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ORIGINAL ARTICLE

Rosmarinic Acid Inhibits Stem-Like Breast Cancer through Hedgehog and Bcl-2/Bax Signaling Pathways

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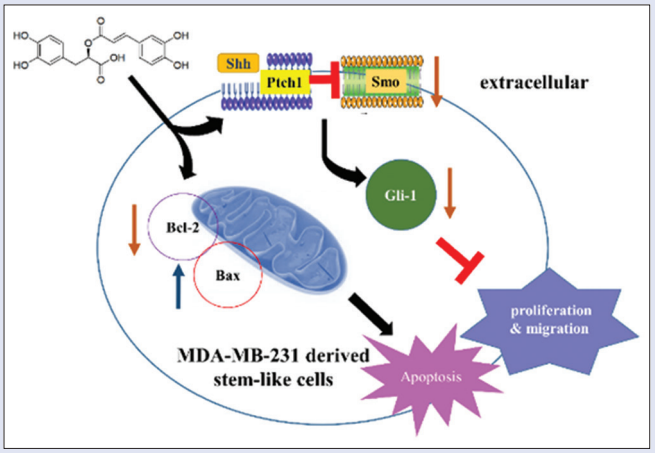
ABSTRACT

Background: Rosmarinic acid (RA) is a natural phenolic acid present in various Lamiaceae herbs. RA shows anti-tumor effects on many tumors but has yet to be tested on triple negative breast cancer and its derived breast cancer stem-like cells (BCSCs). **Objective:** This study aimed to detect whether RA could inhibit the proliferation and migration of BCSCs through hedgehog (Hh) signaling while promoting apoptosis via Bcl-2/Bax. **Materials and Methods:** BCSCs from the human breast cancer cell line MD-MB-231 were isolated by fluorescence-activated cell sorting with the surface markers of $CD44^{+}/CD^{24-/low}$. The viability, migration, and apoptosis of BCSCs were assessed by the CCK-8 assay, cell wound healing test, and flow cytometry for positive staining for Annexin V-FITC and propidium iodine (PI), respectively. mRNA and protein levels of Hh and Bcl-2/Bax signaling pathways were obtained by real-time reverse transcriptase polymerase chain reaction and immunoblots. **Results:** RA inhibited the viability and migration of BCSCs and increased the numbers of late apoptotic cells. Consistent with the increased apoptosis, RA treatment downregulated Bcl-2 while upregulating Bax expression. In line with its effect to limit migration, RA treatment inhibited the expression of Hh-related genes smoothened and glioma-associated oncogene homolog 1. **Conclusion:** The present study suggests that RA exerts anti-cancer effects on BCSCs by inhibiting Bcl-2 and Hh signaling pathways.

Key words: Bax, Bcl-2, breast cancer stem-like cells, hedgehog signaling pathway, rosmarinic acid

SUMMARY

- Rosmarinic acid decreased viability and migration of breast cancer stem-like cells derived from MDA-MB-231
- Rosmarinic acid increased the numbers of late apoptotic cells in breast cancer stem-like cells
- Rosmarinic acid upregulated the expression of Bax and downregulated the expression of Bcl-2 in breast cancer stem-like cells
- Rosmarinic acid inhibited the expression of Hh signaling genes in breast cancer stem-like cells.



Abbreviations used: TNBC: Triple-negative breast cancer, BCSCs: Breast cancer stem-like cells, RA: Rosmarinic acid, Hh: Hedgehog signaling, Shh: Sonic hedgehog, Ptch: Patched Receptor, Smo: phosphorylation of smoothened, Gli-1: Glioma-associated oncogene homolog 1, FACS: Fluorescence-activated cell sorting, RT-PCR: Reverse transcriptase polymerase chain reaction, ANOVA: The analysis of variance.

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INTRODUCTION

Breast cancer is the most common malignancy in women, and the incidence of breast cancer has continued to rise in recent years.[1] Breast tumors can be classified according to three common markers: estrogen receptor, progesterone receptor, and human epidermal growth factor (EGF) receptor type-2. Triple-negative breast cancer (TNBC) lacks all three markers, and it accounts for 10%–15% of invasive breast cancers.[2] The standard chemotherapeutics, such as tamoxifen, trastuzumab, or formestane, are not effective for TNBC, and the treatment for this kind of cancer is still challenging.[3]

Recently, studies indicate the presence of breast cancer stem-like cells (BCSCs) and BCSCs represent a minor subpopulation of tumor cells with the phenotype of $CD44^{+}/CD24^{-/low}$.[4] With the features of long life, high clonogenicity, self-replicating potential, plasticity, resistance to chemotherapy and anti-apoptosis, the BCSCs play a critical role in breast cancer initiation, maintenance and metastasis. However, the drug effect on BCSCs is poor and is closely associated with the patient's prognosis.[5]

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Many reports showed that the MDA-MB-231 cell line could be successfully induced into BCSCs *in vitro*.[6] Here, we isolated BCSCs from the MDA-MB-231 cell line and sought to identify signaling pathways that are associated with BCSCs viability and migration. In particular, we tested how BCSCs respond to Rosmarinic acid (RA; α-o-caffeoyl-3,4-di hydroxyphenyl lactic acid), a natural phenolic compound enriched in a traditional Chinese medicine *Sarcandra glabra* that has been shown to be highly effective for clinical therapy of many kinds of tumors.[7] RA was also reported to suppress the growth of human MCF-7 breast cancer cells and H-22 hepatocellular carcinoma cells.[8,9] However, the effect of RA on TNBC or BCSCs has rarely been investigated.

