Downregulated miR-21 mediates matrine-induced apoptosis via the PTEN/Akt signaling pathway in FTC-133 human follicular thyroid cancer cells

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Received January 20, 2019; Accepted July 3, 2019

DOI: 10.3892/ol.2019.10693

Abstract. Matrine is an alkaloid extracted from the leguminous plant *Sophora flavescens*. Matrine has clinical effects in the treatment of tumors, including those in lung cancer, nasopharyngeal cancer and liver cancer. However, the effect of matrine on follicular thyroid cancer has not been reported. The aim of the present study was to investigate the effect of matrine on follicular thyroid cancer and its potential mechanism. FTC-133 follicular thyroid cancer cells were treated with different concentrations of matrine, and an MTT assay showed that matrine inhibited the growth of FTC-133 cells in a dose- and time-dependent manner with an IC_{50} value of 154.8 μM. Cell apoptosis was analyzed by flow cytometry and the results showed that matrine effectively induced the apoptosis of FTC-133 cells. The expression level of microRNA (miR)-21 was analyzed by reverse transcription-quantitative PCR (RT-qPCR) analysis, and the mRNA and protein expression levels of PTEN, Akt and phosphorylated (p)-Akt were detected by RT-qPCR analysis and western blotting, respectively. The expression of miR-21 was significantly downregulated, PTEN was upregulated at the mRNA and protein expression levels, and p-Akt was downregulated in the FTC-133 cells. The effects of miR-21 mimics and miR-21 inhibitor on the expression of miR-21, PTEN and Akt in FTC-133 cells, and the effect of miR-21 mimics/matrine on the expression of PTEN were also investigated. The results of the present study suggested that matrine inhibited the growth and induced apoptosis of FTC-133 cells via the miR-21/PTEN/Akt signaling pathway.

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Key words: follicular thyroid cancer, matrine, microRNA-21, PTEN, Akt, apoptosis

Introduction

Thyroid cancer is rapidly increasing in incidence (1). Follicular thyroid carcinoma (FTC) and papillary thyroid carcinoma (PTC) account for ~94% of cases of thyroid cancer, with FTC being more aggressive and having a poorer prognosis than PTC (1-3). The clinical therapy of FTC, including surgery, radioactive iodine ablation and thyroid-stimulating hormone suppression, leads to complications, such as developing resistance to hormones, which can be disabling and may be life-threatening (4,5). Therefore, there is an urgent need to examine the mechanism and identify novel targets involved in FTC to improve early diagnosis, develop better treatments and prolong survival time.

The traditional Chinese medicine matrine can induce apoptosis in ^{V600E}BRAF-harboring melanoma, lung cancer, prostate cancer, PTC, breast cancer and T cell lymphoblastic leukemia cells (6-13). The PTEN/Akt pathway serves an important role in the inhibitory effect of matrine on cancer cells (6-8,11,12). However, to the best of our knowledge, the pro-apoptotic effect and underlying mechanism of matrine in FTC have not been investigated previously.

MicroRNA-21 (miR-21) is one of the most frequently upregulated microRNAs in malignant tumors (14,15). Previous studies have shown that miR-21 is a potential regulator of PTEN in various types of cancer, including thyroid cancer (11), breast cancer (12,16), non-small cell lung cancer (14) and human hepatocytes (17). PTEN is a tumor suppressor gene with phospholipase activity, which suppresses the phosphorylation of Akt and is involved in the occurrence and development of cancer (12). In the present study, the action and underlying mechanism of miR-21 on matrine-induced inhibition of the FTC-133 human FTC cell line were investigated.

Materials and methods

Matrine and cell line. Matrine ($C_{15}H_{24}N_2O$; MW 248.36; CAS: 519-02-8, purity ≥98%) was purchased from Shanghai

Aladdin Biochemical Technology Co., Ltd. and was dissolved in 0.9% NaCl solution for use. The FTC-133 human FTC cells were donated by Changchun Institute of Applied Chemistry, Chinese Academy of Sciences (Beijing, China). The cells were cultured in RPMI-1640 medium (Hyclone; GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences) and incubated at 37˚C at a humidity of 95 and 5% CO_2.

MTT assay. The FTC-133 cells were seeded in a 96-well plate (200 µl/well) at a density of $1x10^4$ cells per well. After 12 h, the cells were treated with matrine at concentrations of 0, 40, 80, 200 and 400 µM at 37˚C. Following incubation at 37˚C for 24, 48 and 72 h, 20 µl of 5 mg/ml MTT reagent (Beijing Solarbio Science & Technology Co., Ltd.) was added to each well for 4 h. The medium was then removed and 150 µl DMSO was added to dissolve the formazan crystals. Finally, the absorbance was measured at a wavelength of 570 nm using a SpectraMax Plus 384 microplate reader (Molecular Devices, LLC).

