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期末報告

麥黃酮及其衍生物抑制不同骨癌細胞株轉移、血管新生及誘導細胞凋亡之機制探討

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中 文 摘 要 ： 黃酮類化合物過去被發現大量存在植物中，具有廣泛的生物學作用，而其中一類化合物－五羥黃酮（Tricetin）在許多文獻中被發現具有抑制癌細胞增生，同時誘導癌細胞走向細胞凋亡的效果，包括乳癌及肝癌，但五羥黃酮與骨肉瘤之間的相關性尚未被釐清。因此本實驗擬探討五羥黃酮是否會對骨肉瘤細胞的生長與轉移造成影響。首先本實驗將五羥黃酮劃分為四組濃度（0、20、40、80 μM），將其各別加入骨肉瘤細胞株U2OS及HOS中，並利用MTT assay發現五羥黃酮在此濃度差異下，對U2OS與HOS細胞並無毒殺性。透過Zymography assay發現五羥黃酮會顯著抑制U2OS細胞的MMP-9蛋白活性，亦會抑制HOS細胞的MMP-9蛋白活性。利用Wound healing assay及Boyden chamber assay發現五羥黃酮會顯著抑制U2OS與HOS細胞爬行、轉移及侵襲的能力。上述之結果顯示五羥黃酮能抑制此兩株骨肉瘤細胞的轉移，且對U2OS細胞的MMP-9具較專一的抑制效果，因此本實驗想進一步觀察五羥黃酮對U2OS細胞的作用機制。利用Western blot及RT-PCR、Real time PCR發現五羥黃酮能抑制MMP-9的蛋白表現量及MMP-9 mRNA的表現量。Western blot的結果亦顯示五羥黃酮會抑制磷酸化p38及磷酸化Akt訊息傳遞蛋白之表現。最後利用p38及Akt抑制劑確認五羥黃酮能透過訊息傳遞路徑p38及Akt來調控下游蛋白表現。總結以上結果，本實驗發現在骨肉瘤細胞中，五羥黃酮能透過調控MMP-9的轉錄及p38、Akt訊息傳遞路徑來抑制MMP-9的表現使癌症轉移能力降低。

中 文 關 鍵 詞 ： 五羥黃酮、癌轉移、骨癌細胞株

英 文 摘 要 ： Tricetin, a dietary flavonoid, has cytostatic properties and anti-metastasis activities in various cancer cells; however, we little know whether the effect of tricetin on metastasis of human osteosarcoma. Here, we tested the hypothesis that tricetin possesses the anti-metastatic effects on human osteosarcoma cells and also investigated the underlying mechanisms of such effect. We investigated the effects of tricetin on cell viability, motility, migration and invasion in human osteosarcoma U2OS and HOS cells. Gelatin zymography, western blotting, polymerase chain reaction (PCR) and the luciferase assay were used to further explore the underlying mechanisms involved in anti-metastatic effects in U2OS cells. Our results showed that Tricetin, up to 80 μM without cytotoxicity, attenuated U2OS and HOS cells motility, invasiveness and migration by reducing matrix metalloproteinase (MMP)-9 enzyme activities. In U2OS cells, tricetin decreased MMP-9 protein and mRNA expressions, which was confirmed by real-time PCR. Next, tricetin reduced phosphorylation of p38 and Akt, but no effect on phosphorylation of ERK1/2 and JNK. In conclusion, tricetin possesses the anti-metastatic activity of osteosarcoma cells by transcriptionally repressing MMP-9 via p38 and Akt signaling pathways. This may be potentially useful as ant-metastatic agents for osteosarcoma

chemotherapy.

英文關鍵詞：metastasis, MMP-9, osteosarcoma, tricetin

Tricetin inhibits human osteosarcoma cells metastasis by transcriptionally repressing MMP-9 via p38 and Akt pathways

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Abstract

Tricetin, a dietary flavonoid, has cytostatic properties and anti-metastasis activities in various cancer cells; however, we little know whether the effect of tricetin on metastasis of human osteosarcoma. Here, we tested the hypothesis that tricetin possesses the anti-metastatic effects on human osteosarcoma cells and also investigated the underlying mechanisms of such effect. We investigated the effects of tricetin on cell viability, motility, migration and invasion in human osteosarcoma U2OS and HOS cells. Gelatin zymography, western blotting, polymerase chain reaction (PCR) and the luciferase assay were used to further explore the underlying mechanisms involved in anti-metastatic effects in U2OS cells. Our results showed that Tricetin, up to 80 μM without cytotoxicity, attenuated U2OS and HOS cells motility, invasiveness and migration by reducing matrix metalloproteinase (MMP)-9 enzyme activities. In U2OS cells, tricetin decreased MMP-9 protein and mRNA expressions, which was confirmed by real-time PCR. Next, tricetin reduced phosphorylation of p38 and Akt, but no effect on phosphorylation of ERK1/2 and JNK. In conclusion, tricetin possesses the anti-metastatic activity of osteosarcoma cells by transcriptionally repressing MMP-9 via p38 and Akt signaling pathways. This may be potentially useful as ant-metastatic agents for osteosarcoma chemotherapy.

