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Matrine inhibits TPC-1 human thyroid cancer cells via the miR-21/PTEN/Akt pathway

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Abstract. Papillary thyroid cancer (PTC) is the primary type of thyroid cancer and the most widespread endocrine malignancy. Matrine is a traditional Chinese medicine and has been demonstrated as a promising alternative drug for the treatment of TPC-1 human PTC. In the present study, the therapeutic effects and the underlying molecular mechanisms of matrine on TPC-1 cells were investigated. Treatment with matrine at the concentrations of 1, 2, 5, 10 and 20 mg/ml inhibited TPC-1 cell proliferation by up to 95.8% (for 20 mg/ml matrine). Flow cytometry indicated that treatment with 10 mg/ml matrine induced up to 61.8% apoptosis of the TPC-1 cells and the cell cycle was arrested at the G0/G1 phase following treatment with matrine (2, 5 and 10 mg/ml) for 48 h. Quantitative polymerase chain reaction indicated that the expression of microRNA (miR)-21 was downregulated and phosphatase and tensin homolog (PTEN) mRNA levels increased up to 1.66-fold following treatment with matrine, and RAC-α serine/threonine-protein kinase (Akt) mRNA levels were downregulated 0.34-fold following treatment with 5 mg/ml matrine, compared with the normal control group. Western blot analysis indicated that matrine at 2 and 5 mg/ml increased levels of the miR-21 target PTEN and decreased the levels of phosphorylated (p)Akt. Furthermore, miR-21 mimic transfection decreased the expression levels of PTEN and increased the levels of pAkt. These results suggested that the miR-21/PTEN/Akt pathway may be one of the mechanisms by which matrine induces apoptosis and cell cycle arrest in TPC-1 thyroid cancer cells. Matrine is an alternative potential drug for the treatment of thyroid cancer.

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Introduction

Thyroid cancer is the most widespread endocrine malignancy. Papillary thyroid cancer (PTC) is the most common and accounts for approximately 80-90% of human thyroid cancers (1-4). Study of the treatment and pathogenesis of thyroid cancer aids in increasing the rate of early diagnosis of thyroid cancer and the development of novel therapies, improving the quality of life and prognosis, and reducing the mortality of patients with thyroid cancer.

The dysfunction of microRNA (miRNA/miR) has an important association with human PTC (5-9). Transfection of miR-21 into TPC-1 human PTC cells resulted in an increased proliferation and a decreased apoptosis (8). The plasma exosome miR-21 expression pattern in patients with PTC and follicular thyroid cancer (FTC) is distinct from benign tumors (9). miR-21 acts by targeting the tumor suppressor gene phosphatase and tensin homolog (PTEN), which suppresses the tumor by dephosphorylating RAC-α serine/threonine-protein kinase (Akt) (10-13).

The Chinese traditional medicine matrine exhibits extensive anti-tumor activities through multiple mechanisms including pro-apoptotic action, cell cycle arrest, growth inhibition, alteration of miRNA expression, upregulation of PTEN and suppression of Akt (14-22). Li *et al* (23), reported that the natural medicine matrine may affect miRNA-21 to inhibit MCF-7 breast cancer growth. This result expands the understanding of the antitumor activities of natural medicines.

Considering the extensive anti-tumor activity of matrine and the miR-21 expression pattern in human PTC, the miR-21-regulated antitumor effect of matrine may also exist in PTC. Therefore, the present study was designed to determine the effect of matrine on miR-21 and its target PTEN/Akt pathway in TPC-1 human PTC cells. Proliferation, cell cycle and apoptosis levels were tested. The present study was designed to identify alternative therapeutic methods for treatment of thyroid cancer and provide a foundation for further research on the treatment of thyroid cancer using matrine.

Materials and methods

Cell line and drug treatment. Matrine ($C_{15}H_{24}N_2O$; MW 248.36; CAS:519-02-8; purity >98%) was purchased from Meryer Chemical Company (Shanghai, China). TPC-1

human thyroid cancer cells were donated by CIAC of CAS (Changchun, Jilin, China). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Sijiqing Inc., Hangzhou, Zhejiang, China) at 37˚C in a 5% CO_2 incubator.

