Original Paper

Matrine Inhibits Breast Cancer Growth Via *miR-21/PTEN/Akt* Pathway in MCF-7 Cells

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Key Words

Matrine • Breast cancer • Cell cycle • miR-21 • PTEN • pAkt

Abstract

Background: Matrine is one of the major alkaloids extracted from *Sophora flavescens* and has been used clinically for breast cancer with notable therapeutic efficacy in China. However, the mechanisms are still largely unknown. **Methods:** Cell viability was analyzed by MTT assay. After MCF-7 cells were treated with matrine for 48h, apoptosis was detected by flow cytometry, TUNEL assay and transmission electron microscopy, and the cell cycle distribution was also analyzed by flow cytometry. Further, the expression of PTEN, pAkt, Akt, pBad, Bad, p21^{WAF1/CIP1}, and p27^{KIP1} was determined by Western blot. Changes of miR-21 level were quantified by real-time RT-PCR. After miR-21 was transfected in MCF-7 cells, PTEN protein level was measured by Western blot. **Results:** Matrine inhibited MCF-7 cell growth in a concentration- and time-dependent manner, by inducing apoptosis and cell cycle arrest at G₁/S phase. Matrine up-regulated PTEN by downregulating miR-21 which in turn dephosphorylated Akt, resulting in accumulation of Bad, p21^{WAF1/CIP1} and p27^{KIP1}. **Conclusion:** Our study unraveled, for the first time, the ability of matrine to suppress breast cancer growth and elucidated the miR-21/PTEN/Akt pathway as a signaling mechanism for the anti-cancer action of matrine. Our findings also reinforce the notion that miRNAs can act as mediators of the therapeutic efficacy of natural medicines.

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Introduction

Breast cancer is the most common malignancy and the leading cause of cancer death in women worldwide. There were about 1,383,500 new female breast cancer cases and 458,400 of the total cancer deaths in 2008 [1]. Many patients especially with metastatic breast cancer must accept chemotherapy, such as anthracyclines and taxanes, which made patients encounter cumulative toxicity and additional tolerability problems [2]. Even more, most patients eventually develop resistance to chemotherapy, which frequently results in subsequent recurrence and metastasis.

Accumulating evidence suggests that natural agents open up a new avenue for successful treatment of cancers, especially by combining with conventional therapeutics [3]. Matrine is one of the effective monomers found in *Sophora flavescens*. Its chemical formula is $C_{15}H_{24}N_2O$. Matrine has long been used for the treatment of viral hepatitis, cardiac arrhythmia, and skin inflammations [4]. It has been well documented that matrine inhibits growth and proliferation of various cancer cells through inducing apoptosis and cell cycle arrest [5-7]. Recent observations showed that matrine and its compounds inhibit proliferation, migration, and metastasis of breast cancer both *in vitro* and *in vivo* [8-10], and were used as adjuvant therapy in China to improve the 5-year survival rate and life quality as well as immune functions of patients with breast cancer. However, little is known about the mechanisms underlying the therapeutic effects of matrine.

MicroRNAs (miRNAs) play important roles in the development of various types of cancers [11, 12]. MiRNA-21 (miR-21), as the most overexpressed miRNA in the vast majority of cancers, targets many important tumor suppressors to promote breast cancer growth, proliferation, migration, and metastasis [13, 14]. PTEN (phosphatase and tensin homolog, deleted on chromosome ten), as one of the target genes of miR-21 [15], is a dual lipid and protein phosphatase that can dephosphorylate its downstream targets. Mutation of PTEN has been observed in about 5% of primary breast cancer and loss of expression rate is as high as 48% associated with a poor prognosis. Inactivation of PTEN leads to loss of its lipid phosphatase activity and accumulation of phosphatidylinositol 3,4,5-trisphosphate (PIP_3), resulting in phosphorylation of Akt [16]. As a result, on one hand, inactivation of PTEN stimulates cell cycle progression and proliferation by down-regulating p21^{WAF1/CIP1} and p27^{KIP1}; on the other hand, it prevents cancer cells from apoptosis by inhibiting apoptotic pathways [17, 18]. It appears that the miR-21/PTEN/Akt signaling pathway plays a critical role in tumorigenesis. It is not known, however, whether the anti-cancer effects of matrine are related to this pathway.

