Matrine suppresses breast cancer metastasis by targeting ITGB1 and inhibiting epithelial-to-mesenchymal transition

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Received November 27, 2018; Accepted October 17, 2019

DOI: 10.3892/etm.2019.8207

Abstract. Metastasis can be a fatal step in breast cancer progression. Effective therapies are urgently required due to the limited therapeutic options clinically available. The aim of the present study was to investigate the effect of matrine (MAT), a traditional Chinese medicine, on the proliferation and migration of human breast cancer cells and its underlying mechanisms of action. The proliferation of MDA-MB-231 cells was inhibited and apoptosis was induced following treatment with MAT, as determined by MTT and Annexin -V-FITC/PI assays. Western blot analysis was used to detect the LC-3II/I levels and the results suggested that tumor autophagy is involved in the anti-tumor activity of MAT. To the best of our knowledge, this is the first study to report that MAT inhibits MDA-MB-231 and MCF -7 cell motility, potentially by targeting integrin \( \beta_1 \) (ITGB1) and epithelial-to-mesenchymal transition (EMT), as indicated by Transwell® and siRNA interference assays. In conclusion, ITGB1 and EMT are involved in MAT-induced breast carcinoma cell death and the inhibition of metastasis. This may lead to the development of novel compounds for the treatment of breast cancer metastasis.

Introduction

Breast cancer is the most invasive type of malignancy in females worldwide, leading to >39,000 deaths in the USA each year (1). Although a number of treatments have seen significant improvement over the years, breast cancer remains a paramount health issue and is at the forefront of medical research (2). It can be considered a heterogeneous disease segmented into five molecular subtypes: Luminal A, luminal B, HER2 -enriched, basal -like and claudin -low (3). Treatment options for these cases include surgery, chemotherapy and/or radiotherapy (4). However, breast cancer remains a leading cause of cancer-associated mortality, especially among young women (5). Therefore, the treatments that currently available for patients with breast cancer require urgent improvement.

Chinese traditional herbs can kill tumor cells by acting on multiple targets with few adverse effects, making it an area of great research interest. Matrine (MAT), an alkaloid derived from \( \textit{Sophora Flavescens} \), is a traditional Chinese medicine used for the treatment of aggressive cancers (6). MAT was found to inhibit the progress of hepatic, cervical and gastric cancer (7), with a plethora of studies focusing on the pharmacological and clinical applications of MAT (8-10). To the best of our knowledge, little attention has previously been paid to the effects of MAT on breast cancer metastasis. Migration is the driving process of cancer metastasis and corresponds to poor clinical symptoms, a deterioration in health and eventual death (11). A previous study compared different datasets and identified integrin \( \beta_1 \) (ITGB1) as one of the crucial genes involved in breast cancer cell migration (12). In addition, ITGB1 is reportedly highly expressed in the claudin -low subtype of breast cancer (13). However, whether MAT inhibits the migration of breast cancer cells by mediating ITGB1 expression remains unclear.

In the present study, it was demonstrated that MAT dose-dependently inhibits proliferation and induces apoptosis in MDA-MB-231 cells. In addition, the present data provided novel evidence of MAT-induced inhibition of cell migration by targeting ITGB1 and the epithelial-to-mesenchymal transition (EMT) in breast cancer.

Materials and methods

Reagents. MAT was purchased from Sigma-Aldrich (Merck KGaA) and stored at 4°C. MAT was later dissolved in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) at a concentration of 20 mg/ml and stored at -20°C. Chloroquine diphosphate salt (CQ) was purchased from Sigma-Aldrich (Merck KGaA).

Cell culture. The human breast cancer cell lines MDA-MB-231 and MCF-7 (Shanghai Institute of Cell Biology, Chinese Academy of Sciences) were cultured in RPMI-1640...
medium supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc.) and 100 µg/ml penicillin/streptomycin (HyClone; GE Healthcare Life Sciences) in a humidified atmosphere containing 5% CO₂ at 37°C.

