Cellular and radiobiological effects of carbonic anhydrase IX in human breast cancer cells

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Abstract. Hypoxia-induced carbonic anhydrase IX (CAIX) is involved in intracellular and extracellular pH regulation, which is critical for tumor growth and metastasis. CAIX is overexpressed in breast cancer and is associated with the poor survival of patients after radiotherapy. Therefore, we evaluated the cellular and radiobiological effects of CAIX inhibition in human breast cancer cells. We used *CA9* siRNA and the CA inhibitor (CAI) U104, respectively, to inhibit CAIX expression and activity in basal triple-negative MDA-MB-231 and luminal MCF-7 cells under hypoxic conditions. We investigated the effects of CAIX inhibition on *CA9* mRNA and CAIX protein level, as well as on CAIX activity, intracellular pH, proliferation, apoptosis, clonogenic survival, migration, cell cycle distribution and radiosensitivity. *CA9* siRNA and CAI U104 decreased *CA9* mRNA and CAIX protein level in MDA-MB-231 and MCF-7 cells. Furthermore, incubation with CAI U104 significantly decreased carbonic anhydrase activity and reduced the intracellular pH. Additionally, *CA9* siRNA or U104 reduced clonogenic survival, migration and the number of cells in the G_0/G_1 phase, induced apoptosis and demonstrated additive or synergistic effects in combination with irradiation. In conclusion, combination of CAIX inhibition and irradiation is a promising treatment strategy against breast cancer with hypoxia-induced CAIX expression.

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Abbreviations: CAIX, carbonic anhydrase IX; CAI, carbonic anhydrase inhibitor; pHe, extracellular pH; pHi, intracellular pH; PARP, poly(ADP-ribose)-polymerase; HPRT, hypoxanthine-guanine phosphoribosyltransferase; POLR2A, DNA-directed RNA polymerase II subunit A; BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester; SRB, sulforhodamine B; DMF10, dose-modifying factor 10%; EF, enhancement factor

Key words: carbonic anhydrase IX, inhibition, radiation, breast cancer, CAIX/CA9

Introduction

Solid tumors are often characterized by hypoxic areas with a pO_2 of less than 5 mmHg (equivalent to 0.7% O_2) due to poor vascularization and uncontrolled growth (1). One of the most universal characteristics of solid tumors is the so called 'Warburg effect', i.e. adaption of hypoxic tumor cells' metabolism from oxidative phosphorylation to glycolysis to generate energy in the absence of oxygen (2). This adaptation increases the production of lactic acid and the generation of CO_2 by neutralizing protons (H^+) in the cytoplasm, a process catalyzed by carbonic anhydrases (CA). Consequently, the extracellular environment is acidified because of the export of lactic acid (monocarboxylate transporter, MCT) and CO_2 release from the tumor cells (3,4). Tumor cells are able to adapt to the resulting acidic environment, whereas it is toxic to normal cells (4). In various tumor entities, one of the main mediators of the hypoxic response is the transcriptional factor hypoxia-inducible factor 1 (HIF-1). HIF-1 governs the cellular adaption to oxygen deficiency by regulating tumor-relevant genes involved in important processes such as glucose transport (*GLUT-1*), angiogenesis (*VEGF*), proliferation (*IGF-2*) and pH regulation (*CA9*) (5). Furthermore, the expression of membrane-bound CAIX is highly upregulated by HIF-1α and catalyzes the hydration of carbon dioxide (CO_2) to bicarbonate ions (HCO_3^-) and protons (H^+) (6). CAIX thus contributes to the acidification of the extracellular pH (pHe) under hypoxic conditions. Additionally, bicarbonate is actively transported back into cells by a sodium-dependent co-transporter, where it neutralizes H^+ to maintain a favorable intracellular pH (pHi) (7). Moreover, a low pH of the extracellular environment has been proven to influence CAIX-mediated tumor growth, invasion and metastasis (8-10). *In vivo* CAIX is overexpressed in many tumor entities and is associated with a poor survival of cancer patients (11). Especially in patients with invasive breast cancer, immunohistochemical CAIX expression also correlates with worse relapse-free and overall survival (10,12,13). This poor prognosis may be due to hypoxia-mediated resistance to drug therapy or radiotherapy (3). Hypoxia-induced CAIX expression may also be directly linked to radioresistance. A study of breast cancer patients who received radiotherapy revealed that high CAIX expression in tumor tissue correlates with poor recurrence-free survival (14). This finding was supported by

Table I. siRNAs and primers.

	Gene		Sequence 5'→3'	Localization	Source
siRNAs	*CA9*	Sense	5'-CAGTGCCTATGAGCAGTTG-3'	900-918	Eurofins Genomics,
		Antisense	5'-CAACTGCTCATAGGCACTG-3'		Ebersberg, Germany
	Nonsense	Sense	5'-CGTACGCGGAATACTTCGA-3'		
		Antisense	5'-TCGAAGTATTCCGCGTACG-3'		
Primers	*CA9*	Forward	5'-GAAAACAGTGCCTATGAGCAGTTG-3'	895-918	Sigma-Aldrich; Merck KGaA,
		Reverse	5'-TGCTTAGCACTCAGCATCAC-3'	1,106-1,087	Darmstadt, Germany
	HPRT	Forward	5'-TTGCTGACCTGCTGGATTAC-3'	391-410	
		Reverse	5'-CTTGCGACCTTGACCATCTT-3'	652-633	
	POLR2A	Forward	5'-CTTGCCCCGTGCCATGCAGA-3'	1,358-1,377	
		Reverse	5'-CTCGCACCCGGCCTTCCTTG-3'	1,440-1,421	

Sequences and localization of siRNAs and primers used in this study correspond to the mRNA sequences of *CA9* [NM_001216.2], *HPRT* [NM_000194.2] and *POLR2A* [NM_000937.4]. *CA9*, carbonic anhydrase IX; HPRT, hypoxanthine-guanine phosphoribosyltransferase; *POLR2A*, DNA-directed RNA polymerase II subunit A.

another recent clinical study showing that CAIX overexpression was significantly associated with poor disease-free and overall survival in a cohort of patients with triple-negative (ER^-, PR^- and $Her2^-$) breast cancer treated with radiotherapy, which suggests a correlation between CAIX expression and response to radiotherapy (15). Initial studies in a colorectal carcinoma xenograft model revealed that inhibiting CAIX with different CAIX inhibitors (CAI: acetazolamide, DH348 or 11c) or knockdown of *CA9* with shRNA delayed tumor growth and radiosensitized tumor cells (16,17). Furthermore, it has been supposed that the prognostic significance of CAIX expression differs depending on the breast cancer subtype of patients (18).

