A validation study for the utility of serum microRNA as a diagnostic and prognostic marker in patients with osteosarcoma

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Abstract. In our previous study, osteosarcoma advanced locally, and metastasis was promoted through the secretion of large number of small extracellular vesicles, followed by suppressing osteoclastogenesis via the upregulation of microRNA (miR)-146a-5p. An additional 12 miRNAs in small extracellular vesicles were also detected ≥6x as frequently in high-grade malignancy with the capacity to metastasize as in those with a low metastatic potential. However, the utility of these 13 miRNAs for determining the prognosis or diagnosis of osteosarcoma has not been validated in the clinical setting. In the present study, the utility of these miRNAs as prognostic and diagnostic markers was therefore assessed. In total, 30 patients with osteosarcoma were retrospectively reviewed, and the survival rate was compared according to the serum miRNA levels in 27 patients treated with chemotherapy and surgery. In addition, to confirm diagnostic competency for osteosarcoma, the serum miRNA levels were compared with those in patients with other bone tumors (n=112) and healthy controls (n=275). The patients with osteosarcoma with high serum levels of several miRNAs (miR-146a-5p, miR-1260a, miR-487b-3p, miR-1260b and miR-4758-3p) exhibited an improved survival rate compared with those with low levels. In particular, patients with high serum levels of miR-1260a exhibited a significantly improved overall survival rate, metastasis-free survival rate and disease-free survival rate compared with those with low levels. Thus, serum miR-1260a may potentially be a prognostic marker for patients with osteosarcoma. Moreover, patients with osteosarcoma had higher serum miR-1261 levels than those with benign or intermediate-grade bone tumors and thus may be a potential therapeutic target, in addition to being useful for differentiating whether or not a bone tumor is high-grade. A larger investigation is required to clarify the actual utility of these miRNAs in the clinical setting.

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Abbreviations: miRNA, microRNA; AJCC, American Joint Committee on Cancer; ROC, receiver operating characteristic; NCCH, National Cancer Center Hospital; NCCN, National Comprehensive Cancer Network

Key words: validation, utility, serum miRNA, osteosarcoma, diagnosis, prognosis, marker

Introduction

MicroRNAs (miRNAs) consist of 20-30 bases and play an important role in fostering communication among cells in various organisms (1). Tumor cells also affect the surrounding normal cells via miRNAs, encouraging changes in the tumor microenvironment to facilitate tumor progression (2-6). These miRNAs have been reported to be transmitted to the target cells via tumor-derived small extracellular vesicles (3-6). Thus, these miRNAs in circulating blood may be useful as prognostic or diagnostic markers of tumors (7-11).

Sarcoma is a rare cancer, and osteosarcoma typically occurs in children, adolescents and young adults (12,13). Thus far, the diagnosis of osteosarcoma has generally depended on the histological findings of a biopsy specimen. The prognosis of these patients has been reported to be associated with the

presence of metastasis at presentation, a delayed initiation of treatment, a poor response to chemotherapy and small amounts of mature osteoclasts in biopsy specimens (14-19). Compared with these factors, however, circulating biomarkers assessed using blood tests are simpler and easier to evaluate, especially in children. Previously, several serum miRNAs associated with the prognosis or diagnosis of osteosarcoma have been reported, including circulating miR-663a, miR-25-3p, miR-487a, miR-493-5p, miR-501-3p and miR-502-5p (20-25).

In our previous study, osteosarcoma advanced locally and metastasis was promoted through the secretion of large number of small extracellular vesicles, followed by the suppression of osteoclastogenesis via the upregulation of miR-146a-5p (26). Several other miRNAs were also identified (miR-1260a, miR-487b-3p, miR-6720-3p, miR-1260b, miR-4758-3p, miR-4690-3p, miR-4286, miR-6765-3p, miR-1261, miR-7975, miR-4664-5p and miR-3907) that were included in small extracellular vesicles derived from high-grade osteosarcoma cells with the capacity to metastasize at rates ≥6x than in those with low metastatic potential (26). However, to the best of our knowledge, the utility of the 13 identified miRNAs for determining the prognosis or diagnosis of high-grade osteosarcoma has not yet been validated in the clinical setting.

Therefore, in the present study, the association of these miRNAs with the prognosis of patients with osteosarcoma and their potential utility in the diagnosis of osteosarcoma and differentiation from other bone tumors was investigated.

Materials and methods

Patient enrollment. A total of 43 patients with osteosarcoma were treated at The National Cancer Center Hospital (NCCH) in Tokyo, Japan, from January 2007 to June 2013. Among these patients, 13 were excluded due to a previous history of chemotherapy or surgical treatment at another institution (n=7), metastatic lesions at presentation (n=2), the histology of dedifferentiated osteosarcoma from low-grade osteosarcoma (n=2) and absence of microarray data (n=2). The remaining 30 patients who had no previous history of treatment for the histology of primary osteosarcoma without metastatic lesions at presentation and whose microarray data were sufficiently obtained from their serum samples, were retrospectively reviewed in the present study. A total of 27 patients underwent standard treatment with the combination of chemotherapy and surgery in accordance with the National Comprehensive Cancer Network (NCCN) guidelines (27) for osteosarcoma during the follow-up period at the NCCH. In total, 3 patients did not undergo either neoadjuvant or adjuvant chemotherapy. Of these 3 patients, 1 patient was assumed to have a low-grade malignant bone tumor before surgery, while another patient did not receive adjuvant chemotherapy due to the patient's refusal to receive further treatment after surgery. The other patient opted to receive surgery and radiotherapy instead of chemotherapy.

Patients with a histological diagnosis of osteochondroma (n=15), enchondroma (n=11) and fibrous dysplasia (n=10) as benign tumors, giant cell tumors of the bone (n=28), synovial chondromatosis (n=5) and osteoblastoma (n=3) as intermediate-grade tumors, Ewing sarcoma (n=26), chordoma (n=8) and chondrosarcoma (grade 2-3) (n=6) as high-grade tumors [according to the histological grade in the 2020 WHO classification (12)], who were treated at the NCCH from January 2007 to June 2013 and whose microarray data were sufficiently obtained from their serum samples at presentation before any treatment, were also included in the study.

The present study was conducted in accordance with the 1975 Declaration of Helsinki. It was approved by The NCCH Institutional Review Board (Tokyo, Japan; approval nos. 2004-050, 2013-111 and 2015-266). Written informed consent was obtained from each participant and/or their family.

Data collection on patient and control characteristics. The age (mean and range), sex, tumor location (limb or trunk), tumor size (mean and range), staging [using the American Joint Committee on Cancer (AJCC) 8th edition] (28), surgery, chemotherapy, radiation therapy, distant metastasis, local recurrence, follow-up periods (mean and range), oncological outcomes at the final follow-up time and the levels of 13 serum miRNAs (miR-146a-5p, miR-1260a, miR-487b-3p, miR-6720-3p, miR-1260b, miR-4758-3p, miR-4690-3p, miR-4286, miR-6765-3p, miR-1261, miR-7975, miR-4664-5p and miR-3907) at the first visit were retrospectively investigated in all patients with osteosarcoma selected for study using medical records. The age (mean and range), sex and the level of the 13 serum miRNAs at the first visit in patients with other types of bone tumors (osteochondroma, enchondroma, fibrous dysplasia, giant cell tumors of the bone, synovial chondromatosis, osteoblastoma, Ewing sarcoma, chordoma and chondrosarcoma) and healthy controls were also reviewed. The 13 serum miRNA levels in all patients in the present study were used in microarray experiments in a previous study conducted at the NCCH, which are publicly available (25). A microarray analysis was not newly conducted in the present study.

