Changes in microRNA expression in the MG-63 osteosarcoma cell line compared with osteoblasts

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Abstract. Osteosarcoma (OS) is the most common primary malignant bone tumor, particularly in adolescents and young adults. Early diagnosis remains a significant problem in the clinical treatment of OS as we remain far from a comprehensive understanding of the molecular genetic mechanisms and the biology involved. In addition, microRNAs (miRNAs or miRs), a large family of small non-coding RNAs, may provide a greater understanding of OS as they play a complex role in gene expression regulation *in vitro* and *in vivo*. In the current study, the differential expression profiles of miRNAs between OS and osteoblast cell lines were investigated by miRNA microarrays and real-time quantitative PCR (RT-qPCR). A total of 268 miRNAs were identified that were significantly dysregulated in OS compared with the osteoblast cell line, including miR-9, miR-99, miR-195, miR-148a and miR-181a, which had been validated as overexpressed, and miR-143, miR-145, miR-335 and miR-539, which were confirmed to be downregulated. This differential expression may aid future OS diagnosis and prognosis prediction and illustration of the potential mechanisms in the oncogenesis, development and metastasis of OS. Bioinformatic research on these differentially expressed miRNAs suggests that they are able to regulate the biological behaviors of OS in a complex and effective manner. Further study on the function of these miRNAs is likely to provide new insights into OS biology and treatment.

Introduction

Although it accounts for less than 0.5% of all types of cancer, osteosarcoma (OS) is the most frequent primary malignancy of the bone and occurs mainly in adolescents and young adults (1). The initiation of combinational chemotherapy with aggressive surgical resection has markedly improved the prognosis of OS patients during the last few decades (2). However, the current neoadjuvant chemotherapy outcome for OS remains unsatisfactory in the presence of metastases (3-5). Despite the various efforts of basic research and clinical practice, the molecular genetic mechanisms and the biology involved in OS remain poorly understood. A greater understanding of OS is essential for developing novel approaches to increase survival rates (3).

As a large family of naturally occurring small non-coding RNAs, microRNAs (miRNAs or miRs) employ a post-transcriptional gene regulation mechanism that is involved in numerous cellular processes, playing a role in development regulation, differentiation, cell proliferation, differentiation, apoptosis, cell cycle and tumorigenesis (6). Previous studies have shown that miRNAs may play complex regulatory roles by binding to the 3' untranslated region of mRNAs; a single miRNA affects the expression of hundreds of protein-coding target genes, while a protein-coding target gene is regulated by a variety of miRNAs (7).

There is growing evidence that the aberrant expression of specific miRNAs is correlated with various human tumors, including breast cancer, hepatocellular carcinoma, leukemia and colon cancer (8,9). It has been reported that miRNAs regulate cancer cell apoptosis, cell cycle arrest, migration and invasion. The alteration of specific miRNAs may lead to various responses to the chemotherapy and several miRNAs have been demonstrated to participate in the development of tumor metastasis (10).

The current study was designed to investigate the differential expression profiles of miRs between an OS and osteoblast cell line. miRNA expression levels were determined using bead-based array performing oligonucleotide capture probes specific for miRNAs, which is feasible and attractive for its high speed and heightened accuracy. In this study, the differential miRNAs were explored through screening 1,146 mature miRNAs between the MG-63 and hFOB1.19 (HOB) cell lines and the expression of selected miRNAs was confirmed using real-time quantitative PCR (RT-qPCR) in these two cell lines. The tumor function-associated targeted mRNAs of selected miRNAs by bioinformatics and previous literature were also investigated. These findings provide insights into the role of miRNAs in OS.

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Materials and methods

Cell lines and reagents. Human OS MG-63 and osteoblast HOB cell lines were obtained from the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). MG-63 cells were cultured in MEM/EBSS (Hyclone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone), 50 U/ml penicillin and 50 mg/ml strep-tomycin in a humidified incubator with 5% CO_2 at 37˚C. The HOB cells were maintained in the same conditions, except that DMEM/F12 (v/v: 1:1, Hyclone) supplemented with 10% FBS and 0.3 mg/ml G418 (Sigma, St. Louis, MO, USA) was used.