In the present study, we investigated the cellular and radiobiological effects of CAIX inhibition in two human breast cancer cell lines of different subtypes: The highly invasive metastatic breast cancer cell line MDA-MB-231 (basal, triple-negative) and the less invasive non-metastatic breast cancer cell line MCF-7 (luminal, ER^+, PR^+ and $Her2^-$). In the present study, inhibition of CAIX was performed by two alternative strategies, namely treatment with *CA9* siRNA or exposure to the CAIX/CAXII selective inhibitor U104, which is currently being tested in a phase I clinical trial in patients with advanced solid tumors (19). Our previous study indicated that betulin 3,28-disulfamate, which is described as a CAI by Winum *et al* (20), radiosensitized MDA-MB-231 breast cancer cells (21). Recently, CAI FC9403A but not CAI S4 showed synergistic effects with irradiation in MDA-MB-231 breast cancer spheroids (22). However, to the best of our knowledge, there are no studies regarding the cellular and radiobiological effects of a selective *CA9* knockdown with RNA interference or a CAIX/CAXII specific inhibition in human breast cancer cells. We hypothesized that selective *CA9*/CAIX knockdown/inhibition will decrease the proliferation, migration and clonogenic survival of both breast cancer cell lines and that the ureidosulfonamide U104 (or SLC-0111)-induced inhibition of CAIX and CAXII will cause stronger effects on radiosensitivity in breast cancer cells than RNA interference.

Materials and methods

Cell culture conditions and treatments of breast cancer cells. The breast cancer cell lines MDA-MB-231 and MCF-7 were cultured with RPMI-1640 containing 25 mM HEPES and L-glutamine (Lonza, Walkersville, MD, USA) and supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1% pyruvate, 185 U/ml penicillin and 185 μg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified atmosphere of 3% CO_2 at 37˚C. All experiments were conducted with cells in the logarithmic growth phase.

Twenty-four hours before treatment, the cells were seeded in cell culture flasks (Greiner Bio-One, Kremsmünster, Austria). Breast cancer cells were transfected with 100 nM *CA9* targeting siRNA (Table I) using INTERFERin™ reagent as recommended by the manufacturer (Polyplus Transfection, Illkirch, France). To inhibit CAIX activity, breast cancer cells were incubated with the CAIX inhibitor U104 (R&D Systems, Minneapolis, MN, USA) diluted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich Chemie; Merck KGaA, Darmstadt, Germany). Following treatment, the cells were exposed to hypoxia for 24 to 72 h. To achieve hypoxic conditions (0.1% O_2) we used a gas generator system (Anaerocult P; Merck Millipore, Darmstadt, Germany) as previously described (23).

Irradiation was performed under hypoxic conditions (0.1% O_2) 24 h after treatment with U104 and 72 h after siRNA transfection. The cells were irradiated with 2, 6 and 10 Gy at a dose rate of 2 Gy/min accomplished with 6 MV photons and adequate bolus material on an Elekta Synergy linear accelerator (Elekta AB, Stockholm, Sweden).

Quantitative real-time PCR (qPCR) and western blot hybridization. RNA isolation, cDNA synthesis and qPCR were performed as previously described (24). The primers used are cited in Table I. A no-template reaction was used as a negative control. *HPRT* (hypoxanthine-guanine

phosphoribosyltransferase) and *POLR2A* (DNA-directed RNA polymerase II subunit A) served as housekeeping genes and were used for normalization. We used plasmid DNA standards for each gene (10^7-10^3 copies/μl) to calculated the copy number of gene of interest or the housekeeping gene.

To isolate protein, breast cancer cells were lysed in cell lysis buffer supplemented with protease inhibitors (Cell Signaling Technology, Inc., Danvers, MA, USA) and homogenized by ultrasound. The protein concentration was determined using Bradford method. The proteins were separated by gel electrophoresis [4-12% Bis-Tris mini gels (Invitrogen; Thermo Fisher Scientific, Inc.] and transferred to a polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Darmstadt, Germany). The membranes were blocked with 10% non-fat milk/TBST (50 mM NaCl, 30 mM Tris-HCl pH 8.0, 0.1% Tween-20) and incubated with monoclonal mouse anti-CAIX antibody (dilution 1:2,000; clone no. M75; Bioscience Slovakia, Bratislava, Slovak Republic), rabbit anti-human cleaved PARP (Asp214) antibody (dilution 1:2,000; cat. no. 9541; Cell Signaling Technology, Inc.) or monoclonal mouse anti-β-actin antibody (dilution 1:10,000; cat. no. A5441; Sigma-Aldrich; Merck KGaA), followed by incubation with HRP-conjugated secondary antibodies (goat anti-rabbit, cat. no. P0448; and rabbit anti-mouse, cat. no. P0260; both diluted at 1:5,000; Dako Deutschland GmbH, Hamburg, Germany). Further washing steps were followed by the visualization of immune complexes with an ECL detection system (GE Healthcare, Chicago, IL, USA). Quantification of western blot signals was performed by the use of Image Studio Lite 5.2 software (LI-COR Biosciences, Lincoln, NE, USA).

Measurement of CAIX activity and intracellular pH (pHi). Breast cancer cells were cultured under normoxic or hypoxic conditions, respectively, for 24 h. Afterwards, hypoxic cells were treated with 50 μM U104 for 3 h. To measure CAIX activity, breast cancer cells were washed with cold isotonic buffer (130 mM NaCl, 5 mM KCl, 20 mM Hepes; pH adjusted to pH 8.2 at 4˚C), scraped down and resuspended in 2 ml of isotonic buffer. The pHe was measured with a microelectrode (WTW, Weilheim, Germany) for 1 min before adding 1 ml of CO_2-saturated water while monitoring the pH every 5 sec for 10 min. The CAIX activity was calculated according to the Wilbur-Anderson-method (WAU/mg=2*(T0-T)/T*mg protein). The duration (T) to lower the pH of the isotonic buffer from 8.0 to 6.6 at 4˚C was determined [T0, unanalyzed reaction (isotonic buffer); T, catalyzed reaction (e.g., normoxia, hypoxia)].

