Anti-tumor peptide SA12 inhibits metastasis of MDA-MB-231 and MCF-7 breast cancer cells via increasing expression of the tumor metastasis suppressor genes, CDH1, nm23-H1 and BRMS1

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Abstract. In recent years, there has been progress in the treatment of breast cancer; however, the prognosis is still poor due to recurrence and metastasis following conventional treatment. The anti-tumor peptide SA12 has been demonstrated to inhibit proliferation and arrest the cell cycle in MDA-MB-231 and MCF-7 breast cancer cells. In the present study, whether SA12 was able to inhibit the metastasis of breast cancer cells was investigated. Wound healing and Transwell assays were used to investigate the inhibition of SA12 on cell migration while, reverse transcription-quantitative PCR and western blot assays were used to identify the mechanism of action behind the effects of SA12 on cell migration. Results from the wound healing and Transwell assays revealed that SA12 significantly inhibited the migration of MDA-MB-231 and MCF-7 breast cancer cells following treatment with 100 μM SA12. Compared with that in the controls, the mRNA expression levels of cadherin 1 (CDH1), non-metastasis 23-H1 (nm23-H1) and breast cancer metastasis suppressor 1 (BRMS1) were increased in MDA-MB-231 and MCF-7 cells following treatment with 100 μM SA12. Furthermore, the protein expression levels of E-cadherin, NM23A and BRMS1 were also increased in MDA-MB-231 cells and MCF-7 cells following treatment with 100 μM SA12. In conclusion, SA12 inhibited the migration of MDA-MB-231 and MCF-7 breast cancer cells and enhanced the expression of the tumor metastasis suppressor genes, CDH1, nm23-H1 and BRMS1, which may be responsible for the SA12-induced inhibition of breast cancer cell metastasis.

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Introduction

Breast cancer is one of the most common malignant tumor types and is also one of the leading causes of cancer-associated mortality worldwide (1). Over the past few decades, surgical treatment, radiotherapy, chemotherapy and endocrine therapy have been utilized for the clinical treatment of breast cancer. However, the prognosis for patients with breast cancer remains poor due to the recurrence and metastasis of breast cancer following conventional treatment (2).

Bioactive peptides, which are composed of several amino acids, have been indicated to be effective as a novel therapeutic strategy in cancer therapies (3). Previous studies have revealed that a novel anti-tumor peptide, SA12, inhibits proliferation and arrests the cell cycle in MDA-MB-231 and MCF-7 breast cancer cells (4,5). Tumor metastasis is critical in the progression of breast cancer, which is regulated by a number of genes. Tumor metastasis suppressor genes, including cadherin 1 (CDH1), non-metastasis 23-H1 (nm23-H1) and breast cancer metastasis suppressor 1 (BRMS1) are important negative regulators during tumor metastasis (6,7). E-cadherin, which is the gene encoding the product of CDH1, is downregulated in a number of cancer types during the epithelial-mesenchymal transition and its expression is inversely correlated with metastasis. Overexpression of E-cadherin suppresses the invasion of cancer cells (6). Non-metastatic 23A (NM23A), a potential metastasis suppressor encoded by the nm23-H1 gene, was identified as a metastasis suppressor in melanoma cells. NM23A expression has been identified to be inversely correlated with clinical metastasis of multiple tumors, mediating suppression of metastasis and controlling cellular responses to the microenvironment (7). BRMS1 is currently the only metastasis suppressor that has been identified in models of breast cancer and has since been revealed to suppress metastasis in a broad spectrum of cancer types, regulating the interactions between tumor cells and the tumor microenvironment (6). The present study aimed to explore whether the anti-cancer peptide SA12 inhibited the metastasis of MDA-MB-231 and MCF-7 breast cancer cells and to further investigate the roles of the tumor metastasis suppressor genes, CDH1, nm23-H1 and BRMS1, as well as their gene encoding products, E-cadherin, NM23A and BRMS1, in the inhibition of SA12 in MDA-MB-231 and MCF-7 cells.

