miRNA-218 targets multiple oncogenes and is a therapeutic target for osteosarcoma

KENTARO SATO[1], EIJI OSAKA[2], KYOKO FUJIWARA[3,4], RYOTA FUJII[2], TADATERU TAKAYAMA[3], YASUAKI TOKUHASHI[5] and KAZUYOSHI NAKANISHI[2]

[1]Department of Orthopedic Surgery, Nihon University Hospital, Chiyoda-ku, Tokyo 101-8309; [2]Department of Orthopedic Surgery, Nihon University School of Medicine; [3]Department of Internal Medicine, Nihon University School of Medicine, Itabashi-ku, Tokyo 173-8610; [4]Department of Anatomy, Nihon University School of Dentistry, Chiyoda-ku, Tokyo 101-8310; [5]Department of Orthopedic Surgery, Tachikawa Kinen Hospital, Kasama City, Ibaraki 309-1736, Japan

Received October 25, 2021; Accepted February 18, 2022

DOI: 10.3892/or.2022.8303

Abstract. Survivin is overexpressed in various cancers and is correlated with treatment resistance and prognosis. MicroRNAs (miRNAs) directly regulate several target genes and are potential therapeutic agents for various cancers. The present study evaluated multiple gene targets of miR-218, including survivin, in osteosarcoma and compared the anti-tumor effects of miR-218 with those of YM155, an anti-survivin agent. It assessed the expression levels of miR-218 and survivin in osteosarcoma and osteoblast cell lines, as well as the proliferative, migratory and invasive capacities of cells following treatment with miR-218 or YM155. The form of cell death was assessed using fluorescence-activated cell sorting analysis to examine the expression of invasion ability-related genes. Osteosarcoma cell lines were subcutaneously injected into immunodeficient mice; the mice were then treated with miR-218 or YM155 to assess the anti-tumor effects of these agents. The results showed that miR-218 was downregulated, whereas survivin was overexpressed in the osteosarcoma cell line compared with normal osteoblast cells. The expression of survivin was suppressed upon overexpression of miR-218 (miR-218 group) or administration of YM155 (YM155 group), leading to apoptosis and inhibition of osteosarcoma cell proliferation. Invasion and migration abilities were inhibited in the miR-218 group, but not in the YM155 group. In the animal model, both the miR-218 and YM155 groups showed a reduced tumor volume and decreased survivin expression. In osteosarcoma, miR-218 showed a wider range of therapeutic efficacy compared with YM155, suggesting that miR-218 should be evaluated as a treatment target.

Correspondence to: Dr Eiji Osaka, Department of Orthopedic Surgery, Nihon University School of Medicine, 30-1 Oyaguchikami-cho, Itabashi-ku, Tokyo 173-8610, Japan
E-mail: osaka.eiji@nihon-u.ac.jp

Key words: osteosarcoma, miR-218, survivin, YM155, targeted therapy

Introduction

Osteosarcoma is the most frequent primary bone malignancy affecting the metaphysis of the long bones in children and adolescents. Perioperative chemotherapy combined with surgical treatment has markedly improved five-year disease-free survival and overall survival rates of 59 and 65% (1); however, treatment-resistant and recurrent cases, with metastases and poor prognosis, show a mean survival time of <1 year (2,3). Driver-gene mutations directly involved in tumorigenesis and growth have been discovered in many cancers, leading to the development of molecularly targeted drugs with high efficacy and response rates (4). Although several molecularly targeted drugs for osteosarcoma have been tested in clinical trials, their effectiveness was observed to be insufficient, probably due to the large number of subtypes and non-unified genetic abnormalities of the disease (5,6). Therefore, the mechanisms of tumorigenesis in osteosarcoma must be determined to improve treatment outcomes.

Survivin, a member of the inhibitor of apoptosis protein family, inhibits apoptosis by suppressing caspase activity (7). While it is not expressed in normal tissues, it is overexpressed in almost all types of cancer, including osteosarcoma (8). In addition, patients with high expression of survivin are reported to have a poor prognosis in various cancers (9-15). The present authors previously reported that increased expression of survivin is a poor prognostic factor in osteosarcoma (16).

YM155 is a molecularly targeted drug developed as an inhibitor of survivin expression, leading to the suppression of cell proliferation and exhibiting anti-tumor effects by improving drug sensitivity (17). YM155 is a potential therapeutic target undergoing clinical testing for lung cancer and melanoma; however, no difference in patient survival was

reported between treatment with docetaxel alone or in combination with YM155 in a phase II study of breast cancer (18). Thus, one-to-one drugs have limitations and therapeutic agents that act more broadly to target multiple genes may be needed.

Micro (mi)RNAs are small non-coding RNAs of 18-25 nucleotides that regulate gene expression by binding to the 3'-untranslated region of target mRNAs, thereby regulating various processes such as metabolism, immunity and digestion. Additionally, miRNAs are involved in the progression of numerous diseases, including cancer, making them potential therapeutic targets (19,20).

Of the numerous miRNAs that reportedly target survivin (Table I) (21), the present study focused on miR-218, as it targets genes acting on the NF-κB and the Wnt/β-catenin pathways, which play crucial roles in osteosarcoma tumorigenesis (Table II) (22). miR-218 suppresses survivin expression and exerts antitumor effects in glioblastoma and cervical cancer (23,24). In addition, it improves drug sensitivity and induces apoptosis in lung cancer (25). In osteosarcoma, the mitochondrial apoptotic pathway is thought to be regulated by B-cell lymphoma 2 (Bcl-2) family proteins, including the anti-apoptotic proteins Bcl-2 and Bcl-xl and pro-apoptotic proteins Bax, Bak and Bad (26). Although miR-218 is known to arrest the cell cycle through E2F2 as a tumor suppressor (27), little is known about its role in other processes, such as apoptosis. In addition, the regulation of survivin expression by miR-218 in osteosarcoma has not been evaluated *in vivo*.

A single miRNA simultaneously targets multiple genes and thus, may normalize an entire failed biological network; however, the therapeutic efficacy of miRNAs compared with that of molecularly targeted drugs remains to be elucidated. The present study evaluated the *in vitro* and *in vivo* anti-tumor efficacy of miR-218 in osteosarcoma and compared the effects with those of YM155 to provide a foundation for the development of therapeutic strategies.

Materials and methods

Osteosarcoma and normal osteoblast cell lines. The human osteosarcoma cell lines MG63 and HOS were purchased from the Health Science Research Resource Bank of Japan. The normal osteoblast cell line hFOB1.19 was purchased from ATCC. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Inc.) supplemented with 10% fetal bovine serum (Nichirei Biosciences, Inc.), 0.1 mM non-essential amino acids (Thermo Fisher Scientific, Waltham, MA, USA) and 100 IU/ml penicillin-streptomycin-glutamine (Thermo Fisher Scientific, Inc.) and incubated at 37˚C with 5% CO_2.

Transfection of miRNA-218 mimics, YM155 and controls. Osteosarcoma cell lines ($1x10^5$ cells) were treated with a has-miR-218-5p mimic (sequence, 5'-UUGUGCUUGAUC UAACCAUGU-3'; miR-218 group), designed and purchased from Ambion and negative control miRNA (cat. no. 4464058; Thermo Fisher Scientific, Inc.; NC-miR group), adjusted to a final concentration of 50 nM, using Lipofectamine® 2000 Transfection Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 48 h. Cells were alternately administered YM155 (YM155 group), purchased from Cayman Chemical Company and adjusted to a final concentration of 5 nM. Phosphate-buffered saline (PBS; NC group) was used as a control.

Reverse transcription-quantitative (RT-q) PCR. At 48 h post-treatment (transfection with miRNA-218 or administration of YM155), total RNA was extracted from each cell type ($1x10^5$ cells) using the RNeasy Mini kit (Qiagen GmbH). The extracted total RNA was reverse transcribed using Prime Script RT Master Mix (Takara Bio Inc.) to prepare complementary DNA (cDNA) at a concentration of 500 ng/μl according to the manufacturer's recommendations. RT-qPCR for miR-218 was performed using Takara Prime Script and TaqMan Probe (Takara Bio, Inc.) according to the manufacturer's instructions. The PCR reactions were carried out with an initial denaturation for 30 sec at 95˚C followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. RNU44 (Thermo Fisher Scientific, Inc.) was used as an endogenous control. The synthesized cDNA was used to analyze survivin expression levels using SYBR Premix Ex Taq II (Takara Bio, Inc.). The PCR reactions were carried out with an initial denaturation for 30 sec at 95˚C followed by 40 cycles of 95˚C for 5 sec, 55˚C for 10 sec and 72˚C for 30 sec. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control to correct the expression levels with the $2^{-\Delta\Delta Cq}$ method (28). The experiment was repeated three times. The specific primers used were as follows: miR-218 (assay ID 000521; Thermo Fisher Scientific, Inc.); RNU44 (assay ID 001094; Thermo Fisher Scientific, Inc.); survivin-forward, 5'-ACCGCATCTCTACATTCA AG-3'; survivin-reverse, 5'-CAAGTCTGGCTCGTTCTC-3'; GAPDH-forward, 5'-TCACCAGGGCTGCTTTTAAC-3'; GAPDH-reverse, 5'-TGACGGTGCCATGGAATTTG-3'.

