J Breast Cancer. 2022 Dec;25(6):500-512
https://doi.org/10.4048/jbc.2022.25.e47
pISSN 1738-6756·eISSN 2092-9900

Journal of Breast Cancer JBC

Original Article

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Ceramide Synthase 6 Mediates Triple-Negative Breast Cancer Response to Chemotherapy Through RhoA- and EGFR-Mediated Signaling Pathways

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OPEN ACCESS

Received: Oct 25, 2021
Revised: Apr 8, 2022
Accepted: Oct 24, 2022
Published online: Nov 14, 2022

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Funding
This work was supported by the Health Commissioner of Hubei Province (WJ2017H166) and the Wuhan Scientific and Technology Bureau (2021021661010121).

ABSTRACT

Purpose: Limited treatment options and lack of treatment sensitivity biomarkers make the clinical management of triple-negative breast cancer (TNBC) challenging. Ceramide synthase 6 (CERS6) generates ceramides, which are key intermediates in sphingolipid biosynthesis and play important roles in cancer progression and resistance.
Methods: CERS6 was analyzed to determine its potential as a treatment sensitivity biomarker. CERS6 levels were determined in patients with breast cancer, and the roles and downstream signaling of CERS6 were analyzed using cellular and biochemical assays.
Results: Analysis of CERS6 expression in 195 patients with TNBC and their clinical response to chemotherapy revealed that individuals with CERS6 overexpression experienced significantly inferior responses to chemotherapy than those without CERS6 overexpression. Functional analysis demonstrated that although CERS6 overexpression did not affect TNBC cell growth and migration, it conferred chemoresistance. CERS6 inhibition significantly reduced growth, migration, and survival by suppressing the RhoA- and EGFR-mediated signaling pathways. Compared to control cells, CERS6-depleted cells were consistently less viable at different concentrations of chemotherapeutic agents.
Conclusion: Our study is the first to demonstrate that CERS6 may serve as a treatment sensitivity biomarker in patients with TNBC in response to chemotherapy. In addition, our findings suggested that CERS6 may be a therapeutic target for TNBC treatment.

Keywords: CERS6; Chemoresistance; EGFR; RhoA; Triple Negative Breast Neoplasms

INTRODUCTION

Triple-negative breast cancer (TNBC) lacks the expression of estrogen receptor, human epidermal growth factor receptor 2, and progesterone receptor and accounts for approximately 10%–15% of all breast cancers [1]. Compared with other subtypes of breast cancer, TNBC is highly aggressive, has early recurrence and poor outcomes, and lacks biomarkers and effective therapeutic strategies. Anthracycline/taxane-based chemotherapy

Conflict of Interest
The authors declare that they have no competing interests.

Author Contributions
Conceptualization: Ke F; Data curation: Chen H, He B; Formal analysis: Chen H, He B, Ke F; Funding acquisition: He B, Ke F; Investigation: Chen H, Ke F; Project administration: Chen H; Validation: Chen H; Writing - original draft: Chen H, Ke F; Writing - review & editing: Chen H, He B, Ke F.

regimens remain the only systemic therapeutic options in the adjuvant and metastatic settings of this disease [2]. However, most patients develop resistance rapidly and relapse [3]. Emerging therapies, including antibody (Ab)-drug conjugates, PARP and PI3K/Akt/mTOR inhibitors, and immunotherapy, are currently being investigated in clinical trials [4]. Targeted therapies are required to improve the clinical outcomes of TNBC.

Ceramides are important intermediates in the biosynthesis of sphingolipids, which are bioactive lipids that regulate multiple cellular functions and are synthesized by ceramide synthases (CERS) [5]. Ceramide synthase 6 (CERS6) is a ceramide synthase that predominantly generates C16-ceramide. Recent studies have highlighted that CERS6 predicts poor prognosis of ovarian and gastric cancers and functions as an oncoprotein [6,7]. Other studies have consistently demonstrated that CERS6 confers resistance to chemotherapy in T-cell acute lymphoblastic leukemia [8]. CERS6 is required for cell migration and metastasis in lung cancer [9]. CERS and ceramide levels were higher in breast cancer tissues than in benign and normal tissues. In particular, C16-ceramide displays metastatic potential, as its upregulation is associated with positive lymph node status in breast cancer [10]. In line with previous efforts, this study explored the expression and function of CERS6 in breast cancer, focusing on TNBC and its association with clinical response to chemotherapy.

METHODS

Cell culture, reagents, and western blot

Normal human breast cell lines (MCF-10A and MCF-12A) and human breast cancer cell lines (SK-BR-3, MDA-MB-468, Hs 578T, BT-549, MDA-MB-231, MCF-7, HMT-3522, and BT-483) were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco, Grand Island, USA), doxorubicin, 5-fluorouracil (FU), paclitaxel, and cisplatin (Selleck, Houston, USA). Calpeptin (Tebu-bio, Le Perray-en-Yvelines Cedex, France) was reconstituted according to the manufacturer's recommendations. Western blotting was performed according to the standard protocol. The antibodies used for western blot analysis included p-Akt (#9271; Cell Signaling Technology, Danvers, USA), Akt (#9272; Cell Signaling Technology), p-mTOR (#5536; Cell Signaling Technology), mTOR (#2972; Cell Signaling Technology), p-EGFR (#2234; Cell Signaling Technology), EGFR (#2232; Cell Signaling Technology), p-myosin phosphatase-targeting subunit 1 (MYPT1, #4563; Cell Signaling Technology), MYPT1 (#2634; Cell Signaling Technology), p-myosin light chain (MLC, #3671; Cell Signaling Technology), MLC (#3672; Cell Signaling Technology), β-actin (#4967; Cell Signaling Technology), and CERS6 (#PA5-113036; Thermo Fisher Scientific, Waltham, USA). Active Rho and Rac1 pull-down and detection kits (Thermo Fisher Scientific) were used for RhoA-GTP and Rac1-GTP detection, respectively. The raw experimental images of the western blot membranes are shown in the **Supplementary Figure 1**.

ELISA assays

Snapped frozen tissues were homogenized using a polytron homogenizer in 5 mL ice-cold RIRA buffer (Invitrogen, Waltham, USA) on ice. The mixture was incubated at 4°C for 1 hour in a culture tube rotator. The lysate was then cleared by centrifugation at 4°C, and the supernatant was collected for BCA protein assay (Pierce; Thermo Fisher Scientific) to determine protein concentration. The tissue samples were adjusted to a concentration of 5

https://doi.org/10.4048/jbc.2022.25.e47

mg/mL using phosphate buffered saline (PBS). CERS6 protein levels were measured using Human Ceramide Synthase 6 and CERS6 enzyme-linked immunosorbent assay (ELISA) Kit (BT LAB, Birmingham, USA). Briefly, 40 μL of protein lysate, 10 μL of human CERS6 Ab, and 50 μL of streptavidin-horseradish peroxidase were added to each well. A blank was used as the background control, and standards were used to generate a standard concentration curve. After 1 hour of incubation, the substrates were added, and the optical density of each well was determined on a microplate reader at 450 nm. Cellular RhoA and Rac1 activities were assessed using cell lysates and were measured using RhoA and Rac1 G-LISA Activation Assay Kit (Cytoskeleton, Denver, UA), respectively, according to the manufacturer's protocol.