Materials and methods

Reagents. DMEM and FBS were purchased from Gibco; Thermo Fisher Scientific, Inc. Bovine serum albumin and trypsin were purchased from Invitrogen; Thermo Fisher Scientific, Inc. Cell culture plates and Transwell chambers were purchased from Corning Inc. RNAiso Plus reagent, PrimeScript RT reagent kit and SYBR Premix Ex Taq II were purchased from Takara Biotechnology Co., Ltd. RIPA lysis buffer was purchased from Sangon Biotech Co., Ltd. The mouse anti-human E-cadherin monoclonal antibody (cat. no. ab1416), the rabbit anti-human NM23A polyclonal antibody (cat. no. ab92327) and the rabbit anti-human BRMS1 monoclonal antibody (cat. no. ab134968) were purchased from Abcam. The mouse anti-human β-actin monoclonal antibody (cat. no. TA328071), the horse-radish (HRP)-labelled goat anti-mouse secondary antibody (cat. no. TA130003) and the HRP-labelled goat anti-rabbit secondary antibody (cat. no. TA140003) were purchased from OriGene Technologies, Inc.

Cell lines and culture. Human MDA-MB-231 and MCF-7 cell lines were purchased from the American Type Culture Collection and cultured in DMEM medium containing 10% FBS at 37˚C in an atmosphere of 5% CO_2.

Peptide preparation. The amino acid sequence of SA12 used in the present study was Ser-Val-Pro-Leu-Phe-Asn-Phe-Ser-Val-Tyr-Leu-Ala. The peptide was chemically synthesized and purified by GL Biochem Shanghai, Ltd. The synthetic peptide was dissolved in DMSO using ultrasound and vortexed for 30 sec at 200 x g, and then diluted to 100 μM in DMEM.

Wound healing assay. MDA-MB-231 and MCF-7 cells were seeded into 6-well cell culture plates with a density of 5x10⁵ cells/well at 37˚C overnight. Upon reaching 80% confluence, the cells were scratched using a micropipette tip and the floating cells were removed through PBS solution washes. The cells were treated with 100 μM SA12 diluted in serum-free DMEM, while the serum-free DMEM containing 0.1% DMSO was used as the blank control. Cells were incubated at 37˚C for 48 h without changing the medium, and wound healing was examined using a phase contrast microscope (Olympus Corporation) at x200 magnification. The cell migration distance was measured and the migration distance at 48 h was normalized to the distance at 0 h in each sample.

Transwell assay. A total of 500 μl serum-free DMEM containing 50 μl bovine serum albumin (10 g/l) was added to each Transwell chamber and incubated at 37˚C for 30 min to hydrate the basement membrane. MDA-MB-231 and MCF-7 cells were cultured until 90% confluent at 37˚C, and the serum was removed to starve the cells for 12 h. The cells were then digested with trypsin and centrifuged at 200 x g for 5 min at room temperature. The cell pellets were resuspended in serum-free medium at a cell density of 5x10⁵ cells/ml. The Transwell chambers were placed in the 24-well cell culture plates with 500 μl DMEM per well and 200 μl prepared cell suspension was added into the Transwell chamber. The cells were treated with 100 μM SA12 diluted in DMEM and the control group was treated with serum-free cell culture medium

Table I. Primers designed for reverse transcription-quantitative PCR.

Gene	Primer
CDH1	F: 5'-GAACGCATTGCCACATACAC-3'
	R: 5'-GAATTCGGGCTTGTTGTCAT-3'
nm23-H1	F: 5'-AGAAAGGATTCCGCCTTGTT-3'
	R: 5'-GGCCCTGAGTGCATGTATTT-3'
BRMS1	F: 5'-GCAGCGGAGCCTCAAGATTCG-3'
	R: 5'-GCAGCGTGTCATAGAGCAGCAG-3'
β-actin	F: 5'-GACTTAGTTGCGTTACACCCTTTC-3'
	R: 5'-TGCTGTCACCTTCACCGTTC-3'

F, forward; R, reverse; BRMS1, breast cancer metastasis suppressor 1; CDH1, cadherin 1; nm23-H1, non-metastasis 23-H1.

containing 0.1% DMSO. In order to prevent cell proliferation caused by the serum in the lower chambers, the cells were collected following incubation for 24 h at 37˚C, without medium changes. The cells were fixed with 2% glutaraldehyde for 30 min and stained with 0.2% gentian violet (Baso Diagnostic, Inc.) for 10 min at temperature. The migrated cells were photographed using a phase contrast microscope (Olympus Corporation) at x200 magnification. The number of migrated cells in 5 visual fields was randomly counted using ImageJ 1.8.0 software (National Institutes of Health) and the average number was calculated to determine the cell mobility in cells following SA12 treatment.