miRNA mimics and inhibitor transfection. To determine the effects of miR-1261, 70 pmol of miRNA hairpin-inhibitor transfection control with Dy547 (miR hairpin inhibitor-Ctrl; cat. no. IP-004500-01-05) or hsa-miR-1261 hairpin-inhibitor (cat. no. IH-301388-01-0002) (both from Horizon Discovery Ltd.; PerkinElmer, Inc.) was transfected into 143B cells [highly aggressive, k-ras activated, human osteosarcoma cells (OS)] obtained from the ATCC using Lipofectamine™ RNAiMAX (Thermo Fisher Scientific, Inc.), and 70 pmol of miRNA mimic transfection control with Dy547 (miR mimic-Ctrl; cat. No. CP-004500-01-05) or hsa-miR-1261 mimic (cat. no. C-301388-00-0002) (both from Horizon Discovery Ltd.; PerkinElmer, Inc.) was transfected into HOS cells (non-aggressive, wild-type k-ras, human OS) obtained from the ATCC using Lipofectamine RNAiMAX. These two cell lines were cultured at 37˚C in DMEM (Nacalai Tesque, Inc.) with 10% heat inactivated FBS (Biowest) and 100 units/ml Penicillin G and 100 μg/ml Streptomycin (FUJIFILM Wako Pure Chemical Corporation) for 1 day before transfection. Both cells were cultured at 37˚C in DMEM after transfection for 2 days. To confirm the transfection efficacy of the cells, uptake of the Dy547-labelled control in each cell line was observed using a BZ-9000 phase-contrast and fluorescence microscope (Keyence Corporation) and the positive cells were counted by a BZ-II Analyzer, version 1.42 (Keyence Corporation) (Fig. S1).

Cell viability and cell proliferation assays. 143B and HOS cells were seeded at $3x10^3$ cells/200 μl/96-well (n=3) after the aforementioned miRNA transfection. After incubating for 2 days, cellular viability was evaluated using WST-8 assay reagent (Nacalai Tesque, Inc.) for an hour.

After the culture of cells ($5x10^4$ cells/500 μl/4-well glass slide) for 2 days, the cells were fixed using 4% paraformaldehyde 500 μl/well at room temperature for 20 min. Cells were then blocked using serum-free unconjugated blocking liquid (cat. no. X0909; EnVision Systems; Dako; Agilent Technologies, Inc.) at room temperature for 10 min. For immunocytochemistry, the primary anti-rabbit polyclonal antibody for Ki-67 (1:200; cat. no. NB500-170; Novus Biologicals, LLC) was incubated with the cells at 4˚C for 1 day. Anti-mouse IgG conjugated with peroxidase-labeled polymers (cat. no. K4001; EnVision Systems; Dako; Agilent Technologies, Inc.) was incubated with the cells at room temperature for 30 min as the secondary antibody. DAB-Chromogen staining was conducted for 3 min at room temperature (cat. no. GV825; EnVision Systems; Dako; Agilent Technologies, Inc.). The percentage of the Ki-67$^+$ cells per total cells was measured by phase-contrast and fluorescence microscopy using a BZ-9000 and BZ-II Analyzer version 1.42. (KEYENCE).

Reverse transcription-quantitative PCR (RT-qPCR). To detect mRNA, total RNA extraction was performed on 143B and HOS cells that had been seeded at $6x10^5$ cells/3 ml/60-mm dish after miRNA transfection and cultured at 37˚C for 2 days, using a Fast Gene RNA Basic Kit (Nippon Gene Co., Ltd.). The mRNA was reverse transcribed using 100 ng RNA and 10 μl reaction mix from a ReverTra Ace qPCR RT Kit (Toyobo Life Science) according to the manufacturer's protocol (37˚C for 15 min, 50˚C for 5 min, 98˚C for 5 min and held at 4˚C). After diluting the cDNA with 90 μl dH2O, 4 μl was incubated with 16 μl Fast SYBR Green Master Mix (Thermo Fisher Scientific, Inc.) in a LightCycler96 system (Roche Diagnostics K.K.). The thermocycler conditions were as follows: 95˚C for 20 sec, 45 cycles of 95˚C for 3 sec (denaturation) and 60˚C for 30 sec (combination of annealing and extension). At the end of the qPCR, specificity was confirmed using melting peak analysis. Paired primers are listed in Table SI. mRNA expression of each gene was normalized to GAPDH using $2^{-\Delta\Delta Cq}$ (29).

Statistical analysis. Using receiver operating characteristic (ROC) curves, the optimal threshold values for miR-146a-5p, miR-1260a, miR-487b-3p, miR-6720-3p, miR-1260b, miR-4758-3p, miR-4690-3p, miR-4286, miR-6765-3p, miR-1261, miR-7975, miR-4664-5p and miR-3907 in patients with osteosarcoma were obtained when the Youden index was maximal, from which death from disease, distant metastasis and freedom from disease were predicted. The 27 patients with osteosarcoma who underwent standard treatment with a combination of chemotherapy and surgery in accordance with the NCCN guidelines were divided into two groups based on the area under the curve values (low vs. high level) determined by ROC curves analyses. The overall survival rate, distant metastasis-free survival rate and disease-free survival rate were plotted using the Kaplan-Meier method for each group and compared by the log-rank test. P<0.05 was considered to indicate a statistically significant difference. The overall survival was defined as the period until the death of the patient since the osteosarcoma diagnosis was made. The distant metastasis-free survival was defined as the period until a distant metastasis was detected by an imaging examination. The disease-free survival was defined as the period of no evidence of disease.

For differentiation of osteosarcoma, serum miRNA levels at presentation in 30 patients with osteosarcoma were compared with those in patients with other types of bone tumors (benign, intermediate and high-grade) and healthy controls. Using the level of each miRNA (miR-146a-5p, miR-1260a, miR-487b-3p, miR-6720-3p, miR-1260b, miR-4758-3p, miR-4690-3p, miR-4286, miR-6765-3p, miR-1261, miR-7975, miR-4664-5p and miR-3907) in the 10 bone tumor groups and the osteosarcoma group, the Kruskal-Wallis test was performed to differentiate osteosarcoma and, as a post-hoc test, all 11 groups (including the healthy control group) were compared by Steel-Dwass multiple tests. P<0.05 was considered to indicate a statistically significant difference.

Cell viability using the WST-8 assay, the percentage of Ki-67$^+$ cells, and the change in miRNA expression levels for miR-1261 target genes between HOS cells transfected with hsa-miR-1261 mimic and HOS cells transfected with miRNA mimic control, or between 143B cells transfected with hsa-miR-1261 hairpin-inhibitor and 143B cells transfected with miRNA hairpin-inhibitor control were compared by unpaired Student's t-test. P<0.05 was considered to indicate statistically significant difference.

All statistical analyses were performed using EZR version 1.61 (Saitama Medical Center, Jichi Medical University), which is a graphical user interface for the R software program (The R Foundation for Statistical Computing). More precisely, it is a modified version of R commander designed to add statistical functions frequently used in biostatistics (30,31).

Results

Patient characteristics. The osteosarcoma study population included 20 male and 10 female patients with a mean age of 24 (range, 10-68) years old. The histological findings were high-grade osteosarcoma in all cases. Tumors were located in the pelvis in 3 patients and in the appendicular skeleton in 27 patients. No distant metastasis was observed at presentation in all cases. The mean tumor size was 9.7 cm in the greatest dimension (range, 6-18 cm). The tumor stage was IIA in 13 patients, IIB in 14 patients and III in 3 patients according to the AJCC 8th staging classification. In total, 27 patients underwent surgical treatment after neoadjuvant chemotherapy, and thereafter received adjuvant chemotherapy. The mean follow-up period was 108 (range, 26-182) months. Local recurrence was observed in 1 patient at 48 months after surgery, and distant metastasis was observed in 11 patients at a mean follow-up period of 23 (range, 10-37) months. In total, 6 patients died of disease at a mean follow-up period of 49 (range, 26-90) months. At the final follow-up, a total of 19 patients were free from disease (Table I).

The characteristics of the patients with other types of bone tumors were as follows: i) 8 male and 7 female patients with a mean age of 27 (range, 6-48) years old with osteochondroma;

Table I. Patient characteristics (n=30).