**Cell proliferation assay.** To test the effect of MAT on MDA-MB-231 proliferation, 4x10⁴ cells/well were seeded into 96-well culture plates (Num³™; Thermo Fisher Scientific, Inc.) in 100 µl RPMI-1640 medium and then cultured in a 37°C 5% CO₂ incubator overnight. The supernatant was then changed to one that contained different doses of MAT (0, 1 and 2 mg/ml) and cultured for 24 and 48 h, followed by another 2 h after 20 µl MTT (5 mg/ml; Promega Corporation) was added to each well. Optical density values were obtained using a plate reader at a wavelength of 490 nm.

**Cell apoptosis assay.** Annexin-V-FITC/PI double staining assays were performed to detect the effects of apoptosis on MDA-MB-231 cells. Cells were exposed to MAT (2 mg/ml) or the vehicle control for 48 h in a 24-well plate (3x10⁴ cells/well), after which each group was washed with PBS three times followed by staining at room temperature with an Annexin-V-FITC Apoptosis Detection kit I (RT; BD Biosciences; Becton, Dickinson and Company). The number of apoptotic cells was counted using flow cytometry (FACSCanto™; BD Biosciences; Becton, Dickinson and Company) and the FlowJo Software (version 8.2.4; FlowJo LLC), according to the manufacturer's protocol.

**Cell migration assay.** Migratory abilities of MDA-MB-231 and MCF-7 cells were determined using a chemotaxis chamber (Corning Life Sciences) according to the manufacturer's protocol. In this assay, cell motility was assessed by migration through a membrane (24-well Transwell® plate, 8-µm pore size) towards a chemoattractant. Briefly, cells were seeded into the upper chambers of the Transwell® inserts (3x10⁴ per well) towards a chemoattractant. Following incubation at 37°C for 16 h, cells were stained with Calcein-AM (0.2 µg/ml; cat. no. C3100MP; Invitrogen; Thermo Fisher Scientific, Inc.) at RT for 30 min. The migrated cells were counted using an eclipse Ti inverted microscope (Nikon Corporation). The number of cells that had migrated was determined using MetaMorph image analysis software (version 4.0; Molecular Devices, LLC) and the results are presented as the mean ± standard deviation (n=3).

**RNA interference.** Oligonucleotides for human ITGB1 siRNA kit was purchased from Guangzhou RiboBio Co., Ltd. The kit contains three predesigned duplexes targeting a specific ITGB1 gene. Cells were transfected with ITGB1 siRNA or NC at the concentration of 50 nmol/l using the opti-MEM plus X-treme GENE siRNA transfection reagent (Roche Diagnostics) according to the protocol of the manufacturer. The ITGB1 siRNA sequence was as follows: Forward, 5'-CCA UUCUGAUAUGCUGA-3' and reverse, 5'-UCAGAGAUUC AUCAGAAUGG-3'. After 48 h of post-transfection, western blot analyses were further performed.

**RNA isolation and reverse transcription-quantitative (RT-q)PCR.** Briefly, total cellular RNA was extracted using TRIzol® (Life Technologies; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Total RNA was extracted using TRIzol® and a Total RNA kit (Tiangen Biotech Co., Ltd.), cDNA was generated at 37°C using 1 mg total RNA and a QuantiTect Reverse Transcription kit (Qiagen GmbH). RT-qPCR was performed using the SYBR Green (Bio-Rad Laboratories, Inc.) method on an ABI Prism 7000 Sequence Detection System (Life Technologies; Thermo Fisher Scientific, Inc.). The primer for ITGB1 was 5'-CTCTACTTCT GCAAGATGGAATG-3' (forward) and 5'-CTTTTGATCCACCG TTGTATACC-3' (reverse). The primer for β-actin (control) was 5'-CCACACCCCGCACCAGTTCG-3' (forward) and 5'-TACAGCGGGGGAGCAGCTGT-3' (reverse). All primers were purchased from Sangon Biotech Co., Ltd., and diluted in DEPC water. The qPCR reaction was performed as follows: 95°C For 30 min, 40 cycles of 95°C for 15 sec and 56°C for 20 sec. To confirm the amplification specificity, the PCR products were subjected to melting curve analysis. The relative mRNA level of ITGB1 was normalized to the β-actin mRNA and analyzed by the comparative threshold (Cq) cycle method (2ΔΔCq), according to previous research (14).