MTT assay. TPC-1 cells were seeded in 96-well plates at a density of 10^5/ml (0.2 ml in each well) for 12 h. Cells were exposed to matrine at a final concentration of 0 (M0, control), 1, 2, 5, 10, 20 mg/ml for 24, 48 and 72 h. Methyl thiazolyl tetrazolium (MTT; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at a final concentration of 0.5 mg/ml was incubated in each well for another 4 h. The formazan crystals were dissolved in 150 μl dimethyl sulfoxide and the absorbance was measured at a wavelength of 490 nm by a Multiskan FC microplate reader (Thermo Fisher Scientific, Inc.). All experiments were repeated three times. The percentage growth inhibition vs. the M0 control cells was calculated.

Flow cytometry. For apoptosis analysis, TPC-1 cells in the log phase were collected following treatment with matrine at 0 (control), 2, 5 and 10 mg/ml for 48 h, fixed with 70% ethanol and washed twice with cold PBS. The cells were resuspended in 500 μl binding buffer at a concentration of 10^6/ml, mixed with 10 μl Annexin V (Bioworld Technology Inc., Jiangsu, China) for 15 min in the dark at room temperature and incubated with 5 μl propidium iodide (PI; Bioworld Technology Inc.) for 10 min in the dark. The cells were analyzed using a FACSCalibur flow cytometer with the CellQuest 3.0 sampling software (BD Biosciences, Franklin Lakes, NJ, USA). The apoptosis rate was determined using the FlowJo software (BD Biosciences).

For cell cycle analysis, TPC-1 cells were cultured in the serum-free medium for 12 h for starvation. TPC-1 cells were treated with matrine at 0 (M0, control), 2, 5 and 10 mg/ml for 48 h. Then, $5x10^5$ cells in each group were fixed with 70% ethanol, washed with cold PBS and incubated with RNase and PI (Bioworld Technology Inc.) for 30 min in the dark. The cells were analyzed by a FACSCalibur flow cytometer using CellQuest 3.0 software (BD Biosciences). The proportion of cells in each phase was determined using the FlowJo software (BD Biosciences). Each experiment was performed three times.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). For detection of miR-21 levels, the experiments were performed using the Ambion mirVanaTM qRT-PCR miRNA Detection kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. miR-21 primer sequences for qPCR were 5'-GGGGTAGCTTATCAGACT GATG-3' (forward) and 5'-TGTCGTGGAGCGGCAATT G-3' (reverse) (23). To detect PTEN and Akt mRNA levels, total RNA was extracted from TPC-1 cells by TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The first-strand cDNA was synthesized using FastQuant RT kit (with gDNAase; Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, double-stranded cDNA was synthesized. Primers for the qPCR were 5'-GCAATATGTTCATAACGA TGGCTGTGG-3' (PTEN forward) and 5'-GAACTGGCA GGTAGAAGGCAACTC-3' (PTEN reverse); 5'-GCAGGA TGTGGACCAACGTGAG-3' (Akt forward) and 5'-GCAGGC AGCGGATGATGAAGG-3' (Akt reverse). All primers were synthesized by Sangon Biotech Co., Ltd (Shanghai, China). RT-qPCR was performed on a Roche LightCycler® 96 fluorescence quantification PCR machine (Roche Diagnostics, Basel, Switzerland). The reaction system included 1.5 μl cDNA, 7.5 μl SYBR Green, 0.3 μl of primers and PCR-grade water to the total volume of 15 μl. The PCR thermocycling conditions included a pre-denaturation at 95˚C for 30 sec; 40 cycles of 95˚C for 15 sec and 60˚C for 30 sec for amplification; and a default condition for dissociation (23). The cycle threshold (Ct) values were obtained. Quantitative analyzes of miR-21 or mRNA expression levels were performed following normalization to U6 RNA or GAPDH mRNA (Sangon Biotech Co., Ltd., Shanghai, China). Relative experimental/reference miRNA-21 and mRNA expression was calculated using the following formula: $2^{-\Delta Ct(interest\text{-}reference)}$. The fold change was calculated using $2^{-\Delta\Delta Ct}$ method.

