

# Effects of the overexpression of IFITM5 and IFITM5 c.-14C>T mutation on human osteosarcoma cells

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**Abstract.** The present study aimed to investigate the effects of overexpression of interferon-induced transmembrane protein 5 (IFITM5) and IFITM5 c.-14C>T mutation on osteogenic differentiation, and the proliferation, migration and invasion of SaOS2 cells. SaOS2 cells were transfected with plasmids containing wild type IFITM5 (W) or IFITM5 containing the c.-14C>T mutation (MU). The mRNA and protein expression levels of IFITM5 in SaOS2 cells were respectively detected by reverse transcription quantitative polymerase chain reaction and western blotting. The proliferative, migratory and invasive ability of SaOS2 cells was also examined. In addition, the expression levels of osteogenic differentiation markers alkaline phosphatase (ALP), osteocalcin (OCN) and runt-related transcription factor 2 (Runx2) were detected. Mineralized nodules were detected by Alizarin Red S staining and were quantified by measuring absorbance. The mRNA and protein expression levels of IFITM5 were high in cells transfected with IFITM5 and IFITM5 c.-14C>T mutation, and were higher in cells transfected with IFITM5 c.-14C>T mutation. There was no difference in proliferation between the control group (C) and the W and MU groups. However, overexpression of IFITM5 and IFITM5 c.-14C>T mutation increased apoptotic rate, decreased invasive capacity, increased the expression of ALP, OCN and Runx2, and increased the number of mineralized nodules following osteogenic induction. In addition, compared with C and W groups, cells transfected with IFITM5 c.-14C>T

mutation exhibited decreased migratory ability. In conclusion, overexpression of IFITM5 and IFITM5 c.-14C>T mutation promotes tumor cell apoptosis, inhibits tumor invasion and promotes osteogenic differentiation. These findings may provide a theoretical basis for the development of a novel treatment method that targets IFITM5, and provides a platform for the potential treatment of human osteosarcoma.

## Introduction

Osteosarcoma is a type of malignant bone tumor, which is characterized by pain and bone destruction, and may potentially lead to pathological bone fracture with the progression of tumor growth. Patients with osteosarcoma often exhibit reduced activity in daily life, as well as reduced quality of life. Tumor growth stimulates osteoclast activity, resulting in bone resorption and bone fracture (1). Therefore, promoting osteogenic differentiation over osteoclast activity, without increasing tumor cell proliferation, migration and invasion, may be helpful for patients by reducing pain and improving their quality of life. The SaOS2 human osteosarcoma cell line resembles osteoblasts in their differentiative, proliferative and mineralization ability. SaOS2 cells are able to differentiate and form hydroxyapatite nodules under osteogenic conditions, which are critical for bone formation. In addition, SaOS2 cells can be induced to form mineral nodules, and express several markers of bone differentiation, including osteocalcin (OCN), alkaline phosphatase (ALP) and runt-related transcription factor 2 (Runx2), in medium containing dexamethasone,  $\beta$ -glycerophosphate and ascorbic acid. These previous findings suggested that SaOS2 may possess potent osteogenic capacity (2,3).

Osteosarcoma is generally considered a disease associated with differentiation, which is caused by genetic and epigenetic disruptions in the terminal differentiation of osteoblasts. In addition, >80% of cases of osteosarcoma are poorly differentiated histopathologically; therefore, novel therapeutic strategies based on the non-cytotoxic induction of cell differentiation-responsive pathways may represent a significant advance (4).

Interferon-induced transmembrane protein 5 (IFITM5) encodes bone-restricted IFITM-like protein (BRIL), which is

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involved in mineralization and is expressed in the skeleton. IFITM5 was initially observed at embryonic day 14.5, when undifferentiated cells differentiate into osteoblasts and begin to form mineralized structures (5,6). IFITM5 is highly expressed at the early stage of mineralization and is considered to serve an essential role in bone formation. Furthermore, IFITM proteins are involved in early development, cell adhesion and cell growth regulation; and therefore may be considered to act as tumor suppressors (7). The C>T transition at position-14 of the 5' untranslated region of IFITM5 has previously been identified as the underlying cause of Type V osteogenesis imperfecta (OI) (8,9). It has been reported that Type V OI primary osteoblasts display increased mineralization, despite decreased collagen type I,  $\alpha 1$  expression (10). However, to the best of our knowledge, there are currently no reports on the effects of IFITM5 on proliferation, migration and osteogenic differentiation of human osteosarcoma cells. Therefore, there remains a need to investigate the effects of IFITM5 and IFITM5 c.-14C>T mutation on cancer cells. The present study investigated the effects of IFITM5 and IFITM5 c.-14C>T mutation transfection on tumor proliferation, migration, invasion and osteogenic differentiation in human osteosarcoma cells.

## Materials and methods

**Materials.** Dexamethasone, ascorbate acid, Alizarin Red S, 10% cetylpyridinium chloride and  $\beta$ -glycerophosphate sodium were purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). McCoy's 5A medium and fetal bovine serum (FBS) were obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Penicillin and streptomycin (P/S) were purchased from Beyotime Institute of Biotechnology (Haimen, China).

For transfection, X-tremeGENE™ HP DNA Transfection Reagent was purchased from Roche Diagnostics (Basel, Switzerland). Endo-Free Plasmid Mini kit II was purchased from Omega Bio-Tek, Inc. (Norcross, GA, USA). Wild type IFITM5 (pcDNA4-IFITM5-E12-W) and IFITM5 c.-14C>T mutation (pcDNA4-IFITM5-E12-MU) plasmids were derived from pcDNA4 plasmids obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China).