RNA extraction. Total RNA was extracted from each cell line using an miRNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. This effectively recovered mRNA and miRNA. RNA concentration and quality were measured using the spectrophotometer (ND-2000, NanoDrop, Wilmington, DE, USA).

miRNA expression profiling by Illumina miRNA microassay. The Illumina® TotalPrep™ RNA amplification kit (Ambion, Austin, TX, USA) was used in cDNA synthesis and purifica-tion with 200 ng total RNA from each treated cell, followed by hybridizing on Human MicroRNA Expression profiling v2 panels (Illumina, San Diego, CA, USA) according to the manufacturer's instructions (Illumina MicroRNA Expression Profiling Assay Guide) and the method described previ-ously (11). The Human v2 MicroRNA Expression Profiling kit contains 1,146 assays for detecting >97% of the miRNAs described in the miRBase database, plus additional novel content derived using Illumina sequencing technology.

Array data processing and analysis were performed with Illumina BeadStudio software (www.illumina.com). The microRNA expression array was scanned and extracted using BeadScan, with the data corrected by background subtraction in the GenomeStudio module. The array intensity data were imported into BeadStudio v3.2 (Illumina), a software package that permits visualization and normalization of the data. The 'Average' normalization method was used for all analyses reported, with the exception of assay reproducibility, given the number of replicates. The normalized intensities and detec-tion P-values were exported and further analyzed using the R environment (version 2.6), in combination with Bio-conductor packages (12).

miRNAs were considered significantly differentially expressed if the P-values were <0.05 and the fold change ratio (FCR) was >2.

RT-qPCR of specific miRNAs. Validation of differential gene expression was performed for selected miRNAs, including miR-181a, miR-148a, miR-99a, miR-195, miR-9, miR-335, miR-143, miR-145 and miR-539. These miRNAs were amplified using the Bulge-Loop™ miRNA qRT-PCR Primer Set (Ribobio, Guangzhou, China) (13). The thermal profile for the RT-qPCR was at 95˚C for 1 min, followed by 40 cycles of 95˚C for 10 sec, 60˚C for 20 sec and 72˚C for 5 sec on a Bio-Rad CFX96 RT-qPCR system (Bio-Rad, Hercules, CA, USA). All qPCR reactions, including no-template controls, were performed in triplicate. Expression levels of each miRNA were evaluated using a comparative threshold cycle (Ct) method normalized to that of U6. The fold changes of each miRNA were calculated from the expression levels in the MG-63 and HOB cell lines.

Bioinformatics analysis. The TarBase 6.0 database (http:// diana.cslab.ece.ntua.gr/) was used to investigate the validated target genes in cancer research (14). Moreover, following the collection of all the validated genes, the function of these genes was analyzed by previous literature and bioinformatics research. The chromosome location of these miRNAs and their target genes was investigated to exclude the potential bias of sex chromosomes and illustrate the complex and comprehensive mechanisms in miRNA regulation of the target gene expression.

Results

miRNA expression in the MG-63 and HOB cell lines. The fold changes (MG-63/HOB) were auto-analyzed by software (BeadStudio v3.2, Illumina). Of the 1,146 miRNAs detected in the microarray, 159 miRNAs were shown to be decreased and 109 miRNAs as increased. The various miRNAs were selected for further analysis as follows: i) the minimum value should be >100 in the two cell lines to eliminate the background value; ii) the fold changes should be >5 for improved accuracy. As Fig. 1 shows, 46 miRNAs were selected as differentially expressed between the MG-63 and HOB cell lines, of which 26 were underexpressed and 20 were overexpressed. The fold change was mainly <10, while several miRNAs in MG-63 cells were markedly changed compared with the HOB cell line, including miR-335, miR-493, miR-494, miR-195 and miR-9.

Validation of miRNAs in the MG-63 and HOB cell lines. The RT-qPCR was employed to validate the differential expression of selected miRNAs. As Fig. 2 shows, it was revealed that 9 specific miRNAs were differentially expressed between the OS MG-63 and HOB cell lines and the differences are consistent with the microarray results shown. Therefore, it was demonstrated that these are the differentially expressed miRNAs in OS MG-63 compared with the HOB cell line.

Bioinformatics research on these differential miRNAs and their target genes. Tables I and II show the target genes involved in the biological behavior of cancer and validated by previous literature. The functions of these target genes are complex as they are correlated with various cellular processes, including cell proliferation, differentiation, cell cycle, apop-tosis, signaling, migration and invasion. Moreover, miRNAs may regulate the expression and function of their target genes although they are located on various chromosomes.

