

Original Article

MiR-29a is down-regulated and inhibits cell proliferation in osteosarcoma: a meta analysis

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Abstract: MiRNAs are a type of small noncoding RNA molecules with 18-25 nucleotides in length. Previous studies demonstrated miRNAs played as key regulators of gene expression and translation by binding to 3'UTR. MiRNA were differently expressed in human cancers and involved in cancer initiation and progression. In this study, in order to identify differentially expressed miRNAs in OS, we analyzed two public databases, including GSE69470 and GSE70367. We identified 17 differentially expressed miRNAs in OS by analyzing public databases. Bioinformatics analyses showed differentially expressed miRNAs were involved in regulating OS cell adhesion, cell cycle, PI3K-Akt signaling pathway, and mTOR signaling pathway. According to our analysis, we found miR-29a and miR-137 played as key miRNAs in OS. Functional validation showed that miR-29a overexpression significantly inhibited OS proliferation and cell cycle progression. We believed this study would provide a potential new therapeutic and prognostic target for OS.

Keywords: miR-29a, osteosarcoma, bioinformatics analysis, expression profiling, proliferation

Introduction

MiRNAs are a type of small non-coding RNA molecules with 18-25 nucleotides in length. Previous studies demonstrated miRNAs played as key regulators of gene expression and translation by binding to 3'UTR [1, 2]. MiRNAs were differentially expressed in human cancers and involved in cancer initiation and progression by regulating cancer cell proliferation [3, 4], metastasis [5, 6], apoptosis [7, 8] and autophagy [9, 10]. By using high-throughput methods, including RNA-sequence and microarray, a series of disease related miRNAs were identified. For example, Ines et al. reported miR-548c-5p, miR-7-5p, miR-210-3p, and miR-128-3p could serve as biomarkers for systematically untreated breast cancer by using miRNA microarray [11]. Zhang et al. found microRNAs in CD4 (+) T cell subsets are markers of T cell dysfunction in individuals at risk for type 1 diabetes. Analyzing public microarray datasets could provide useful information to identify novel biomarkers for human diseases.

Osteosarcoma is one of the most common primary malignant bone tumors in children and teenagers. However, the mechanisms underlying osteosarcoma progression were still unclear. Of note, a few miRNAs were found to be involved in OS progression. For example, microRNA-124 suppressed osteosarcoma proliferation by inhibiting Snail2 expression [12]. MiR-340 alleviated chemoresistance through regulating ZEB1 in osteosarcoma cells [13]. MiR-378 induced osteosarcoma proliferation via suppressing the expression of KLF9 [14]. Therefore, understanding the functional roles of miRNAs regulating OS progression could provide prognostic and therapeutic biomarkers for OS.

In this study, to identify differentially expressed miRNAs in OS, we analyzed two public databases, including GSE69470 [15] and GSE70367. To explore the molecular mechanisms of aberrantly expressed miRNAs, we conducted GO and KEGG analysis. According to our analysis, we found miR-29a played as a key miRNA in OS. Then, we explored its functional roles in OS.

Material and methods

Microarray data and data preprocessing

Total of two miRNA expression databases of Osteosarcoma including GSE69470 and GSE70367 were downloaded from NCBI GEO databases. GSE69470 was submitted by Monks A et al. and contained 5 normal samples and 72 sarcoma samples [16]. GSE70367 was submitted by Tanaka K et al. and contained five OS cell lines and human mesenchymal stem cells (hMSCs).

GO & KEGG pathway analysis

MAS system provided by CapitalBio company (Molecule Annotation System, <http://bioinfo.capitalbio.com/mas3/>) was used to analyze the biological roles of differentially expressed mRNAs. Gene functions were classified into three subgroups namely biological processes (BP), cell component (CC), and molecular function (MF). The enriched GO terms were presented by enrichment scores. KEGG pathway analysis was carried out to determine the involvement of differentially expressed mRNAs in different biological pathways. The recommend *P*-value (Hypergeometric-*P* value) cut-off is 0.05.

Cell culture and transfection

HOS were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). All experiments were carried out with cell lines at passages below 25. HOS cell line was cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% FBS (Hyclone, USA) at 37°C in 5% CO₂.

Synthetic miR-29a mimic and negative control (NC) were designed and supplied by HuaGene (China). HOS was seeded in a 6-well plate and incubated for 24 h. Then HOS cells were subsequently transfected with 50 nM using Lipofectamine® 3000 (Life Technologies). Total RNAs were extracted at 48 h after transfection.

RNA extraction and quantitative RT-PCR

Total RNA for RT-qPCR was extracted using TRNzol Universal (TianGen). Reverse transcription (RT) was performed with TIANScript II RT Kit (TianGen) following the manufacturer's instructions. For analysis of microRNA expression, RT-qPCR was performed using Talent SY-

BR Green Reagents (TianGen) on the Light-CyclerR 480. The expression level of miR-29a was normalized to U6. The PCR primers for mature miR-29a and U6 were designed by HuaGene. Relative miRNA expression was calculated using the 2- $\Delta\Delta$ Ct method. Each sample was assayed in triplicate to ensure quantitative accuracy.

