Identification and validation of microRNAs and their targets expressed in osteosarcoma

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Abstract. Osteosarcoma (OS) is the most common type of bone cancer in children and adolescents, and has a poor prognosis. Previous studies have demonstrated that a number of microRNAs (miRNAs) were deregulated in OS, and that the expression of certain miRNAs was correlated with the stage of OS. Therefore, miRNAs may serve a role as a diagnostic and prognostic biomarker of OS. miRNA and mRNA integrated analysis of public expression profiles in the Gene Expression Omnibus database for OS was performed, and the regulated targets of miRNA in OS were predicted. Next, the regulatory network of miRNAs/genes was constructed and verified by reverse transcription-quantitative polymerase chain reaction in tissues and MG-63 cell lines. Two miRNA expression profiling studies and four eligible mRNA expression profiling studies were selected. Ten upregulated miRNAs, 5 downregulated miRNAs and 5 DGEs were identified in OS compared with normal tissues. hsa-miR-346 was inversely correlated with the target gene c-FLIP, which was consistent with the results of integrated analysis. *In vitro*, pre-miRNA-346 can downregulate the protein expression of c-FLIP, while not changing the mRNA level of c-FLIP. In the regulatory network, hsa-miR-346 and its target gene, c-FLIP, can be used as biomarkers for an earlier diagnosis of OS.

Introduction

Osteosarcoma (OS) is the most common type of bone cancer encountered in children and adolescents (1). The incidence rates of OS for all races and sexes are 4/1,000,000/year for individuals aged 0-14 years and 5/1,000,000/year for individuals aged 0-19 years (2). This tumor is highly aggressive and metastasizes primarily to lungs. It occurs in the metaphyseal regions of the proximal humerus, proximal tibia and distal femur with a male predominance. At present, the pathogenesis of OS remains unclear. Due to the early occurrence of distant metastasis and insensitivity to chemotherapy, the 5-year survival rate of the patients was only 50-60%. Therefore, it is important to investigate novel treatments for OS. Numerous studies have indicated that miRNA serves an important role in the occurrence, development, invasion and metastasis of OS (3,4). miRNA has been developed as a biomarker for OS (5-7).

MicroRNA (miRNA) is a type of small non-coding RNA, containing 18-21 nucleotides. It can regulate gene expression at the post-transcriptional level by base pairing to the complementary sequences in the 3'-untranslated region (UTR) of the target mRNA. miRNAs occur, not only in the cell, but also in the serum, plasma, saliva, urine, milk and other body fluids or secretions (8). Ma *et al* (9) detected the expression levels of circulating miR-148a by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and revealed that expression of circulating miR-148a in the peripheral blood have clinical potential as a novel diagnostic biomarker for OS (9). Previous studies have revealed that in the OS tissues, the expression levels of certain miRNAs increased while the expression levels of certain miRNAs decreased. miR-27a functions as an oncogene by targeting MAP2K4 in the OS MG63 cell line. Inhibition of miR-27a can inhibit the proliferation and migration of MG63 cells (10). However, the expression levels of miR-145 and miR-32 were significantly lower in the OS tissues than in the adjacent normal tissues (11,12). Overexpression of miR-145 or miR-32 can inhibit the proliferation of human OS cells. Therefore, research into the expression of miRNAs in OS is required.

The development of gene expression profiles may aid in elucidating the function of miRNAs in OS. Hu *et al* (4) identified 268 miRNAs that were significantly dysregulated in OS by miRNA microarrays and RT-qPCR. Among these miRNAs, miR-9, miR-99, miR-195, miR-148a and miR-181a were overexpressed, whereas miR-143, miR-145, miR-335 and miR-539 were downregulated (4). In another miRNA microarray of 19 human OS cell lines, the expression levels of miR-9, miR-21, miR-31, miR-196a/b, miR-374a, miR-29 and miR-130 were increased, while the expression levels of miR-126, miR-486-5p, miR-150, miR-142-3p, miR-223, miR-144, miR-1, miR-195 and miR-206 were decreased (13). Collectively, these altered miRNAs may function as valuable diagnostic and predictive tools for OS,

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but the complexity of OS, difference in sample datasets and diversity of analysis methods serve as limitations, as the expression levels of certain miRNAs in different studies are inconsistent. Therefore, it is necessary to identify certain specific miRNAs as biomarkers for the diagnosis of OS. This study presented an integrative strategy for identifying OS-associated miRNAs/mRNAs by analyzing miRNA and mRNA expression profiles from the Gene Expression Omnibus database (GEO). To the best of our knowledge, the present study predicted differentially expressed miRNA target genes by intersecting the datasets from the GEO database, and constructed an miRNA-targets regulatory network. Certain important miRNAs would be used as biomarkers for the diagnosis, prediction and prognosis of therapeutic response.