Discussion

The significance of miRNAs in the regulation of cellular processes has been increasingly noted (43). Up- and/or downregulation of miRNA expression in cancer suggests that miRNAs function as classical tumor suppressor genes or oncogenes (6-8). The expression fold changes of several miRNAs may aid in tumor stratification and clinical outcome prognosis (44,45). Previous research shows that specific miRNA expression may be correlated with cancer recur-

Table I. Validated target genes of the miRNAs in the increased group.

| miRNA	Location	Validated targets (ref.)	Location	Functions in cancer
miR-195	17p13.1	CDK6 (15)	7q21-q22	Cell cycle and arrest
		E2F3 (15)	6p22	Cell cycle and arrest
		CCND1 (15)	11q13	Cell cycle and arrest
		VEGFA (16)	6p12	Angiogenesis and metastasis regulation
		Bcl-2 (17)	18q21.3	Apoptosis regulation
		SKI (18)	1q22-q24	Proto-oncogene
		BCL2L11 (18)	2q13	Apoptosis regulation
		CDK4 (19)	12q14	Cell cycle and arrest
miR-99a	21q21.1	MTOR (20)	1p36.2	Response to anti-cancer drugs
		FGFR3 (20)	4p16.3	Mitogenesis and differentiation
		SKI (21)	1q22-q24	Proto-oncogene
		IGF1 (22)	12q23.2	Anti-apoptosis
miR-9	1q22	NF-KB1 (23)	4q24	Transcription regulation
		CDH1 (24)	16q22.1	Metastasis regulation
		VEGFA (24)	6p12	Angiogenesis and metastasis regulation
		VIM (24)	10p13	Cell attachment, migration and signaling
		MMP13 (24)	11q22.3	Invasion and metastasis regulation
		BIK (25)	22q13.31	Apoptosis regulation
miR-148a	7p15.2	CDC25B (26)	20p13	Cell cycle and arrest
		PTPN4 (21)	2q14.2	Cell growth, differentiation, mitotic cycle and oncogenic transformation
		CDK19 (21)	6q21	Cell cycle and arrest
		ROCK1 (27)	18q11.1	Invasion and metastasis
miR-181a	1q32.1	CDKN1B (28)	12p13.1-p12	Cell cycle and arrest
		Bcl-2 (29)	18q21.3	Apoptosis regulation
		Hras (30)	11p15.5	Signal transduction
		CDX2 (31)	13q12.3	Cell growth and differentiation
		S100A1 (31)	1q21	Cell cycle and differentiation
		KLF6 (32)	10p15	Tumor suppressor

miR, miRNA, microRNA.
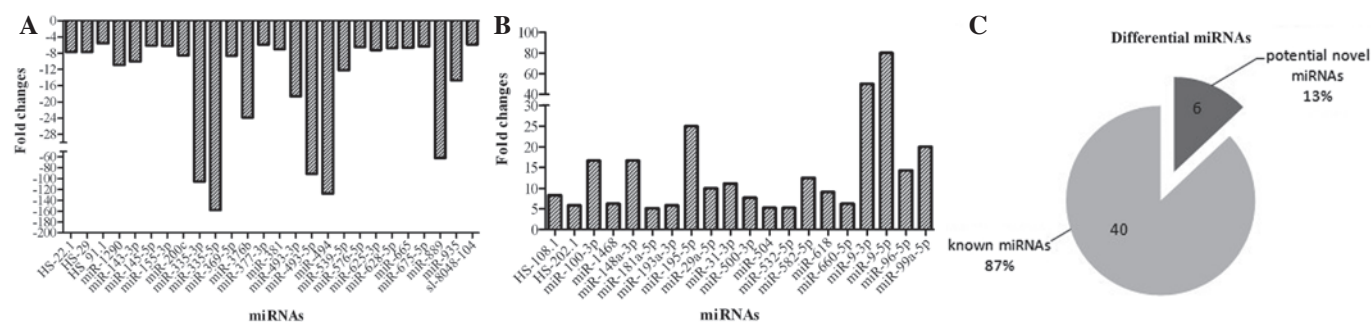
Figure 1. Alterations of miRNA expression in the OS MG-63 cell line compared with the osteoblast hFOB1.19 by microarray analysis. (A) There are 26 miRNAs in the decreased group. Negative values indicate downregulation in the MG-63 cell line compared with the hFOB1.19 cell line. (B) There are 20 miRNAs in the increased group. (C) These 46 miRNAs are identified as the differentially expressed miRNAs in OS compared with the osteoblast cell line. Of these, 40 miRNAs are known miRNAs in the miRBase, while the remaining 6 miRNAs are potentially novel miRNAs altered in OS. This suggests that there are new miRNAs involved in the carcinogenesis and development of OS. OS, osteosarcoma. miR, miRNA, microRNA.

rence. Therefore, the distinct difference between normal and abnormal cells may be correlated with the early diagnosis and treatment of the primary cancer or its recurrence.

