MicroRNA-133a, downregulated in osteosarcoma, suppresses proliferation and promotes apoptosis by targeting Bcl-xL and Mcl-1

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A R T I C L E  I N F O

Article history:
Received 11 February 2013
Revised 16 May 2013
Accepted 19 May 2013
Available online 10 June 2013

Edited by: J. Rubin

Keywords:
MicroRNA
miR-133a
Apoptosis
Bcl-xL
Mcl-1
Osteosarcoma

A B S T R A C T

Deregulated microRNAs and their roles in cancer development have attracted much attention. Although miR-133a has been shown to be important in osteogenesis, its roles in osteosarcoma carcinogenesis and progression remain unknown. Hence, we focused on the expression and mechanisms of miR-133a in osteosarcoma development in this study. We found that miR-133a was downregulated in osteosarcoma cell lines and primary human osteosarcoma tissues, and its decrease was significantly correlated with tumor progression and prognosis of the patients. Functional studies revealed that restoration of miR-133a could reduce cell proliferation, promote cell apoptosis, and suppress tumorigenicity in osteosarcoma cell lines. Furthermore, bioinformatic prediction and experimental validation were applied to identify target genes of miR-133a, and the results revealed that the anti-tumor effect of miR-133a was probably due to targeting and repressing of Bcl-xL and Mcl-1 expression. Taken together, our data elucidate the roles of miR-133a in osteosarcoma pathogenesis and implicate its potential in cancer therapy.

Original Full Length Article

Introduction

MicroRNAs (miRNAs) are an abundant class of 17–25 nucleotide small noncoding RNAs. They posttranscriptionally regulate gene expression through binding to the 3′ untranslated regions (3′UTR) of target mRNAs. Since the initial observation, about 1000 miRNA sequences have been determined in mammals [1], but their detailed roles in physiology and pathiology still need investigation. Recently, growing evidences have suggested that miRNAs participate in the regulation of diverse biological processes [2], and their deregulation or dysfunction plays critical roles in cancer development and clinical outcomes of cancer patients [3]. However, evaluating the deregulated miRNAs and their roles in cancer development, especially in osteosarcoma, is still an ongoing process [4].

Osteosarcoma is the most common type of human primary malignant bone tumor characterized by an aggressive clinical course [5]. It usually develops in children and young adults. The mechanisms that orchestrate the multiple oncogenic insults required for osteosarcoma carcinogenesis and progression are still largely unclear. To date, deregulated miRNAs and their roles in osteosarcoma development have attracted much attention. Some of them, including miR-31, miR-34, miR-20a, miR-140 and miR-143, have been reported to participate in the initiation and progression of osteosarcoma and modulate the biological properties of cancer cells [6–15]. However, the detailed roles of miRNAs in cancer biology, especially in osteosarcoma, still need to be further investigated.

miR-133a has been recognized as a muscle specific miRNA which may regulate myoblast differentiation and participate in myogenic and heart diseases [16–18]. And recently, miR-133a is also reported to be an important regulator in osteogenesis, as its expression is downregulated in bone morphogenetic protein (BMP)-induced osteogenesis and it can target and suppress RunX2 expression to inhibit osteoblast differentiation [19]. But whether miR-133a is deregulated in osteosarcoma and its potential roles in osteosarcoma carcinogenesis and progression are still unknown.

In this study, we have taken efforts to explore the potential roles of miR-133a in osteosarcoma development. The expression of miR-133a in clinically resected human osteosarcoma tissues was evaluated, and the correlation between miR-133a deregulation and osteosarcoma progression was analyzed. Furthermore, the roles of miR-133a in osteosarcoma development and the underlying mechanisms were investigated. Our data indicate the roles of miR-133a in the control of cell growth and apoptosis in osteosarcoma, and suggest the potential therapeutic application of miR-133a for osteosarcoma patients.
Materials and methods

Patients and osteosarcoma tissues

Surgically resected paired osteosarcoma tumor tissues and adjacent normal tissues used in qRT-PCR and Western blot were collected from 92 primary osteosarcoma patients who received operations between 2006 and 2009 at Changhai Hospital (Shanghai, China), and the detailed information of these patients were shown in Supplementary Table 1. Surgically removed tissues were quickly frozen in liquid nitrogen until analysis. All samples were collected with the informed consents of the patients and the experiments were approved by the ethics committee of Second Military Medical University, Shanghai, China. The investigations were conducted according to the Declaration of Helsinki principles.