Cellular assays

Cell proliferation was determined using the BrdU Cell Proliferation Assay Kit (Abcam, Cambridge, UK). Cell apoptosis was determined using cellular DNA fragmentation ELISA (Roche, Basel, Switzerland). Cell viability was measured using an MTT assay kit (Abcam). The migration assay was performed using a Boyden chamber, which consists of a Falcon cell culture insert with 8-μm pore size polycarbonate membrane filters. The medium in the lower chamber contained 10% FBS. Cells (5×10^4) in serum-free medium were plated in the upper chamber and incubated for 24 hours at 37°C, 5% CO_2. After incubation, the non-migrating cells were removed from the upper surface of the membrane using a cotton swab, and the migrated cells on the lower surface of the culture inserts were stained with 0.4% crystal violet. The number of cells in 5 random fields of each membrane was counted under a microscope.

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded sections were proceeded for antigen retrieval with autoclave heating and Ab staining with rabbit polyclonal anti-CERS6 Ab (1:200 dilution, PA5-113036; Thermo Fisher Scientific) at 4°C overnight. Tissue sections were incubated with secondary Ab and stained using the avidin–biotin immunoperoxidase method.

Phalloidin staining

Transfected cells were fixed with 4% paraformaldehyde. After washing with PBS, the fixed cells were incubated with 100 nM TRITC-conjugated phalloidin (Molecular Probes, Eugene, USA) and 100 nM Dapi solution in PBS for 30 minutes in the dark. Staining was performed using fluorescence microscopy.

Ethics committee approval

Clinical samples were obtained from patients at Xiangyang Central Hospital. Written informed consent and Institutional Review Board approval (document No. 87582) were obtained from all the patients who contributed to this study. All the included patients were treatment-naïve at the time of diagnosis. All TNBC tissues and their adjacent normal breast tissues were stored at –80°C and were used for protein extraction. Some were fixed, embedded, and used for IHC.

Transfection of small interfering RNA (siRNA) or overexpression plasmid

The cells (2×10^5 cells/well) were plated in 12-well plates in DMEM. Upon reaching 80% confluence, cells were transfected with 100 nM non-targeting siRNA, specific CERS6-targeted siRNA (Sigma, Livonia, USA), or 2 μg human CERS6 cDNA ORF and empty vectors (OriGene, Rockville, USA) using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocol. The sequences of siRNAs CERS6a and CERS6b were (5'-->3'): CAA CUG ACC UUC ACU ACU AUA GUA GUG AAG GUC AGU UG and UAC GGU ACU AUU UCA CAC UUA CGC UAC ACU CAA, respectively. Stable clones that overexpressed CERS6 were selected in the presence of G418.

https://doi.org/10.4048/jbc.2022.25.e47

Statistical analyses

We compared the controls and test variables using Student's *t*-test in all cell assays. Data are presented as mean values with corresponding standard deviations (SDs). To establish the significance of chemosensitive and chemoresistant groups, we tabulated the ratios of CERS6 tumor/normal values. Subsequently, Kaplan-Meier analysis was performed to determine the odds ratio (OR) and 95% confidence intervals (CIs). In all analyses, we confirmed statistical significance with *p*-values < 0.05. All statistical analyses were conducted using the PRISM v9.0 software (GraphPad Inc., San Diego, USA).

RESULTS

CERS6 expression in patients with TNBC predicts their response to chemotherapy

To evaluate the expression pattern of CERS6 in breast cancer, we first performed IHC analysis of CERS6 in paired tumor and adjacent normal (at least 5 cm away from the tumor) breast tissues, focusing on patients with TNBC. The overall cellularity was moderate to high in all examined cases. Representative IHC samples are shown in **Figure 1A**. We observed weak CERS6 staining intensity in normal breast tissues. The ductal epithelial and stromal cells were CERS6-positive.