Materials and methods

Search strategy. The NCBI GEO database (available at http://www.ncbi.nlm.nih.gov/geo) was carefully searched until January 8, 2016, to identify relevant data, including four mRNA expression profiling (GSE70414, GSE42572, GSE56001 and GSE36001) and two microRNA expression profiling (GSE28424 and GSE70415) datasets. Two distinct sets of key words were used simultaneously, namely 'osteosarcoma' and 'Homo sapiens'.

Data were used if they met the following criteria: i) They studied the patients with expression profiles or non-coding RNA profiles by array; ii) the selected dataset included genome-wide mRNA transcriptome data and miRNA expression data; iii) these data came from biopsy tissues or cultured cells of patients with OS and a control group; iv) standardization and original datasets were considered. A total of 4 sets of mRNA datasets and 2 sets of miRNA datasets were incorporated into the present study following selection.

Screening differentially expressed miRNAs and mRNAs. Following background correction and normalization of raw data, the differentially expressed miRNAs and mRNAs between OS and controls were identified by Student's t-tests using the Limma package in R (Bioconductor 3; http://www.bioconductor.org) (14). Next, the χ^2 test was used to combine P-values of multiple studies. The random effects model was used to calculate effects from multiple studies. The criterion of selection of differently expressed miRNAs and mRNAs was FDR <0.01.

Targets of miRNA. The targeted genes for human miRNA were downloaded from the miRTarBase database (http://mirtarbase.mbc.nctu.edu.tw/), and the transcriptional targets of the identified miRNAs in OS were predicted. Since the miRNAs and the mRNAs targeted by miRNA exhibit an inverse expression association, in the present study, the miRNA and the mRNAs that exhibited inverse expression correlations with each other were subjected to further study.

Gene annotation. The Database for Annotation, Visualization and Integrated Discovery (DAVID; https://david.ncifcrf.gov/) (15) is the most efficient and commonly used tool to analyze gene functional enrichment. To fully understand the biological functions of miRNA target genes, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed (http://www.genome.ad.jp/kegg/). P<0.05 was considered to indicate a statistically significant difference.

Human OS tissue samples and the MG-63 cell line. Five frozen OS tissue samples and 5 normal bone samples from individuals of similar age groups were obtained from Xiangya Hospital of Central South University (Changsha, China). The mean ages of the OS patients and normal patients were 55±5 and 58±6, years, respectively. All patients were male. The excised primary OS tumors were obtained prior to the initiation of chemotherapy or radiotherapy following the receipt of written informed consent and approval from the Institutional Review Board of Xiangya Hospital of Central South University. The MG-63 cell line was cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with streptomycin (100 μg/ml) and penicillin (100 U/ml) (Life Technologies; Thermo Fisher Scientific, Inc.).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols, and reverse transcribed using a Transgen reverse transcription kit (Beijing Transgen Biotech Co., Ltd., Beijing, China). The resultant cDNA was used for subsequent qPCR analysis. qPCR was performed in a ABI7500 Real-Time PCR system with a PCR Master mix (SYBRGreen) reagent kit (Beijing Transgen Biotech Co., Ltd.). The thermal cycling conditions were as follows: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min, annealing at 55˚C for 30 sec and elongation at 72˚C for 3 min. The results were analyzed using the $2^{-\Delta\Delta Cq}$ method (16). GAPDH was used as an internal control gene and each reaction was performed in triplicate. The primers used in the RT-qPCR are listed in Tables I and II.

Western blotting. Total proteins were isolated using an active protein extraction kit (KGP1050, Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). A BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used to determine the protein concentration. A total of 30 μg protein per lane was separated using 10% SDS-PAGE, transferred onto polyvinylidene difluoride membranes and then blocked with 5% fat-free milk at room temperature for 2 h. Membranes were then incubated with primary antibodies detecting c-FLIP (ab8421; 1:1,000 dilution; Abcam, Cambridge, MA) and GAPDH (ab8226; 1:2,000 dilution; Abcam, Cambridge, MA) at 4˚C overnight. Following two washes with Tris-buffered saline with Tween 20 (0.5%) (TBS-T), the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG (1:5,000; ZB-2306 or ZB-2304; OriGene Technologies, Inc., Beijing, China) for 2 h at room temperature and then washed two times with TBS-T. Proteins were detected using enhanced chemiluminescence RapidStep™ ECL, according to the manufacturer's protocol

Table I. Primers of the genes.