Reverse transcription-quantitative PCR (RT-qPCR) analysis. The MDA-MB-231 and MCF-7 breast cancer cell lines were seeded into 6-well plates at a density of 5x10⁵ cells/well and cultured at 37˚C. Cells were treated with 100 μM SA12 diluted in DMEM for 48 h at 37˚C, and cells were treated with DMEM containing 0.1% DMSO as a control for 48 h at 37˚C. RNAiso plus reagent (Takara Bio, Inc.) was used to extract total RNA and subsequently, the obtained RNA was dried naturally at room temperature and dissolved in 50 μl RNase-free water. PrimeScript RT reagent kit (Takara Bio, Inc.) was used to reverse transcribe mRNA to generate cDNA at 37˚C for 15 min and 85˚C for 5 sec. CDH1, nm23-H1 and BRMS1 cDNA were amplified using SYBR Premix Ex Taq II (Takara Bio, Inc.), with primers provided in Table I. β-actin was used as the internal control. The following thermocycling conditions were used for the qPCR: Initial denaturation at 95˚C for 3 min; 40 cycles of 95˚C for 15 sec, 54˚C for 20 sec and 72˚C for 20 sec; and a final extension at 72˚C for 10 min. The relative mRNA expression levels were calculated as fold changes using the $2^{-\Delta\Delta Cq}$ method (8) to reflect the changes of CDH1, nm23-H1 and BRMS1 genes following treatment with SA12.

Western blot analysis. The MDA-MB-231 and MCF-7 breast cancer cell lines were seeded into 6-well plates at a density of 5x10⁵ cells/well and treated with 100 μM SA12 diluted in DMEM for 48 h at 37˚C. Cells treated with DMEM containing 0.1% DMSO were used as the control. The cells

were harvested following digestion with trypsin, centrifuged at 200 x g for 5 min at room temperature, lysed with 200 μl RIPA lysis buffer (Beyotime Institute of Biotechnology) containing 2 μl PMSF (100 mM) (Beyotime Institute of Biotechnology), and incubated for 30 min on ice. The lysate was centrifuged at 14,000 x g for 15 min at 4°C, the supernatant was extracted, and the concentration of total protein was calculated using a bicinchoninic acid assay (Beyotime Institute of Biotechnology). Subsequently, 80 μg protein/lane was separated via 12% SDS-PAGE (Beyotime Institute of Biotechnology). Subsequently, the proteins were transferred onto nitrocellulose membranes and blocked with 5% skimmed milk for 30 min at room temperature. The membranes were then incubated with E-cadherin (1:50), NM23A (1:1,000) and BRMS1 (1:1,000) primary antibodies overnight at 4°C, and subsequently with the goat anti-mouse or goat anti-rabbit HRP-labeled secondary antibodies (1:2,000) for 2 h at room temperature. The protein signals were visualized using ECL (EMD Millipore). The density of bands in SA12 treated cells and controls were compared and normalized to β-actin (1:1,000), which was used as the internal control. The relative protein expression levels of E-cadherin, NM23A and BRMS1 after SA12 treatment were calculated using Gel-Pro Analyzer 4.0 software (Media Cybernetics, Inc.).

Statistical analysis. All the experiments were repeated at least 3 times and the results are presented as the mean ± standard deviation. Data were analyzed using unpaired or paired Student's t-tests. P<0.05 was considered to indicate a statistically significant difference. SPSS v14.0 software (SPSS, Inc.) was used for statistical analysis.

Results

SA12 inhibits the migration of MDA-MB-231 and MCF-7 cells in wound healing assays. To examine the inhibitory effect of SA12 on the migration of MDA-MB-231 and MCF-7 breast cancer cells, wound healing assays were performed. The cells were treated with 100 μM SA12, while the control group was treated with 0.1% DMSO. The cells in each group were cultured for 48 h and images were obtained. The results revealed that SA12 inhibited the migration of MDA-MB-231 and MCF-7 breast cancer cells, and the cell migration distances were reduced compared with the control group (P<0.05; Fig. 1). The percentage of migrated cells was 51.3 and 34.6% in MDA-MB-231 and MCF-7 cells, compared with the control group, respectively (data not shown).

