Journal of Medicinal Plants Research Vol. 5(16), pp. 3896-3903, 18 August, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Tetramethylpyrazine (TMP) promotes chondrocyte proliferation via pushing the progression of cell cycle

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Accepted 8 July, 2011

Tetramethylpyrazine (TMP) is the major bioactive constituent of Rhizoma Chuanxiong which has long been used as an important component in several Chinese medicine formulations for the clinical treatment of osteoarthritis. However, the molecular mechanism of the therapeutic effect of TMP remains unclear. In the present study, we investigated the cellular effects of TMP in cultured primary chondrocytes. Chondrocytes isolated from the knee articular cartilage of SD rats were cultured and identified using toluidine blue staining. The second generation of chondrocytes was treated with or without TMP. We found that TMP treatment could promote chondrocyte proliferation via pushing the progression of cell cycle. Furthermore, using RT-PCR and Western blotting analyses we observed that the mRNA and protein levels of Cyclin D1 and CDK4 were significantly enhanced after TMP treatment, whereas those of p21 were significantly decreased. Our study suggests that promoting the proliferation of chondrocytes is one of the mechanisms by which TMP treats osteoarthritis.

Key words: Tetramethylpyrazine, chondrocyte, osteoarthritis, cell cycle.

INTRODUCTION

Osteoarthritis (OA) is a chronic degenerative disease, which is the leading cause of disability among Chinese. OA is best characterized by a basic pathology of cartilage degradation caused by the mutual influence of mechanical and biological factors (Heinegård and Saxne, 2011; Li et al., 2010). Articular cartilage is composed of chondroctyes and extracellular matrix (ECM), in which chondroctyes can rapidly respond to extracellular signals and regulate the dynamic equilibrium between the degradation and synthesis of the ECM, which is crucial to the maintenance of the cartilage function (Okamoto and Atsuta, 2010; Yuan et al., 2010). Therefore, the functional changes of chondrocytes contribute to the degradation of the articular cartilage and thus the pathologenesis of OA. Previous study reported that enhancing chondrocyte function by promoting chondrocytes proliferation might be one efficient treatment to cure OA or delay the progression of OA. The cell cycle takes place in chondrocytes leading to its division and duplication, which plays important roles contributing to chondrocyte proliferation. The cell cycle is the series of events that consists of four distinct phases: G_1 phase, S phase, G_2 phase and M phase (Li et al., 2010; Kamel et al., 2009). Activation of each phase is dependent on the proper progression and completion of the previous one. G1/S transition, which is one of the two main checkpoints, is a rate-limiting step in the cell cycle and regulates the cell proliferation. G1/S progression is highly regulated by Cyclin D1 and Cyclin-dependent kinase 4 (CDK4). Cyclin

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Abbreviations: OA, osteoarthritis; **TMP,** tetramethylpyrazine; **TCM,** traditional Chinese medicine; **DMSO,** dimethyl sulfoxide; **MTT,** 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide.

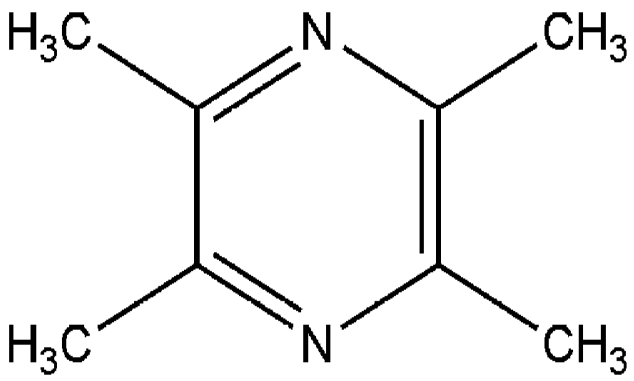

Figure 1. The chemical structure of TMP.

D1, which is the first cyclin produced in the cell cycle, binds to existing CDK4, forming the active Cyclin D1-CDK4 complex, which in turn phosphorylates the retinoblastoma susceptibility protein (Rb) (Ferrer et al., 2006; Homma and Homma, 2008). The phosphorylated Rb dissociates from the E2F/DP1/Rb complex, activating E2F. Activation of E2F results in transcription of various genes, such as Cyclin E and Cyclin A, regulating other cell cycle phases. p21 is a inhibitory protein of cell cycle, which prevents cell cycle progression by binding to and inactivating, Cyclin D1-CDK4 complex (Parafioriti et al., 2009; Sesselmann et al., 2009). Therefore, up-regulation of Cyclin D1 and CDK4 expression and/or down-regulation of p21 expression will contribute to cell cycle progression and promote chondrocyte proliferation.

