SPANDIDOS PUBLICATIONS

INTERNATIONAL JOURNAL OF MOLECULAR MEDICINE 41: 1845-1854, 2018

Honokiol suppresses proliferation and induces apoptosis via regulation of the miR-21/PTEN/PI3K/AKT signaling pathway in human osteosarcoma cells

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Received March 6, 2017; Accepted November 15, 2017

DOI: 10.3892/ijmm.2018.3433

Abstract. Honokiol (HNK) is a small biphenolic compound, which exerts antineoplastic effects in various types of cancer. However, the mechanism underlying the antitumor effects of HNK in osteosarcoma (OS) cells is not yet fully understood. Emerging evidence has indicated that microRNAs (miRNAs/miRs) serve key roles in numerous pathological processes, including cancer. It has previously been reported that Chinese medicinal herbs harbor anticancer properties via modulating miRNA expression. Therefore, the present study aimed to determine whether HNK could suppress OS cell growth by regulating miRNA expression. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometric analysis were used to evaluate the cell proliferation and apoptosis in human OS cells after treatment with HNK, respectively. The results demonstrated that HNK inhibits proliferation and induces apoptosis of human OS cells in a dose-dependent manner. Furthermore, HNK-induced apoptosis was characterized by upregulation of proapoptotic proteins, including cleaved-caspase-3, cleaved-poly (ADP-ribose) polymerase and B-cell lymphoma 2 (Bcl-2)-associated X protein, and downregulation of the anti-apoptotic protein Bcl-2. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) verified that HNK was able to induce aberrant expression of miRNAs in human OS cells, and miR-21 was one of the miRNAs that was most significantly downregulated. To further investigate miR-21 function, the present study validated that HNK reduces miR-21 levels in a dose-dependent manner. In addition, restoration of miR-21 expression abrogated the suppressive effects of HNK on OS cells. Luciferase assay and western blot analysis identified that miR-21 inhibits the expression of phosphatase and tensin homolog (PTEN) by directly targeting its 3'-UTR. Notably, HNK was able to suppress the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway; however, it was reactivated by miR-21 overexpression. Taken together, these data indicated that HNK may inhibit proliferation and induce apoptosis of human OS cells by modulating the miR-21/PTEN/PI3K/AKT signaling pathway. Therefore, miR-21 may be considered a potential therapeutic target for the treatment of osteosarcoma with HNK.

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Key words: honokiol, osteosarcoma, microRNA-21, phosphatase and tensin homolog/phosphoinositide 3-kinase/protein kinase B signaling

Introduction

Osteosarcoma (OS) is the most frequent primary malignant bone tumor, which is commonly diagnosed in children and young adolescents, with a male predominance (1). OS is highly aggressive and primarily metastasizes to the lung (2). Surgical tumor resection and multi-agent chemotherapy are the main current therapeutic strategies used to treat OS. It has previously been reported that chemotherapy may increase the 5-year survival rate for localized disease by >50% compared with surgery alone. Conversely, patients diagnosed with metastases exhibit a poor prognosis, with a 5-year survival rate of 20-30% following surgical resection and/or radiotherapy (3,4). Furthermore, currently approved agents exhibit severe side effects (3,4); therefore, the development of a novel agent with increased efficiency and reduced toxicity in OS treatment is required.

Honokiol (HNK) is a biphenolic compound extracted from the magnolia tree, which has been used to treat anxiety, thrombotic stroke and gastrointestinal symptoms in traditional Chinese and Japanese medicine (5). HNK has long been known to exert antimicrobial (6), anti-inflammatory (7) and antiangiogenic (8,9) effects. Increasing evidence has revealed that HNK exerts antineoplastic functions in various types of cancer, including angiosarcoma (8), colorectal carcinoma (10), breast cancer (11) and gastric cancer (12). Furthermore, HNK may trigger apoptotic pathways that result in mitochondrial dysfunction (13), influence retinoblastoma function and E2F transcription factor 1 transcriptional activity (14), and suppress the phosphoinositide 3-kinase

(PI3K)/mammalian target of rapamycin (mTOR) pathway (15). However, the molecular mechanism underlying the anticancer effects of HNK on OS cells remains to be elucidated.

MicroRNAs (miRNAs/miRs) are a class of small (19-24 nucleotide) noncoding RNAs that mediate post-transcriptional regulation of target genes by suppressing translation or promoting RNA degradation. miRNAs have crucial functions in various biological and pathological processes, including cellular proliferation, differentiation, apoptosis and carcinogenesis (16). In recent years, it has been demonstrated that some natural products are able to control tumor-suppressive and oncogenic miRNAs, including curcumin (diferuloylmethane), which inhibits hepatocellular cancer cell proliferation via modulating miRNA expression (17). Furthermore, previous studies have reported that Chinese medicinal herbs exert antitumor effects by modulating miRNA expression (18,19). Zhang *et al* demonstrated that HNK suppresses bladder tumor growth by inhibiting the enhancer of zeste homolog 2/miR-143 axis (20). Avtanski *et al* also revealed that HNK rescued leptin-induced tumor progression by suppressing the Wnt1-metastasis associated 1-β-catenin signaling pathway in a miR-34a-dependent manner (11). Therefore, it may be hypothesized that HNK inhibits proliferation and induces apoptosis, via the modulation of miRNA expression, in human OS cells.

The present study investigated the effects of HNK on OS tumor growth inhibition and explored the underlying molecular mechanisms. The results indicated that HNK may inhibit growth and promote apoptosis of human OS cells in a dose-dependent manner. Furthermore, the results verified that HNK induces aberrant expression of miRNAs in human OS cells, and miR-21 suppresses phosphatase and tensin homolog (PTEN) by directly targeting its 3'-untranslated region (3'-UTR). Notably, the results indicated that HNK blocks the PI3K/protein kinase B (AKT) signaling pathway by inhibiting miR-21 expression in human OS cells. Collectively, these results suggested that the molecular mechanism by which HNK induces apoptosis was modulated by the miR-21/PTEN/PI3K/AKT axis in human OS cells.