The Hedgehog (Hh) signaling pathway controls cell growth, differentiation, and viability, and aberrant activation of the Hh pathway induce formation, progression, and invasion of human breast cancer.[10] Sonic Hh (Shh), Patched Receptor (Ptch), phosphorylation of smoothened (Smo) and glioma-associated oncogene homolog 1 (Gli-1) are the key regulators of the Hh pathways. Increased levels of Smo and Gli-1 have been found in TNBC or BCSC, and this is closely related to a poor prognosis.[10] Inhibitors targeting the Hh pathway also decrease tumor cell proliferation.[11]

Bcl-2 and Bax are two key members of the Bcl-2 family, the main regulators of the mitochondrial apoptosis pathway.[12] Bcl-2 is an anti-apoptotic gene that helps cells evade normal death, whereas Bax gene is a pro-apoptotic gene that sentences the cell to death. Dysregulation of Bcl-2 and Bax plays critical roles in the initiation, maintenance, and metastasis of breast cancer. Elevated Bcl-2 protein and reduced Bax protein were frequently detected in TNBC and BCSCs.[12] Increased Bcl-2 in residual tumor cells containing rich BCSCs has been identified in postchemotherapy breast cancer specimens.[13] Drugs targeting Bcl-2/Bax could effectively induce apoptosis of TNBC by suppressing Bcl-2 and elevating Bax.[14]

Here, we showed that RA inhibited the viability and migration and induced apoptosis of stem-like cells (BCSCs) isolated from the human breast cancer cell line MDA-MB-231. The beneficial effect of RA on BCSCs is associated with decreasing the Bcl-2/Bax ratio and suppressing Hh signaling. These data identified potential therapeutic targets for TNBC and BCSCs.

MATERIALS AND METHODS

Materials

The purity of RA extracted from *S. glabra* is more than 98% (identified by UPLC, Saychun Biotechnology co. Ltd., Hubei, China). RA was dissolved in 0.1% dimethyl sulfoxide (DMSO) at the final concentrations of 0, 10, 30, 90, 270, and 810 μmol/L.

Cell culture and stem-like cells enrichment

The TNBC MDA-MB-231 cell line (ATCC) and normal breast MCF-10A cell line were obtained from the Shanghai Institute of Cell Biology and the Life Science Center (Shanghai, China). MDA-MB-231 Cells were seeded in RPMI-1640 medium (HyClone; cat. #SH30809.01) and MCF-10A cells were seeded in DMEM medium (Gibco; cat. #11965-092) and 100 ng/ml cholera toxin. All the mediums were supplemented with 10% fetal calf serum (PAN Biotech, Aidenbach, German) in the presence of streptomycin and penicillin. MDA-MB-231 Cells were enzymatically dissociated in a 0.05% trypsin/0.025% EDTA solution and cell suspensions were cultured in a bottle with low surface attachment (Corning) in serum-free RPMI-1640 medium containing 10 ng/mL basic fibroblast growth factor (Peprotech, Rocky Hill, NJ; cat. #AF-100-15), 20 ng/mL EGF (Peprotech, Rocky Hill, NJ; cat. #100-18B) and 2% B-27 Supplement (Gibco, New York, US; cat. #17504-044). Such culture conditions help the cell suspensions convert to sphere-forming cells. All these cells were incubated in a humidified 5% CO_2 incubator at 37°C.

Flow cytometric sorting

The stem-like CD44+/CD24$^{-/low}$ subpopulation of the MDA-MB-231 cells was separated by fluorescence-activated cell sorting (FACS). The sphere-forming cells were washed once with phosphate-buffered saline (PBS) and then harvested with trypsin/EDTA. Detached cells were washed with PBS containing 1% calf serum (wash buffer), centrifuged, and resuspended in wash buffer (10^7 cells/ml). Then, the cells were incubated with fluorochrome-conjugated monoclonal antibodies (BD Biosciences, San Diego, CA, USA) such as anti-CD44 (FITC; cat. #555478), anti-CD24 (PE; cat. #555428), combinations of anti-CD44 and anti-CD24, and their respective isotype controls. After incubation with the combinations of antibodies at 4°C in the dark for 30 min, cells were sorted with PBS as sheath fluid, at 12–15 p. s. i. using FACS AriaIII (BD Biosciences, US). The BCSCs gated by FACS was the lowest quintile of CD24-positive cells plus all the CD24-negative cells.