Gene symbol	Forward (5'-3')	Reverse (5'-3')
ESR1	CCCACTCAACAGCGTGTCTC	CGTCGATTATCTGAATTTGGCCT
BTK	TCTGAAGCGATCCCAACAGAA	TGCACGGTCAAGAGAAACAGG
Foxp1	ATGATGCAAGAATCTGGGACTG	AGCTGGTTGTTTGTCATTCCTC
c-FLIP	TCAAGGAGCAGGGACAAGTTA	GACAATGGGCATAGGGTGTTATC
MAOA	GAATCAAGAGAAGGCGAGTATCG	GGCAGCAGATAGTCCTGAAATG
EGR1	GGTCAGTGGCCTAGTGAGC	GTGCCGCTGAGTAAATGGGA
GAS1	ATGCCGCACCGTCATTGAG	TCATCGTAGTAGTCGTCCAGG
TSC22D3	AACACCGAAATGTATCAGACCC	TGTCCAGCTTAACGGAAACCA
MEOX2	GCACCCGTTCTCCCAATCC	TCCCGCGATTATGCAAGATGA
SLC2A3	GCTGGGCATCGTTGTTGGA	GCACTTTGTAGGATAGCAGGAAG
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

Table II. Primers of the miRNAs.

miRNA	Stem loop primer (5'-3')	Forward (5'-3')	Reverse (5'-3')
hsa-miR-22	GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACTAAAGC	GAGTCTTCAGTGGCAA	GTGCAGGGTCCGAGGT
hsa-miR-134	GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACCCCCTC	GGATGTGACTGGTTGACC	GTGCAGGGTCCGAGGT
hsa-miR-493	GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACAATGTT	GGGTTGTACATGGTAGGCT	GTGCAGGGTCCGAGGT
hsa-miR-346	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACAGAGGC	GGGTGTCTGCCCGCATGCCT	GTGCAGGGTCCGAGGT
hsa-miR-494	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACAGAGAA	GGGAGGTTGTCCGTGTTGTC	GTGCAGGGTCCGAGGT
hsa-miR-541	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACAGTGGG	GGGAAAGGATTCTGCTGTCGGT	GTGCAGGGTCCGAGGT
hsa-miR-182	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACAGTGTG	GGGTTTGGCAATGGTAGAACT	GTGCAGGGTCCGAGGT
hsa-miR-183	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACAGTGAA	GGATATGGCACTGGTAGAA	GTGCAGGGTCCGAGGT
hsa-miR-301a	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACAGTAGT	GGAGCTCTGACTTTATTGC	GTGCAGGGTCCGAGGT
hsa-miR-596	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACCCCGAG	GGGAAGCCTGCCCGGCTC	GTGCAGGGTCCGAGGT
U6	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACAAAATG	GCGCGTCGTGAAGCGTTC	GTGCAGGGTCCGAGGT

miR/miRNA, microRNA.

(cat. no. 345818; Merck KGaA). ImageJ 1.8.0 (National Institutes of Health, Bethesda, MD, USA) was applied to quantify the relative protein levels.

Statistical analysis. Data are presented as the mean ± standard deviation. The statistical analysis was performed using Student's t-test or analysis of variance (ANOVA) in SPSS13.0 (SPSS, Inc., Chicago, IL, USA). Bonferroni's post-hoc test was used when $P<0.05$ by one-way ANOVA. All experiments were repeated ≥3 times, and representative experiments are shown.

Results

Collecting differentially expressed miRNAs and mRNAs in OS. A total of two miRNA expression profiling studies and

Table III. Characteristics of mRNA expression profiling in osteosarcoma.

GEO accession	Platform	Sample	Samples (N:C)	Country	Year	First author	(Refs.)
GSE70414	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	Cell line	1:5	Japan	2015	Kawano *et al*	(17)
GSE42572	GPL13376 Illumina HumanWG-6 v2.0 expression beadchip	MSC from donor	5:7	USA	2015	Buddingh *et al*	(18)
GSE56001	GPL10558 Illumina HumanHT-12 V4.0 expression beadchip	Primary human osteosarcoma cells	3:3	Taiwan	2014	Wang *et al*	(19)
GSE36001	GPL6102 Illumina human-6 v2.0 expression beadchip	Cell line	6:19	Norway	2012	Kresse *et al*	(20)

GEO, Gene Expression Omnibus.

Table IV. Characteristics of microRNA expression profiling of osteosarcoma.

GEO accession	Platform	Sample	Samples (N:C)	Country	Year	First author	(Refs.)
GSE28424	GPL13376 Illumina HumanWG-6 v2.0 expression beadchip	Cell line	4:19	Norway	2012	Namløs *et al*	(13)
GSE70415	GPL16384 [miRNA-3] Affymetrix Multispecies miRNA-3 Array	Cell line	1:3	Japan	2015	Wang *et al*	(21)

GEO, Gene Expression Omnibus.

four mRNA expression profiling studies were included. The characteristics of the aforementioned studies are presented in Tables III and IV, respectively. After normalizing the raw miRNA expression datasets and mRNA expression datasets, 15 differentially expressed miRNAs were identified. Among these 15 differentially expressed miRNAs, 5 were upregulated and 10 were downregulated (Table V). A set of 452 differentially expressed genes (DGEs) were identified in OS, including 359 upregulated and 93 downregulated.