To investigate the effect of matrine on FTC-133 cells with a high expression of miR-21, following treatment of the FTC-133 cells with matrine and miR-21 mimics, cell viability was measured again using an MTT assay. Each MTT assay was repeated three times. The results are expressed as the percentage growth inhibition with respect to the normal control cells.

Flow cytometry. The FTC-133 cells were treated with matrine at concentrations of 0, 80, 200 and 400 µM in a 6-well plate (Corning, Inc.) for 24 h. Cell apoptosis was detected using the Annexin V-FITC Detection kit according to the manufacturer's protocol (Bioworld Technology, Inc.). Following staining, the samples were analyzed using a Guava easyCyte6-2L flow cytometer (Merck KGaA).

Transfection of miR-21 mimics and miR-21 inhibitor. The FTC-133 cells were seeded at a density of $1x10^5$ cells/ml for 24 h. When the cells reached 80% confluence, they were transfected either with miR-21 mimics (sense, 5'-UAG CUUAUCAGACUGAUGUUGA-3'; antisense, 5'-UCA ACAUCAGUCUGAUAAGCUA-3') or miR-21 inhibitor (5'-UCA ACAUCAGUC UGAUAAGCUA-3'; Biomics Biotechnologies Co., Ltd.) at final concentrations of 0 (negative control; NC), 30 and 50 nM using X-tremeGENE siRNA transfection reagent following the manufacturer's protocol (Roche Diagnostics). Based on our preliminary experiments, transfection with a low concentration had no significant effects, whereas a high concentration of transfection produced cytotoxicity. Therefore, the transfection concentrations of 30 and 50 nM were selected. To examine the biological effects of transfection on FTC-133 cells, the expression of miR-21 was determined by reverse transcription-quantitative PCR (RT-qPCR) analysis 48 h after transfection, and the mRNA and protein levels of PTEN, Akt and phosphorylated (p)-Akt were detected.

RT-qPCR analysis of miR-21, PTEN and Akt. Total RNA was extracted from FTC-233 cells with TRIzol® reagent following the manufacturer's instructions (Ambion; Thermo Fisher Scientific, Inc.). The concentration of RNA was determined using the Nanodrop 2000 instrument (Thermo Fisher Scientific, Inc.). The optical density 260/280 rate was between 1.8 and 2.0. Nucleic acid electrophoresis was performed for quality analysis of RNA in Tris-Borate-EDTA solution.

To perform RT-qPCR of miR-21, 25 ng RNA was reverse transcribed to cDNA using the mirVana RT-qPCR miRNA Detection kit (Ambion; Thermo Fisher Scientific, Inc.). The reaction conditions were as follows: 37˚C for 30 min and 95˚C for 10 min. The levels of miR-21 were determined by RT-qPCR using the mirVana RT-qPCR miRNA Detection kit following the manufacturer's instructions (Ambion; Thermo Fisher Scientific, Inc.) and normalized to U6 as the internal reference. The reactions were performed on the Aligent Mx3000P PCR system (Agilent Technologies, Inc.). The primer sequences used for RT-qPCR were as follows: miR-21, 5'-GCGCGCGTAGCTTATCAGACTGA-3' (forward) and 5'-ATCCAGTGCAGGGTCCGAGG-3' (reverse); U6, 5'-CTC GCTTCGGCAGCACA-3' (forward) and 5'-AACGCTTCA CGAATTTGCGT-3' (reverse). The amplification program was as follows: 95˚C for 3 min, 40 cycles of 95˚C for 15 sec and 60˚C for 30 sec.

To investigate the mRNA expression levels of PTEN and Akt, 50 ng RNA was reverse transcribed to cDNA using the FastQuant RT kit (Beijing Tiangen Biotech Co. Ltd.). The reaction conditions were as follows: 42˚C for 15 min and 95˚C for 3 min. RT-qPCR was performed with SYBR Premix Ex Taq (Takara Bio, Inc.) and normalized to GAPDH as the internal reference. The primer sequences used were as follows: PTEN, 5'-GCAATATGTTCATAACGATGGCTGTGG-3' (forward), 5'-GAACTGGCAGGTAGAAGGCAACTC-3' (reverse); Akt, 5'-GCAGGATGTGGACCAACGTGAG-3' (forward), 5'-GCA GGCAGCGGATGATGAAGG-3' (reverse). The thermocycling conditions for PTEN and Akt was as follows: 95˚C for 30 sec, 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. The mRNA expression level was calculated using the $2^{-\Delta\Delta Cq}$ method (18), normalizing to the Cq value of the reference gene GAPDH.