RNA extraction and qRT-PCR

Total RNA, including miRNA, was extracted using miRNeasy kit (Qiagen) according to the manufacturer’s instructions. To detect miRNA expression, real-time quantitative RT-PCR (qRT-PCR) analysis was performed using LightCycler (Roche) and SYBR RT-PCR kit (Takara). For miRNA analysis, stem-loop RT primers for miR-133a were 5′-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT AGC ACC AGG T-3′; and quantitative PCR primers were 5′-TTT GGT CCC CTT CAA CC-3′ (forward) and 5′-CTG CAG GGT CCG AGG T-3′ (reverse) [15]. Primers for detecting internal control U6 expression were described previously [15]. The relative expression level of miR-133a was normalized to that of internal control U6 by using 2−ΔΔCt cycle threshold method [20].

Cell culture and transfection

Human normal osteoblastic cell line hFOB 1.19, and human osteosarcoma cell lines MG63 and U2OS were obtained, cultured, seeded, and transfected as we described previously [15]. In brief, 5 × 10^3 or 5 × 10^5 cells were seeded into each well of 96-well plate or 6-well respectively and incubated overnight, then transfected with miRNA mimics or inhibitor at a final concentration of 20 nM or 50 nM respectively using INTERFERin transfection reagent (Polyplus-transfection) following the manufacturer’s instructions. Negative control (NC) RNA or miR-133a mimics, and negative control inhibitor or miR-133a inhibitor were all 2′-O-methyl modified to improve RNA stability and synthesized by GenePharma (Shanghai, China). siRNAs targeting human Bcl-xl were 5′-GGU AUU GGU GAG UCG GAU CddTdT-3′ and 5′-GAU CCG ACC CAC CAA UAC CddTdT-3′; siRNAs targeting human Mcl-1 were 5′-GAA ACG CGG UUA UCG GAC UddTdT-3′ and 5′-AGU CCG AUU ACC GGC UUU CddTdT-3′.

Analysis of cell proliferation in vitro

The in vitro cell proliferation of MG63 or U2OS cells transfected with NC or miR-133a was measured using the MTT method [15]. Briefly, cells were seeded into 96-well plates and transfected. In the indicated time periods, spent medium was replaced with fresh medium containing 0.5 mg/ml MTT. Cells were then incubated at 37 °C for 4 h and resolved by DMSO (Sigma). The absorbance was measured at 570 nm.

Detection of apoptosis

Osteosarcoma MG63 or U2OS cells were transfected with NC or miR-133a mimics respectively. At 48 h post transfection, spent cell culture medium was replaced with serum free DMEM and placed in 1% oxygen incubator. In the indicated time periods post serum deprivation, cells were harvested, washed, resuspended in the staining buffer, and examined with Vybrant Apoptosis Assay kit (Invitrogen).

Stained cells were detected by FACS Calibur and data were analyzed with CellQuest software (both from Becton Dickinson). The Annexin V-positive cells were regarded as apoptotic cells.

Tumorigenicity assay in nude mice

All experiments involving animals were undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University, Shanghai, China. The tumorigenicity assay was performed as reported previously [15,21,22]. In detail, NC or miR-133a transfected osteosarcoma MG63 or U2OS cells (1 × 10^6) were suspended in 0.1 ml PBS and then injected subcutaneously into either side of the posterior flank of the same BALB/c athymic nude mice at 4 wk of age. Tumor growth was measured using caliper daily, and tumor volume was calculated according to the formula: volume = length × width^2 × 0.5.

3′UTR luciferase reporter assay

The human B-cell lymphoma-extra large (Bcl-xl) and myeloid cell leukemia-1 (Mcl-1) 3′UTR luciferase reporter constructs and these constructs with miR-133a target site deletion were made as we described previously [15]. All constructs were confirmed by DNA sequencing. Luciferase reporter assay was performed as reported [15]. Briefly, at 36 h post transfection, luciferase activities were detected using Dual-Luciferase Reporter Assay System (Promega) following the manufacturer’s instructions. Data were normalized by dividing Firefly luciferase activity with that of Renilla luciferase.

Western blot

Cells and grinded human tissues were lysed using M-PER Protein Extraction Reagent (Pierce) supplemented with protease inhibitor cocktail (Calbiochem). Protein concentrations of the extracts were measured with BCA assay (Pierce) and equalized with the extraction reagent. Equal amounts of the extracts were loaded and subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and then blotted as reported [15]. Antibodies specific to Bcl-xl, Mcl-1, β-actin, and horseradish peroxidase-coupled secondary antibodies were purchased from Cell Signaling Technology. Densitometric analysis was done with Labworks Image Acquisition and Analysis Software (UVP, Upland, CA). The background was subtracted, and the signals of the detected bands were normalized to the amount of loading control β-actin band.

