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ARHGAP29 Is Involved in Increased Invasiveness of Tamoxifen-resistant Breast Cancer Cells and its Expression Levels Correlate With Clinical Tumor Parameters of Breast Cancer Patients

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Abstract. *Background/Aim: Aggressive breast cancer (BC) cells show high expression of Rho GTPase activating protein 29 (ARHGAP29), a negative regulator of RhoA. In breast cancer cells in which mesenchymal transformation was induced, ARHGAP29 was the only one of 32 GTPase-activating enzymes whose expression increased significantly. Therefore, we investigated whether there is a correlation between expression of ARHGAP29 and tumor progression in BC. Since tamoxifen-resistant BC cells exhibit increased mesenchymal properties and invasiveness, we additionally investigated the relationship between ARHGAP29 and increased invasion rate in tamoxifen resistance. The question arises as to whether ARHGAP29 is a suitable prognostic marker for the progression of BC. Materials and Methods: Tissue microarrays were used to investigate expression of ARHGAP29 in BC and adjacent normal breast tissues. Knockdown experiments using siRNA were performed to investigate the influence of ARHGAP29 and the possible downstream actors RhoC and pAKT1 on invasive growth of tamoxifen-resistant BC spheroids in vitro. Results: Expression of ARHGAP29 was frequently increased in BC tissues compared to adjacent normal breast tissues. In addition, there was evidence of a correlation between high ARHGAP29 expression and advanced clinical tumor stage. Tamoxifen-resistant BC cells show a significantly higher expression of ARHGAP29 compared to their parental wild-type cells. After knockdown of ARHGAP29 in tamoxifen-resistant BC cells, expression of RhoC was significantly reduced. Further, expression of pAKT1 decreased significantly. Invasive growth of three-dimensional tamoxifen-resistant BC spheroids was reduced after knockdown of ARHGAP29. This could be partially reversed by AKT1 activator SC79. Conclusion: Expression of ARHGAP29 correlates with the clinical tumor parameters of BC patients. In addition, ARHGAP29 is involved in increased invasiveness of tamoxifen-resistant BC cells. ARHGAP29 alone or in combination with its downstream partners RhoC and pAKT1 could be suitable prognostic markers for BC progression.*

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Rho GTPase activating protein 29 (ARHGAP29) also known as PTPL1-associated RhoGAP protein 1 (PARG1) is a RhoGTPase regulating protein (GAP). It increases the intrinsic GTPase activity of the respective Rho protein and thus leads to a switch from the active GTP form to the inactive GDP form. It, thus, negatively regulates Rho proteins. ARHGAP29 has a strong affinity for RhoA and a weaker affinity for Rac2 and Cdc42 (1). In addition to its GAP domain, ARHGAP29 has a C-terminal amino acid residue specifically for interaction with the protein tyrosine kinase PTPL1 and additionally a cysteine-rich domain with similarity to the Zn^{2+}- and diaglycerol-binding domain of the protein kinase C (PKC) family. Another region in the structure of ARHGAP29 that shows homologies to other proteins is the ZPH (ZK669.1a-PARG homology) region. The gene sequence in this region is homologous to that of the nematode Caenorhabditis elegans gene ZK669.1a (1).

Expression of ARHGAP29 was detected in various tissues. The intracellular protein was highly expressed in skeletal muscle and heart tissue. Medium expression was found in placenta, liver and pancreas, while ARHGAP29 was only expressed at very low levels in brain, lung and kidney

(1). In oncology, high expression of ARHGAP29 in renal cell carcinoma, gastric carcinoma and prostate carcinoma was found to be a poor prognostic marker (2-4). Increased expression correlated with increased invasion and proliferation of cells *in vitro* and advanced tumor stages (staging and grading) in the analysis of tissue samples (2, 4).

An inhibitory effect of ARHGAP29 on the RhoA-Rho-associated protein kinase (ROCK) signaling pathway has been described as the underlying mechanism (2). Due to its inhibitory effect on RhoA, ARHGAP29 is an important component of various signaling cascades. Qiao *et al*., for example, showed a connection between ARHGAP29 and the yes-associated protein (YAP) signaling pathway in a gastric carcinoma cell line. Accordingly, YAP inhibits the RhoA-LIM domain kinase (LIMK) cofilin signaling pathway *via* increased ARHGAP29 expression. This leads to the destabilization of F-actin *via* reduced phosphorylation of cofilin, which leads to cytoskeletal remodeling and promotes cell migration and metastasis (3). The inhibitory effect of ARHGAP29 on the RhoA-cofilin signaling pathway was confirmed in a study of pancreatic cancer cell lines (4).

Other publications have also identified the transcription cofactor YAP as a central node of various signaling pathways in tumor biology. YAP is inhibited by the Hippo signaling pathway, which is considered an important tumor suppressor signaling pathway (5). Overexpression of YAP, which is typical for carcinoma cells, stimulates cell proliferation and thus tumor growth and induces further pro-metastatic processes, such as cell transformation and migration (6-8). The TEA domain (TEAD) and connective tissue growth factor (CTGF) were identified as further downstream targets (9). YAP has also been associated with chemotherapeutic resistance and CSC formation (6, 10). The role of YAP in breast cancer (BC) is unclear. YAP has been described as both a tumor suppressor and an oncogene (11).

In addition to its involvement in the YAP-RhoA signaling pathway and the associated metastatic potential, the influence of ARHGAP29 has also been established in other contexts. For example, it was shown that ARHGAP29 expression is also increased in circulating tumor cells (CTC) and in motility-stimulated glioblastoma cells in gastric carcinoma compared to the primary tumor (3, 12). Another publication identified ARHGAP29 (=PARG1) by deoxyribonucleic acid (DNA) microarray analysis as a possible tumor suppressor gene in mantle cell lymphomas (13).