Keywords: metastasis, MMP-9, osteosarcoma, tricetin

Introduction

Osteosarcoma consists of approximately 20% of all primary bone cancers and it is the most common malignant bone tumor affecting children, adolescents, and young adults in the world (Mirabello et al. 2009; Ottaviani and Jaffe 2009). Complete radical en bloc resection is generally the typical treatment of choice, but approximately 80% of osteosarcoma may finally gain metastatic potential (Hayden and Hoang 2006). The modern treatment that combine advanced surgical techniques and new chemotherapy protocols has enabled promoted frequency of limb-sparing surgery and the long-term survival to an approximate 68% in 2009 in the treatment of osteosarcoma (Oertel et al. 2010; Ottaviani and Jaffe 2009). However, the high metastatic ability of osteosarcoma is accountable for poor prognoses and high mortality rates and pulmonary metastases are the most common cause of mortality. At present, it is a reasonable strategy to develop new and alternative approaches in anti-metastasis or complementary therapies, including dietary supplements and phytotherapeutic agents with nominal cytotoxicity to normal tissues and potent anti-cancer properties.

Metastasis is a complex process that includes several events collectively termed the invasion-metastasis cascade (Valastyan and Weinberg 2011), including damaging intercellular interaction, increasing cancer cells and extracellular matrix (ECM) interaction, damaging ECM components, and increasing invasion and migration of

cancer cells. Break-down of the ECM of the basement membrane for cell invasion by cancer cells is mediated through proteases, particularly matrix metalloproteinases (MMPs) (Kessenbrock et al. 2010). Molecularly, MMP-2 (gelatinase A, 72 kDa), MMP-9 (gelatinase B, 92 kDa) and tissue inhibitors of metalloproteinases (TIMPs) are particularly thought to be involved in tumor cell progression, invasion and metastasis (Chien et al. 2013; Liotta and Kohn 2001; Yeh et al. 2016).

The steroid receptor coactivator (Src) phosphorylates focal adhesion kinase (FAK) and the activated p-FAK in turn activates the signaling transduction pathway of phosphatidylinositol 3-kinase (PI3K)/Akt, mitogen/extracellular signal-regulated kinase (MEK)/extracellular signal-regulated protein kinase (ERK), and c-Jun N-terminal kinase (JNK) to regulate the adhesion, migration, and invasion of cancer cells. Thus, the protein expression and phosphorylation of FAK are usually recognized as one of the biomarkers of cancer metastasis (Guo and Giancotti 2004).

Many flavonoids, which are a class of polyphenolic chemicals with a three-ring structure, have cytostatic (Fotsis et al. 1997) and apoptotic (Plaumann et al. 1996) properties to contribute possible pharmaceutical applications. Tricetin (5,7,3′,4′,5′-pentahydroxyflavone), a dietary flavonoid in Myrtaceae pollen and Eucalyptus honey (Campos et al. 2002; Martos et al. 2000a; Martos et al. 2000b; Yao et al. 2004), has potent anti-inflammatory (Geraets et al. 2007), anticancer activities

(Hsu et al. 2010; Hsu et al. 2009), and suppression of the metastasis in human non-small lung cancer cells (Hung et al. 2015) and human gliobasttoma multiforme cells (Chao et al. 2015). However, the anti-metastastic effect of tricetin in human osteosarcoma is yet not to be investigated. Therefore, the purpose of the present study was to characterize the inhibitory effects of tricetin on the cell motility, the migratory property and invasiveness of osteosarcoma cells and their underlying mechanisms.

Material and methods

Cell culture and tricetin treatment

U2OS (human osteosarcoma 15-yr-old female) and HOS (human osteosarcoma; 13-yr-old female) cell lines, obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan), were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen Corp., Life Technologies, Carlsbad, California) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Inc-Logan, UT) and 1% penicillin (100 U/mL)/streptomycin (100 μg/mL) (Sigma, St. Louis, MO), and 5 mL glutamine. The cell culture was maintained at 37 °C in a humidified atmosphere of 5% CO_2 incubator as described elsewhere (Cheng et al. 2016b). U2OS and HOS cells were plated in 24-well plates at a density of 3×10^4 cells per well and were added at different concentrations (0, 20, 40, and 80 μM) of tricetin (Extrasynthese, Genay, France) at 37◦C for 24 h for the subsequent assays.

Microculture tetrazolium (MTT) assay

For the cell viability experiment in the concentration range, a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay was performed to determine the cytotoxicity of different concentrations (0, 20, 40, and 80 μM) of tricetin on 8×10^4/well of U2OS cells and 1.2×10^5/well of HOS cells

for 24 h. After the exposure period, the media were removed and the cells were washed with phosphate-buffered saline (PBS). Afterwards, the medium was changed and the cells were incubated with MTT reagent (0.5 mg/mL) for 4 h as described elsewhere (Hsieh et al. 2007). The viable cell number per dish was directly proportional to the production of formazan, which could be measured spectrophotometrically at 563 nm following solubilization with isopropanol. The results were rechecked by morphological analysis of apoptosis.

Wound healing assay

To measure the possibility that tricetin alter cell motility of U2OS and HOS cells, we plated 5×10^5/well U2OS and 4×10^5/well HOS cells in 6-well plates for 16 h, wounded by scratching with a pipette tip, then incubated with DMEM containing 0.5% FBS and treated with different concentrations (0, 20, 40, and 80 μM) of tricetin for 0 and 24 h. We used a phase-contrast microscope (×100) to photograph the cells as described elsewhere (Lu et al. 2013; Yang et al. 2013).