Materials and methods

Reagents and cell culture. HNK was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HNK was dissolved in 10 μM dimethyl sulfoxide (DMSO) and was maintained at 4˚C. The human OS cell lines Saos-2 and MG-63 were obtained from the American Type Culture Collection (Manassas, VA, USA) and were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 50 U/ml penicillin and 50 μg/ml gentamicin (both Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 2.5 μg/ml amphotericin B, 1% glutamine and 2% HEPES at 37˚C in a humidified incubator containing 5% CO_2.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT assay was used to investigate the antiproliferative effects of HNK on OS cells. Briefly, $1x10^4$ cells were seeded into 96-well plates overnight. After treatment with 1-100 μM HNK for 24 h at 37˚C, the cells were washed with PBS and incubated for 48 h at 37˚C in fresh medium. The cells in the control group were only treated with 100 μl DMSO for 24 h at 37˚C. Subsequently, 20 μl 5 mg/ml MTT (Sigma-Aldrich; Merck KGa) solution was added to each well and incubated at 37˚C for an additional 4 h. The supernatant was then discarded and 150 μl DMSO was added to each well. Finally, absorbance of the samples was measured at 490 nm using a microplate reader (Sunrise™; Tecan Group Ltd., Männedorf, Switzerland).

Apoptosis analysis. Flow cytometric analysis was used to detect cell apoptosis. Briefly, the cells were treated with 1-100 μM HNK for 24 h at 37˚C, after which $5x10^5$ cells were obtained from the culture and were washed with cold PBS. The cells in the control group were only treated with 100 μl DMSO for 24 h at 37˚C. Cell apoptosis was evaluated using the Annexin V/propidium iodide (PI) staining kit (BioVision, Inc., Milpitas, CA, USA) according to the manufacturer's protocol. Flow cytometry was conducted at the Flow Cytometry Core Facility at Cedars-Sinai Medical Center (Los Angeles, CA, USA) using FACScan (BD Biosciences, San Jose, CA, USA). Data were analyzed using the Cell Quest program version 6.0 (FACScan; BD Biosciences).

Western blot analysis. Cells were lysed as described previously (21). Subsequently, a bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used to measure protein concentration. Total proteins (60 μg) were separated by 10% SDS-PAGE (Sigma-Aldrich; Merck KGaA) and were then transferred onto polyvinylidene difluoride (PVDF) membranes (BD Biosciences). After blocking with 5% non-fat milk at room temperature for 1 h, PVDF membranes were incubated with primary antibodies (dilution 1:500) for 2 h at room temperature and followed with a horseradish peroxidase conjugated secondary antibody (dilution 1:1,000) for 1 h at room temperature. Mouse anti-PTEN (sc-7974), mouse anti-AKT (sc-6546) and mouse anti-p-AKT (Ser473; sc-33437) primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Rabbit anti-mTOR (39408), rabbit anti-p-mTOR (32199), rabbit anti-p70S6K (32505), rabbit anti-p-p70S6K (66134) and β-actin (79467) antibodies were purchased from Abcam (Cambridge, MA, USA). Antibodies of apoptosis-associated proteins were as follows: cleaved PARP (ab32064), Bax (ab25901), cleaved caspase-3 (ab13847) and Bcl-2 (ab32503), purchased from Abcam. The corresponding rabbit anti-mouse (315-065-003) and goat anti-rabbit (305-065-003) secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Subsequently, protein bands were scanned on X-ray film using the enhanced chemiluminescence detection system (PerkinElmer, Inc., Waltham, MA, USA). AlphaImager software version 2000 (ProteinSimple, San Jose, CA, USA) was used to measure relative intensity of each band on the blots. Measurements were conducted independently at least three times with similar results.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total cellular RNA was extracted using ZR RNA MicroPrep™ kit (Zymo Research Corp., Irvine, CA,

USA) according to the manufacturer's protocol. RNA concentration was measured using a spectrophotometer (Eppendorf, Hamburg, Germany). The high capacity cDNA synthesis kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to synthesize cDNA using miRNA-specific primers according to the manufacturer's protocol. The primers for miR-188-5p, miR-202, miR-623, miR-21, miR-532-5p, miR-628-3p and the internal control RNU44 gene were obtained from Ambion (Thermo Fisher Scientific, Inc.). The primers were as follows: miR-188-5p forward, 5'-TGTGGCTATCTTGCTGCCC-3' and reverse, 5'-GAGT CATTCTCCTTCCCACC-3'; miR-202 forward, 5'-TTAGG CCAGATCCTCAAAGAAG-3' and reverse, 5'-ATAGGAAA AAGGAACGGCGG-3'; miR-623 forward, 5'-ATCCCTT GCAGGGGCTGTTGGGT-3' and reverse, 5'-GCCAGCAC AGAATTAATACGAC-3'; miR-21 forward, 5'-TAGCTTA TCAGACTGATGTTGA-3' and reverse, 5'-GCCAGCA CAGAATTAATACGAC-3'; miR-532-5p forward, 5'-GCCCA TGCCTTGAGTGTAG-3' and reverse, 5'-GTGCGTGTC GTGGAGTCG-3'; miR-628-3p forward, 5'-GGGGGATG CTGACATATTTAC-3' and reverse, 5'-CAGTGCGTG TCGTGGAGT-3'; RNU44 forward, 5'-CCTGGATGATG ATAGCAAATGC-3' and reverse, 5'-GAGCTAATTA AGACCTTCATGTT-3'. qPCR was conducted using TaqMan Gene Expression assay (Applied Biosystems; Thermo Fisher Scientific, Inc.) on an Applied Biosystems 7500 real-time PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR reaction was performed at 95˚C for 5 min followed by 40 cycles of 95˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 30 sec. The $2^{-\Delta\Delta Cq}$ method was used to analyze relative miRNA expression (22). All reactions were performed in triplicate.

Transfection assay. Saos-2 and MG-63 ($5x10^3$) cells were seeded into each well of 6-well plates, maintained in DMEM containing 10% FBS, and treated with either control vehicle (DMSO) or HNK. Subsequently, the cells were transfected with miR-21 mimics or miR-21 inhibitor (Guangzhou RiboBio Co., Ltd., Guangzhou, China) at a final concentration of 50 μM using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Cells were collected 48 h post-transfection. RT-qPCR was used to confirm that miR-21 expression was specifically upregulated/knocked down following transfection with mimics/inhibitor (Fig. 1). Subsequently, OS cell proliferation and apoptosis were assessed by MTT assay and flow cytometric analysis, respectively.