**Western blot analysis.** Protein concentration was measured using a bicinchoninic acid Protein Assay Reagent (Pierce; Thermo Fisher Scientific, Inc.). Total protein (20 µg/well) was separated via 10% SDS-PAGE and then transferred onto PVDF membranes at 250 mA for 1 h. Membranes were blocked at 37°C for 2 h with 5% non-fat milk in Tris-buffered saline/0.1% Tween-20 and then incubated at 4°C overnight with the following primary antibodies: Anti-ITGB1 (1:1,000; cat. no. ab183666; Abcam), anti-β-LC3 (1:1,000; cat. no. ab128025; Abcam), anti-epithelial (E)-cadherin, anti-neural (N)-cadherin, anti-vimentin (1:1,000; cat. no. 9782T; EMT Antibody Sampler kit Cell; Cell Signaling Technology, Inc.), anti-β-galactosidase (1:5,000; cat. no. 10494-1-AP; ProteinTech Group, Inc.) and anti-β-actin (1:5,000; cat. no. 20536-1-AP; ProteinTech Group, Inc.). Subsequently, membranes were incubated at 37°C for 2 h with anti-rabbit IgG secondary antibodies (1:3,000; cat. no. 14708; Cell Signaling Technology, Inc.) and the immunoblotted proteins were then detected using an Odyssey Western Blotting Detection System (Genetech Co., Ltd.) and Odyssey software (version 1.2).

**Statistical analysis.** All data are expressed as the mean ± standard deviation based on experiments performed in triplicate, and were analyzed using SPSS 18.0 statistical analysis software (SPSS, Inc.). One-way analysis of variance followed by Student-Newman-Keuls post hoc test was used. P<0.05 was considered to indicate a statistically significant difference.

**Results**

MAT inhibits MDA-MB-231 cell growth by inducing apoptosis. To determine the role of MAT in breast cancer, MDA-MB-231 cells were treated with various concentrations of MAT for 24 and 48 h, following which an MTT assay was performed to evaluate proliferation (Fig. 1A). The data demonstrated that
MAT inhibited the proliferation of MDA-MB-231 cells in a dose- and time-dependent manner. Tumor growth was not only associated with abnormal proliferation, but was also dependent on a reduction in apoptosis. To confirm that the apoptosis observed in the cancer cells was induced by MAT, an Annexin-V-FITC/PI apoptosis assay was performed (Fig. 1B).

For flow cytometry, MDA-MB-231 cells were treated with or without MAT (2 mg/ml) for 48 h. Cells in the late and early stages of apoptosis were observed in the upper and lower right quadrant of the plots (Q2 and Q4 areas), respectively. These results indicated that treatment with MAT was able to impair proliferation and induce apoptosis in breast cancer cells.

Role of autophagy in MAT-induced decrease in cell growth. Previous studies have demonstrated that both autophagy and apoptosis are involved in the effects observed in cancer cells treated with MAT, including acute myeloid leukemia (15) and osteosarcoma (16) cells. It was verified that MAT inhibits cell proliferation and induces apoptosis. In addition, the fact that apoptosis often occurs simultaneously with autophagy prompted the present study to investigate the association between MAT and autophagy. First, the autophagy inhibitor CQ, a small alkaline molecule that accumulates in lysosomes and reduces hydrolysis (17), was used. As shown in Fig. 2A and B, when MDA-MB-231 and MCF-7 cells were exposed to various doses of MAT with and without CQ, proliferation was significantly decreased in the MAT+CQ group, as compared with the MAT alone group (P<0.05).

Next the expression of LC3-II/I, one of the main autophagy regulatory proteins (18), was investigated following exposure to MAT in cells pre-treated with CQ for 1 h. The results showed that the expression of LC3-II/I was accumulated in MDA-MB-231 and MCF-7 cells treated with MAT or with MAT+CQ. In addition, LC3-II/I in cells treated with MAT+CQ was significantly upregulated, as compared with cells treated with MAT alone (P<0.001; Fig. 2C and D). These
results therefore suggested that autophagy is involved in MAT-induced breast cancer cell apoptosis.