Transfection of miR-21 mimic and western blotting. TPC-1 cells were transfected with miR-21 mimics using X-treme reagent following the manufacturer's protocol (Roche Diagnostics, Basel, Switzerland). At 48 h after transfection and/or 48 h after the treatment with 0 (M0, control), 2 and 5 mg/ml matrine, the expression of PTEN and p-Akt protein was measured by western blotting. Briefly, TPC-1 cells were washed with cold PBS and lysed in lysis radioimmunoprecipitation assay buffer (Pierce; Thermo Fisher Scientific, Inc.). The concentration of proteins was determined using a bicinchoninic acid Protein Assay kit (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China). The proteins were separated on 10% SDS-PAGE gels. Gels were transblotted to polyvinylidene difluoride membranes (Beyotime Institute of Biotechnology, Jiangsu, China). The membranes were incubated with the mouse anti-PTEN and anti-pAkt monoclonal antibodies (1:1,000 diluted; Cell Signaling Technology, Inc., Danvers, MA, USA) at 4˚C overnight, and then with the goat horseradish peroxidase-labeled second antibody (1:1,000 diluted; TransGen Biotech, Beijing, China) at 25˚C for 2 h. Chromophore DAB reagent (Beyotime Institute of Biotechnology) was used to develop color. The grayscale ratio of PTEN or Akt to the internal control GAPDH was calculated using the software Image Pro 6.0 (Media Cybernetics, Rockville, MD, USA). The grayscale of test protein bands was normalized to the GAPDH bands.

Statistical analysis. Data are presented as the mean ± standard deviation. SPSS software, v.16.0 (SPSS Inc., Chicago, IL, USA) was used to perform statistical analysis. Student's t-test was used for comparisons between two groups and one-way analysis of variance followed by Dunn's test was used for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cell viability. MTT assay was performed to determine the growth of thyroid cancer TPC-1 cells following treatment with matrine at 0, 1, 2, 5, 10 and 20 mg/ml for 24, 48 and 72 h, respectively. Matrine inhibited the growth of TPC-1

Table I. TPC-1 cell percentage apoptosis and cell cycle distribution.

Matrine (mg/ml)	0	2	5	10
Apoptosis %	15.1±2.1	16.1±1.9	25.9±2.8[a]	61.8±5.4[a]
Cycle phase %				
G0/G1	51.36±2.07	60.63±2.57[a]	62.96±1.98[a]	74.49±3.65[a]
S	23.37±3.25	10.95±2.54[a]	11.26±3.47[a]	2.42±1.63[a]
G2/M	18.49±2.46	18.23±4.64	18.32±2.88	18.65±3.19

[a]P<0.05 vs. M0 control.

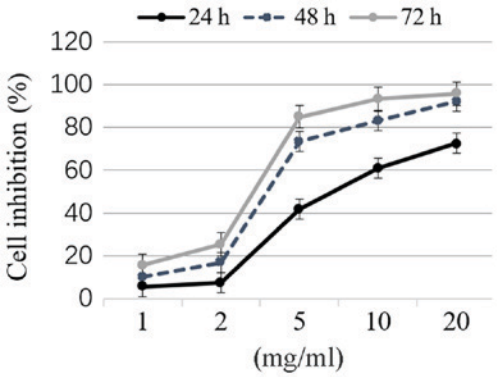

Figure 1. Percentage of TPC-1 cell inhibition following treatment with 0, 1, 2, 5, 10 and 20 mg/ml matrine for 24, 48 and 72 h. The percentage growth inhibition vs. the untreated normal cells was calculated.

cells in a concentration- and time-dependent manner (Fig. 1). The half maximal inhibitory concentration (IC_{50}) represents the concentration of a drug that is required for 50% inhibition *in vitro* and is a measure of the effectiveness of a drug in inhibiting cell growth (24). In the present study, the IC_{50} of treatment with matrine for 48 h was 3.54 mg/ml.