The pHi was measured with BCECF (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester) (Thermo Fisher Scientific, Inc.) as previously described (25). The cells were seeded on poly-lysine-treated coverslips and cultured under hypoxic conditions for 24 h. Subsequently, the cells were incubated with pH 6.6 ringer solution with/without 50 μM U104 for 3 h before measuring the pHi.

Sulforhodamine B assay. The SRB assay was performed as previously described (21). MDA-MB-231 and MCF-7 cells were seeded in 96-well plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and treated with *CA9* siRNA or CAI U104 (10, 100 and 250 μM) under hypoxic conditions for 72 h. The cells were then fixed, washed and dyed with 0.4% sulforhodamine, and absorbance was measured at 540 nm using a GENios microplate reader (Tecan Group AG, Männedorf, Switzerland).

Clonogenic survival assay and radiosensitivity. The colony-forming assay was performed 24 h after incubation with the CAIX inhibitor U104 or 72 h after siRNA transfection under hypoxic conditions. For determination of radiosensitivity, the cells were irradiated 24 h (U104) and 72 h (siRNA) after treatment and the clonogenic survival assay was performed 1 h after irradiation, as previously described (26). The survival fraction is the ratio of the plating efficiencies of treated cells (e.g., siRNA treatment, irradiation) to that of control cells (e.g., nonsense siRNA, non-irradiated). Additionally, the DMF10 (dose-modifying factor 10%), i.e., the ratio of radiation doses with or without treatment resulting in 10% survival, and the EF_{10Gy} (enhancement factor), i.e., the ratio of survival fraction of treated and control cells, were determined. Data were fitted to a linear quadratic model ($-\ln S=\alpha D + \beta D^2$) using OriginPro 8G (OriginLab Corp., Northampton, MA, USA).

Migration assay and cell cycle analysis. To analyze migration, we performed a wound scratch assay as previously described (26). In brief, cells were seeded in 24-well plates (Greiner Bio-One) and cultured until they reached 100% confluency. In detail, 24 h after U104 treatment and 48 h after siRNA transfection, respectively, the cells were wounded by creating a cell-free area with a 200-μl pipette tip and washed twice to remove detached cells. Images were captured immediately (0 h) and 16 h after scratching to quantify the extent of the wounded area using the software Photoshop (Adobe Systems Inc., San Jose, CA, USA).

The cell cycle was analyzed 72 h after siRNA transfection or incubation with U104 as previously described (24). Briefly, propidium iodide (PI) was used to label DNA, and the DNA content was measured by flow cytometry on a FACScan instrument (BD Biosciences, Franklin Lakes, NJ, USA). The cell cycle phase distribution was then analyzed using the software ModFit (Verity Software House, Topsham, ME, USA).

Statistical analysis. Data represent at least three independent experiments. All data represent the mean value and standard deviation (+ SD). The significance of differences was assessed using one-way ANOVA followed by Tukey's post hoc test or Dunnett's post hoc test, or unpaired two-sided Student's t-test. A P-value <0.05 was considered to indicate a significant difference in reference to the population of negative control cells (nonsense siRNA or DMSO), if not otherwise indicated.

Results

CAIX expression under different cell culture conditions. We investigated *CA9* mRNA and CAIX protein expression levels under different oxygen conditions (21 vs. 0.1% O_2) and different pH values (pH 6.6 vs. 7.4) in the breast cancer cell lines MDA-MB-231 and MCF-7. As expected, the expression of CA9 mRNA and CAIX protein increased in both cell lines under hypoxic conditions compared to levels observed under normoxic conditions (Fig. 1). The *CA9* mRNA level was significantly elevated 100-fold under hypoxia in both breast cancer