Western blotting. The cells were lysed using Pierce RIPA buffer (Thermo Fisher Scientific, Inc.) containing a protease inhibitor (Roche Applied Science, Basel, Switzerland). After sonication, the cells were centrifuged and the supernatant was collected for protein extraction. The protein concentration was measured using Bio-Rad DC kits (Bio-Rad Laboratories, Inc.). Cell lysates (20 μg of protein) were electroblotted with 4-12% NuPAGE Bis-Tris gel (Thermo Fisher Scientific, Inc.) at 30 mA. The primary antibodies rabbit anti-survivin (cat. no. 2808; 1:1,000), anti-MMP-2 (cat. no. 13132; 1:1,000) and anti-MMP-9 (cat. no. 13667; 1:1,000; all from Cell Signaling Technology, Inc.) were transferred to difluoride membranes by the semi-dry method and blocked at 4˚C for 1 h with Blocking One (Nacalai Tesque, Inc.) to block non-specific binding. Each primary antibody was diluted with Can Get Signal Immunoreaction Enhancer Solution 1 (Cytiva) and incubated with the membrane for 12 h at 4˚C. The anti-GAPDH rabbit polyclonal antibody (cat. no. ab9485; Abcam,) was used as an endogenous control. After primary antibody treatment, the membranes were washed with 0.1% Tween-containing PBS and incubated with secondary horseradish peroxidase-conjugated anti-rabbit IgG antibodies (cat. no. NA934; Cytiva), each diluted in 0.1% Tween-containing PBS at 1:2,000, for 1 h at 25˚C. Chemiluminescence was induced using Chemi-Lumi One Super assay kit (Nacalai Tesque, Inc.) before bioimaging. Data were visualized and photographed using the analyzer LAS-4000 (Fujifilm). Protein expression was semi-quantified

Table I. miRNAs that regulate survivin.

miRNA	Type of cancer	Target gene	Effect
-494	Acute lymphoblastic leukemia	TEL-AML1	Apoptosis
-16	Colorectal cancers	Direct survivin	Apoptosis
-320a	Acute lymphoblastic leukemia	TEL-AML1	Apoptosis
-542-3p	Lung cell carcinoma cell line A549	Direct survivin	Cell cycle arrest
-708	Renal cell carcinoma	Direct survivin	Activation caspase-3, -9
-218	Nasopharyngeal carcinoma	Direct survivin	Apoptosis
-143	Breast cancer cell line MCF-7	Direct survivin	Inhibited E2-induced cell proliferation
-203	Prostate cancer	Direct survivin	Cell death
	Laryngeal cancer	Direct survivin	G1 phase cell cycle arrest
	Hepatocellular carcinoma	Direct survivin	Suppressed proliferation
	Lung cancer	Direct survivin	Inhibits proliferation and invasion
	Pancreatic cancer cells	Direct survivin	Apoptosis, cell cycle arrest
	Osteosarcoma	Direct survivin	Apoptosis, cell cycle arrest
-150	Burkitt's lymphoma cell line DG75	Direct survivin	Apoptosis
-34a	Non-small cell lung cancer	Notch-1	Apoptosis
	Neuroblastoma	MYCN	Apoptosis
	Laryngeal squamous cell carcinoma	Direct survivin	Cell cycle arrest
	Gastric cancer	PI3K/AKT/survivin pathway	Improve the sensitivity of cisplatin
	Melanoma cell lines	Direct survivin	Apoptosis
	Osteosarcoma	Direct survivin	Apoptosis, cell cycle arrest

miRNA, microRNA; TEL-AML, ETV6-RUNX1, Ets variant gene 6-Runt-related transcription factor 1; MYCN, N-myc proto-oncogene protein.

using ImageJ software (ver. 1.53; National Institute of Health) and quantified by correcting for the band concentration of GAPDH for each of the visualized proteins.

Water-soluble tetrazolium salts 8 cell proliferation assay. Post-treatment (transfection with miRNA-218 or administration of YM155), the cells were seeded into 96-well plates and incubated for 24, 48, or 72 h. Next, the Cell Count Reagent SF (Nacalai Tesque, Inc.) was added to the culture medium and incubated at 37˚C for 1 h. Absorbance was measured using Wallac 1420 ARVO MX (PerkinElmer, Inc.).

Fluorescence-activated cell sorting analysis. Following transfection with miRNA-218 or NC-miRNA or administration of YM155 or PBS, the cells were seeded into 6-well plates, incubated for 48 h, treated with trypsin/EDTA and centrifuged at 300 x g for 3 min at 4˚C. The pellet was dissolved in 500 μl of 1X binding buffer, transferred to a fluorescence-activated cell sorting (FACS) tube, Cells were stained with Annexin V-FITC and propidium iodide using Annexin V-FITC Apoptosis Detection kit (cat. no. K101-25, Abcam) according to the manufacturer's protocols. The cells were sorted by FL1-H (Annexin V channel) and FL2-H (PI channel) on a FACSCalibur (BD Biosciences) flow cytometer and analyzed by CellQuest (v. 3.3; BD Biosciences). The apoptotic rate was calculated as the percentage of early and late apoptotic cells.

Animal model. A total of 24 eight-week-old male nude mice (26.4±1.4 g, BALB/cAJcl-nu/nu) were purchased from CLEA Japan, Inc. and kept in an environment of 22˚C and 60% humidity under a 12-h dark/light cycle, with free access to a food and water. Osteosarcoma cells were lysed in 100 μl Matrigel and 5.0x10^6 cells were injected subcutaneously into the mice. Tumor size was calculated as the long diameter x short diameter x height/2 and experiments were begun when the tumor volume was >120 mm^3. AteloGene containing miRNAs, miR-218, or NC-miRNAs was injected subcutaneously at the recommended concentration of 25 μM, whereas YM155 (3 mg/kg) and PBS were injected intratumorally (n=6 per group). Each treatment was applied three times at 0, 7 and 14 days. Tumor volumes were measured every 2-3 days until tumor resection at 18 days. Animals were sacrificed with intraperitoneal administration of sodium pentobarbital (100 mg/kg). The tumor tissue was subjected to survivin immunostaining according to the manufacturer's protocols (cat. no. 2808, 1:200; Cell Signaling Technology, Inc.) and the immunohistochemistry score was calculated as the area x density (0-15 points), where the area is the percentage of tumor cells per slide (0, 0%; 1, <5%; 2, 5-20%; 3, 21-50%; 4, 51-75%; 5, >75%) and density can be absent, weak, moderate, or strong, ranging from 0-3, respectively. Each slide was observed once by three individuals. The animal study was approved by the Animal Experiment Committee of Nihon University School of Medicine, Japan (approval number AP16M012) and humane endpoints were also in accordance with this approval.

Table II. Target genes of micro-RNA 218.