However, ARHGAP29 and its inhibitory effect on RhoA do not only play a role in tumor biology. ARHGAP29 has also been linked to angiogenesis and migration of endothelial cells. Induced by Ras-related protein 1 (Rap1), it has an inhibitory effect with afadin on the RhoA-ROCK axis and thus increases vascular endothelial growth factor (VEGF)-induced angiogenesis (14). The interaction between Rap1 and ARHGAP29 has also been described in the context of endothelial cell barrier function as the Rap1-ras-interacting protein 1 (Rasip1)/Radil-ARHGAP29-RhoA signaling pathway. Activation of Rap1 resulted in decreased RhoA activity manifested by cell spreading and stabilization of barrier function (15). The Rasip1-ARHGAP29 signaling pathway was also shown to be essential for endothelial tubulogenesis (=formation of blood vessel lumen) of blood vessels (16, 17). In addition to Rap1, ARHGAP29 has also been identified as an effector protein of Rap2 (18). In addition, ARHGAP29 is associated with the differentiation of myofibroblasts. Hypoxia induces ARHGAP29 *via* hypoxia-inducible factor 1-alpha (HIF-1α) and prevents the differentiation of fibroblasts into myofibroblasts by inhibiting RhoA (19). Another described interaction partner of ARHGAP29 is the interferon regulatory factor 6 (Irf6), which influences cell migration of keratinocytes (20). ARHGAP29 is also thought to play an important role in craniofacial development and is thus associated with the development of non-syndromic cleft lip and/or palate (21-23).

In the gynecological context, ARHGAP29 has been previously mentioned in connection with intrauterine adhesions. Xu Q *et al*. showed that microRNA-1291 stimulates ARHGAP29 and leads to endometrial fibrosis *via* inhibition of the RhoA-ROCK axis (24). A recent publication describes ARHGAP29 in the context of BC. Kolb *et al*. showed that the expression of ARHGAP29 is increased in mesenchymal-transformed BC cells and TNBC (25). They discussed that after knockdown of ARHGAP29, the invasiveness of BC cells decreased significantly. This could be explained by a consequent increase in RhoA activity and thus stabilization of the cytoskeleton. In contrast, proliferation increased in cells with ARHGAP29 knock-down. Thus, a reduced invasion of the BC cells due to reduced proliferation could be excluded. In addition, Kolb *et al*. identified AKT1 as a possible interaction partner of ARHGAP29 and showed that the expression of AKT1 decreased in ARHGAP29-altered cells (25). The mechanism behind this remained unclear.

In summary, ARHGAP29 may play an important role in the invasion and metastasis of BC. ARHGAP29 has been linked to several signaling pathways that influence cytoskeletal organization. Rasip1 and YAP, for example, have been identified as upstream targets of ARHGAP29 (3, 15). RhoA was found to be a frequent downstream interaction partner and an effect of ARHGAP29 on the expression of AKT1 was described (3, 25). The underlying mechanism has not yet been clarified. Another possible interaction partner of ARHGAP29 could be RhoC. RhoC has been ascribed an important role in the invasion of BC cells. In addition, RhoC has been identified as a substrate of AKT1 (26). The effect of ARHGAP29 on AKT1 could possibly be related to RhoC.

The question arose as to whether there is a correlation between expression of ARHGAP29 and tumor progression

in BC. Since tamoxifen-resistant BC cells exhibit increased mesenchymal properties and invasiveness, the influence of ARHGAP29 expression on the invasiveness of tamoxifen-resistant BC cells and the role of downstream partners of ARHGAP29 are of interest.

Materials and Methods

Human tissues. To analyze the expression of ARHGAP29 in specimens of human BCs we used a human tissue array (BR8011a, US Biomax, Inc., Rockville, MD, USA) containing paraffin-embedded human normal and malignant breast tissue specimens whose characteristics are outlined in Table I. Informed consent for the use of human tissues was obtained in accordance with the ethics guidelines that were effective at the time of collection and processing.

Immunohistochemistry. The tissue array slides were deparaffinized and rehydrated. Antigens were retrieved by incubation with 0.01 M citrate buffer (pH 6.0) in a microwave (700 W) for 5 min. Endogenous peroxidase activity was quenched by treatment with 3% hydrogen peroxide solution for 6 min. After washing in PBS, the slides were treated with polyclonal rabbit anti-human ARHGAP29 receptor antiserum (#NBP1-05989, Novus Biologicals, Centennial, CO, USA) in a 1:50 dilution in 1% BSA in 10 mM Tris, pH 8.0, 500 mM NaCl and 0.1% Tween-20 (TBST) over night. The next day, after being washed, incubation with the secondary antibody (donkey anti-rabbit IgG Fluor 488, #R37118, Invitrogen, Darmstadt, Germany) and nuclear staining with DAPI solution (1 μg/ml) was performed. Finally, the slides were covered with fluorescence mounting medium. The immunohistochemically labeled slides were examined with the Olympus IX51 fluorescence microscope (Olympus Germany, Hamburg, Germany). Photos were taken using the CellSens software (Olympus). To reduce background fluorescence, DAPI or GFP (ARHGAP29) filters were set in CellSens software. Exposure times were set to 250 ms for DAPI and 1000 ms for GFP. Fluorescence of each tissue sample was evaluated in a double-blind manner without knowledge of tumor entity or tumor classification (Table I).

Cell culture. The human BC cell lines MCF-7 and T-47D were obtained from the American Type Cell Collection (ATCC; Manassas, VA, USA) and cultured in minimum essential medium (MEM; biowest, Nuaillé, France) supplemented with 10% fetal bovine serum (FBS; biochrom, Berlin, Germany), 1% Penicillin/Streptomycin (P/S; Gibco, Carlsbad, CA, USA), 0.1% Transferrin (Sigma, St. Louis, MO, USA), and 26 IU Insulin (Sanofi, Frankfurt, Germany). To retain the identity of cell lines, purchased cells were expanded and aliquots were frozen in liquid nitrogen. A new frozen stock was used every half year and mycoplasma testing of cultured cell lines was performed routinely using PCR Mycoplasma Test Kit I/C (PromoCell GmbH, Heidelberg, Germany). All cells were cultured in a humidified atmosphere with 5% CO_2 at 37˚C.

Generation of tamoxifen-resistant MCF-7 and T-47D cells. 4-hydroxytamoxifen (4-OHT) resistant sublines MCF-7-TR and T47D-TR were developed as previously described using a concentration of 4-OHT (Sigma, Deisenhofen, Germany) of 1.25 μM in culture medium (27). The cells were cultured as previously described (27).

Small interfering RNA (siRNA) transfection. The BC cells (1×10^6 cells/ml) were seeded in 2 ml of MEM with 10% FBS (-P/S) in a 25 cm^2 cell culture flask. Cells were transiently transfected with siRNA specific to ARHGAP29 (#sc-78491; Santa Cruz Biotechnology, Dallas, TX, USA) in OPTI-MEM I medium (Gibco, Carlsbad, CA, USA) with siRNA transfection reagent (sc-29528; Santa Cruz Biotechnology). A non-targeting control was used as a control (#sc-37007; Santa Cruz Biotechnology). In order to evaluate the transfection efficiency, a fluorescein-labeled siRNA control (#sc-36869; Santa Cruz Biotechnology) was used. After an incubation period of 6 h, MEM supplemented with 20% FBS and 20% penicillin/streptomycin was added.