Luciferase assay. The potential binding site between PTEN and miR-21 was identified using TargetScan (http:/www.targetscan.org). The miR-21 mimics/inhibitor and corresponding negative control (NC) were synthesized by Guangzhou RiboBio Co., Ltd. Wild-type (wt) PTEN-3'-UTR and mutant (mut) PTEN-3'-UTR containing the putative binding site of miR-21 were established and cloned into the firefly luciferase-expressing vector pMIR-REPORT (Ambion; Thermo Fisher Scientific, Inc.). For the luciferase assay, Saos-2 cells at a density of $2x10^5$/well were seeded into 24-well plates and were co-transfected with 0.8 μg pMIR-PTEN-3'-UTR or pMIR-PTEN-mut-3'-UTR and 50 nM miR-21 mimic/inhibitor or corresponding NC using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 48 h post-transfection, luciferase activity was measured using the dual-light luminescent reporter gene assay (Applied Biosystems; Thermo Fisher Scientific, Inc.). Each experiment was repeated at least three times in independent experiments. The ratio of *Renilla* luciferase to firefly luciferase was calculated for each well.

Choice of differentially expressed miRNAs list using heat map analysis. We obtained the microarray date from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/), and the GEO accession no. is GSE85871. Observations with adjusted P-values ≥0.05 were removed, and thus excluded from further analysis. The heat map of the miRNAs most obvious differences was created using a method of hierarchical clustering by GeneSpring GX, version 7.3 (Agilent Technologies, Santa Clara, CA, USA).

Statistical analysis. All statistical analyses were performed using SPSS 14.0 software (SPSS, Inc., Chicago, IL, USA). Each experiment was repeated at least three times. Numerical data are presented as the mean ± SD. For numerical variables, the results were evaluated by the Student's t-test (comparison between 2 groups) or one way ANOVA to make multiple-group comparisons followed by the post hoc Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

Results

HNK inhibits growth of human OS cells. To investigate the antiproliferative effects of HNK on OS cells, Saos-2 and MG-63 cells were treated with various concentrations of HNK for 24 h, and the MTT assay was used to evaluate cell viability. The results indicated that treatment with 1-100 μM HNK reduced cell viability of Saos-2 and MG-63 cells in a dose-dependent manner (Fig. 2A and B). The half maximal inhibitory concentration (IC_{50}) values of HNK were 37.85 μM in Saos-2 and 38.24 μM in MG-63 cells. Similar IC_{50} values of HNK were detected in human Saos-2 and MG-63 OS cells.

HNK induces apoptosis of human OS cells. It has been widely reported that HNK may induce apoptosis of various malignant cell types (9,23). To examine HNK-induced apoptosis of OS cells, the cells were analyzed by Annexin V-PI staining following treatment with HNK. The results demonstrated that the proportion of apoptotic cells was markedly increased following HNK treatment compared with in the control group (P<0.01). Furthermore, following treatment with 10 or 40 μM HNK, the number of apoptotic cells increased in a dose-dependent manner (Fig. 2C and D). With regards to apoptotic induction, Saos-2 and MG-63 had similar results (Fig. 2D). To further explore the apoptotic mechanism, the intracellular apoptotic signaling pathway was investigated in OS cells following treatment with various concentrations of HNK. The results revealed that the protein expression levels of cleaved-caspase-3, cleaved-PARP and Bax were significantly upregulated, and Bcl-2 was significantly downregulated following HNK treatment. Furthermore, HNK regulated

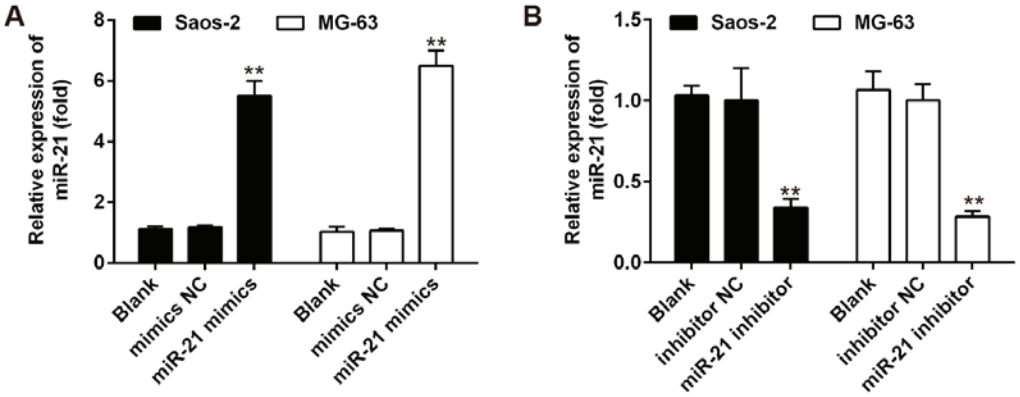

Figure 1. miR-21 expression in Saos-2 or MG-63 cells was detected post-transfection with (A) miR-21 mimics or (B) miR-21 inhibitor. **P<0.01 vs. the Blank group. miR-21, microRNA-21; NC, negative control.