Pathway	Gene name	Official name	Functions
RTK pathway	RTKs	Receptor tyrosine kinase	Cell surface receptors for growth factors, cytokines and hormones
	PLCy1	Phosphoinositide phospholipase C-gamma-1	Intracellular transduction of receptor
	ARAF	A-Raf proto-oncogene	Involvement of cell growth and development
AKT/mTOR pathway	PIK3C2A	Phosphoinositide 3-kinase-C2-alpha	Cell proliferation, migration and intracellular protein trafficking
	RICTOR	RPTOR independent companion of MTOR complex 2	Embryonic growth and development
Focal adhesion pathway	Laminin 5	-	High-molecular weight proteins of the extracellular matrix
	Integrin	-	Transmembrane receptors to facilitate cell-extracellular matrix adhesion
	CAV2	Caveolin 2	Main component of the inner surface of caveolae
	ACTN1	Alpha-actinin-1	Spectrin gene superfamily
	PXN	Paxillin	Actin-membrane attachment of cell adhesion to ECM
	LASP1	LIM and SH3 domain protein 1	cAMP and cGMP dependent signaling protein
	SH3GL1	SH3 domain containing GRB2 like 1	Endocytosis and cell cycle
wnt/β-catenin pathway	FZ	frizzled	Receptors in the Wnt signaling pathway
	LEF1	Lymphoid enhancer-binding factor 1	Forming a complex with β-catenin and promotes transcriptional activity
	Survivin	Baculoviral inhibitor of apoptosis repeat-containing 5	Inhibitor of apoptosis (IAP) family
	HMGB1	High mobility group box 1 protein	Cell differentiation and tumor cell migration
NF-κB pathway	ECOP	EGFR-coamplified and overexpressed protein	Increasing NF-κB transcriptional activity
	IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta	Controlling activation of NF-kappa-B
Cell cycle	CCND1	Cyclin D1	Regulation of the G1/S phase transition
	TET2	Tet methylcytosine dioxygenase 2	Involvement of myelopoiesis
	BMI1	B lymphoma Mo-MLV insertion region 1 homolog	Oncogene by regulating p16 and p19
	CDK6	Cyclin dependent kinase 6	G1 phase progression and G1/S transition
Slit-robo pathway	Robo1	Roundabout homolog 1	Regulation of cell migration and cell adhesion
Epigenetic pathway	HOXB3	Homeobox B3	Involvement of development and biologic subset of AML

Wound healing assay. The osteosarcoma cells were seeded into 24-well plates at $1.0x10^5$ cells/200 μl/well and a gap was created by scraping the centre of the bottom of the dish 24 h post-incubation using a cell scratcher scratch stick (AGC Techno Glass). Following treatment with miRNA-218, NC-miRNA, YM155, or PBS, medium containing 10% FBS) and mitomycin C (MMC, 0.25 μg/ml) was added to eliminate the growth effect. The pore area at 0, 24, 48 and 72 h was imaged by phase-contrast microscope Leica DM IL (Leica Microsystems GmbH), calculated using ImageJ software as follows: migration rate=[(pore area at 0 h)-(pore area at 24, 48, or 72 h)]/(pore area at 0 h) x100.

Matrigel invasion assay. Invasion chambers (Corning, Inc.) containing miR-218, YM155, or NC-miRNA were used to

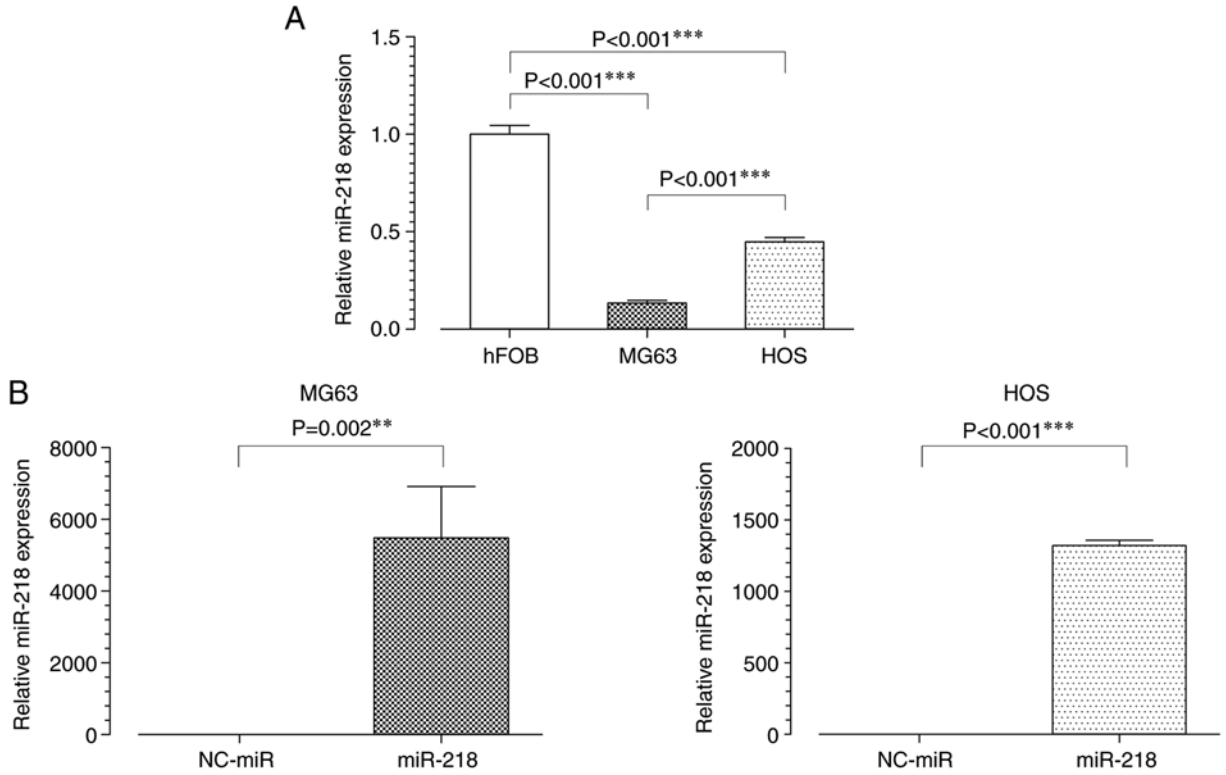

Figure 1. Expression levels of miR-218 in normal and osteosarcoma cell lines. (A) miR-218 expression levels in normal cell lines (hFOB) and osteosarcoma cell lines. One-way ANOVA with post-hoc Bonferroni-corrected t-test. (hFOB vs. MG63, hFOB vs. HOS, MG63 vs. HOS: P<0.001. (B) Change in miR-218 expression levels after transfection of MG63 or HOS with miR-218. (MG63: P=0.002, HOS: P<0.001) **P<0.01, ***P<0.001. miR, microRNA.

study the invasive capacity of the cells, which is the ability to degrade and migrate extracellular substrates beyond the basement membrane. Osteosarcoma cells ($5x10^5$ cells/well) were suspended in minimum essential medium (MEM) or DMEM, seeded into the Matrigel-coated upper chamber and incubated with MMC (0.25 μg/ml) in the presence of 10% fetal bovine serum. After 72 h of treatment, cells that had passed to the bottom of the chamber were fixed in 4% paraformaldehyde for 15 min, stained with Giemsa (24˚C, 2 min) and counted under phase-contrast microscope Leica DM IL (Leica Microsystems GmbH) using x20 objective and measurements were taken in 10 fields of view per chamber.

Statistical analysis. Each experiment was performed at least three times. Data are presented as the means ± standard deviation or median and range. Student's t-test and Mann-Whitney U test were used for comparing two groups and one-way ANOVA or mixed repeated measures ANOVA was used for comparing multiple groups where the Bonferroni's correction was used to compare differences between groups. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-218 expression is downregulated in osteosarcoma cell lines compared with normal cells. To analyze the function of miR-218 in osteosarcoma, its expression levels in osteosarcoma and normal osteoblast cells was first assessed using RT-qPCR. miR-218 expression levels were significantly lower in osteosarcoma cell lines, MG63 and HOS, compared with normal osteoblast cells, hFOB (Fig. 1A). After transfection with miR-218, MG63 and HOS cells overexpressed miR-218 (Fig. 1B). Although differences in transfection efficiency between the two osteosarcoma cell lines were observed, the amount of original miR-218 were significantly lower in MG63 cells than in HOS cells. Therefore, MG63 cells were used in subsequent experiments as it was easier to evaluate the effects of miR-218.

miR-218 overexpression and YM155 administration suppresses survivin expression. The half-maximal inhibitory concentration (IC_{50}) was measured in a water-soluble tetrazolium salts 8 (WST 8) cell proliferation assay 48 h post-treatment with miR-218 or YM155. The IC_{50} of miR-218 was 50 nM, whereas that of YM155 was 5 nM; these concentrations were used in further experiments involving MG63 cells (Fig. 2A). The RT-qPCR results showed that survivin transcription levels were decreased in both experimental groups compared with those in the negative control groups (Fig. 2B). Western blot analysis confirmed these results, revealing decreased protein expression levels of survivin in the miR-218 and YM155 groups (Fig. 2C and D).

miR-218 overexpression and YM155 administration inhibits cell proliferation. To investigate the effects of miR-218 overexpression and YM155 administration on cell proliferation in osteosarcoma, a WST 8 assay and FACS analysis were performed. The WST 8 assay showed that the miR-218 and

