverse transcription was used as a negative control to distinguish cDNA and genomic DNA amplification.

Bioinformatical analysis

For correlation of *icb-1* expression with *ESR1*, *TFF1*, *CCND1*, *CCNA2*, and *MKI67* in 1085 breast cancer patients, we used the correlation function of the GEPIA online tool analyzing publicly available gene expression data <http://gepia.cancer-pku.cn/index.html> [16]. For survival analyses with regard to *icb-1* expression, we used the Kaplan–Meier Plotter online tool <http://kmplot.com/analysis/> including gene expression data of 3951 breast cancer patients [17].

Statistical analysis

Statistical analysis of gene expression between cells transfected with *icb-1* siRNA and control siRNA was performed by means of Student's *t* test. Multiple comparisons were analyzed using ANOVA F-test to test the null hypothesis, followed by Tukey's test. Correlation analyses were performed by means of Spearman's rank correlation. For statistics, we used Graph Pad Prism Version 7.04 Software (Graph Pad, San Diego, USA). Statistical significance was stated in case of *p* values being lower than 0.05.

Results

ICB-1 knockdown increases basal and E2-triggered growth of T-47D breast cancer cells

In a previous study, we observed knockdown of *icb-1* to strongly increase basal and E2-triggered proliferation of

SK-OV-3 ovarian cancer cells and to a weaker extent of MCF-7 breast cancer cells [8]. To corroborate these data using an additional cell line and to prepare the fulvestrant experiments performed in this study, we knocked down *icb-1* expression in a second ER α -positive breast cancer cell line, T-47D, by means of siRNA transfection. 96 h after transfection, *icb-1* protein levels by means of Western blot analysis were found to be reduced down to 24.8%, and transcript levels as assessed by RT-qPCR were decreased down to 18.6% when compared to cells transfected with negative control (NC) (both $p < 0.01$) (Fig. 1). This knockdown of *icb-1* raised the number of viable cells 2.16-fold 5 days after transfection (Fig. 2a) and increased the growth-stimulatory effect of E2 (3 nM) by up to 69.4% on day 6 ($p < 0.001$), when cells were cultured in DMEM with serum- and hormone-free serum replacement (SR2, Sigma) (Fig. 2b).

Knockdown of *icb-1* increases the growth-inhibitory effect of fulvestrant

In dose–response experiments, T-47D cells transfected with *icb-1* or NC-siRNA were now treated with different concentrations of fulvestrant (1, 3, 10, 30, 100, 300, and 1000 nM) in serum-free SR2 medium in the presence of 3 nM E2. *Icb-1* knockdown cells exhibited a significantly increased response to this SERD when used in concentrations between 10 and 1000 nM compared to cells transfected with NC-siRNA (Fig. 3b). After 5 days of treatment, the strongest increase of fulvestrant response was observed at concentrations of 30, 100, and 300 nM (all $p < 0.01$) and 1000 nM ($p < 0.05$), ranging from a decrease of viable cell numbers by 21.7% (NC-siRNA) to 36.7% (*icb-1* siRNA) ($p < 0.01$) at 30 nM fulvestrant to a reduction from 44.8% (NC-siRNA) to 63.5% (*icb-1* siRNA) at 1000 nM ($p < 0.05$). To avoid off-target effects of the highest fulvestrant concentrations tested, we now performed a time-course analysis with the lowest concentration of this drug strongly affected by *icb-1* knockdown. Treatment of T-47D cells with 30 nM fulvestrant in serum-free medium containing 3 nM E2 resulted in a time-dependent decrease of viable cell numbers from day 4 to 6, which was significantly more pronounced in cells transfected with *icb-1* siRNA (Fig. 3a). The maximum effect was observed on day 6, when the growth-inhibitory effect of 30 nM fulvestrant on control cells (26.4%) was increased to 43.7% after *icb-1* knockdown ($p < 0.01$).