Cell proliferation assay

CCK-8 assays were performed to evaluate the effect of miR-29a mimic or NC on cell proliferation. Four thousand transfected cancer cells were plated in 96-well plates at 100 μ l medium/well. Proliferation was assessed at 0 day, 1 day, 2 day, 3 day and 4 day. The plates were monitored at specific time points using a Microplate reader after incubated 10 μ l CCK-8/well 1.5 h, which measured absorbance at 450 nm. The absorbance at 630 nm was used as a reference. Each experiment was performed at least in triplicate.

Cell cycle assay

Transfected HOS cells in the log phase of growth were collected and fixed in 0.03% Triton X-100 and propidium iodide (PI) (50 ng/mL) for 15 min. For the cell cycle analysis, the transfected cells were examined with a fluorescence activated cell sorting (FACS) flow cytometer and analyzed with ModFit software (Verity Software House, ME, USA). Each test was performed in triplicate.

Statistical analysis

The numerical data were presented as the mean \pm standard deviation (SD) of at least three determinations. Statistical comparisons between groups of normalized data were performed using T-test or Mann-Whitney U-test according to the test condition. A *P* < 0.05 was considered statistical significance with a 95% confidence level.

Result

Identification of the significantly differentially expressed miRNAs in osteosarcoma

In order to identify significantly differentially expressed miRNAs in OS, we analyzed two miRNA expression profiles, GSE69470 and GSE70367, which were download from the GEO dataset. A total of 1 control cell line and 5 OS cell lines