3D tumor spheroid assay. In order to investigate the effects of suppression of ARHGAP29 on invasive growth, a 3D tumor spheroid assay was performed based on a method developed by Vinci *et al*. (28). The cells were seeded into 96 well, ultra-low attachment plates (Corning Life Sciences, Corning, NY, USA) to form spheroids, three-dimensional *in vitro* equivalents of micrometastases (28). The spheroids were coated with Matrigel and the invasion of the spheroids into the Matrigel was then analyzed over a period of 48 hours. The spheroids were photographed and analyzed using the Olympus IX51 microscope using CellSens software (Olympus).

Western blot analysis. In Western Blot analysis, cells were lysed in cell lytic M buffer (Sigma) supplemented with 0.1% phosphatase-inhibitor (Sigma) and 0.1% protease-inhibitor (Sigma). Isolated proteins (40 μg) were fractioned using 12% SDS gel and electro-transferred to a polyvinylidene difluoride membrane (Merck Millipore, Cork, Ireland). Primary antibodies against ARHGAP29 1:2000 (#NBP1-05989, Novus Biologicals, Centennial, CO, USA), RhoC 1:2,000 (#GTX100546, GeneTex, Irvine, CA, USA), pAKT1 1:1,000 (#9271, Cell Signaling, Danvers, MA, USA), and GAPDH 1:2,000 (#5174S, Cell Signaling) were used. The membrane was washed and incubated in horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Buckinghamshire, UK). Antibody-bond protein bands were assayed using a chemiluminescent luminol enhancer solution (Cyanagen, Bologna, Italy).

Statistical analysis. All experiments were performed on at least three biological and technical replicates. Data were analyzed by 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 the respective control) by assuming both populations had the same standard derivation. $p<0.05$ was considered statistically significant.

Results

Expression of ARHGAP29 in BC and adjacent normal breast tissue. To evaluate ARHGAP29 as a potential therapeutic target in BC, 79 tissue samples were analyzed for ARHGAP29 expression. In addition, it was analyzed whether progression of BCs correlates with the expression of ARHGAP29. Twenty-five tissue samples (32%) came from benign mammary gland tissue taken from cancer-adjacent sections. The remaining 54 samples (68%) were from BC tissue, including 17 sections from intraductal BCs, 33 from

Table I. *Expression of Rho GTPase activating protein 29 (ARHGAP29) in breast cancer (BC) tissue and adjacent normal breast tissue (AT).*

No.	Age	Pathology diagnosis	TNM	Grade	Stage	Type	ARHGAP29
1	41	Intraductal carcinoma	TisN0M0	–	0	Malignant	++
2	54	Intraductal carcinoma	TisN0M0	–	0	Malignant	++
3	68	Intraductal carcinoma	TxNxMx	–	–	Malignant	–
4	45	Intraductal carcinoma	TisN0M0	–	0	Malignant	–
5	47	Intraductal carcinoma	TisN0M0	–	0	Malignant	++
6	45	Intraductal carcinoma	TisN0M0	–	0	Malignant	–
7	53	Intraductal carcinoma	T2N1M0	–	IIB	Malignant	++
8	57	Intraductal carcinoma	TisN0M0	–	0	Malignant	++
9	48	Intraductal carcinoma	T1aN0M0	–	IA	Malignant	++
10	30	Intraductal carcinoma	TisN0M0	–	0	Malignant	++
11	67	Intraductal carcinoma	TisN0M0	–	0	Malignant	+
12	42	Intraductal carcinoma	T1N0M0	–	I	Malignant	–
13	39	Intraductal carcinoma	TisN0M0	–	0	Malignant	+
14	36	Intraductal carcinoma	T3N0M0	–	IIB	Malignant	+
15	47	Intraductal carcinoma	T3N1M0	–	IIIA	Malignant	+
16	62	Intraductal carcinoma	T3N0M0	–	IIB	Malignant	+
17	63	Intraductal carcinoma	T2N0M0	–	IIA	Malignant	++
18	55	Invasive ductal carcinoma	T3N2M0	1	IIIA	Malignant	+
19	50	Invasive ductal carcinoma	T2N0M0	1	IIA	Malignant	++
20	58	Invasive ductal carcinoma	T3N1M0	1	IIIA	Malignant	+++
21	50	Invasive ductal carcinoma	T2N1M0	1	IIB	Malignant	+
22	57	Invasive ductal carcinoma	T3N1M0	–	IIIA	Malignant	+
23	40	Invasive ductal carcinoma	T2N1M0	1	IIB	Malignant	+
24	63	Invasive ductal carcinoma	T2N1M0	1	IIB	Malignant	++
25	68	Invasive ductal carcinoma	T2N1M0	1	IIB	Malignant	–
26	59	Invasive ductal carcinoma	T2N1M0	2	IIB	Malignant	+++
27	57	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	+
28	57	Invasive ductal carcinoma	T2N1M0	2	IIB	Malignant	++
29	45	Invasive ductal carcinoma	T2N1M0	2	IIB	Malignant	+
30	68	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	–
31	52	Invasive ductal carcinoma	T3N0M0	2	IIB	Malignant	+++
32	54	Invasive ductal carcinoma	T3N1M0	2	IIIA	Malignant	++
33	39	Invasive ductal carcinoma	T2N1M0	2	IIB	Malignant	+++
34	79	Invasive ductal carcinoma	T4N1M0	2	IIIB	Malignant	+
35	44	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	++
36	61	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	++
37	49	Invasive ductal carcinoma	T3N1M0	2	IIIA	Malignant	+
38	52	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	++
39	56	Invasive ductal carcinoma	T2N1M0	2	IIB	Malignant	–
40	46	Invasive ductal carcinoma	T3N2M0	2	IIIA	Malignant	+++
41	59	Invasive ductal carcinoma	T2N0M0	3	IIA	Malignant	+++
42	42	Invasive ductal carcinoma	T2N0M0	–	IIA	Malignant	–
43	47	Invasive ductal carcinoma	T2N2M0	3	IIIA	Malignant	++
44	48	Invasive ductal carcinoma	T2N2M0	3	IIIA	Malignant	++
45	54	Invasive ductal carcinoma	T3N0M0	3	IIB	Malignant	++
46	50	Invasive ductal carcinoma	T3N2M0	3	IIIA	Malignant	++
47	66	Invasive ductal carcinoma	T2N0M0	3	IIA	Malignant	+++
48	53	Invasive ductal carcinoma	T2N0M0	3	IIA	Malignant	++
49	46	Invasive ductal carcinoma	T3N1M0	3	IIIA	Malignant	–
50	47	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	+
51	48	Lobular carcinoma in situ	T2N0M0	–	IIA	Malignant	++
52	47	Invasive lobular carcinoma	T2N1M0	–	IIB	Malignant	–
53	37	Invasive lobular carcinoma	T4N1M0	–	IIIB	Malignant	+++
54	70	Invasive lobular carcinoma	T4N0M0	–	IIIB	Malignant	++
55	50	Cancer adjacent breast tissue	–	–	–	AT	+
56	32	Cancer adjacent breast tissue	–	–	–	AT	–
57	49	Cancer adjacent breast tissue	–	–	–	AT	–
58	28	Cancer adjacent breast tissue	–	–	–	AT	–