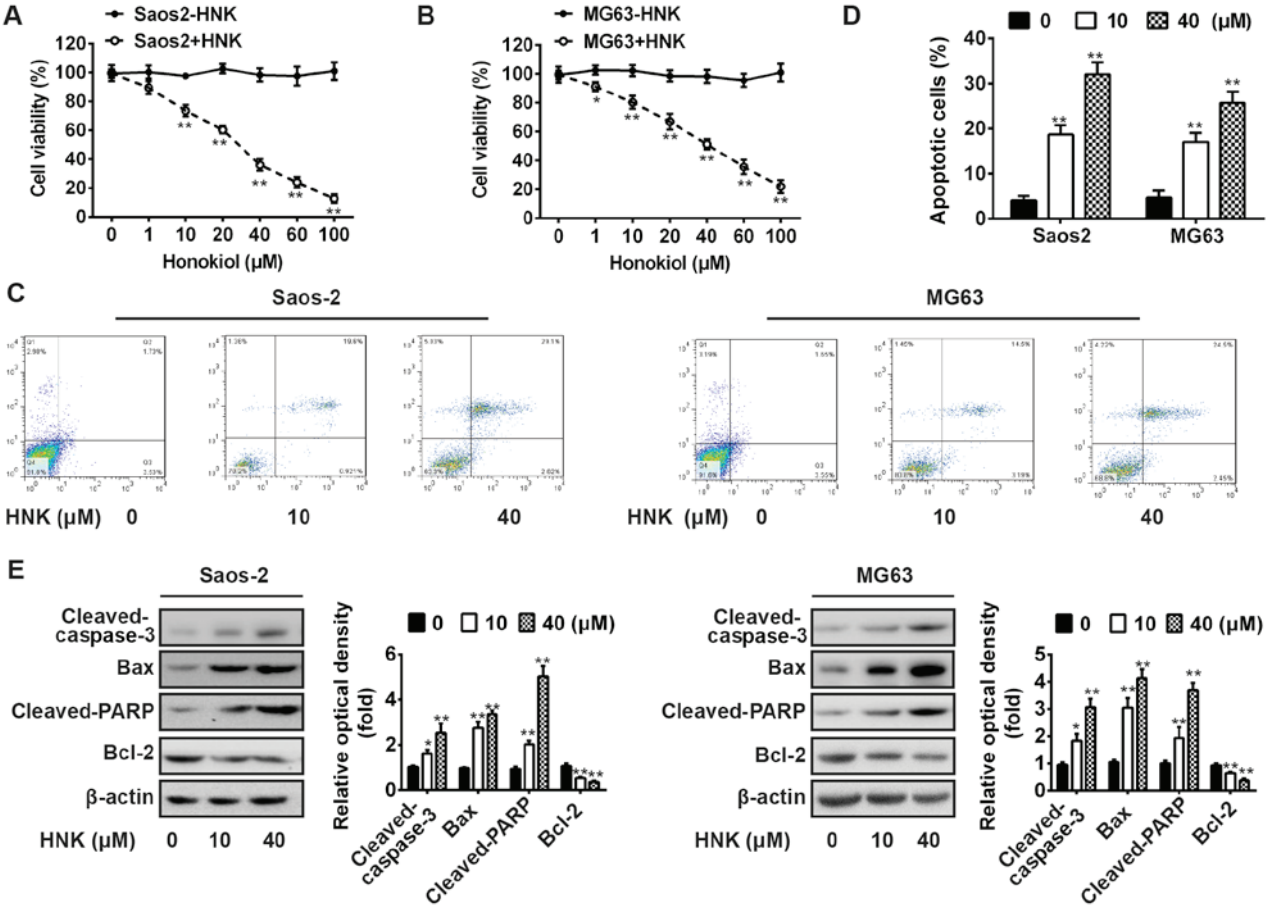

Figure 2. HNK inhibits proliferation and induces apoptosis of human OS cells. (A and B) Human Saos-2 and MG-63 OS cells were treated with or without 1-100 μM HNK for 24 h, and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was conducted to assess viability. (C) Saos-2 and MG-63 cells were treated with or without 10 or 40 μM HNK for 24 h, and apoptosis was measured using flow cytometry. (D) Proportion of apoptotic cells was quantified in three independent experiments. (E) Saos-2 and MG-63 cells were treated with or without 10 or 40 μM of HNK for 24 h, and the protein expression levels of cleaved-caspase-3, cleaved-PARP, Bax and Bcl-2 were analyzed by western blot analysis. Representative data from one of three individual experiments with similar results are presented. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01 vs. 0 μM group. Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; HNK, honokiol; OS, osteosarcoma; PARP, poly (ADP-ribose) polymerase.

these protein expression levels in a dose-dependent manner in human OS cells (Fig. 2E). These data suggested that HNK may induce apoptosis of human OS cells by activating the intracellular apoptotic signaling pathway.

HNK induces miRNA aberrant expression in human OS cells. A recent study revealed that some miRNAs were upregulated in the T24 human bladder cancer cell line following treatment with 9.6 μg/ml HNK (20). Microarray data obtained from