Down-regulated miRNA-29a in osteosarcoma

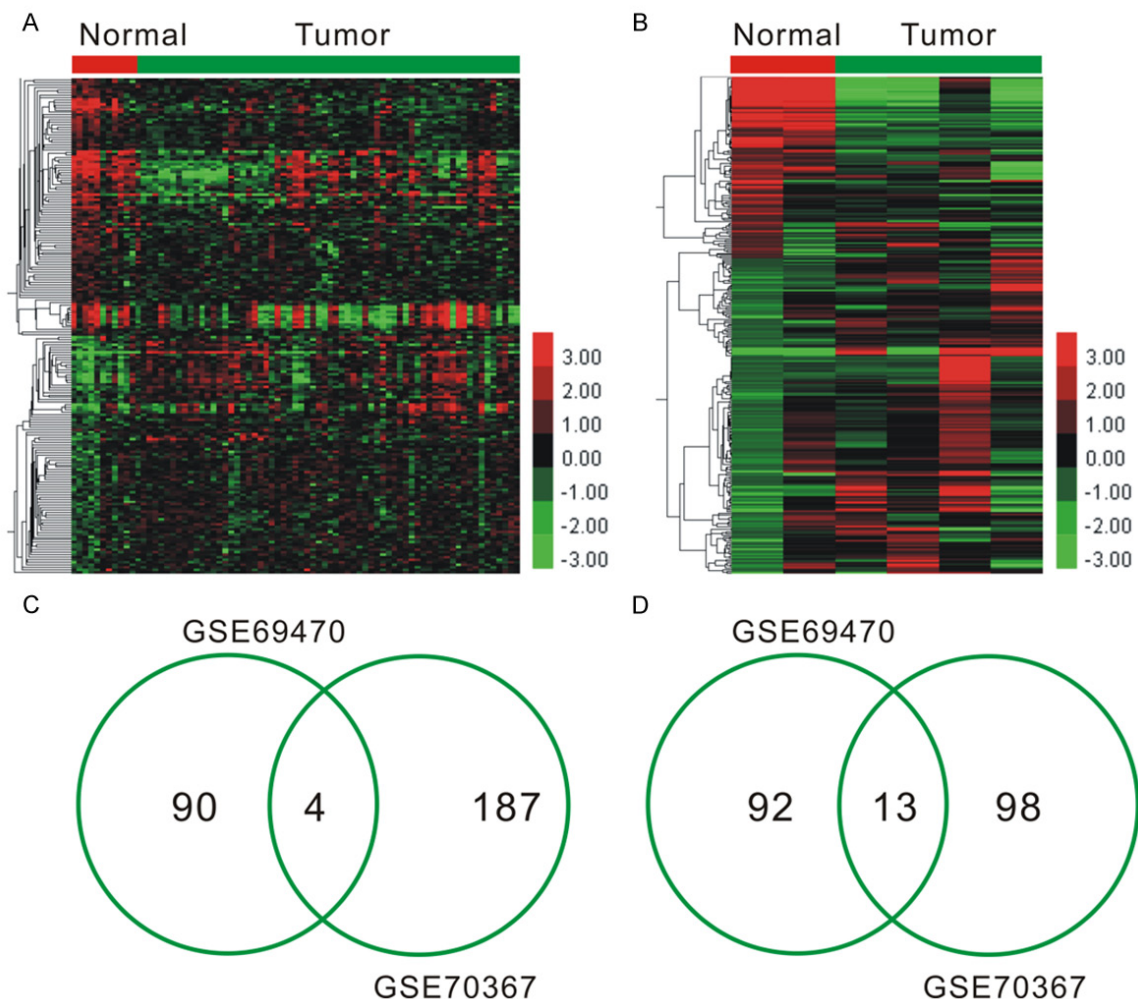


Figure 1. The significantly differentially expressed miRNAs in Osteosarcoma. A. GSE69470, 169 up-regulated miRNAs, and 70 down-regulated miRNAs were detected in OS compared with normal samples. B. GSE70367, we identified 234 up-regulated miRNAs and 198 down-regulated miRNAs were detected in OS compared with normal samples. C. miRNAs that are up-regulated in both GSE69470 and GSE70367. D. miRNAs that are down-regulated in both GSE69470 and GSE70367. (Fold change ≥ 1.5 , $P < 0.05$).

were included in GSE70367, and 5 normal samples and 72 sarcoma samples were included in GSE69470. As shown in the **Figure 1A**, by analyzing GSE69470, 94 miRNAs were upregulated and 105 miRNAs were downregulated in OS compared with normal samples with fold change ≥ 1.5 and $P < 0.05$. Meanwhile, we identified 191 up-regulated miRNAs and 111 down-regulated miRNAs by analyzing GSE70367 (**Figure 1B**). Clustering analysis was subsequently performed for all abnormally expressed miRNAs in OS (**Figure 1C, 1D**).

Construction of differentially expressed miRNA-targets networks

Furthermore, we constructed differentially expressed miRNA-target networks by using differ-

ent databases including TargetScan, miRDB, and starbase. A total of 613 genes were identified as potential targets of differentially expressed miRNAs. Next, we constructed miRNA-targets networks pairs using Cytoscape v3.2.1 (<http://www.cytoscape.org/>) software (**Figure 2**).

GO and KEGG analysis of differentially expressed miRNAs

We performed GO analysis for differentially expressed miRNAs by using the target mRNAs. According to the GO analysis, differentially expressed miRNAs were enriched in homophilic cell adhesion, positive regulation of protein localization to nucleus, protein K48-linked ubiquitination, regulation of cell cycle, protein