For proliferation and invasion assays, 3-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and Wright-Giemsa stain were obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Hoechst kit was purchased from Beyotime Institute of Biotechnology. MTT solution was dissolved in PBS to ensure the final concentration reached 5 mg/ml.

For western blotting, bicinchoninic acid (BCA) reagent kit and Tris-buffered saline-0.1% Tween 20 (TBST) were purchased from Beyotime Institute of Biotechnology. SDS-polyacrylamide gel electrophoresis gels were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Polyvinylidene difluoride (PVDF) membranes were purchased from EMD Millipore (Billerica, MA, USA). Rabbit polyclonal antibodies against IFITM5 (SAB2105607) and GAPDH (SAB4300645) were obtained from Sigma-Aldrich; Merck Millipore. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG; A0208) was obtained from Beyotime Institute of Biotechnology.

For reverse transcription quantitative polymerase chain reaction (RT-qPCR), TRIzol® reagent was purchased from Invitrogen; Thermo Fisher Scientific, Inc. Random Primers, dNTP mixture, 5X PrimeScript buffer, RNase inhibitor (40 U/ $\mu$ l) and PrimeScript reverse transcriptase (200 U/ $\mu$ l) used in the reverse transcription were obtained from Takara Bio, Inc. (Otsu, Japan). SYBR Green used for qPCR was purchased from Roche Diagnostics.

**Cell culture, osteogenic differentiation and transfection.** SaOS2 cells were obtained from the Chinese Academy of Sciences Cell Bank. The cells were stored in liquid nitrogen and were thawed in a 37°C water bath prior to culture. The cells were cultured in McCoy's 5A medium supplemented with 10% FBS and 1% P/S. The cultures were incubated at 37°C in a 95% humidified atmosphere containing 5% CO<sub>2</sub>. Once the cells reached 90% confluence, they were detached by mild treatment with trypsin.

SaOS2 cells were plated at a density of  $2 \times 10^4$  cells/well in 96-well plates for 24 h prior to transfection. Transfection was performed once the cells reached 80-90% confluence. The pcDNA4 plasmids were obtained from *Escherichia coli*, according to the Endo-Free Plasmid Mini kit II protocol. Construction of the plasmid IFITM5 3'untranslated region (UTR) including the predicted binding site of miR-762 was amplified by RT-PCR and inserted into multiple cloning sites of the T-Vector pMD19 (pMD19-UTR) (Takara Bio, Inc.) using the *SacI* and *XbaI* restriction sites. A site-directed gene mutagenesis kit (Takara Bio, Inc.) was used to construct a mutant type of miR-762-binding site vector (pMD19-mUTR) with 4 base mutations within the seed region in accordance with the manufacturer's protocol. SaOS2 cells were transfected with 0.01  $\mu$ g/ $\mu$ l pcDNA4-IFITM5-E12-W, which contains IFITM5, and pcDNA4-IFITM5-E12-MU, which contains IFITM5 c.-14C>T mutation, using X-tremeGENE™ HP DNA Transfection Reagent. The control (C) group was treated with the same volume of X-tremeGENE™ HP DNA Transfection Reagent. The cells were harvested for protein and mRNA expression analyses at 24, 48 and 72 h post-transfection.

In addition, SaOS2 cells ( $8 \times 10^4$ ) were cultured overnight in 24-well plates and were transfected with pcDNA4-IFITM5-E12-W or pcDNA4-IFITM5-E12-MU using X-treme GENE™ HP DNA Transfection Reagent for 3 days. The control group was just treated with the transfection reagent for 3 days. Subsequently, the cells were induced under osteogenic conditions (McCoy's 5A medium supplemented with 10% FBS, 1% P/S,  $10^{-8}$  M dexamethasone, 50  $\mu$ g/ml ascorbate acid and 10 mmol/l  $\beta$ -glycerophosphate sodium). After 3 days, the cells were harvested for protein and mRNA analysis.

**SaOS2 cell proliferation assay.** To determine the effects of IFITM5 and IFITM5 c.-14C>T mutation overexpression on cell proliferation, an MTT assay was carried out, according to the manufacturer's protocol. The assay was conducted 24, 48 and 72 h post-transfection of cells plated in a 96-well plate. Briefly, 20  $\mu$ l MTT was added to each well and incubated at 37°C for 4 h. After draining off the solution in the well, 120  $\mu$ l DMSO was added to each well and thoroughly mixed. Subsequently, absorbance of the plate was read at 492 nm, after complete elution, using a microtiter plate reader.

**SaOS2 cell migration assay.** SaOS2 cells were plated at a density of  $3 \times 10^5$ /well in 6-well plates and were cultured until confluent. The confluent cells were scraped with a pipette tip and cells scratched off were washed with PBS. The wells of each 6-well plate were divided into three groups: W group, which was transfected with pcDNA4-IFITM5-E12-W; MU group, which was transfected with pcDNA4-IFITM5-E12-MU; and C group, which remained untransfected. Cell migration into the wound surface was observed at 0 and 48 h post-transfection by microscopy. Cells that migrated into the wound surface were counted and the average number of nuclei was determined by counting five random fields per well under an optical microscope. Each experiment was performed in duplicate.