Although there are several studies concerning specific miRNAs as the key biomarkers for the diagnosis, treatment and evaluation of the chemoresponse or chemoprevention in

Table II. Validated target genes of the miRNAs in the decreased group.

| miRNA	Location	Validated targets (ref.)	Location	Functions in cancer
miR-145	5q32	FSCN1 (33)	7p22	Cell migration, motility, adhesion and cellular interactions
		MMP1 (34)	11q22.3	Invasion and metastasis regulation
		MMP12 (34)	11q22.3	Invasion and metastasis regulation
		MMP14 (34)	14q11-q12	Invasion and metastasis regulation
		TP53 (35)	17p13.1	Proliferation and apoptosis regulation
miR-143	5q32	MAPK7 (36)	17p11.2	Proliferation, differentiation, transcription regulation
		MMP13 (37)	11q22.3	Invasion and metastasis regulation
		Bcl-2 (38)	18q21.3	Apoptosis regulation
		Hras (39)	11p15.5	Signal transduction
		TP53 (35)	17p13.1	Proliferation and apoptosis regulation
miR-335	7q32.2	SP1 (40)	12q13.1	Cell proliferation, differentiation, apoptosis regulation
		IGF1R (40)	15q26.3	Anti-apoptosis
		BRCA1 (40)	17q21	Tumor suppressor
		BIK (41)	22q13.31	Apoptosis regulation
		SMAD3 (41)	15q22.33	Carcinogenesis
		SMAD9 (41)	2q26	Carcinogenesis
		PML (41)	15q22	Tumor suppressor
miR-539	14q32.31	MITF (42)	3p14.2-p14.1	Cell proliferation

miR, miRNA, microRNA.
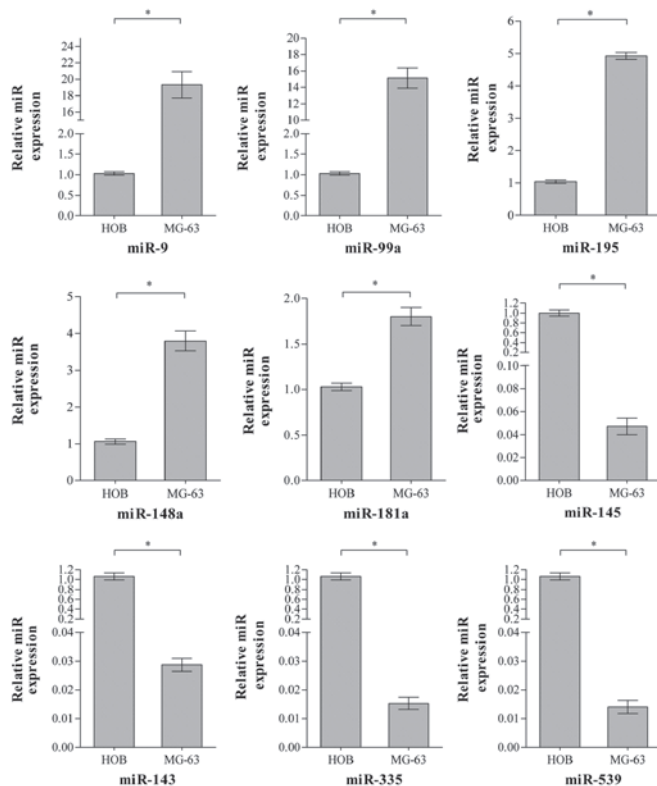
Figure 2. Validation of the selected miRNAs by real-time PCR. Differences of these 9 miRNAs between the OS and osteoblast cell lines are consistent with results of the microarray analysis. $2^{-\Delta\Delta CT}$ is used to analyze the relative expression of miRNAs in MG-63 compared with hFOB 1.19 cells. The CT value is the fractional cycle number at which the fluorescence passes the fixed threshold. All data are expressed as mean ± SEM of three independent experiments. *$P<0.05$. OS, osteosarcoma; HOB, hFOB1.19 cell line; miR, miRNA, microRNA.

cancer therapy (46,47), little is known about miRNA profiling and its signature in OS. The explosion of microarray technology has led to its wide application in miRNA expression analysis. The miRNA microarray was employed to detect the miRNA profiling in the OS and HOB cell lines, respectively, and the real-time PCR was employed to validate the miRNA of interest or marked differences between these two cell lines. In the current study, 1,146 miRNAs were detected by the microarray, revealing 159 miRNAs as being part of the decreased group and 109 miRNAs as the increased group. Following further analysis of the miRNA microarray result, 46 miRNAs were selected as the differentially expressed miRNAs between the MG-63 and HOB cell lines.