Down-regulated miRNA-29a in osteosarcoma

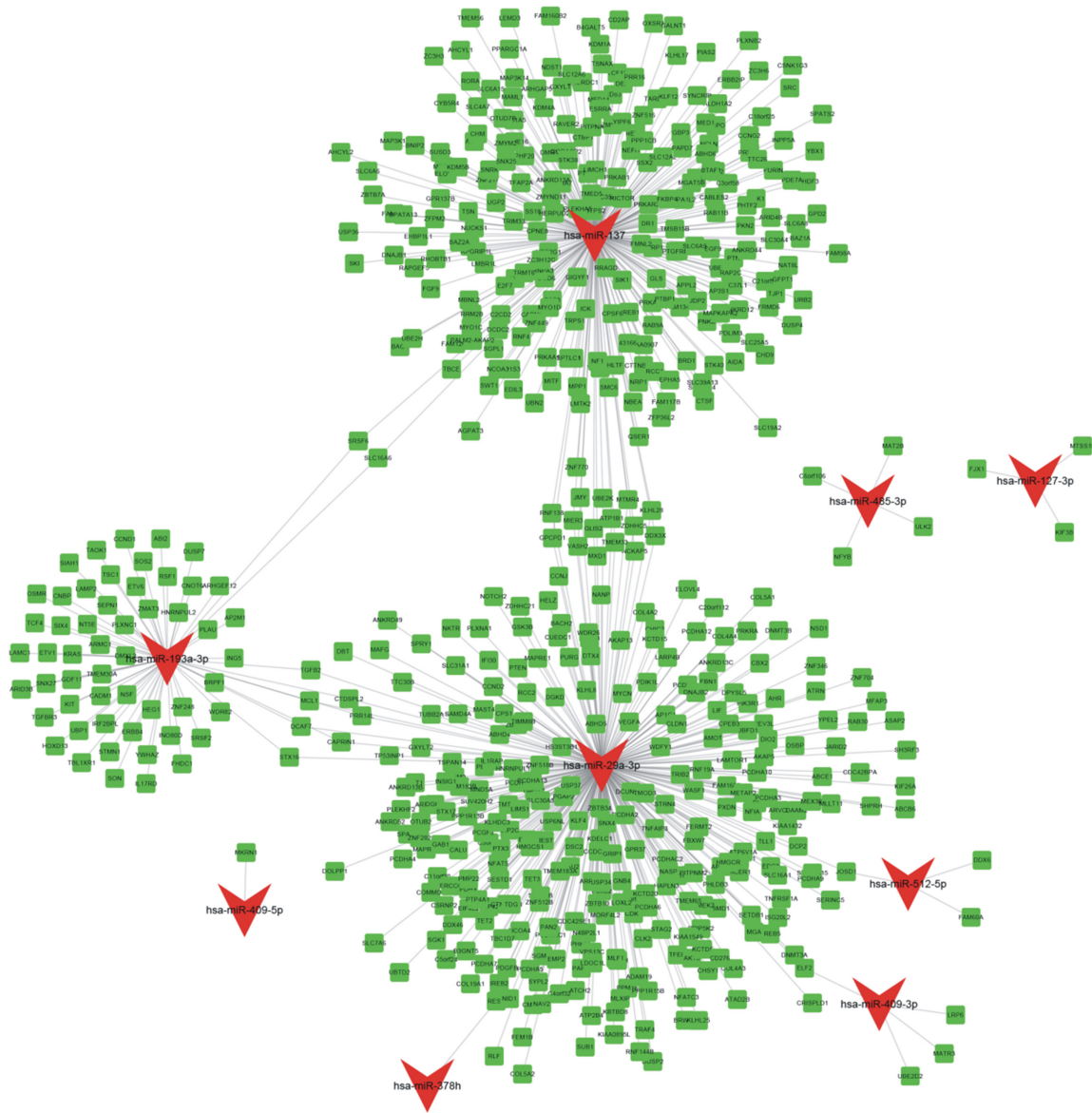


Figure 2. The differentially expressed miRNA-targets networks according to negative-related pairs.

K11-linked deubiquitination, 5-methylcytosine catabolic process, positive regulation of transcription (**Figure 3A**).

KEGG pathway analysis revealed that differentially expressed miRNAs mainly participated in regulating PI3K-Akt signaling pathway, Thyroid hormone signaling pathway, mTOR signaling pathway, FoxO signaling pathway, Insulin signaling pathway, Pathways in cancer, AMPK signaling pathway, ErbB signaling pathway, Prolactin signaling pathway, p53 signaling pathway (**Figure 3B**). The effects of differentially expressed miRNAs on PI3K-Akt signaling path-

way and mTOR signaling pathway were shown in **Figure 3C** and **3D**.

MiR-29a and miR-137 were the key regulators in network

In this network, we observed miR-29a and miR-137 were the key regulators, which potentially regulated more than 200 targets. In order to predict the functions of miR-29a and miR-137 in the network, we performed Cytoscape plug-in ClueGo analysis. In miR-29a mediated functional network analysis, 303 mRNAs were conducted to analyze their functions. The