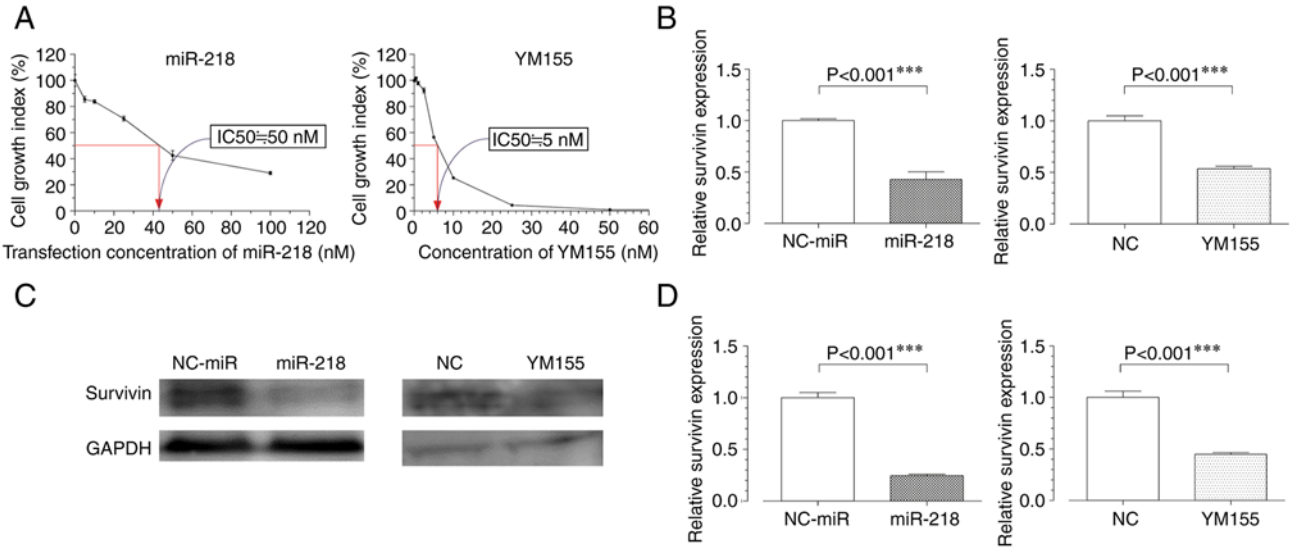

Figure 2. Survivin expression analysis following miR-218 overexpression and YM155 treatment (MG63). (A) Median inhibition concentration (IC_{50}) determined using WST8 cell proliferation assay. The horizontal axis is the concentration of miR-218 at the time of transfection. (B) Transcription levels of survivin using reverse transcription-quantitative PCR. (miR-218: P<0.001, YM155: P<0.001) (C) Western blot analysis of survivin expression 48 h post-treatment. (D) Quantification of western blotting by band concentration. (miR-218: P<0.001, YM155: P<0.001) ***P<0.001. miR, microRNA.

YM155 groups had reduced proliferative capacity compared with the control groups 72 h post-treatment (Fig. 3A). Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.), which was used in gene transfer, has been reported as toxic (29) and probably caused a decrease in the cell number in the NC-miR group. For the same reason, the miR-218 group failed to show a 50% suppression in the cell number compared with the NC-miR group 48 h post-treatment, even though the concentration was set to the IC_{50}. However, the NC group was not affected by gene transfer and reached the cell number limit 48 h post-treatment. To evaluate apoptosis 48 h post-treatment, Annexin V-PI staining was performed. FACS analysis showed an increase in the rightward migration of each cell in the miR-218 and YM155 groups, indicating early and late apoptosis (Fig. 3B).

miR-218 and YM155 inhibits tumor volume growth. The effect of miR-218 overexpression on tumorigenesis in osteosarcoma was evaluated *in vivo*. In nude mice, miR-218 and YM155 significantly inhibited tumor growth compared with that in the NC-miR and NC groups 18 days post-treatment (Fig. 4A and B). Survivin immunostaining was performed on tumor specimens and both, the miR-218 and YM155 groups, showed decreased immunohistochemistry scores (Fig. 4C).

Overexpression of miR-218, but not YM155, inhibits cell migration and invasion capacity. Effect of the overexpression of miR-218 in osteosarcoma was assessed using wound healing and migration assays. The former assay showed a decrease in the pore area of the miR-218 group and consequently in its migration capacity; in contrast, no significant difference was observed between the YM155 and NC groups (Fig. 5). Consistently, the migration assay showed inhibition of the invasive capacity in the miR-218 group, but no significant difference was observed between the YM155 and NC groups (Fig. 6). The expression level of MMP-2/9, which is involved in the migration and invasion ability of cells was further assessed. In western blotting analysis, MMP-2/9 levels were found to be decreased in the miR-218 group but showed no significant difference between the YM155 and NC groups (Fig. 7).

Discussion

The present study showed that in osteosarcoma, miR-218 overexpression and YM155 treatment both led to apoptosis and inhibited the cell proliferative capacity *in vivo* and *in vitro*. However, miR-218 inhibited cell invasion and migration, whereas YM155 did not. There is no direct comparison in the literature between the multi-target miR-218 and single-target molecularly targeted drugs. In osteosarcoma, which shows a non-unified genetic abnormality, it was demonstrated that miR-218 has a broader effect and is more effective than single-target drugs.

Cancer is a disease caused by genetic abnormalities; however, the mechanism of cancer development remains unclear. Mutations in driver genes directly involved in tumorigenesis and growth have been discovered in certain types of cancer (such as breakpoint cluster region-Abelson tyrosine kinase in chronic myeloid leukemia and genetic mutations of epidermal growth factor receptor in lung cancer) (30,31). Molecularly targeted drugs (such as gefitinib used in lung cancer) are highly efficient against cancers caused by driver-gene mutations and increase the median survival of patients (32,33). Clinical trials of various molecularly targeted agents have been conducted in osteosarcoma. Although antitumor activity of one such agent, cabozantinib, has been reported, its long-term action and effect on metastasis remain unclear, probably due to the non-unified genetic abnormality inherent to osteosarcoma (5,34).

Clinical trials with miRNAs for treating malignant mesothelioma and hepatitis C showed positive outcomes (35,36);