**SaOS2 cell apoptosis assay.** SaOS2 cells were plated at a density of  $3 \times 10^5$ /well in 6-well plates containing cell slides, and were cultured until they reached confluence. The W group was transfected with pcDNA4-IFITM5-E12-W, the MU group was transfected with pcDNA4-IFITM5-E12-MU, and the C group was not transfected. A total of 48 h post-transfection, cells were fixed with 0.5 ml fix solution (C0003-1; Beyotime Institute of Biotechnology) per well for 10 min. After three washes, 0.5 ml Hoechst 33258 dye solution was added to each well. A drop of anti-fade mounting medium was added to the slides before they were covered. The average number of nuclei was assessed by counting five random fields per well under an optical microscope. Each experiment was performed in duplicate.

**SaOS2 cell Transwell migration assay.** Cells were seeded in the upper chamber of Transwell plates and cultured at a density of  $2 \times 10^4$ /well. After, 24 h cells were transfected with pcDNA4-IFITM5-E12-W or pcDNA4-IFITM5-E12-MU for 48 h at  $37^\circ\text{C}$ . The medium in the upper chamber was then replaced with FBS-free medium, and medium supplemented with 20% FBS was added to the lower chamber. After a 24 h incubation at  $37^\circ\text{C}$ , the upper sides of the filters were carefully washed three times with PBS, and cells remaining on the upper sides were removed with a cotton wool swab. The Transwell filters were then fixed with 4% paraformaldehyde for 30 min, washed three times with PBS, and stained with Wright-Giemsa stain for 1 h. Cells that had migrated to the bottom side of the filter were counted under an optical microscope, and the average number of cells was determined by counting five random fields per filter. Each experiment was conducted in duplicate.

**Alizarin Red S staining.** After 3 days of culturing in osteogenic medium, group C, W and MU cells were washed three times with PBS and fixed with  $500 \mu\text{l}$  4% paraformaldehyde for 20 min. The fixed cells were then stained with  $500 \mu\text{l}$  0.1% Alizarin red S solution (pH 8.3). For quantification, 10% cetylpyridinium chloride solution was added to each well to elute the dye. The absorbance of the eluted solution was measured at 560 nm using a microtiter plate reader after complete elution.

**RT-qPCR.** Total RNA was isolated from the cells using TRIzol® reagent and was reverse-transcribed into cDNA, according to the manufacturer's protocol.

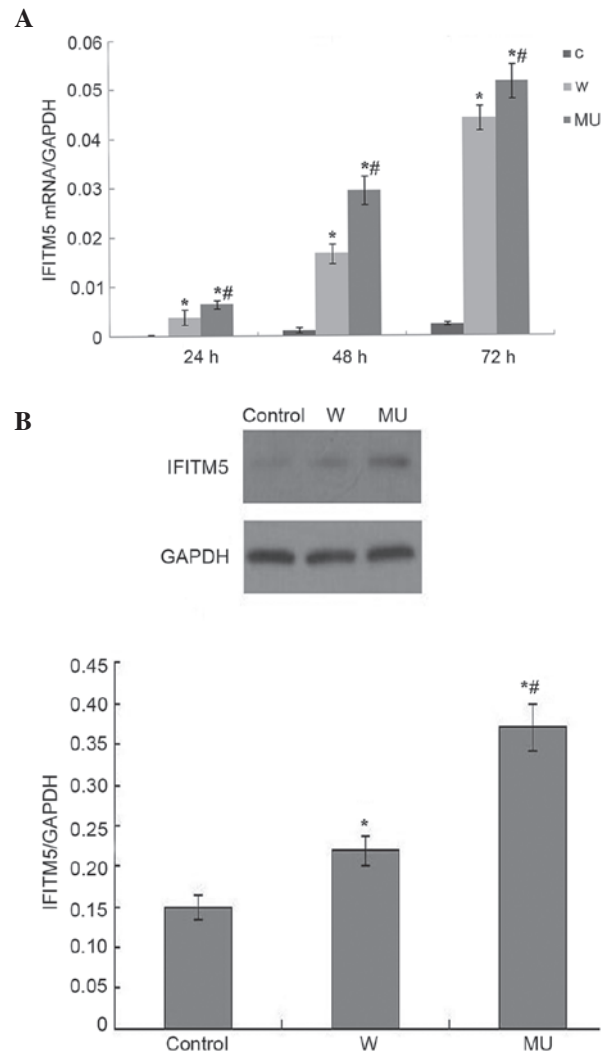


Figure 1. Protein and mRNA expression levels of IFITM5 were detected 24, 48 and 72 h post-transfection. (A) IFITM5 mRNA expression was detected by quantitative polymerase chain reaction using GAPDH as a housekeeping gene. (B) IFITM5 protein expression was evaluated by western blotting in control, W and MU groups. Data are presented as the mean  $\pm$  standard deviation of at least three independent experiments. \* $P < 0.05$  vs. control group (untransfected SaOS2 cells); \*\* $P < 0.05$  vs. W group (SaOS2 cells transfected with pcDNA4-IFITM5-E12-W). IFITM5, interferon-induced transmembrane protein 5; W, pcDNA4-IFITM5-E12-W-transfected cells; MU, pcDNA4-IFITM5-E12-MU-transfected cells.