Regulatory network of miRNAs and targets in OS. The present study used the miRTarBase to predict the putative transcriptional targets of upregulated and downregulated miRNAs in OS. Compared with the putative targets with DGEs in OS, 452 miRNA-target gene pairs with an inverse association were obtained. Among them, 359 miRNA-target gene pairs were upregulated and 93 miRNA-target gene pairs were downregulated.

According to the miRNA-target gene pairs, a miRNA-target gene regulatory network was constructed (Fig. 1). The regulatory networks consisted of 452 miRNA-target interactions between 13 miRNAs and 452 targets in the context of OS. The top ten downregulated miRNAs in the regulatory network were hsa-miR-541, hsa-miR-22, hsa-mir-520f-x-st, hsa-miR-134, hsa-miR-493, hsa-miR-22-st, hsa-miR-494, hsa-miR-633, hsa-miR-541-st

Table V. List of differentially expressed miRNAs in osteosarcoma.

miRNAs	P-value	LogFC
Downregulated miRNAs		
hsa-miR-541	1.19×10^{-4}	-1.45
hsa-miR-22	2.78×10^{-4}	-1.05
hsa-mir-520f_x_st	1.97×10^{-2}	-1.04
hsa-miR-134	7.49×10^{-12}	-4.37
hsa-miR-493	5.05×10^{-3}	-1.36
hsa-mir-22_st	2.29×10^{-3}	-1.38
hsa-miR-494	1.24×10^{-3}	-1.33
hsa-miR-633	1.79×10^{-4}	-0.82
hsa-miR-541_st	4.30×10^{-2}	-0.99
hsa-miR-605	3.22×10^{-3}	-1.86
Upregulated miRNAs		
hsa-miR-182_st	1.31×10^{-4}	2.70
hsa-miR-183	1.04×10^{-3}	2.88
hsa-miR-596_st	9.22×10^{-3}	1.07
hsa-miR-301a	2.66×10^{-7}	5.55
hsa-miR-346	7.27×10^{-3}	1.82

miRNAs, microRNAs; FC, fold-change.