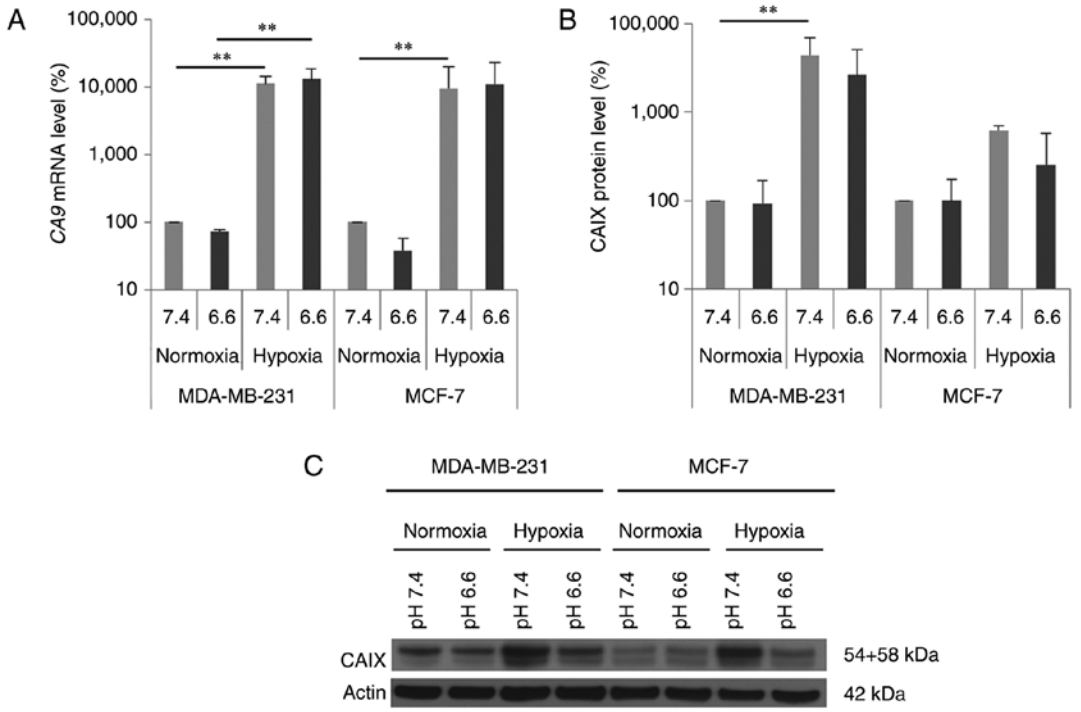

Figure 1. *CA9* mRNA and CAIX protein expression level under different cell culture conditions. MDA-MB-231 and MCF-7 breast cancer cells were cultured under different conditions varying in oxygen concentration (21% normoxia, 0.1% hypoxia) and pH (physiological pH: 7.4 and acidic pH: 6.6). The *CA9* mRNA (A) and CAIX protein expression levels (B) were determined with qPCR and western blotting. Intensity of CAIX western blot bands was quantified. The protein level of CAIX was normalized to equivalent protein levels of actin of each western blot band and is represented as a relative value to normoxia pH 7.4 (set as 1.0). Data represent mean values and standard deviation (+ SD) of at least three independent experiments (**P<0.01). (C) A representative western blot analysis of CAIX protein level in MDA-MB-231 and MCF-7 breast cancer cells cultured under normoxia and hypoxia with pH 7.4 and 6.6. is shown. CAIX, carbonic anhydrase IX.

cell lines (Fig. 1A, pH 7.4: MDA-MB-231: P=0.002; MCF-7: P=0.009), whereas the increase in CAIX protein level was higher in the MDA-MB-231 cells (pH 7.4: 40-fold; P=0.002) than that noted in the MCF-7 cells (pH 7.4: 6-fold; P=0.5). In addition, hypoxia-induced CAIX expression was independent of the extracellular pH (pHe 6.6 and 7.4) (Fig. 1B and C). Under normoxic and hypoxic conditions, *CA9* mRNA levels were not significantly altered by acidic conditions (pHe 6.6) in both breast cancer cell lines (Fig. 1A). The CAIX protein level was also not influenced by the acidic medium under normoxic conditions. However, hypoxic conditions produced a trend towards reduced CAIX protein levels in both cell lines cultured in acidic medium (Fig. 1B and C).

Effects of CA9 siRNAs in breast cancer cells. RNA interference (RNAi) technique was used to transfect chemically synthesized siRNAs against *CA9* into breast cancer cells and the expression of *CA9* mRNA and CAIX protein expression levels were examined by real-time PCR and via western blot analyses. In MDA-MB-231 and MCF-7 cells, the *CA9* mRNA expression levels were reduced by 79 to 87% (P<0.01) 48 h after transfection with *CA9* siRNA (Fig. 2A). Moreover, *CA9* siRNA continued to suppress *CA9* mRNA expression 72 h after siRNA transfection (data not shown). Due to the long half-life of CAIX protein, we observed a strong reduction in CAIX protein level in both cell lines 72 h after transfection with *CA9* siRNA (Fig. 2B and C). Additionally, the sulforhodamine B (SRB) assay showed that *CA9* knockdown did not affect the proliferation of breast cancer cells (Fig. 2E). However, poly(ADP-ribose) polymerase (PARP) cleavage, a marker of apoptosis, increased after treatment with *CA9* siRNA in both cell lines (Fig. 2D). In MDA-MB-231 cells, *CA9* siRNA reduced clonogenic survival by 54% (P=0.05), and in MCF-7 cells, treatment with *CA9* siRNA resulted in reduced clonogenic survival of ~40% (P=0.3) (Fig. 3F).

In a wound scratch assay, the poorly invasive MCF-7 cells migrated to a lower extent compared to highly invasive MDA-MB-231 cells (data not shown). The migration of MDA-MB-231 cells was significantly inhibited by 50% (P=0.008) after *CA9* knockdown (Fig. 2G). In contrast, migration of MCF-7 cells was reduced by 20% after transfection with *CA9* siRNA (P=0.02). A subsequent flow cytometric analysis revealed that CAIX inhibition decreased the number of MDA-MB-231 cells in the G_0/G_1 phase (P=0.005) and increased the number of cells in the G2/M phase (P=0.2) (Fig. 2H). In contrast, inhibition of CAIX expression in MCF-7 cells did not affect cell cycle distribution (Fig. 2H).

CAIX inhibition with U104. Inhibition of CAIX was also performed by an alternative strategy, namely treatment with the CAIX inhibitor U104, which specifically inhibits CAIX activity. Cytotoxicity assays revealed that U104 was more cytotoxic in MDA-MB-231 cells than in MCF-7 cells (Fig. 3A). The concentration that reduced survival by half (IC_{50}) was determined using a dose-response curve fitting. Specifically, the IC_{50} of U104 was 112.6±21.8 μM in MDA-MB-231 cells, whereas in MCF-7 cells the IC_{50} was much higher (306.9±37.9 μM; P<0.001). The measurement of the CAIX