Cell viability assay

Cell viability was determined using the CCK-8 assay kit (BeyotimeInst Biotech, China). Briefly, 1×10^4 MDA-MB-231, BCSCs and MCF-10A cells/well were treated with different concentrations of RA for 24, 48, or 72 h. 10 μL CCK-8 solution was added to each well and incubated at 37°C for 3 h, and the absorbance was determined at 450 nm from 5 replicates using a microplate reader Bio-RAD 680 (USA). Densitometric analysis was performed, and the levels of RA-treated cells were normalized against the levels of the DMSO vehicle group. Each experiment was independently replicated at least three times. The IC_{50} of RA on MDA-MB-231 cells or BCSCs cells was calculated using the GraphPad Prism program.

Wound healing assay for cell migration analysis

MDA-MB-231 and BCSCs cells were seeded at a density of 2.5×10^5 cells/well in 6-well culture plates and allowed to form a confluent monolayer. The layer of cells was scraped with a 200 μl micropipette tip to create a wound. Cells were washed twice with PBS and replaced with 1% serum medium containing various concentrations of RA. At 0, 24, 48 h (3 wells per group), the width of the wound (3 wounds per well) was monitored under a phase-contrast microscope at ×100 and measured using an image analysis system (Image-Pro Plus 5.0; Media Cybernetics). The reduced width of cell wound was equal to the average width of cell wound at 0 h minus that at 24 or 48 h and compared between control and RA groups.

Cell apoptosis detection

5×10^5 BCSCs cells were harvested and incubated with different concentrations of RA for 48 h (3 wells per group). Cells were washed once with cold PBS, resuspended in 1 ml PBS, then dual stained with 5 μL of Annexin V-FITC and 10 μL of PI and then incubated in the dark for 30 min. Flow Cytometry was carried out to identify apoptotic populations of the BCSCs cells using the FACSVerse (BD Biosciences, US).

RNA isolation and quantification

The mRNA levels were determined using reverse transcriptase polymerase chain reaction (RT-PCR). After the BCSCs (5 wells of a 6-well plate per group) were treated with different concentrations of RA for 48 h, total RNA was extracted using the TRIzol reagent (Takara Inc., Dalian, China; cat. #9109) and then reverse-transcribed to complementary DNA (cDNA) using the RNase Hi (Takara Inc., Dalian, China; cat. #RR037A). Real-time quantitative PCR was then conducted

using the SYBR Green I fluorescent dye reagent (Roche Inc., Shanghai, China; cat. #19317900) with the ABI System Sequence Detector 7500. β-actin was used as an internal standard. PCR amplifications were performed for all samples under the following conditions: (stage 1, 1 cycle) 95°C for 30 s; (stage 2, 40 cycles) 95°C for 5 sec, 60°C for 32 s. For each sample, triplicate of PCR experiments was conducted and averaged to eliminate loading errors. The oligonucleotide sequences of the primers are presented in Table 1.

Immunoblots

After the BCSCs (5 wells per group) were treated with different concentrations of RA for 48 h, the total protein of the BCSCs was extracted using a total protein extraction kit (Applygen Technologies Inc., MA, US). Samples containing equal amounts of protein (40 μg) were size fractionated by electrophoresis and proteins were transferred from a gel to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was then incubated with Tris-buffered saline (pH 7.5) and dehydrated skim milk to block binding of nonspecific proteins. The PVDF membranes were then incubated overnight at 4°C with primary antibodies specific to Bcl-2 (1:1000, Abcam Inc., cat. #ab196495), Bax (1:1000, Abcam Inc., cat. #ab32503), Shh (1:1000, Abcam Inc., cat. #ab53281), Ptch (1:1000, Abcam Inc., cat. #ab109407), Smo (1:1000, Bioss Biotechnology Co; cat. # bs-2801R), and Gli-1 (1:1000, Abcam Inc., cat. #ab49314). The membranes were washed in in TBST, incubated with secondary HRP-linked antibodies (1:2000, Zhongshan Biotechnology Inc., cat. #zb-2305) and then imagined with the Gel Imaging System (Bio-Rad). The relative levels of proteins of interest were calculated after normalized to the β-actin levels that serve as loading controls.

Statistical analysis

Data were presented as the mean with standard deviation. Statistical analyses were performed using the analysis of variance (ANOVA) method after testing for the homogeneity of variance. To compare the significance of inhibition rate, a repeated-measures ANOVA was used. The *P* value was calculated by ANOVA using the Statistical Package for Social Sciences software version 17.0 (International Business Machines Corporation, Newyork, USA), and a value <0.05 was considered statistically significant.