*MAT decreases the migratory capacity of MDA-MB-231 and MCF-7 cells potentially by targeting ITGB1.* Metastasis is a primary cause of morbidity and mortality in patients with cancer (19), and cell migration and invasion are the most important steps in this complex process. Therefore, transwell assays were performed to detect the migratory capacity of breast cancer cells and found that MAT (2 mg/ml) significantly inhibited the migration of MDA-MB-231 and MCF-7 cells (P<0.05; Fig. 3A), indicating that MAT may be a promising anti-metastatic agent for breast cancer.

ITGB1 is reportedly highly expressed in breast cancer and correlates with cell migration (20). Therefore the levels of ITGB1 in MDA-MB-231 and MCF-7 cells following treatment with MAT were investigated. As hypothesized, following incubation with MAT, the relative mRNA expression of ITGB1 was significantly decreased to 64.3 and 60.3% in MDA-MB-231 and MCF-7 cells, respectively (P<0.05). The protein activity of ITGB1 also decreased to 53 and 61.6% in MDA-MB-231 and MCF-7 cells, respectively compared with the control (CTL) group (Fig. 3B). To further confirm that ITGB1 is involved in MTA-impaired migration, siRNA was used to silence ITGB1 expression in MDA-MB-231 and MCF-7 cells. ITGB1-silencing was verified by RT-qPCR and western blot analysis (Fig. 3C and D). Furthermore, transfection with ITGB1 siRNA decreased the migratory capacity of MDA-MB-231 and MCF-7 cells (Fig. 3E). Overall, these data indicated that ITGB1 is involved in MAT-induced inhibition of breast cancer cell motility.
MAT regulates EMT in breast cancer cells. During EMT, cells lose epithelial characteristics and obtain mesenchymal properties, including decreased E-cadherin and increased N-cadherin and vimentin. Considering the significant effect of EMT on tumor cell migration, as well as that the process can be mediated by ITGB1 (21), the levels of certain...
EMT-associated markers were detected. MDA-MB-231 cells exhibited a mesenchymal phenotype, while MCF-7 cells exhibited the properties of epithelial cells. Thus, the expression of E-cadherin, N-cadherin and vimentin between MDA-MB-231 and MCF-7 cells was different in Fig. 4. In addition, the western blot assays of Fig. 4A and B were not performed on the same PVDF membranes, so the levels of these proteins in these two cells cannot be compared due to variation in experimental conditions. Incubation with MAT can markedly increase the expression of E-cadherin and reduce the levels of N-cadherin and vimentin compared with their CTL group. These changes demonstrated that EMT in MAT-treated MDA-MB-231 and MCF-7 breast cancer cells is blocked, reducing cell metastasis.

Discussion

Natural resources, especially traditional plant-based medicines, are being increasingly investigated as anti-tumor agents (22). MAT is a component of one such traditional plant (Sophora Flavescens), which has been shown to exert therapeutic effects on various types of solid tumors (23,24). In the present study, it was demonstrated that MAT exerts therapeutic effects on MDA-MB-231 and MCF-7 breast cancer cells through inhibiting proliferation and migration. Mechanistically, MAT induces apoptotic cell death, influences ITGB1 expression and blocks EMT to produce these anti-cancer effects.

Previous reports have demonstrated that MAT inhibits the growth of various types of tumors by inducing apoptosis and cell cycle arrest (25,26). The present study reinforced this by demonstrating the ability of MAT to inhibit MDA-MB-231 and MCF-7 cell growth and induce apoptosis. To investigate the mechanisms of MAT-induced cell growth inhibition, cell autophagy and LC3-II/I, two forms of LC3 were focused on. Cytoplasmic LC3-I is conjugated to phosphatidylethanolamine to form LC3-II, which is closely associated with autophagosome membranes and serves as a reliable marker for the monitoring of autophagy (27). First, it was found that cell growth inhibition was increased following the application of the autophagy inhibitor CQ, indicating that impaired autophagy aggravates MAT-induced cell growth inhibition. Secondly, the expression levels of LC3-II/I were further examined and the present data showed that the level of LC3-II/I was elevated following MAT treatment. In addition, its expression markedly increased with CQ co-treatment, suggesting that cancer cell autophagy and apoptosis could be targets for enhancing the anti-tumor effects of MAT.