Table I. *Continued*

Table I. *Continued*

No.	Age	Pathology diagnosis	TNM	Grade	Stage	Type	ARHGAP29
59	42	Cancer adjacent breast tissue	–	–	–	AT	–
60	48	Cancer adjacent breast tissue	–	–	–	AT	+
61	47	Cancer adjacent breast ductal tissue	–	–	–	AT	+
62	48	Cancer adjacent breast tissue	–	–	–	AT	–
63	47	Cancer adjacent breast tissue	–	–	–	AT	–
64	31	Cancer adjacent breast tissue	–	–	–	AT	–
65	44	Cancer adjacent breast tissue	–	–	–	AT	–
66	47	Cancer adjacent breast tissue	–	–	–	AT	+
67	49	Cancer adjacent breast tissue	–	–	–	AT	–
68	38	Cancer adjacent breast tissue	–	–	–	AT	–
69	45	Cancer adjacent breast tissue	–	–	–	AT	–
70	40	Cancer adjacent breast tissue	–	–	–	AT	+
71	48	Cancer adjacent breast tissue	–	–	–	AT	–
72	34	Cancer adjacent breast tissue	–	–	–	AT	–
73	57	Cancer adjacent breast tissue	–	–	–	AT	–
74	47	Cancer adjacent breast tissue	–	–	–	AT	+
75	38	Cancer adjacent breast tissue	–	–	–	AT	+
76	41	Cancer adjacent breast tissue	–	–	–	AT	++
77	77	Cancer adjacent breast tissue	–	–	–	AT	–
78	53	Cancer adjacent breast tissue	–	–	–	AT	+
79	29	Cancer adjacent breast tissue with fibroadenoma	–	–	–	AT	+

Information on tissue of origin, pathology, grading, staging, TNM classification and assessment of ARHGAP29 expression. (–) not expressed, (+) slightly expressed, (++) moderately expressed and (+++) strongly expressed.

invasive ductal carcinomas, one sample from a lobular carcinoma *in situ* and three from invasive lobular carcinomas.

Ten tissue samples (40%) from normal mammary gland tissue (n=25) showed a positive signal for ARHGAP29, while 44 (81%) of the samples from BC tissue (n=54) showed a positive signal for ARHGAP29 (Figure 1A). Thus, the majority (60%) of sections from normal breast tissue showed no expression of ARHGAP29, while only 19% of sections from carcinoma tissue were negative for ARHGAP29. A more detailed breakdown of ARHGAP29-positive tissue samples based on the fluorescence intensity of ARHGAP29-positive antibody labeled tissue samples into (+) slightly fluorescent, (++) strongly fluorescent and (+++) whole tissue array very strongly fluorescent, showed a shift towards stronger ARHGAP29 expression in BC tissue, compared to normal mammary gland tissue (Figure 1B). Thus, the majority of positive sections in normal mammary gland tissue showed only a slight signal (+) for ARHGAP29 (90%). Sections from carcinoma tissue showed a significantly higher proportion of strong expression [50% (++)] and 18% of the sections showed very strong expression of ARHGAP29 across the entire TMA (+++).

To investigate a possible correlation between the progression of cancer and the expression of ARHGAP29, tissue microarrays were analyzed for their ARHGAP29 expression with indication of the TNM classification (Table I). On closer examination of the lymph node status, the sections can be divided into 24 lymph node-positive (N1 or N2) samples and 29 lymph node-negative (N0) samples. No difference in the expression of ARHGAP29 was found between the two groups. Thus, in both lymph node-positive and lymph node-negative samples, the proportion expressing ARHGAP29 was 83% (Figure 2A).

On closer examination of the lymph node-positive tissue samples and division according to the TNM classification into N1 (=involvement of mobile axillary lymph nodes level I-II) and N2 (=involvement of fixed axillary lymph nodes level I-II or clinically diagnosed involvement of ipsilateral lymph nodes of the internal mammary artery), a shift towards more frequent and stronger expression of ARHGAP29 in advanced carcinomas is also evident here (Figure 2B). In sections with a lower lymph node status (N1), *i.e.*, less advanced carcinoma, there is a larger proportion of ARHGAP29-negative samples and a smaller proportion with very strong expression of ARHGAP29 (+++) than in sections with lymph node status N2.

Expression of ARHGAP29 in tamoxifen-resistant BC cells. First, sublines of ERα-positive breast cancer cell lines T47D (Figure 3A) and MCF-7 (Figure 3B) with secondary resistance against 4-hydroxytamoxifen (4-OHT) were generated according to Günthert *et al*. (27). Treatment of parental T47D