Cell invasion and migration assays

A modified Boyden chamber invasion assay coating with a layer of 100 μg/cm^2 Matrigel was used as described elsewhere (Cheng et al. 2016a; Hsieh et al. 2007; Lu

et al. 2013) and applied to 8-μm-pore-size polycarbonate membrane filters to study the effect of tricetin on invasiveness and migration of U2OS and HOS cells in vitro. After treatment with the indicated concentrations (0, 20, 40, and 80 μM) of tricetin, U2OS and HOS cells were seeded into the upper section of the Boyden chamber (Neuro Probe, Cabin John, MD) at a density of 5×10^3 U2OS cells per well, and then incubated for 24 h at 37 °C. Non-invasive cells stayed in the upper chamber, while invasive cells passed through the basement membrane layer and clung to the bottom of the Boyden chamber membrane. Finally, we used a light microscope to count the cells. Using the Boyden chamber on membrane filters without Matrigel, we investigated the effect of tricetin on cell migration. Migration of cells treated with indicated concentrations of (0, 20, 40, and 80 μM) of tricetin was measured as described in the cell invasion assay previously (Lin et al. 2016a).

Gelatin zymography

After plating 8×10^4/well U2OS and HOS cells in 24-well plates for 16 h, cells were treated with different concentrations (0, 20, 40, and 80 μM) of tricetin for 24 h. We used gelatin zymography to test the effect of tricetin on gelatinases in U2OS and HOS cells as described elsewhere (Hsieh et al. 2007). Briefly, prepared 16 μL samples containing 10 μg of total protein were loaded onto a precast sodium dodecyl

sulfate–polyacrylamide gel containing 0.1% gelatin. After electrophoresis, gels were processed. The non-staining band representing the level of gelatinases measured by spot density measurement using a densitometer (AlphaImager 2000, Alpha Innotech Corp., San Leandro, CA).

Western blot analysis

Western blotting was used to explore the molecular mechanism of signaling pathways involved in U2OS cells. After treatment with different concentrations (0, 20, 40, and 80 μM) of tricetin for 6 h, the total cell lysates of 5.5×10^5 U2OS cells were prepared as described elsewhere (Hsieh et al. 2007; Lu et al. 2013; Yang et al. 2013). The total cell lysates or nuclear extracts were incubated with the primary antibodies, washed and monitored by immunoblotting using specific secondary antibodies. The relative photographic densities were quantitated by scanning the photographic negatives using a gel documentation and analysis system (Alpha-Imager 2000, Alpha Innotech Corporation, San Leandro, CA, USA). After measuring the intensity of each band by densitometry, relative intensities were calculated by normalizing to β-actin from the corresponding sample.

Reverse transcriptase–polymerase chain reaction (RT-PCR) and TaqMan

quantitative real-time PCR

Total RNA was isolated using Total RNA mini kit (Geneaid) and reverse transcribe into cDNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Procedures of complementary DNA (cDNA) synthesis and PCR amplification and quantitative real-time PCR assays were performed as described elsewhere (Hsieh et al. 2007; Hsieh et al. 2004; Lu et al. 2013). Specific primers and the fluorogenic probe were used for the MMP-9 gene as described previously (Yeh et al. 2016).

Luciferace reporter assay

Luciferase reporter assay analyzed luciferase activities by Luciferase Assay System (Promega). The MMP-9 promoter/reporter plasmid was provided from Prof. Ko (Chung Shan Medical University, Taichung, Taiwan). The construction method was performed as described previously (Lin et al. 2016b). U2OS cells were seeded (5×10^4 cells per well) in 24-wells culture plates. After 24 h of incubation, pGL-3-basic and MMP-9 promoter plasmids were co-transfected with a β-galactosidase expression vector pCH110 (Pharmacia, Piscataway, NJ, USA) into cells using Lipofectamine™ 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA). After 16 h of transfection, cells were treated with tricetin (0, 20, 40, and 80 μM)

for 24 h. The cell lysates were harvested, and luciferase activity was determined using a luciferase assay kit (Sigma chemical Co.). The value of the luciferase activities was normalized to a β-galactosidase internal control.

Statistical analysis

For all of the measurements, analysis of variance followed by with Student's t-test was used for more than two groups. Each experiment was performed in triplicate and 3 independent experiments were performed. p values < 0.05 was considered statistically significant.

Results

Cytotoxicity of tricetin in U2OS and HOS cells

After 24 h treatment, 8×10^4/well U2OS and 1.2×10^5/well HOS cells viability in the presence of concentrations of 20, 40, and 80 μM tricetin was not significantly different to that of controls in the MTT assay (Figs. 1A and 1B). Like the study reported by others (Chao et al. 2015), a 24 h treatment with tricetin up to 80 μM had no cytotoxic effect on human osteosarcoma U2OS and HOS cells, although the concentrations of tricetin were reported to show significantly toxic effects against breast cancer cells (Hsu et al. 2009). We used the concentration range for tricetin in all subsequent experiments to investigate its anti-metastatic properties.

Tricetin inhibits cells motility, migration and invasiveness of U2OS and HOS

As illustrated in figures 1C and 1D, tricetin significantly attenuated human osteosarcoma U2OS and HOS cells motility dose-dependently in the wound healing assay. To examine whether tricetin also suppresses U2OS and HOS cellular migration potential and invasive activity, migration and invasion assays were subsequently tested. Both modified Boyden chamber with or without Matrigel assays showed that tricetin significantly inhibited the migration activity and invasive potential in U2OS and HOS cells dose-dependently (Figs. 2A-2D). Therefore, tricetin could be

considered to decrease the activity of metastasis in both U2OS and HOS cells.

Tricetin inhibits MMP-9 enzyme activities, protein expression and mRNA levels of U2OS cells

In gelatin zymography, tricetin decreased the MMP-9 enzyme activity in both U2OS and HOS cells dose-dependently (Figs. 3A and 3B). In order to validate the inhibitory results on gelatinases in U2OS cells, western blot analysis was performed. We observed that tricetin decreased the MMP-9 expression (Fig. 3C). Subsequently, RT-PCR analysis was used to verify the change in U2OS cells and showed that tricetin dose-dependently repressed the mRNA expression of MMP-9 ($p < 0.001$) (Fig. 3D), which was further confirmed by quantitative real-time PCR ($p < 0.001$) (Fig. 3E). To further demonstrate the inhibitory effect on MMP-9, a luciferase reporter assay showed that tricetin significantly reduced MMP-9 promoter activity dose-dependently (Fig. 3F). Therefore, tricetin downregulates the mRNA of MMP-9 in U2OS cells at least partially at a transcriptional level.