Western blotting. The cells were collected and washed with cold PBS and then lysed in lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.) supplemented with protease inhibitor, phosphatase inhibitor and PMSF (lysis buffer: Protease inhibitor: Phosphatase inhibitor: PMSF=100:1:1:1). The concentrations of proteins were determined using a Bicinchoninic Acid Protein Assay kit (Beijing Solarbio Science & Technology Co., Ltd.). In total, 30 µg protein was separated by SDS-PAGE on 10% gels and electroblotted onto PVDF membranes (Beijing Solarbio Science & Technology Co., Ltd.). The membranes were blocked with 5% non-fat milk in TBS with Tween-20 at 37˚C for 1 h and incubated with primary antibodies of PTEN (26H9) mouse mAb (cat. no. 9556, 1:1,000; Cell Signaling Technology, Inc.), p-Akt (Ser-473) mouse mAb (cat. no. 12694, 1:1,000; Cell Signaling Technology, Inc.) and Akt1 (2H10) mouse mAb (cat. no. 2967, 1:1,000; Cell Signaling Technology, Inc.) overnight at 4˚C. Following washing with TBST, the membranes were incubated with horseradish peroxidase-labeled horse anti-mouse secondary antibodies (cat. no. 7076, 1:1,000; Cell Signaling Technology, Inc.) at 37˚C for 1 h. The protein signal was

visualized using 3,3'diaminobenzine reagent (Beijing Solarbio Science & Technology Co., Ltd.). GAPDH (TransGen Biotech Co., Ltd.) was used as the internal reference.

Statistical analysis. All data are presented as the mean ± SD. Analyses were performed with GraphPad Prism 5.0 (GraphPad Software, Inc.). Statistical significance of the results were analyzed by Student's t-test for two group comparison and one-way ANOVA followed by Dunnett's test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Matrine inhibits the growth of FTC-133 cells. To detect the biological effect of matrine on FTC-133 cells, an MTT assay was performed. The FTC-133 cells were treated with matrine at concentrations of 0, 40, 80, 200 and 400 μM for 24, 48 and 72 h. The results showed that matrine had an inhibitory effect on FTC-133 cells in a dose- and time-dependent manner with an IC_{50} of 154.8 μM at 48 h (Fig. 1A). Compared with the control, the treatment group with a low concentration of 40 μM had no significant inhibitory effect (P>0.05). The inhibition rate was ~80% for 200 μM at 72 h. When the concentration of matrine reached 400 μM, growth of the FTC-133 cells was almost completely inhibited.

Following transfection of the FTC-133 cells with miR-21 mimics at a final concentration of 50 nM, the cells were treated with matrine at concentrations of 0, 40, 80, 200 and 400 μM for 24, 48 and 72 h. Cell viability was measured again using an MTT assay. Compared with the control group, the marked inhibitory effect of matrine on the transfected FTC-133 cells was still observed. However, compared with the matrine treatment group, the inhibition rate was decreased significantly (Fig. 1B). This suggested that high expression of miR-21 reduced the inhibitory effect of matrine on FTC-133 cells.

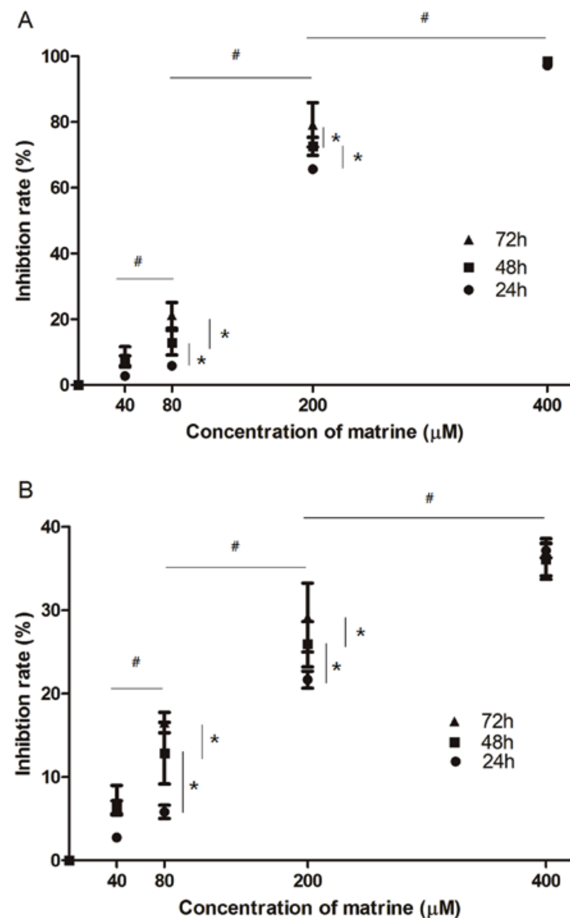

Figure 1. MTT results. (A) FTC-133 cells were treated with matrine at a series of concentrations (0, 40, 80, 200 and 400 μM) for 24, 48 and 72 h. The inhibitory rate was assayed using an MTT assay. The IC_{50} at 48 h was calculated to be 154.8 μM. (B) High expression of miR-21 inhibited matrine-induced FTC-133 apoptosis. All experiments were performed three times. Data are expressed as the mean ± SD. *P<0.05 compared between two time points within each concentration; #P<0.05 between two concentrations within each time point. miR-21, microRNA-21.