Down-regulated miRNA-29a in osteosarcoma

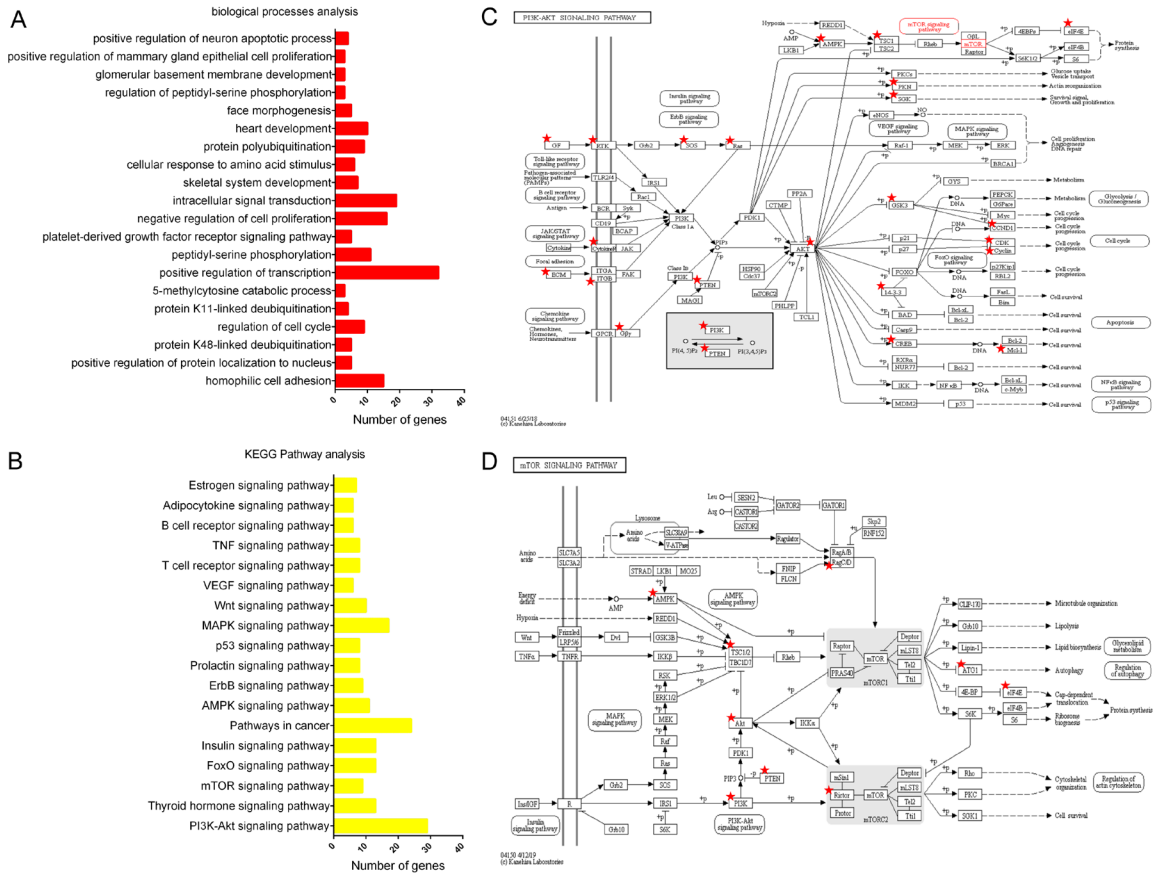


Figure 3. Molecular function analysis of the differentially expressed miRNAs. (A) KEGG analysis (B) Gene Ontologies analysis (GO) for the differentially expressed miRNAs. The differentially expressed miRNA was involved in regulating P53 signaling pathway (C) and mTOR signaling pathway (D).

results revealed that miR-29a was involved in regulating intrinsic apoptotic signaling pathway, mTOR signaling pathway, DNA methylation or demethylation and cell-cell adhesion via plasma-membrane adhesion molecules (**Figure 4A**). In miR-137 mediated functional network analysis, 259 mRNAs were conducted to analyze their functions. The results revealed that miR-137 was involved in regulating amino acid transport, regulation of activating receptor signaling, regulation of epithelial cell proliferation and regulation of cellular response to insulin stimulus (**Figure 4B**).

MiR-29a inhibited OS proliferation and cell cycle

Considering that miR-137 was reported to be a tumor suppressor in OS by Feng et al. and Li et al., we performed gain of function assay to explore miR-29a's roles in OS in order to further validate our analysis.

We performed cell proliferation assay to evaluate the effects of miR-29a on OS growth by using CCK-8 kit. As shown in **Figure 5A**, the expression levels of miR-29a were significantly induced after transfecting HOS cells with miR-29a. We observed ectopic miR-29a expression significantly decreased the growth rate of HOS cells (**Figure 5B**).

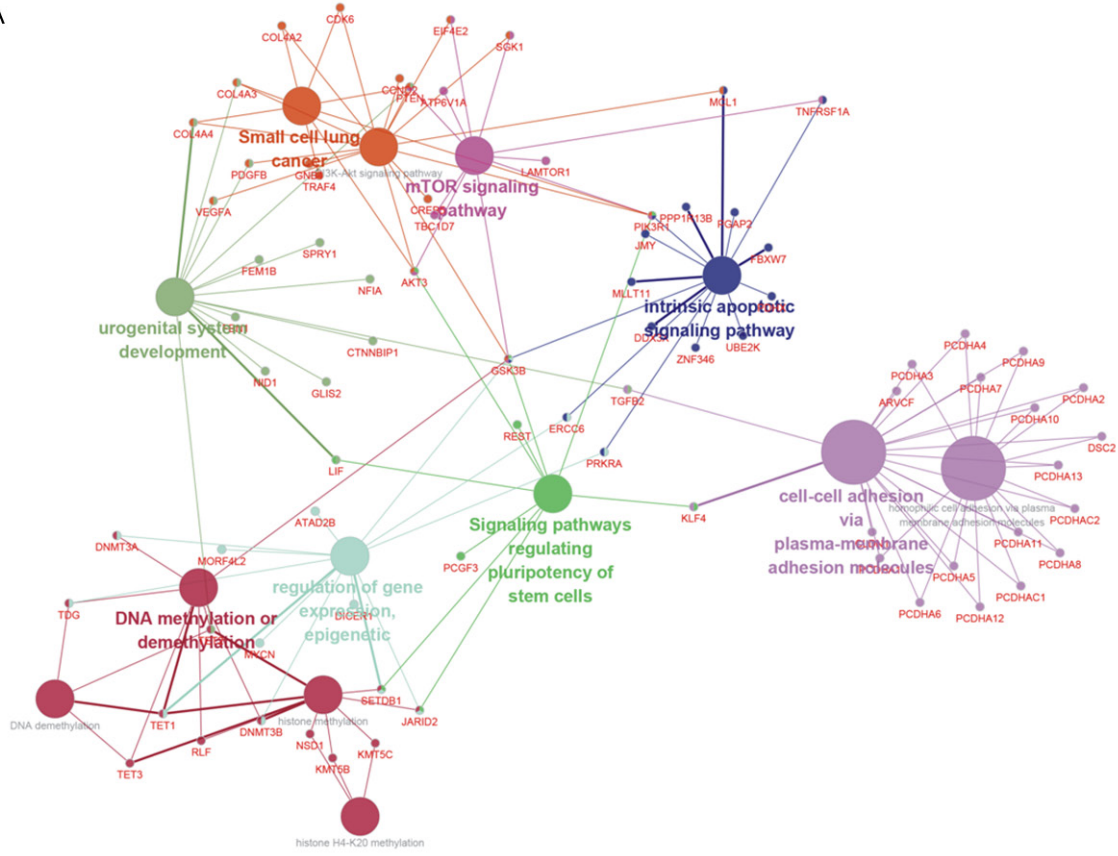
We further assessed the effect of miR-29a on cell cycle of HOS cells by using flow cytometric analysis. We found miR-29a could induce OS cell cycle arrest. The proportion of cells in G1 phase significantly reduced and the proportion of cells in S phase were increased after overexpressing miR-29a in HOS cells (**Figure 5C**). These results suggested miR-29a acted as a tumor suppressor in OS.