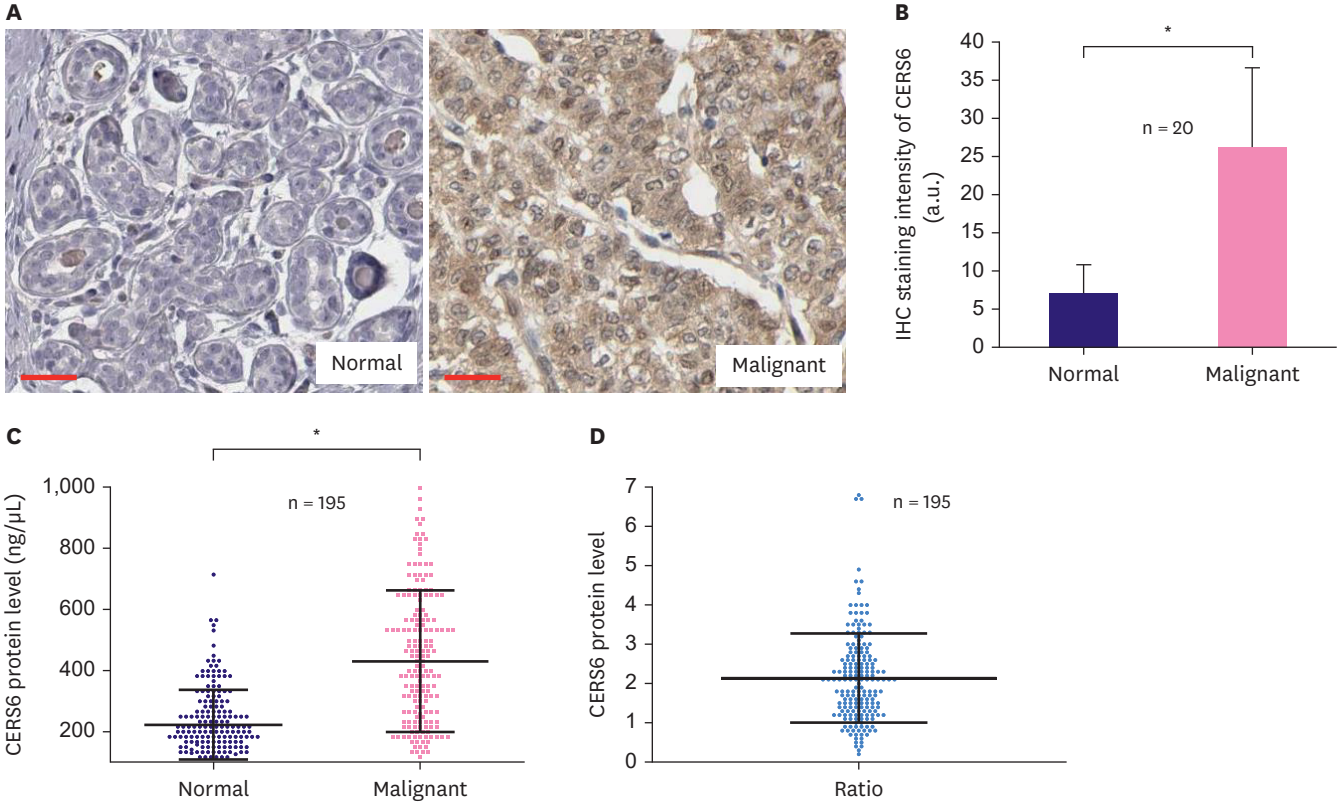

Figure 1. CERS6 is significantly increased in tumor compared to normal breast tissue in patients with TNBC. (A) Representative IHC imaging showing CERS6 staining on malignant and adjacent normal breast tissues from a patient with TNBC. Scale bar represents 50 μm. (B) Average of CERS6 IHC staining density on malignant and adjacent normal breast tissues from 20 patients with TNBC. IHC staining intensity was calculated using IHC profiler plugin in ImageJ software and displayed as absolute intensity in a.u. (C) Scatter plot of CERS6 protein in paired TNBC malignant tissues and adjacent normal tissues (n = 195). CERS6 from TNBC tissues was quantified using enzyme-linked immunosorbent assay. The line shown in the scatter plot indicates the average ± standard deviation of CERS6 protein level in TNBC malignant and normal tissue. (D) Average of CERS6 protein ratios in the tumor and normal tissues of patients with TNBC (n = 195).
CERS6 = ceramide synthase 6; TNBC = triple-negative breast cancer; IHC = immunohistochemistry; a.u. = arbitrary unit.
*$p < 0.05$, compared to normal samples.

https://doi.org/10.4048/jbc.2022.25.e47

Table 1. Establishing CERS6 upregulation and chemoresistance in TNBC

Variables	Ratio < 2.1 (n = 96)	Ratio > 2.1 (n = 99)	OR (95% CI)	*p*-value
Resistance	37 (38.5)	78 (78.8)	3.16 (1.02–7.69)	0.03
Sensitive	59 (61.5)	21 (21.2)		

Values are presented as number (%). Study subjects were newly diagnosed TNBC cases and divided into 2 groups based on the ratio of CERS6(tumor)/(normal) > 2.1 and those with CERS6(tumor)/(normal) < 2.1. Disease outcomes of chemo-sensitive or chemoresistance (based on the Response Evaluation Criteria in Solid Tumors version 1.1 criteria) were analyzed in these 2 groups. Using the Kaplan-Meier estimator, the OR was determined to assess clinical significance.
CERS6 = ceramide synthase 6; TNBC = triple-negative breast cancer; OR = odds ratio; CI = confidence interval.

In contrast, moderate to strong CERS6 staining intensity was observed in the breast cancer tissues. Quantification of staining density showed that CERS6 was significantly increased in the tumor by 3-fold compared to that in the adjacent normal tissue (**Figure 1B**).

To further confirm this finding, we measured CERS6 protein levels in a larger cohort of patients with TNBC (n = 195) using ELISA. Meanwhile, we attempted to correlate CERS6 levels with chemotherapy response in patients with TNBC. Patients included in this perspective analysis were treatment-naïve when examining CERS6 levels. Consistent with the IHC results, the average CERS6 level was significantly higher in TNBC tumors than in adjacent normal tissues (**Figure 1C**). In addition, 168 of 195 patients demonstrated that the tumor/normal ratio of CERS6 was > 1 (**Figure 1D**), suggesting that CERS6 upregulation is a persistent phenomenon in patients with TNBC. The average ± SD of CERS6 tumor/normal ratio value was 2.1 ± 1.3 in all tested TNBC samples. In total, 99 and 96 patients displayed CERS6 tumor/normal ratio value ≥ 2.1 and < 2.1, respectively (**Table 1**).

Patients were then administered chemotherapy as the first-line treatment after diagnosis (**Supplementary Table 1**). After 6 months, clinical response was documented as "complete response," "partial response," "stable disease," and "progressive disease" based on the Response Evaluation Criteria in Solid Tumors (RECIST) [11]. We defined resistant individuals as "suboptimal responders" or "failures," which included subjects who had never achieved either complete response or partial response. In contrast, sensitive individuals were defined as individuals who corresponded to RECIST-defined "optimal responders." As shown in **Table 1**, 38% of patients with a CERS6 ratio < 2.1 and 79% of patients with a CERS6 ratio > 2.1 were resistant to chemotherapy. When analyzed together, the overall OR for resistant disease among subjects with CERS6 ratio > 2.1 compared with those with < 2.1 was 3.16 (95% CI, 1.02–7.69; p= 0.03). In comparison, we did not find any significant differences between the 2 groups with respect to other potential prognostic factors, including age, grade, and level of plasminogen activator inhibitor (**Table 2**). In addition, CERS6 expression is likely to be an independent biomarker for predicting TNBC responses to chemotherapy.

TNBC cells with CERS6 overexpression are more resistant to chemotherapy

As shown in **Figure 2A**, immunoblotting analysis of CERS6 demonstrated that CERS6 was upregulated in TNBC cell lines (MDA-MB-468, Hs578T, and BT-549) and other breast cancer cell lines (MCF-7, HMT-3522, SK-BR-3, and BT-483) compared to that in normal breast cell lines (MCF-10A and MCF-12A). Next, we performed ELISA, a quantitative assay to determine CERS6 levels in TNBC and normal breast cell lines. CERS6 level was significantly increased by 2–4-fold in TNBC cell lines compared to that in normal breast cell lines (**Figure 2B**). Although overexpression of CERS6 resulted in a 3-fold increase in CERS6 protein levels (**Figure 2C and D**), the time-course analysis showed that CERS6 did not affect TNBC growth (**Figure 2E**). We also did not observe any significant difference in migration between CERS6-

https://doi.org/10.4048/jbc.2022.25.e47

Table 2. Subject demographics and correlations with CERS6 ratio

Variables	Resistant (n = 108)	Sensitive (n = 87)	*p*-value	
			Univariate	Multivariate
Age				
> 50	56 (28.7)	44 (22.6)	-	-
≤ 50	52 (26.7)	43 (22.1)	0.46	-
Stage				
I	11 (5.6)	14 (7.2)	-	-
II	54 (27.7)	47 (24.1)	0.28	-
III	43 (22.1)	26 (13.3)	0.06	-
PAI-1				
< 14	47 (24.1)	39 (20.0)	-	-
≥ 14	61 (31.3)	48 (24.6)	0.18	-
CERS6 ratio				
< 2.1	37 (19.0)	59 (30.3)	-	-
≥ 2.1	71 (36.4)	28 (14.4)	< 0.01	0.03

The proportion of subjects in each category is listed as percentage in parentheses.
PAI-1 = plasminogen activator inhibitor-1; CERS6 = ceramide synthase 6.