Patient characteristic	Value
Mean age (range), years	24 (10-68)
Sex, n	
Male	20
Female	10
Mean follow-up period (range), months	108 (26-182)
Tumor location, n	
Pelvis	3
Femur	17
Tibia	10
Mean tumor size (range), cm	9.7 (6-18)
Tumor stage, n	
IIA	13
IIB	14
III	3
Treatment, n	
Surgery	30
Chemotherapy (neoadjuvant + adjuvant)	27
Neoadjuvant	28
Doxorubicin + cisplatin + MTX	19
Doxorubicin + cisplatin + ifosfamide	9
Adjuvant	28
Radiotherapy	2
Clinical outcome, n	
Progression-free	18
Local recurrence	1
Distant metastasis	11
Oncological outcome at final follow-up, n	
DOD	6
AWD	5
Disease-free	19

MTX, methotrexate; DOD, dead of disease; AWD; alive with disease.

ii) 7 male and 4 female patients with a mean age of 42 (range, 11-75) years old with enchondroma; iii) 7 male and 3 female patients with a mean age of 31 (range, 12-68) years old with fibrous dysplasia; iv) 15 male and 13 female patients with a mean age of 35 (range, 10-67) years old with giant cell tumor of the bone; v) 3 male and 2 female patients with a mean age of 52 (range, 36-63) years old with synovial chondromatosis; vi) 1 male and 2 female patients with a mean age of 16 (range, 15-18) years old with osteoblastoma; vii) 19 male and 7 female patients with a mean age of 27 (range, 8-65) years old with Ewing sarcoma; viii) 6 male and 2 female patients with a mean age of 60 (range, 26-92) years old with chordoma; and ix) 3 male and 3 female patients with a mean age of 48 (range, 19-76) years old with chondrosaroma [Grade 2-3; graded using the WHO Classification of Tumors, Soft Tissue and Bone Tumors, 5th edition (12)]. The healthy controls were 150 male and 125 female individuals with a mean age of 52 (range, 35-80) years old.

Survival rates. The optimal cut-off points and areas under the curve of the miRNAs (miR-146a-5p, miR-1260a, miR-487b-3p, miR-6720-3p, miR-1260b, miR-4758-3p, miR-4690-3p, miR-4286, miR-6765-3p, miR-1261, miR-7975, miR-4664-5p and miR-3907) for predicting death from disease, distant metastasis or freedom from disease are shown in Table II.

A comparison of the overall survival according to miRNA levels is shown in Figs. 1A and 2. The patients with high serum levels of miR-146a-5p (P=0.04), miR-1260a (P=0.04) and miR-487b-3p (P=0.04) were significantly associated with an improved overall survival compared with those with low levels. By contrast, the serum levels of miR-6720-3p, miR-1260b, miR-4758-3p, miR-4690-3p, miR-4286, miR-6765-3p, miR-1261, miR-7975, miR-4664-5p and miR-3907 were not associated with the overall survival.

A comparison of the distant metastasis-free survival according to miRNA values is shown in Figs. 1B and 3. The patients with high serum levels of miR-1260a (P=0.04), miR-487b-3p (P=0.04), miR-1260b (P=0.03) and miR-4758-3p (P=0.04) were significantly associated with an improved distant metastasis-free survival compared with those with low levels. By contrast, the serum levels of miR-146a-5p, miR-6720-3p, miR-4690-3p, miR-4286, miR-6765-3p, miR-1261, miR-7975, miR-4664-5p and miR-3907 were not associated with the distant metastasis-free survival.

A comparison of the disease-free survival according to miRNA level is shown in Figs. 1C and 4. The patients with high serum levels of miR-1260a (P=0.04), miR-1260b (P=0.02) and miR-4758-3p (P=0.04) were significantly associated with an improved disease-free survival compared with those with low levels. By contrast, the serum levels of miR-146a-5p, miR-487b-3p, miR-6720-3p, miR-4690-3p, miR-4286, miR-6765-3p, miR-1261, miR-7975, miR-4664-5p and miR-3907 were not associated with the disease-free survival.

Differentiation from other types of bone tumors. A comparison of serum miRNAs levels in patients with bone tumors and healthy controls is shown in Figs. 1D and 5. The levels of serum miR-1261 in patients with osteosarcoma were significantly higher than those in the healthy controls (P<0.05) and patients with benign (osteochondroma, P<0.05) or intermediate-grade bone tumor (giant cell tumor of the bone, P<0.05). In addition, the levels of serum miR-1261 tended to be lower for patients with other benign bone tumors (enchondroma and fibrous dysplasia) and other intermediate-grade bone tumors (synovial chondromatosis and osteoblastoma) compared with patients with osteosarcoma, although significant differences were not observed. The serum levels of miR-1261 were not significantly associated with those in patients with other high-grade bone tumors. By contrast, the serum levels of miR-146a-5p, miR-1260a, miR-487b-3p, miR-6720-3p, miR-1260b, miR-4758-3p, miR-4690-3p, miR-4286, miR-6765-3p, miR-7975, miR-4664-5p and miR-3907 in patients with osteosarcoma were not markedly different from those in patients with benign bone tumors, intermediate-grade bone tumors or other high-grade bone tumors, although the levels were significantly higher than those in healthy controls (P<0.05).

Table II. Optimal cut-off values, AUC, sensitivity and specificity of variables to predict death from disease, distant metastasis or freedom from disease.

	Overall survival rate				Distant metastasis-free survival rate				Disease-free survival rate			
miRNA	Cut-off value	AUC	Sensitivity, %	Specificity, %	Cut-off value	AUC	Sensitivity, %	Specificity, %	Cut-off value	AUC	Sensitivity, %	Specificity, %
miR-146a-5p	6.621	0.881	100	67	6.693	0.718	80	59	6.693	0.694	80	59
miR-1260a	6.621	0.881	100	62	6.693	0.753	90	59	6.693	0.741	90	59
miR-487b-3p	6.621	0.889	100	67	6.693	0.741	90	59	6.621	0.682	70	65
miR-6720-3p	6.695	0.833	100	62	6.695	0.629	70	59	6.695	0.747	80	65
miR-1260b	7.735	0.802	100	48	6.693	0.694	60	82	6.693	0.724	60	82
miR-4758-3p	6.621	0.865	100	62	6.693	0.735	90	59	6.693	0.782	90	59
miR-4690-3p	7.849	0.633	100	40	7.346	0.510	78	59	7.346	0.438	70	56
miR-4286	7.378	0.579	100	48	7.378	0.653	90	53	7.378	0.700	90	53
miR-6765-3p	6.285	0.746	67	81	6.693	0.688	80	59	6.693	0.612	70	53
miR-1261	9.984	0.675	83	62	9.984	0.565	60	59	9.984	0.665	70	65
miR-7975	6.621	0.778	100	57	6.693	0.671	90	53	6.693	0.724	90	53
miR-4664-5p	6.019	0.762	67	81	6.621	0.612	70	59	6.019	0.659	50	82
miR-3907	6.621	0.841	100	57	6.693	0.700	90	53	6.693	0.753	90	53

AUC, area under the curve; miR, microRNA; miRNA, microRNA.