Rhizoma Chuanxiong is a Chinese medicinal herb which has long been used as an important component in several Chinese medicine formulations for the clinical treatment of osteoarthritis. Tetramethylpyrazine (TMP) is one of the active components of Rhizoma Chuanxiong and is an amide alkaloid with a molecular formula of $C_8H_{12}N_2$, the chemical structure shown in Figure 1 (Ju et al., 2010; Li et al., 2010). In order to extend the clinical observations of the potential anti-cartilage degeneration effect of Rhizoma Chuanxiong and help to establish a scientific foundation for further research, in this study we evaluated the effect of TMP on the cell cycle of cultured primary chondrocytes, and investigated the possible molecular mechanisms mediating its biological effect. We found that TMP facilitated chondrocyte proliferation in a dose-dependent manner. In addition, TMP treatment promoted the cell cycle G1/S progression in chondrocytes, which was accompanied by up-regulating the expression of Cyclin D1 and CDK4 as well as down-regulating the expression of p21. Our finding suggests that promotion of chondrocyte proliferation via pushing the cell from G1 to S phase probably is one of the mechanisms by which Rhizoma Chuanxiong can be effective in the treatment of OA.

MATERIALS AND METHODS

Materials and reagent

Dulbecco's modified eagle media (DMEM), fetal bovine serum (FBS), trypsin-EDTA and TRIzol reagent were all purchased from Invitrogen (Grand Island, NY, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and type II collagenase were obtained from the Sigma Chemical Co. (St. Louis, MO, USA). Rabbit anti-rat Cyclin D1, CDK4, p21, β-actin and HRP secondary goat anti-rabbit antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). Cell cycle assay kit was provided by Becton Dickinson (San Jose, CA, USA). Toluidine blue was obtained from the Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). DNA primers were synthesized by Sangon Biotech (Shanghai, China). TMP (HPLC≥98%) was produced by Nanjing Zelang Medical Technological Co., Ltd (Nanjing, China). Stock solutions of TMP were prepared by dissolving the TMP powder in DMSO to a concentration of 1 mg/ml, and stored at -20°C. The working concentrations of TMP were made by diluting the stock solution with the culture medium. The final concentration of DMSO in the medium was <0.5%.

Animals

Male Sprague-Dawley (SD) rats at 4 weeks of age (total 36) were purchased from the Super-BK Laboratory Animal Inc. (Shanghai, China). The study protocol was approved by Fujian University of Traditional Chinese Medicine. The disposal of animals was in line with the Ministry of Science and Technology of the People's Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals, 2006.

Isolation, culture and identification of rat chondrocytes

SD rats were killed by cervical dislocation and both knee joints were dissected out under sterile conditions. The knee joints were immersed in 75% alcohol for 5 min and placed phosphate buffered saline (PBS). After the separation of muscles and tendons from knee joints and the clearance of synovial fluid on the cartilage surface, the cartilage was rinsed in PBS and in DMEM three times. The cut 1 mm^3 cartilage pieces were placed in dishes containing 0.2% Type II collagenase and transferred to a 37°C incubator. The supernatant was centrifuged and the resulting pellet was digested with renewal digestive juice. This procedure was repeated four times at 60 min intervals, centrifuged at 800 rpm for 5 min to obtain a cell pellet. Cells were re-suspended in DMEM completed culture medium containing 10% FBS, 50 mg/L vitamin C and 100 U/ml penicillin and 100 µg/ml streptomycin to a concentration of (2 to 3) $\times 10^5$ cells/ml (measured with a hemacytometer counting plate) for culture inoculation (termed P1). The cells were subcultured at 80 to 90% confluency, sequentially termed P2 and P3.

A cell climbing sheet of P2 chondrocytes (at 3 days) was washed with PBS, fixed with 10% neutral formaldehyde for 30 min and stained with 1% toluidine blue for 30 min. Samples were quickly washed with anhydrous ethyl alcohol, dried and sealed for an observation. Cell morphology was observed using a phase-contrast microscope (Olympus, Japan). The photographs were taken at a magnification of 200x.

Evaluation of cell viability by MTT assay

Chondrocytes viability was assessed by MTT colorimetric assay.

The passage 2 chondrocytes were seeded into 96-well plates at a density of 1.0×10^4 cells/well in 0.1 ml 10% FBS DMEM, cultured for 24 h and were starved for 24 h in serum-free DMEM medium. The chondrocytes were treated with final TMP concentrations of 25, 50 and 100 µg/ml for 48 h, and the vehicle control cells were treated with 0.5% DMSO. After treatment, 10 µl MTT (5 mg/ml in phosphate buffered saline, PBS) were added to each well, and the samples were incubated at 37°C for 4 h.