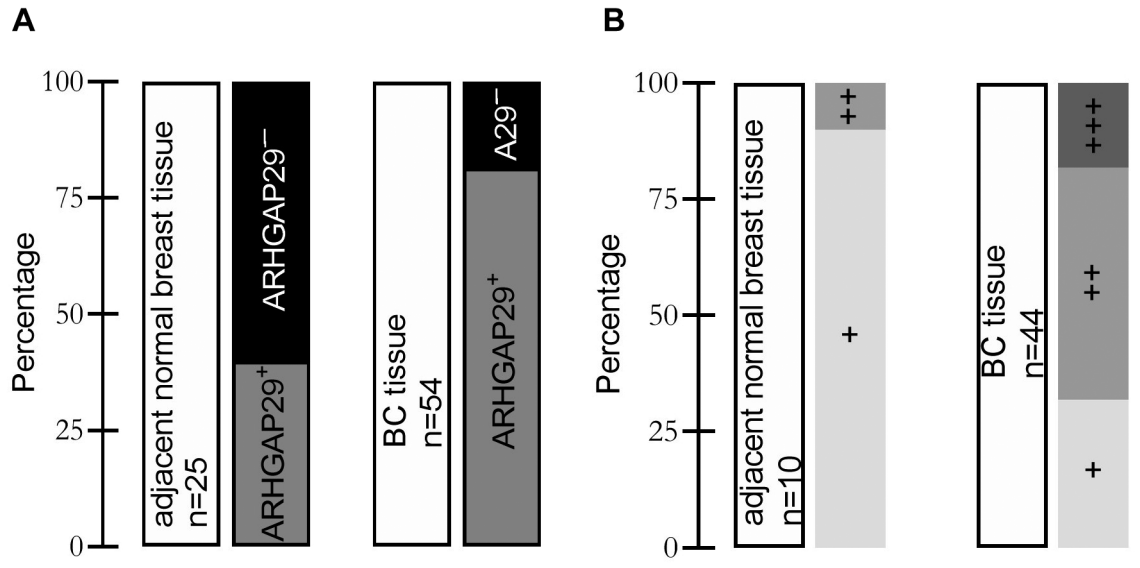

Figure 1. *Expression of Rho GTPase activating protein 29 (ARHGAP29) (A29) in breast cancer (BC) and adjacent normal breast tissue. Tissue samples (n=79) were analyzed for ARHGAP29 expression by immune fluorescence. (A) Percentage of ARHGAP29-positive (ARHGAP29+) and ARHGAP29-negative (ARHGAP29-) samples is shown, divided into normal breast tissue and BC tissue. (B) More detailed breakdown of ARHGAP29-positive sections from the tissue groups examined in (A) according to (+) mild expression, (++) medium expression, (+++) strong expression.*

cells with 5 μM 4-OHT resulted in a reduction of viability to 54.80±5.23% SEM ($p<0.001$ *vs*. control=100%, n=3; Figure 3A), while viability of tamoxifen-resistant T47D-TR cells remained unchanged (95.37±5.78% SEM, n=3; Figure 3A). Viability of parental MCF-7 cells treated with 5 μM 4-OHT was reduced to 62.08±6.41% SEM ($p<0.01$ *vs*. control=100%, n=3; Figure 3B). Tamoxifen-resistant MCF-7-TR cells treated with 5 μM 4-OHT showed no changes in viability (92.49±7.86% SEM, n=3; Figure 3B).

In the next step, expression of ARHGAP29 in the parental T47D and MCF-7 BC cells was compared with their tamoxifen-resistant sublines (Figure 3C). In tamoxifen-resistant T47D BC cells, expression of ARHGAP29 was increased to 168.48±20.74% SEM ($p<0.05$ *vs*. T47D =100%, n=4; Figure 3C). Tamoxifen-resistant MCF-7-TR cells showed an increased expression of ARHGAP29 to 193.11±23.49% SEM ($p<0.01$ *vs*. MCF-7=100%, n=4; Figure 3C).

Successful suppression of ARHGAP29 expression by siRNA was verified by Western blot. Both tamoxifen-resistant BC cell lines showed a significantly reduced expression of ARHGAP29 after knockdown (T47D-TR: 68.48±11.17% SEM *vs*. control =100%; $p<0.001$; n=4; MCF-7-TR: 49.87±11.55% SEM *vs*. control=100%; $p<0.05$; n=4; Figure 3D).

Effect of ARHGAP29 suppression by siRNA on expression of RhoC and pAKT1 in tamoxifen-resistant BC cells. The effect of ARHGAP29 suppression on the suspected downstream partners RhoC (Figure 4A) and pAKT1 (Figure 4B) was investigated. In the tamoxifen-resistant BC cell lines T47D-TR and MCF-7-TR, a significant reduction of RhoC expression to 36.02±6.34% SEM (T47D-TR: $p<0.001$ *vs*. siRNA control=100%, n=3) or to 64.81±8.75% SEM (MCF-7-TR: $p<0.01$ *vs*. control=100%, n=3) was observed after suppression of ARHGAP29 expression (Figure 4A). The influence of ARHGAP29 suppression on expression of pAKT1 was less pronounced, but still significant. In the tamoxifen-resistant BC cell line T47D-TR, expression of pAKT1 was significantly reduced to 63.67±9.75% SEM ($p<0.05$ *vs*. siRNA control=100%, n=3) after suppression of ARHGAP29 (Figure 4B). In the tamoxifen-resistant BC cell line MCF-7-TR, a significant reduction of pAKT1 expression to 72.12±9.94% SEM ($p<0.05$ *vs*. siRNA control=100%, n=3) was observed after suppression of ARHGAP29 expression (Figure 4B).

Effect of suppression of ARHGAP29 by siRNA on invasive growth of tamoxifen-resistant BC spheroids. In addition to the effects on expression of RhoC and pAKT1, we also investigated whether the suppression of ARHGAP29 has an effect on the invasive growth of tamoxifen-resistant BC spheroids. After suppression of ARHGAP29 expression, the invasive growth of tamoxifen-resistant T47D-TR (Figure 4C) and MCF-7-TR (Figure 4D) BC spheroids was significantly reduced. The invasive growth of T47D-TR spheroids with suppressed ARHGAP29 was significantly reduced to 62.79±8.60% SEM ($p<0.01$ *vs*. siRNA control=100%, n=6; Figure 4C) compared to the siRNA control. In MCF-7-TR