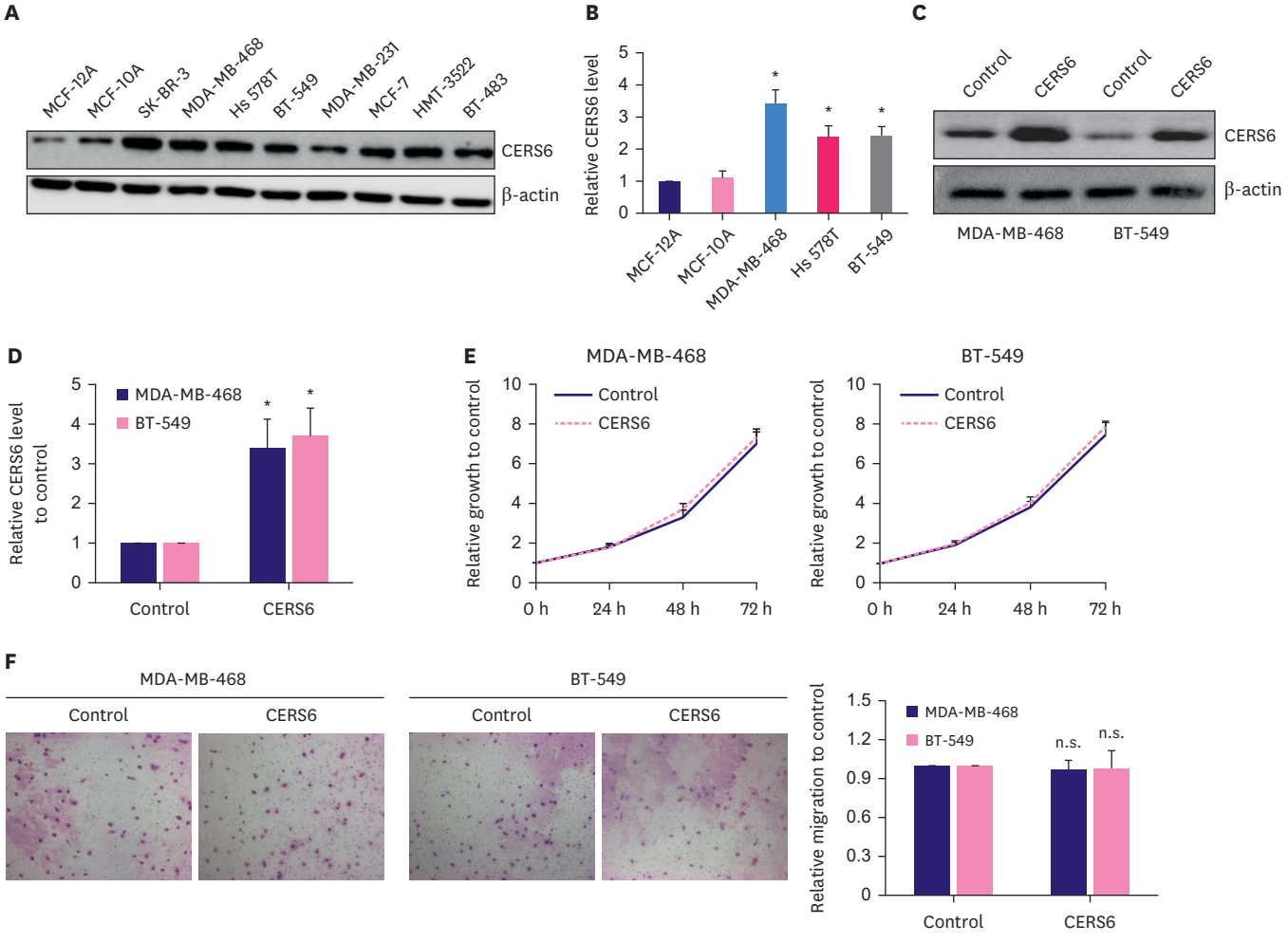

Figure 2. CERS6 overexpression does not affect proliferation and migration in TNBC cells. Representative western blot image (A) and ELISA analysis (B) showing CERS6 staining in normal (MCF-12A and MCF-10A) and malignant breast cancer cell lines. Western blot image (C) and ELISA analysis (D) of CERS6 in TNBC cells. (E) CERS6 overexpression does not affect proliferation and migration in MDA-MB-468 and BT-549 cells. (F) Migration in control and CERS6-overexpressing TNBC cells. Representative migration images were taken under microscope.
CERS6 = ceramide synthase 6; TNBC = triple-negative breast cancer; n.s. = not significant (compared to control); ELISA = enzyme-linked immunosorbent assay.
*$p < 0.05$, compared to control or normal samples.

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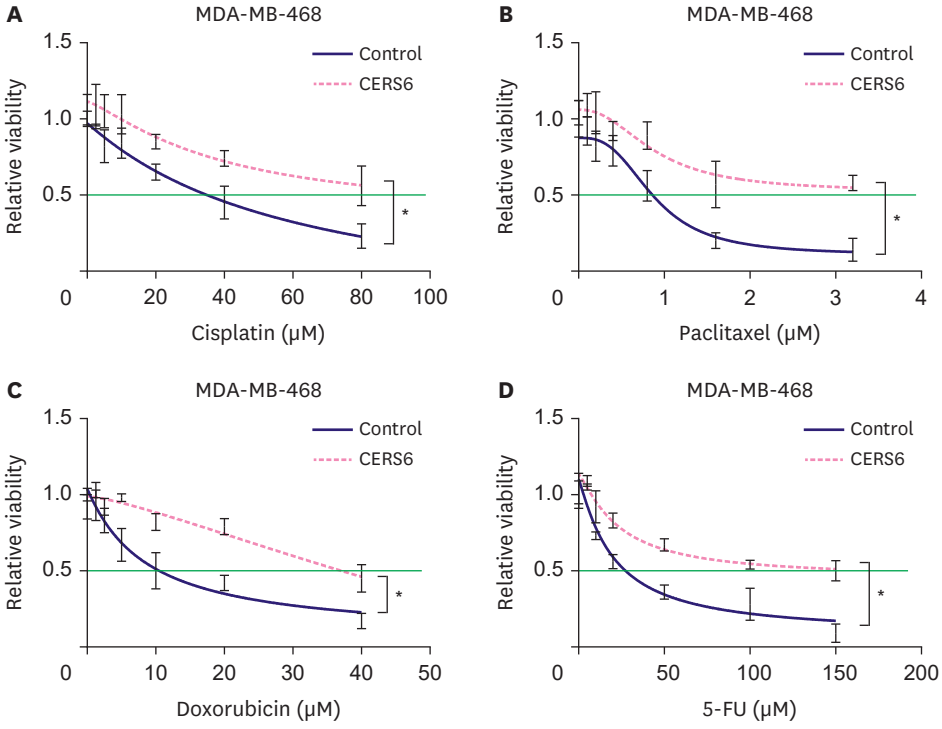