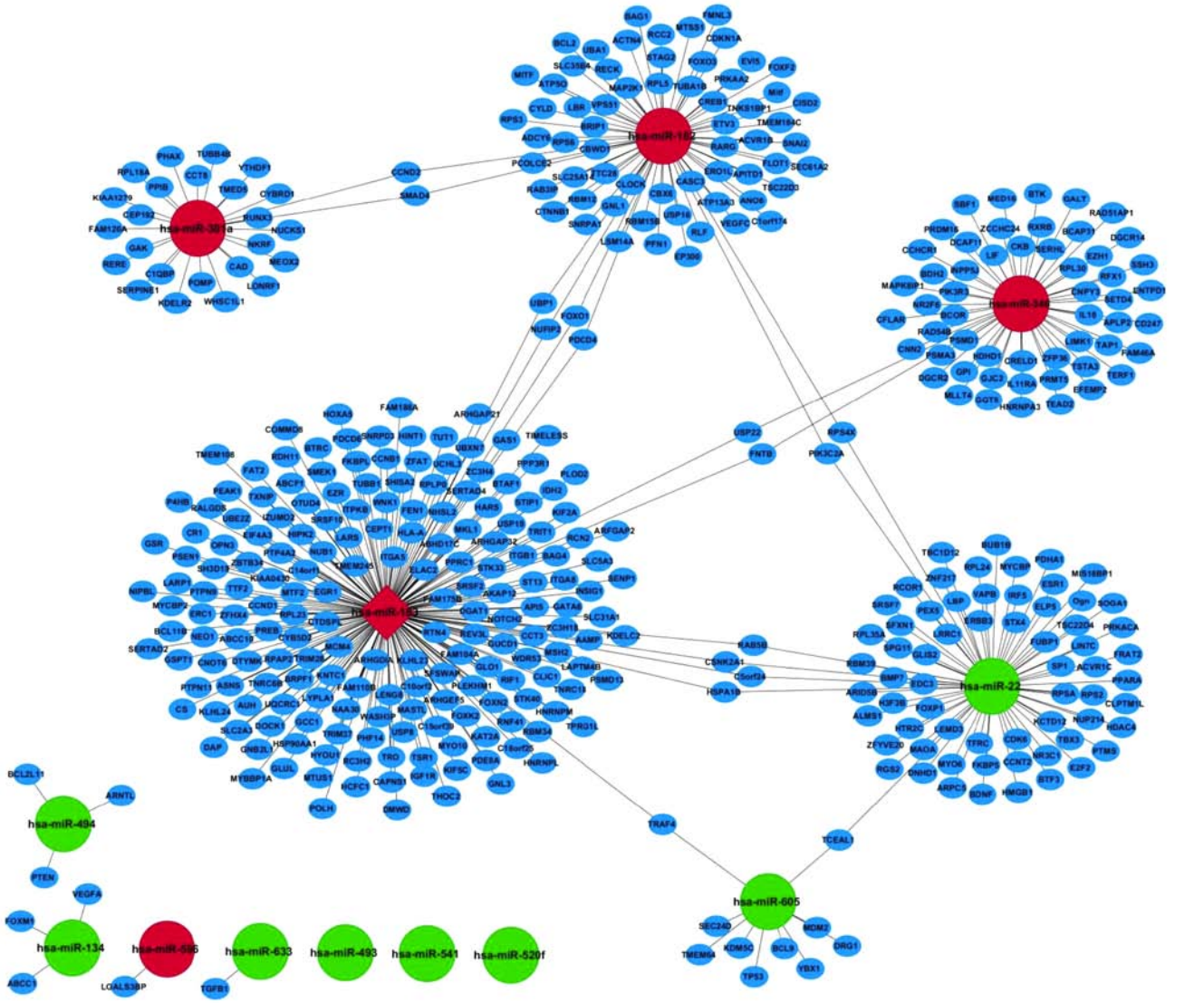

Figure 1. The regulatory network between miRNAs and target genes in osteosarcoma. Red represents upregulation and green represents downregulation. Ellipse represents target genes, blocks represent miRNAs and circles represent miRNAs. miRNA, microRNA.

and hsa-miR-605. The top five upregulated miRNAs in the network were hsa-miR-182, hsa-miR-183, hsa-mir-596, hsa-miR-310a and hsa-miR-346.

Enrichment analysis. To assign putative biological functions and pathway involvement to the differentially expressed target genes, enrichment analysis was performed. It was revealed that the significantly enriched GO terms for molecular functions were transcription regulator activity, transcription activator activity, enzyme binding, transcription factor activity, sequence-specific DNA binding, transcription factor binding, structure-specific DNA binding, RNA binding, nucleotide binding and DNA binding. The significantly enriched GO terms for cellular component were cytosol, organelle lumen, membrane-enclosed lumen, intracellular organelle lumen, nuclear lumen, nucleoplasm, non-membrane-bounded organelle, intracellular non-membrane-bounded organelle, ribonucleoprotein complex and cytosolic part, while those for biological processes were regulation of cell cycle, regulation of apoptosis, regulation of cell death, regulation of transcription, positive regulation of macromolecule metabolic process, negative regulation of apoptosis and negative regulation of cell death (Table VI).

The results of KEGG pathway enrichment analysis indicated that the most significantly enriched pathways were prostate cancer, pancreatic cancer, chronic myeloid leukemia, colorectal cancer, pathways in cancer, melanoma, ribosome, glioma, cell cycle and bladder cancer, which may have a significant impact on OS (Table VII).

RT-qPCR validation. To validate the association between miRNAs and their target genes in the regulatory network, the OS tissues from 5 patients and 5 healthy controls were used. A total of 10 miRNA-target pairs were selected for RT-qPCR, including hsa-miR-22 and ESR1, hsa-miR-22 and MAOA, hsa-miR-22 and FOXP1, hsa-miR-346 and BTK, hsa-miR-346 and c-FLIP, hsa-miR-182 and TSC22D3, hsa-miR-183 and EGR1, hsa-miR-183 and GAS1, hsa-miR-183 and SLC2A3, and hsa-miR-301a and MEOX2. The RT-qPCR results indicated that hsa-miR-346 was

Table VI. Top 10 GO terms of differentially expression microRNA target genes.