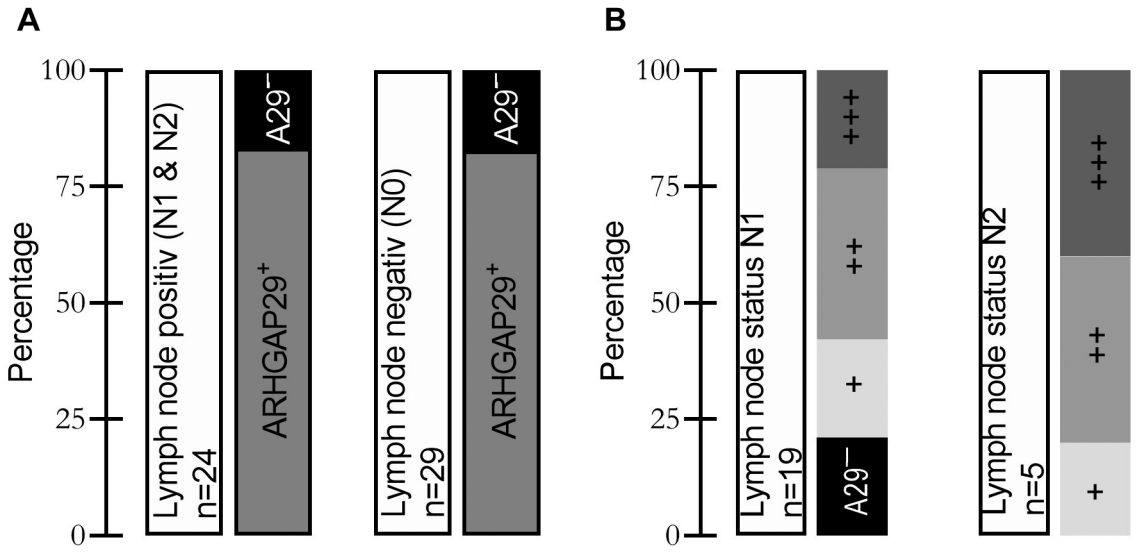

Figure 2. *Expression of Rho GTPase activating protein 29 (ARHGAP29) in breast cancer (BC) tissues taking into account the lymph node status. (A) 53 tissue samples with known TNM classification were analyzed for ARHGAP29 expression by immune fluorescence. The percentage of ARHGAP29-positive and ARHGAP29-negative samples is shown, divided into positive (N1 or N2) and negative lymph node status (N0). (B) Lymph node positive cancer samples (n=24) were analyzed for ARHGAP29 expression by immune fluorescence. (–) no, (+) mild, (++) medium, (+++) strong expression of ARHGAP29. The percentage of ARHGAP29 expression is shown according to lymph node status N1 (n=19) or N2 (n=5).*

spheroids, suppression of ARHGAP29 led to a reduction in invasive growth to 72.99±5.51% SEM ($p<0.001$ *vs*. siRNA control=100%, n=6; Figure 4D).

Subsequently, the influence of AKT1 activation on invasive growth of tamoxifen-resistant BC spheroids with suppressed ARHGAP29 was investigated. The activity of AKT1 was stimulated by the synthetic AKT activator SC79. In T47D-TR spheroids with suppressed ARHGAP29 expression (Figure 4C), as well as in MCF-7-TR spheroids with suppressed ARHGAP29 expression (Figure 4D), invasive growth was still significantly reduced after treatment with AKT activator SC79 to 86.20±4.89% SEM (T47D-TR: $p<0.05$ *vs*. siRNA control =100% and *vs*. siRNA control + SC75, n=6) or to 90.86±4.09% SEM (MCF-7-TR: $p<0.05$ *vs*. siRNA control =100% and *vs*. siRNA control + SC75, n=6). However, this reduction was now significantly less pronounced than without SC79 treatment (T47D-TR: ARHGAP27 knockdown with SC75 treatment *vs*. ARHGAP27 knockdown without SC75 treatment, $p<0.05$, n=6; MCF-7-TR: ARHGAP27 knockdown with SC75 treatment *vs*. ARHGAP27 knockdown without SC75 treatment, $p<0.05$, n=6). Thus, the inhibition of the invasive growth of the spheroids, triggered by suppression of ARHGAP29, could be partially reversed by activation of AKT1.

Discussion

ARHGAP29 and associated signaling pathways are the subject of current research in various areas of gynecology and oncology. Research topics related to ARHGAP29 include uterine adhesions in Asherman syndrome and tumor progression in renal cell carcinoma, gastric carcinoma and prostate cancer (2-4, 24). This study investigated the role of ARHGAP29 in breast cancer (BC) invasiveness.

ARHGAP29 as a prognostic marker in BC. Miyazaki *et al*. identified PARG1 (=ARHGAP29) as a poor prognostic marker in renal cell carcinoma. High expression of the protein correlated with higher TNM stages, higher grading, a high Ki67 score and a lower survival rate (2). A correlation between high ARHGAP29 expression and low patient survival was also found in gastric, prostate and BC (3, 4, 25). Using Kaplan-Meier survival analysis, Kolb *et al*. showed that high expression of ARHGAP29 in Luminal A-type BC is associated with a lower survival rate than in BC cells of the same type with low ARHGAP29 expression (25).

We analyzed 79 tissue samples of a tissue microarray consisting of tissue samples from normal mammary gland tissue and BC tissue for ARHGAP29 expression by immunofluorescence. The sections from BC tissue showed a higher expression of ARHGAP29 compared to tissue sections from normal mammary gland tissue. Not only was the proportion of ARHGAP29-positive sections greater, but also the expression of ARHGAP29 was more pronounced in the sections from carcinoma tissue than in the samples from benign mammary gland tissue. These results are consistent with data from Kolb *et al*. showing that ARHGAP29 expression is