Table 1: Primer sequences used in this study

Genes	Forward primer (5′-3′)	Reverse primer (5′-3′)	Length (bp)
β-ACTIN	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT	223
Bcl-2	GTGTGTGGAGAGCGTCAACC	AGAAATCAAACAGAGGCCGCA	167
Bax	AGCGACTGATGTCCCTGTCT	TCCAGATGGTGAGTGAGGCG	115
Shh	GCTGCTAGTCCTCGTCTCCT	GGGTCTTCTCGGCCACATTG	143
Ptch	TGCGGCAAGTTCTTGGTTGT	CACGTTGGTCTCGAGGTTCG	84
Smo	CTGCGCTACAACGTGTGCC	CCTCCTGGGAGTCCGAGTCT	85
Gli-1	GCAAGTCAAGCCAGAACAGG	GGGGGTAATGGGAAAAGAGA	122

Shh: Sonic hedgehog; Ptch: Patched receptor; Smo: Smoothened; Gli-1: Glioma-associated oncogene homolog 1

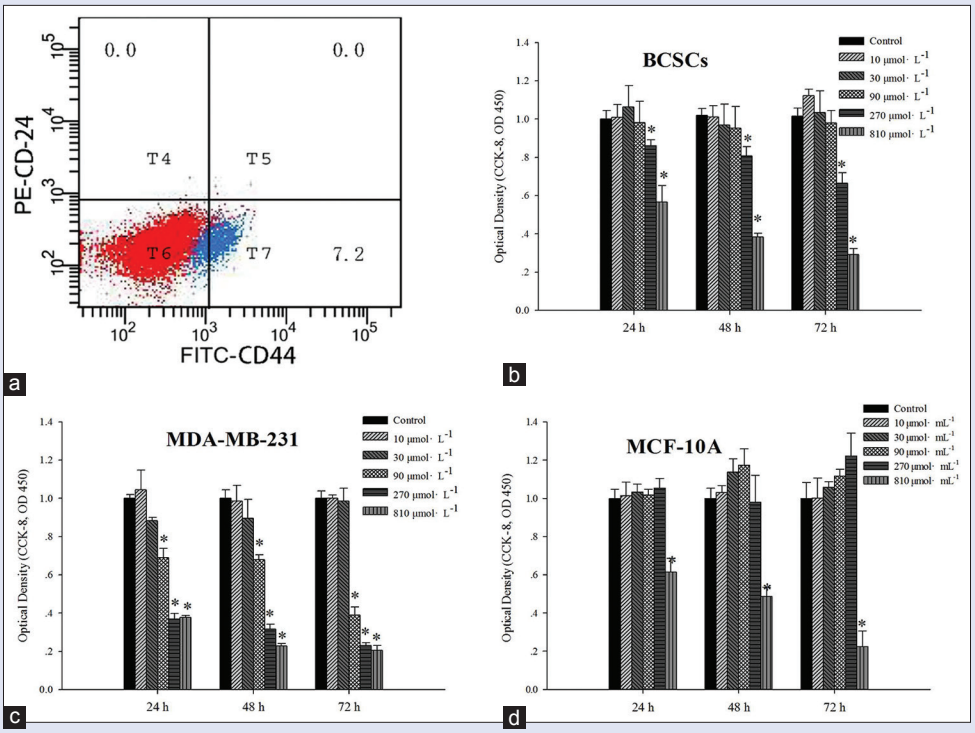

Figure 1: Effects on cell viability assay between MDA-MB-231, breast cancer stem-like cells and MCF-10A after rosmarinic acid treatment. (a) The CD44+/CD24-/low subpopulation of MDA-MB-231 cells were sorted by the Flow cytometer. Cell viability of MDA-MB-231 (b), breast cancer stem-like cells (c) and MCF-10A (d) in response to rosmarinic acid treatment at different concentrations (0, 10, 30, 90, 270 and 810 μmol/L) was measured with the CCK8 assay at 24, 48 and 72 h. DMSO was used as vehicle control. *$P < 0.05$, compared with the control

RESULTS

Rosmarinic acid decreased the viability of MDA-MB-231 and breast cancer stem-like cells

The CD44+/CD24$^{-/low}$ subpopulation cells constitutes 3%–6% of the MDA-MB-231 cells [Figure 1a]. The effects of RA on cell viability of MDA-MB-231, BCSCs, and MCF-10A were determined by CCK-8 assay. At all three time points tested, 24, 48 and 72 h, RA inhibited the viability of BCSCs at the concentrations of 270 and 810 μmol/L [Figure 1b] whereas 90–810 μmol/L RA is effective to inhibit the viability of MDA-MB-231 [Figure 1c]. Only 810 μmol/L RA inhibits the cell viability of MCF-10A and no decreases of cell viability were observed at concentrations below 270 μmol/L [Figure 1d]. For BCSCs, the IC_{50} at 24, 48, and 72 h were 873, 650, and 453 μmol/L, respectively. For MDA-MB-231, the IC_{50} at 24, 48 and 72 h were 206, 156 and 117 μmol/L, respectively.