GO ID	GO term	Count	P-value	FDR
A, Biological process				
GO:0051726	Regulation of cell cycle	32	1.62×10^{-10}	2.85×10^{-7}
GO:0042981	Regulation of apoptosis	51	1.12×10^{-9}	1.96×10^{-6}
GO:0043067	Regulation of programmed cell death	51	1.55×10^{-9}	2.72×10^{-6}
GO:0010941	Regulation of cell death	51	1.73×10^{-9}	3.04×10^{-6}
GO:0010604	Positive regulation of macromolecule metabolic process	51	9.35×10^{-9}	1.64×10^{-5}
GO:0007049	Cell cycle	46	6.73×10^{-8}	1.18×10^{-4}
GO:0045449	Regulation of transcription	105	8.03×10^{-8}	1.41×10^{-4}
GO:0043066	Negative regulation of apoptosis	28	1.95×10^{-7}	3.43×10^{-4}
GO:0043069	Negative regulation of programmed cell death	28	2.58×10^{-7}	4.52×10^{-4}
GO:0060548	Negative regulation of cell death	28	2.74×10^{-7}	4.81×10^{-4}
B, Cellular component				
GO:0005829	Cytosol	74	3.48×10^{-12}	1.31×10^{-9}
GO:0043233	Organelle lumen	83	2.64×10^{-9}	9.92×10^{-7}
GO:0031974	Membrane-enclosed lumen	84	2.90×10^{-9}	1.09×10^{-6}
GO:0070013	Intracellular organelle lumen	81	4.88×10^{-9}	1.83×10^{-6}
GO:0031981	Nuclear lumen	70	8.13×10^{-9}	3.06×10^{-6}
GO:0005654	Nucleoplasm	47	3.18×10^{-7}	1.20×10^{-4}
GO:0043228	Non-membrane-bounded organelle	97	1.58×10^{-6}	5.95×10^{-4}
GO:0043232	Intracellular non-membrane-bounded organelle	97	1.58×10^{-6}	5.95×10^{-4}
GO:0030529	Ribonucleoprotein complex	30	1.42×10^{-5}	5.31×10^{-3}
GO:0044445	Cytosolic part	15	1.51×10^{-5}	5.67×10^{-3}
C, Molecular function				
GO:0030528	Transcription regulator activity	71	4.60×10^{-7}	2.91×10^{-4}
GO:0016563	Transcription activator activity	29	2.27×10^{-6}	1.44×10^{-3}
GO:0019899	Enzyme binding	31	3.06×10^{-5}	1.92×10^{-2}
GO:0003700	Transcription factor activity	46	7.68×10^{-5}	4.74×10^{-2}
GO:0043565	Sequence-specific DNA binding	33	8.57×10^{-5}	5.28×10^{-2}
GO:0008134	Transcription factor binding	29	1.31×10^{-4}	7.99×10^{-2}
GO:0043566	Structure-specific DNA binding	13	3.45×10^{-4}	$0.1.96\times10^{-1}$
GO:0003723	RNA binding	35	3.90×10^{-4}	2.19×10^{-1}
GO:0000166	Nucleotide binding	82	5.57×10^{-4}	2.97×10^{-1}
GO:0003677	DNA binding	84	7.06×10^{-4}	3.61×10^{-1}

GO, Gene Ontology.

inversely associated with the target gene, c-FLIP (Fig. 2). By contrast, the mRNA expression of BTK, MAOA, ESR1, FOXP1, GAS1, SLC2A3, MEOX2, TSC22D3 and EGR1 were not consistent with the results of integrated analysis (Fig. 2). Notably, the expression of hsa-miR-22 in OS was significantly different to that in the control, while the expression of target genes, MAOA, ESR1 and FOXP1, was not significantly different to that in the control; and the expression of EGR1 changed while the expression of hsa-miR-183 did not change. These results suggested that the mRNA and miRNA relative levels between OS tissues and cell lines were a little different. Another possible reason for this phenomenon was that one single target mRNA can be regulated by several miRNAs.

Validation of miRNA-346 and c-FLIP in MG-63 cell lines. The association between miRNA-346 and c-FLIP was verified in OS tissues obtained from 4 patients; however, the association between miRNA-346 and c-FLIP requires further investigation due to the limited number of OS

Table VII. Top 10 KEGG pathways of differentially expression miRNA target genes.