Tricetin has no effect on the Src-FAK pathway in U2OS cells

For determining the molecular mechanisms further, western blot analysis was used. Compared with the control group, neither p-FAK Tyr397 and p-FAK Tyr925 nor

p-Src was changed by tricetin (Fig. 4A). These data indicated that p-Src and p-FAK did not mediate the anti-metastastic mechanism of tricetin in U2OS cells.

Tricetin decreases phosphorylation of JNK and p38 of U2OS cells

In depth investigation for the molecular mechanism, mitogen-activated protein kinases (MAPK) and PI3K-Akt pathways were detected in each group using western blot analysis. We found that phosphorylation of p38 and Akt were dose-dependently reduced by tricetin in U2OS cells, while there was no obvious influence on phosphorylation of ERK, and JNK (Figs. 4B and 4C). These results suggested involvement of p38 and Akt signal pathways in the effects of tricetin on U2OS cells. Using inhibitors of p38 (SB203580) and Akt (LY294002), the inhibition of cell motility in the wound healing assay by 20 μM tricetin in U2OS cells was further believed through down-regulation of p38 and Akt phosphorylation (Figs. 4D and 4E).

Discussion

The purpose of this study was to investigate the anti-metastatic effects of tricetin on osteosarcoma U2OS and HOS cells. We provided clear evidence that tricetin, at concentrations without cytotoxicity (up to 80 μM), inhibited tumor cell motility, invasiveness and migratory potential in U2OS and HOS cells. Because tumor cell invasion requires both cell migration and digestion of the basement membrane by MMPs (Cheng et al. 2016a; Cheng et al. 2016b; Foroni et al. 2012; Kessenbrock et al. 2010), we hypothesized that tricetin could affect one or the other of these two mechanisms in osteosarcoma cells. MMP-9 is found to involve in early cancer development, progression and metastasis in breast cancer patients (Garbett et al. 2000; Hanemaaijer et al. 2000; Jinga et al. 2006; Somiari et al. 2006; Talvensaari-Mattila and Turpeenniemi-Hujanen 2005; Voorzanger-Rousselot et al. 2006; Wu et al. 2008). Actually, tricetin inhibited the MMP-9 enzyme activities in both U2OS and HOS cells and protein and mRNA expressions of MMP-9 at a transcriptional level in U2OS cells.

In various kinds of cells, different stimuli induce MMP-9 expression through activation of the MEK-ERK or PI3K-Akt signaling pathways, which subsequently activate AP-1 and NF-κB (Cheng et al. 2006; Chung et al. 2004; Genersch et al. 2000; Gum et al. 1997; Han et al. 2006; Moon et al. 2004; Woo et al. 2004). Also, p38

MAPK up-regulates MMP-9 expression in Raw 264.7 cells stimulated with LPS (Woo et al. 2004) and CpG oligodeoxynucleotide (Lim et al. 2007). In western blot analysis, down-regulation of p38 and Akt phosphorylation was believed to be involved in tricetin-mediated suppression of cell motility, migration and invasion as well as MMP-9 expressions of U2OS cells.

Although the nonreceptor protein tyrosine kinase Src is capable of modulating cell migration and invasion through interaction with integrins, the FAK, and regulators of the family of Rho-GTPases (Huveneers and Danen 2009), our data indicated that p-Src and p-FAK could not mediate the anti-metastastic mechanism of tricetin in U2OS cells. Therefore, the tricetin-induced anti-metastatic ability and transcriptional suppression of MMP-9 on U2OS cells are not through FAK and Src.

Recently, natural plant products have gained increasing attention for potential use in adjuvant treatment against angiogenesis, proliferation, progression, and metastasis of malignant cancer cells (Ravindranath et al. 2009; Shankar et al. 2008). Tricetin, one of natural flavonoid derivatives which are cytoprotective and present in dietary plants and vegetables, causes cell cycle arrest of human breast adenocarcinoma MCF-7 cells at the G2/M phase by blocking cell cycle progression, increasing p21 expression in a p53-dependent manner, and decreasing the expression of cdc2, cdc25C and cyclin B, and inducing apoptosis (Hsu et al. 2009). Also, tricetin

induces an apoptotic response with changes in the expression of Bax and Bak, decreasing levels of Bcl-2 and Bcl-XL, and subsequently triggering the mitochondrial apoptotic pathway apoptotic response. In liver cancer cells, tricetin induces apoptotic cell death by triggering ROS generation and, subsequently, JNK activation as well as both intrinsic and extrinsic apoptotic pathways (Hsu et al. 2010). Notably anti-metastatic ability, tricetin suppresses benzo(a)pyrene-induced bone metastasis of human non-small cell lung cancer H460 cells (Hung et al. 2015) and suppresses the metastasis of human glioblastoma multiforme cells by inhibiting MMP-2 through the ERK pathway and modulation of the expression and transcriptional activity of SP-1 (Chao et al. 2015).

In conclusion, this is the first study to demonstrate that tricetin inhibits human osteosarcoma HOS and U2OS cell-matrix and cell-cell interactions, migration potential, and the invasive activity, and transcriptionally represses MMP-9 expression via p38 and Akt signaling pathways. Certainly, our work reinforces the idea that tricetin, a highly promising osteosarcoma anti-metastatic agent, may be of value in the development of novel therapeutic approaches for the anti-metastatic treatment of osteosarcoma.