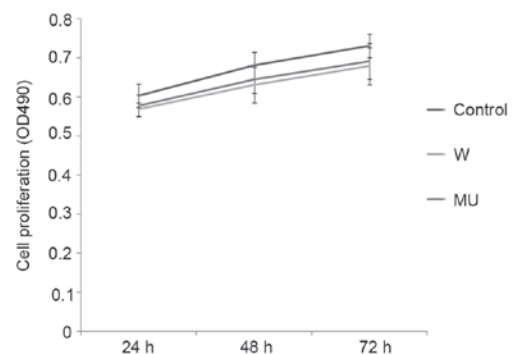


Figure 2. Effects of IFITM5 on cell proliferation. Cell number was increased in a time-dependent manner, with little difference between W and MU groups ( $P > 0.05$ ). There were no significant differences between the groups ( $P > 0.05$ ). IFITM5, interferon-induced transmembrane protein 5; W, pcDNA4-IFITM5-E12-W-transfected cells; MU, pcDNA4-IFITM5-E12-MU-transfected cells; OD, optical density.



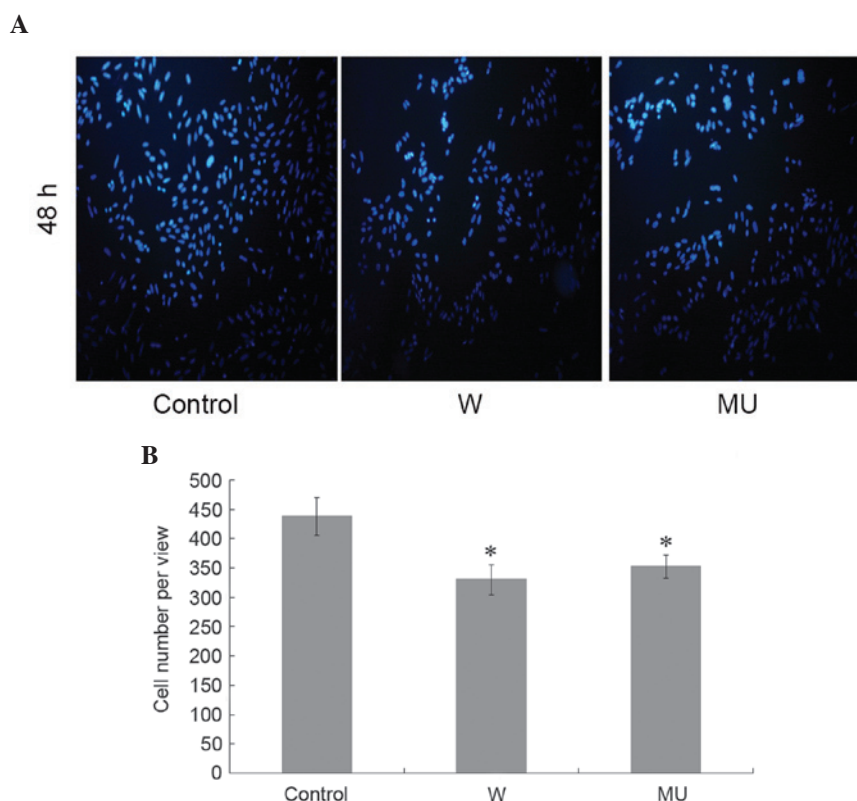


Figure 3. Effects of IFITM5 on cell apoptosis. (A) Effects of IFITM5 on cell apoptosis were determined using Hoechst dye solution 48 h post-transfection. Magnification,  $\times 100$ . (B) Number of nuclei was consistent with cell number. Cell number was markedly decreased in W and MU groups compared with in the control group. There was no significant difference between the W and MU groups. \* $P < 0.05$  vs. control group (untransfected SaOS2 cells). IFITM5, interferon-induced transmembrane protein 5; W, pcDNA4-IFITM5-E12-W-transfected cells; MU, pcDNA4-IFITM5-E12-MU-transfected cells.

qPCR was performed using specific primers, the SYBR<sup>®</sup> PrimeScript<sup>®</sup> RT-PCR kit, and with cDNA as a template, using an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The qPCR reaction mixture consisted of 5  $\mu$ l SYBR Green PCR Master mix (Roche Diagnostics), 1  $\mu$ l cDNA, 1  $\mu$ l forward primer (Beijing Genomics Institute, Shenzhen, China), 1  $\mu$ l reverse primer (Beijing Genomics Institute) and 2  $\mu$ l RNase-free ddH<sub>2</sub>O (Tiangen Biotech Co., Ltd., Beijing, China). The total PCR reaction volume was 10  $\mu$ l. cDNA was used to conduct gene-specific PCR for IFITM5, Runx2, ALP and OCN. The primers used were as follows: IFITM5, forward 5'-TTGATCTGGTCGGTGTTCAG-3', reverse 5'-GTCAGTCATAGTCCGCGTCA-3'; Runx2, forward 5'-GCCGGAATGATGAGAACTA-3', reverse 5'-GGTGAAACTCTTGCCTCGTC-3'; ALP, forward 5'-TGGCTCTGCCTTTATCCCTAGT-3', reverse 5'-AAATAAGGTGCTTTGGGAATCTGT-3'; OCN, forward 5'-GCCATCACCCTGTCTCCTAA-3', reverse 5'-GCTGTGGAGAAGACACACGA-3'; and GAPDH, forward 5'-CACCATCTTCCAGGAGC-3' and reverse 5'-AGTGGACTCCACGACGTA-3'. The qPCR was carried out according to the following cycling conditions: 45 cycles at 94°C for 2 min, 94°C for 30 sec, 60°C for 30 sec, 72°C for 40 sec and 72°C for 5 min. The relative levels of target gene transcripts were normalized to the control gene GAPDH by  $2^{-\Delta\Delta C_q}$  (11).