Matrine induces cell apoptosis. To investigate the pro-apoptotic effect of matrine on FTC-133 cells, the cells were exposed to matrine at concentrations of 0, 80, 200 and 400 μM. Cell apoptosis was detected by flow cytometry and the results are presented in Fig. 2. When the cells were treated with a high concentration of 200 μM, the microscopic morphology revealed no necrosis (Fig. S1). Therefore, the impact of matrine on cell survival was not associated with necrosis but apoptosis. The apoptosis fractions for 0, 80, 200 and 400 μM were 0.396, 3.22, 62.9 and 97.0%, respectively. The pro-apoptotic effect was in a matrine concentration-dependent manner. Therefore, in the following experiments, matrine was used at the concentrations of 80 and 200 μM.

Effect of matrine, miR-21 mimics and miR-21 inhibitor on the expression of miR-21 in FTC-133 cells. The FTC-133 cells were exposed to matrine at concentrations of 0, 80 and 200 μM for 48 h. The expression level of miR-21 was examined by RT-qPCR analysis. As shown in Fig. 3A, the expression level of miR-21 was significantly downregulated when the cells were treated with 200 μM matrine for 48 h (M200/NC: 0.56±0.026-fold). The expression of miR-21 was examined following transfection of the cells with 0 (NC), 30 and 50 nM of either miR-21 mimics or miR-21 inhibitor. As shown in Fig. 3B and C, as expected, the overexpression and inhibition of miR-21 were achieved by transfection with miR-21 mimics or miR-21 inhibitor, respectively. miR-21 mimics increased the level of miR-21 (30 nM/NC: 1.72±0.08-fold; 50 nM/NC: 2.71±0.13-fold). The miR-21 inhibitor decreased the level of miR-21 (50 nM/NC: 0.44±0.10-fold). These results suggested that the inhibitory effect of matrine on FTC-133 cells was associated with the downregulation of miR-21.

Effects of matrine, miR-21 mimics and miR-21 inhibitor on the expression of PTEN and Akt in FTC-133 cells. Following treatment of the FTC-133 cells with matrine at 0, 80 and 200 μM for 48 h, the mRNA and protein expression levels of PTEN and Akt (or p-Akt) were examined by RT-qPCR analysis and western blotting (Fig. 4A and 4B), respectively. As shown in Fig. 4A, the mRNA expression levels of PTEN were upregulated 1.46±0.35-fold for M80/NC and 2.74±0.30-fold for M200/NC. There was no significant change in the mRNA level of Akt (P>0.05). The western blotting results are presented in Fig. 4B; the protein expression levels of PTEN were markedly upregulated in the matrine treatment