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Figure 1

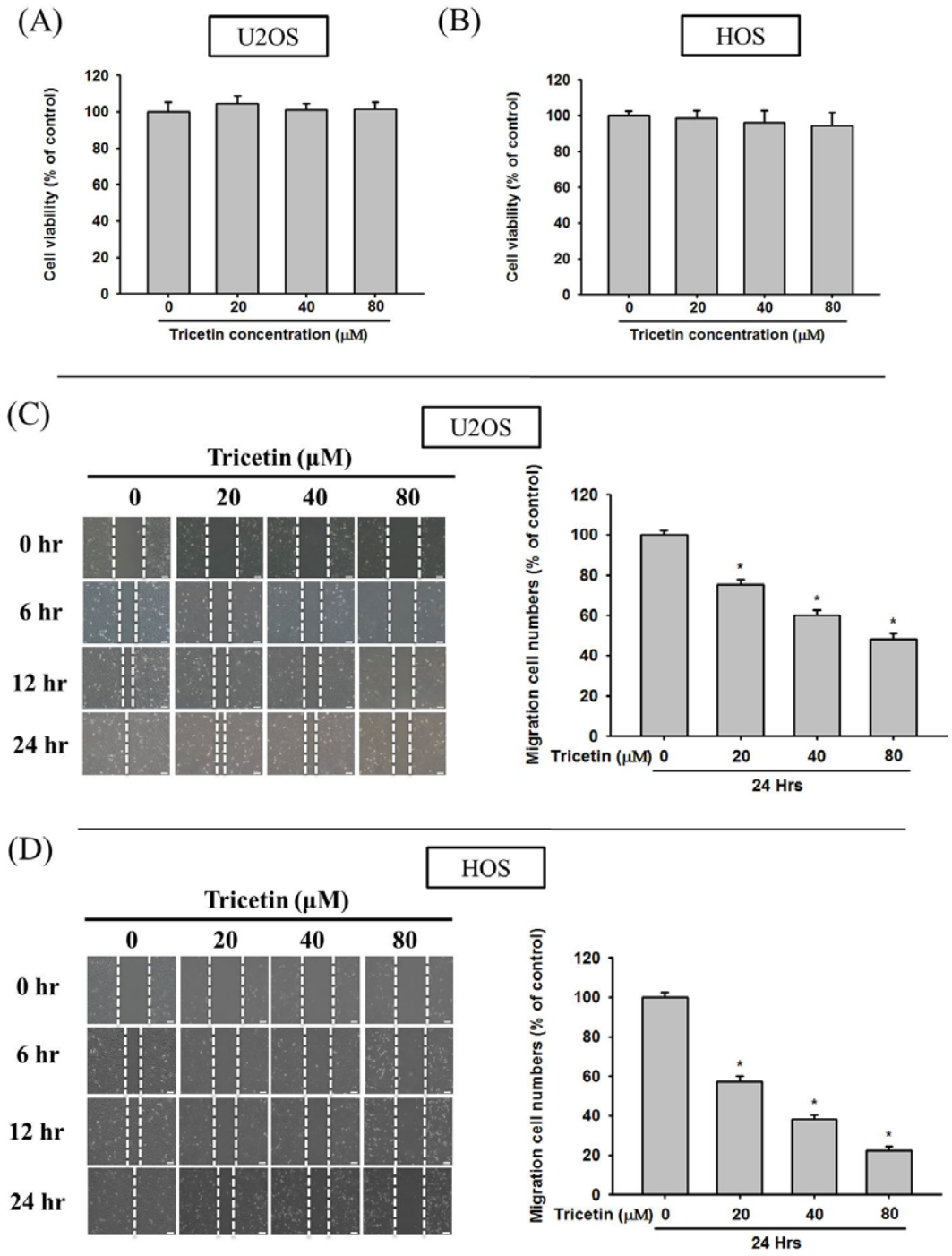

Figure 1: Tricetin inhibited *in vitro* wound closure in the U2OS and HOS cells. Human osteosarcoma (A) U2OS and (B) HOS cells were treated with tricetin (0, 20, 40 and 80 μM) in a serum-free medium for 24 h by using an MTT assay. (C–D) U2OS and HOS cells were wounded and then treated with tricetin (0, 20, 40 and 80 μM) for 24 h in a serum-containing medium. At 0, 6, 12, and 24 h, phase-contrast photographs of the wounds at four locations were taken. *Significantly different, $p < 0.05$, compared with the vehicle group.