ICB-1 knockdown affects expression of estrogen pathway and proliferation genes

To examine molecular mechanisms which might underlie the observed effect of *icb-1* gene expression on basal and E2-triggered growth as well as the fulvestrant sensitivity of T-47D cells, we analyzed the expression of six candidate

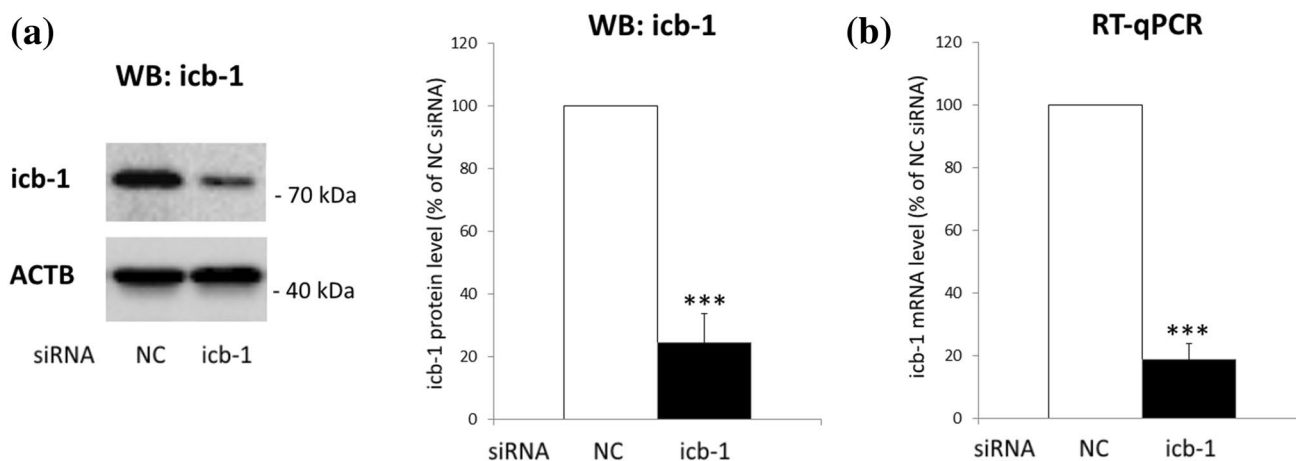


Fig. 1 Knockdown of *icb-1* expression. **a** Western blot analysis of ICB-1 protein expression 96 h after transfection with 20 nM *icb-1*-specific siRNA (Silencer Select siRNA s18156, ThermoFisher) or negative control siRNA (NC) using anti-ICB-1 antibody (C-term) (Biomol, WA-AP9910b) (1:100). Left panel: representative Western blot result. Right panel: illustration of mean band densities (meas-

ured with ImageJ software) of three independent experiments normalized to respective ACTB levels and expressed in percentage of *icb-1* expression in NC-siRNA transfected cells. **b** RT-qPCR analysis of *icb-1* mRNA levels using the $\Delta\Delta(C_T)$ method [14, 15]. Data are expressed in percentage of *icb-1* levels in cells transfected with negative control (NC) siRNA. ($n=3$)

genes, three of them part of the ER α pathway [ESR1, PR, and TFF1 (pS2)] and three proliferation genes (CCND1, CCNA2, and KI67). Knockdown of *icb-1* in T-47D cells increased mRNA expression of ESR1 (1.6-fold, $p < 0.01$) and TFF1 (2.37-fold, $p < 0.01$) (Fig. 4a), but did not affect PR expression (data not shown). In serum-free medium, addition of 3 nM E2 resulted in a 1.63-fold increase of TFF1 (pS2) mRNA expression in *icb-1* knockdown cells when compared to control cells, but did not affect ESR1 or PR (Fig. 4b). In cells transfected with *icb-1* siRNA, E2 strongly increased Ki67 expression (6.07-fold, $p < 0.001$) and to a lower extent mRNA levels of cell cycle regulators CCND1 (1.59-fold) and CCNA2 (1.50-fold) (both $p < 0.05$) when compared to control cells.

ICB-1 negatively correlates with expression of ESR1, TFF1, and CCND1 in breast cancer tissue

To examine to what extent our in vitro gene expression results from T-47D knockdown experiments could be verified in breast cancer tissue, we next analyzed correlation between expression of *icb-1* and the genes affected by *icb-1* knockdown in 1085 breast cancer tissues by means of the GEPIA online tool for meta-analysis of publicly available DNA microarray data [16]. Spearman rank correlation analyses between expression of *icb-1* and these genes revealed a negative correlation of *icb-1* with mRNA expression of ESR1 (Spearman's $\rho = -0.38$, $p = 7.8e-38$) in breast cancer tissues and a weaker, but statistically highly significant negative correlation with ER α target TFF1 (pS2) (Spearman's $\rho = -0.26$, $p = 1.2e-18$). With

regard to the proliferation-associated genes tested, these analyses revealed no significant correlation of *icb-1* with CCNA2 or KI67, but a moderate negative association with CCND1 (Spearman's $\rho = -0.25$, $p = 1.7e-16$).