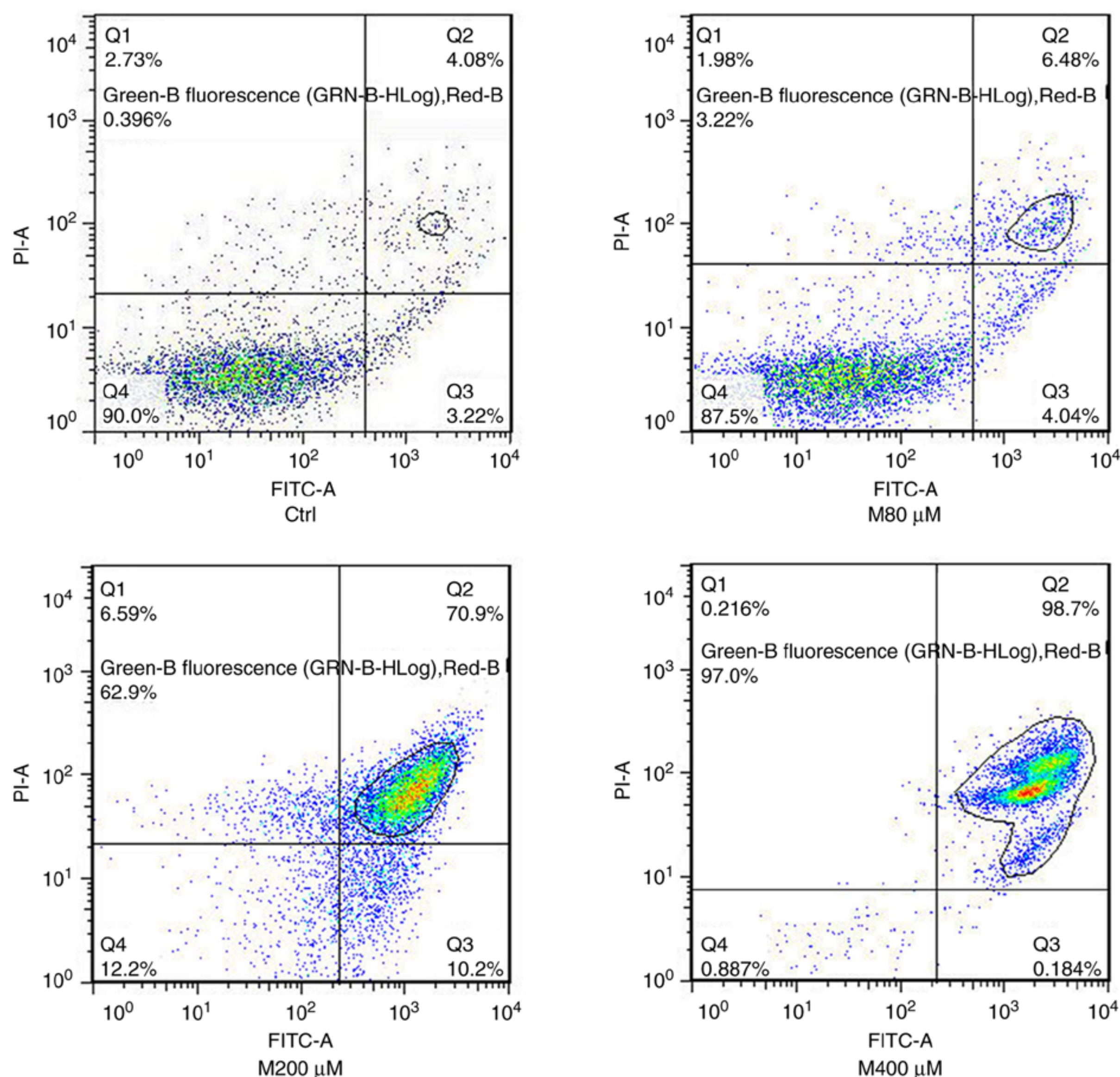

Figure 2. Effects of matrine on the apoptosis of FTC-133 cells. FTC-133 cells were treated with matrine at 0 (Ctrl), 80, 200 and 400 μM. The apoptotic rate was measured by flow cytometry with Annexin V-FITC/PI staining. Compared with that in the control group, the apoptotic rate was increased significantly in FTC-133 cells. All experiments were performed three times. Ctrl, control; PI, propidium iodide.

groups (M80/NC: 2.37±0.12-fold; M200/NC: 2.94±0.24-fold). The protein expression levels of Akt did not change between groups. p-Akt (Ser-473 phosphorylation) was detected and the p-Akt/Akt ratio levels were downregulated compared with those in the control (M80/NC: 0.76±0.06-fold; M200/NC: 0.27±0.06-fold; Fig. 4B). Matrine upregulated the expression of PTEN at the transcriptional and translation levels, suggesting that PTEN was an important member of the cellular signaling pathway in which matrine affects FTC-133 cells. The results showed that the PTEN/Akt signaling pathway may serve an important role in matrine inhibiting cell growth and inducing cell apoptosis.

To further determine the association between miR-21 and the PTEN/Akt signaling pathway in FTC-133 cells, the mRNA and protein expression levels of PTEN and Akt (or p-Akt) following transfection with either miR-21 mimics or miR-21 inhibitor were examined by RT-qPCR analysis and western blotting (Fig. 4C-F). The mRNA levels of PTEN were downregulated by the miR-21 mimics (30 nM/NC: 0.50±0.06-fold; 50 nM/NC: 0.40±0.03-fold; Fig. 4C) and upregulated by the miR-21 inhibitor (50 nM/NC: 2.28±0.17-fold; Fig. 4E). The transfection had no effect on the mRNA levels of Akt (P>0.05; Fig. 4C and E).

At the protein expression level (Fig. 4D and F), miR-21 mimics suppressed the expression of PTEN (30 nM/NC: 0.55±0.02-fold; 50 nM/NC: 0.41±0.05-fold), increased the p-Akt/Akt ratio (50 nM/NC: 1.97±0.39-folds) but had no effect on the expression levels of Akt. The miR-21 inhibitor increased the expression levels of PTEN (30 nM/NC: 1.87±0.05-fold; 50 nM/NC: 3.08±0.13-fold) and suppressed the p-Akt/Akt ratio (30 nM/NC: 0.38±0.02-fold; 50 nM/NC: 0.33±0.13-fold). However, the miR-21 inhibitor did not affect the levels of Akt. Combining the results of the transcriptional and translational levels of PTEN and Akt in FTC-133 cells, it was identified that miR-21 directly affected the expression of PTEN, and PTEN suppressed the phosphorylation of Akt at the Ser-473