Apoptosis and cell cycle. Flow cytometry with FITC-A/PI staining was performed to detect apoptosis in TPC-1 thyroid cancer cells exposed to 0, 2, 5 and 10 mg/ml matrine. The total percentage of apoptotic cells is described as the summation of percentages of both early and late apoptotic subpopulations; the annexin V-FITC positive cells (25). The effects of matrine on cell apoptosis are presented in Fig. 2. Matrine at the concentrations of 2, 5 and 10 mg/ml led to 16.1±1.9, 25.9±2.8 and 61.8±5.4% apoptosis, respectively, all elevated compared with 15.1±3.1% in the M0 control group (Table I). Matrine significantly induced TPC-1 cell apoptosis.

Effects of matrine on cell cycle distribution were analyzed by flow cytometry in TPC-1 cells. In the M0 control group, a total of 51.36±2.07% cells were in the G1 phase, 23.37±3.25% in the S phase and 18.49±2.46% in the G2/M phase (Table I). Following treatment with matrine at 10 mg/ml for 48 h, the percentage of cells in the G1 phase increased to 74.49±3.65%, and those in the S phase decreased to 2.42±1.63%. No significant alterations in the percentage of cells in the G2/M phase were observed (Table I). These results demonstrated that matrine induced cell cycle arrest at the G0/G1 phase in thyroid cancer cells.

Expression levels of miR-21, PTEN and Akt. The expression level of miR-21 was determined by qPCR after TPC-1 cells were treated with matrine at 0, 2 and 5 mg/ml (M0, M2 and M5) for 48 h. As shown in Fig. 3, matrine downregulated the expression level of miR-21 in TPC-1 cells (0.75±0.09 for M2/M0 and 0.44±0.13 for M5/M0). PTEN mRNA levels increased by 1.21-fold for M2/M0 and 1.66-fold for M5/M0, respectively. Akt mRNA levels decreased by 0.61-fold for M2/M0 and 0.34-fold for M5/M0.

The protein levels of PTEN and phosphorylated (p)Akt were detected by western blotting. As presented in Fig. 4, PTEN was significantly upregulated by matrine compared with the M0 control (by 1.64-fold for M5/M0 and 1.21-fold for M2/M0). Levels of pAkt were downregulated (0.61 for M2/M0 and 0.24 for M5/M0). The miR-21 mimic markedly reduced the expression level of PTEN protein to 0.83 vs. the M0 blot value and increased the level of pAkt by 1.24-fold vs. M0 blot value. These results demonstrated that matrine regulated the miR-21/PTEN/Akt pathway to induce TPC-1 apoptosis and cell cycle arrest.

Discussion

In the present study, matrine induced apoptosis and G1 cell cycle arrest through downregulating miR-21 to affect the PTEN/Akt signaling in TPC-1 human thyroid cancer cells. Matrine can inhibit cancer cell proliferation by multiple mechanisms in various tumors (14-23). However, the effect of matrine on thyroid cancer has not been previously tested. The present study demonstrated that matrine inhibited growth of TPC-1 cells, suggesting that this medication may be used to treat thyroid cancer. Furthermore, matrine induced apoptosis and cell cycle arrest in TPC-1 cells. These results were consistent with those previously obtained in studies investigating other cancers (14,15,17,23,26,27).

Dysfunctional miR-21 regulates a tumor suppressor PTEN to promote cancer cell growth (5,9,11,23,27,28). In the present study, miR-21 level in TPC-1 cells treated with matrine was downregulated, and subsequently, expression of its target PTEN was increased at both mRNA and protein levels. Furthermore, miR-21 mimic was transfected into TPC-1 cells and it was determined that overexpression of miR-21 downregulated PTEN protein levels. These results indicated that matrine can regulate miR-21 to prevent PTEN inhibition in TPC-1 thyroid cancer cells. A similar matrine/miR-21/PTEN interaction was observed in MCF-7 breast cancer cells as