SA12 inhibits the migration of MDA-MB-231 and MCF-7 cells in Transwell assays. To further determine the inhibitory effect of SA12 on the migration of MDA-MB-231 and MCF-7 breast cancer cells, Transwell assays were performed. The cells were treated with 100 μM SA12, with 0.1% DMSO-treated cells used as the control. The cells were harvested following 24 h incubation and the number of cells migrated from the basement membrane of the upper chambers in 5 visual fields was counted and the average number was calculated. The results revealed that SA12 significantly inhibited the migration of MDA-MB-231 and MCF-7 breast cancer cells following 24 h of treatment, and the number of migrated cells were
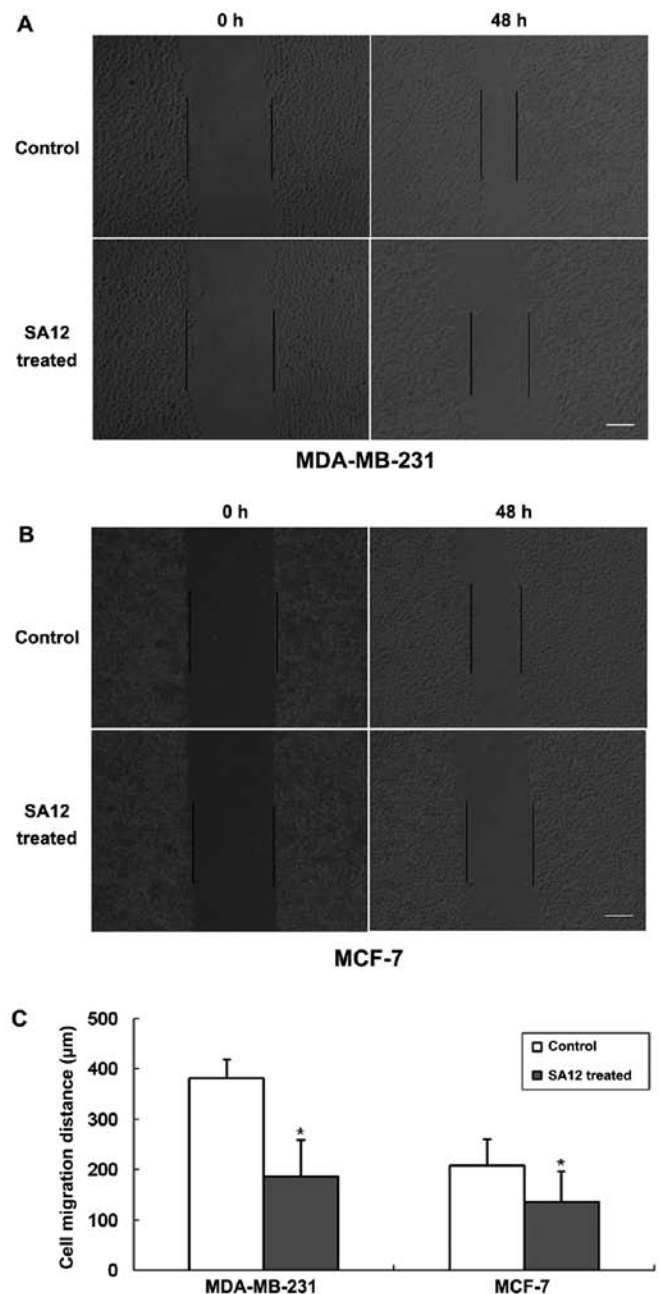

Figure 1. SA12 inhibited the migration of breast cancer cells in wound healing assays. SA12 inhibited the migration of (A) MDA-MB-231 and (B) MCF-7 breast cancer cells following 48 h of treatment. (C) Quantification of the extent of wound closure. Scale bars represent 200 μm. *P<0.05 vs. the corresponding control group.

decreased compared with the control groups (P<0.05; Fig. 2). The percentage of migrated cells was 38.2 and 29.8% in MDA-MB-231 and MCF-7 cells, respectively, compared with the controls (data not shown). These results revealed that SA12 inhibited the migration of breast cancer MDA-MB-231 and MCF-7 cells.