In view of the established anti-breast-cancer effects of matrine and the tumorigenic role of the miR-21/PTEN/Akt pathway in breast cancer, we proposed that matrine may inhibit breast cancer growth via the miR-21/PTEN/Akt pathway. This study was designed to test this hypothesis.

Materials and Methods

Materials

Matrine (purity >95%) was purchased from Kangjiu Chemical Co, China, and was dissolved in physiological saline to prepare a 20 mM stock solution which was stored at 4°C in the dark.

Cell culture

Human breast cancer cell line MCF-7 was purchased from Shanghai cell bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in BPMI 1640 (Hyclone, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

MTT assay

Cells were seeded in 96-well plates at a density of 2×10^4 /ml (0.2 ml/well) for 12 h. After exposure to matrine for 24, 48 and 72 hours, respectively, 20 μ l methyl thiazolyl tetrazolium (MTT) solution (5 mg/ml) was added to each well and cells were incubated for another 4h. The formazan crystals were dissolved in 150 μ l dimethylsulfoxide (DMSO) and the absorbance of samples was measured at 490 nm by a microplate reader (Model 3550, Bio-Rad Laboratories, CA). All experiments were performed in triplicate for three times.

Cell apoptosis by flow cytometry

MCF-7 cells were collected after they had been treated with matrine at 0 (control), 0.4 mM (M1 group), 0.8 mM (M2 group) for 48h, and then washed twice with cold PBS. The cells were resuspended in 500 μ l binding buffer at a concentration of 10^6 /ml and then mixed with 10 μ l Annexin V (Beijing Biosea Biotechnology, CO.LTD, China) for 15 min in the dark at room temperature (RT), followed by the addition of 5 μ l PI (Beijing Biosea Biotechnology, CO.LTD, China). After incubation at RT in the dark for 5 min, samples were analyzed by a FACSria flow cytometry (BD Biosciences, San Jose, CA, USA).

TUNEL assay

TUNEL assay was carried out according to the manufacturer's protocol (In Situ Cell Death Detection Kit, POD, Roche, Germany). Briefly, MCF-7 cells were fixed in 4% paraformaldehyde in PBS after treatment with matrine at 0, 0.4, or 0.8 mM for 48h, then washed with PBS three times followed by 3% H_2O_2 in methanol for 10 min at RT. Next, cells on slides were incubated with 0.1% triton X-100 in 0.1% sodium citrate for 2 min on ice. Finally, cells were incubated with TUNEL reaction mixture for 1h, followed by DAPI for 5 min, at RT in the dark. Cells were analyzed by a fluorescence microscope (Eclipse 80I, Nikon, Tokyo, JP).

Examination of cell ultrastructure by transmission electron microscopy

After treatment with matrine at 0, or 0.8 mM for 48h, MCF-7 cells were fixed in 2.5% glutaraldehyde overnight at 4°C. The cells were then post-fixed with 1% osmium tetroxide for 2h, washed in PBS, and progressively dehydrated in a 10% graded series of 50%-100% ethanol and propylene oxide. The embedded blocks were cut into ultrathin sections with a microtome and stained with saturated uranyl followed by lead citrate. The ultrastructure of cells were examined by a transmission electron microscope (Hitachi H-7650, Tokyo, Japan), as previously described in detail [19].

Cell cycle analysis by flow cytometry

After starvation in the serum-free medium for 12h, MCF-7 cells were treated with matrine at 0, 0.4, and 0.8 mM for 48h in BPME 1640 supplemented with 10% fetal bovine serum. The cells were incubated with RNase (Sigma, USA) and PI (Sigma, USA) [20], and the DNA content was determined on a FACSCalibur system (BD Biosciences, San Jose, CA, USA) by using ModFitLT V3.2.1. To provide valid quantitative data, 10^5 individual cells were analyzed in each experiment.

Quantification of miR-21

Total RNA was extracted from MCF-7 cells by TRIzol® Reagent (Invitrogen, USA) following the manufacturer's protocol. MiR-21 levels were measured using the *mirVana*™ qRT-PCR miRNA Detection Kit (Ambion, USA) described elsewhere [21]. Primers for real-time PCR were 5'-GGGGTAGCTTATCAGACTGATG-3' (miR-21 forward) and 5'-TGTCGTGGAGCGCAATTG-3' (miR-21 reverse). Variations in expression of miR-21 between different RNA samples were calculated after normalization to U6.