Statistical analysis

Data are shown as mean ± s.d. Statistical comparisons between groups were analyzed using Student’s t-test and a two-tailed p < 0.05 was considered to indicate statistical significance. The correlation between miR-133a expression and clinical osteosarcoma stages was analyzed using Spearman’s rank correlation coefficient assay in SPSS 17.0. Analysis of overall survival in osteosarcoma patients was performed using log-rank test in SPSS 17.0. The correlation between miR-133a expression and Bcl-xl or Mcl-1 protein levels was analyzed using Pearson’s correlation coefficient assay in SPSS 17.0.

Results

miR-133a is downregulated in osteosarcoma

In order to investigate the roles of miR-133a in human osteosarcoma development, we compared miR-133a expression in human normal osteoblastic cell line hFOB 1.19 with that in human osteosarcoma cell lines MG63 and U2OS by real-time qRT-PCR. And miR-133a expression was significantly decreased in osteosarcoma MG63 and
U2OS cells (Fig. 1A). Furthermore, in the 92 pairs of human primary osteosarcoma tumor and adjacent normal tissue samples, miR-133a expression was significantly suppressed in tumor tissues as compared to that in adjacent normal tissues (Fig. 1B). These results suggest that miR-133a is downregulated in osteosarcoma cells, which might be involved in human osteosarcoma development.

Downregulated miR-133a is correlated with osteosarcoma progression and prognosis

We next investigated whether the downregulation of miR-133a was correlated with osteosarcoma progression. By using Spearman’s rank correlation assay, we found that miR-133a expression in tumor tissues was significantly reverse-correlated with osteosarcoma clinical tumor stages in tested human samples (Fig. 2A). These results suggest that decreased miR-133a expression may participate in the progression of osteosarcoma. Furthermore, the Kaplan–Meier survival analysis also revealed that low miR-133a expression in tumor tissues was significantly correlated with the reduced overall survival of osteosarcoma patients (Fig. 2B). Together, these results indicate the important roles of miR-133a in both progression and prognosis of osteosarcoma.

miR-133a reduces cell proliferation, promotes cell apoptosis, and suppresses tumorigenicity

Decreased expression of miR-133a in tumor samples inspired us to investigate whether miR-133a functions as a tumor suppressor in osteosarcoma. In MG63 and U2OS cells, transfection of miR-133a mimics significantly restored intracellular miR-133a expression (Supplementary Fig. 1A), and restoration of miR-133a reduced cell proliferation in both osteosarcoma cell lines (Fig. 3A). Furthermore, miR-133a restoration promoted cell apoptosis upon serum deprivation and hypoxia in the osteosarcoma cells (Fig. 3B). These results demonstrate that miR-133a inhibits osteosarcoma growth in vitro.

Next, an in vivo model was applied to evaluate the effect of miR-133a restoration on tumorigenicity. In miR-133a transfected MG63 and U2OS cells, exogenous miR-133a expression could be maintained for 5 to 10 days in osteosarcoma cells after inoculation in nude mice (Supplementary Fig. 1B). Notably, miR-133a mimic transfected osteosarcoma MG63 and U2OS cells revealed delayed tumor formation and dramatic reduction of tumor sizes as compared to that of the negative control transfectants (Fig. 3C). As exogenous miR-133a expression could be maintained only in the early period post osteosarcoma inoculation, we presume that the proliferation-inhibiting and apoptosis-promoting
effect of miR-133a mainly occurs in the first week after inoculation, which in turn results in the observed suppressed tumorigenicity of miR-133a transfectants. Together, these results further suggest the tumor suppressive effect of miR-133a on osteosarcoma.

As expression of miR-133a is relatively higher in human normal osteoblast cell line hFOB 1.19, we further evaluated the effects of miR-133a inhibition on cell proliferation and apoptosis in hFOB 1.19. As shown in Supplementary Fig. 2, transfection of miR-133a inhibitor significantly inhibited miR-133a expression, and miR-133a inhibition enhanced cell proliferation as well as inhibited the serum deprivation and hypoxia induced cell apoptosis. These results validated the roles of miR-133a in cell proliferation and apoptosis.

miR-133a targets Bcl-xL and Mcl-1

In order to further investigate the molecular basis for the apoptosis promoting effect of miR-133a on osteosarcoma, we next worked on identifying the molecular targets of miR-133a. The predicted target genes of miR-133a in TargetScan database (http://www.targetscan.org) were all retrieved and subjected to the enrichment analysis of cell signaling pathways using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.genome.jp/kegg/) [3]. We found that KEGG pathway “pathway in cancer” was the most significantly enriched by the predicted targets of miR-133a (p = 0.001) (Supplementary Fig. 3 and Table 2), suggesting that miR-133a may play an important role in the inhibition of osteosarcoma intracellular signaling. Interestingly, to elucidate the apoptosis promoting role of miR-133a in osteosarcoma cells, we observed that Bcl-xL and Mcl-1, which are well-accepted anti-apoptotic molecules in osteosarcoma [23,24], were both potential targets of miR-133a (Fig. 4A).