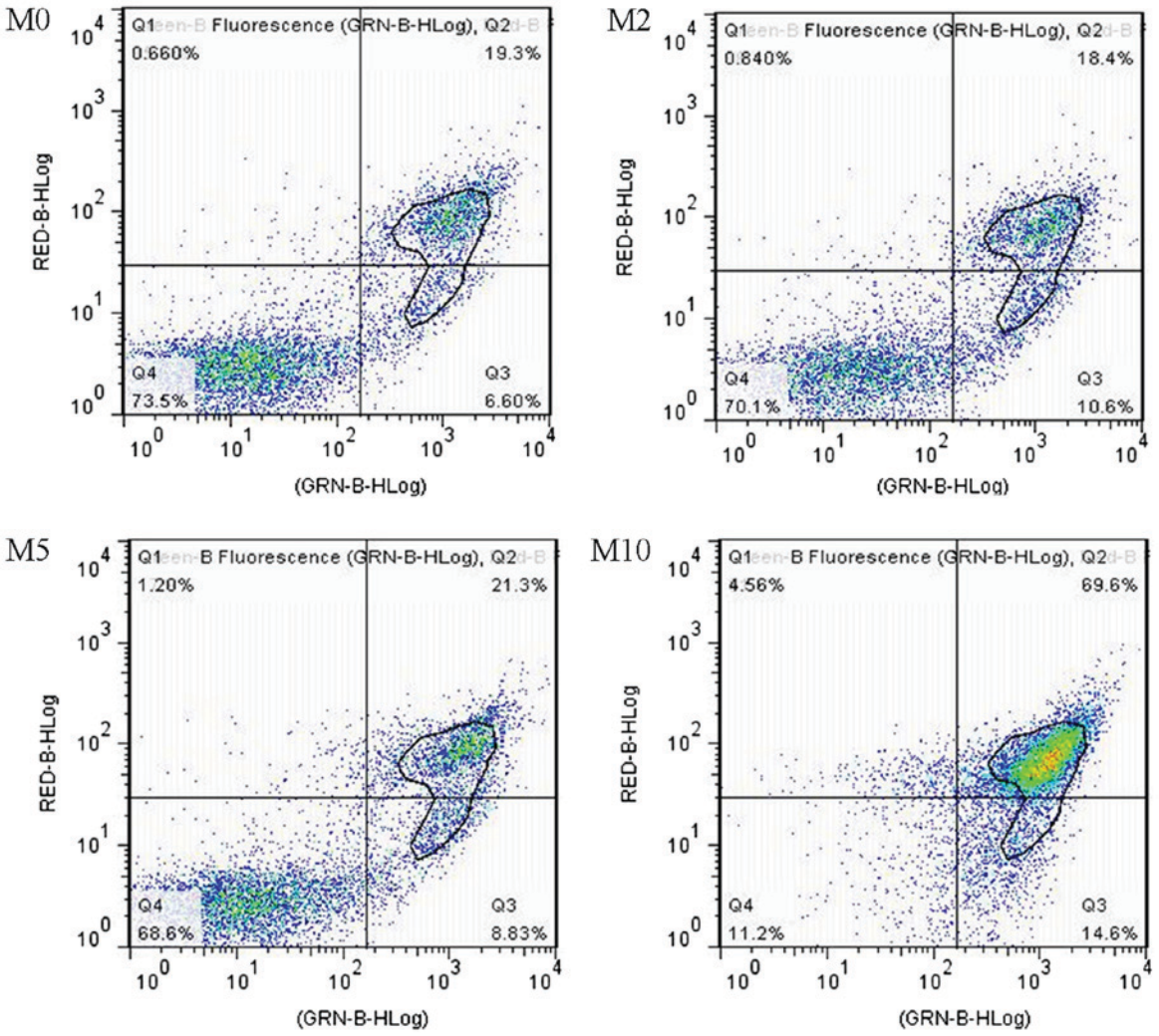

Figure 2. Flow cytometry with FITC-A/PI double staining to detect TPC-1 apoptosis. M, matrine, at 0 (M0 control), 2, 5, 10 mg/ml.