SA12 increases the mRNA expression levels of CDH1, nm23-H1 and BRMS1. The tumor metastasis suppressor genes, CDH1, nm23-H1 and BRMS1 are important negative regulators in tumor metastasis (6). To investigate the possible mechanism of action behind how SA12 inhibited the metastasis of MDA-MB-231 and MCF-7 breast cancer cells, the
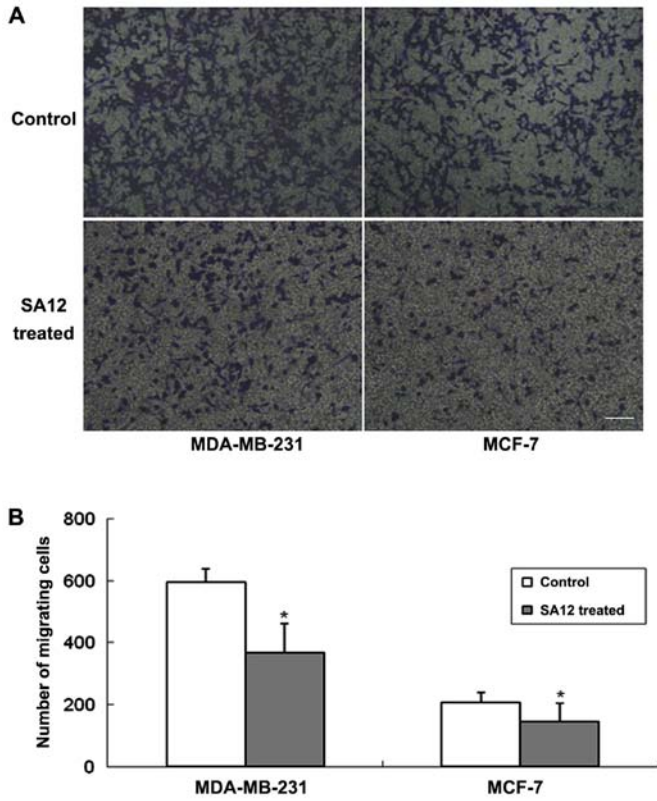
Figure 2. SA12 inhibited the migration of breast cancer cells in Transwell assays. (A) SA12 inhibited the migration of MDA-MB-231 and MCF-7 breast cancer cells after 24 h of treatment. (B) The number of migrating cells was found to significantly decrease in both cell lines after treatment. Scale bar represents 200 μm. *P<0.05 vs. the corresponding control group.
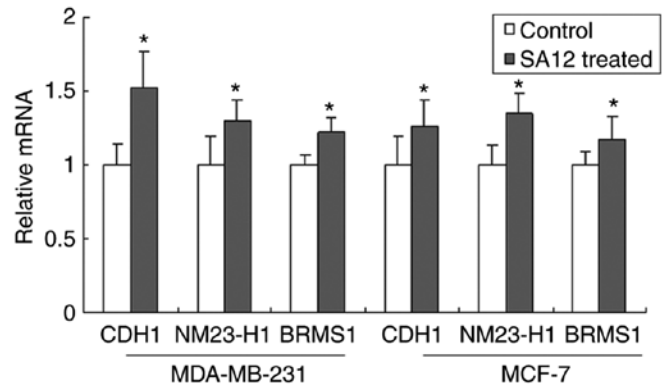