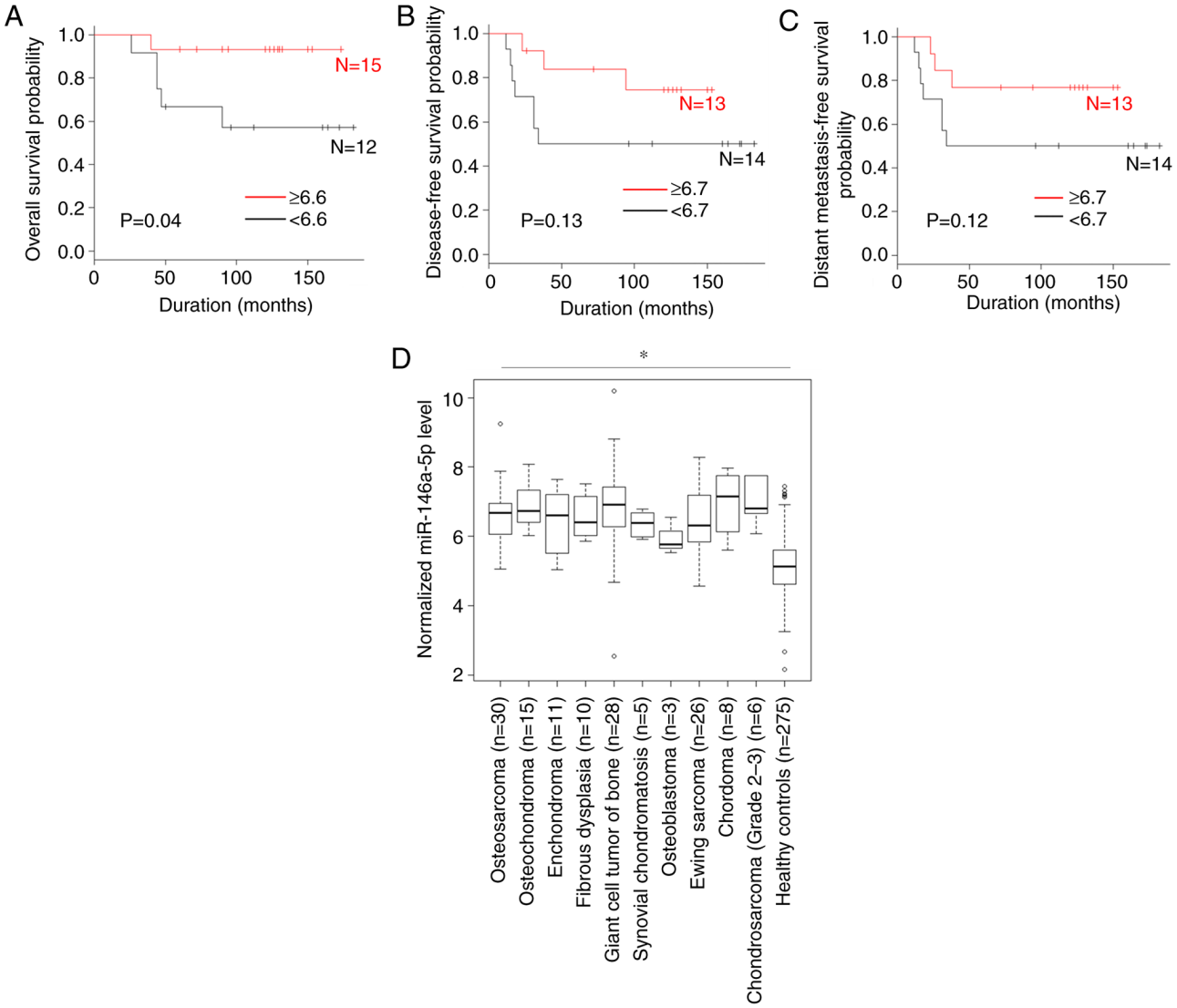

Figure 1. A comparison of the overall rate, distant metastasis-free rate and disease-free survival rate according to the levels of miR-146a-5p, and the serum levels of miR-146a-5p in patients with bone tumors and healthy controls. (A) Overall survival, (B) distant metastasis-free survival and (C) disease-free survival., (D) A comparison of serum levels of miR-146a-5p in patients with bone tumors and healthy controls. Only the level in patients with osteosarcoma was significant compared with the healthy controls. *P<0.05. miR, microRNA.

Effect of miR-1261 on cellular viability and proliferation. The inhibition of miR-1261 in 143B cells did not suppress cell viability (P=0.35; Fig. 6A), and the addition of miR-1261 to HOS cells did not promote cell viability (P=0.42; Fig. 6B). The amount of Ki-67$^+$ 143B cells appeared to decrease with the inhibition of miR-1261 (Fig. 7A-C), and the amount of Ki-67$^+$ HOS cells appeared to increase with the transfection of miR-1261 (Fig. 7D-F), although neither assay showed a statistically significant difference (P=0.11 and P=0.19, respectively).

Effect of miR-1261 on target genes. In reference to the list of predicted targets for hsa-miR-1261 (32), three genes associated with osteosarcoma were selected for screening, cadherin 6 (CDH6) (33), kinesin family member 26B (KIF26B) (34) and SET domain containing 2, histone lysine methyltransferase (SETD2) (35,36). The inhibition of miR-1261 in 143B cells significantly increased the mRNA levels of CDH6 and SETD2, but there was no significant difference in the mRNA level of KIF26B (Fig. 8A). The addition of miR-1261 mimic to HOS cells significantly decreased the mRNA levels of KIF26B and SETD2, but there was no significant difference in the mRNA levels of CDH6 (Fig. 8B).

Discussion

The present study is a retrospective observational study to validate the utility of 13 serum miRNA levels as prognostic and diagnostic markers in the clinical setting using pretreatment patient data, as was first noted in our previous basic study (26). The patients with osteosarcoma with high serum levels of several types of miRNA (miR-146a-5p, miR-1260a, miR-487b-3p, miR-1260b and miR-4758-3p) exhibited an improved survival rate compare with those with low levels. In particular, patients with high serum levels of miR-1260a exhibited an improved overall survival rate, metastasis-free survival rate and disease-free survival rate compared with those with low levels. Of further note, patients with osteosarcoma exhibited higher levels of serum miR-1261 than patients with benign or intermediate-grade bone tumors.