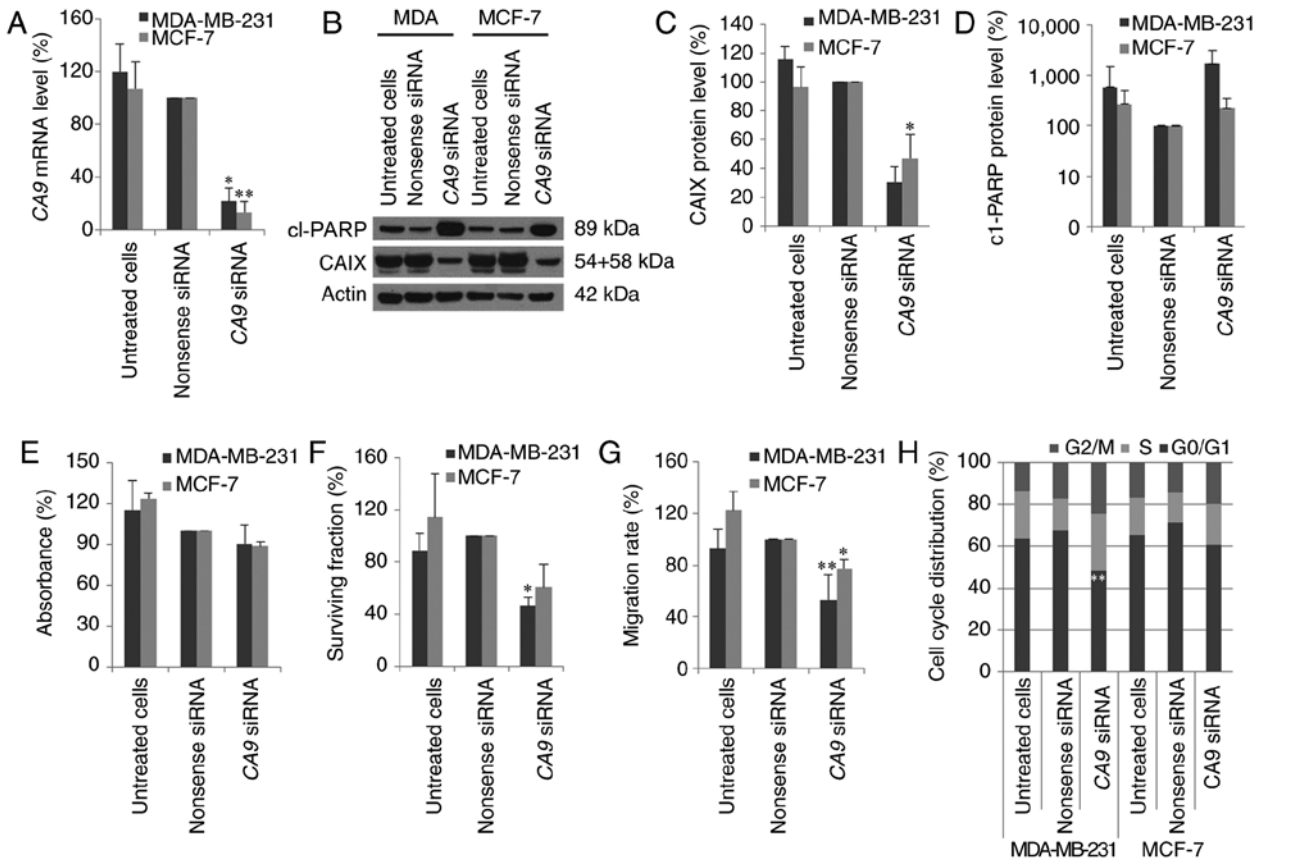

Figure 2. Effects of *CA9* siRNAs. MDA-MB-231 (grey bar) and MCF-7 (light grey bar) cells were transfected with *CA9* siRNA for 72 h under hypoxic conditions. (A) qPCR was performed to measure *CA9* mRNA expression level and (B) western blotting was conducted to determine the protein level of CAIX and cleaved PARP (cl-PARP), a marker of apoptosis. For western blot analysis, one representative result of three independent experiments is shown. (C and D) Quantification of CAIX and cl-PARP protein levels is shown. Data represent protein levels of cl-PARP or CAIX, which were normalized to equivalent protein levels of actin of each western blot band and are represented as relative values to nonsense siRNA (set as 1.0). (E) The cytotoxicity of siRNA treatment was examined using SRB assay and (F) clonogenic survival using clonogenic survival assay. Additionally, the migration (G) and cell cycle distribution (H) of breast cancer cells transfected with *CA9* siRNA were analyzed. Data represent mean values and standard deviation (+ SD) of at least three independent experiments (cell cycle: MCF-7: n=2) (*P<0.05, **P<0.01).

activity in MDA-MB-231 cells revealed a 2.5-fold increase in the CAIX activity under hypoxic conditions compared to normoxic conditions (P=0.002). Treatment with 50 μM U104 reduced CAIX activity to basal normoxic CAIX activity level (P=0.002) (Fig. 3B). In MCF-7 cells, no change in the CAIX activity was detected after exposure to hypoxia (data not shown). Additionally, we investigated the pHi after U104 treatment. The extracellular pH (pHe) was adjusted to pH 6.6, and untreated MDA-MB-231 cells had a pHi of 6.71 (Fig. 3C). Treatment with 50 μM U104 reduced the pHi by 0.13 pH units to 6.58 (P=0.2). Furthermore, in MCF-7 cells a lower effect of treatment with CAI U104 on pHi was observed compared to MDA-MB-231 cells. The untreated MCF-7 cells had a pHi of 6.52, which was reduced by 0.09 pH units (P=0.4) after incubation with U104 (Fig. 3C).

Moreover, inhibition of CAIX with U104 reduced the *CA9* mRNA expression level (Fig. 3D). In particular, the *CA9* mRNA level in MDA-MB-231 cells was reduced by 60 and 80% 72 h after treatment with 100 and 250 μM U104, respectively. In MCF-7 cells, the *CA9* mRNA level was reduced to a lesser extent by 50 and 70% 72 h after treatment with 100 and 250 μM, respectively (Fig. 3D). Additionally, the CAIX protein level was decreased in both cell lines 72 h after incubation with 10 and 100 μM U104, and CAIX protein could no longer be detected after incubation with 250 μM U104 (MDA-MB-231: P=0.0009) (Fig. 3E and F). PARP cleavage was significantly increased after treatment with U104 in MDA-MB-231 cells (P=0.001) (Fig. 3E and G).

CAIX inhibition by U104 did not affect the clonogenic survival of MDA-MB-231 cells but reduced the clonogenic survival of MCF-7 cells by 55% (P=0.2) (Fig. 4A). Incubation with U104 reduced the migration rate of MDA-MB-231 and MCF-7 cells by 52% (P=0.008) and 44% (P=0.8), respectively (Fig. 4B). Investigation of the cell cycle distribution of MDA-MB-231 cells revealed an increased number of cells in the S phase and a statistically significantly reduced number of cells in G_0/G_1 (P=0.02 and P=0.01, respectively) after treatment with CAI U104 (Fig. 4C). However, CAIX inhibition with U104 increased the number of MCF-7 cells in G2/M and reduced the number of MCF-7 cells in G_0/G_1 (P=0.1 and P=0.1, respectively) (Fig. 4C).