Rosmarinic acid inhibited the migration of MDA-MB-231 and breast cancer stem-like cells

Wound healing test of cultured cells was carried out to investigate how RA affects the migration; the decrease of the gap between two sides of cells after mechanically removing cells from the center of cell culture by scrapping is positively correlated with the speeds of the cells migrating over the time after cell stripping [Figure 2a]. The migration of the BCSCs was significantly blocked after treatment with high concentrations of RA (270 or 810 μmol/L) [Figure 2b]. The migration of MDA-MB-231 cells was effectively blocked with 90–810 μmol/L RA at 24 and 48 h, compared to control [Figure 2c]. Of note, atrophy (marked shrinkage in cell size) of both cell types was observed after treatment with the highest concentration (810 μmol/L) of RA [Figure 2a].

Rosmarinic acid treatment reduced hedgehog signaling in breast cancer stem-like cells

Real-time RT-PCR analysis was conducted to measure the mRNA levels of Shh, Ptch, Smo, Gli-1 genes, and immunoblot analysis was carried out to measure their protein levels. Compared to the control, the 270 and 810 μmol/L RA treatment significantly lowered the levels of mRNA and protein of Smo and Gli-2. However, no significant change in the mRNA or protein levels of Shh and Ptch was detected after RA treatment at any of the tested concentrations [Figure 3].

Rosmarinic acid-induced apoptosis of breast cancer stem-like cells

Flow cytometry was used to assess the apoptosis of BCSCs after RA treatment by staining for the annexin V-FITC/PI; FITC-conjugated annexin V labels the externalization of phosphatidylserine in apoptotic