Figure 3. CERS6 overexpression alleviates chemotherapy-induced toxicity in MDA-MB-468 cells. Viability increased in CERS6-overexpressing than control TNBC cells exposed to paclitaxel (A), cisplatin (B), doxorubicin (C), and 5-FU (D). The IC50 of cisplatin was 60.57 μM and 38.84 μM on the control and CERS6 groups; the IC50 of paclitaxel was 0.9 μM and 3.3 μM on the control and CERS6 groups; the IC50 of doxorubicin was 8.6 μM and 39 μM on the control and CERS6 groups; the IC50 of 5-FU was 36 μM and 153 μM on the control and CERS6 groups. After 3 days of treatment, cell viability was assessed. IC50 is indicated by the green line.
CERS6 = ceramide synthase 6; TNBC = triple-negative breast cancer; FU = fluorouracil; IC = inhibitory concentration.
*$p < 0.05$.

overexpressing and control MDA-MD-468 and BT-549 cells (**Figure 2F**). We observed that 4 chemotherapeutic agents, paclitaxel, cisplatin, doxorubicin, and 5-FU, were less effective in decreasing the viability of CERS6-overexpressing MDA-MB-468 cells compared to the control (**Figure 3**, **Supplementary Figure 2A and B**). Notably, BT-549 cells responded similarly (**Supplementary Figure 2C and D**, **Supplementary Figure 3**). These results demonstrate that CERS6 overexpression did not affect TNBC cell growth and migration. However, chemotherapy-induced toxicity was alleviated by CERS6 overexpression.

CERS6 inhibition is active against TNBC

To investigate the effects of CERS6 inhibition in TNBC, we used 2 independent siRNAs targeting CERS6 mRNA and performed proliferation and apoptosis assays in the absence and presence of chemotherapeutic agents. We observed minimal protein levels of CERS6 in MDA-MD-468 and BT-549 cells after siRNA transfection (**Figure 4A and B**). In addition, we found that CERS6 depletion significantly decreased proliferation and migration and induced apoptosis in TNBC cells (**Figure 4C-E**). There was up to 75% inhibition of migration and up to 50% inhibition of the growth of TNBC cells. Notably, siRNA-CERS6 cells were consistently less viable under different concentrations of chemotherapeutic agents than control cells (**Figure 5**, **Supplementary Figure 4**).

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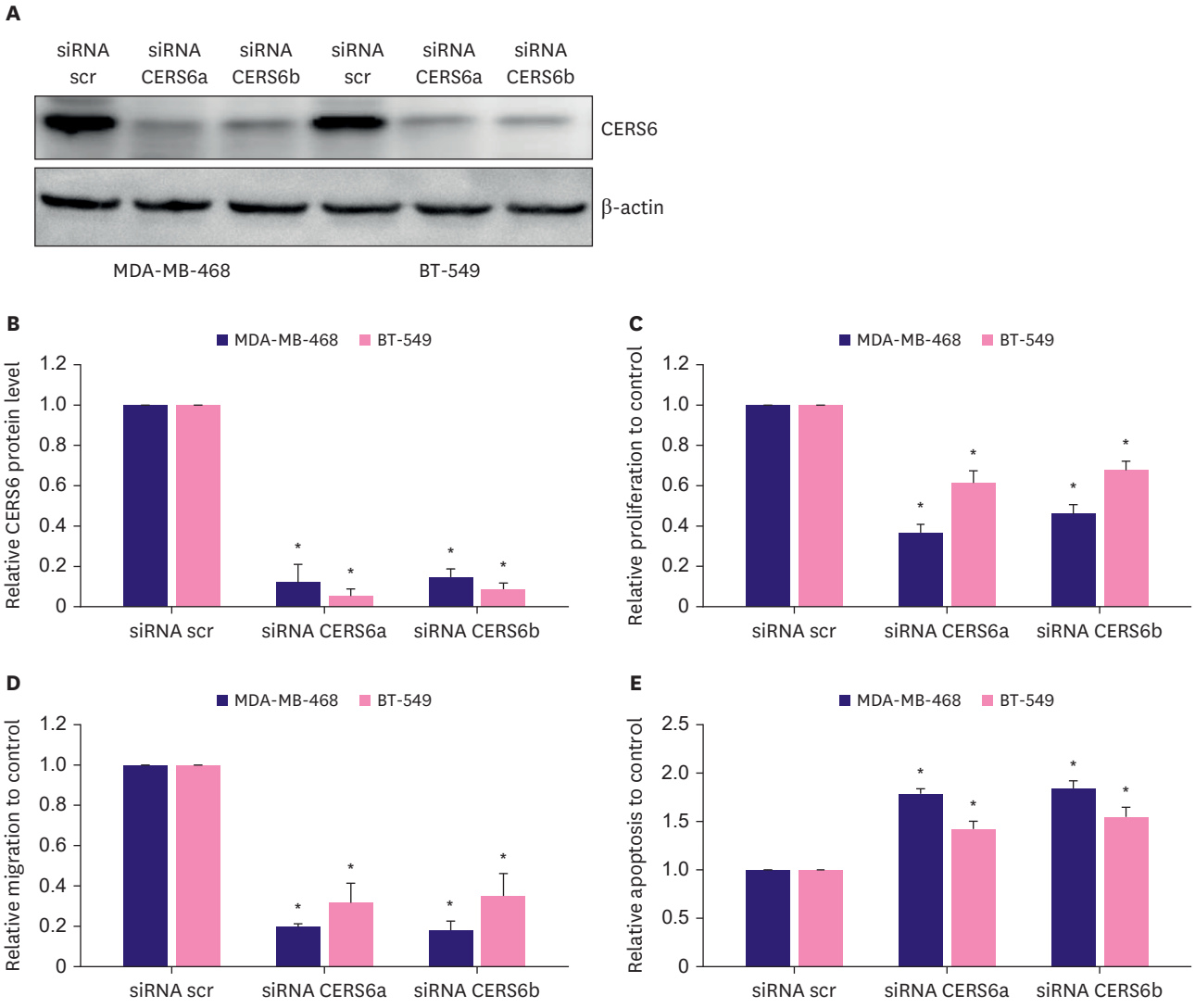

Figure 4. CERS6 depletion suppresses growth and migration and induces apoptosis in TNBC cells. Western blot analysis (A) and enzyme-linked immunosorbent assay (B) show CERS6 level in TNBC cells after siRNA knockdown. CERS6 siRNA knockdown significantly inhibits growth (C), decreases migration (D), and induces apoptosis (E) in TNBC cells.
CERS6 = ceramide synthase 6; TNBC = triple-negative breast cancer; siRNA = small interfering RNA.
*$p < 0.05$, compared to control.