Furthermore, based on previous research and the potential biological targets predicted by the various databases, including Targetscan and PicTar, 9 miRNAs were selected to validate their expression and demonstrate the difference between the two cell lines. The stem-loop RT-PCR method described in the present study is designed to detect and analyze mature miRNAs in a fast, specific, accurate and reliable manner (48). Therefore, RT-qPCR was employed to validate the expression of specific miRNAs of interest. Fig. 2 shows the expression of the selected 9 miRNAs quantified in the MG-63 and HOB cell lines. The differences of these miRNAs between the two cell lines are consistent with the microarray results shown. Therefore, these 9 miRNAs are accepted as the differentially expressed miRNAs between the MG-63 and HOB cell lines.

It is well known that the miRNAs involved in complex cancer-related cellular processes by regulating the various target mRNA expression and miRNAs are thought to be components of vast regulatory networks (49,50). Previous research

has confirmed that multiple miRNAs target the same gene, suggesting that the correlation between miRNAs and target genes are complex and interactive (51). Therefore, analysis of the target genes of these differential miRNAs may reveal their functions as oncogenes or anti-oncogenes. Furthermore, this analysis is likely to aid the fuller understanding of the biological function of specific miRNAs through analysis of their target genes and vice versa (52-54).

The validated target genes of these 9 miRNAs were obtained from bioinformatics research. Tables I and II show the validated target genes which have been demonstrated by previous research to be involved in various cellular process in cancer biology, including proliferation, differentiation, cell cycle, apoptosis, signaling, migration and invasion. These target genes are located on different chromosomes, suggesting that miRNAs may regulate the expression and function of mRNA although they are located on various chromosomes. These 9 miRNAs may play a significant role in the biological behavior of cancer, although they alter the target gene expression in different directions (7).

A total of 9 miRNAs have been reported that may act as biomarkers in the diagnosis, treatment and prediction of the prognosis of cancer. Previous evidence has demonstrated that miR-143 and miR-145, that belong to the same miRNA cluster, regulate the expression and function of various target genes. It is well known that the underexpression of the miR-143/145 cluster, the expression of which was decreased in OS in the current study, are strongly associated with carcinogenesis in various tumor types, suggesting that they may act as significant tumor suppressors (37). As Tables I and II demonstrate, tumor protein p53(TP53), fascin homolog 1(FSCN1), several matrix metallopeptidases (MMPs) and mitogen-activated protein kinases (MAPKs), which are regulated by the miR-143/145 cluster, may be involved in cancer cell proliferation, differentiation, gene transcription, apoptosis, migration and invasion (55). The similar effect of miR-143 and/or miR-145 has been demonstrated in OS cell lines by previous research (56). Therefore, we may conclude that the aberrant expression levels of the miR-143/145 clusters are correlated with the carcinogenesis and development of OS compared with the HOB cell line.

miR-9 is another of the widely-researched miRNAs in cancer biology (23-25). It has been shown that miR-9 is overexpressed in various tumor types, particularly in tumors with micro-metastasis. The validated target genes (NF-KB1, CDH1, VEGFA, VIM, MMP13 and BIK) shown in Table I suggest that miR-9 may act as a significant regulatory miRNA, which was also increased in OS in the current study compared with the HOB cell line.

A previous study demonstrated that 6 other miRNAs that were validated in the current study are also involved in the biology of cancer. Of these, miR-99a may play a significant role in cell growth and correlates with the prognosis of patients with specific tumors (57,58). The aberrant expression of miR-195 in certain types of cancer may be an effective biomarker in diagnosis (59,60). The differential expression of miR-148a has been reported to be a potential marker for colorectal cancer screening and prognosis (61). miR-181a, which has been recently demonstrated to be overexpressed miRNA in OS tissue, is correlated with cancer development, apoptosis evasion and cell proliferation (62). With regard to the reduced expression group, miR-335,

which is also underexpressed in certain types of cancer, has also been demonstrated to be significant as a biomarker of metastatic tumor and maintain differentiation (63-65). Recently, it has been demonstrated that miR-539 may inhibit cell proliferation through suppressing the MITF expression (42). However, the functional study of the 6 miRNAs in the OS cell line are limited and ongoing research concerning their function may illustrate the further mechanism of these miRNAs in OS oncogenesis, development and metastasis.

In conclusion, the aberrant expression levels of specific miRNAs, including miR-9, miR-99a, miR-195, miR-148a, miR-181a, miR-143, miR-145, miR-335 and miR-539, may act as potential biomarkers in the diagnosis, treatment and prognosis prediction of OS. Further research on the function of their target genes may provide new insights into the biology and treatment of OS.

Acknowledgements

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