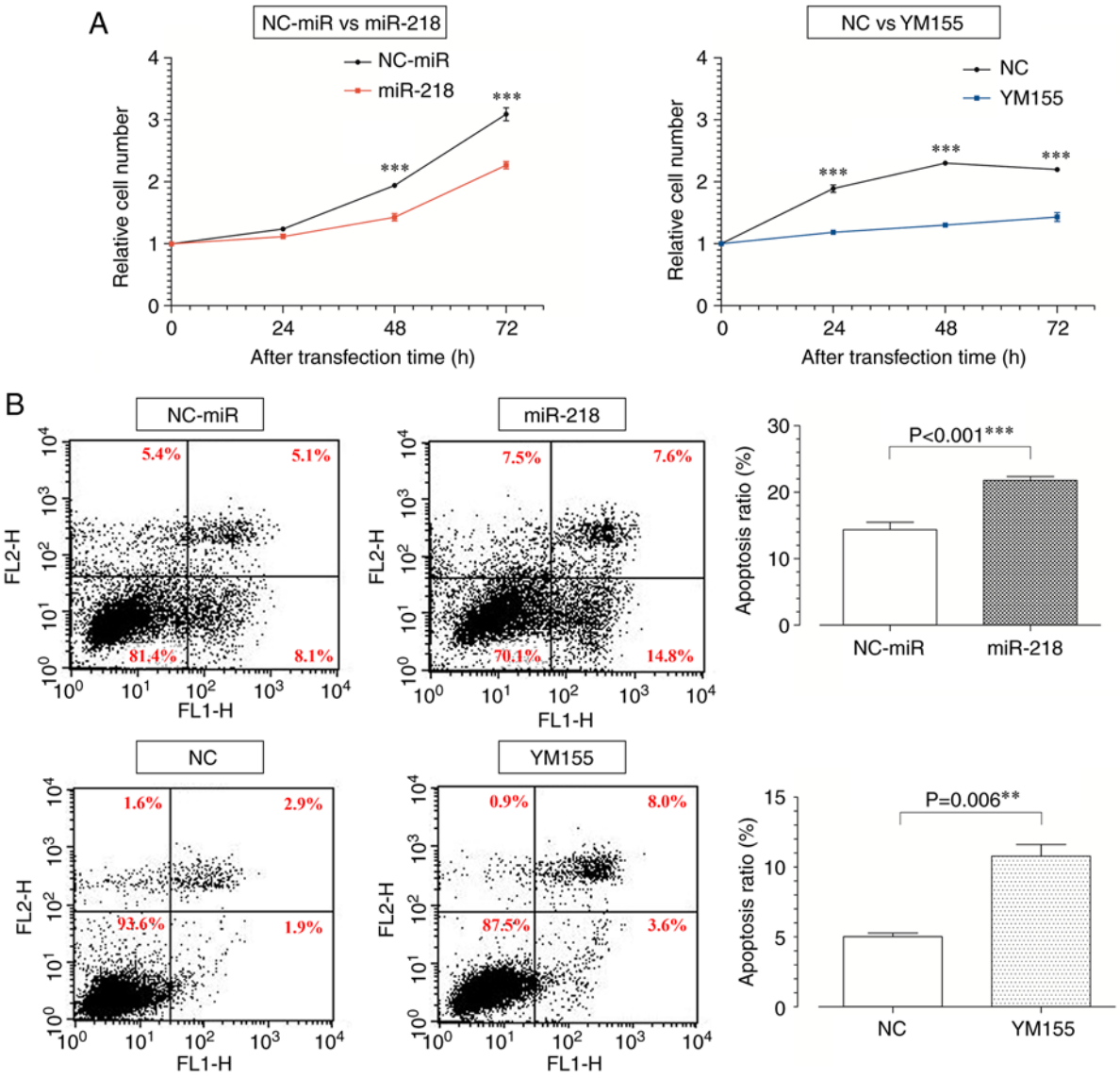

Figure 3. Analysis of proliferation and cell death following miR-218 overexpression and YM155 treatment (MG63). (A) Changes in proliferative capacity at 24, 48 and 72 h post-treatment. Mixed repeated measures ANOVA with post hoc Bonferroni-corrected t-test. (miR-218: 48, 72 h, P<0.001; YM155: 24, 48, 72 h, P<0.001). (B) Fluorescence-activated cell sorting analysis 48 h post-treatment. (miR-218: P<0.001, YM155: P=0.006) **P<0.01, ***P<0.001. miR, microRNA.

however, the use of miRNAs for treating osteosarcoma has not been evaluated. The present study focused on survivin, a therapeutic target gene for anti-tumor drugs, which is not expressed in normal tissues but is overexpressed in tumor cells. In osteosarcoma, miR-34a and miR-203 suppress the survivin gene, causing cell cycle arrest in the G_2-M phase and inducing apoptosis and improving sensitivity to cisplatin (37). However, their effects on the cell invasive and migratory capacity, as well as their *in vivo* anti-tumor potency are unknown.

miR-218 is a molecular switch that, in addition to its other actions shown in Table II, suppresses survivin and the Wnt/β-catenin pathway. Lu *et al* (22) and Fernández *et al* (38) show that lymphoid enhancer-binding factor (lef) 1, β-catenin and T-cell factor (tcf)/lef are involved in survivin expression. Therefore, miR-218 may directly and indirectly regulate survivin, which is highly expressed in osteosarcoma. Bmi-1, a constituent gene of the NF-κB pathway, is associated with the development, extension and prognosis of osteosarcoma. The Bmi-1 antibody, AbBmi-1, suppresses NF-κB and MMP-9 expression and reduces the proliferative and migratory potential of osteosarcoma cells; thus, it may be useful as a novel therapeutic target (39). In addition, epithelial to mesenchymal transition has been extensively studied for the migratory and invasive potential of tumor cells. It has been reported that miR-218 targets and suppresses the expression of roundabout homolog 1 and epidermal growth factor receptor-coamplified and overexpressed protein, thereby decreasing NF-κB transcriptional activity and suppressing epithelial to mesenchymal transition (40). Wnt/β-catenin-mediated complexes with transcription factors, such as tcf and lef, as well as transcriptional coactivators, are intimately involved in osteosarcoma tumorigenesis, resulting in transcriptional activity of multiple downstream target oncogenes (41). In summary, the present study focused on miR-218 because it is closely involved in osteosarcoma tumorigenesis and has many targets in the NF-κB and Wnt/β-catenin pathways, along with affecting the expression of survivin.

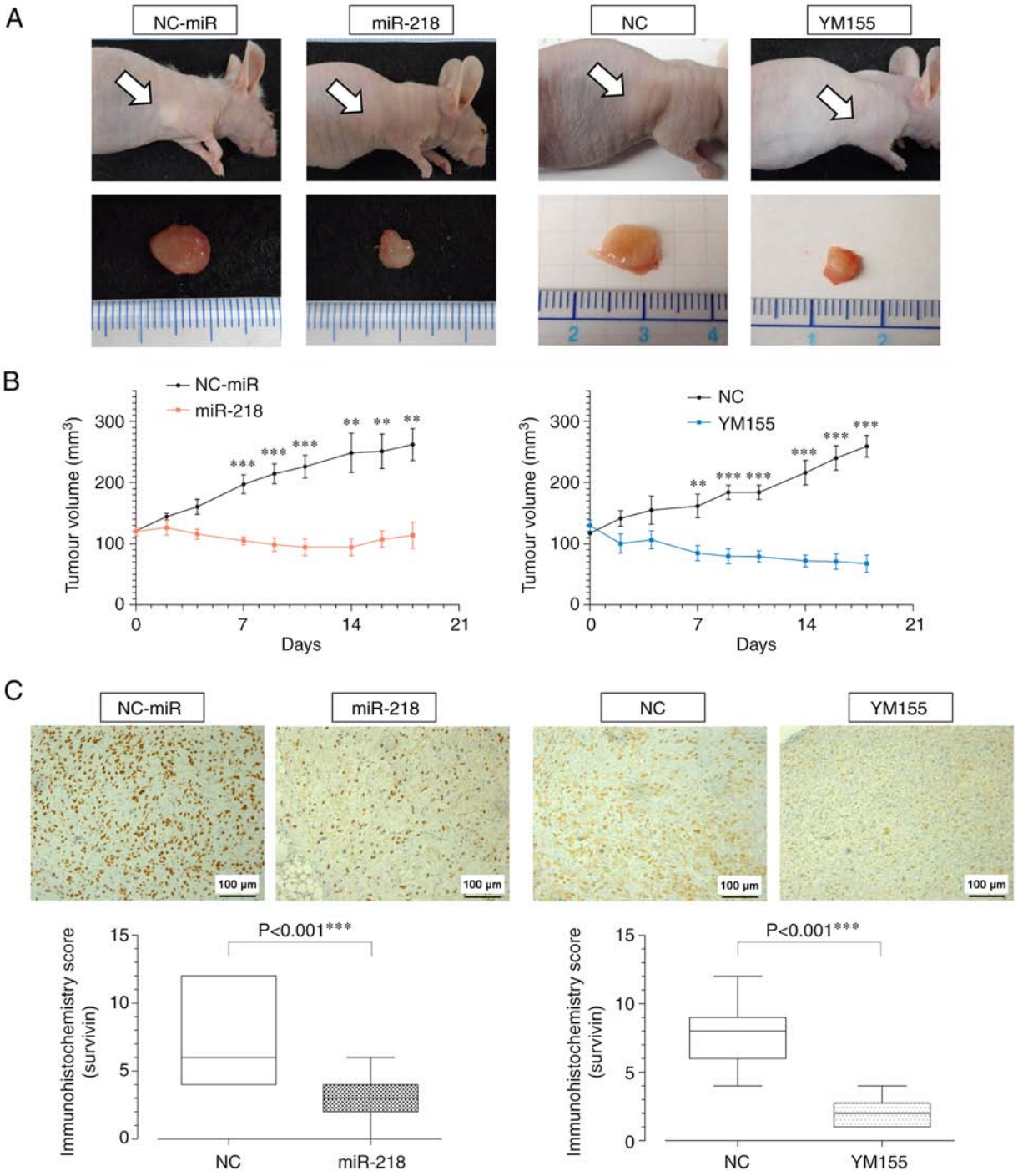

Figure 4. Tumor volume and survivin expression following miR-218 overexpression and YM155 treatment (MG63) in an immunodeficient mouse model. (A) Gross tumor at 18 days post-treatment. (B) Changes in tumor volume over time. Mixed repeated measures ANOVA with post hoc Bonferroni-corrected t-test. (miR-218: 7, 9, 11 days, P<0.001, 14 days, P=0.002, 16 days, P=0.002, 18 days, P=0.002, days; YM155: 7 days, P=0.009, 9, 11, 14, 16, 18 days, P<0.001) (C) Survivin staining of tumor tissues and immunohistochemistry score. (miR-218: P<0.001, YM155: P<0.001). Scale bar=100 μm. **P<0.01, ***P<0.001. miR, microRNA.