Transfection of miRNAs

MCF-7 cells were transfected with miR-21 mimics using X-treme (Roche, Germany) following the manufacturer's protocol. After 48h transfection, the expression of PTEN protein was measured by Western blot.

Western blot

Western blot analysis was carried out as described elsewhere [21]. Cells were washed with cold PBS and lysed in lysis buffer (western blot lysate: protease inhibitor cocktail=100:1). The concentration of proteins was determined by BCA Protein Assay Kit. The samples were electrophoresed in 12% SDS-PAGE gels. After electrophoresis, gels were transferred to nitrocellulose membranes by electroblotting and blocked in 5% nonfat milk in PBS at RT for 2h. The primary antibodies including anti-PTEN (Cell Signaling,

Fig. 1. Effects of matrine on cell viability of MCF-7 cells. After cells had been treated with matrine at 0, 0.1, 0.2, 0.4, 0.8, and 1.6 mM for 24h, 48h and 72h respectively, cell viability of MCF-7 cells was determined by MTT assay. To determine the optimal concentrations, IC₅₀ was calculated from the dose-response curve with sigmoidal regression constructed using GraphPad 9.0 Software.

USA), anti-pAkt (Cell Signaling, USA), anti-Akt (Cell Signaling, USA), anti-pBad-Ser136 (Santa Cruz, USA), anti-Bad (Santa Cruz, USA), anti-p21^{WAF1/CIP1} (Santa Cruz, USA), and anti-p27^{KIP1} (Cell Signaling, USA) were used, with GAPDH as an internal control.

Data analysis

All data are presented as mean $\pm$ SD. Student's *t* test was used for two group comparison and One-way ANOVA followed by Bonferroni test for multiple comparisons. A two-tailed *P* < 0.05 was considered as statistically significant.

Results

Matrine inhibits breast cancer cells growth

The growth of breast cancer cell line MCF-7 was inhibited, as determined by MTT assay, after cells had been treated with matrine at 0.1, 0.2, 0.4, 0.8, and 1.6 mM for 24h, 48h, and 72h, respectively. It was found that matrine inhibited the growth of MCF-7 cells in a concentration- and time-dependent manner with an IC₅₀ (48h) of ~0.8 mM (Fig. 1).

Matrine induces apoptosis in breast cancer cells

We used three different approaches, flow cytometry, TUNEL assay and electron microscopy, to examine the effects of matrine on MCF-7 cell apoptosis. As shown in Figure 2, matrine induced significant apoptotic cell death in MCF-7 cells. Measurements from our flow cytometry experiments indicated that matrine at concentrations of 0.4 and 0.8 mM caused $13.98 \pm 0.98\%$ and $25.6 \pm 4.8\%$ apoptotic cell death, respectively (Fig. 2A & 2B). TUNEL assay for chromosomal fragmentation yielded the results in support of above data: matrine remarkably increased the number of TUNEL-positive cells or apoptotic cells (Fig. 2C). Transmission electron microscopic examination showed severe shrinking of cell morphology, nuclear fragmentation and apoptotic bodies in the presence of matrine in MCF-7 cells (Fig. 2D).

Matrine induces cell cycle arrest at G₁/S phase in breast cancer cells

Effects of matrine on cell cycle distribution were analyzed by flow cytometry in MCF-7 cells. The cells were found $63.96 \pm 0.67\%$ in G₁ phase, $23.53 \pm 1.13\%$ in S phase and $12.51 \pm 0.78\%$ in G₂ phase under control conditions (Fig. 3A). After being treated with matrine at 0.8 mM for 48h, the number of cells in G₁ phase was increased to $84.62 \pm 1.55\%$ (*P* < 0.001),