The purple-blue MTT formazan precipitate was dissolved in 100 µl DMSO and cells were shaken for 10 min. The absorbance was measured at 570 nm using an ELISA reader (BioTek, Model EXL800, USA).

TMP intervention

The passage 2 chondrocytes were seeded into 6-well plates at a density of 2.0×10^5 cells/well in 2 ml 10% FBS DMEM, cultured for 24 h and starved for 24 h in serum-free DMEM medium. The chondrocytes were treated with 25, 50 and 100 µg/ml concentrations of TMP for 48 h, and the vehicle control cells treated with 0.5% DMSO. After treatment, the cell cycle distribution of the chondrocytes was analyzed by flow cytometry, the mRNA and protein expression levels of Cyclin D1, CDK4 and p21 in chondrocytes were detected using RT-PCR and Western blotting, respectively.

Detection of the cell cycle by flow cytometry analysis with PI staining

After treated with various concentrations of TMP, cell cycle of chondrocytes was determined by flow cytometry analysis using a fluorescence-activated cell sorting (FACS) caliber (Becton-Dickinson, CA, USA) and cell cycle assay kit. Staining was performed according to the manufacturer's instructions. DNA content data was analyzed with Mod Fit to count the number of chondrocytes at G_0/G_1, S and G_2/M phases.

RNA extraction and RT-PCR analysis

The passage 2 chondrocytes were treated with various concentrations of TMP for 48 h. Total RNA from chondrocytes was isolated with TRIzol reagent. Oligo(dT)-primed RNA (1 µg) was reverse transcribed with SuperScript II reverse transcriptase according to the manufacturer's instructions. The obtained cDNA was used to determine the mRNA amount of CyclinD1, CDK4 and p21 by PCR with Taq DNA polymerase. β-actin was used as an internal control.

The primers used for CyclinD1 (247 bp, sense 5′-TGA CTG CCG AGA AGT TGT GC-3′, antisense 5′-GAG GGT GGG TTG GAA ATG AA-3′), CDK4 (274 bp, sense 5′-GCT CCG AGA ATG GCT ACC AC-3′, antisense 5′-CCT TGA TGT CCC GAT CAG TT-3′), p21 (304 bp, sense 5′-CCC GTG GAC AGT GAG CAG TT-3′, antisense 5′-GGG CGT GAG ACA CCA GAG TG-3′) and β-actin (432 bp, sense 5′-TCA GGT CAT CAC TAT CGG CAA T -3′, antisense 5′-AAA GAA AGG GTG TAA AAC GCA -3′). PCR was performed under the following conditions: 94°C for 5 min, followed by 94°C for 30 s, 52 /52 /55 /52°C for 30 s and 72°C for 30 s for a total of 35 cycles, followed by 72°C for 10 min.

Amplified products were electrophoresed on a 1.5% agarose gel, imaged, and analyzed with a gel imaging system to yield a quantitative measurement of the optical density ratio of values of CyclinD1, CDK4 and p21 to β-actin.

Western blotting analysis

The passage 2 chondrocytes treated with various concentrations of TMP for 48 h. The treated chondrocytes were lysed and protein concentrations were determined by the BCA assay using bovine serum albumin as a standard.

The samples were loaded with 20 µg and separated by electrophoresis on 12% SDS-polyacrylamide gels under a reducing condition using 200 V for 1 h. After electrophoresis, the proteins were transferred to PVDF membranes in a Tris-glycine transfer buffer (48 mM Tris, 39 mM glycine, 0.05% w/v SDS, 10% v/v methanol) using a semidry blotting system, which was detected with antibodies against Cyclin D1, CDK4, p21 and β-actin (1:1000) overnight at 4°C with rocking. After PVDF membranes were washed in TBST, secondary horseradish peroxidase (HRP)-conjugated antibodies were added at 1:2000 dilution for 1 h at room temperature and the PVDF membranes were washed again in TBST. Blots were developed using Super Signal Pico Substrate, and images were taken using a Kodak image station. Protein bands were analyzed using the Fluor-s Gel imaging analysis system and normalized to β-actin in the sample.

Statistical analysis

Data were processed with SPSS 13.0. The quantitative data were expressed as mean ± standard deviation (SD). Statistical analysis of the data was performed with Student's t-test and ANOVA. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Morphological observation and identification of chondrocytes

After isolated from the knee joints of SD rats, chondrocytes were completely digested after three treatments with 0.2% type II collagenase. The primary chondrocytes (termed P1) grew relatively slow. It took 8 days for P1 chondrocytes to proliferate into fusiform overlapping monolayer with typical chondrocytic morphology, that is, an irregular “flagstone” stereo shape (Figure 2A).