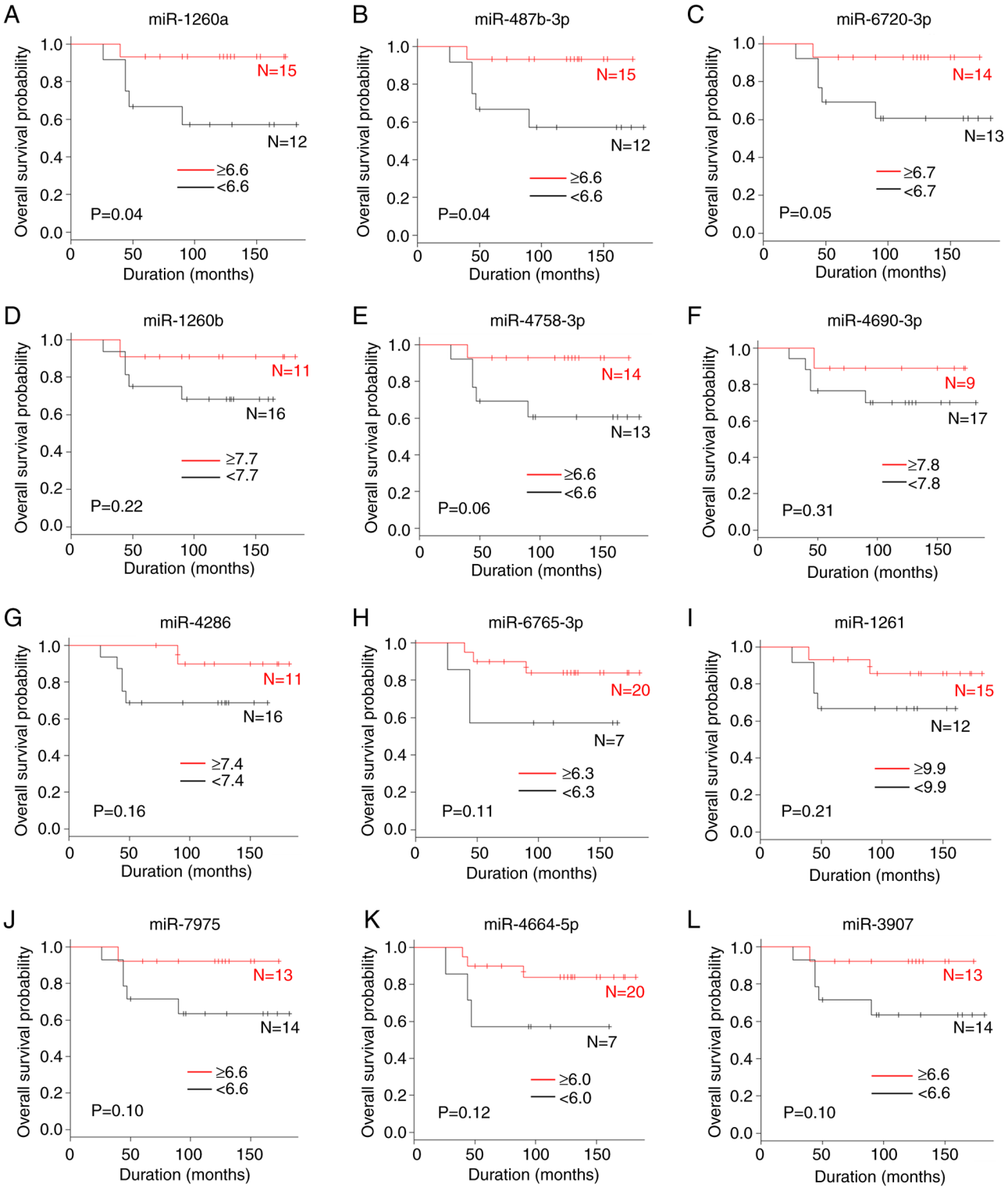

Figure 2. A comparison of the overall survival rate according to miRNA levels. (A) miR-1260a, (B) miR-487b-3p, (C) miR-6720-3p, (D) miR-1260b, (E) miR-4758-3p, (F) miR-4690-3p, (G) miR-4286, (H) miR-6765-3p, (I) miR-1261, (J) miR-7975, (K) miR-4664-5p and (L) miR-3907. miR, microRNA.

In our previous *in vivo* and *in vitro* study, highly aggressive osteosarcoma advanced locally and promoted metastasis via the secretion of large number of small extracellular vesicles (26). 143B cells, k-ras-activated human osteosarcoma cells with the capacity to metastasize, secreted greater amounts of small extracellular vesicles (exosomes) than HOS cells, which are k-ras-wild-type human osteosarcoma cells with a low metastatic potential. The tumor-derived exosomes contained various types of miRNAs, including miR-146a-5p (37,38). miR-146a-5p suppressed osteoclastogenesis in the tumor bone microenvironment, and patients with osteosarcoma with few mature osteoclasts on histology of a biopsy specimen exhibited a worse prognosis and higher rates of lung metastasis than those with numerous

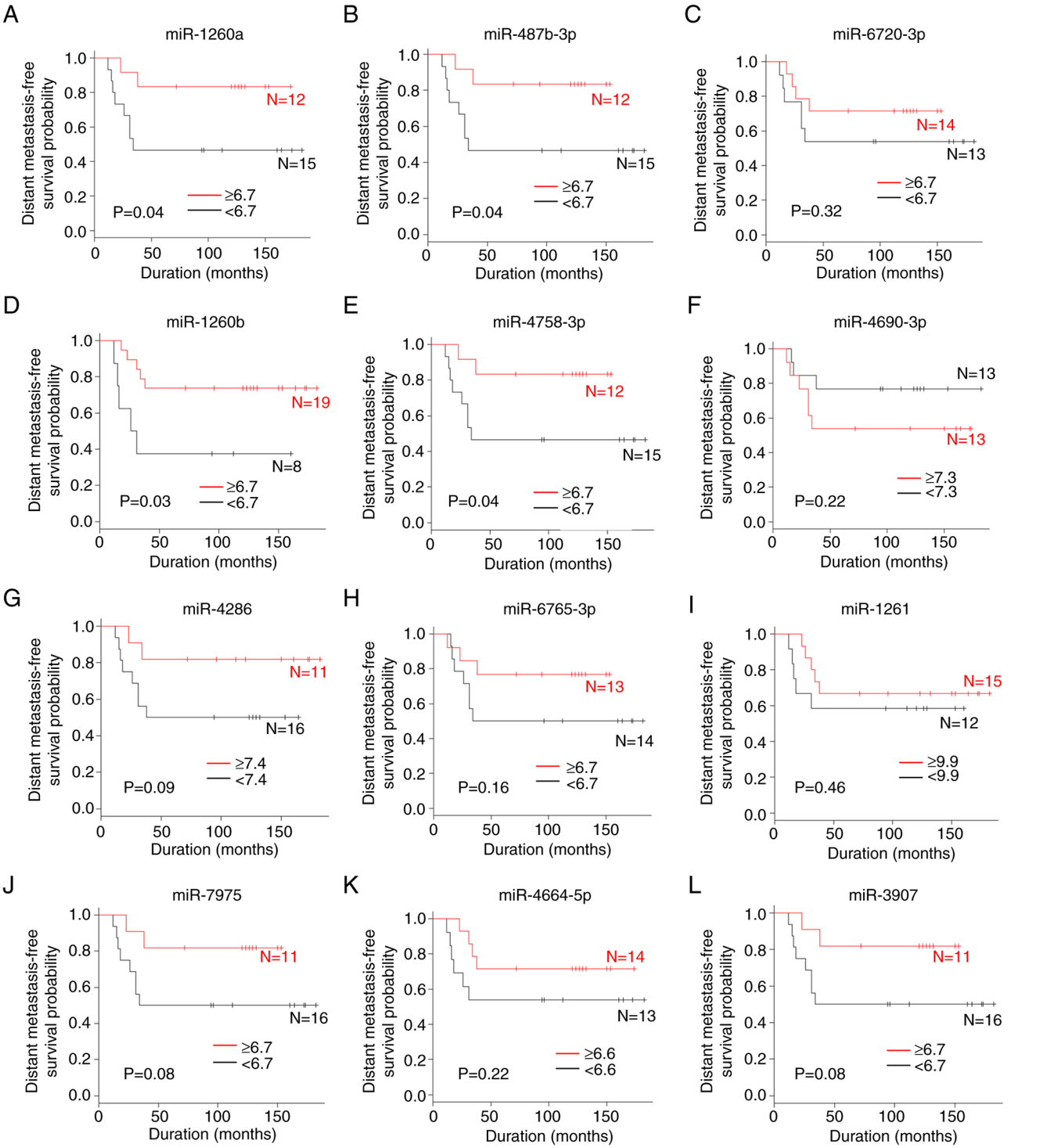

Figure 3. A comparison of the distant metastasis-free survival rate according to miRNA levels. (A) miR-1260a, (B) miR-487b-3p, (C) miR-6720-3p, (D) miR-1260b, (E) miR-4758-3p, (F) miR-4690-3p, (G) miR-4286, (H) miR-6765-3p, (I) miR-1261, (J) miR-7975, (K) miR-4664-5p and (L) miR-3907. miR, microRNA.

mature osteoclasts (19,26). Thus, a poor prognosis was expected in those patients with high levels of miR-146a-5p compared with those with low levels. The other 12 miRNAs described in the present study were also identified in the k-ras-activated 143B cells ≥6 times more frequently than in the k-ras-wildtype HOS cells in our previous study (26). Similarly, high levels of the other 12 miRNAs were expected to reflect a poor prognosis.