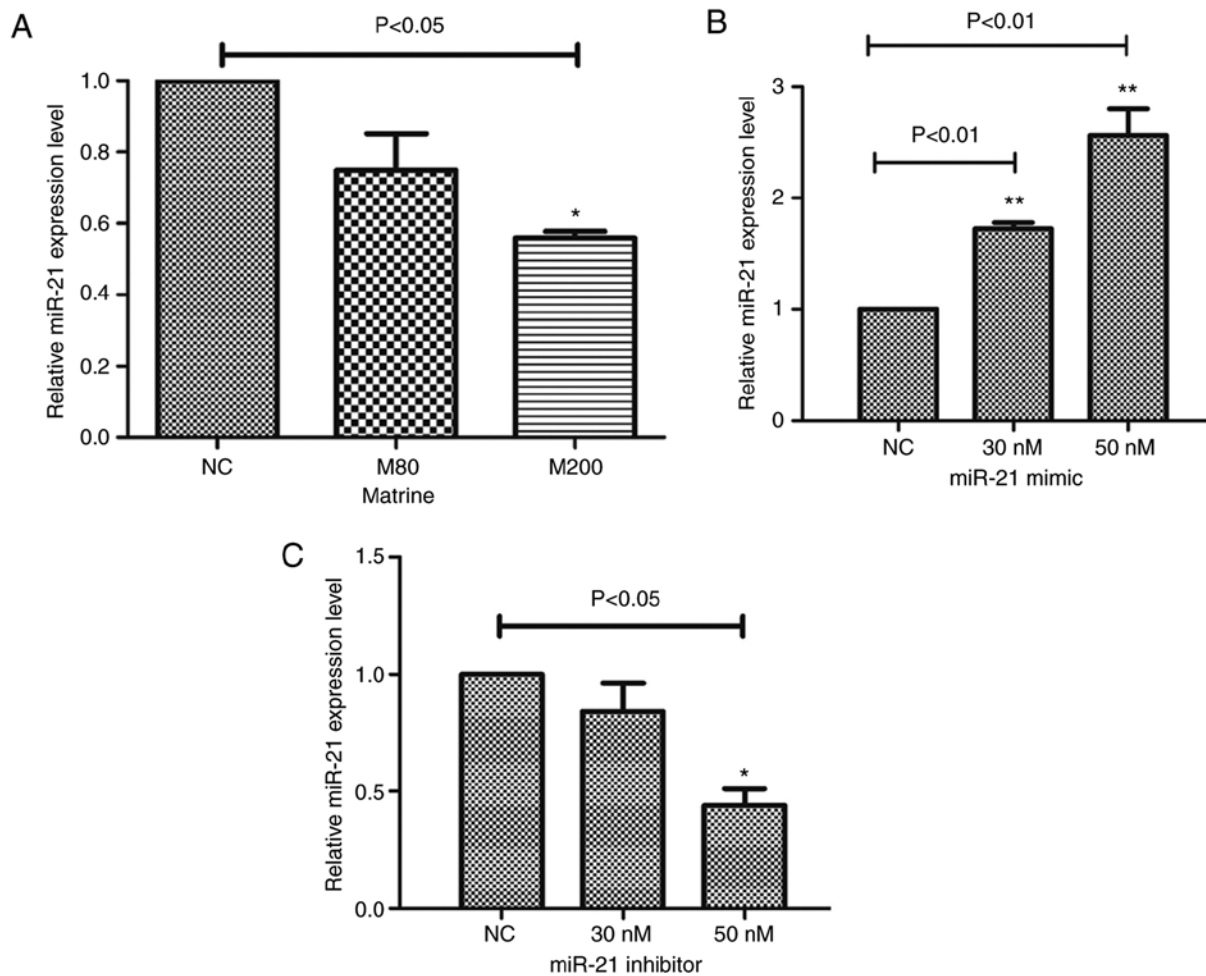

Figure 3. Expression level of miR-21 determined by reverse transcription-quantitative PCR analysis. (A) Expression level of miR-21 following treatment of the cells with matrine. Expressions level of miR-21 following transfection of the cells with either (B) miR-21 mimics or (C) miR-21 inhibitor. U6 was used for normalization. Data are expressed as the mean ± SD. *P<0.05 and **P<0.01 vs. NC group. miR-21, microRNA-21; NC, negative control.

site. Additionally, matrine significantly reduced the expression of miR-21 and increased the expression of PTEN in FTC-133 cells. These results suggested that matrine inhibited the growth of FTC-133 cells and induced cell apoptosis through the miR-21/PTEN/Akt signaling pathway. To further validate this result, the expression of PTEN was detected in FTC-133 cells treated with miR-21 mimics/matrine. The western blotting results showed that there was no significant change in the expression of PTEN in the miR-21 mimics/matrine group compared with that in the control group (Fig. 4G). Simultaneously, compared with the matrine treatment group, the expression of PTEN in the miR-21 mimics/matrine group was reduced. This suggested that PTEN, which was reduced by miR-21 mimics, was replenished by matrine. It was demonstrated that matrine exerted inhibitory effects on the cells through the miR-21 and PTEN pathway.

Discussion

The morbidity and mortality rates of thyroid cancer are increasing year by year worldwide. Surgical resection is the main treatment for thyroid cancer. The prevention, diagnosis and treatment of thyroid cancer, as with other cancerous diseases, have become an increasingly concerning problem. At present, research on the antitumor effects of the active ingredients of traditional Chinese medicine has attracted increasing attention. Additionally, the antitumor effect of traditional Chinese medicine has been confirmed in clinical practice.