The second and third generations of chondrocytes (P2 and P3, respectively) grew much faster, cells formed fusiform overlapping monolayer within 4 to 5 days. However, the morphology of P3 chondrocytes has changed to a certain extent, whereas P2 chondrocytes kept the typical chondrocytic morphology (Figure 2B and C). Toluidine blue staining is a classic method for identification of chondrocytes. Toluidine blue can interact with proteoglycan that is a major element of ECM produced by chondroctyes; and the coupling of the proteoglycan with toluidine blue forms a purplish-red multimer. As shown in Figure 2D, after toluidine blue staining, the purplish red metachromatic granules were observed both in the cytoplasm of P2 chondrocytes and in the extracellular space. Therefore, in this study we used P2 chondrocytes in the following experiments.

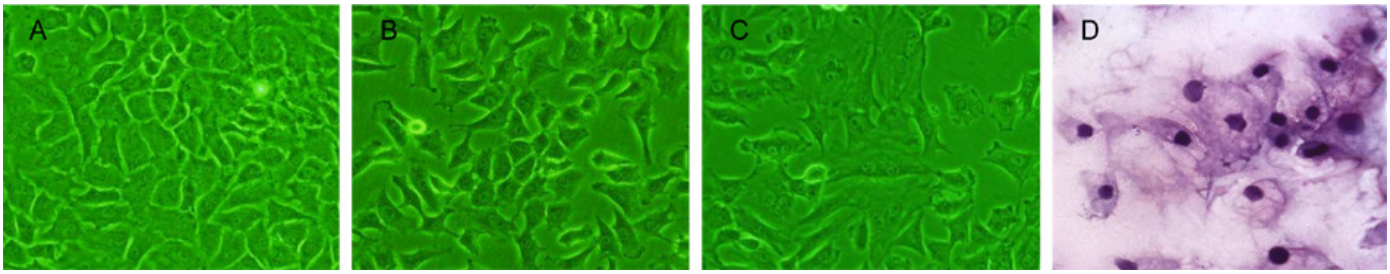

Figure 2. Morphological observation and identification of chondrocytes. (A) P1 chondrocytes cultured for 8 days were spindly and polygonal in an irregular "flagstone" stereo shape. (B) P2 chondrocytes cultured for 3 days. (C) P3 chondrocytes cultured for 3 days. (D) P2 chondrocytes cultured for 3 days, stained with toluidine blue.

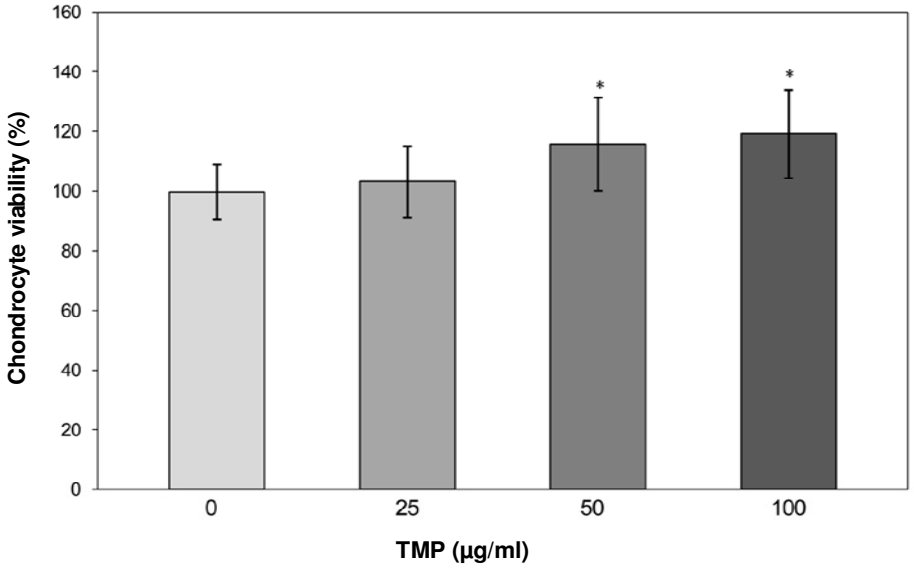

Figure 3. Effect of TMP on chondrocytes viability. P2 chondrocytes were treated with various concentrations of tetramethylpyrazine for 48 h. Chondrocytes viability was determined by the MTT assay. Data are averages with S.D. (error bars) from at least three independent experiments. *P<0.05, significant versus untreated control cells.