However, the present study demonstrated unexpectedly contrary outcomes concerning the prognosis for patients with high levels of several types of miRNAs (miR-146a-5p, miR-1260a, miR-487b-3p, miR-1260b and miR-4758-3p). For example, patients with high serum levels of these miRNAs had an improved survival rate compared with those with low levels, possibly due to the small number of patients included in the analysis, the different cut-off values of miRNAs used, the

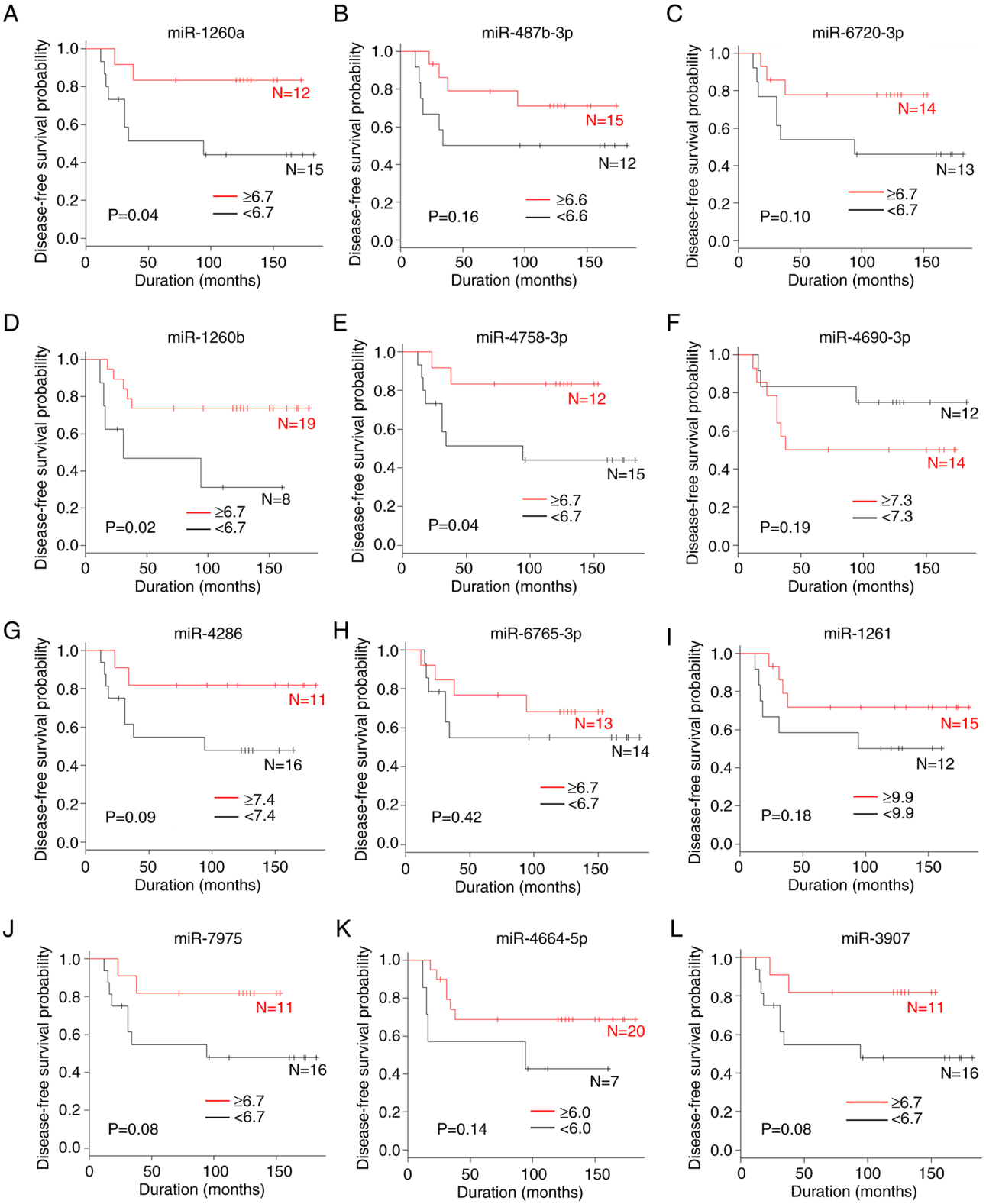

Figure 4. A comparison of the disease-free survival rate according to miRNA levels. (A) miR-1260a, (B) miR-487b-3p, (C) miR-6720-3p, (D) miR-1260b, (E) miR-4758-3p, (F) miR-4690-3p, (G) miR-4286, (H) miR-6765-3p, (I) miR-1261, (J) miR-7975, (K) miR-4664-5p and (L) miR-3907. miR, microRNA.

decomposition of miRNAs in the serum (39) or the inconsistency in miRNA levels in the serum and tumor tissues.

The upregulation of miR-1260a was previously reported to be a biomarker of lung adenocarcinoma (40). However, the serum levels of miR-1260a were shown to be decreased in ovarian and prostate cancer and increased in the postoperative state for metastatic melanoma (41-43). Furthermore, miR-1260a was also reported to improve osteogenesis and angiogenesis (44), so high serum levels of this miRNA may contribute to an improved survival rate and have potential utility as a prognostic marker for some types of cancer, including osteosarcoma. In a recent study, 143B cells had higher levels of

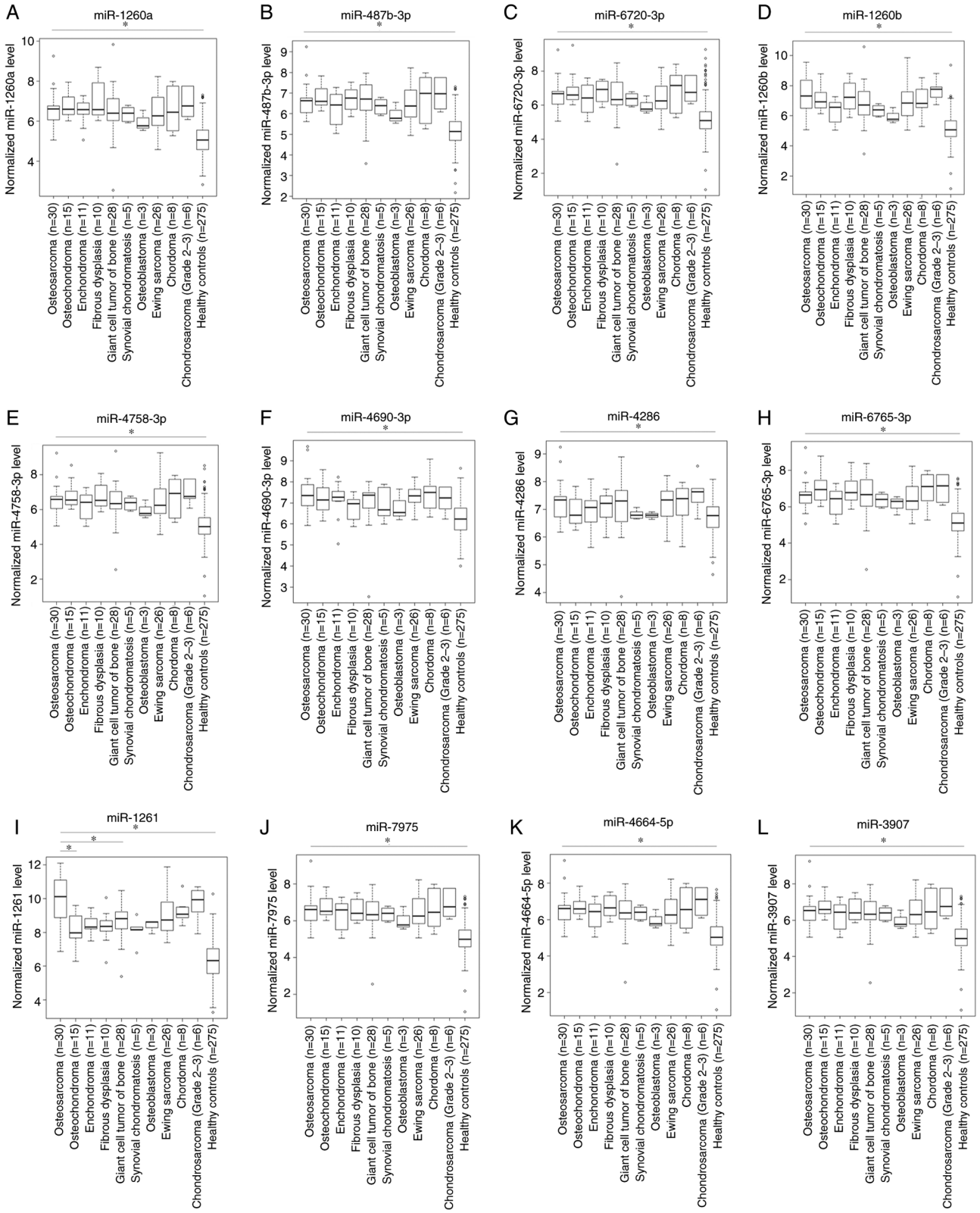

Figure 5. A comparison of serum miRNAs levels in patients with bone tumors and healthy controls. (A) miR-1260a, (B) miR-487b-3p, (C) miR-6720-3p, (D) miR-1260b, (E) miR-4758-3p, (F) miR-4690-3p, (G) miR-4286, (H) miR-6765-3p, (I) miR-1261, (J) miR-7975, (K) miR-4664-5p and (L) miR-3907. *P<0.05. miR, microRNA.

miR-1260a than HOS cells (26), however, high-grade malignant cells may also generate some miRNAs that have a health benefit, not just those that negatively influence health (45-48). Serum miR-1260a may be a potential prognostic marker for patients with osteosarcoma, although a further investigation is required to validate this.