The present study investigated the underlying cause of the suppression of invasion and migration in the miR-218 group, but not in the YM155 group, and focused on MMPs, which serve a central role in cancer metastasis, particularly MMP-2/9, which are target genes of miR-218. miR-218 and not YM155, suppressed the expression of MMP-2/9, which is in contrast with a previous study suggesting that migration and invasion were suppressed by YM155 treatment (42,43). This discrepancy may be attributed to the considerably higher concentrations of YM155 used in previous studies (42). Typically, the anti-tumor effect is proportional to the concentration and duration of administration of a drug. In clinical practice, high drug concentrations that show anti-tumor potency often cause strong side effects. Additionally, MMCs were not administered to prevent proliferation in previous studies in the assessment of migration capacity (43). Addition of MMCs can eliminate the impact of the proliferative capacity, allowing accurate assessment of migration and

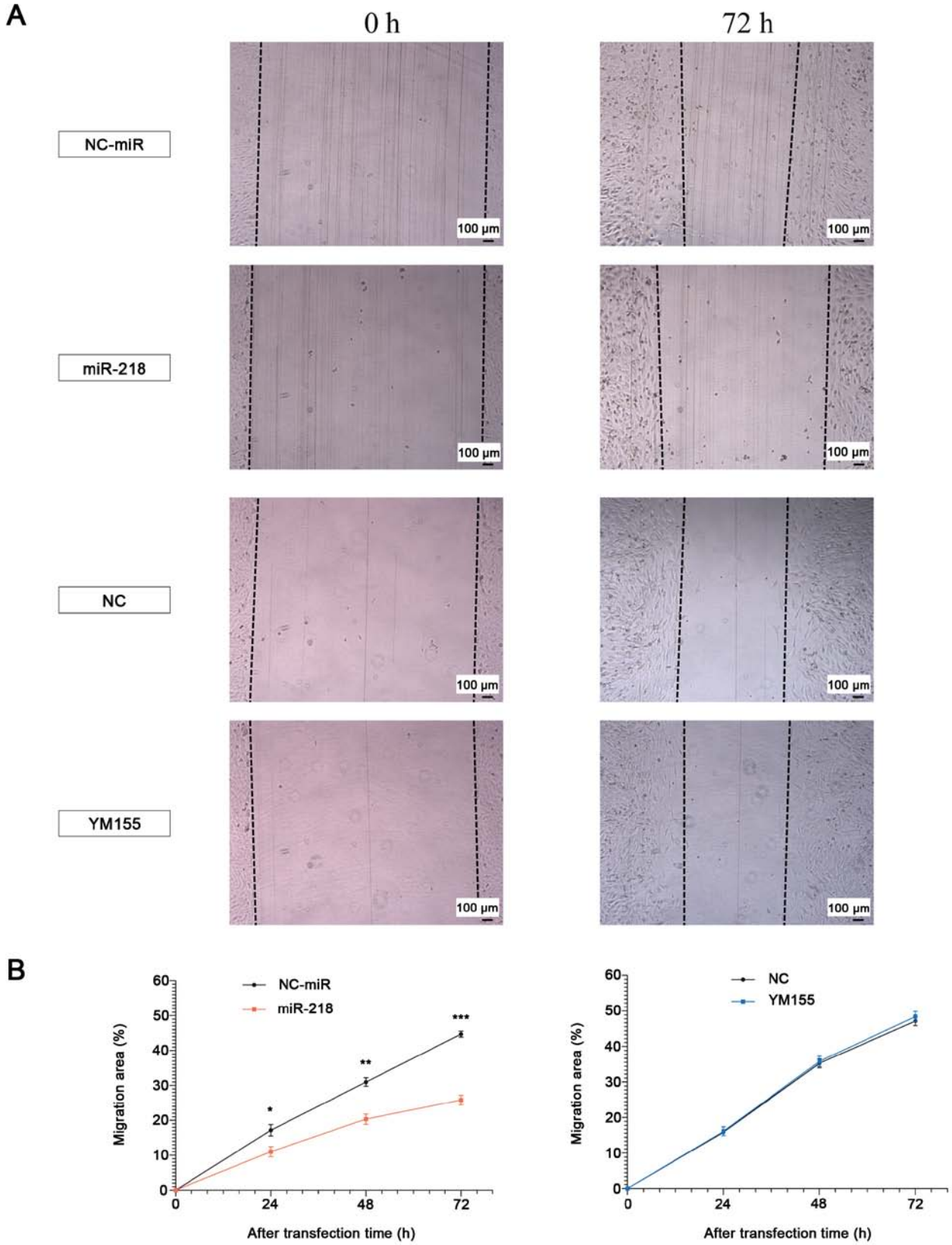

Figure 5. Migration ability following miR-218 overexpression and YM155 treatment (MG63). (A and B) Changes in migration capacity 24, 48 and 72 h post-treatment. Mixed repeated measures ANOVA with post hoc Bonferroni-corrected t-test. (miR-218: 24 h, P=0.049, 48 h, P=0.005, 72 h, P<0.001, YM155: 24 h, P=0.835, 48 h, P=0.774, 72 h, P=0.547) Scale bar: 100 μm. *P<0.05, **P<0.01, ***P<0.001. miR, microRNA.

invasion capacity (44). The present study revealed a significant difference between the miR-218 and YM155 groups in the IC_{50} for the migration and invasion potentials at the same degree of proliferative capacity inhibition migration and invasion ability. Furthermore, MMC administration minimized the effect of the proliferative capacity, demonstrating

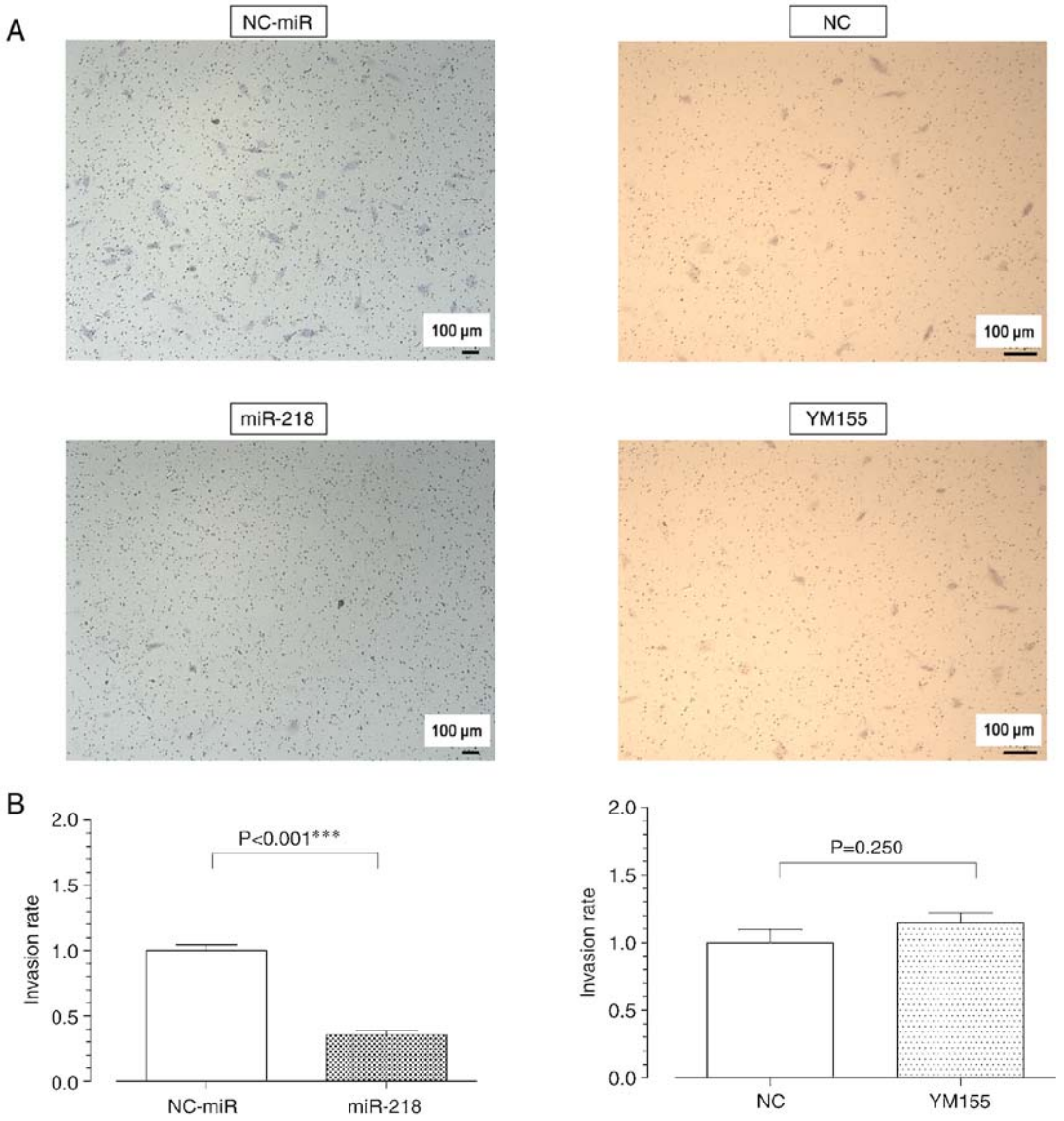

Figure 6. Invasion ability following miR-218 overexpression and YM155 treatment (MG63). (A and B) Changes in invasive capacity 72 h post-treatment. (miR-218: P<0.001, YM155: P=0.250). Scale bar=100 μm. ***P<0.001. miR, microRNA.