Figure 2

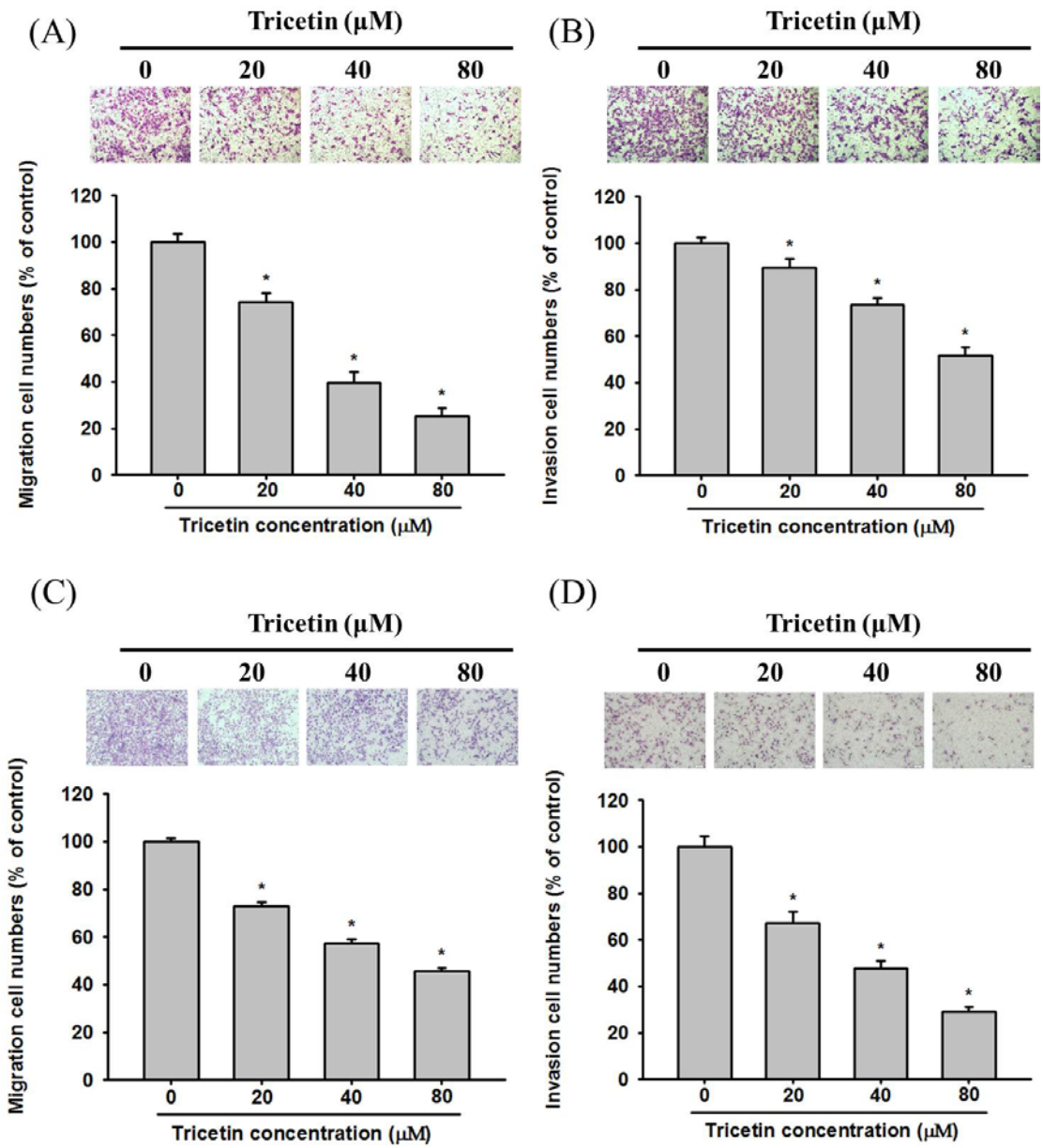

Figure 2: Tricetin inhibited migration and invasion in the U2OS and HOS cells. (A-B) Human osteosarcoma U2OS cells were treated with tricetin (0, 20, 40, and 80 μM); cell migration and cell invasion was subsequently measured using a Boyden chamber and a Matrigel-coated Boyden chamber as described in material and methods section. Migration abilities of the U2OS cells were quantified by determining the number of cells that invaded the underside of the porous polycarbonate. (C-D) Human osteosarcoma HOS cells were treated with tricetin (0, 20, 40, and 80 μM); cell migration and cell invasion was subsequently measured using a Boyden chamber and a Matrigel-coated Boyden chamber as described in material and methods section. *Significantly different, $p < 0.05$, compared with the vehicle group.