Effect of TMP on chondrocyte viability

The effect of TMP on the viability of chondrocytes was determined by MTT assay. As shown in Figure 3, treatment with 25, 50 and 100 μg/ml of TMP for 48 h respectively increased cell viability by 5.14, 15.85 and 19.24%, compared to untreated control cells (P<0.05), suggesting that TMP promotes the growth of chondrocytes in dose-dependent manners.

Effect of TMP on the cell cycle of chondrocytes

To determine the mechanism of the pro-proliferative activity of TMP, we examined its effect, the G1 to S progression in chondrocytes via PI staining followed by FACS analysis. As shown in Figure 4, after stimulation for 48 h, the percentage proportion of cells in G_0/G_1 phase cells following treatment with 25, 50 and 100 μg/ml of TMP 68.27, 63.54 and 60.87%, all of which were significantly lower than that of untreated control cells (74.45%; P<0.01 or P<0.05). Consistently, the percentage proportion in S-phase cells showed an opposite trend after TMP treatment. Taken together, it is suggested that TMP can enhance chondrocyte proliferation by promoting the cell cycle G1 to S progression.

Effect of TMP on expression of Cyclin D1, CDK4 and p21

Cyclin D1, CDK4 and p21 proteins are key regulators of

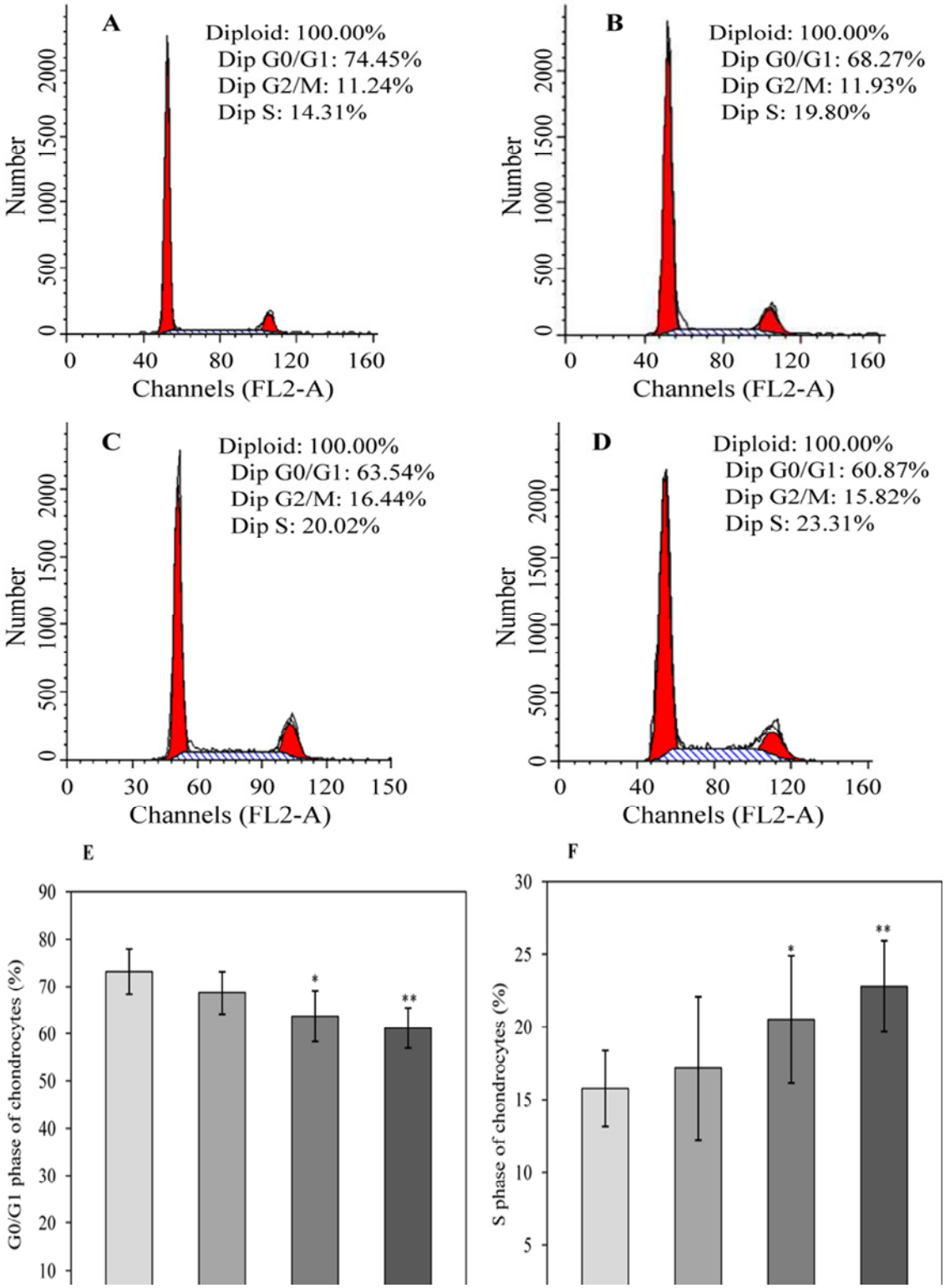

Figure 4. Effect of TMP on the cell cycle of chondrocytes. P2 chondrocytes were treated with the indicated concentrations of TMP for 48 h. Chondrocytes were collected and stained with PI followed by FACS analysis. The data shown are