Numerous miRNAs have been reported to be associated with the diagnosis and prognosis of patients with

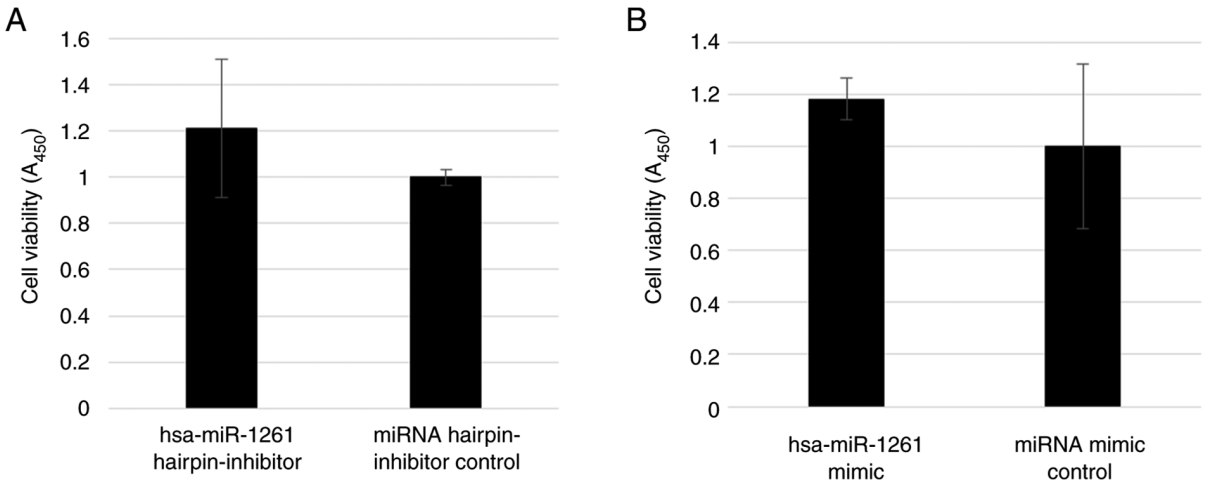

Figure 6. Cell viability. (A) 143B cells transfected with hsa-miR-1261 hairpin-inhibitor and miRNA hairpin-inhibitor control. (B) HOS cells transfected with hsa-miR-1261 mimic and miRNA mimic control. miR, microRNA.

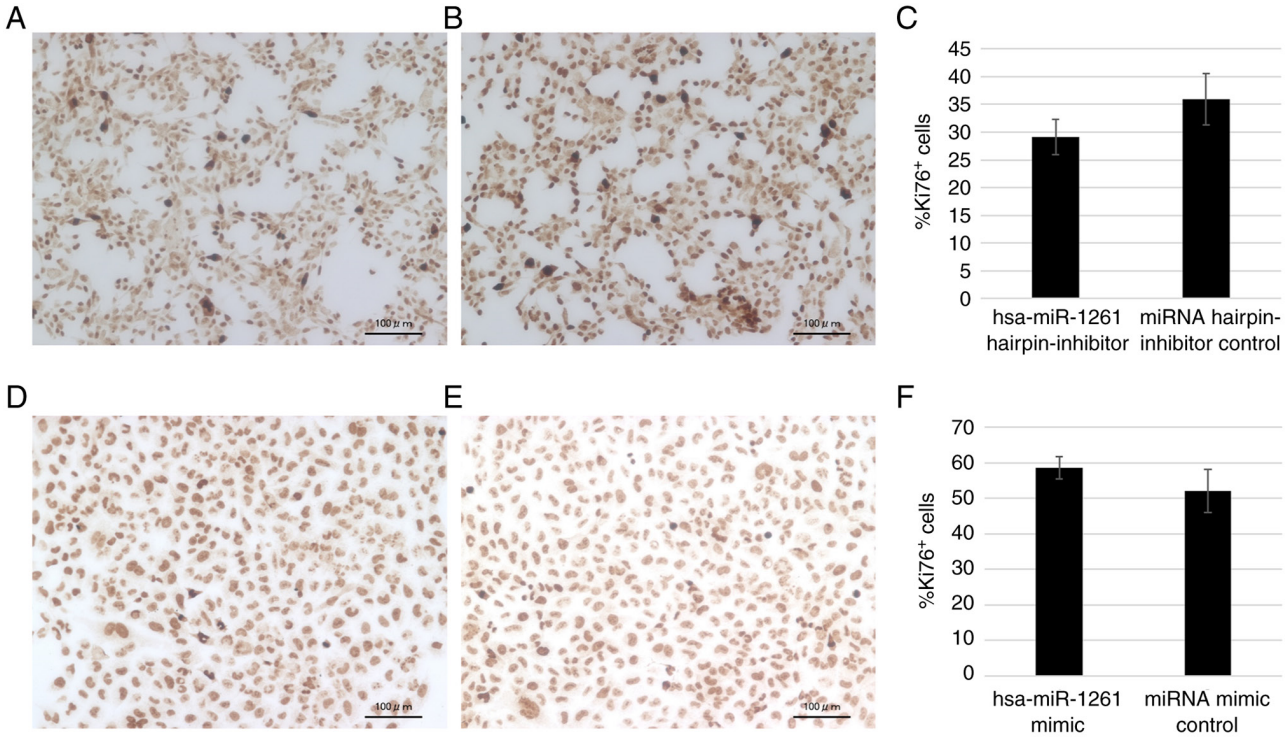

Figure 7. Immunocytochemical staining of Ki-67. (A) 143B cells transfected with hsa-miR-1261 hairpin-inhibitor. (B) 143B cells transfected with miRNA hairpin inhibitor control. (C) Comparison of the Ki-67$^+$ level between 143B cells transfected with hsa-miR-1261 hairpin-inhibitor or miRNA hairpin-inhibitor control. (D) HOS cells transfected with hsa-miR-1261 mimic. (E) HOS cells transfected with miRNA mimic control. (F) Comparison of the Ki-67$^+$ level between HOS cells transfected with hsa-miR-1261 mimic or miRNA mimic control. miR, microRNA.

cancer, but very few miRNAs are clinically used as specific markers for the disease (49). One reason for this might be due to the diverse functions of the miRNAs, depending on the disease. miR-487b-3p improves chemoresistance or metastasis in osteosarcoma but impairs osteoblastgenesis, and promotes tumorigenesis in colon cancer, prostate cancer and glioma (50-54). miR-1260b promotes tumorigenesis in breast cancer and lung adenocarcinoma and also inhibits bone loss (55-57). miR-4758-3p promotes tumorigenesis in glial tumor, gastric cancer and endometrial tumors (58-60), but its association with osteosarcoma is unknown.

In the human body, normal cells located around tumor cells might absorb some tumor-derived miRNAs that negatively influence cell survival (45). As a result of this, only a small amount of the tumor-derived miRNAs that exist in the stromal tissue enter the bloodstream. Some miRNAs have also been shown to be secreted by normal cells, including inflammatory cells, in response to the presence of tumor cells (46-48). Thus, identifying the origin of serum miRNAs might be difficult, and further investigations are required to confirm the utility of the 13 miRNAs analyzed in the present study as prognostic markers in the clinical setting.