Fig. 2. Effects of matrine on apoptosis in MCF-7 cells. (A) After cells had been treated with matrine at 0 (Ctrl), 0.4 (M1), and 0.8 (M2) mM for 48h, apoptosis of MCF-7 was detected by flow cytometry. (B) Quantitative analysis was performed and data are expressed as mean $\pm$ SD. n=6 * p <0.05 versus Ctrl. (C) Apoptosis of MCF-7 cells was detected by TUNEL assay. TUNEL staining of corresponding cells as shown in the images. Apoptotic cells were significantly increased by 0.8 mM matrine. (D) Morphological alterations of cells were examined by electron microscopy. Normal cells contained an irregular nucleus and abundant ribosomes and rough endoplasmic reticulum. The cytoplasm was rich in mitochondria, and there were a small number of microvilli on the surface in Ctrl group. The cell with matrine showed an apoptotic appearance of nuclei (karyopyknosis) disappearance of nuclear pores, and karyorrhexis.

whereas that in S phase was decreased to $3.31\pm0.38\%$ ($P<0.001$), and no significant changes of the number of cells in G_2 phase were observed ($P>0.05$) (Fig. 3B).

Matrine down-regulates expression of miR-21

The level of miR-21 was determined by real-time RT-PCR after MCF-7 cells were treated with matrine for 48h. Figure 4A showed that matrine decreased the level of miR-21 in MCF-7

Fig. 3. Effect of matrine on cell cycle distribution of MCF-7. (A) After cells had been treated with matrine at 0 (Ctrl) and 0.8 (M2) mM for 48h, respectively, cell cycle distribution of MCF-7 was detected by flow cytometry. (B) Quantitative assay was performed and data are expressed as mean $\pm$ SD. n=6 *** p <0.001 vs Ctrl.

cells (0.76 ± 0.12 for M1/Ctrl and 0.46 ± 0.34 for M2/Ctrl). Forced expression of miR-21 by transfection markedly reduced the level of PTEN protein to 0.76 ± 0.11 of the control values (Fig. 4B).

Matrine regulates the PTEN/Akt pathway to induce apoptosis and cell cycle arrest

As miR-21 has been shown to control the expression and activities of PTEN and Akt [15] which in turn controls its downstream targets pBad, Bad, p21^{WAF1/CIP1}, p27^{KIP1} to regulate apoptosis and cell cycle arrest, we investigated whether the miR-21/ PTEN/Akt pathway plays a role in the matrine-induced apoptosis and cell cycle arrest in MCF-7 cells by measuring the changes of the protein levels of PTEN and phosphorylated Akt (pAkt) by Western blot analysis. As shown in Figure 5A & 5B, PTEN was significantly up-regulated by matrine compared with control (1.75 ± 0.72 fold M2 over Ctrl), while pAkt was down-regulated (0.63 ± 0.22 for M1/Ctrl and 0.58 ± 0.14 for M2/Ctrl). Consistently, pBad was downregulated by matrine (0.49 ± 0.17 for M2/ Ctrl), whereas Bad, p21^{WAF1/CIP1} (1.75 ± 0.52 M2/Ctrl) and p27^{KIP1} (2.87 ± 1.30 M2/Ctrl) were up-regulated (Fig. 5C & 5D).

Fig. 4. (A) Matrine downregulates miR-21 in MCF-7 cells. (B) Effect of miR-21 on the expression of PTEN protein in MCF-7 cells. After MCF7 cells had been transfected with miR-21, the expression of PTEN protein level was measured by Western blotting. Quantitative analysis was performed with GAPDH as an internal control. Data are expressed as mean $\pm$ SD normalized to Ctrl. n=6 * p <0.05 vs Ctrl, *** p <0.001 vs Ctrl.

Discussion

In the present study, we demonstrate that matrine induces apoptotic cell death and cell cycle arrest likely via downregulating miR-21 to alter the expression and activities of PTEN/Akt and the downstream molecules in MCF-7 human breast cancer cells. Our findings uncovered the miR-21/PTEN/Akt signaling pathway as a novel mechanism by which natural agents like matrine produce anti-cancer effects.