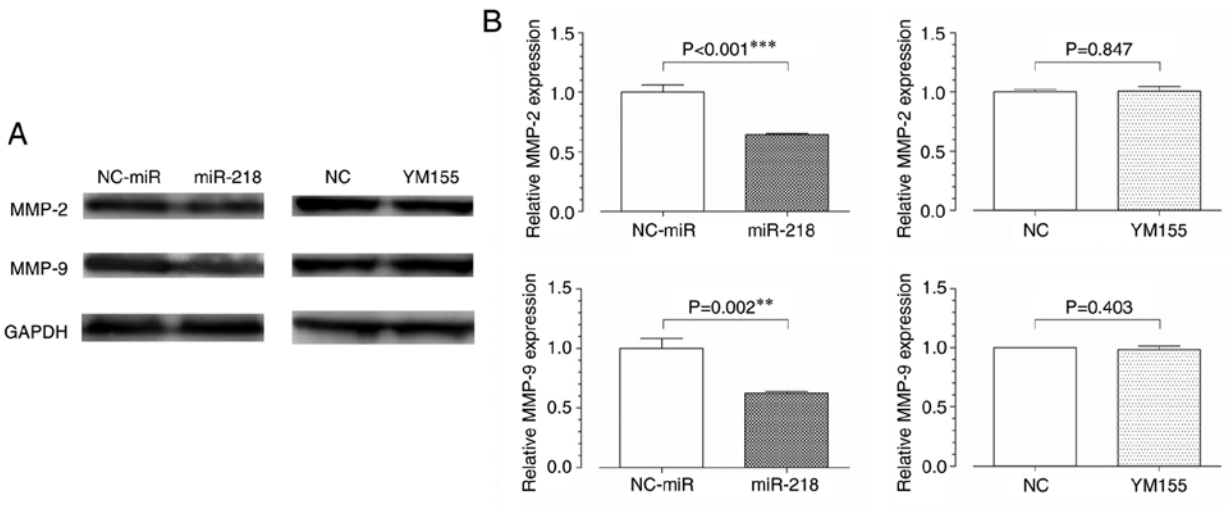

Figure 7. Analysis of proteins related to migration and invasion ability after miR-218 overexpression and YM155 treatment (MG63). (A and B) Western blotting of related proteins and semi-quantification of western blots by band concentration. (miR-218: MMP-2, P<0.001, MMP-9, P=0.002; YM155: MMP-2, P=0.847, MMP-9, P=0.403) **P<0.01, ***P<0.001. miR, microRNA.

further efficacy and potential of miRNA-targeted treatment methods.

One limitation of the present study is that it used only MG63 cells to compare the antitumor effects of miR-218 and YM155. MG63 cells form highly malignant tumors. It has been reported that osteosarcoma cell lines show characteristic labelling profiles, producing extracellular matrices with different compositions and that differences in tumor malignancy exist among different cell lines (45). Among them, MG63 cells show a higher methylation rate of WW domain-containing oxidoreductase and higher proliferative and invasive capacity compared with HOS cells (46). Additionally, MG63 cells showed high efficiency of miR-218 transfection, which was suitable for the comparative study with YM155. The amount of original miR-218 in MG63 cells was significantly lower than that in HOS cells (Fig. 1A) and the expression of miR-218 following transfection was higher in MG63 cells (Fig. 1B). As the purpose of the present study was to investigate the anti-tumor effect of miR-218 overexpression in comparative analysis with YM155 via target genes including survivin, the MG63 cell line was chosen for the present study. However, further studies in other cell lines with varying levels of malignancy might help to elucidate the role of miR-218 in the treatment of osteosarcoma. The second limitation is that the present study was not able to conduct experiments with clinical specimens of osteosarcoma. In the future, it will be necessary to verify the antitumor effects of miR-218 and YM155 in clinical specimens of osteosarcoma.

Overall, miRNAs may have broad clinical applications as therapeutic molecules, as they re-express originally intrinsic genes. Due to the non-unified genetic abnormality inherent to osteosarcoma, the antitumor effect of miR-218, which regulates more genes than molecularly targeted drugs, may be enhanced in osteosarcoma compared with other carcinomas.

Acknowledgements

The authors would like to express our deepest gratitude to the members of the Department of Orthopedic Surgery, Nihon University School of Medicine.

Funding

The present study was supported by Nihon University School of Medicine Alumni Association 60th Anniversary Fund Research Grant (2016) to Dr Eiji Osaka (Department of Orthopedic Surgery, Nihon University School of Medicine).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KS, EO, KF, TT, YT and KN proposed the concept and design of the study. KS and RF made substantial contributions to the study execution and acquisition of data. KS and EO confirm the authenticity of all the raw data. All authors critically revised the manuscript, commented on drafts and approved the final manuscript. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

The animal study was approved by the Animal Experiment Committee of Nihon University School of Medicine, Japan (approval number AP16M012).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Eaton BR, Schwarz R, Vatner R, Yeh B, Claude L, Indelicato DJ and Laack N: Osteosarcoma. Pediatr Blood Cancer 68 (Suppl 2): e28352, 2021.
2. Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, Kotz R, Salzer-Kuntschik M, Werner M, Winkelmann W, *et al*: Prognostic factors in high-grade osteosarcoma of the extremities or trunk: An analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol 20: 776-790, 2002.
3. Kempf-Bielack B, Bielack SS, Jürgens H, Branscheid D, Berdel WE, Exner GU, Göbel U, Helmke K, Jundt G, Kabisch H, *et al*: Osteosarcoma relapse after combined modality therapy: An analysis of unselected patients in the cooperative osteosarcoma study group (COSS). J Clin Oncol 23: 559-568, 2005.
4. Lee YT, Tan YJ and Oon CE: Molecular targeted therapy: Treating cancer with specificity. Eur J Pharmacol 834: 188-196, 2018.
5. Szuhai K, Cleton-Jansen AM, Hogendoorn PC and Bovée JV: Molecular pathology and its diagnostic use in bone tumors. Cancer Genet 205: 193-204, 2012.
6. Shaikh AB, Li F, Li M, He B, He X, Chen G, Guo B, Li D, Jiang F, Dang L, *et al*: Present advances and future perspectives of molecular targeted therapy for osteosarcoma. Int J Mol Sci 17: 506, 2016.
7. Liu Y, Teng Z, Wang Y, Gao P and Chen J: Prognostic significance of survivin expression in osteosarcoma patients: A meta-analysis. Med Sci Monit 21: 2877-2885, 2015.
8. Mita AC, Mita MM, Nawrocki ST and Giles FJ: Survivin: Key regulator of mitosis and apoptosis and novel target for cancer therapeutics. Clin Cancer Res 14: 5000-5005, 2008.
9. Islam A, Kageyama H, Hashizume K, Kaneko Y and Nakagawara A: Role of survivin, whose gene is mapped to 17q25, in human neuroblastoma and identification of a novel dominant-negative isoform, survivin-beta/2B. Med Pediatr Oncol 35: 550-553, 2000.
10. Kami K, Doi R, Koizumi M, Toyoda E, Mori T, Ito D, Fujimoto K, Wada M, Miyatake S and Imamura M: Survivin expression is a prognostic marker in pancreatic cancer patients. Surgery 136: 443-448, 2004.
11. Kappler M, Köhler T, Kampf C, Diestelkötter P, Würl P, Schmitz M, Bartel F, Lautenschläger C, Rieber EP, Schmidt H, *et al*: Increased survivin transcript levels: An independent negative predictor of survival in soft tissue sarcoma patients. Int J Cancer 95: 360-363, 2001.
12. Miyachi K, Sasaki K, Onodera S, Taguchi T, Nagamachi M, Kaneko H and Sunagawa M: Correlation between survivin mRNA expression and lymph node metastasis in gastric cancer. Gastric Cancer 6: 217-224, 2003.
13. Monzó M, Rosell R, Felip E, Astudillo J, Sánchez JJ, Maestre J, Martín C, Font A, Barnadas A and Abad A: A novel anti-apoptosis gene: Re-expression of survivin messenger RNA as a prognosis marker in non-small-cell lung cancers. J Clin Oncol 17: 2100-2104, 1999.