Figure 3. SA12 enhanced mRNA expression levels of CDH1, nm23-H1 and BRMS1. Compared with that in the controls, the mRNA expression levels of CDH1, nm23-H1 and BRMS1 were increased in MDA-MB-231and MCF-7 cells after treatment with 100 μM SA12. *P<0.05 vs. the corresponding control group. BRMS1, breast cancer metastasis suppressor 1; CDH1, cadherin 1; nm23-H1, non-metastasis 23-H1.
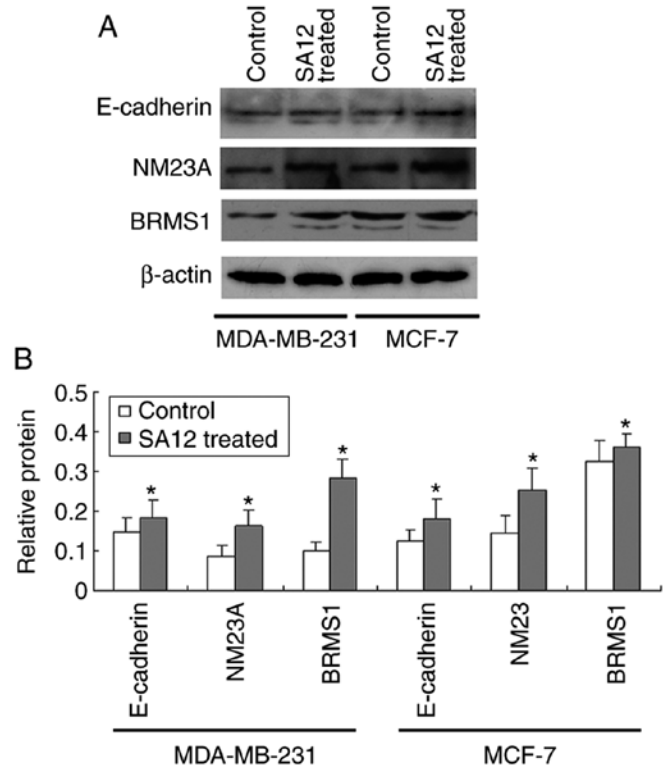

Figure 4. SA12 enhanced protein expression levels of E-cadherin, NM23A and BRMS1. (A) After treatment with 100 μM SA12 for 48 h, the protein expression levels of E-cadherin, NM23A and BRMS1 in MDA-MB-231 and MCF-7 cells were increased. (B) The percentage of E-cadherin, NM23A and BRMS1 protein expression levels were increased in MDA-MB-231 and MCF-7 cells compared with that in the controls. *P<0.05 vs. the corresponding control group. BRMS1, breast cancer metastasis suppressor 1; NM23A, non-metastatic 23A..

mRNA expression levels of CDH1, nm23-H1 and BRMS1 were detected using RT-qPCR. As presented in Fig. 3, the mRNA expression levels of CDH1, nm23-H1 and BRMS1 were significantly increased after treatment with 100 μM SA12 for 48 h (all P<0.05). The percentage increase in the mRNA levels of the aforementioned markers in MDA-MB cells compared with that in the controls was 51.9, 30.2 and 22.3%, respectively, and 26.1, 34.4 and 17.2%, respectively, in MCF-7 cells. These results suggested that treatment with SA12 significantly increased the mRNA expression levels of CDH1, nm23-H1 and BRMS1 in MDA-MB-231 and MCF-7 breast cancer cells.

SA12 enhances the protein expression levels of E-cadherin, NM23A and BRMS1. To further verify the aforementioned results, the effect of SA12 on the protein expression levels of tumor metastasis suppressors E-cadherin, NM23A and BRMS1 in MDA-MB-231 and MCF-7 breast cancer cells were examined using western blot analysis. As presented in Fig. 4, following treatment with 100 μM SA12 for 48 h, the protein expression levels of E-cadherin, NM23A and BRMS1 in MDA-MB-231 and MCF-7 cells was significantly increased (all P<0.05). The percentage increase in the aforementioned protein levels in MDA-MB-231 cells compared with that in the controls was 42.1, 76.3 and 41.9%, respectively, and 25.2, 32.8 and 23.0%, respectively, in MCF-7 cells. The results indicated that the protein expression levels of E-cadherin, NM23A and BRMS1 were also increased following SA12 treatment. SA12 increased the expression of the tumor metastasis suppressors E-cadherin, NM23A and BRMS1 in MDA-MB-231 and MCF-7 cells. The upregulation of E-cadherin, NM23A and BRMS1 may be responsible for the SA12-induced inhibition of metastasis to the MDA-MB-231 and MCF-7 cells.

Discussion

Malignant tumor metastasis is a complex biological process, involving a number of cellular interactions between tumor and stroma cells. Metastasis occurs when tumor cells break away from the primary tumor and migrate through the extracellular matrix (9). Tumor metastasis suppressor genes, including CDH1, nm23-H1 and BRMS1 are important negative regulators, which serve important roles in inhibiting tumor metastasis (6,7,10). A previous study identified an anti-tumor peptide SA12, which was indicated to inhibit proliferation and arrest the cell cycle of MDA-MB-231 and MCF-7 breast cancer cells (4,5). In the present study, the anti-tumor peptide SA12 inhibited the migration of MDA-MB-231 and MCF-7 breast cancer cells and enhanced the expression of the tumor metastasis suppressor genes, CDH1, nm23-H1 and BRMS1. These genes were also demonstrated to be associated with the SA12-induced inhibition of breast cancer cell migration.