Discussion

The mechanisms underlying osteosarcoma progression was still unclear. Previous studies had

Down-regulated miRNA-29a in osteosarcoma

A



B

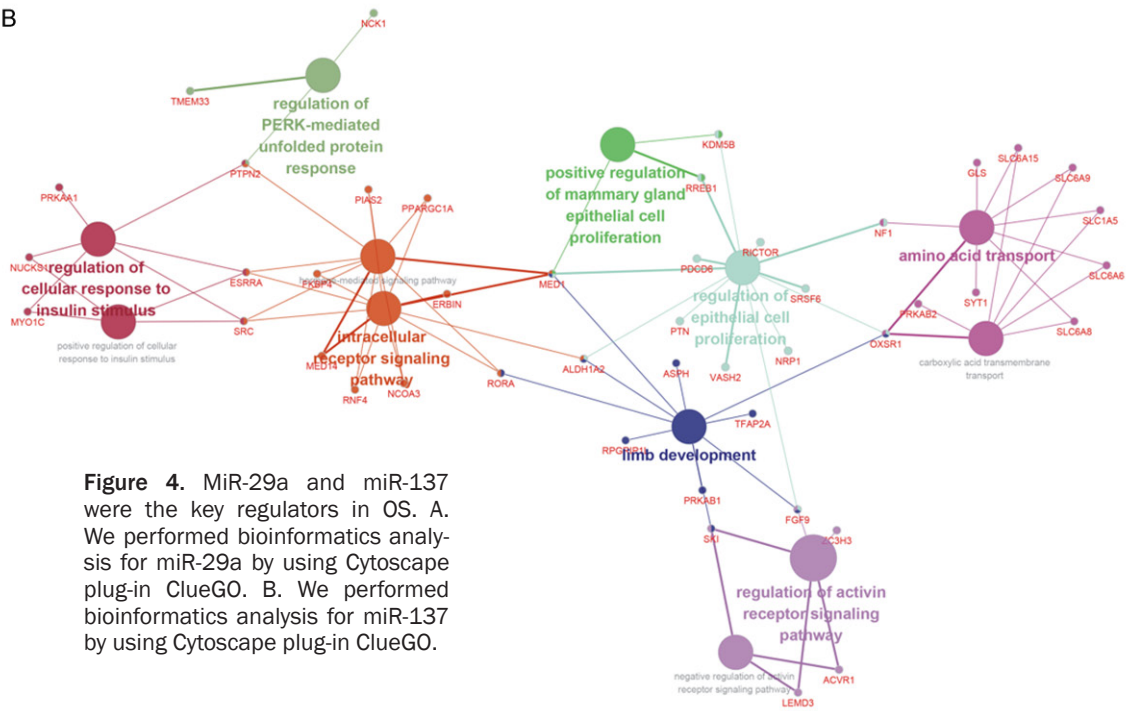


Figure 4. MiR-29a and miR-137 were the key regulators in OS. A. We performed bioinformatics analysis for miR-29a by using Cytoscape plug-in ClueGO. B. We performed bioinformatics analysis for miR-137 by using Cytoscape plug-in ClueGO.

indicated miRNAs played important roles in human cancer progression by regulating a series of biological processes, such as cell cy-

cle, apoptosis, and metastasis. Of note, a few miRNAs were reported to be involved in OS progression and diagnosis. For example, microR-