KEGG ID	KEGG term	Count	FDR	Genes
hsa05215	Prostate cancer	15	0.00083	E2F2, HSP90AA1, MAP2K1, CREB1, TP53, FOXO1, PTEN, CTNNB1, IGF1R, CCND1, CDKN1A, EP300, BCL2, MDM2, PIK3R3
hsa05212	Pancreatic cancer	13	0.00286	E2F2, MAP2K1, TP53, SMAD4, CDK6, TGFB1, RALGDS, ACVR1C, ACVR1B, VEGFC, CCND1, VEGFA, PIK3R3
hsa05220	Chronic myeloid leukemia	13	0.00448	E2F2, MAP2K1, TP53, SMAD4, CDK6, TGFB1, PTPN11, ACVR1C, ACVR1B, CCND1, CDKN1A, MDM2, PIK3R3
hsa05210	Colorectal cancer	13	0.01505	MAP2K1, MSH2, TP53, SMAD4, TGFB1, RALGDS, CTNNB1, ACVR1C, ACVR1B, IGF1R, CCND1, BCL2, PIK3R3
hsa05200	Pathways in cancer	27	0.01618	E2F2, HSP90AA1, MAP2K1, MSH2, RXRB, MITF, TP53, SMAD4, FOXO1, CDK6, ITGB1, PTEN, RALGDS, TGFB1, ACVR1C, CTNNB1, IGF1R, VEGFC, ACVR1B, CCND1, CDKN1A, EP300, BCL2, VEGFA, MDM2, PIK3R3, TRAF4
hsa05218	Melanoma	11	0.09229	E2F2, IGF1R, CDKN1A, CCND1, MAP2K1, MITF, TP53, MDM2, CDK6, PIK3R3, PTEN
hsa03010	Ribosome	12	0.11107	RPSA, RPL35A, RPL30, RPL23, RPL18A, RPLP0, RPL5, RPL24, RPS2, RPS4X, RPS6, RPS3
hsa05214	Glioma	10	0.18967	E2F2, IGF1R, CDKN1A, CCND1, MAP2K1, TP53, MDM2, CDK6, PIK3R3, PTEN
hsa04110	Cell cycle	14	0.19870	E2F2, TP53, SMAD4, CDK6, MCM4, TGFB1, CCNB1, CCND1, CDKN1A, EP300, CCND2, BUB1B, MDM2, STAG2
hsa05219	Bladder cancer	8	0.39189	E2F2, VEGFC, CDKN1A, CCND1, MAP2K1, VEGFA, TP53, MDM2

KEGG, Kyoto Encyclopedia of Genes and Genomes.

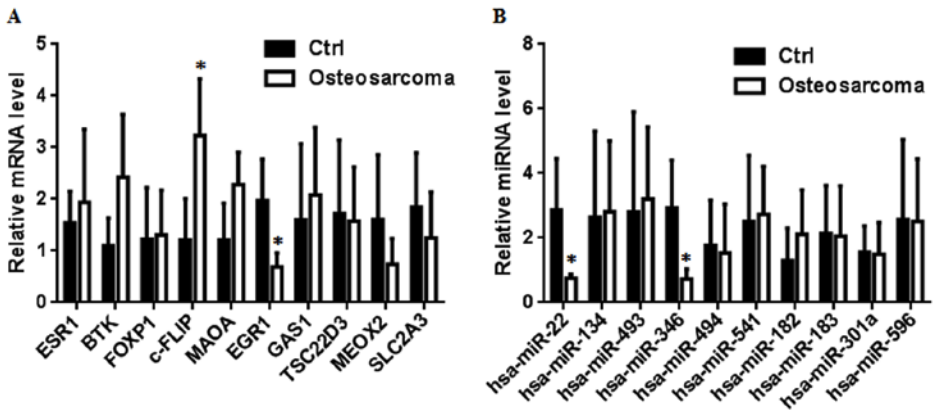

Figure 2. Reverse transcription-quantitative polymerase chain reaction validation of miRNAs-target genes. (A) Relative mRNA expression of ESR1, BTK, FOXP1, c-FLIP, MAOA, EGR1, GAS1, TSC22D3, MEOX2 and SLC2A3. The mRNA expression of c-FLIP was upregulated in OS and the mRNA expression of EGR1 was downregulated in OS. (B) Relative miRNA expression of hsa-miR-22, hsa-miR-134, hsa-miR-493, hsa-miR-346, hsa-miR-494, hsa-miR-541, hsa-miR-182, hsa-miR-183, hsa-miR-301a and hsa-miR-596, the miRNA expression of hsa-miR-22 and hsa-miR-346 were downregulated in OS. *P<0.05 vs. control. miRNA, microRNA; OS, osteosarcoma; ctrl, control.

tissues. Therefore, the OS MG-63 cell line was used to verify the results. Pre-miRNA-346 was used to produce mature miRNA-346. The mRNA expression of c-FLIP did not differ significantly between control and pre-miRNA-346 (Fig. 3). Western blot analysis revealed that the protein content of c-FLIP was lower in the pre-miRNA-346 than in the control (Fig. 3). These results indicated that pre-miRNA-346 did not affect the mRNA level of c-FLIP, and that it changed c-FLIP expression at the protein level.