Matrine is a major alkaloid extracted from the leguminous plant *Sophora flavescens*, it has shown antitumor activity against a variety of cancer cells, including gastric, breast, lung cancer, pancreatic and colon cancer. However, to the best of our knowledge, the effect of matrine on FTC has not been reported previously. In the present study, it was found that matrine exhibited an anticancer effect and pro-apoptotic activity on FTC-133 cells. The results of MTT assays showed that matrine inhibited the growth of FTC-133 cells in a dose- and time-dependent manner; this result is consistent with those of previous studies (6-13). Furthermore, previous studies showed that the sensitivity of different cancer cells to matrine is varied. In the present study, FTC-133 cells had a moderate IC_{50} of 154.8 μM. Several previous studies have shown that the pro-apoptotic activity of matrine contributes to cancer inhibition (6,7,11,12). The pro-apoptotic activity of matrine on FTC-133 cells was also determined in the present study by flow cytometry; the result showed that the pro-apoptotic effect also occurred in a matrine concentration-dependent manner. Therefore, the inhibitory

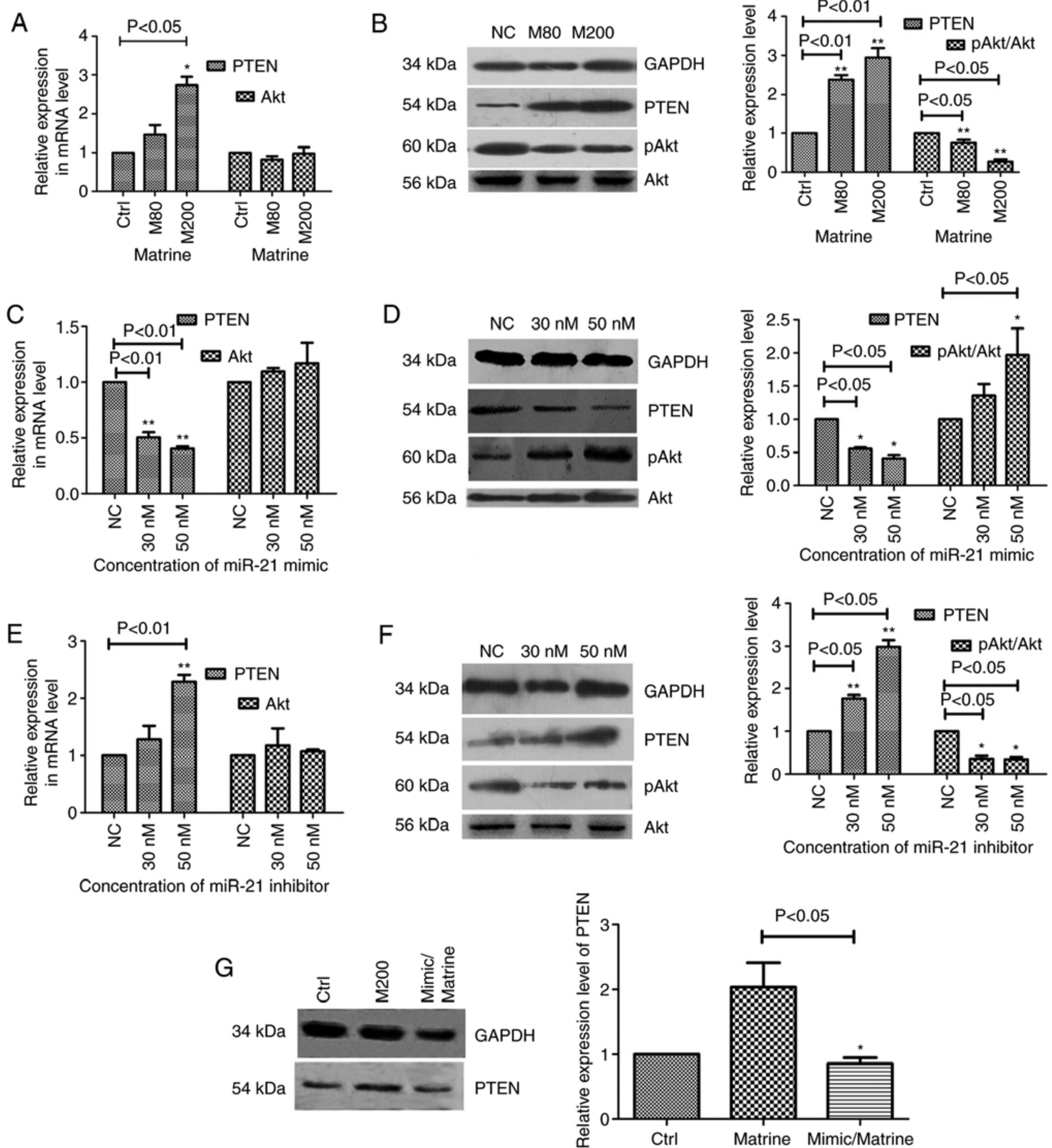

Figure 4. Expression of PTEN and p-Akt determined by RT-qPCR analysis and western blotting. Expression of PTEN and Akt were determined at the mRNA level by RT-qPCR and at the protein level by western blotting. (A) mRNA and (B) protein expression in cells treated with matrine; (C) mRNA and (D) protein expression in cells treated with miR-21 mimic; (E) mRNA and (F) protein expression in cells treated with miR-21 inhibitor. (G) Expression of PTEN at the protein level was determined by western blotting. GAPDH was used as an internal reference. Data are expressed as the mean ± SD. *P<0.05 and **P<0.01 vs. control group. miR, microRNA; NC, negative control; Ctrl, control; p, phosphorylated; RT-qPCR, reverse transcription-quantitative PCR.