Radiosensitivity after CAIX inhibition. Combination of CAIX inhibition by siRNA and irradiation had little or no effects on the radiosensitivity of MDA-MB-231 and MCF-7 cells, respectively (Fig. 5A and C). Specifically, a DMF10

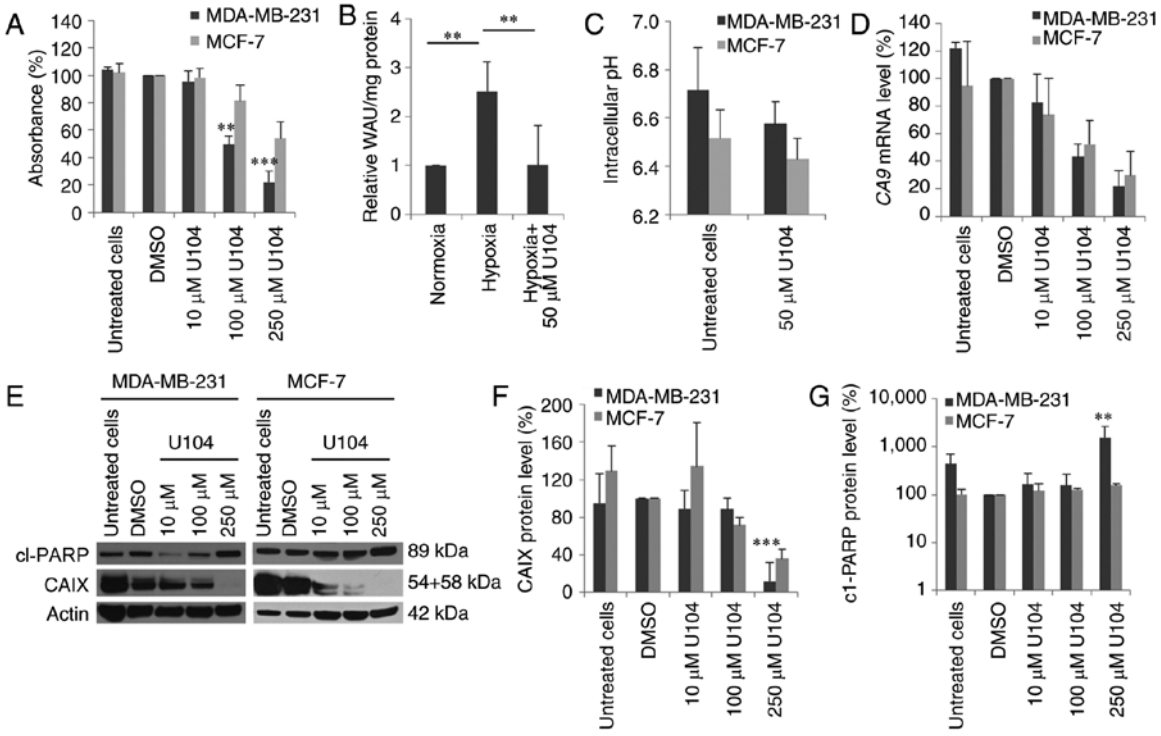

Figure 3. Effect of CAI U104 on proliferation, intracellular pH, *CA9* mRNA and CAIX protein level and apoptosis. MDA-MB-231 (grey box) and MCF-7 (light grey box) cells were treated with CAI U104 under hypoxic conditions (0.1% O_2). (A) SRB assay was performed to determine the cytotoxicity of treatment with CAI U104. For the CAIX activity assay (B: MDA-MB-231) and intracellular pH measurement (C) breast cancer cells were treated with U104 for 3 h. (D) qPCR was performed 72 h after treatment to measure the *CA9* mRNA expression level. (E) Western blotting was performed to measure the protein level of CAIX and cleaved PARP, a marker of apoptosis. For western blot analysis, one representative result of three independent experiments is shown. (F and G) Quantification of CAIX and cl-PARP protein level is shown. Data represent protein levels of cl-PARP or CAIX, which were normalized to equivalent protein levels of actin of each western blot band and represented as relative values to DMSO (set as 1.0). Data represent mean values and standard deviation (+ SD) of at least three independent experiments (**P<0.01, ***P<0.001).

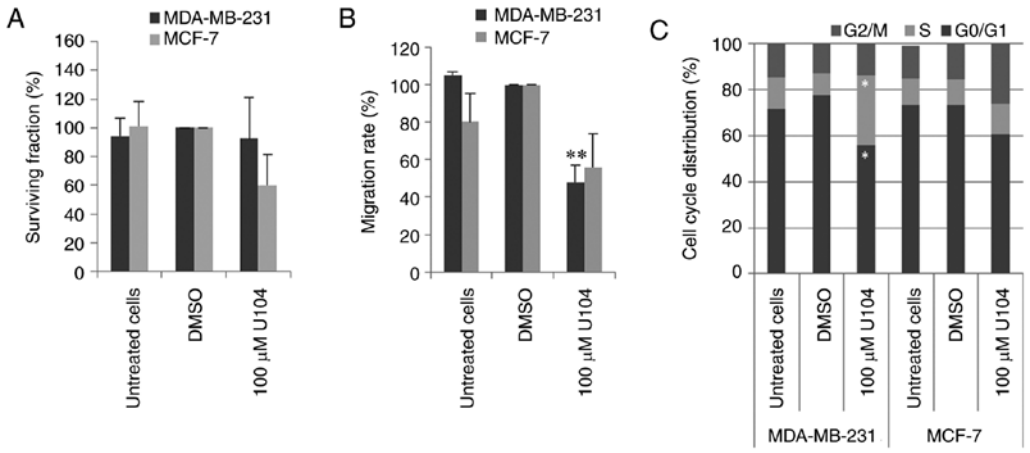

Figure 4. Effects of CAI U104 on clonogenic survival, migration and cell cycle distribution. (A) The clonogenic survival and (B) migration were assessed 24 h after treatment with the CAIX inhibitor U104. (C) FACS analysis was performed to specify cell cycle distribution after the inhibition of CAIX activity with U104 under hypoxic conditions in MDA-MB-231 and MCF-7 cells. Data represent mean values and standard deviation (± SD) of at least three independent experiments (*P<0.05, **P<0.01).

and EF_{10Gy} of 1.10±0.17 (P=0.9) and 1.79±0.36 (P=0.4) were calculated for MDA-MB-231 cells and a DMF10 and EF_{10Gy} of 0.97±0.36 (P=0.9) and 0.82±0.7 (P=0.9) for MCF-7 cells. Inhibition of CAIX with CAI U104 revealed stronger effects on the radiosensitivity of MDA-MB-231 and MCF-7 cells (Fig. 5B and D). The DMF10 and EF_{10Gy} were 1.57±0.16 (P=0.05) and 2.62±0.09 (P=0.02) in MDA-MB-231 cells. In MCF-7 cells a DMF10 of 1.77±0.65 (P=0.2) and an EF_{10Gy} of 4.27±2.80 (P=0.1) was calculated.