averages with S.D. (error bars) from three independent experiments. *P<0.05, **P<0.01, significant versus untreated control cells. (A) Untreated control chondrocytes, (B to D) chondrocytes were treated with TMP 25, 50, 100 µg/ml for 48 h)

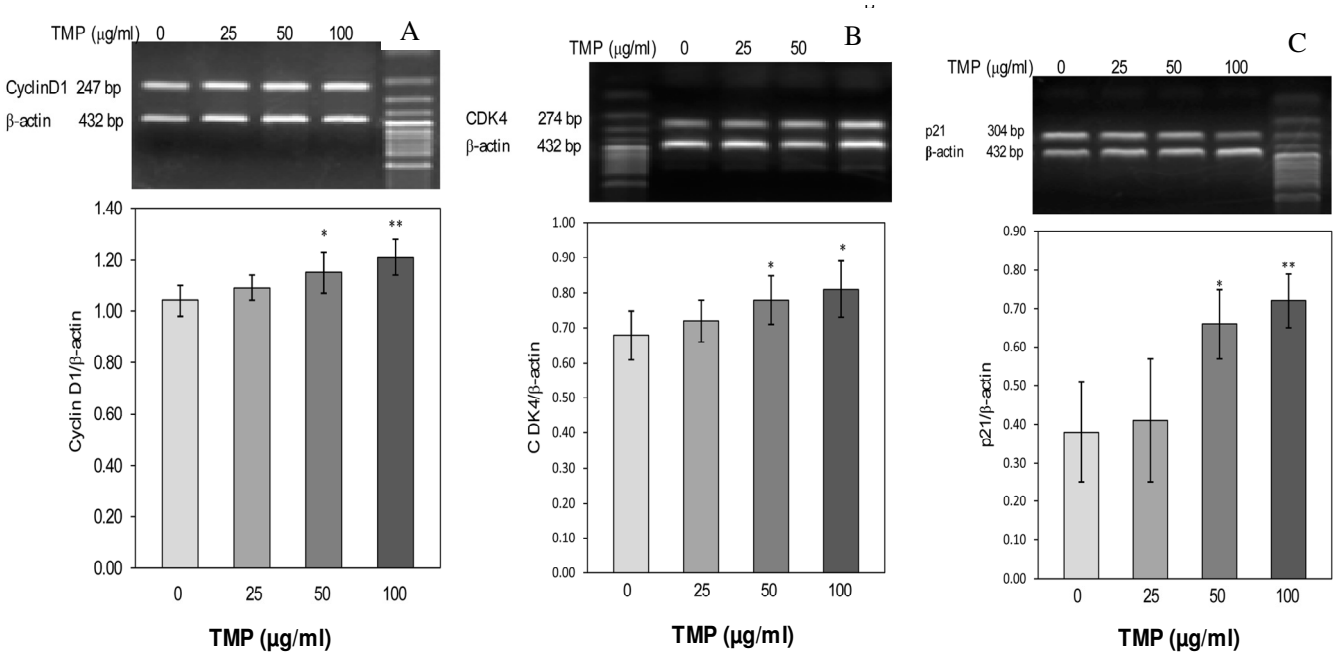

Figure 5. Effect of TMP on the mRNA expression of Cyclin D1, CDK4 and p21 in chondrocytes. P2 chondrocytes were treated with the indicated concentrations of TMP for 48 h. The mRNA levels of Cyclin D1, CDK4 and p21 in TMP-treated and untreated chondrocytes were determined by RT-PCR. β-actin was used as the internal controls for the RT-PCR assays. Data are averages with S.D. (error bars) from at least three independent experiments. *P<0.05, **P<0.01, significant versus untreated control cells.

cell cycle progression. The Cyclin D1-CDK4 complexes formed at the end of G1 phase can promote S phase entry, regulating mitosis and enhancing cell proliferation, whereas p21 inhibits the activity of the Cyclin D1-CDK4 complexes. To further explore the mechanism of TMP's activity, we analyzed the mRNA and protein expression levels of Cyclin D1, CDK4 and p21 after TMP treatment using RT-PCR and Western blotting, respectively. The results of the RT-PCR assay showed that TMP treatment significantly increased Cyclin D1, CDK4 and reduced p21 mRNA expression in chondrocytes (P<0.01 or P<0.05) (Figure 5A to C); and the protein expression pattern of Cyclin D1, CDK4 and p21 was similar to that of their respective mRNA level (Figure 6A to D).