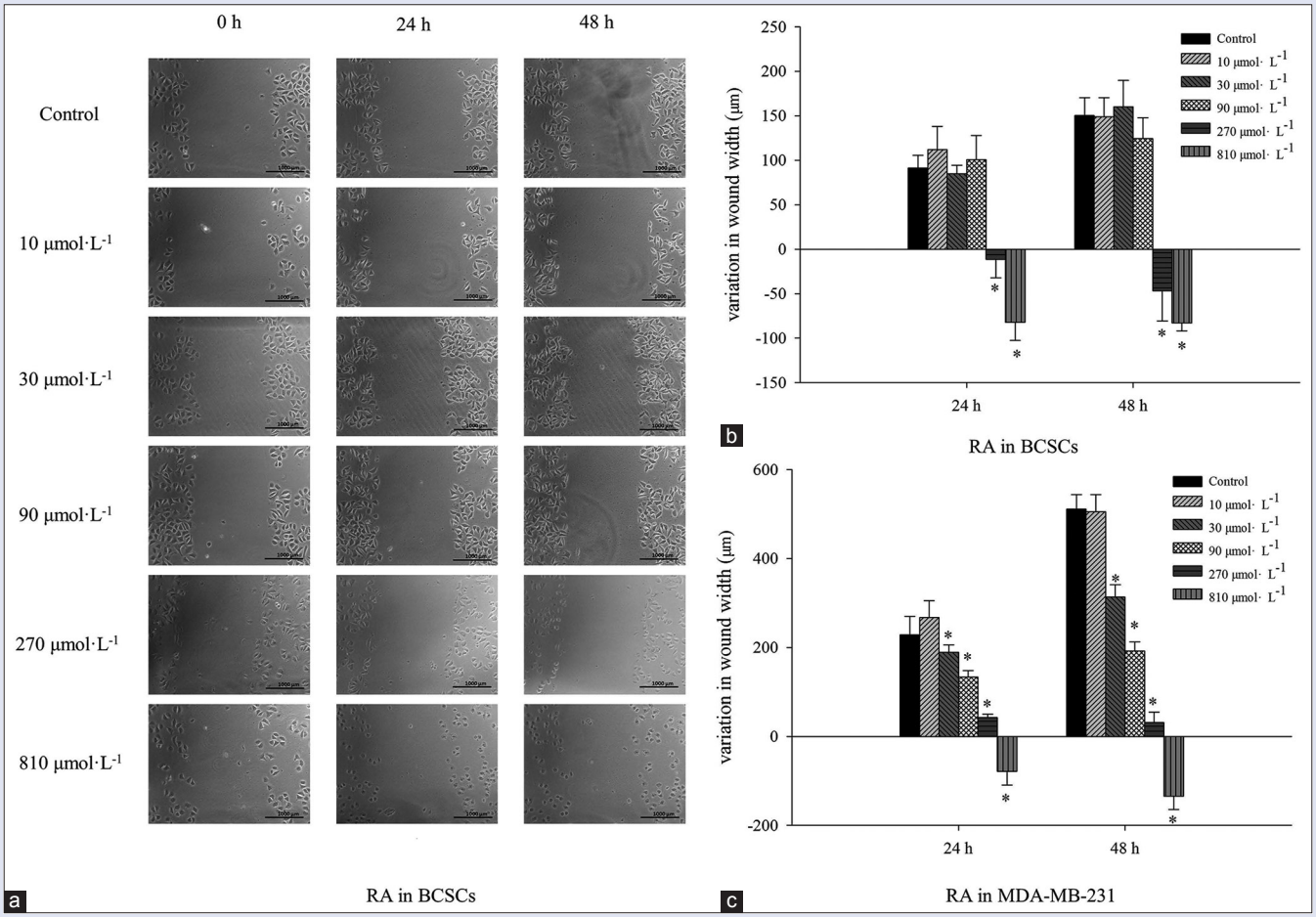

Figure 2: Inhibition on wound healing comparison in MDA-MB-231 and breast cancer stem-like cells after rosmarinic acid treatment. (a and b) Variation of wound width in cultured breast cancer stem-like cells after 0, 10, 30, 90, 270 and 810 μmol/L rosmarinic acid treatment for 24 and 48 h. (c) Variation of wound width in cultured MDA-MB-231 after rosmarinic acid treatment for 24 and 48 h. *$P < 0.05$, compared with the control

cells whereas PI labels nuclei of all cells, including healthy and dying cells. A dose-dependent increase in apoptosis was observed at higher doses of RA treatment [Figure 4], with 270 and 810 μmol/L RA significantly increasing the numbers of apoptotic cells, including those at the early (annexin V-FITC +, PI−) or the late (annexin V-FITC +, PI+) apoptotic stages.

Rosmarinic acid treatment alters Bcl-2, Bax in breast cancer stem-like cells

Compared to the control, the 270 and 810 μmol/L RA treatment significantly lowered the levels of mRNA and protein of Bcl-2, and the same treatments significantly increased the mRNA and protein levels for Bax. Both the mRNA and protein expression ratio of Bcl-2/Bax exhibited a decreasing trend with an increasing RA dose [Figure 5].

DISCUSSION

The prognosis in TNBC is the worst among breast cancer subtypes, and little chemotherapy progress has been achieved over the past several decades.[15] The MDA-MB-231 is a typical TNBC cell line that is commonly used for clinical research. The relatively high proportion of BCSCs with the characteristic resistance to commonly used therapies present in the TNBC further increases the challenge to treat this subtype of breast cancer.

Some natural Chinese medicine has shown promising clinical outcomes in treating breast cancer, hence studying the signaling pathways affected by these medicines may identify therapeutic targets for obstinate TNBC and BCSCs. *S. glabra* has low toxicity and good prognosis in clinic tumor treatment.[16] RA is a phenolic acid enriched in *S. glabra* and phenolic compound has been shown to inhibit breast cancer progression.[17]

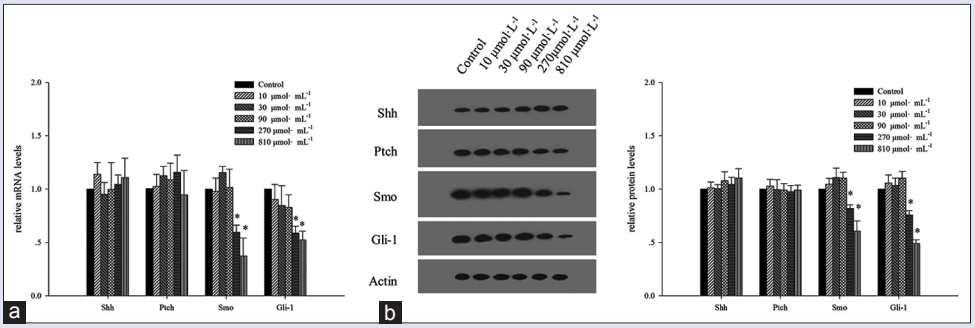

Figure 3: Changes of the mRNA and protein levels of Shh, Ptch, Smo and glioma-associated oncogene homolog 1 genes in the breast cancer stem-like cells after rosmarinic acid treatment. (a) Quantitative reverse transcriptase polymerase chain reactionwas used to compare the changes of the mRNA levels after 0, 10, 30, 90, 270 and 810 μmol/L rosmarinic acid treatment for 48 h. β-actin was used as an internal standard to calculate the relative amounts ($2^{-\Delta\Delta CT}$) of the mRNA of genes of interests. (b) Immunoblot analysis was used to compare the protein levels after 0, 10, 30, 90, 270 and 810 μmol/L rosmarinic acid treatment for 48 h. Left, the representative immunoblots. Right, protein levels of the immunoblot analysis were quantified and normalized to controls. *$P < 0.05$ compared to the control