ICB-1 expression affects survival of breast cancer patients

To examine the role of *icb-1* gene in breast cancer survival, we used an online tool for meta-analysis of publicly available DNA microarray data from 3951 breast cancer patients [17]. Kaplan–Meier plotter software revealed a longer relapse-free survival (RFS) of patients with high *icb-1* expression (HR = 0.87, $p = 0.015$) (Fig. 5), whereas their overall survival (OS) was not affected. With regard to the four “classical” molecular intrinsic subtypes of breast cancer [18, 19], high levels of *icb-1* expression increased both RFS and OS of patients with basal-like breast cancer (HR = 0.7, $p = 0.0059$ and HR = 0.52, $p = 0.0093$, respectively) (Fig. 6). In patients with luminal B-type cancer, high *icb-1* expression considerably increased RFS (HR = 0.62, $p = 1.3e-06$), but not their OS. In the HER2-enriched intrinsic subgroup, high *icb-1* expression notably elevated RFS of the patients (HR = 0.53, $p = 0.0014$). Whereas a similar trend also was observed with regard to OS, it did not reach statistical significance due to low case numbers. In contrast, in patients with Luminal A breast cancer, no effect of *icb-1* expression on RFS or OS was observed.

Fig. 2 Icb-1 knockdown effect on proliferation of T-47D cells. **a** Time-dependent increase of viable cell numbers after transfection with icb-1 or NC (negative control) siRNA. Values are expressed in arbitrary units. **b** Effect of an icb-1 knockdown on E2 response, compared to cells with normal icb-1 levels (transfected with NC-siRNA). Experiments were performed in serum- and hormone-free defined SR2 medium (Sigma). Relative numbers of viable cells were measured using the Cell Titer Blue assay (Promega) and values are expressed in percent of E2 vehicle. ($n=4$), $*p < 0.05$, $**p < 0.01$ vs. NC-siRNA. NC-siRNA negative control siRNA