DISCUSSION

Osteoarthritis (OA) is a multifactorial disease characterized by the breakdown of hyaline articular cartilage and the formation of osteophytes. The only cell type present in mature cartilage is the chondrocyte, which is responsible for extracellular signals and regulate the dynamic equilibrium between the degradation and synthesis of the ECM (Lahm et al., 2010; Aung et al., 2011). Many studies have reported that there is a very low proliferative activity in osteoarthritic chondrocytes, which might be due to better access of chondrocytes to proliferative factors from the synovial fluid due to the damage to the collagen matrix itself or due to fissuring or loosening of the collagen network (Li et al., 2011; Braun et al., 2010). In any case, chondrocytes proliferation is most probably the biological activity that causes chondrocytes clustering. Therefore, promoting proliferation of chondrocytes is an efficient treatment to delay the progression of cartilage degradation.

Complementary and alternative medicine is a current focus of interest for the general public and the medical profession, which includes traditional Chinese herbal medicines (TCM). Chinese herbs have relatively fewer side effects as compared to modern chemotherapeutics and have long been used clinically to treat OA. Rhizoma Chuanxiong, a famous traditional Chinese herbal medicine with properties of promoting blood circulation and removing blood stasis, has been reported to have many pharmacological applications (Liang et al., 2010; Tian et al., 2010). Recently, Rhizoma Chuanxiong has been demonstrated to be clinically effective in treating OA. However, the mechanism of its treatment of OA is still largely unknown. Therefore, the present study was to examine whether TMP, which is one of the major active components of Rhizoma Chuanxiong, promotes chondrocytes proliferation. The cell cycle plays an important role in the proliferation of chondrocytes, which consists of four distinct phases. G_0 phase is a resting phase where the cell has left the cycle and has stopped