Figure 3

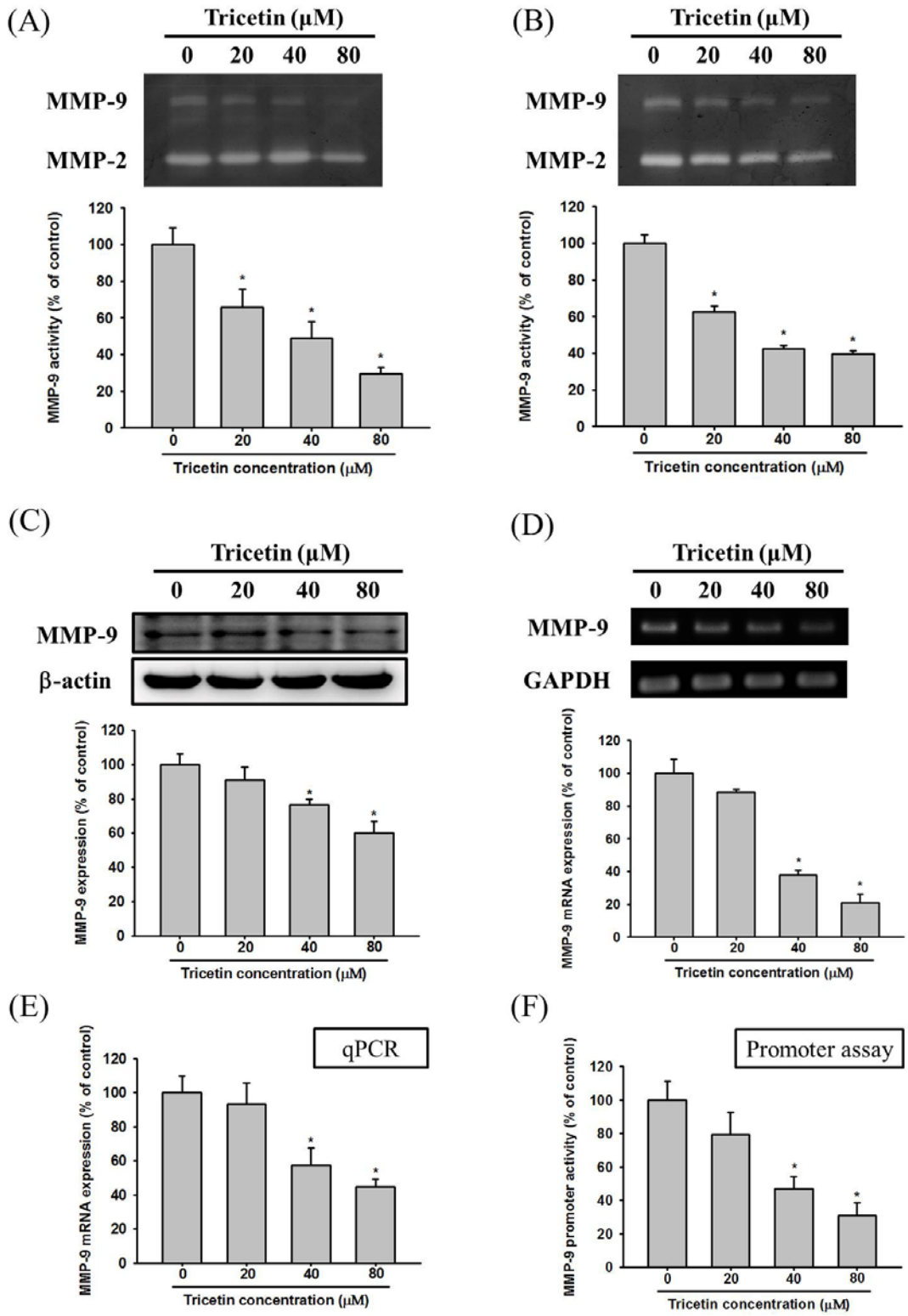
(A)
Tricetin (μM)
0 20 40 80
MMP-9
MMP-2
MMP-9 activity (% of control)
Tricetin concentration (μM)
(B)
Tricetin (μM)
0 20 40 80
MMP-9
MMP-2
MMP-9 activity (% of control)
Tricetin concentration (μM)
(C)
Tricetin (μM)
0 20 40 80
MMP-9
β-actin
MMP-9 expression (% of control)
Tricetin concentration (μM)
(D)
Tricetin (μM)
0 20 40 80
MMP-9
GAPDH
MMP-9 mRNA expression (% of control)
Tricetin concentration (μM)
(E)
qPCR
MMP-9 mRNA expression (% of control)
Tricetin concentration (μM)
(F)
Promoter assay
MMP-9 promoter activity (% of control)
Tricetin concentration (μM)

Figure 3: Tricetin inhibited MMP-9 proteolytic activity, protein expression and mRNA levels in the U2OS cells. (A) U2OS cells and (B) HOS cells were treated with tricetin (0, 20, 40, and 80 μM) for 24 h in a serum-free medium and then subjected to gelatin zymography to analyze the activity of MMP-2 and MMP-9. (C) Western blotting was employed to analyze the protein levels of MMP-9 in the U2OS cells. Quantitative results of MMP-9 protein levels were adjusted with the β-actin protein level. *Significantly different, $p < 0.05$, compared with the vehicle group. (D) Semiquantitative RT-PCR was performed to compare MMP-9 mRNA levels. (E) The mRNA levels of MMP-9 were quantified using a quantitative real-time PCR assay. The values represented the means ± SD of at least three independent experiments. *Significantly different, $p < 0.05$, compared with the vehicle group. (F) U2OS cells were transfected with pGL3 basic or a MMP-9 promoter/reporter plasmid, and then treated with various concentrations (0, 20, 40, and 80 μM) of tricetin. After 24-hour incubation, luciferase activities were determined and normalized to β-galactosidase activity. The values represented the means ± SD of at least three independent experiments. *Significantly different, $p < 0.05$, compared with the vehicle group.