effect of matrine on FTC-133 cells may be achieved mainly by promoting apoptosis.

miR-21 has been reported to be related to the formation of cancer (19). In several types of cancer, the abnormal expression level of miR-21 increases markedly. Furthermore, the overexpression of miR-21 can initiate the apoptosis of breast cancer cells (16). At present, miR-21 has become one of the important markers of several types of cancer (20). PTEN is a target gene of miR-21 (17). As a classical anti-oncogene, similar to p53, PTEN has been investigated for many years. The inhibition of PTEN is key in the process of cell apoptosis, mainly relying on the phosphorylation and dephosphorylation of Akt. As an important protein kinase in several signaling pathways, activated Akt has anti-apoptotic activity and promotes proliferation, invasion and angiogenesis. PI3K can activate Akt through a series of cascade reactions. PI3K can

also be dephosphorylated by PTEN, thereby, avoiding the activation of Akt, which likely activates Bad to trigger intrinsic apoptotic cascades (21-23). The mechanism underlying the effect of matrine on other cancer cells predominantly involves the miR-21/PTEN/Akt signaling pathway (11,12). Therefore, it was hypothesized that the miR-21/PTEN/Akt signaling pathway may be involved in the regulatory mechanism of matrine inhibiting FTC cells. In the present study, when FTC-133 cells were treated with matrine, the expression of miR-21 was significantly downregulated and the expression of PTEN was upregulated. The expression of p-Akt was also downregulated. The regulatory effect of miR-21 on PTEN/Akt in FTC-133 cells was investigated, and the FTC-133 cells were transfected with miR-21 mimics and inhibitor. The results showed that the miR-21 mimics downregulated the expression of PTEN and upregulated the level of p-Akt, whereas the miR-21 inhibitor, similar to matrine, upregulated PTEN and downregulated p-Akt. The phosphorylation level of Akt at Ser-473 increased but the level of Akt did not alter significantly, suggesting that PTEN regulated the activity of Akt in the present study. The results showed that the miR-21/PTEN/Akt signaling pathway may be important in the effect of matrine inhibiting cell growth and inducing cell apoptosis. To further validate the results, the expression of PTEN was detected in FTC-133 cells treated with miR-21 mimics/matrine and it was determined that PTEN, which was reduced by miR-21 mimics, was increased by matrine. These results demonstrated that matrine inhibited the growth of FTC-133 cells and induced cell apoptosis through the miR-21/PTEN/Akt signaling pathway. The results of the present study were consistent with the previously reported effect of matrine in PTC and breast cancer cells (11,12).

In the process of the transcription of miR-21, STAT3 regulates the upstream enhancer encoding miR-21 (24). In addition, previous studies have demonstrated that the expression of p-STAT3 was significantly downregulated by matrine in human cholangiocarcinoma cells and H1975 cells (25,26). According to these results, it was hypothesized that matrine may inhibit the expression of miR-21 at the transcriptional level by decreasing the phosphorylation of STAT3.

In conclusion, the present study showed that matrine inhibited the growth of FTC-133 cells and induced cell apoptosis via the miR-21/PTEN/Akt signaling pathway. Matrine may be a potential drug for the treatment of FTC and miR-21 may be a potential target. The present study provided a theoretical and experimental basis for drug development and the treatment of FTC.

Acknowledgements

Not applicable.

Funding

The current study was supported by the Special Foundation for New Pharmaceutical Health Development of Jilin Province of China (grant no. YYZX201725), Development and Reform Commission Project of Jilin Province of China (grant nos. 3J115AK93429 and 2014G072), Jilin Agricultural University Doctorate Fund of China (grant no. 2015016), Science and Technology Project of Jilin Province of China (grant nos. 20180101260JC and 20160312008ZG) and the National Undergraduate Innovation and Entrepreneurship Project of China (grant no. 201810193033).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

TL and QL designed the experiment. QL performed the MTT assay, flow cytometry, RT-qPCR analysis and western blotting and composed the manuscript. SZ and MW performed the MTT assay, cell transfection and RT-qPCR analysis. YW and SD collected data and performed statistical analysis. TL and YF critically reviewed and revised the manuscript. YF also conceived and designed the experiment and performed the flow cytometery. XW, SL and GC were responsible for flow cytometry and image editing. All authors agreed to the final version.

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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