Discussion

Hypoxia-regulated protein carbonic anhydrase IX (CAIX) is associated with tumor-relevant processes, such as migration,

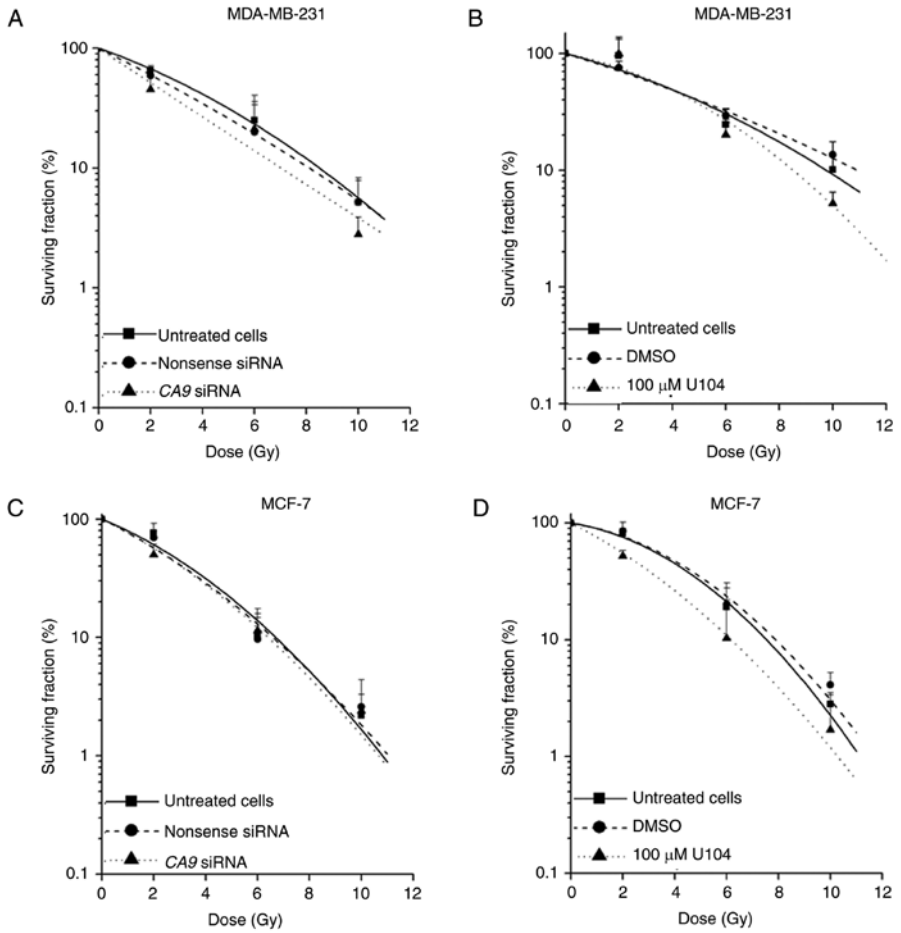

Figure 5. Radiosensitivity of breast cancer cells. (A and B) MDA-MB-231 and (C and D) MCF-7 cells were irradiated with 2, 6 and 10 Gy under hypoxic conditions after CAIX inhibition with *CA9* siRNA (A and C) and CAI U104 (B and D). Data represent mean values and standard deviation (± SD) of at least three independent experiments.

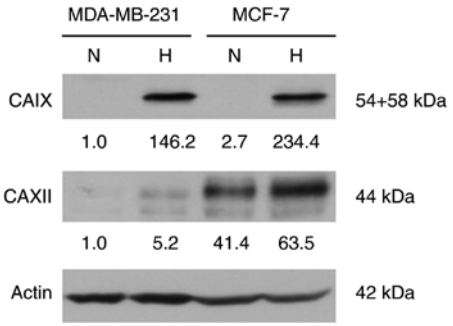

Figure 6. CAIX and CAXII protein expression in breast cancer cell lines. MDA-MB-231 and MCF-7 cells were cultured under normoxic (N) and hypoxic (H) conditions for 72 h. Western blotting was conducted to determine the protein levels of CAIX and CAXII. Four independent experiments were conducted. Representative western blots are shown. Quantified western blot bands of CAIX and CAXII were normalized to actin protein levels and represented as relative values to protein level of MDA-MB-231 cells under normoxic conditions set as 1.0.

proliferation and invasion and its expression correlates with the prognosis of cancer patients (27,28). Although CAIX is primarily responsible for the acidification of the tumor environment, we did not identify an altered *CA9* mRNA expression but a slightly reduced CAIX protein level in response to acidic (pH 6.6) and hypoxic (0.1% O_2) culture conditions in both breast cancer cell lines (Fig. 1). Recently, we detected similar effects in AT1 prostate cancer cells (29). In contrast to our investigations, Tang *et al* observed a decrease in *CA9* mRNA level in MCF-7 cells after incubation with lactic acid under hypoxic conditions (30). On the other hand, we previously showed that the response of CAIX expression to acidic conditions (upregulated or downregulated) depends on the cell line (31). For example, culturing glioblastoma and osteosarcoma cells under acidic conditions (pH 6.4) increased *CA9* gene transcription and CAIX protein level under normoxic and hypoxic conditions (32,33). Therefore, acidic conditions-in contrast to hypoxia-have different, cell-type specific consequences in human tumor cells.