**Western blot analysis.** Cells were harvested for IFITM5 protein expression analysis 72 h post-transfection with

pcDNA4-IFITM5-E12-W or pcDNA4-IFITM5-E12-MU. radioimmunoprecipitation lysis buffer (Beyotime Institute of Biotechnology) was added to cells and lysed for 30 min on ice. Protein was obtained from the cell lysates by centrifugation at 16,363  $\times g$  for 15 min at 4°C, and protein concentrations were determined using the BCA reagent. The samples were heated at 95°C for 5 min, separated by 10% SDS-PAGE, and transferred to methanol-activated PVDF membranes. After blocking with 5% defatted milk in TBST for 2 h at room temperature, the membranes were incubated with rabbit anti-IFITM5 and anti-GAPDH polyclonal antibodies (diluted 1:1,000 in TBSB) at 4°C overnight, followed by incubation with HRP-conjugated IgG secondary antibodies (diluted 1:5,000 in TBST) at 4°C for 1 h. The protein bands were observed by UMAX PowerLook 2100XL-USB (Umax Technologies, Dallas, TX, USA) and quantitatively analyzed for pixel value by ImageJ analysis system (version 1.48u; National Institutes of Health, Bethesda, MD, USA). The relative level of target protein was presented as the ratio of pixel value for the target protein to pixel value for GAPDH.

**Statistical analysis.** Data are presented as the mean  $\pm$  standard deviation. One way analysis of variance (for initial multiple comparisons) and post-hoc Least Significant Difference tests (comparison between two groups) were conducted for data analysis. Statistical analyses were performed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA)  $P < 0.05$  was considered to indicate a statistically significant difference.

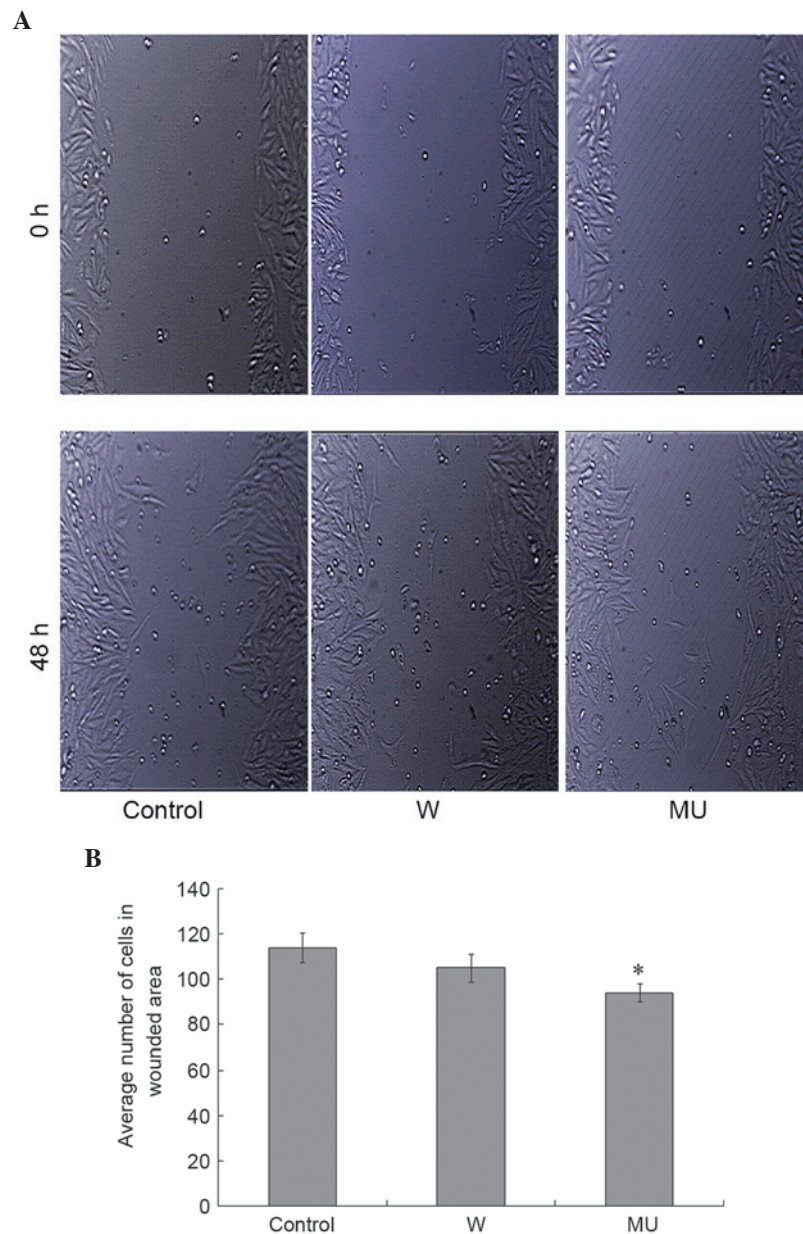


Figure 4. pcDNA4-IFITM5-E12-MU transfection reduced cell migration. (A) Migratory ability of each group was monitored by microscopy at 0 and 48 h post-transfection. Magnification,  $\times 100$ . (B) Quantification was conducted by determining the number of cells that migrated into the wounded area. Transfection with pcDNA4-IFITM5-E12-MU resulted in a significant reduction in SaOS2 cell migration. Cells transfected with pcDNA4-IFITM5-E12-W and control cells were not affected. Data are presented as the mean  $\pm$  standard deviation of triplicate samples. \* $P < 0.05$  vs. control group (untransfected SaOS2 cells). IFITM5, interferon-induced transmembrane protein 5; W, pcDNA4-IFITM5-E12-W-transfected cells; MU, pcDNA4-IFITM5-E12-MU-transfected cells.