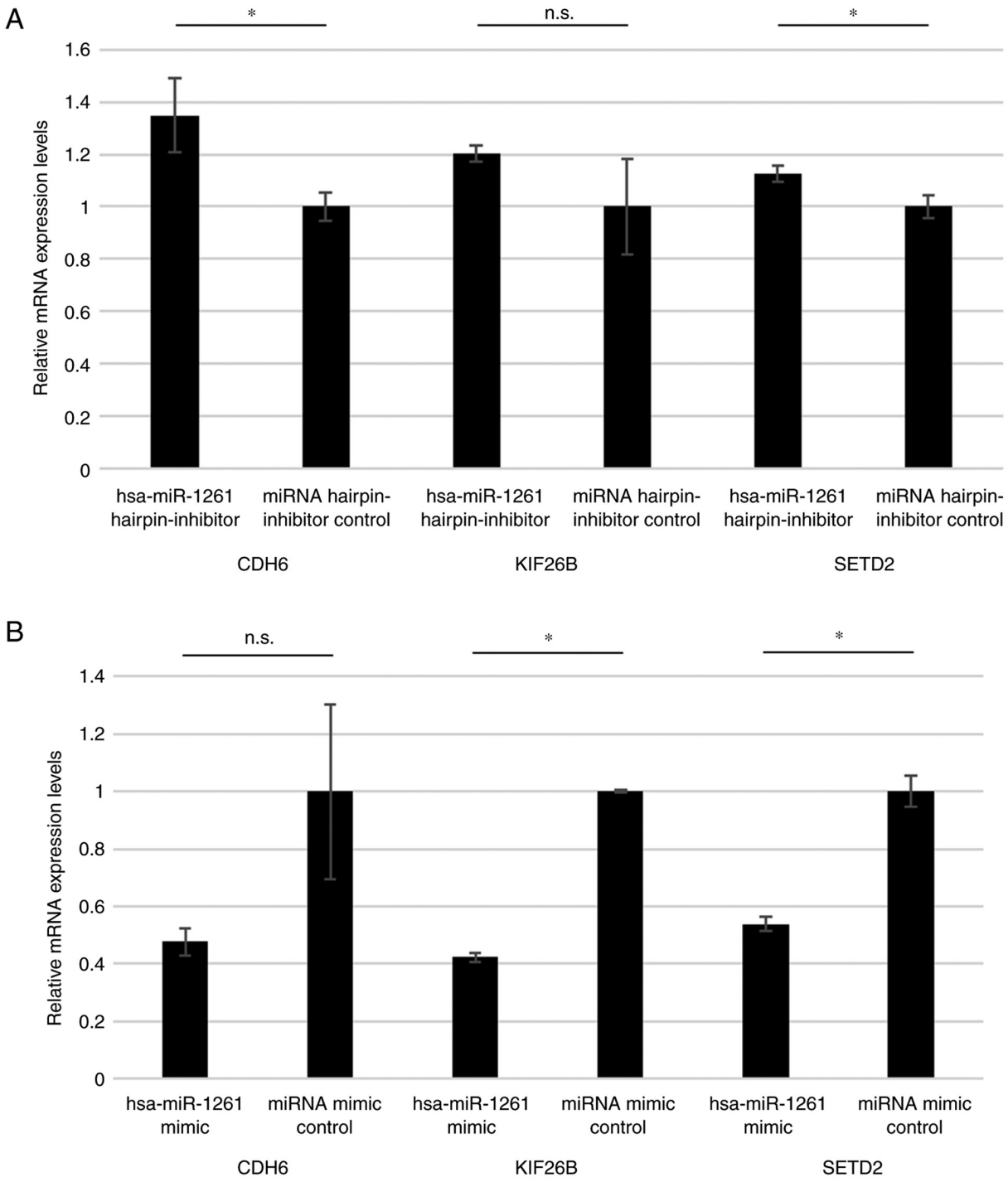

Figure 8. Reverse transcription-quantitative PCR of miR-1261 target genes. (A) mRNA levels of CDH6, KIF26B and SETD2 after the addition of has-miR-1261 hairpin-inhibitor and miRNA hairpin-inhibitor control in 143B cells. (B) mRNA levels of CDH6, KIF26B and SETD2 after the addition of has-miR-1261 mimic and miRNA mimic control in HOS cells. *P<0.05. CDH6, cadherin 6; KIF26B, kinesin family member 26B; miR, microRNA; n.s., no significant difference; SETD2, SET domain containing 2, histone lysine methyltransferase. *P<0.05. miR, microRNA.; n.s., no significant difference.

miR-1261 has been reported to be associated with migration and invasion of prostate cancer, progression of lung adenocarcinoma and as a diagnostic marker of hepatocellular carcinoma (61-64). However, its association with osteosarcoma is unclear. In the present study, the serum levels of miR-1261 in patients with osteosarcoma were significantly higher than those in healthy controls, patients with benign bone tumors and patients with intermediate-grade bone tumors, although there were no significant differences between patients with osteosarcoma and those of patients with other high-grade bone tumors. The presence of histological high-grade malignancies, including osteosarcoma, might be reflected by the high serum levels of miR-1261 in patients with bone tumors. In the present study, *in vitro* experiments were performed to investigate the effect of miR-1261 on cellular viability and proliferation. Following the transfection of HOS cells with an miR-1261 mimic and 143B cells with an miR-1261 hairpin-inhibitor, the cell viabilities were similar, but cell proliferation appeared to

be associated with miR-1261. Furthermore, the mRNA levels of CDH6, KIF26B and SETD2 [target genes of miR-1261 (32)] increased by inhibiting miR-1261 and, by contrast, these target genes decreased upon the addition of an miR-1261 mimic. The SETD2 gene has been described as a tumor suppressor in various types of cancers, including renal, gastric and lung cancer (65,66). However, there are only a few studies associated with osteosarcoma, and the involvement of miR-1261 and SETD2 has not yet been previously described (35,36,65,66). Thus, miR-1261 is potentially a novel therapeutic target in addition to being useful for differentiating whether a bone tumor is high-grade, although a further investigation is necessary.

Several limitations associated with the present study warrant mention. Firstly, this was a retrospective, small-scale, single-institution study. Secondly, the peripheral blood findings were not compared between the preoperative and postoperative periods. Changes in the levels of miRNA after surgery may indicate tumor-derived miRNAs (21-24), but, in the present study, the postoperative blood findings for miRNAs were not investigated as the timing of surgery differed among patients. Thirdly, the characteristics concerning age and sex in healthy patients and patients with other types of bone tumors were not compared with those in patients with osteosarcoma since there was only a small number of patients, and also because the predominance of age and sex depends on the histological type of bone tumors (12).

In conclusion, regarding the 13 serum miRNAs evaluated in the present study, serum miR-1260a may be a potential prognostic marker for patients with osteosarcoma. Patients with osteosarcoma had higher levels of serum miR-1261 than those with benign or intermediate-grade bone tumors, which may be a potential novel therapeutic target in addition to being useful for differentiating whether or not a bone tumor is high-grade. A larger investigation is required to clarify the utility of these miRNAs in the clinical setting.

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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

HT, NY, AK, NA and YAr conceived and designed the study. YAr carried out data acquisition. AK and NA provided assistance for data acquisition. AK and NA managed the patients for the appropriate treatment and observed them at the follow-up outpatient clinic. YAr and TY performed the *in vitro* experiment. YAr, TY, RH, KH, AT, SMi, KI, TH, KA, HY, SMo and YAs analyzed and interpretated all the patient's data. HT, NY, JM, TO and YT contributed to the analysis of the data and critical appraisal. YAr, TY, NA and NY confirm the authenticity of all the raw data. YAr wrote the manuscript. KH, AT, SMi, KI, TH, KA, HY, SMo and YAs supervised the analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients underwent standard treatment during the follow-up period at The National Cancer Center Hospital in Tokyo, Japan. The present study was conducted in accordance with the 1975 Declaration of Helsinki. It was approved by The NCCH Institutional Review Board (Tokyo, Japan; approval nos. 2004-050, 2013-111 and 2015-266). Written informed consent was obtained from each participant and/or their family.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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