CERS6 depletion suppresses TNBC by inhibiting RhoA- and EGFR-mediated pathways

Rho and Rac GTPases play critical roles in regulating the migration of almost all cell types [12]. We found that CERS6 depletion significantly decreased RhoA activity in MDA-MB-468 and BT-549 cells (**Figure 6A**). In contrast, CERS6 depletion did not affect Rac1 activity (**Figure 6B**). Pull-down followed by western blotting analysis demonstrated that RhoA-GTP, but not Rac1-GTP, decreased in CERS6-depleted cells (**Figure 6C**). In addition, total RhoA and Rac1 levels did not change. Consistently, CERS6 depletion led to decreased stress fiber formation, as shown by phalloidin staining, which was labelled F-actin (**Figure 6D**). As expected, we observed decreased phosphorylation of MYPT1 and MLC, which are 2 downstream effectors of the RhoA pathway [13], in CERS6-depleted TNBC cells (**Figure 6E**). The levels of phosphorylated molecules involved in the EGFR/Akt/mTOR pathway decreased in cells after CERS6

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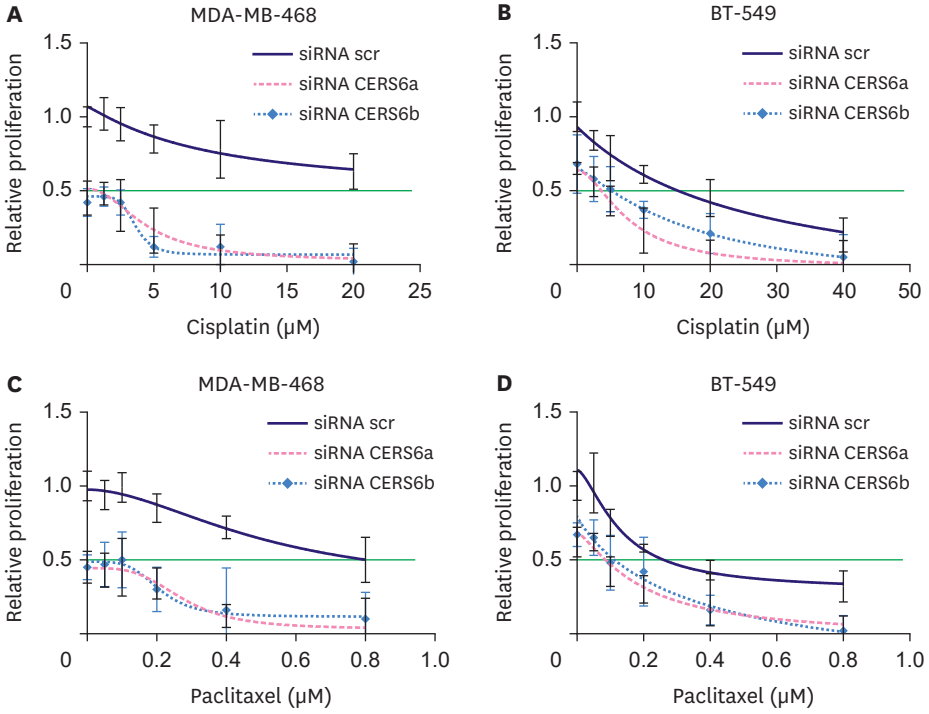

Figure 5. CERS6 depletion significantly augments anti-proliferative effect of chemo drugs in TNBC cells. Proliferation is significantly decreased in CERS6-depleted MDA-MB-468 and BT-549 cells exposed to cisplatin (A, B) and paclitaxel (C, D). After 24 hours of transfection with siRNA, chemo drugs were added to the cell medium and incubated for 3 days followed by proliferation measurement. Inhibitory concentration 50% is indicated by the green line.
CERS6 = ceramide synthase 6; TNBC = triple-negative breast cancer; siRNA = small interfering RNA.

depletion (**Figure 6E**). Rescue studies using the Rho activator I calpeptin [14] significantly reversed the anti-migratory but not anti-proliferative effects of CERS6 depletion in TNBC cells (**Figure 6F and G**), demonstrating that CERS6 depletion inhibits TNBC cell migration through the RhoA-mediated signaling pathway.

DISCUSSION

Patients with TNBC exhibit significant heterogeneity with regard to the speed and depth of their responses to chemotherapy, which likely reflects an underlying biological heterogeneity at the molecular level in chemoresistance factors. Here, we used an unbiased approach to identify CERS6, which predicts or correlates with clinical responses to chemotherapy in patients newly diagnosed with TNBC. We found that upregulation of the CERS6 protein level was a persistent feature in 168 out of 195 patients with TNBC. This is supported by the RNA sequencing expression data of 1,084 breast tumor and 291 normal breast samples from The Cancer Genome Atlas and GTEx projects that CERS6 mRNA levels are significantly increased by approximately 4-fold in breast tumors compared to normal tissues [15,16]. Our findings revealed that CERS6 upregulation affects clinical outcomes among patients with molecularly defined TNBC who are treated with chemotherapy and that individuals with CERS6 upregulation are significantly more resistant to chemotherapy. Increased CERS6 mRNA levels in breast cancer tissues are correlated with sphingosine kinase expression [17]. An earlier study reported that elevated levels of C16-ceramide, a ceramide mainly generated by CERS6,

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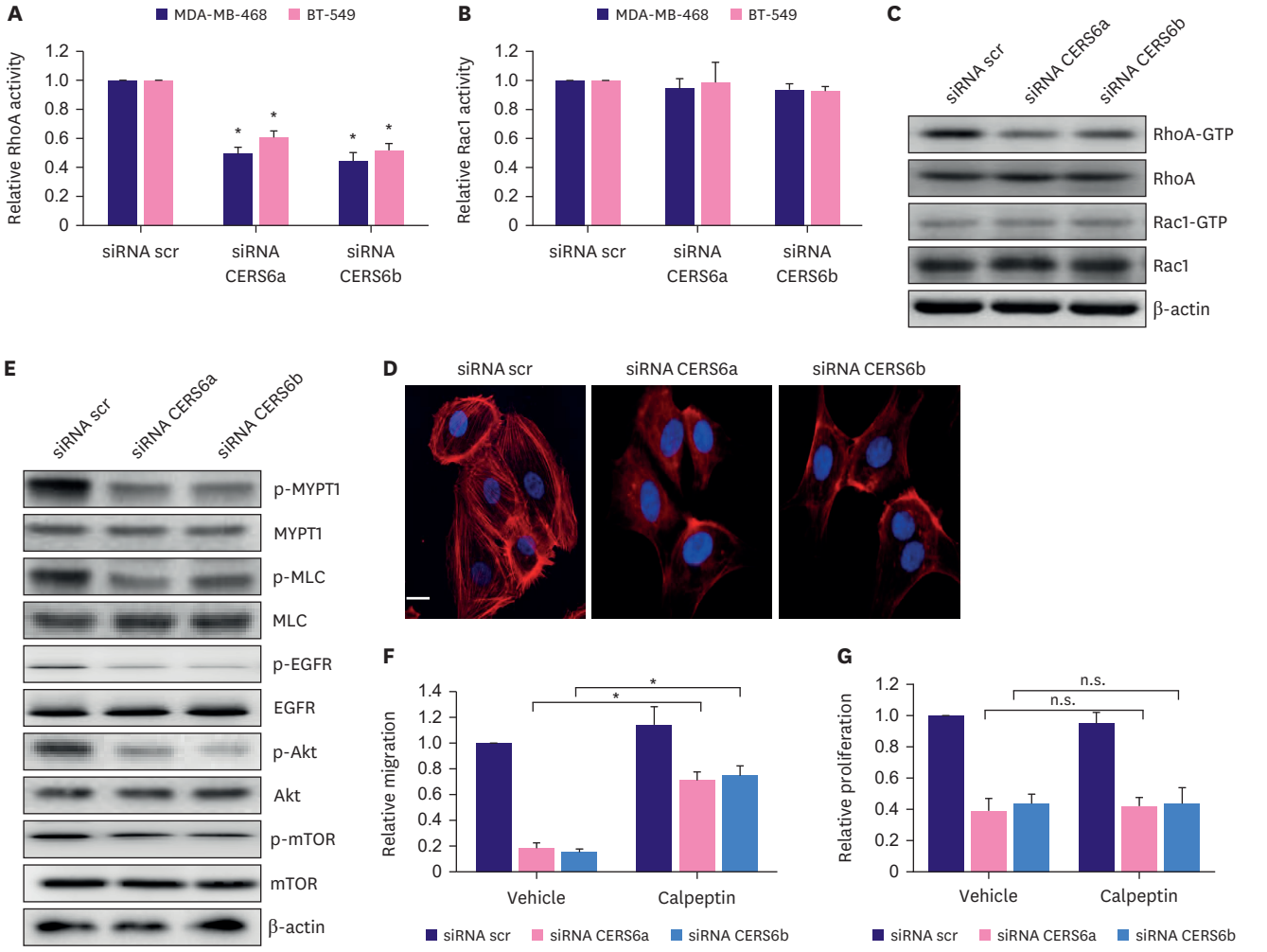