## Results

**IFITM5 expression in vitro.** RT-qPCR and western blotting indicated that IFITM5 was stably expressed in IFITM5 and IFITM5 c.-14C>T mutation-transfected SaOS2 cells (Fig. 1A and B). The mRNA expression levels of IFITM5 were increased in a time-dependent manner. Protein expression was examined 72 h post-transfection. The mRNA and protein expression levels were markedly higher in W and MU groups compared with in C group. SaOS2 cells expressed the highest levels of IFITM5 72 h post-transfection. In addition, there was a statistically significant difference in IFITM5 expression between MU group and the other two groups ( $P < 0.05$ ).

**Effects of IFITM5 on cell proliferation and apoptosis.** Proliferative ability was measured by MTT assay 24, 48 and 72 h post-transfection in the three groups. Cell number increased in a time-dependent manner, with little difference between the W and MU groups, and the C group. There were no significant differences between the groups (Fig. 2).

Hoechst dye solution was used to detect apoptosis in the different groups 48 h post-transfection. The number of nuclei was markedly decreased in W and MU groups compared with in C group. There was no significant difference between groups W and MU (Fig. 3A and B). These results indicate that transfection with pcDNA4-IFITM5-E12-W or pcDNA4-IFITM5-E12-MU enhance the apoptosis of SaOS2 cells. However, the c.-14C>T mutation of IFITM5 appears to

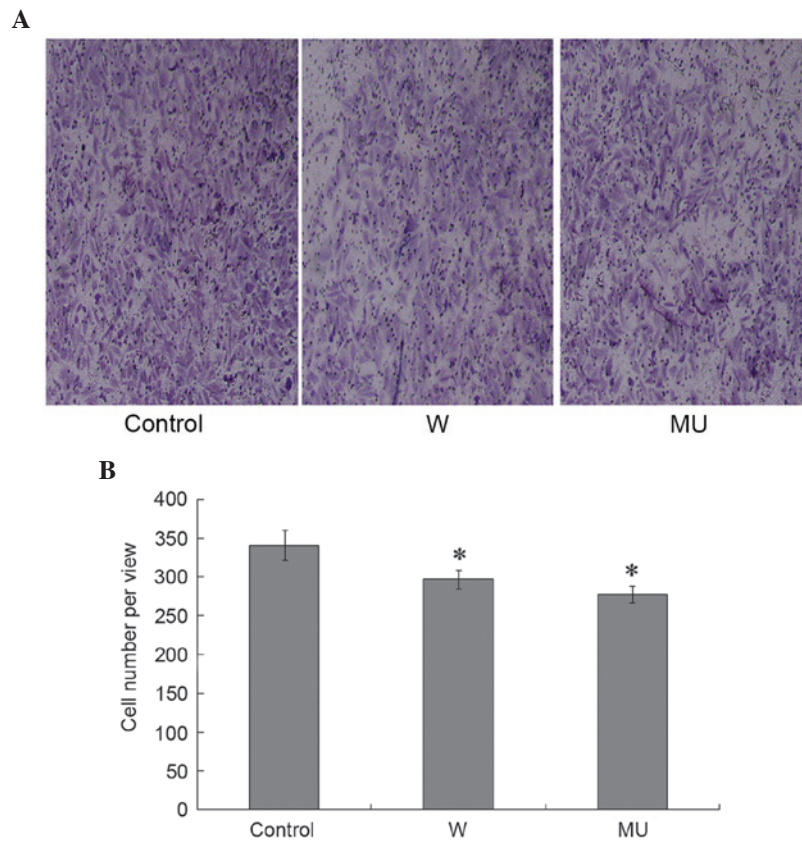


Figure 5. IFITM5 reduced cell migration. (A) Cells that traversed the membranes were counted using a microscope at 48 h post-transfection. Magnification, x40. (B) Cell number on the bottom of the filter was decreased in W and MU groups compared with in the control group. There were no differences between W and MU groups. \* $P < 0.05$  vs. control group (untransfected SaOS2 cells). IFITM5, interferon-induced transmembrane protein 5; W, pcDNA4-IFITM5-E12-W-transfected cells; MU, pcDNA4-IFITM5-E12-MU-transfected cells.

have no additional effects on cell proliferation and apoptosis compared with IFITM5. Furthermore, the treatments have no influence on the proliferation of cells.

**Effect of IFITM5 on cell migration.** In order to investigate the effects of pcDNA4-IFITM5-E12-W and pcDNA4-IFITM5-E12-MU transfection on cell migration, the number of cells that migrated into the wound surface were counted 48 h post-transfection. The results indicated that pcDNA4-IFITM5-E12-MU, rather than pcDNA4-IFITM5-E12-W, significantly decreased SaOS2 cell migration compared with in C group. There was no significant difference between W and MU groups (Fig. 4A and B).

**Effect of IFITM5 on Transwell migration.** Cell migration is a characteristic of malignant tumor cells. The present study adopted a Transwell migration assay to measure this ability. Cells that traversed the membrane were counted under an optical microscope, in order to determine tumor cell invasion. The number of migrated cells was significantly decreased in W and MU groups compared with in C group. There were no significant differences between W and MU groups (Fig. 5A and B). These results indicate that IFITM5 and IFITM5 c.-14C>T mutation may suppress cell migration.