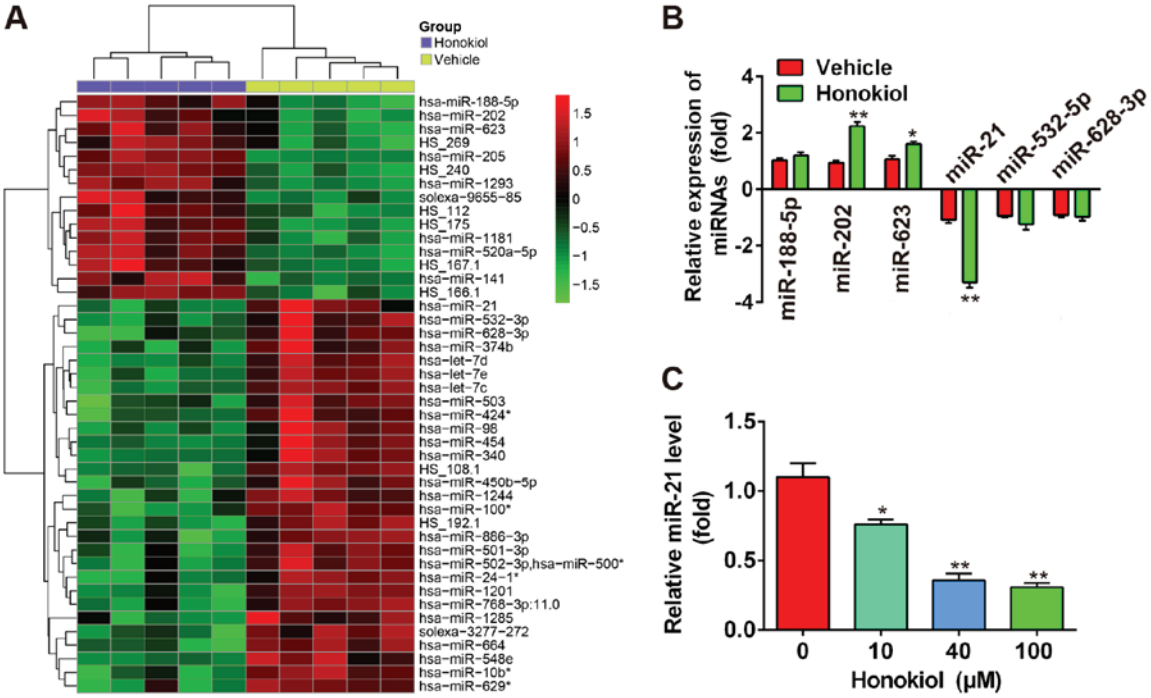

Figure 3. HNK induces aberrant miRNA expression in human OS cells. (A) Microarray data obtained from the Gene Expression Omnibus database (accession no. GSE85871). Heat map shows significant expression alterations of 45 miRNAs in a breast cancer cell line treated with HNK or vehicle. Green represents low expression and red represents high expression. A color change from green to red indicates upregulation and a color change from red to green indicates downregulation. (B) Based on these microarray data, six miRNAs (miR-188-5p, miR-202 and miR-623 were the most significantly upregulated; miR-21, miR-532-5p and miR-628-3p were the most markedly downregulated) were selected and verified by RT-qPCR in OS cells treated with or without HNK. (C) Saos-2 cells were treated with or without 10-100 μM HNK for 24 h, and RT-qPCR was performed to detect the expression levels of miR-21. Data are presented as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. the vehicle group. HNK, honokiol; miRNA/miR, microRNA; OS, osteosarcoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

the Gene Expression Omnibus (GEO) database (accession no. GSE85871, http:/www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE85871) indicated that HNK resulted in aberrant expression of miRNAs in a breast cancer cell line (Fig. 3A). To determine whether HNK also induces aberrant expression of miRNAs in human OS cells, six miRNAs (miR-188-5p, miR-202 and miR-623 were the most significantly upregulated; miR-21, miR-532-5p and miR-628-3p were the most significantly downregulated) were selected based on the microarray data, and were verified by RT-qPCR. The results indicated that miR-202 and miR-623 were markedly upregulated, and miR-21 was significantly downregulated in human Saos-2 OS cells following HNK treatment (P<0.01), whereas miR-188-5p, miR-532-5p and miR-628-3p were not significantly different compared with in the vehicle group (Fig. 3B). miR-21 has previously been reported to be associated with cell apoptosis and proliferation (24). Therefore, the present study further investigated the function of miR-21 in human OS cells. Saos-2 cells were treated with 10-100 μM HNK for 24 h and the expression levels of miR-21 were determined by RT-qPCR. The results demonstrated that HNK reduced miR-21 levels in a dose-dependent manner in human OS cells (Fig. 3C). These results indicated that HNK may exert antitumor effects via modulating miR-21 expression in human OS cells.

Overexpression of miR-21 rescues the suppressive effects of HNK on OS cells. The present study revealed that miR-21 was downregulated in Saos-2 cells following HNK treatment. Furthermore, mounting evidence has confirmed that miR-21 may serve a crucial role in regulating the expression of gene products involved in phenotypic characteristics of cancer cells, including cell proliferation, apoptosis and cell cycle (24,25). Therefore, it may be hypothesized that HNK suppresses cell growth and induces apoptosis of OS cells by modulating miR-21 expression. Saos-2 and MG-63 cells were transfected with miR-21 mimics following HNK treatment for 24, 48 and 72 h (HNK + miR-21 group); treatment with vehicle (vehicle group) or HNK alone (HNK group) served as negative and positive control groups, respectively. Subsequently, cell proliferation in each group was investigated by MTT assay, the results indicated that HNK significantly suppressed cell proliferation; however, the suppressive effects of HNK on OS cells were significantly rescued post-transfection with miR-21 mimics (P<0.01; Fig. 4A and B). To further validate these results, cell apoptosis was measured by flow cytometry. As expected, the proportion of apoptotic cells was significantly decreased in the HNK + miR-21 group compared with in the HNK group in Saos-2 and MG-63 cells (P<0.01; Fig. 4C and D). These data indicated that HNK may exert suppressive effects on human OS cells via downregulating miR-21.

miR-21 suppresses PTEN expression by directly targeting its 3'-UTR. Previous studies have reported that miR-21 may post-transcriptionally suppress PTEN expression in numerous human cancer cells, including lung cancer and esophageal cancer cells (25,26); however, whether PTEN is a direct target of miR-21 in human OS cells remains to be further