With RNA interference and CAI U104, two different strategies were used to target CAIX under hypoxic conditions in two breast cancer cell lines. Both methods resulted in decreased *CA9* mRNA and CAIX protein levels (Figs. 2 and 3). These effects are in line with previous reports that also demonstrated reductions in *CA9* mRNA and CAIX protein level in tumor cells in response to treatment with RNA interference or CAI U104 (10,21,22,34-36). CAI-induced changes were attributed to the possible degradation or internalization of CAIX (21,36). In addition, treatment with CAI U104 decreased the pHi and reduced CAIX activity in MDA-MB-231 breast cancer cells (Fig. 3B and C). Accordingly, the pHi of CAIX-expressing

fibroblasts was previously shown to be more alkaline than the pHi of fibroblasts that do not express CAIX (8). Contrary to MDA-MB-231 cells, a hypoxia-induced increase in CAIX activity was lacking in MCF-7 breast cancer cells. It is conceivable that the CAIX activity assay is insufficiently sensitive for measurement of this slight increase of CAIX protein level in MCF-7 cells after hypoxic exposure. In agreement with this, Meehan *et al* detected only partial activation of HIF signaling in acutely hypoxic MCF-7 cells (22).

In the present study, RNA interference or CAI U104 affected migration, clonogenic survival, cell cycle distribution and apoptosis in both breast cancer cell lines (Figs. 2-4). Other studies investigating various ureido-sulfamate CAIX inhibitors also revealed a reduction in the migration, invasion or metastasis of breast cancer cells *in vitro* or in xenograft models (10,37). In soft tissue sarcoma cells (HT-1080), the depletion of CAIX expression decreased migration, invasion and expansive growth (9). CAIX influences the expression of genes that regulate several processes, such as cell motility, cell-cell contact, focal adhesion formation and epithelial-mesenchymal transition (38). In cervical carcinoma cells, CAIX increased migration and invasion via the *CA9*-dependent inactivation of Rho-GTPase (39). However, *CA9* knockdown did not affect the proliferation of breast cancer cells after 3 days (Fig. 2E). Initial studies of breast cancer cell lines revealed that silencing of *CA9* with siRNAs inhibited long-term proliferation (after 6 days) and clonogenic survival under hypoxic conditions (34). In confirmation with our results concerning ureidosulfonamide U104, Dubois *et al* observed a decrease in proliferation and induction of apoptosis after inhibiting CAIX in colorectal cancer (16). The decreased pHi may be responsible for the decreased proliferation and increased apoptosis rate (16,28). This study of rat prostate cancer cells confirmed the intracellular acidification, anti-proliferative and pro-apoptotic effects of CAIX inhibition with CAI U104 (29). In addition, recently, our previous results confirmed CAI-induced apoptosis for betulinyl sulfamates in MDA-MB-231 and MCF7 cells using different methods (21,40). A further study detected CAI-induced apoptosis in different cancer cell lines (41). However, the molecular mechanism underlying this effect remains to be elucidated.

The effects of RNA interference or CAI U104 were even stronger in the basal triple-negative MDA-MB-231 breast cancer cells compared to luminal MCF-7 cells (Figs. 2-4). In agreement with our results, silencing of *CA9* with shRNA revealed a stronger inhibition of invasion and increased doxorubicin-mediated reduction of spheroid-forming ability in triple-negative MDA-MB-231 cells compared to breast cancer cell lines with other subtypes (18). In addition, the prognostic significance of the *CA9* mRNA level differed depending on the subtype of breast cancer (18). For generalization of these effects, further breast cancer cell lines with each intrinsic breast cancer subtype must be analyzed.

We determined additive/synergistic effects after *CA9* knockdown or U104-induced CAIX inhibition in combination with irradiation in MDA-MB-231 and MCF-7 breast cancer cells under hypoxic conditions (Fig. 5). Initial studies of a colorectal carcinoma xenograft model revealed that CAIX inhibition with different CAIX inhibitors (CAI: Acetazolamide, DH348 or 11c) or *CA9* knockdown by shRNA delayed tumor growth and radiosensitized tumor cells (16,17). These findings corroborate other studies showing that inhibition of carbonic anhydrases with different CAIs radiosensitized different tumor cell lines (21,35,42). This effect may be due to the reduced acidification of the extracellular environment (16,17) or the intracellular acidification (Fig. 3C) caused by CAIX inhibition. Fibroblasts lacking CAIX were strongly radiosensitized when cultured under acidic conditions (pH 7.0) compared to normal conditions (pH 7.5) because they cannot maintain their intracellular pH (42). However, this effect could be abrogated by stably transfecting cells with CAIX. In further studies we plan investigations with stable knockdown of the CAIX gene in mammary carcinoma cell lines. It should be noted that the effects of CAI U104 on radiosensitization were stronger than the effects caused by RNAi, especially in MCF-7 cells. The isoenzyme CAXII is expressed at higher levels in MCF-7 cells compared to MDA-MB-231 cells under normoxic and hypoxic conditions (Fig. 6). The effects of CAIX inhibition by siRNA may be compensated by CAXII, since siRNA selectively inhibited *CA9*, not *CA12* mRNA expression. However, CAI U104 inhibited activity of both isoenzymes CAIX and CAXII and therefore caused stronger effects on radiosensitivity (10). In accordance with that, combined knockdown of *CA9* and *CA12* gene expression revealed stronger radiosensitization of colon carcinoma cells than single knockdown of *CA9* or *CA12* gene expression (42). Due to possible compensatory effects of CAXII, investigation of CAXII function in tumor relevant processes is warranted.

In summary, it was demonstrated that specifically targeting *CA9* or inhibiting CAIX/CAXII activity influences intracellular and extracellular pH that is important for clonogenic survival, apoptosis, migration and radiosensitivity of both breast cancer cell lines. CAIX alone and in combination with CAXII are significant targets for novel combination strategies with radiotherapy in breast cancer.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

AG designed the study, performed the experimental procedures, analyzed the data and drafted the manuscript. KT

performed the experimental procedures and analyzed the data. AR, HW and JK substantially contributed to the data acquisition and interpretation and reviewed the manuscript. OT, MB and DV designed the study, substantially contributed to the acquisition and interpretation of the data and reviewed the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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