Several reports have demonstrated that matrine inhibits cancer cell growth and proliferation by inducing apoptosis and cell cycle arrest in various types of tumors [5-7, 22]. Our data reinforce this point by showing that the ability of matrine to inhibit MCF-7 cells growth by at least partially inducing apoptosis and cell cycle arrest, suggesting that matrine could be used for the treatment of breast cancer. Previous studies have reported that matrine is able to up-regulate proapoptotic proteins including BH3 only proteins (Bim, Puma, Bad, Bik), Bok, Bak and Bax, and inhibit expression of anti-apoptotic proteins like Bcl-2 and Bcl-xl [5, 8, 23]. Our study indicates the miR-21/PTEN/Akt pathway as an alternative mechanism underlying the effects of matrine.

Interestingly, miR-21 is one of the most anomaly and commonly increased miRNAs in cancer, and plays important roles in cancer growth, proliferation, migration, and metastasis by targeting PTEN, PDCD4, Spry1 etc, or by up-regulating proteins like Bcl-2 indirectly [13-15, 24]. It was demonstrated that anti-miR-21 suppressed the growth and proliferation of MCF-7 cells *in vitro* and *in vivo* [13, 14]. PTEN is an important downstream target of miR-21 [15], and overexpression of PTEN in a tetracycline-controlled inducible system induces apoptosis and blocks cell cycle progression in MCF-7 cells [17]. Besides, ectopic expression of PTEN suppresses tumorigenicity and cell growth through apoptosis, G₁ cell cycle arrest or both [17, 18, 25, 26]. However, matched analysis of breast tissues from normal and invasive ductal carcinoma indicated that there is no clear inverse correlation between miR-21 and PTEN [27]. We found that matrine inhibited the expression of miR-21 in MCF-7 cells to induce overexpression of PTEN and subsequently inactivate Akt, which likely activated Bad to trigger intrinsic apoptotic cascades [28]. The possible mechanisms by which matrine produces anti-cancer effects in MCF-7 is illustrated in Figure 6.

Fig. 5. Effects of matrine on the protein levels of PTEN, pAKT, AKT, pBad, Bad, p21, p27 in MCF-7 cells, as measured by Western blotting. Cells were treated with matrine at 0 (Ctrl), 0.4 (M1), and 0.8 (M2) mM for 48h. (A) Example of Western blotting bands; (B) Quantitative assay was performed with GAPDH as an internal control. Data are expressed as mean $\pm$ SD normalized to Ctrl. n=6 * p <0.05 vs Ctrl.

Based upon our hypothesis, Bad might be dephosphorylated when pAkt is inhibited, and is released through interacting with anti-apoptotic Bcl-2 family members. In our study, miR-21 was transfected in MCF-7 and found that PTEN was down-regulated significantly, but had no effect on Bcl-2 (data was not shown), which may result from overexpression of endogenous miR-21 in MCF-7 cells. It was demonstrated that matrine induced apoptosis without influence on the total and phosphorylation of Bad in gastric cancer [5], indicating that matrine may induce apoptosis in a cell specific manner.

It is well known that p21^{WAF1/CIP1} is one of universal cyclin-CDK inhibitors that inhibit all cyclin-CDK complexes by its N-terminal homologous sequences to induce cell cycle arrest

Fig. 6. Proposed model of molecular mechanisms for the anticancer actions of matrine in MCF-7 cells. In the presence of matrine, expression of miR-21 is decreased, which in turn upregulates its target gene PTEN in breast cancer cells. PTEN upregulation results in a decrease in pAKT, which activates p21^{WAF1/CIP1} and p27^{KIP1} along with dephosphorylation of pBad, leading to cell cycle arrest at G1/S phase accompanied by apoptosis.

at G₁, S, or G₂ phase [29]. Although p21^{WAF1/CIP1}-null mice develop normally, they were more susceptible to tumors caused by chemical agents and irradiation [30, 31]. Importantly, p21^{WAF1/CIP1} is reported as a transcriptional target of p53 after DNA damage and is regulated through p53 dependent or independent pathways to induce apoptosis [29]. On the other hand, accumulating studies indicated that loss of functional p27^{KIP1} plays an important role in tumorigenesis and development of human cancers [32-34]. During cell cycle progression the expression of p27^{KIP1} was changed and was highest in senescence, but it was reduced during G₁ and S phase [35]. In accordance with previous studies, our data showed that overexpression of PTEN can up-regulate p21^{WAF1/CIP1} and p27^{KIP1} by dephosphorylating Akt.

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Disclosure Statement

The authors declare that they have no competing interests.

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