14. Sarela AI, Macadam RC, Farmery SM, Markham AF and Guillou PJ: Expression of the antiapoptosis gene, survivin, predicts death from recurrent colorectal carcinoma. Gut 46: 645-650, 2000.
15. Wang H, Xi X, Kong X, Huang G and Ge G: The expression and significance of survivin mRNA in urinary bladder carcinomas. J Cancer Res Clin Oncol 130: 487-490, 2004.
16. Osaka E, Suzuki T, Osaka S, Yoshida Y, Sugita H, Asami S, Tabata K, Sugitani M, Nemoto N and Ryu J: Survivin expression levels as independent predictors of survival for osteosarcoma patients. J Orthop Res 25: 116-121, 2007.
17. Gao JZ, Chen FH, Wang L, Wei H and Meng SL: YM155 inhibits tumor growth and enhances chemosensitivity to cisplatin in osteosarcoma. Eur Rev Med Pharmacol Sci 19: 2062-2069, 2015.
18. Clemens MR, Gladkov OA, Gartner E, Vladimirov V, Crown J, Steinberg J, Jie F and Keating A: Phase II, multicenter, open-label, randomized study of YM155 plus docetaxel as first-line treatment in patients with HER2-negative metastatic breast cancer. Breast Cancer Res Treat 149: 171-179, 2015.
19. Di Leva G, Garofalo M and Croce CM: MicroRNAs in cancer. Annu Rev Pathol 9: 287-314, 2014.
20. Tutar L, Tutar E, Özgür A and Tutar Y: Therapeutic targeting of microRNAs in cancer: Future perspectives. Drug Dev Res 76: 382-388, 2015.
21. Huang J, Lyu H, Wang J and Liu B: MicroRNA regulation and therapeutic targeting of survivin in cancer. Am J Cancer Res 5: 20-31, 2014.
22. Lu YF, Zhang L, Waye MM, Fu WM and Zhang JF: MiR-218 mediates tumorigenesis and metastasis: Perspectives and implications. Exp Cell Res 334: 173-182, 2015.
23. Tong X, Yang P, Wang K, Liu Y, Liu X, Shan X, Huang R, Zhang K and Wang J: Survivin is a prognostic indicator in glioblastoma and may be a target of microRNA-218. Oncol Lett 18: 359-367, 2019.
24. Kogo R, How C, Chaudary N, Bruce J, Shi W, Hill RP, Zahedi P, Yip KW and Liu FF: The microRNA-218~survivin axis regulates migration, invasion and lymph node metastasis in cervical cancer. Oncotarget 6: 1090-1100, 2015.
25. Zarogoulidis P, Petanidis S, Kioseoglou E, Domvri K, Anestakis D and Zarogoulidis K: MiR-205 and miR-218 expression is associated with carboplatin chemoresistance and regulation of apoptosis via Mcl-1 and survivin in lung cancer cells. Cell Signal 27: 1576-1588, 2015.
26. Dai G, Zheng D, Guo W, Yang J and Cheng AY: Cinobufagin induces apoptosis in osteosarcoma cells via the mitochondria-mediated apoptotic pathway. Cell Physiol Biochem 46: 1134-1147, 2018.
27. Xuan C, Jin M, Gao Y, Xu S, Wang L, Wang Y, Han R and An Q: miR-218 suppresses the proliferation of osteosarcoma through downregulation of E2F2. Oncol Lett 17: 571-577, 2019.
28. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
29. Deng W, Fu M, Cao Y, Cao X, Wang M, Yang Y, Qu R, Li J, Xu X and Yu J: Angelica sinensis polysaccharide nanoparticles as novel non-viral carriers for gene delivery to mesenchymal stem cells. Nanomedicine 9: 1181-1191, 2013.
30. Waller CF: Imatinib mesylate. Recent Results Cancer Res 212: 1-27, 2018.
31. Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, Gemma A, Harada M, Yoshizawa H, Kinoshita I, *et al*: Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. N Engl J Med 362: 2380-2388, 2010.
32. Kataoka K, Osaka E, Shimizu T, Okamura Y, Yoshida Y and Tokuhashi Y: Lung squamous cell carcinoma with brachial soft tissue metastasis responsive to gefitinib: Report of a rare case. Thorac Cancer 7: 676-680, 2016.
33. Pakkala S and Ramalingam SS: Personalized therapy for lung cancer: Striking a moving target. JCI Insight 3: 120858, 2018.
34. Italiano A, Mir O, Mathoulin-Pelissier S, Penel N, Piperno-Neumann S, Bompas E, Chevreau C, Duffaud F, Entz-Werlé N, Saada E, *et al*: Cabozantinib in patients with advanced Ewing sarcoma or osteosarcoma (CABONE): A multicentre, single-arm, phase 2 trial. Lancet Oncol 21: 446-455, 2020.
35. van Zandwijk N, Pavlakis N, Kao SC, Linton A, Boyer MJ, Clarke S, Huynh Y, Chrzanowska A, Fulham MJ, Bailey DL, *et al*: Safety and activity of microRNA-loaded minicells in patients with recurrent malignant pleural mesothelioma: A first-in-man, phase 1, open-label, dose-escalation study. Lancet Oncol 18: 1386-1396, 2017.
36. Janssen HL, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, Patel K, van der Meer AJ, Patick AK, Chen A, Zhou Y, *et al*: Treatment of HCV infection by targeting microRNA. N Engl J Med 368: 1685-1694, 2013.
37. Chen X, Chen XG, Hu X, Song T, Ou X, Zhang C, Zhang W and Zhang C: MiR-34a and miR-203 inhibit survivin expression to control cell proliferation and survival in human osteosarcoma cells. J Cancer 7: 1057-1065, 2016.
38. Fernández JG, Rodríguez DA, Valenzuela M, Calderon C, Urzúa U, Munroe D, Rosas C, Lemus D, Díaz N, Wright MC, *et al*: Survivin expression promotes VEGF-induced tumor angiogenesis via PI3K/Akt enhanced β-catenin/Tcf-Lef dependent transcription. Mol Cancer 13: 209, 2014.
39. Liu J, Luo B and Zhao M: Bmi-1-targeting suppresses osteosarcoma aggressiveness through the NF-κB signaling pathway. Mol Med Rep 16: 7949-7958, 2017.
40. Li YJ, Zhang W, Xia H, Zhang BS, Chen P, Zhao YL and Li J: miR-218 suppresses epithelial-to-mesenchymal transition by targeting Robo1 and Ecop in lung adenocarcinoma cells. Future Oncol 13: 2571-2582, 2017.
41. Lin CH, Ji T, Chen CF and Hoang BH: Wnt signaling in osteosarcoma. Adv Exp Med Biol 804: 33-45, 2014.
42. Zhang W, Liu Y, Li YF, Yue Y, Yang X and Peng L: Targeting of survivin pathways by YM155 inhibits cell death and invasion in oral squamous cell carcinoma cells. Cell Physiol Biochem 38: 2426-2437, 2016.
43. Zhang Z, Ma L and Wang J: YM155 exerts a growth inhibitory effect on human osteosarcoma in vitro and in vivo. Oncol Rep 34: 1074-1080, 2015.
44. Imai M, Muraki M, Takamatsu K, Saito H, Seiki M and Takahashi Y: Spontaneous transformation of human granulosa cell tumours into an aggressive phenotype: A metastasis model cell line. BMC Cancer 8: 319, 2008.
45. Pautke C, Schieker M, Tischer T, Kolk A, Neth P, Mutschler W and Milz S: Characterization of osteosarcoma cell lines MG-63, Saos-2 and U-2 OS in comparison to human osteoblasts. Anticancer Res 24: 3743-3748, 2004.
46. Liu Y, Wang Q, Yu P, Miao W, Liu C, Pu Y and Zhang C: Methylation of WWOX gene promotes proliferation of osteosarcoma cells. J BUON 25: 2708-2713, 2020.