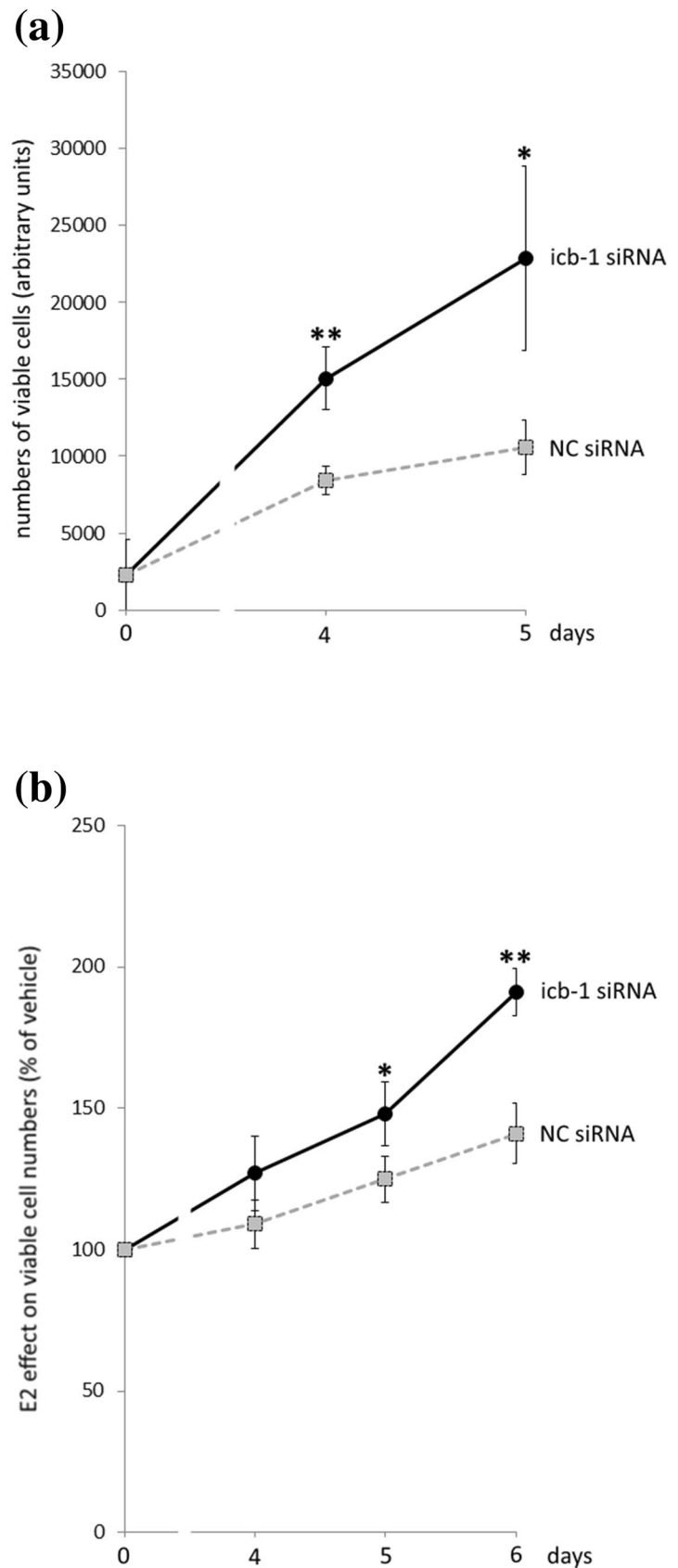


Fig. 3 Effect of an *icb-1* knock-down on fulvestrant response of T-47D breast cancer cells. **a** Time-course analysis of the effect of an *icb-1* knockdown on fulvestrant (30 nM) action in the presence of 3 nM E2. **b** Dose–response analysis of the influence of an *icb-1* knockdown on the effect of the indicated concentrations of fulvestrant in the presence of 3 nM E2 5 days after treatment. Relative numbers of viable cells were measured using the Cell Titer Blue assay (Promega) and are expressed in percentage of cells cultured in E2 plus vehicle. ($n=5$), * $p < 0.05$, ** $p < 0.01$ vs. NC-siRNA. *NC-siRNA* negative control siRNA