Figure 6. CERS6 depletion suppresses TNBC cells through inhibiting RhoA- and EGFR-mediated signaling pathways. (A) CERS6 significantly decreases the activity of RhoA in MDA-MB-468 cells. (B) CERS6 knockdown does not affect Rac1 activity in MDA-MB-468 cells. (C) Representative western blot image of active RhoA and Rac1 in CERS6-depleted MDA-MB-468 cells. (D) Phalloidin staining of control and CERS6-depleted MDA-MB-468 cells. F-actin is indicated by red and nuclear is indicated by blue. Scale bar represents 10 μm. (E) Representative western blot image showing the levels of phosphorylated molecules involved in RhoA and EGFR signaling pathways in CERS6-depleted MDA-MB-468 cells. (F) The inhibitory effects of CERS6 depletion on TNBC cell migration are abolished by RhoA activator calpeptin (1 mg/mL). (E) Calpeptin does not affect proliferation in CERS6-depleted cells.
CERS6 = ceramide synthase 6; TNBC = triple-negative breast cancer; n.s. = not significant; siRNA = small interfering RNA; MYPT = myosin phosphatase-targeting subunit; MLC = myosin light chain.
*$p < 0.05$.

are associated with a positive lymph node status in breast cancer, suggesting that patients with breast cancer with C16-ceramide are likely to display metastatic potential [10]. However, based on gene expression profiling and interactive analyses provided by GEPIA, CERS6 is not involved in overall survival in breast cancer. Our findings suggest that it might be worthwhile to examine the association between CERS6 and overall patients with TNBC. Ceramide kinase, another lipid kinase that regulates ceramide levels, has recently been shown to mediate intrinsic resistance and inferior response to chemotherapy in TNBC [18]. We and others have highlighted the central role of molecules involved in ceramide/sphingolipid metabolism in mediating chemotherapy sensitivity in TNBC. The prognostic value of CERS6 has also been demonstrated in gastric and ovarian cancer [6,7]. We anticipate that the list of cancers in which CERS6 influences treatment response will expand to include others.

https://doi.org/10.4048/jbc.2022.25.e47

We further demonstrated that CERS6 is a potential therapeutic target for TNBC. In doing so, we determined that CERS6 overexpression leads to TNBC cells becoming more resistant to commonly used chemotherapeutic agents such as cisplatin and paclitaxel. CERS6 upregulation increases TRAIL sensitivity in colon cancer cells by increasing the C16-ceramide level [19]. Interestingly, CERS6 upregulation confers resistance to chemotherapy by binding to CD95/Fas in T-cell acute lymphoblastic leukemia [8]. Although ceramide and ceramide synthases have been initially linked to the promotion of apoptosis and drug sensitivity [20], our findings, together with other studies, support the increasingly recognized cytoprotective role of CERS6 in chemotherapeutic drugs in cancer and that CERS6 functions as an oncoprotein [6,8,9].

In agreement with previous findings that CERS6 is required for cancer cell migration and metastasis [9,21], we found that CERS6 depletion remarkably decreased TNBC cell migration, which was achieved through inhibition of the RhoA-mediated signaling pathway. Both RhoA and Rac1 are small GTPases and their signal transmission is modulated by plasma membrane lipids [22]. CERS6 knockdown suppresses Rac1-positive ruffling formation and attenuates lung metastasis in mice [9]. CERS6 depletion does not affect Rac1 activity in TNBC cells. These results suggest that CERS6 inhibition affects the activity of small GTPases and influences cell migration, which is cancer cell type-specific. In addition, the rescue of anti-migratory but not anti-proliferative effects of CERS6 depletion by RhoA activators suggests that other mechanisms are involved. Indeed, CERS6 knockdown suppressed EGFR/Akt/mTOR signaling in TNBC cells. EGFR inhibitors are among the agents being developed for the treatment of TNBC because they are particularly overexpressed in TNBC, and targeting EGFR enhances the chemosensitivity of TNBC cells [23,24]. Our study is the first to demonstrate the biological implications of CERS6 in EGFR-mediated pathways in cancer.

In conclusion, we have identified CERS6 as an important factor in mediating chemotherapy responses in TNBC and described a pathway to RhoA- and EGFR-mediated signaling pathways that proceed via CERS6. Given that CERS6 expression levels are upregulated in breast cancer, CERS6 may account for treatment resistance in other subtypes of breast cancer. Our findings also highlight the therapeutic value of CERS6 inhibition in overcoming chemoresistance in TNBC.

SUPPLEMENTARY MATERIALS

Supplementary Table 1

Clinical and pathologic characteristics of patients with TNBC

Click here to view

Supplementary Figure 1

Uncropped gel for **Figure 2A and C**, **4A**, **6C and E**.

Click here to view

Supplementary Figure 2

CERS6 overexpression alleviates chemotherapy-induced toxicity in TNBC cells. (A, B) Viability is significantly increased in CERS6-overexpressing than control TNBC cells exposed

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to paclitaxel (1 μM) and cisplatin (30 μM) for 3 days. (C, D) Viability is significantly increased in CERS6-overexpressing than control TNBC cells exposed to doxorubicin (10 μM) and 5-FU (50 μM) for 3 days.