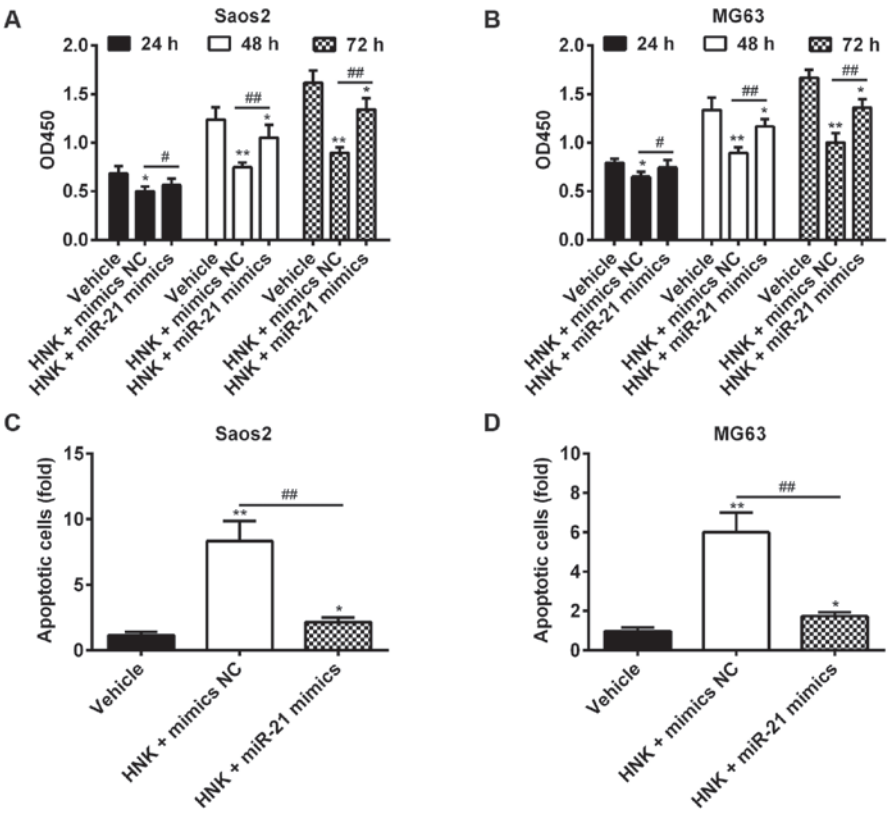

Figure 4. Overexpression of miR-21 rescues the suppressive effects of HNK on OS cells. (A and B) Saos-2 and MG-63 cells were transfected with miR-21 mimics following HNK treatment for 24, 48 and 72 h (HNK + miR-21 mimics group); treatment with vehicle (vehicle group) or HNK alone (HNK + mimics NC group) served as negative and positive control groups, respectively. Cell proliferation in each group was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. (C and D) Saos-2 and MG-63 cells were transfected with miR-21 mimics following HNK treatment for 48 h, and cell apoptosis was determined using flow cytometric analysis. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05, **P<0.01 vs. the vehicle group. $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. HNK + mimics NC group. HNK, honokiol; miR-21, microRNA-21; OD, optical density; OS, osteosarcoma.

elucidated. In the present study, luciferase-reporter plasmids containing wt or mut type 3'-UTR segments of PTEN were constructed (Fig. 5A). The reporters were cotransfected alongside miR-21 mimics/inhibitor or NC into Saos-2 cells, after which luciferase activity was measured. The results demonstrated that miR-21 mimic significantly suppressed luciferase activity compared with mimic NC; however, the miR-21 inhibitor markedly enhanced luciferase activity compared with inhibitor NC in the presence of wt 3'-UTR (P<0.01; Fig. 5B). In addition, miR-21 did not affect luciferase activity of the reporter vector containing mut PTEN-3'-UTR (Fig. 5B). These findings indicated that miR-21 inhibits PTEN by directly targeting PTEN-3'-UTR. To further confirm that PTEN levels are modulated by miR-21, the Saos-2 human OS cell line was transfected with miR-21 mimic/inhibitor or NC, and the protein expression levels of PTEN were determined using western blot analysis. The results indicated that miR-21 inhibited PTEN expression in OS cells compared with the NC (Fig. 5C). To further verify whether PTEN expression was modulated by HNK, Saos-2 cells were treated with 10-100 μM HNK for 24 h and PTEN expression was measured by western blotting. The results demonstrated that HNK enhanced PTEN expression in a dose-dependent manner in human OS cells (Fig. 5D).

HNK suppresses the PI3K/AKT signaling pathway via modulating miR-21 expression in human OS cells. It has previously been reported that the PI3K/AKT signaling pathway serves a critical role in cell survival, and exerts protective effect against tumorigenesis-associated apoptosis in cancer cells (27). Furthermore, PI3K and AKT are negatively modulated by PTEN, which is a key molecule in various diseases that modulates cell proliferation, survival, apoptosis and metabolism. A recent study revealed that miR-21 may mediate proliferation, apoptosis, migration, invasion and cell cycle progression in human esophageal cancer cells by targeting key proteins of the PTEN/PI3K/AKT signaling pathway (25). Therefore, the present study aimed to determine whether HNK-mediated miR-21 modulation regulates the PI3K/AKT signaling pathway in OS cells. Saos-2 and MG-63 cells were transfected with or without miR-21 mimics following treatment with or without HNK, and western blot analysis was used to determine the expression levels of p-AKT, p-mTOR and p-p70S6K, which are major components of the PI3K/AKT signaling pathway (28). The results indicated that the expression levels of AKT, mTOR and p70S6K were significantly downregulated following HNK treatment compared with mock vehicle-treated cells or miR-21 transfection in Saos-2 and MG-63 cells; however, the expression levels of these proteins were significantly upregulated in HNK-treated OS cells post-transfection with miR-21 mimics compared with transfection without miR-21 mimics (P<0.01; Fig. 6). These results demonstrated that HNK may suppress the PI3K/AKT signaling pathway in human OS cells; however, it could be activated by miR-21 overexpression. Taken together, these data suggested that HNK suppresses the PI3K/AKT