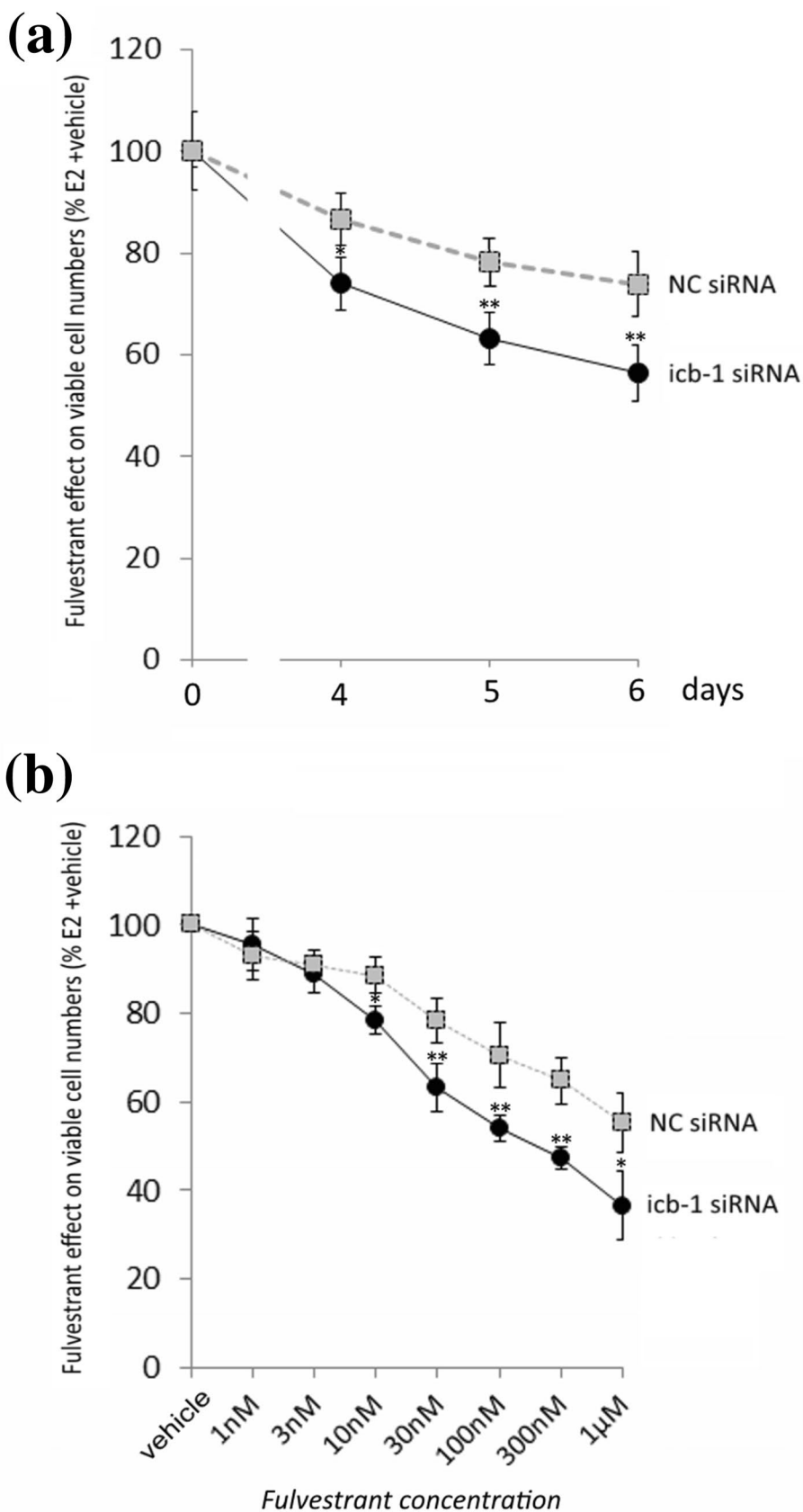
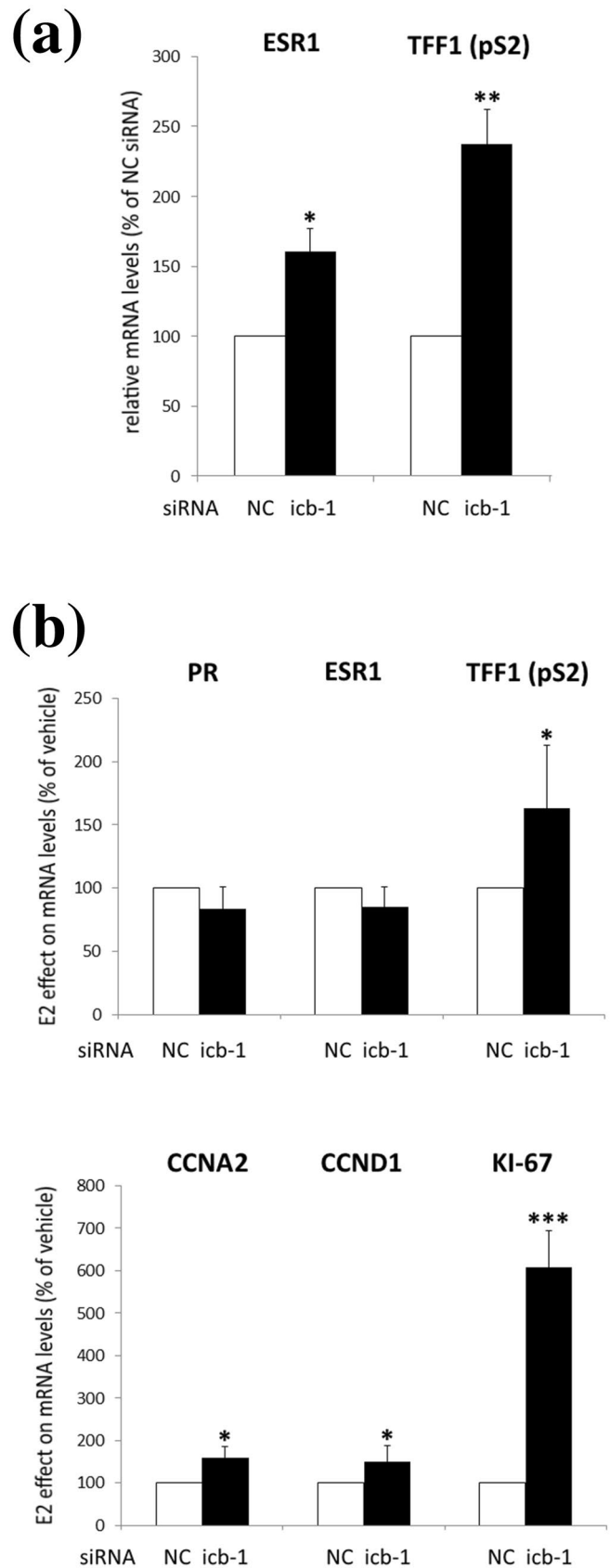


Fig. 4 Gene expression after *icb-1* knockdown, as assessed by RT-qPCR analysis. **a** Effect of *icb-1* knockdown. **b** Effect of E2 stimulation (3 nM) on gene expression. Expression values were processed in relation to housekeeping gene *ACTB* and the $\Delta\Delta C(t)$ method and are expressed in percent of the values of cells treated with negative control (NC) siRNA