**IFITM5 and IFITM5 c.-14C>T mutation stimulates osteogenic differentiation of SaOS2 cells.** To determine whether IFITM5 and IFITM5 c.-14 C>T mutation could affect

mineralization in SaOS2 cells, pcDNA4-IFITM5-E12-W and pcDNA4-IFITM5-E12-MU were transfected into SaOS2 cells. Subsequently, cells in the three groups were induced under osteogenic condition for 72 h. Alizarin Red S staining was performed to detect calcification during differentiation (Fig. 6A and B). Calcium nodes in the cells transfected with pcDNA4-IFITM5-E12-W and pcDNA4-IFITM5-E12-MU were demonstrated increased staining compared with the control cells. This suggested that IFITM5 and IFITM5 c.-14C>T mutation may promote mineralization of SaOS2 cells. Previous studies have reported that osteogenic differentiation is characterized by the synthesis of ALP, OCN and Runx2 (12,13). Therefore, the expression levels of ALP, Runx2 and OCN were detected. Compared with the control cells, the expression levels of ALP, Runx2 and OCN were increased following transfection with pcDNA4-IFITM5-E12-W or pcDNA4-IFITM5-E12-MU. Compared with cells transfected with pcDNA4-IFITM5-E12-W, the expression levels of ALP, Runx2 and OCN were significantly increased in cells transfected with pcDNA4-IFITM5-E12-MU (Fig. 6C-E). The results of the osteogenic gene marker detection were concordant with the results of Alizarin Red S staining (Fig. 6A and B).

## Discussion

Osteosarcoma results in progressive pain and pathological fracture or spinal cord compression, since a part of intact