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Supplementary Figure 3

CERS6 overexpression alleviates chemotherapy-induced toxicity in BT-549 cells. Cell viability increased in CERS6-overexpressing TNBC cells exposed to paclitaxel (A), cisplatin (B), doxorubicin (C), and 5-FU (D). After 3 days of treatment, cell viability was assessed. The Inhibitory concentration 50% is indicated by the green line.

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Supplementary Figure 4

CERS6 depletion significantly augments anti-proliferative effect of chemo drugs in TNBC cells. Proliferation significantly decreased in CERS6-depleted MDA-MB-468 (A) and BT-549 (B) cells exposed to cisplatin (5 μM) and paclitaxel (0.5 μM). After 24 hours of transfection with siRNA, chemotherapeutic drugs were added to the cell medium and incubated for 3 days, followed by proliferation measurement.

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REFERENCES

1. Burguin A, Diorio C, Durocher F. Breast cancer treatments: updates and new challenges. J Pers Med 2021;11:808.
PUBMED | CROSSREF
2. Ge J, Zuo W, Chen Y, Shao Z, Yu K. The advance of adjuvant treatment for triple-negative breast cancer. Cancer Biol Med 2021;19:187-201.
PUBMED | CROSSREF
3. Bai X, Ni J, Beretov J, Graham P, Li Y. Triple-negative breast cancer therapeutic resistance: where is the Achilles' heel? Cancer Lett 2021;497:100-11.
PUBMED | CROSSREF
4. Agostinetto E, Eiger D, Punie K, de Azambuja E. Emerging therapeutics for patients with triple-negative breast cancer. Curr Oncol Rep 2021;23:57.
PUBMED | CROSSREF
5. Piazzesi A, Afsar SY, van Echten-Deckert G. Sphingolipid metabolism in the development and progression of cancer: one cancer's help is another's hindrance. Mol Oncol 2021;15:3256-79.
PUBMED | CROSSREF
6. Uen YH, Fang CL, Lin CC, Hseu YC, Hung ST, Sun DP, et al. Ceramide synthase 6 predicts the prognosis of human gastric cancer: it functions as an oncoprotein by dysregulating the SOCS2/JAK2/STAT3 pathway. Mol Carcinog 2018;57:1675-89.
PUBMED | CROSSREF
7. Shi Y, Zhou C, Lu H, Cui X, Li J, Jiang S, et al. Ceramide synthase 6 predicts poor prognosis and activates the AKT/mTOR/4EBP1 pathway in high-grade serous ovarian cancer. Am J Transl Res 2020;12:5924-39.
PUBMED
8. Verlekar D, Wei SJ, Cho H, Yang S, Kang MH. Ceramide synthase-6 confers resistance to chemotherapy by binding to CD95/Fas in T-cell acute lymphoblastic leukemia. Cell Death Dis 2018;9:925.
PUBMED | CROSSREF
9. Suzuki M, Cao K, Kato S, Mizutani N, Tanaka K, Arima C, et al. CERS6 required for cell migration and metastasis in lung cancer. J Cell Mol Med 2020;24:11949-59.
PUBMED | CROSSREF

10. Schiffmann S, Sandner J, Birod K, Wobst I, Angioni C, Ruckhäberle E, et al. Ceramide synthases and ceramide levels are increased in breast cancer tissue. Carcinogenesis 2009;30:745-52.
PUBMED | CROSSREF

11. Nishino M, Jackman DM, Hatabu H, Yeap BY, Cioffredi LA, Yap JT, et al. New Response Evaluation Criteria in Solid Tumors (RECIST) guidelines for advanced non-small cell lung cancer: comparison with original RECIST and impact on assessment of tumor response to targeted therapy. AJR Am J Roentgenol 2010;195:W221-8.
PUBMED | CROSSREF

12. Ridley AJ. Rho GTPase signalling in cell migration. Curr Opin Cell Biol 2015;36:103-12.
PUBMED | CROSSREF

13. Aburima A, Wraith KS, Raslan Z, Law R, Magwenzi S, Naseem KM. cAMP signaling regulates platelet myosin light chain (MLC) phosphorylation and shape change through targeting the RhoA-Rho kinase-MLC phosphatase signaling pathway. Blood 2013;122:3533-45.
PUBMED | CROSSREF

14. Schoenwaelder SM, Burridge K. Evidence for a calpeptin-sensitive protein-tyrosine phosphatase upstream of the small GTPase Rho. A novel role for the calpain inhibitor calpeptin in the inhibition of protein-tyrosine phosphatases. J Biol Chem 1999;274:14359-67.
PUBMED | CROSSREF

15. Tang Z, Li C, Kang B, Gao G, Li C, Zhang Z. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. Nucleic Acids Res 2017;45:W98-102.
PUBMED | CROSSREF

16. Makoukji J, Raad M, Genadry K, El-Sitt S, Makhoul NJ, Saad Aldin E, et al. Association between CLN3 (neuronal ceroid lipofuscinosis, CLN3 type) gene expression and clinical characteristics of breast cancer patients. Front Oncol 2015;5:215.
PUBMED | CROSSREF

17. Erez-Roman R, Pienik R, Futerman AH. Increased ceramide synthase 2 and 6 mRNA levels in breast cancer tissues and correlation with sphingosine kinase expression. Biochem Biophys Res Commun 2010;391:219-23.
PUBMED | CROSSREF

18. Zhu S, Xu Y, Wang L, Liao S, Wang Y, Shi M, et al. Ceramide kinase mediates intrinsic resistance and inferior response to chemotherapy in triple-negative breast cancer by upregulating Ras/ERK and PI3K/Akt pathways. Cancer Cell Int 2021;21:42.
PUBMED | CROSSREF

19. White-Gilbertson S, Mullen T, Senkal C, Lu P, Ogretmen B, Obeid L, et al. Ceramide synthase 6 modulates TRAIL sensitivity and nuclear translocation of active caspase-3 in colon cancer cells. Oncogene 2009;28:1132-41.
PUBMED | CROSSREF

20. Haimovitz-Friedman A, Kolesnick RN, Fuks Z. Ceramide signaling in apoptosis. Br Med Bull 1997;53:539-53.
PUBMED | CROSSREF

21. Edmond V, Dufour F, Poiroux G, Shoji K, Malleter M, Fouqué A, et al. Downregulation of ceramide synthase-6 during epithelial-to-mesenchymal transition reduces plasma membrane fluidity and cancer cell motility. Oncogene 2015;34:996-1005.
PUBMED | CROSSREF

22. Zhou Y, Hancock JF. Deciphering lipid codes: K-Ras as a paradigm. Traffic 2018;19:157-65.
PUBMED | CROSSREF

23. Masuda H, Zhang D, Bartholomeusz C, Doihara H, Hortobagyi GN, Ueno NT. Role of epidermal growth factor receptor in breast cancer. Breast Cancer Res Treat 2012;136:331-45.
PUBMED | CROSSREF

24. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, et al. Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res 2007;13:4429-34.
PUBMED | CROSSREF