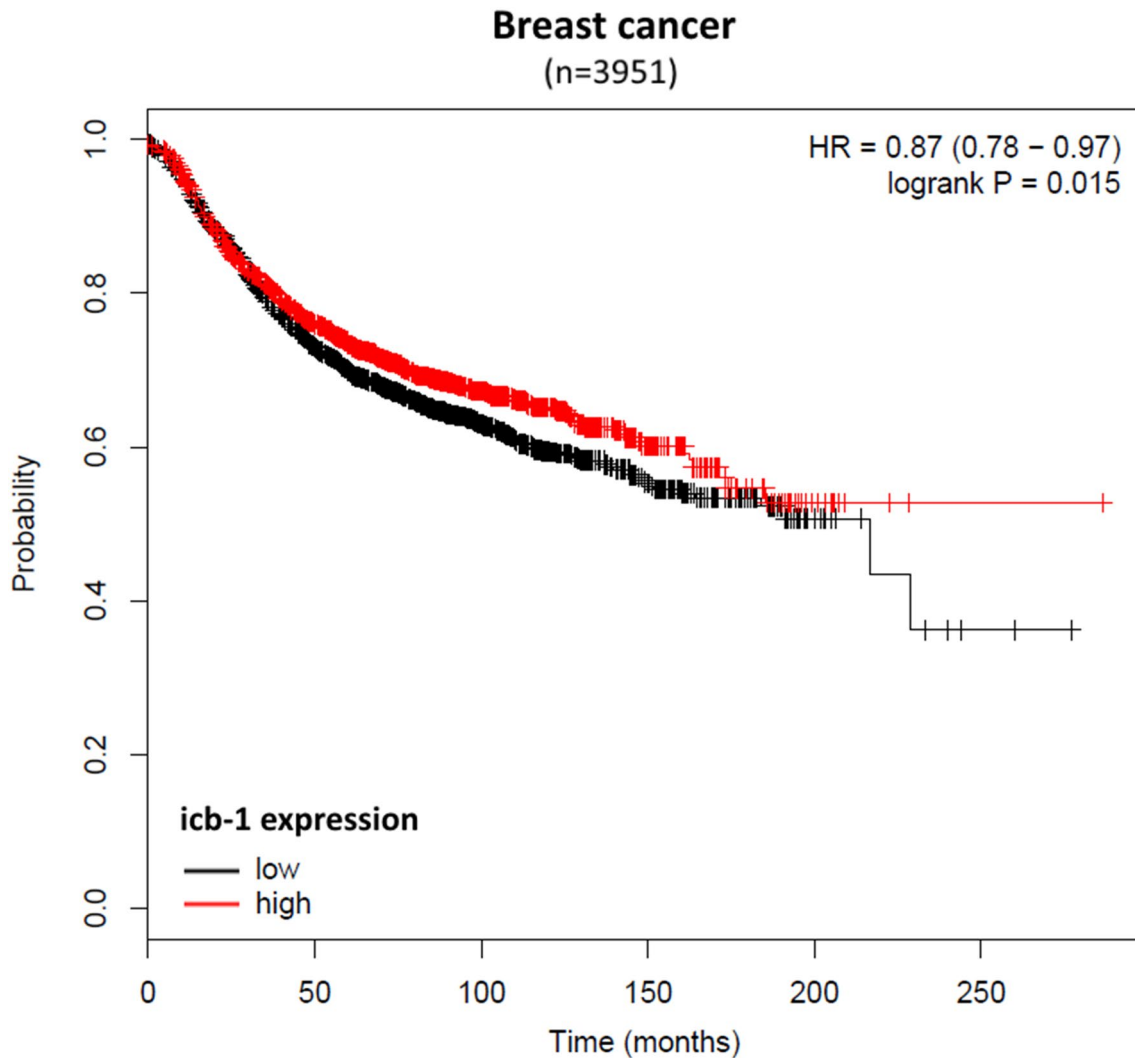


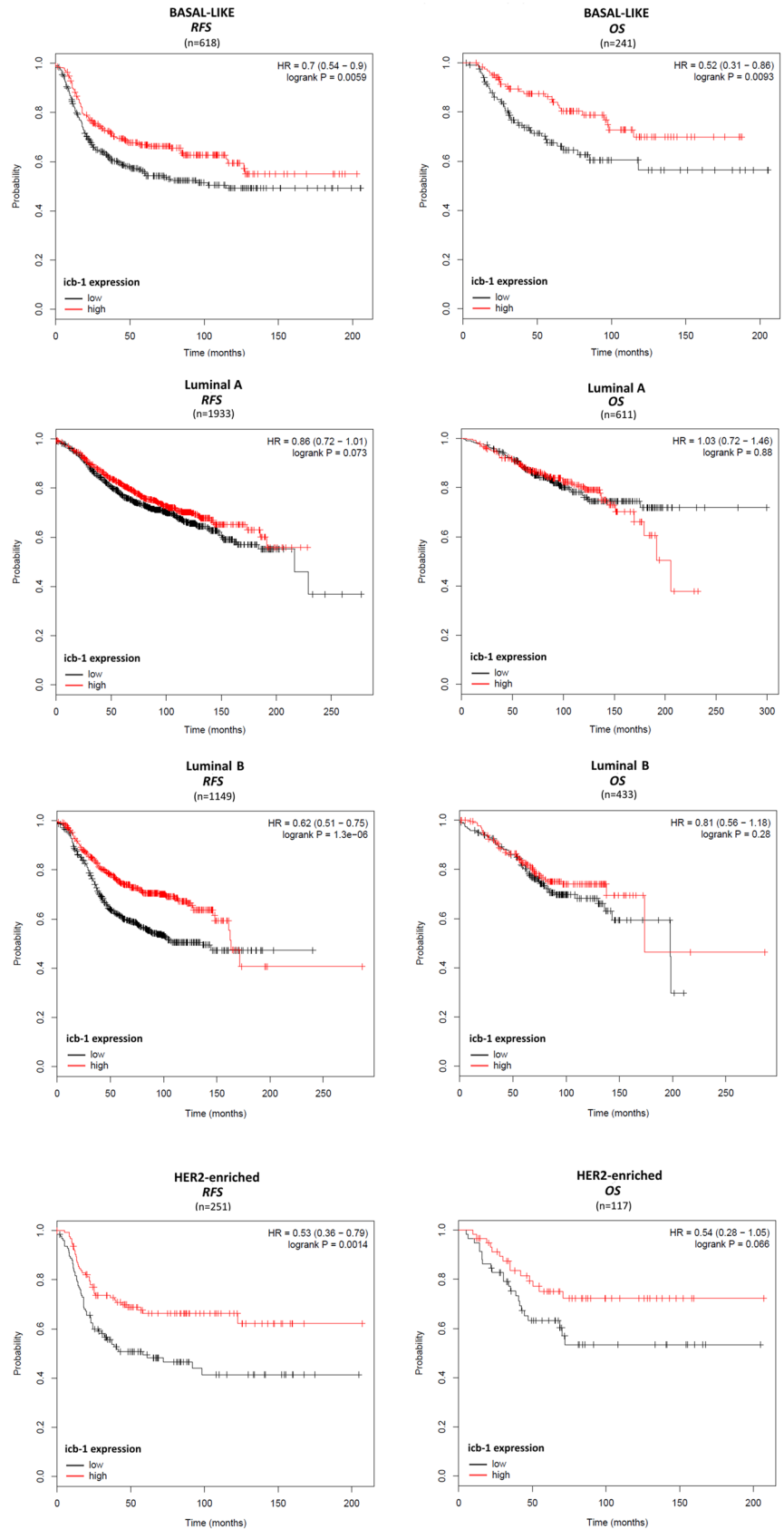
Fig. 5 Kaplan–Meier analysis of icb-1 expression in 3951 breast cancer patients and their relapse-free survival using the online tool <http://kmplo.t.com/analysis/>

Discussion

For the first time, we demonstrate that knockdown of icb-1 is able to increase the response of ER α -positive breast cancer cells to antiestrogen fulvestrant in vitro, which supports the results of a previous study suggesting icb-1 to be part of a gene expression score predicting response of breast cancer patients to this SERD [12]. Our observation that knockdown of icb-1 strongly increases both basal growth and the effect of E2 on T-47D breast cancer cell growth is in line with a previous study reporting the same effects on MCF-7 cells [8]. Our analysis of genes of the ER α pathway as well as of proliferation-associated genes in icb-1 knockdown cells provided possible molecular mechanisms underlying the observed increased fulvestrant response of these cells. The observed effects of an icb-1 knockdown on T-47D gene expression, like