Taken together the previous reports which determined that both Bcl-xL and Mcl-1 were upregulated in osteosarcoma and exerted the anti-apoptotic and pro-survival function of osteosarcoma cells [23,24], we presumed that miR-133a may promote cell apoptosis of osteosarcoma through targeting Bcl-xL and Mcl-1 expression. To verify whether Bcl-xL and Mcl-1 are direct targets of miR-133a, a dual-luciferase
miR-133a target site in 3′ UTR of Bcl-xL
Position 766-771 of Bcl-xL 3′ UTR
5′- AUGCACAUACUGAGACCAAC- 3′
mir-133a GUCGACCAACUCCCCUGGUUU
miR-133a target site in 3′ UTR of Mcl-1
Position 2724-2730 of Mcl-1 3′ UTR
5′- GCCAGGAAGGGUAGGACCAAC- 3′
mir-133a AUGCACACUCCCCUGGUUU

Fig. 4. Anti-apoptotic Bcl-xL and Mcl-1 are directly targeted by miR-133a. (A) Sequence alignment of miR-133a and its predictive target sites in 3′ UTR of Bcl-xL and Mcl-1 was shown as indicated, and the data were downloaded from TargetScan (http://www.targetscan.org). (B) MG63 cells were co-transfected with Bcl-xL or Mcl-1 3′ UTR Firefly luciferase reporter plasmid, or miR-133a target sites deleted reporter construct, together with Renilla plasmids, control RNA or miR-133a mimics as indicated. After 36 h, Firefly luciferase activity was measured and normalized by Renilla luciferase activity. (C) MG63 and U2OS cells were transfected with control RNA or miR-133a mimics. Expression of Bcl-xL, Mcl-1, and internal control (β-actin) was detected by Western blot. Data are shown as mean ± s.d. (n = 4) of one representative experiment. Similar results were performed in three independent experiments. **, p < 0.01.

The pro-apoptotic effect of miR-133a may be through targeting Bcl-xL and Mcl-1

To determine the roles of Bcl-xL and Mcl-1 in miR-133a-promoted apoptosis, we assessed whether knockdown of the endogenous Bcl-xL or Mcl-1 was able to mimic the effect of miR-133a restoration. Transfection of Bcl-xL or Mcl-1 siRNAs significantly suppressed their expression respectively (Fig. 6A), and promoted cell apoptosis upon serum deprivation and hypoxia in osteosarcoma cells (Fig. 6B). These data suggest that the pro-apoptotic effect of miR-133a may be through inhibiting its target genes Bcl-xL and Mcl-1.

Discussion

Osteosarcoma is the most common human primary malignant bone tumor characterized by an aggressive clinical course. Thus, in recent years, it has become one of the most promising fields to investigate molecular mechanisms contributing to osteosarcoma carcinogenesis and progression, especially identification and investigation of the deregulated miRNAs in osteosarcoma development. Several deregulated miRNAs, such as upregulated miR-21 and miR-140; downregulated miR-34, miR-143, and miR-34 members, have been reported and remarked in osteosarcoma development [4]. However, it is still an ongoing process to elucidate new important deregulated miRNAs and their detailed roles in cancer biology, especially in osteosarcoma carcinogenesis and progression.