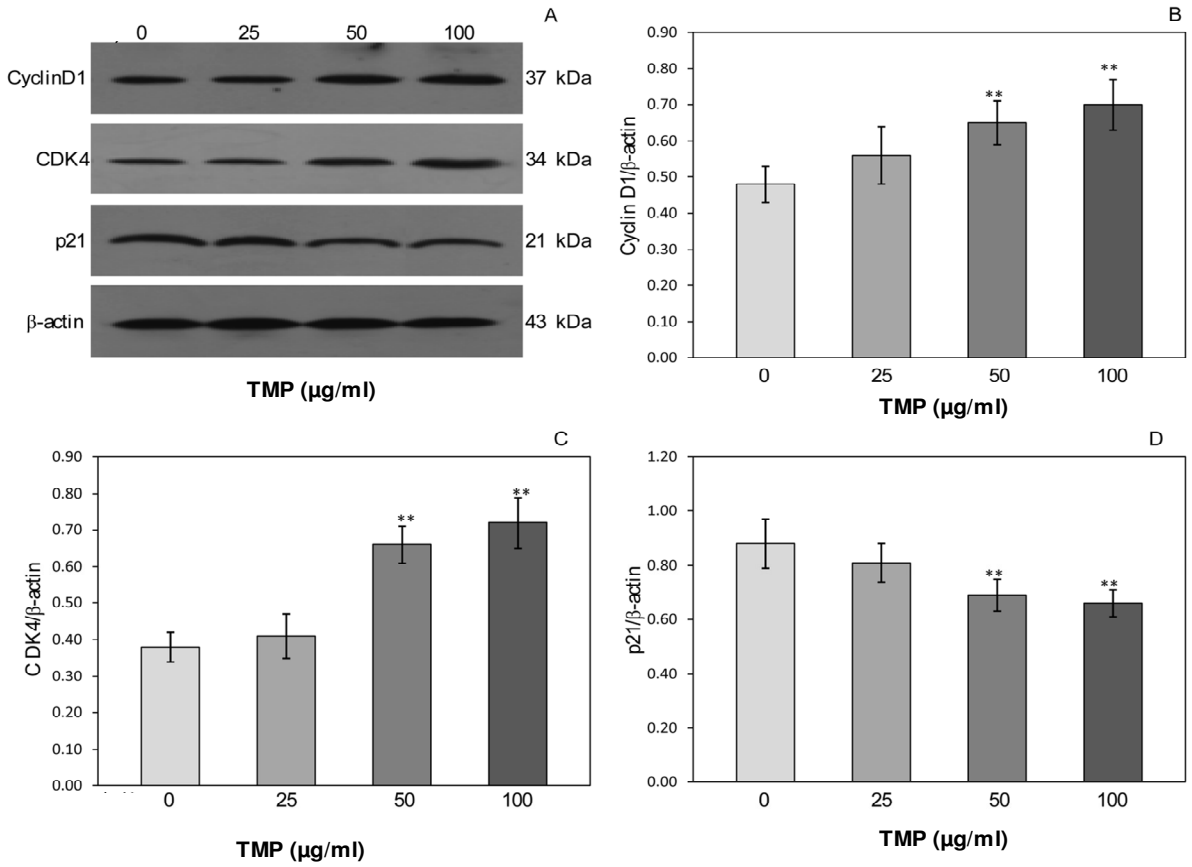

Figure 6. Effect of TMP on the protein expression levels of Cyclin D1, CDK4 and p21 in chondrocytes. P2 chondrocytes were treated with the indicated concentrations of TMP for 48 h. The protein expression levels of Cyclin D1, CDK4 and p21 in TMP-treated and untreated chondrocytes were determined by Western blotting. β-actin was used as the internal controls for the Western blotting assays. Data are averages with S.D. (error bars) from at least three independent experiments. **P<0.01, significant versus untreated control cells.

dividing, G_1 phase is ready for DNA synthesis, S phase occurs DNA replication, G_2 phase is collectively known as interphase, M phase is ready to complete cell division (Legerski, 2010; Biggar and Storey, 2009). The DNA content of cells is a constant parameter that varies over the progression of the cell cycle. Our MTT data showed that TMP promoted chondrocyte viability in a dose-dependent fashion. Flow cytometry detected changes in cell cycle with greater sensitivity than MTT, the results showed that the proportion of chondrocytes in G_0/G_1 phase was significantly reduced, the percentage of chondrocytes in S phases was significantly increased, indicating that TMP pushes the progression of the cell cycle of chondrocytes *in vitro*, thus promoting chondrocyte proliferation.

G1/S transition is one of the two main checkpoints used by cell to regulate the progression of cell cycle, the following cell can pass through S phase and mitosis independent of mitogens (Onumah et al., 2009; Simmons et al., 2008). It is tightly regulated by the expression and phosphorylation of a number of well-characterized cyclins, cyclin-dependent kinases and members of the retinoblastoma gene family (Zhou et al., 2010). Cyclin D1 and CDK4, respectively represent two different classes of G1-specific CDKs whose activation leads to the transcription of genes required for entry into S phase (Hwang et al., 2007; Zhang et al., 2009). p21, is one of upstream inhibitor members, modulate the activity of the Cyclin D1-CDK4 complexe (Mehrara et al., 2010). Some of these inhibitors are induced upon stresses such as DNA damage and nucleotide depletion. For an example, the DNA damage checkpoint pathway via upregulating the expression of p21 through the post-translational modification of p53, which preserves the association of Rb with E2F and arrests cells in the G1 phase until feedback from the DNA repair machinery promotes transition into the S phase (Yanagino et al., 2009).

Therefore, differential expression of the cell cycle regulatory factors including Cyclin D1, CDK4 and p21

may regulate the G1/S transition in chondrocytes. In this study, we demonstrated that TMP treatment dose-dependently enhances Cyclin D1 and CDK4 mRNA expression and reduces p21 mRNA expression in chondrocytes. This indicates that TMP enhances chondrocytes proliferation by affecting Cyclin D1, CDK4 and p21 at transcriptional level. We further studied the role of TMP on the expression of proteins involved Cyclin D1, CDK4 and p21. The results showed that TMP treatment up-regulates Cyclin D1 and CDK4 protein expression and down-regulates p21 protein expression, which is in accordance with the pattern of their mRNA expression after TMP treatment.

In conclusion, our data demonstrate that TMP promotes chondrocytes proliferation via up-regulating the expression of Cyclin D1, CDK4, down-regulating the expression of p21, suggesting that Rhizoma Chuanxiong may be a potential novel therapeutic agent for the treatment of OA.

ACKNOWLEDGEMENTS

This work was supported by the Project supported by the Major Project Foundation of Fujian Provincial Administration of Science and Technology (No. 2009Y0029), the National Natural Science Foundation of China (No. 81072826).

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