Metastasis is a complex multistep process that involves cell growth, migration and transportation via the blood vessels. Therefore, the effects of MAT on cell migration, a crucial step in breast cancer metastasis, were detected. The Transwell® assay showed that MAT (1 mg/ml) may have inhibited cell migration, but no significant differences were observed. However, MAT (2 mg/ml) exhibited a marked
ability to reduce cell migration in both MDA-MB-231 and MCF-7 cells. Consistent with present results, other studies reported that MAT is able to inhibit cell migration in multiple types of cancer (28-30), suggesting that MAT may be a promising anti-metastatic drug. Among metastasis-related genes, integrins are considered to mediate cell-cell crosstalk across the cellular membrane and play an important role in the maintenance of extracellular matrix (ECM) molecules (31). Furthermore, ITGB1 plays critical roles in breast cancer cell proliferation and motility, is highly expressed in aggressive breast tumors and drives metastasis (32,33). ITGB1 is a major adhesion receptor for various ECM components; therefore, the present study investigated the expression of ITGB1 in breast cancer cells following treatment with MAT. The mRNA and protein expression of ITGB1 was impaired in MDA-MB-231 and MCF-7 cells following MAT treatment. Based on siRNA analysis results, the present study hypothesized that ITGB1 and its downstream signaling network is regulated by MAT. The present study therefore provided a new target through which MAT can exert anti-cancer effects.

EMT, a phenotypic cellular process, leads to the loss of cell-cell adhesion. Consequently, cancer cell motility, migration and metastasis is triggered (34). Alterations in cadherin expression are typical in EMT, as is the downregulation of E-cadherin and upregulation of N-cadherin (35) and vimentin (as well as other mesenchymal proteins). A western blot experiment was performed in mesenchymal-like MDA-MB-231 cells and the effects of MAT on EMT were examined. The results showed that incubation of MAT in MDA-MB-231 cells increased the expression of epithelial markers and decreased the expression of mesenchymal marker. Collectively, these data showed that MAT interfered EMT in breast cancer cells. Previous studies also showed ZO1 (36) and E-cadherin (37) were upregulated during EMT process in MDA-MB-231 cells. In the present study, treatment with MAT resulted in the upregulation of E-cadherin and downregulation of N-cadherin and vimentin, strongly indicating that EMT is blocked by MAT. In addition, studies have demonstrated that ITGB1 and ITGB3 exhibit tumor-promoting effects via facilitating EMT in breast cancer and nasopharyngeal carcinoma (38,39). The knockdown of ITGB1 partly increased the expression of E-cadherin and decreased that of vimentin, fibronectin and N-cadherin in BT549 and Hs578T breast cancer cells (40). In the present study, it was shown that the induction and regulation of EMT by MAT may involve multiple molecular mechanisms, including the inhibition of ITGB1 expression.

The limitation of the present study is the lack of specific mechanism by which MAT regulates ITGB1. The authors will measure whether ITGB1 is transcriptionally regulated by MAT via luciferase reporter assays and explore the possibility of reversing MAT-inhibited cellular proliferation and migration by overexpressing ITGB1 in MDA-MB-231 and MCF-7 cells in the following study.

In conclusion, the present results revealed that MAT exerts modulatory effects on apoptotic cell death and that the inhibition of MAT-induced migration is potentially affected through the attenuation of ITGB1 and EMT. Therefore, MAT may serve as a novel suppressor of breast cancer.

Acknowledgements
The authors would like to thank Chief Attending Physician Dr Qinghua Yao, Zhejiang Cancer Hospital, for her durable support and constructive guidance.

Funding
The present study was supported by Zhejiang Medical and Health Science and Technology Plan (grant no. 2013KYB04).

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors’ contributions
LR wrote the manuscript and performed the experiment. WM and LW collected and interpreted the data. XW obtained funding and designed the study. All authors have read and approved the manuscript for publication.

Ethics approval and consent to participate
All experimental protocols were performed in accordance with the regulation of the Helsinki Declaration and were approved by Ethics Committee of our hospital. Written consent of the participants was obtained.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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