Breast cancer metastasis is one of the leading causes of mortality in women and is regulated by adhesion molecules such as the cadherin superfamily (11). E-cadherin, which is the gene encoding product of CDH1, is an important member of the cadherin superfamily, which maintains the balance of cell-cell and cell-extracellular matrix adhesion (12). E-cadherin is a transmembrane glycoprotein expressed in human epithelial tissues, which connects epithelial cells together and serve a role in cell adhesion, cell morphology and cell movement (13). Intercellular adhesion serves an important role in the invasion and metastasis of tumor cells (14). Dysregulation of E-cadherin in tumors is associated with the epithelial-mesenchymal transition and subsequently induces tumor cell dissemination as well as promoting cell migration and invasion (15). E-cadherin supports the function of cell adhesion molecules in the repression of tumor metastasis in breast cancer (11,16). Studies from a meta-analysis revealed that reduced E-cadherin expression levels are associated with a poorer prognosis in breast cancer tissue; therefore, may be a potential therapeutic target for treating breast cancer (17). In the present study, the anti-tumor peptide SA12 was indicated to enhance the expression of the tumor metastasis suppressor gene CDH1 in MDA-MB-231 and MCF-7 cells. These results are consistent with a previous report that revealed that E-cadherin influences the imbalance of homogeneous adhesion between tumor cells and heterogeneous adhesion between tumor cells and stromal cells (18). This imbalance is crucial for the detachment of tumor cells from the primary tumor and for the invasion or metastasis of independent tumor cells (19-21).

Furthermore, the present study revealed that SA12 treatment increased the expression of nm23-H1 and BRMS1 in MDA-MB-231 and MCF-7 cells, which are also tumor metastasis suppressor genes. NM23A, which is a potential metastasis suppressor encoded by the nm23-H1 gene (22,23), was indicated to be highly expressed in differentiated tissues, but at lower levels in breast cancer, gastric cancer, bladder cancer, intestinal cancer and osteosarcoma tissues (24,25). The expression levels of nm23-H1 are negatively correlated with lymph node metastasis and reflect the metastatic ability of tumors (26-28). Phosphorylation of kinase suppressor of Ras1 (KSR1)-serine392 by NM23A is known to reduce ERK activation and inhibit the ERK-MAPK signaling pathway, which serves a crucial role in cancer metastasis (29). Another

important tumor metastasis suppressor gene, BRMS1, which was discovered in breast carcinoma cells, has since been identified to suppress metastasis in a variety of cancer types (30). A previous study demonstrated that BRMS1 regulates the interactions between tumor cells and the tumor microenvironment, affecting the key events of metastasis, including the inhibition of migration and invasion, as well as the initiation of growth by single cells and promotion of anoikis (31). Increased expression of BRMS1 restores the homotypic and heterotypic intercellular communication of gap junctions (30). Additionally, a role for BRMS1 in transcriptional regulation has also been revealed since the identification of BRMS1 in the mammalian Sin3 histone deacetylase complex (30,31).

In conclusion, the anti-tumor peptide SA12 was revealed to inhibit the metastasis of MDA-MB-231 and MCF-7 breast cancer cells in the current study. SA12 enhanced the expression of the tumor metastasis suppressor genes, CDH1, nm23-H1 and BRMS1, and maybe responsible for SA12-induced inhibition of metastasis in MDA-MB-231 and MCF-7 cells. To more conclusively demonstrate that CDH1, nm-23-H1 and BRMS1 are involved in this suppression, knockdown experiments should be performed, which is a limitation of the present study and a future direction for research. A more in-depth study should focus on the detailed molecular mechanisms of action behind the reduction in angiogenesis induced by SA12. Further studies to reveal the molecular mechanisms of action underlying the SA12-induced inhibition of breast cancer cell metastasis and the involvement of tumor metastasis suppressor genes in the cell signal pathways are required to expand on the findings of the present study.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LY designed the study, acquired the data and drafted the manuscript; FL performed the statistical analysis; ZG processed the data; XC analyzed the results; KD and HZ interpreted the data and revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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