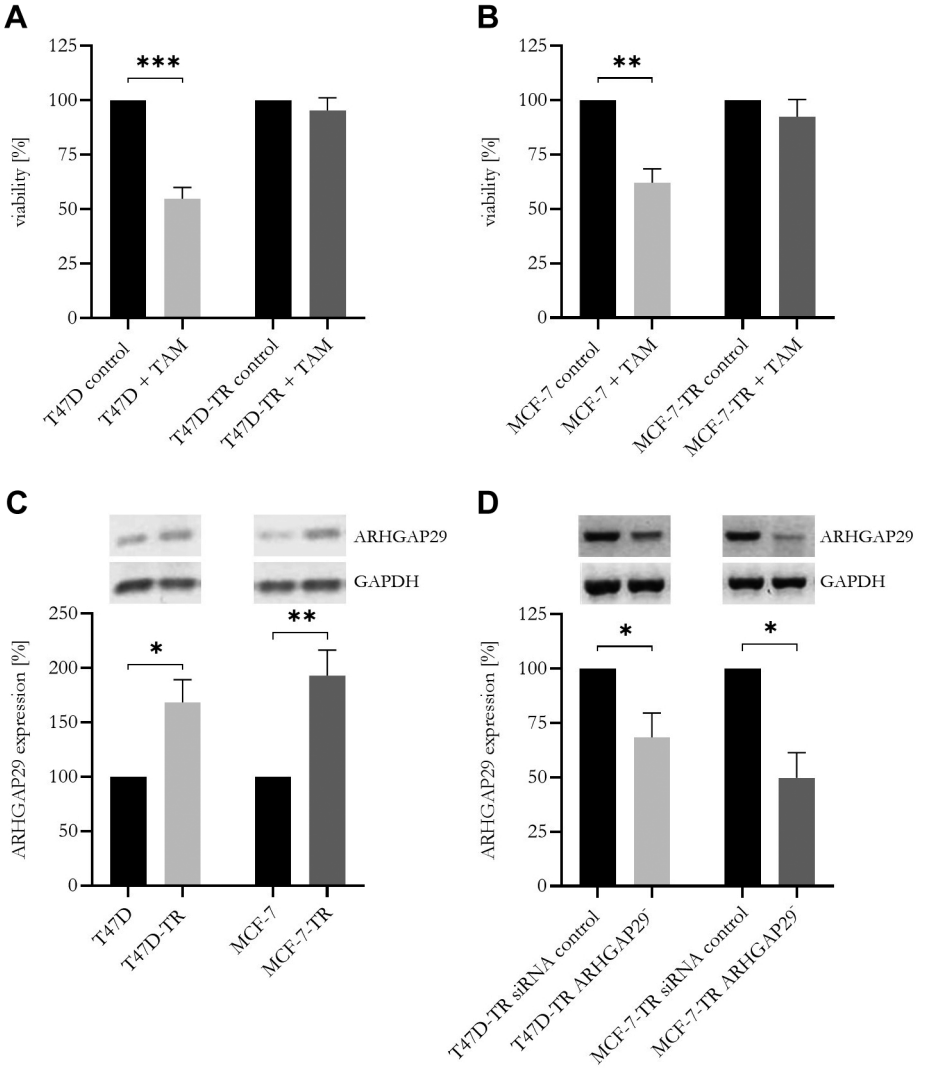

Figure 3. *Effect of tamoxifen treatment on viability of the breast cancer (BC) cell lines T47D (A) and MCF-7 (B) compared to the tamoxifen-resistant BC sublines T47D-TR (A) and MCF-7-TR (B). Expression of Rho GTPase activating protein 29 (ARHGAP29) in tamoxifen-resistant BC cells compared to their parental wild-type cells (C). Knockdown of ARHGAP29 in tamoxifen-resistant T47D-TR and MCF-7-TR BC cells (D). Unpaired t-test, two-tailed, mean±SEM; (A, B) n=3, (C, D) n=4; *p<0.05, **p<0.01, ***p<0.01.*

increased in invasive TNBC cells and mesenchymal-altered cells compared to wild type (25). Of 223 genes upregulated during mesenchymal transformation of the MCF-7 cell line, ARHGAP29 was the only GTPase-activating protein (25). Mesenchymal-transformed BC cells (29) and also tamoxifen-resistant BC cells (30) show a higher invasiveness compared to their wild type. Increased invasiveness of cells is associated with epithelial-mesenchymal transition (EMT) and local spread in the context of cancer. For this reason, this study further investigated whether there is also a correlation between ARHGAP29 expression and clinicopathological factors, such as the spread of carcinoma cells to the regional lymph nodes.

With the help of additional data on the TMA, ARHGAP29 expression could be visualized taking into account the lymph node status (N). There was no difference in ARHGAP29 expression between lymph node-positive and lymph node-negative tissue samples. In both groups, the proportion of ARHGAP29-positive sections was 83%. The breakdown of the lymph node-positive tissue samples into N1 and N2 stages provided an indication of a possible correlation between ARHGAP29 expression and cancer progression. It could be shown that there is a shift towards stronger expression of ARHGAP29 with increasing N-stage. This could also be an indication of ARHGAP29 as a possible

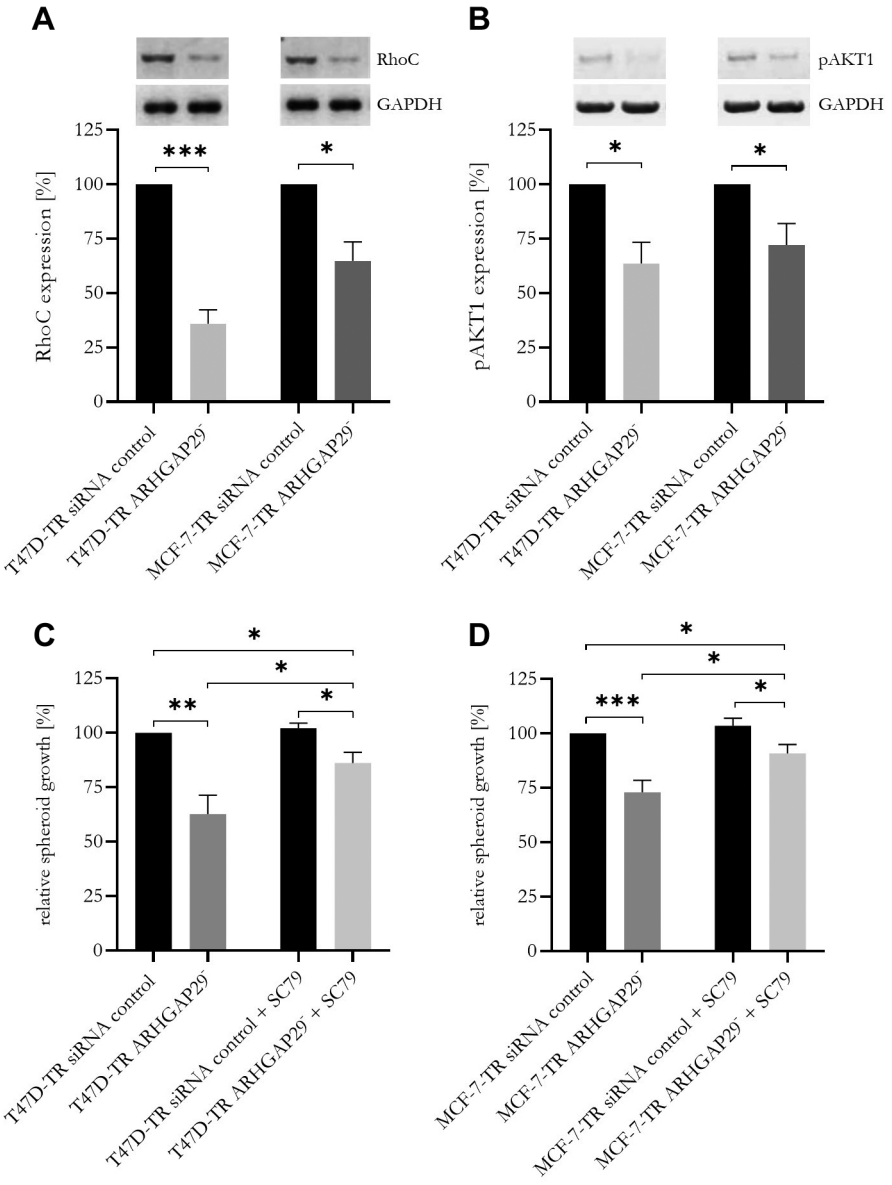

Figure 4. *Expression of RhoC (A) and pAKT1 (B) after suppression of Rho GTPase activating protein 29 (ARHGAP29) by siRNA in tamoxifen-resistant T47D-TR and MCF-7-TR breast cancer (BC) cells. Effect of ARHGAP29 suppression without or with AKT1 activator SC79 treatment on invasive growth of BC spheroids derived from tamoxifen-resistant T47D-TR (C) and MCF-7-TR (D) BC cells. Unpaired t-test, two-tailed, mean±SEM; (A, B) n=3, (C, D) n=6; *p<0.05, **p<0.01, ***p<0.01.*