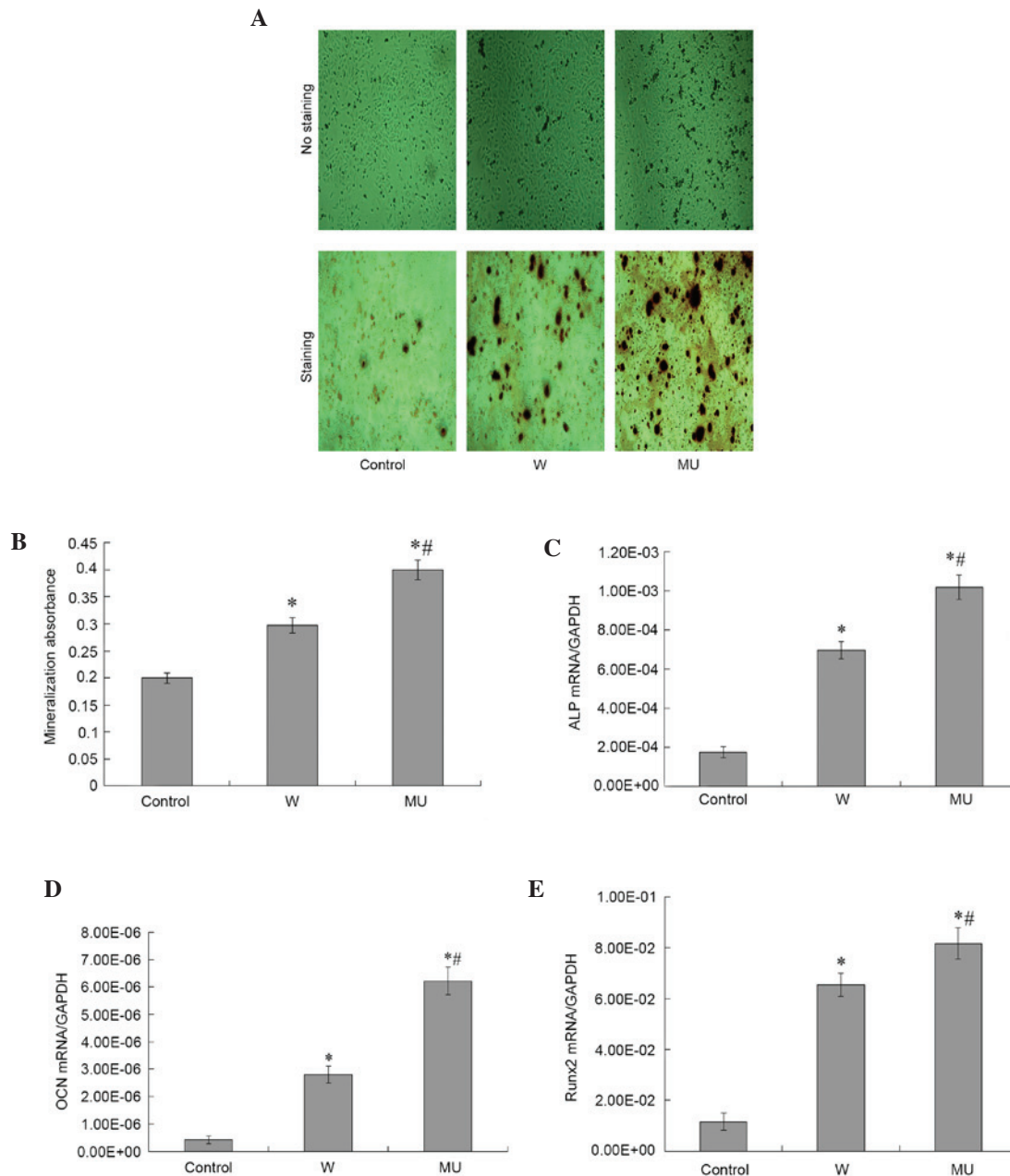


Figure 6. Effects of IFITM5 on osteoblast mineralization in SaOS2 cells. (A) Mineralized nodules were measured by Alizarin Red S staining 72 h post-transfection with pcDNA4-IFITM5-E12-MU or pcDNA4-IFITM5-E12-W. Magnification, x100. (B) For quantification, 10% cetylpyridinium chloride solution was added to each well to elute Alizarin Red S. After complete elution, the absorbance of the eluted solution was measured at 560 nm on a microtiter plate reader. The absorbance was significantly decreased in W and MU groups compared with in the control group. The absorbance was markedly increased in MU group compared with W group. (C-E) Effects of IFITM5 on the expression of osteogenic markers. The expression of (C) ALP was compatible with the results of Alizarin Red S staining. \* $P < 0.05$  vs. control group (untransfected SaOS2 cells), # $P < 0.05$  vs. W group. The expression of (D) OCN and (E) Runx2 were compatible with the results of Alizarin Red S staining. \* $P < 0.05$  vs. control group (untransfected SaOS2 cells), # $P < 0.05$  vs. W group IFITM5, interferon-induced transmembrane protein 5; W, pcDNA4-IFITM5-E12-W-transfected cells; MU, pcDNA4-IFITM5-E12-MU-transfected cells; ALP, alkaline phosphatase, OCN, osteocalcin; Runx2, runt-related transcription factor 2.

bone is substituted with malignant cells with the growth of metastatic bone tumor. During tumor growth, the balance of osteoclast and osteoblast activity is disturbed. It is well-known that stimulation of osteoclasts results in bone resorption, which can be suppressed by the bone formation that results from osteoblast differentiation. Bone resorption should be coupled to bone formation in order to maintain skeletal homeostasis, and imbalances in this process lead to various human diseases, such as osteoporosis and osteopetrosis. Tumor growth stimulates activation of osteoclasts, leading to bone resorption

rather than bone formation, thus resulting in bone destruction. Therefore, the identification of a method that promotes SaOS2 cell mineralization, without increasing tumor cell proliferation, migration and invasion, is important.

IFITM5 encodes BRIL, which is involved in mineralization and is expressed in the skeleton. Type V OI is characterized by C>T transition at position-14 of the 5' untranslated region of IFITM5. It has previously been reported that Type V OI primary osteoblasts display increased mineralization (10). The effects of pcDNA4-IFITM5-E12-W and pcDNA4-IFITM5-E12-MU on

mineralization in SaOS2 cells were investigated in the present study. Furthermore, the effects of pcDNA4-IFITM5-E12-W and pcDNA4-IFITM5-E12-MU were examined on tumor cell proliferation, migration and invasion.

Osteosarcoma cells share several similar features to undifferentiated osteoprogenitor cells, including a high proliferative capacity and similar expression profiles of osteogenic markers, such as Runx2, ALP and OCN (14,15). Osteosarcoma cells can be induced to differentiate into mature osteoblasts by certain compounds (16). Clinically, all-trans retinoic acid-based differentiation therapy in acute promyelocytic leukemia has achieved great success, and differentiation-based approaches for the treatment of other malignant tumors has garnered attention (17,18). Differentiation therapy may be considered a promising alternative to conventional chemotherapy for some malignancies (18). The aim of this type of therapy is to activate endogenous differentiation programs in cancer cells, resulting in cellular maturation of the tumor and concurrent loss of the tumor phenotype (4). In the present study, osteoblast differentiation of SaOS2 cells transfected with IFITM5 and c.-14C>T mutation IFITM5 was induced by certain reagents. The markers of osteoblast differentiation and mineralized bone nodules were subsequently detected.

Compared with C group, W and MU groups exhibited increased mRNA and protein expression levels of IFITM5. These results indicate that IFITM5 was stably expressed in IFITM5- and IFITM5 c.-14C>T mutation-transfected SaOS2 cells. Furthermore, the mRNA and protein expression levels of IFITM5 were increased in MU group compared with in W group. The stability of expression is required for the subsequent steps.

The present study demonstrated that overexpression of IFITM5 and IFITM5 c.-14C>T mutation had no effect on the proliferation of SaOS2 cells; however, they did induce apoptosis. Overexpression of IFITM5 and IFITM5 c.-14C>T mutation decreased the migratory and invasive ability of tumor cells, and also induced osteoblast differentiation of SaOS2 cells alongside increased bone mineralization. These results may alleviate symptoms associated with excessive bone resorption, and may promote tumor cell differentiation and maturation. Previous studies have been conducted regarding the therapeutic potential of treatments that overcome differentiation defects associated with osteosarcoma and prevent tumorigenesis (4,19). The results of the present study may shed light on improving osteosarcoma treatment; however, the mechanisms underlying IFITM5 and IFITM5 c.-14C>T mutation overexpression-mediated osteoblast differentiation and mineralization of SaOS2 cells remains unclear.

In conclusion, the present study examined the effects of IFITM5 and IFITM5 c.-14C>T mutation overexpression on SaOS2 osteosarcoma cells, with regards to tumor features, and osteoblast differentiation and mineralization. IFITM5 is involved in osteoblast differentiation and mineralization, and exerted beneficial effects on tumor cell proliferation, apoptosis, migration and invasion. These results may provide information regarding the development of a novel treatment method that targets IFITM5, and may provide a platform for future treatments of human osteosarcoma. Future studies aim to develop a detailed understanding of the role of IFITM5 in tumor biological characteristics and osteogenic differentiation.

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