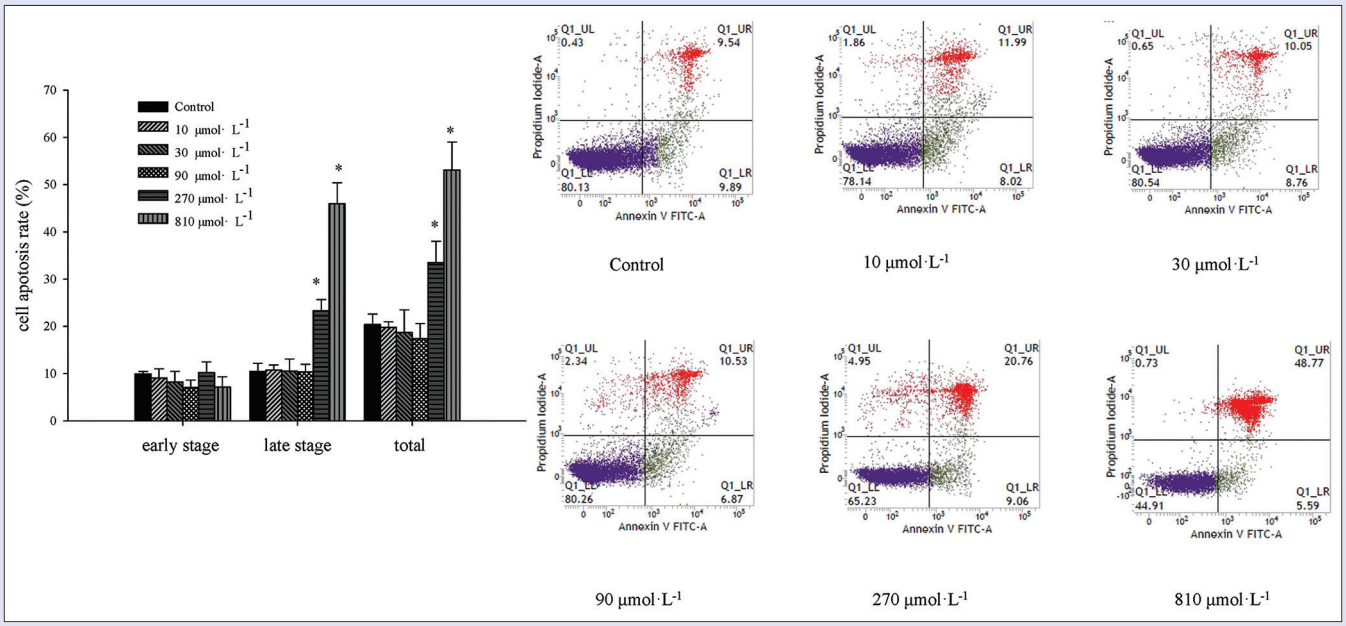

Figure 4: Rosmarinic acid treatment causes apoptosis of the breast cancer stem-like cells. Annexin V-FITC and propidium iodine dual staining of the flow cytometry was carried out to detect apoptotic cells after 0, 10, 30, 90, 270 and 810 μmol/L rosmarinic acid treatment for 48 h. *$P < 0.05$, compared to the control

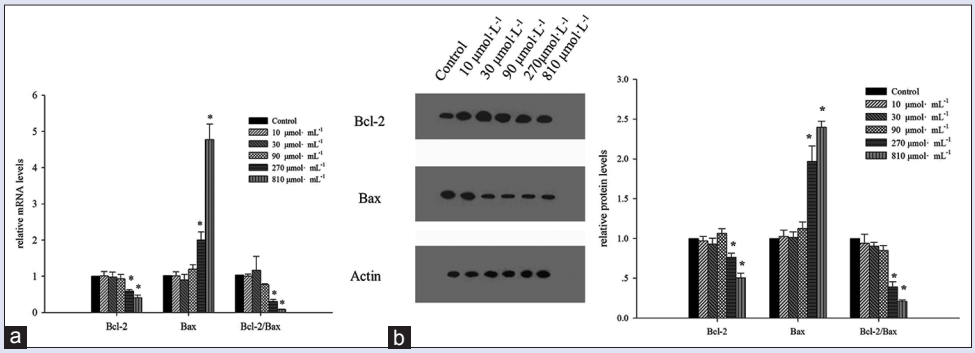

Figure 5: Changes of the mRNA and protein levels of Bcl-2 and Bax genes in the breast cancer stem-like cells after rosmarinic acid treatment. (a) Quantitative reverse transcriptase polymerase chain reactionwas used to compare the changes of the mRNA levels after 0, 10, 30, 90, 270 and 810 μmol/L rosmarinic acid treatment for 48 h. (b) Immunoblot analysis was used to compare the protein levels after 0, 10, 30, 90, 270 and 810 μmol/L rosmarinic acid treatment for 48 h. Data presented as bar representing mean value and error bars showing standard deviation. * $P < 0.05$ compared to the control

Our results here further showed that RA could both inhibit the viability and migration of MDA-MB-231 and its BCSCs.