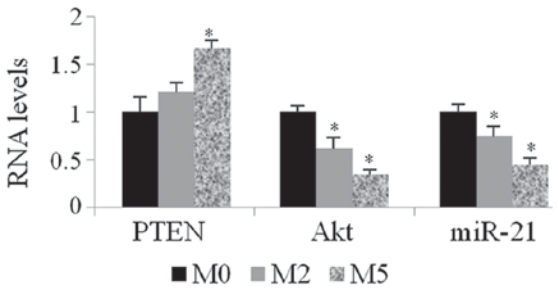

Figure 3. Matrine at 2 and 5 mg/ml downregulates miR-21, increases the expression levels of PTEN mRNA and reduces the levels of Akt mRNA. Quantitative analysis was performed with GAPDH as an internal reference. Data are presented as the mean ± standard deviation normalized to the M0 group. *P<0.05 vs. the M0 group, n=3. M0, 0 mg/ml matrine; M2, 2 mg/ml matrine; M5, 5 mg/ml matrine; miR-21, microRNA 21; PTEN, phosphatase and tensin homolog; Akt, RAC-α serine/threonine-protein kinase.

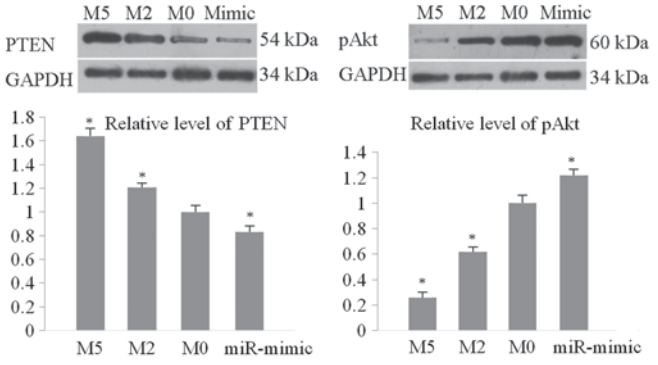

Figure 4. Western blot analysis. Matrine at 2 and 5 mg/ml increases the expression levels of PTEN and reduces the levels of pAkt. miR-21 mimic downregulates PTEN and increases the levels of pAkt. Quantitative analysis was performed with GAPDH as an internal reference. Data are presented as the mean ± standard deviation normalized to the M0 group. *P<0.05 vs. the M0 group, n=3. M0, 0 mg/ml matrine; M2, 2 mg/ml matrine; M5, 5 mg/ml matrine; miR-21, microRNA 21; PTEN, phosphatase and tensin homolog; Akt, RAC-α serine/threonine-protein kinase; p, phosphorylated.

reported in a previous study (23). PTEN as a tumor suppressor can dephosphorylate Akt to induce apoptosis and cell cycle arrest at the G1 and S phase (10,11,20,23,26-28). In the present study, upregulation of PTEN suppressed phosphorylation of Akt to cause apoptosis and cell cycle arrest in the G1 phase in TPC-1 cells.

In addition to the miR-21/PTEN/Akt pathway, matrine may inhibit cancer cells in other ways. Matrine can upregulate proapoptotic proteins including B cell lymphoma (Bcl)-2

associated agonist of cell death, Bcl-2 antagonist/killer 1 and Bcl-2 associated X, apoptosis regulator, and inhibit expression of anti-apoptotic Bcl-2 and Bcl-xl (18,29,30). In addition to the PTEN/Akt signaling, miR-21 serves important roles in cancer growth, proliferation, migration and metastasis by targeting programmed cell death 4 and Sprouty RTK signaling antagonist 1, or by upregulating Bcl-2 indirectly (10,13,31). Overexpression of PTEN upregulates the p21/WAF1/CIP1 and p27/KIP1 pathways by dephosphorylating Akt to increase cell apoptosis and/or induce G1 phase arrest (17,23). These issues should be further investigated in thyroid cancer cells.

In conclusion, the data presented in the present study suggested that the miR-21/PTEN/Akt pathway may be one of the mechanisms for matrine to inhibit TPC-1 thyroid cancer cells. Matrine may be an alternative potential drug for the treatment of thyroid cancer.

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Availability of data and materials

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

Authors' contributions

ZL cultured cells and performed flow cytometry, PCR, western blotting and transfection assays. ZX also cultured cells and performed the MTT assay and statistical analysis. CS designed the experiment, revised the manuscript and approved the submission.

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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