Discussion

The development of suitable biomarkers is vital for diagnosing cancer or predicting patient prognoses. miRNAs have attracted a great deal of attention as they are highly stable and may be a potential biomarker for tumors and other diseases (22,23). The prognostic and diagnostic value of miRNA expression in OS has been studied intensely in recent years (24,25). However, due to the sample origin,

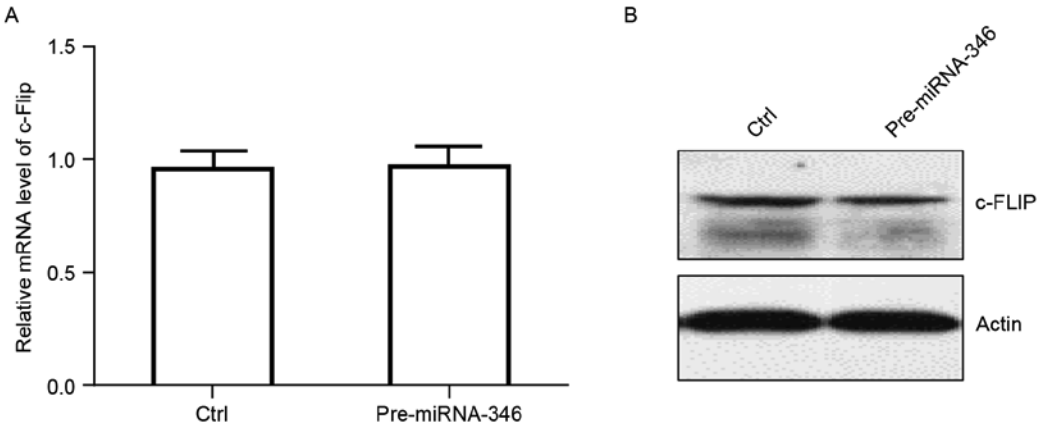

Figure 3. Pre-miRNA-346 downregulated the expression of c-FLIP at the protein level. (A) mRNA level of c-FLIP did not have significant difference between pre-miRNA-346 and control. (B) Protein content of c-FLIP in MG-63 cells with pre-miRNA-346 was lower than that in control cells. miRNA, microRNA; ctrl, control.

small sample size and application of different platforms, the miRNA expression profiles have yielded inconsistent results (26). Therefore, it is necessary to intersect the data to identify miRNAs that are significantly associated with OS. The present study searched the GEO databases, and only identified 4 mRNA expression profiling studies and 2 miRNA expression profiling studies in the GEO database, while no data associated with OS were identified in the TCGA database. In the present study, 4 mRNA expression profiling studies and 2 miRNA expression profiling studies obtained from the GEO database were integrated and 15 differentially expressed miRNAs were identified. In the miRNA-targets regulatory network, the top 5 upregulated and top 10 downregulated miRNAs were identified. These 15 differentially expressed miRNAs may be associated with OS.

Compared with non-cancerous bone tissues, miR-22 was downregulated in OS tissues. Patients with a lower miR-22 expression level exhibited a poorer overall survival and disease-free survival (27). Upregulation of miR-22 can cause DNA repair and impaired genomic integrity by inhibiting MDC1 in OS cells (28). The present study confirmed the downregulation of miR-22 in OS tissues. miR-22 may serve as a promising biomarker for predicting the prognosis of OS. miR-346 has been proven to be associated with numerous types of cancer. In the tissues of patients with cancer, the expression of miR-346 is upregulated and this is consistent with the results of the present study, suggesting that this may be a target site for the OS therapy.

miR-182 exhibits altered expression in various types of cancer. miR-182 expression may have predictive value for patients with cancer. In certain studies, miR-182 has been reported to be downregulated in OS tissues and cell lines (29,30). Overexpression of miR-182 inhibited tumor growth, migration and invasion. However, in the present study, the expression of miR-182 was upregulated in OS tissues. This result is inconsistent with those of previous studies, and may be caused by the small sample size. Zhu *et al* (31) revealed that the expression of miR-183 was significantly downregulated in OS tissues and cells, and statistical analysis demonstrated that the strong association between miR-183 expression and pulmonary metastasis and local recurrence of OS. Therefore, miR-183 serves an important role in the metastasis of OS.

The miR-301a serves an important role in various biological and pathological processes, including inflammation, cellular development and differentiation, apoptosis and cancer (32-34). Upregulation of miR-301a contributed toward chemoresistance of OS cells since miR-301a reduced doxorubicin-induced cell apoptosis, whereas anti-miR-301a enhanced apoptosis in OS cells (35). Consistently, the results of the present study demonstrated that miR-301a was upregulated in OS compared with the control. The results of the present study suggested that miR-301a may be a potential biomarker for OS.

In conclusion, in the regulatory network, hsa-miR-22 and hsa-miR-301a were inversely correlated with target genes ESR1 and MEOX2, respectively, and may be used as biomarkers for an earlier diagnosis of OS.

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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

CZ contributed to data analysis and the majority of the experiments; JW contributed to the RT-qPCR assay; FL contributed to the western blot assay; QL contributed to the data analysis; and HH contributed to the project design.

Ethics approval and consent to participate

The present study was approved by Ethics Committee of Xiangya Hospital of Central South University (Changsha, China).

Patient consent for publication

Consent for publication was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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