prognostic marker in the progression of BC. It was not possible to make any statements about the T or M stage with the help of the available supplementary data on TMA. This needs to be investigated further in larger-scale studies. In addition to analyses of the TNM classification, it should also be investigated whether there are correlations in relation to grading, Ki67 score or hormone receptor status.

In summary, it can be said that increased expression of ARHGAP29 in the tissue microarrays examined could indicate progressive cancer. Comparable results were found in other cancer entities, such as prostate or renal cell carcinoma. However, ARHGAP29 is also expressed in benign mammary gland tissue, as data from the investigated TMA show. One challenge could be to determine a suitable cut-off value in order to be able to consider ARHGAP29 as a prognostic marker in BC. This would require the results to be confirmed in further, larger-scale studies and the function of ARHGAP29 to be investigated in more detail.

RhoC and pAKT1 act as possible downstream partners of ARHGAP29. Tamoxifen-resistant BC cells show an increased invasive behavior compared to their tamoxifen-sensitive parental cells (30). We could demonstrate that expression of ARHGAP29 is increased in tamoxifen-resistant BC cells. This has already been shown for mesenchymal-transformed BC cells and for triple-negative BC cells, which also show increased invasion (25). When investigating possible signaling pathways associated with ARHGAP29, RhoC was identified as a possible further indirect downstream partner. An interaction with RhoA, also from the RhoGTPase family, has already been demonstrated in the literature.

Mesenchymal-transformed BC cells show increased invasion (29). Against this background, Kolb *et al*. already showed that suppression of ARHGAP29 led to reduced invasiveness of mesenchymal-altered BC cells and triple-negative BC cells (25). A decrease in proliferation could be ruled out as the cause (25). This effect has been shown in different cancer entities in connection with RhoA and YAP as well as the ROCK-LIMK signaling pathway. It is assumed that ARHGAP29 inhibits RhoA and thus eliminates the stabilizing effect on the cytoskeleton through the ROCK-LIMK-cofilin signaling pathway. Cell migration is driven by increased remodeling of actin filaments and thus a more flexible cytoskeleton (3, 25, 31). We could demonstrate that suppression of ARHGAP29 led to a significant reduction in RhoC expression. At the same time, ARHGAP29 knockdown resulted in a significant reduction in invasive growth. RhoC plays an important role in the context of breast cancer and metastasis (32, 33). In inflammatory breast cancer (IBC), RhoC is considered a particularly relevant RhoGTPase (34). In addition, RhoC expression has been confirmed as a potential marker for the metastatic potential of small tumors (35). As for RhoA, RhoC also shows a correlation between high expression and advanced tumor stage and thus poor prognosis (35, 36). The phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling pathways have been identified as being associated with RhoC (37, 38). RAC-alpha serine/threonine-protein kinase (AKT1, PKB) has also been associated with RhoC. Lehman *et al*. showed that RhoC acts as a substrate of AKT1. The phosphorylation of RhoC by AKT1 and thus activation is essential for cell motility and invasion in IBC (26). These discoveries were complemented by a study of Joglekar *et al*., who identified caveolin-1 as an activator of AKT1 in this signaling pathway (39).

Further studies showed that inhibition of RhoA and RhoC using specific siRNA reduces the proliferation and invasiveness of breast cancer cells (36, 40). It appears that RhoC (and not RhoA) is essential for the invasive properties of invasive breast cancer cells (41, 42). In addition, it was shown that RhoC also has an influence on the formation of tumor stem cells. A knockout of RhoC led to reduced tumor formation (43). In contrast, increased expression promoted the formation of a stem cell-like state (43). In mesenchymal transformed BC cells, AKT1 has also been identified as a possible downstream partner of ARHGAP29 (25). We have now investigated whether ARHGAP29 affects the active form of AKT1 in tamoxifen-resistant BC. We could demonstrate that expression of pAKT1 was also significantly reduced after knockdown of ARHGAP29 in tamoxifen-resistant BC cells, although somewhat less pronounced than the reduction in RhoC. The influence of AKT1 activation using AKT activator SC79 on the invasion behavior of aggressive ARHGAP29-deficient breast carcinoma cells was then investigated. The reduced invasive growth due to ARHGAP29 suppression was partially reversed by the activation of AKT1. In melanoma, it has already been shown that inhibition of RhoC leads to reduced expression of pAKT and consequently to reduced invasion (44). In general, it is helpful to know which signaling pathways are involved in tumor progression and especially in the development of resistance in order to be able to predict such events accordingly. Using CHO cells, Ogata *et al*. were able to predict the genes involved in irinotecan resistance and their functional properties (45).

The results show that there is a causal relationship between ARHGAP29, RhoC and pAKT1 in increased invasiveness of tamoxifen-resistant BC cells. The reduced invasion of tamoxifen-resistant BC cells caused by the suppression of ARHGAP29 could be partially reversed by activation of the potential ARHGAP29 downstream partner AKT1.

Conclusion

The expression of ARHGAP29 correlates with clinical tumor parameters of BC patients. Furthermore, ARHGAP29 is involved in increased invasiveness of tamoxifen-resistant BC cells. In the signaling cascade of ARHGAP29, RhoC and pAKT1 are presumably downstream partners in the regulation of invasiveness. ARHGAP29 alone or in combination with RhoC and pAKT1 could therefore be suitable prognostic markers for the progression of BC.

Conflicts of Interest

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

Authors' Contributions

Conceptualization, Carsten Gründker; Investigation, Maike Kansy, Katharina Wert, and Katharina Kolb; Project administration, Carsten Gründker; Writing original draft, Carsten Gründker; Review & editing, Julia Gallwas.

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