E2-independent up-regulation of ESR1 mRNA and of ER α target gene TFF1 (pS2), are also in line with our previous study using MCF-7 breast cancer cells [8, 20]. When we examined the effects of icb-1 siRNA on E2-triggered expression of proliferation genes, in this study, we broadened gene selection. The finding that icb-1 knockdown enhanced E2-triggered up-regulation of its target gene CCND1 gene in T-47D cells is in line with similar results of experiments using MCF-7 cells [8, 20]. In the present study, we additionally examined the E2 effect on proliferation-associated genes CCNA2 and MKI67 subject to icb-1 expression, which demonstrated up-regulation of both genes, which was most pronounced with regard to MKI67, suggesting that icb-1 suppresses ER α activity, which not only affects its target gene TFF1 (pS2), but also E2-triggered induction of CCND1, CCNA2, and MKI67 expression [20]. Thus, the increased fulvestrant response

Fig. 6 Kaplan–Meier analysis of icb-1 expression in tumors of four different intrinsic subtypes and patients' relapse-free survival (RFS) and overall survival (OS) using the online tool <http://kmpplot.com/analysis/>



in *icb-1* knockdown cells is suggested to be the consequence of increased ER α levels, resulting in an elevated E2 effect on expression of proliferation genes leading to a considerable increase of T-47D cell proliferation. Thus, *icb-1* is suggested to suppress E2-triggered proliferation via restriction of ER α expression, leading to a limitation of the growth-inhibitory response to fulvestrant.

To verify our *in vitro* results of an *icb-1* knockdown on gene expression of T-47D cells, we examined the correlation of *icb-1* expression with ESR1, TFF1 (pS2), CCND1, CCNA2, and MKI67 in 1085 breast cancer tissues by means of the GEPIA online tool for meta-analysis of publicly available DNA microarray data [16]. The fact that we found a considerable negative correlation of *icb-1* with ESR1 and TFF1 (pS2) in this high number of breast cancer tissues clearly suggests that the restrictive action of *icb-1* on ESR1 levels and activity we found by *in vitro* knockdown experiments with T-47D cells is also present in the *in vivo* situation. The observed negative correlation of *icb-1* with CCND1 in breast cancer tissues might support a previous hypothesis that *icb-1* exerts tumor-suppressing properties in this cancer entity [11]. The fact that we did not find significant correlations of *icb-1* with CCNA2 and MKI67 is suggested to result from the fact that these proliferation genes are not regulated by ER α only, but by a multitude of other growth-regulatory pathways [21, 22].

Finally, the *in silico* analysis of gene expression data of 3951 breast cancer patients using an online survival tool (Kaplan–Meier Plotter) [17], revealing a longer RFS of patients with high *icb-1* expression for the first time, demonstrated a significant beneficial role of *icb-1* gene expression in breast cancer survival. This finding strongly supports the proposed tumor-suppressing role of this gene in breast cancer [11]. With regard to the “classical” intrinsic molecular subtypes of breast cancer [18, 19], which could be analyzed by means of the Kaplan–Meier Plotter online tool [17], in the luminal B subgroup, high *icb-1* expression significantly increased patients’ RFS, which might be explained by its restricting effect on ESR1 and MKI67 highly expressed in this subtype. In contrast, in luminal A cancer, which also expresses ESR1 but low levels of MKI67, *icb-1* expression did not affect patients’ survival, which might be due to the fact that in this low-grade and slowly growing cancer subgroup, the inhibiting effect of this gene on E2-triggered proliferation might be too low to be visible. The fact that high *icb-1* expression is associated with a longer RFS in the HER2-enriched, ESR1-negative subtype and with a longer RFS and OS in basal-like cancer, which also lacks ESR1 expression, might be explained by the fact that *icb-1* has been reported to also exert ESR1-independent anti-tumoral effects in breast cancer, like up-regulation of apoptosis genes [11].

Conclusion

The results of this study suggest that *icb-1* not only decreases the sensitivity of T-47D breast cancer cells to antiestrogen fulvestrant, but also exerts considerable beneficial effects on the survival of breast cancer patients. Considering the gene expression data from our knockdown experiments and the observed correlations in breast cancer tissue, our data suggest both findings to be mediated by *icb-1* triggered restriction of ER α expression and activity.

Acknowledgements We would like to thank Mrs. Bettina Federhofer for expert technical assistance.

Author contributions MR: data collection and management, and data analysis. MS, SS: data analysis and manuscript editing. OO: providing essential project support and manuscript editing. OT: protocol/project development, data collection and management, data analysis, and manuscript writing/editing.

Funding None.

Data availability Data and materials are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest All authors state that they do not have any conflicts of interest with regard to this study.

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