Down-regulated miRNA-29a in osteosarcoma

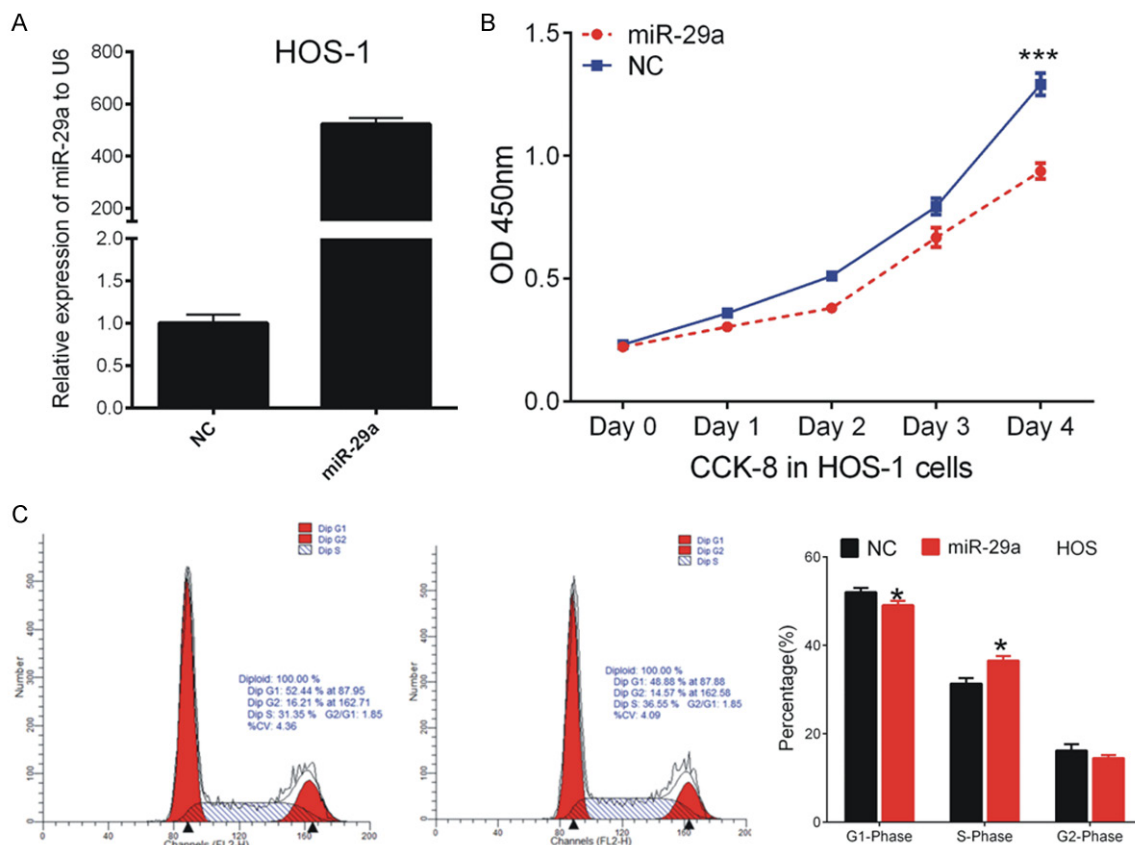


Figure 5. MiR-29a inhibited OS proliferation and cell cycle. A. The transfection efficiency. B. Overexpression of miR-29a significantly inhibited proliferation of HOS cells. C. The proportion of cells in G1 phase significantly reduced and the proportion of cells in S phase were significantly increased after overexpressing miR-29a in HOS cells.

NA-935 could suppress osteosarcoma proliferation and invasion by inhibiting HMGB1 [16] and microRNA-129-5p inhibited OS cell proliferation and metastasis via targeting ROCK1 [17]. However, these studies lacked systems-level identification of OS-related miRNAs in a large sample size.

In the present study, we analyzed two miRNA expression profiles, GSE69470 and GSE70367, to identify differentially expressed miRNAs in OS. A total of 4 up-regulated and 13 down-regulated miRNAs were identified in OS. Furthermore, we performed GO and KEGG analysis of these miRNAs. GO analysis showed differentially expressed miRNAs were enriched in homophilic cell adhesion, positive regulation of protein localization to nucleus, protein K48-linked deubiquitination, and regulation of cell cycle. KEGG pathway analysis revealed that the differentially expressed miRNAs mainly participated in regulating PI3K-Akt signaling pathway, Thyroid hormone signaling pathway, mTOR sig-

naling pathway, FoxO signaling pathway, and Insulin signaling pathway.

In this network, we observed miR-29a and miR-137 were the key regulators in osteosarcoma, which potentially regulated more than 200 targets. MiR-29a was reported as a tumor suppressor, however, the roles of miR-29a in osteosarcoma remained largely unclear. MiR-137 was reported to play crucial roles in certain types of cancer, including cervical cancer, breast cancer, and non-small cell lung cancer. In OS, miR-137 was downregulated in human osteosarcoma and acted as a tumor suppressor by targeting FXYD6 and EZH2. Our results revealed that miR-29a was involved in regulating intrinsic apoptotic signaling pathway, mTOR signaling pathway, DNA methylation or demethylation and cell-cell adhesion via plasma-membrane adhesion molecules, and miR-137 was involved in regulating amino acid transport, regulation of activating receptor signaling, regulation of epithelial cell proliferation and regulation of cellular response to insulin stimulus.