The cell viability study with the CCK-8 assay and the migration study with the cell wound healing test provide strong evidence that RA inhibits the viability and migration of MDA-MB-231 and BCSCs. 270 and 810 μmol/L RA not only reduced the viability of MDA-MB-231 and BCSCs but also inhibited their migration. Cellular atrophy was observed for the highest doses of RA. Our results were consistent with a previous study reporting high cytotoxicity of two aqueous-ethanol extracts (both containing RA) in MDA-MB-361 and MDA-MB-451 breast cancer cells.[18] In addition, RA treatment is also effective to lower the viability of estrogen receptor-positive human breast cancer (MCF-7) cells.[8] Based on our results of the IC_{50} and cell wound healing tests, the sensitivity of BCSCs to RA was much lower than that of MDA-MB-231, consistent with its notorious characteristic of drug-resistance.[19] Cell viability assay also showed that 270 μmol/L RA had no inhibitory effects on normal breast cell MCF-10A, suggesting that RA has the prospect for the breast cancer treatment.

Binding of Shh to its receptor Ptch activates the Hh signaling cascade that frees the Smo to enable transcription of Gli-1 and then elicits several downstream growth effectors that contribute to the growth of the breast cancer.[20] The genes of Hh signaling cascade were generally highly expressed both in TNBC and BCSCs.[21] Shh promotes the growth and migration of MDA-MB-231[20] whereas the Hh signaling inhibitors suppressed the growth and migration of TNBC and BCSCs.[22] Our data showed that 270 and 810 μmol/L RA treatment could decrease the expression of Smo and Gli-1 in BCSCs, suggesting that RA inhibits the proliferation and migration of BCSCs by inhibiting the Hh pathway. This novel observation from our study is consistent with another report showing that RA inhibits Wnt signaling, a direct downstream target of the Hh pathway.[23]

Apoptosis is a distinctive mode of programmed cell death that involves activation of a well-defined signaling cascades to eliminate cells. Apoptosis is also the key process that is targeted by chemotherapy drugs to suppress the growth of tumor cells. RA induces apoptosis of tumor cells such as human glioma and colon carcinoma.[24,25] In our study, a significantly higher apoptotic rate was detected in BCSCs after 270 and 810 μmol/L RA treatment. This result suggests that the apoptotic pathway is an important target for the anti-tumor effect of RA, consistent with the results of other breast cancer cell lines.[18,26] Significantly more BCSCs cells at the late apoptotic stage after RA treatment suggests that the induced cell death was irreversible.

Bcl-2 and Bax are the two key regulators in the mitochondria apoptotic pathway with opposite functions.[12] Bcl-2 protein locates in the mitochondrial membrane and restrain multi-factor induced apoptosis and prolong the life of the tumor cells when over-expressed, whereas Bax is pro-apoptotic and induce cell death.[12] Bax protein is homologous to Bcl-2 and Bcl-2, and Bax regulate apoptosis through the formation of homo-or heterodimers. When Bcl-2 expression increases, the Bax homodimer separates, and Bax binds to Bcl-2 to form a more stable heterodimer, inhibiting apoptosis induced by Bax homodimers. In contrast, when Bcl-2 expression decreases, the Bax homodimer increases and promotes apoptosis. An unbalanced BcL2/BAX ratio breaks the structural and functional stability of mitochondria and the endoplasmic reticulum, inducing apoptosis. High Bcl-2/Bax ratio was reported in MDA-MB-231 and BCSCs, reflecting their active growth and drugs that target the Bcl-2/Bax signaling are more effective in TNBC treatment.[14,27] Therefore, we postulate that the Bcl-2/Bax ratio may also be another target contributing to the beneficial inhibitory effects of RA on BCSCs. Indeed, we observed decreased Bcl-2 and increased Bax at their mRNA and protein levels after RA treatment. The phenolic acids could inhibit the breast cancer cells by reducing the expression of Bcl-2 and RA is a typical phenolic acid.[28] Further evidence of RA treatment in lowering Bcl-2 and elevating Bax were also reported in human colon adenocarcinoma and mice skin cancer.[29,30] Multiple signal pathways exist in apoptosis, which can also be induced by death receptors related protein such as Fas and Casepase 3. Our previous study has shown that no significant changes of the gene expression for Fas and Caspase 3 were detected after MDA-MB-231 exposed to RA, suggesting Bcl-2/Bax pathway might have a more general effect.[31]

In this study, the relative mRNA and protein expression levels of genes after RA treatment were generally consistent, suggesting that altered expression of genes occurs mainly at the level of transcriptional regulation.

CONCLUSION

In summary, our study demonstrates that RA inhibits the viability and migration and induced apoptosis of the BCSCs. Our observations of correlated reductions of the Bcl-2/Bax ratio and Hh signaling (i.e., less Smo and Gli-1) suggest these pathways are useful targets for drug development to treat TNBC.

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Conflicts of interest

There are no conflicts of interest.

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