Figure 4

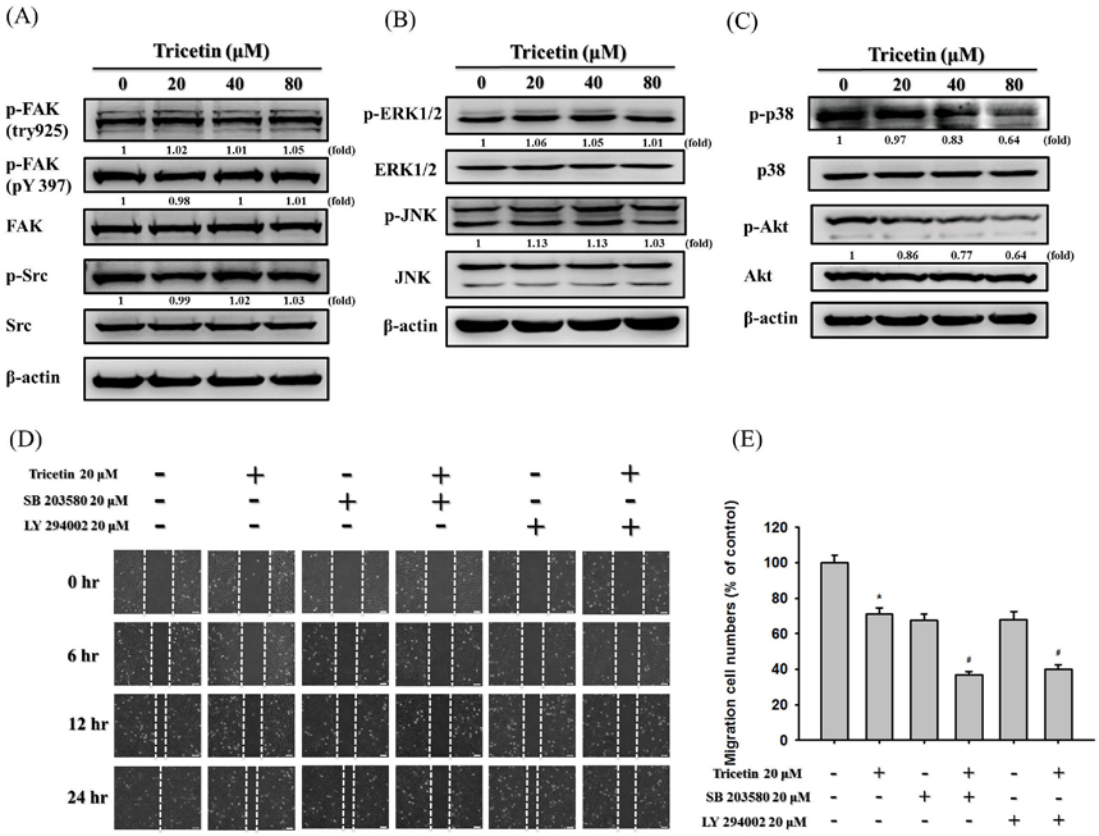

Figure 4: Effects of tricetin on the FAK, Src, MAPK and Akt pathways. U2OS cells were treated with tricetin (0, 20, 40, and 80 μM) for 24 h, and the total cell lysates were then subjected to Western blotting to analyze the phosphorylation of (A) FAK, and Src, (B) ERK 1/2 and JNK 1/2, (C) p38 and Akt. (D-E) U2OS cells were wounded and then pretreated with SB203580 (20 μM) and LY294002 (20 μM) for 30 min, and then incubated in the presence or absence of tricetin (20 μM). At 0, 6, 12, and 24 h, phase-contrast photographs of the wounds at four locations were taken. *p < 0.05, compared with the control group. $^{\#}p$ < 0.05, compared with the tricetin-treated group.

科技部補助計畫衍生研發成果推廣資料表

日期:2016/10/12

科技部補助計畫	計畫名稱: 麥黃酮及其衍生物抑制不同骨癌細胞株轉移、血管新生及誘導細胞凋亡之機制探討
	計畫主持人: 呂克修
	計畫編號: 104-2314-B-040-006- 學門領域: 醫工、骨科、幹細胞

無研發成果推廣資料

104年度專題研究計畫成果彙整表

計畫主持人：呂克修	**計畫編號：**104-2314-B-040-006-
計畫名稱：麥黃酮及其衍生物抑制不同骨癌細胞株轉移、血管新生及誘導細胞凋亡之機制探討	

成果項目					量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
國內	學術性論文	期刊論文			0	篇	
		研討會論文			2		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		
	技術移轉	件數			0	件	
		收入			0	千元	
國外	學術性論文	期刊論文			0	篇	
		研討會論文			0		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		

	技術移轉	件數	0	件	
		收入	0	千元	
參與計畫人力	本國籍	大專生	0	人次	
		碩士生	1		
		博士生	0		
		博士後研究員	0		
		專任助理	0		
	非本國籍	大專生	0		
		碩士生	0		
		博士生	0		
		博士後研究員	0		
		專任助理	0		
其他成果 （無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）					

科技部補助專題研究計畫成果自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否具有政策應用參考價值及具影響公共利益之重大發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以100字為限）
   　□實驗失敗
   　□因故實驗中斷
   　□其他原因
   說明：

2. 研究成果在學術期刊發表或申請專利等情形（請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊）
   論文：□已發表　■未發表之文稿　□撰寫中　□無
   專利：□已獲得　□申請中　■無
   技轉：□已技轉　□洽談中　■無
   其他：（以200字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性，以500字為限）
   黃酮類化合物過去被發現大量存在植物中，具有廣泛的生物學作用，而其中一類化合物－五羥黃酮（Tricetin）在許多文獻中被發現具有抑制癌細胞增生，同時誘導癌細胞走向細胞凋亡的效果，包括乳癌及肝癌，但五羥黃酮與骨肉瘤之間的相關性尚未被釐清。因此本實驗擬探討五羥黃酮是否會對骨肉瘤細胞的生長與轉移造成影響。首先本實驗將五羥黃酮劃分為四組濃度，將其各別加入骨肉瘤細胞株U2OS及HOS中，並利用MTT assay發現五羥黃酮在此濃度差異下，對U2OS與HOS細胞並無毒殺性。透過Zymography assay發現五羥黃酮會顯著抑制U2OS細胞的MMP-9蛋白活性，亦會抑制HOS細胞的MMP-9蛋白活性。利用Wound healing assay及Boyden chamber assay發現五羥黃酮會顯著抑制U2OS與HOS細胞爬行、轉移及侵襲的能力。Western blot的結果亦顯示五羥黃酮會抑制磷酸化p38及磷酸化Akt訊息傳遞蛋白之表現。總結以上結果，本實驗發現在骨肉瘤細胞中，五羥黃酮能透過調控MMP-9的轉錄及p38、Akt訊息傳遞路徑來抑制MMP-9的表現使癌症轉移能力降低。

4. 主要發現
   本研究具有政策應用參考價值：■否　□是，建議提供機關
   （勾選「是」者，請列舉建議可提供施政參考之業務主管機關）
   本研究具影響公共利益之重大發現：■否　□是

說明：（以150字為限）