MiR-29a was reported to be aberrantly expressed in various cancers, including prostate cancer, breast cancer. Increasing evidence showed miR-29a was involved in regulating cell proliferation, cell cycle, cell senescence and differentiation, cell apoptosis, cell metastasis and epigenetic modulation. In this study, we also evaluated the effect of miR-29a on OS proliferation. We observed ectopic miR-29a expression significantly decreased the growth rate of HOS cells. Moreover, we found miR-29a could induce OS cell cycle arrest by increasing the proportion of cells in S phase and decreasing the proportion of cells in G1 phase. These results suggested miR-29a acted as a tumor suppressor in OS.

Conclusion

In conclusion, we identified 17 differently expressed miRNAs in OS by analyzing public databases. Bioinformatics analysis showed differentially expressed miRNAs were involved in regulating cell adhesion, cell cycle, PI3K-Akt signaling pathway, and mTOR signaling pathway in OS. According to our analysis, we found miR-29a and miR-137 played as key miRNAs in OS. Functional validation showed miR-29a significantly inhibited OS proliferation and cell cycle. We believed this study would provide a potential new therapeutic and prognostic target for OS.

Disclosure of conflict of interest

None.

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References

- [1] Ambros V. The functions of animal microRNAs. *Nature* 2004; 431: 350-5.
- [2] Lai EC. Micro RNAs are complementary to 3'UTR sequence motifs that mediate negative post-transcriptional regulation. *Nat Genet* 2002; 30: 363-4.
- [3] Hayashita Y, Osada H, Tatematsu Y, Yamada H, Yanagisawa K, Tomida S, Yatabe Y, Kawahara K, Sekido Y, Takahashi T. A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. *Cancer Res* 2005; 65: 9628-32.
- [4] Hwang HW, Mendell JT. MicroRNAs in cell proliferation, cell death, and tumorigenesis. *Br J Cancer* 2006; 94: 776-80.
- [5] Baranwal S, Alahari SK. miRNA control of tumor cell invasion and metastasis. *Int J Cancer* 2010; 126: 1283-90.
- [6] Chang RM, Xiao S, Lei X, Yang H, Fang F, Yang LY. miRNA-487a promotes proliferation and metastasis in hepatocellular carcinoma. *Clin Cancer Res* 2017; 23: 2593-604.
- [7] Su Z, Yang Z, Xu Y, Chen Y, Yu Q. MicroRNAs in apoptosis, autophagy and necroptosis. *Oncotarget* 2015; 6: 8474-90.
- [8] Pileczki V, Cojocneanu-Petric R, Maralani M, Neagoe IB, Sandulescu R. MicroRNAs as regulators of apoptosis mechanisms in cancer. *Clujul Med* 2016; 89: 50-55.
- [9] Aredia F, Scovassi AI. A new function for miRNAs as regulators of autophagy. *Future Med Chem* 2017; 9: 25-36.
- [10] Frankel LB, Lund AH. MicroRNA regulation of autophagy. *Carcinogenesis* 2012; 33: 2018-2025.
- [11] Block I, Burton M, Sorensen KP, Andersen L, Larsen MJ, Bak M, Cold S, Thomassen M, Tan Q, Kruse TA. Association of miR-548c-5p, miR-7-5p, miR-210-3p, miR-128-3p with recurrence in systemically untreated breast cancer. *Oncotarget* 2018; 9: 9030-42.
- [12] Huang J, Liang Y, Xu M, Xiong J, Wang D, Ding Q. MicroRNA-124 acts as a tumor-suppressive miRNA by inhibiting the expression of Snail2 in osteosarcoma. *Oncol Lett* 2018; 15: 4979-87.
- [13] Yan H, Zhang B, Fang C, Chen L. miR-340 alleviates chemoresistance of osteosarcoma cells by targeting ZEB1. *Anticancer Drugs* 2018; 29: 440-8.
- [14] Peng N, Miao Z, Wang L, Liu B, Wang G, Guo X. MiR-378 promotes the cell proliferation of osteosarcoma through down-regulating the expression of kruppel-like factor 9. *Biochem Cell Biol* 2018; 96: 515-521.
- [15] Teicher BA, Polley E, Kunkel M, Evans D, Silvers T, Delosh R, Laudeman J, Ogle C, Reinhart R, Selby M, Connelly J, Harris E, Monks A, Morris J. Sarcoma cell line screen of oncology drugs and investigational agents identifies patterns associated with gene and microRNA expression. *Mol Cancer Ther* 2015; 14: 2452-62.
- [16] Liu Z, Li Q, Zhao X, Cui B, Zhang L, Wang Q. MicroRNA-935 inhibits proliferation and invasion of osteosarcoma cells by directly targeting High mobility group box 1. *Oncol Res* 2018; [Epub ahead of print].
- [17] Han C, Wang W. MicroRNA-129-5p suppresses cell proliferation, migration and invasion via targeting ROCK1 in osteosarcoma. *Mol Med Rep* 2018; 17: 4777-4784.