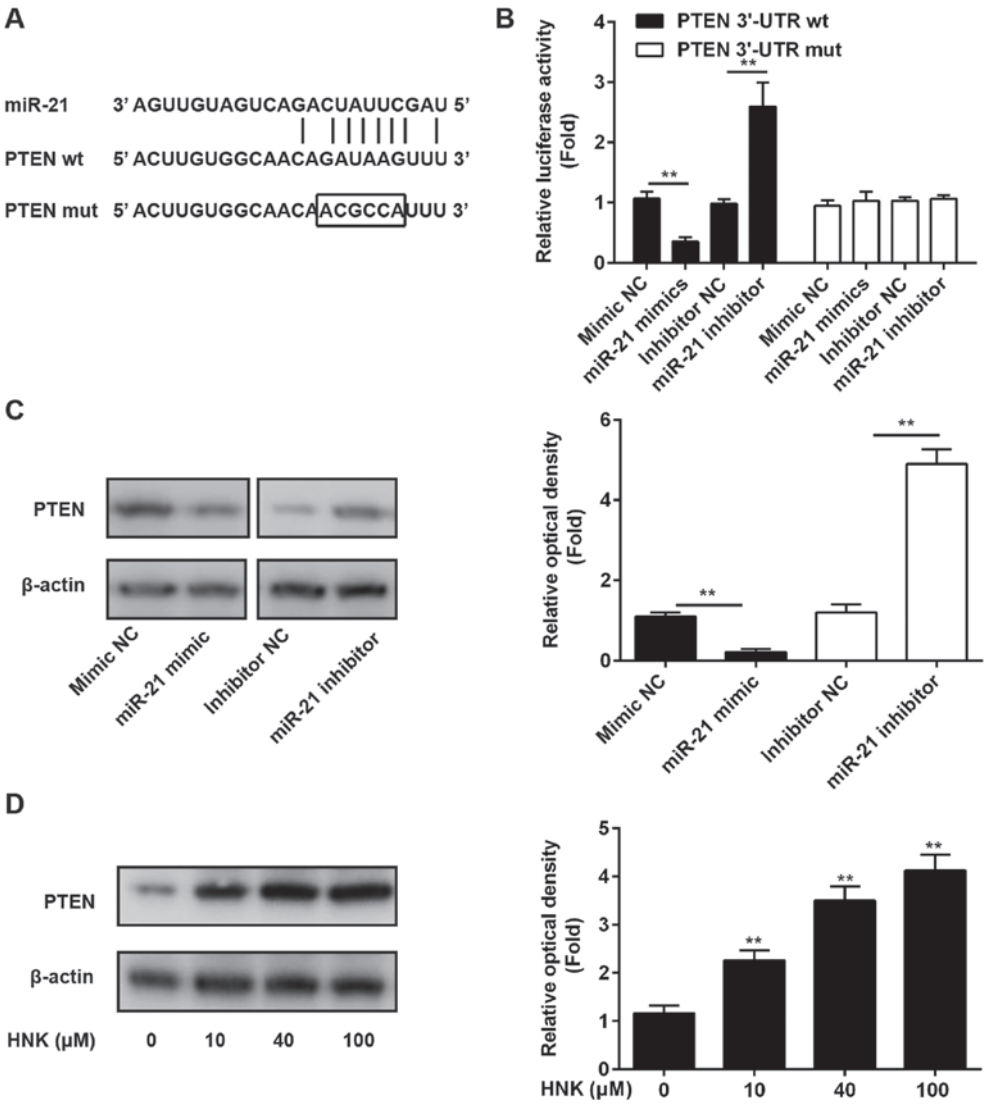

Figure 5. miR-21 suppresses PTEN expression by directly targeting its 3'-UTR. (A) PTEN 3'-UTR region containing wt or mut binding site for miR-21. (B) Relative luciferase activity of wt or mut PTEN 3'-UTR in Saos-2 cells post-transfection with miR-21 mimic/inhibitor or corresponding NC. (C) Western blot analysis was conducted to detect the protein expression levels of PTEN in Saos-2 cells post-transfection with miR-21 mimic/inhibitor or corresponding NC; β-actin was used as an internal control. (D) Saos-2 cells were treated with or without 10-100 μM HNK for 24 h, and western blot analysis was used to measure PTEN expression. Data are presented as the mean ± standard deviation of three independent experiments. **P<0.01 vs. corresponding NC group. 3'-UTR, 3'-untranslated region; HNK, honokiol; miR-21, microRNA-21; mut, mutant; NC, negative control; PTEN, phosphatase and tensin homolog; wt, wild-type.

signaling pathway by inhibiting miR-21 expression in human OS cells.

Discussion

HNK has been widely used to treat various diseases in traditional Chinese medicine, and it has been reported to exert anticancer functions (9,23). However, it remains unclear whether HNK may be used as a stand-alone natural compound to exert strong anticancer effects against human OS. In the present study, the potential of HNK-induced apoptosis of human OS cells was examined and the underlying molecular mechanisms were investigated. The results demonstrated that HNK inhibited cell growth in a dose-dependent manner in Saos-2 and MG-63 cells. Furthermore, the results indicated that HNK-induced apoptosis was dependent upon caspase activation, and that HNK increased expression of the proapoptotic protein Bax, and reduced expression of the anti-apoptotic protein Bcl-2. Notably, the results verified that HNK may induce aberrant miRNA expression in human OS cells, and miR-21 inhibits PTEN by directly targeting its 3'-UTR. Furthermore, HNK was revealed to induce apoptosis through modulating the miR-21/PTEN/PI3K/AKT signaling pathway in human OS cells.

Natural products that are able to prevent and treat cancers are the source of numerous medically beneficial drugs, including camptothecin, curcumin, isoflavone, luteolin, matrine, HNK, phenolic leaf extract of *Heimia myrtifolia* (Lythraceae) and xanthoangelol (29,30). A recent study demonstrated that xanthoangelol, which is isolated from *Angelica keiskei* roots, may inhibit tumor growth, metastasis to the lung and liver, and tumor-associated macrophage expression in tumors (30). In addition, it is well known that some natural compounds possess anticancer effects in human OS (31-33). Steinmann *et al* revealed that HNK exhibits prominent antimetastatic activity in OS and is able to induce rapid cell death *in vitro* (34). In the

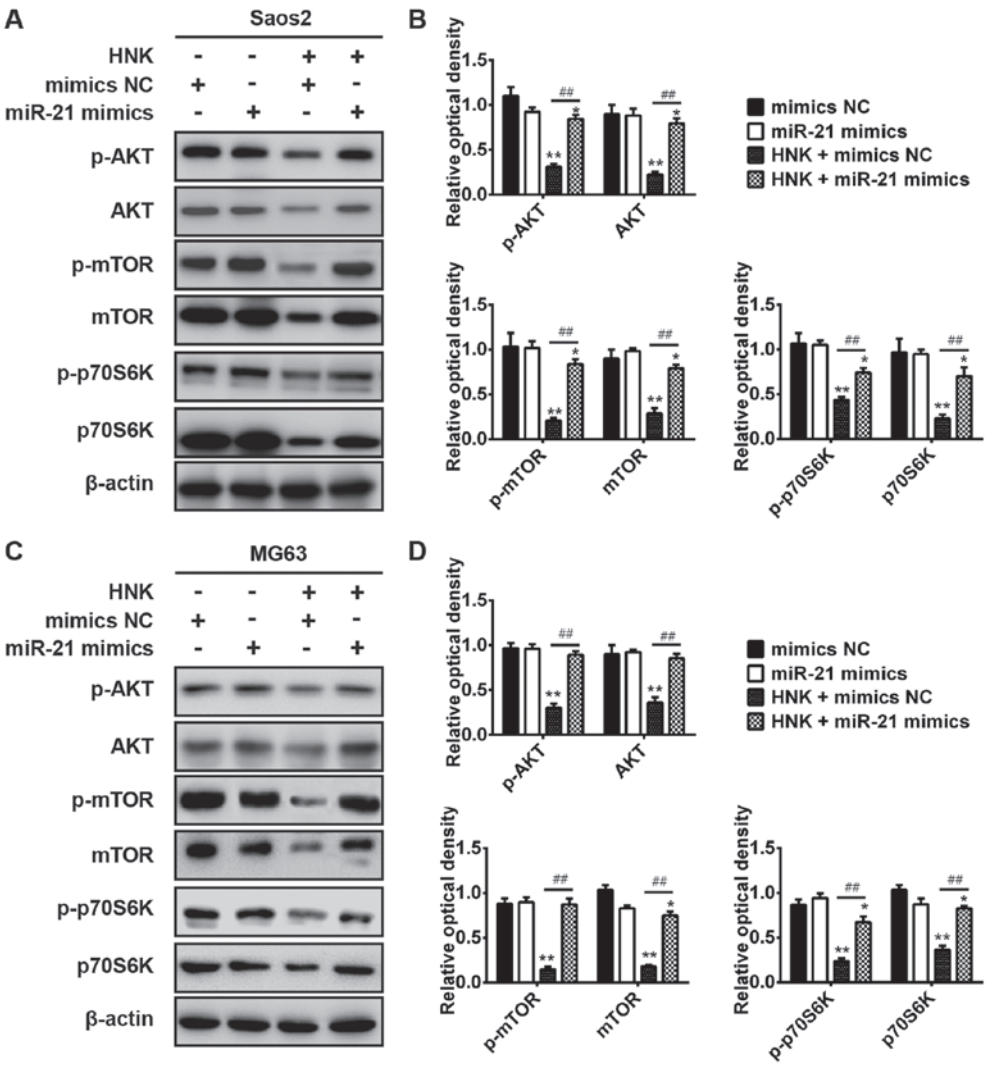

Figure 6. HNK suppresses the phosphoinositide 3-kinase/AKT signaling pathway via modulating miR-21 expression in human OS cells. Saos-2 and MG-63 cells were transfected with or without miR-21 mimics following treatment with or without HNK, and the expression levels of p-AKT, p-mTOR, p-p70S6K, AKT, mTOR and p70S6K were detected by western blot analysis in (A and B) Saos-2 and (C and D) MG-63 cells. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05, **P<0.01 vs. mimics NC group; $^{\#\#}$P<0.01 vs. HNK + mimics NC group. AKT, protein kinase B; HNK, honokiol; miR-21, microRNA-21; mTOR, mammalian target of rapamycin; OD, optical density; OS, osteosarcoma; p70S6K, p70S6 kinase; p-, phosphorylated.

present study, the MTT assay and flow cytometry were used to determine cell viability and apoptosis in HNK-treated human OS cells; the results indicated that HNK significantly reduced cell viability and induced apoptosis of OS cells. Furthermore, it has been reported that HNK may trigger apoptosis via activation of the intrinsic or extrinsic apoptotic pathways (9,35). HNK-induced apoptosis may also enhance caspase expression and PARP cleavage (23). In the present study, the results further confirmed that HNK significantly upregulated the expression levels of proapoptotic proteins (caspase-3, cleaved-PARP and Bax) and downregulated the levels of the anti-apoptotic protein Bcl-2. These data suggested that HNK may serve a crucial role in the apoptosis of OS cells.

Mounting evidence has demonstrated that miRNAs have important roles in numerous types of cancer, whereas some Chinese medicinal herbs harbor anticancer effects by targeting miRNAs. Liu *et al* revealed that berberine may regulate cisplatin sensitivity via mediating the miR-21/programmed cell death 4 axis in the ovarian cancer cells (18). Zeng *et al* demonstrated that camptothecin promotes cancer cell apoptosis via miRNA-mediated mitochondrial pathways (36). Furthermore, a recent study identified that HNK triggers the liver kinase B1-miR-34a axis and resists the oncogenic effects of leptin in breast cancer (11). Previous studies have also indicated that miR-34a modulates tumor cell growth and cell cycle progression in OS (37,38). The present study revealed that HNK induced aberrant miRNA expression in a breast cancer cell line, according to microarray data obtained from the GEO database (accession no. GSE85871; http:/www.ncbi.nlm.nih.gov/geo/query/acc. cgi?acc=GSE85871). Based on these data, six miRNAs, which were most significantly upregulated or downregulated in breast cancer cells following HNK treatment, were selected and RT-qPCR was conducted to validate these results in human OS cells. The results demonstrated that miR-202 and miR-623 were significantly upregulated, whereas miR-21 was significantly downregulated in the Saos-2 human OS cell line, whereas miR-188-5p, miR-532-5p and miR-628-3p were not significantly different compared with in the vehicle group. In addition, the present study indicated that HNK treatment reduced miR-21 expression in a dose-dependent

manner in human OS cells. Conversely, restoration of miR-21 expression abrogated the suppressive effects of HNK on OS cells. These data indicated that HNK may reduce cell viability and induce apoptosis via inhibiting miR-21 expression in human OS cells. Previous studies also reported that HNK induces apoptosis and G_1 cell cycle arrest in various types of cancer cell (39-42). Furthermore, miR-21 has been identified as an important regulator of diverse cellular functions via the regulation of cancer cell growth (43-45). However, the possible molecular mechanism underlying HNK-induced apoptosis of human OS cells requires further research.

Increasing evidence has confirmed that miR-21 modulates PTEN by directly targeting its 3'-UTR in various types of cancer, including hepatocellular cancer (24) and lung cancer (46). PTEN is a tumor suppressor gene that regulates the cell cycle and apoptosis in numerous solid tumors (47). Consistent with these results, the present study confirmed that miR-21 inhibits PTEN by directly targeting its 3'-UTR in human OS cells. Datta *et al* revealed that the PI3K/AKT signaling pathway acts as a key oncogenic pathway to induce cell growth and survival (48), which is negatively regulated by PTEN (49). Furthermore, Bai *et al* reported that the PI3K/AKT signaling pathway may be modulated by miR-21 in hepatocytes (50). The present study revealed that HNK suppresses the PI3K/AKT signaling pathway; however, it could be reactivated by miR-21 overexpression. Taken together, these data indicated that HNK induced apoptosis of human OS cells via modulating the miR-21/PTEN/PI3K/AKT signaling pathway.

In conclusion, the present study provided a novel insight into the molecular mechanism underlying HNK-induced apoptosis of human OS cells. Notably, the present results confirmed that HNK induces aberrant miRNA expression and HNK-induced apoptosis was modulated by the miR-21/PTEN/PI3K/AKT signaling pathway in human OS cells. These results suggested that HNK may exert anticancer effects to prevent OS progression.

Competing interests

The authors declare there is no competing interest.

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