Here, we presented the downregulation of miR-133a in osteosarcoma and suggested the anti-tumor effect of miR-133a in osteosarcoma pathogenesis. As previously reported, miR-133a expression was proved to play an important role during osteoblast differentiation, by the finding that BMP2 treatment could decrease the expression of miR-133a during osteoblast lineage commitment and osteogenesis [19]. Together with our finding that miR-133a is further decreased in osteosarcoma, we presume that miR-133a expression is decreased during osteoblast commitment but further miR-133a decrease may contribute to osteosarcoma development. In combination with previous reports revealing the roles of miR-133a in some other types of cancer, such as bladder cancer, esophagus cancer, and prostate cancer [25–27], we further confirmed that miR-133a might function as a tumor suppressor or an
antionco-miR in cancer carcinogenesis and progression. Among them, miR-133a expression is decreased in all these types of cancer, but the underlying mechanisms which mediate the downregulation of miR-133a in cancer are still elusive. We have tried to figure out the mechanisms responsible for miR-133a decrease in osteosarcoma. Two miR-133a gene locus (has-miR-133a-1, Chr 18; and has-miR-133a-2, Chr 20) was detected in osteosarcoma genome, and we found that equal amounts of the two miR-133a genes were detected as compared to those in the matched adjacent normal tissues (data not shown), thus suggesting that the two miR-133a genes are less likely to be deleted in osteosarcoma genome. Furthermore, we treated osteosarcoma MG63 and U2OS cells with DNA methylation inhibitor 5′-aza or HDAC inhibitor TSA, and found that miR-133a expression is rarely changed (data not shown), thus suggesting that miR-133a decrease in osteosarcoma may be less likely to be mediated by the enhanced DNA methylation or decreased histone acetylation. Hence, we presume that the mechanisms responsible for miR-133a decrease may be complicated or even the accumulation of various factors, such as epigenetic modifications, transcriptional factors, signaling cascades, and miRNA degrading routes. We will keep on investigating this issue in our future work.

Recently, identification of the molecular biomarkers correlating with progression and prognosis of cancer patients has attracted much attention. We presented here that the down-regulated miR-133a expression in osteosarcoma tissues was correlated with the cancer stages and overall survival of osteosarcoma patients, thus suggesting the potential roles of miR-133a in osteosarcoma development and outlining a potential biomarker of prognosis prediction for these patients.

Fig. 5. Correlation between miR-133a expression and Bcl-xL or Mcl-1 protein levels in osteosarcoma. (A) Shown are the protein expression of Bcl-xL, Mcl-1, and internal control β-actin in human normal osteoblastic hFOB 1.19 cell line versus osteosarcoma MG63 and U2OS cell lines detected by Western blot. (B) Expression of Bcl-xL and Mcl-1 examined by Western blotting was densitometrically quantified and normalized to β-actin expression. miR-133a expression was measured by qRT-PCR. Statistical analysis was performed using Pearson’s correlation coefficient assay in SPSS 17.0. miR-133a expression and Bcl-xL or Mcl-1 protein levels were shown as standardized values, r and p values were shown as indicated.

Fig. 6. Inhibition of Bcl-xL and Mcl-1 might be responsible for the tumor suppressive effect of miR-133a. (A) MG63 cells were transfected with control RNA, Bcl-xL siRNA, or Mcl-1 siRNA as indicated. After 48 h, expression of Bcl-xL, Mcl-1, and internal control β-actin was detected by Western blot. (B) MG63 cells were transfected as in (A) and treated with serum deprivation and hypoxia for 48 h. Cells were stained with PI and Annexin V, and analyzed using FACS. Data are shown as representative pictures. Similar results were performed in three independent experiments.
Additionally, previous reports have showed that the expression of some coding genes, including Bcl-xL, is also correlated with overall survival of osteosarcoma patients [23]. Hence, combined detection of the deregulated miRNAs and coding genes, including miR-133a, may be valuable to predict the prognosis of osteosarcoma patients more accurately.

The anti-tumor effect of miR-133a in osteosarcoma is validated both in vitro and in vivo. Restoration of miR-133a expression significantly reduces cell proliferation, promotes cell apoptosis, and suppresses tumorigenicity. Together with the reports that Bcl-xL and Mcl-1 are both involved in the progression of osteosarcoma, our findings lead to the thoughts that development of small molecule inhibitors to Bcl-2 family members may bear considerable potential for the targeted therapy of osteosarcoma patients, especially for those who respond poorly to radiotherapy or chemotherapy.

Human important anti-apoptotic molecules Bcl-xL and Mcl-1 are identified to be new direct targets of miR-133a in osteosarcoma, suggesting that miR-133a may exert its pro-apoptotic function via inhibiting Bcl-xL and Mcl-1 expression. Bcl-2 family members are well-accepted to be directly involved in serum deprivation and hypoxia induced apoptosis, especially that Bcl-xL and Mcl-1 can prevent mitochondrial cytochrome c release and subsequent caspase-9-dependent cell death, by which inhibiting apoptosis signaling [28]. Together with the result that miR-133a can repress Bcl-xL and Mcl-1 expression, the effects of miR-133a on promotion of serum deprivation and hypoxia induced apoptosis are suggested to be mediated by inhibition of Bcl-xL and Mcl-1 expression. Previous reports also showed that human EGFR, TAGLN2, and FSCN1 are molecular targets of miR-133a in other types of cancer [25–27]. In combination with our data, cancer pathways may be tightly regulated by miR-133a expression, and miR-133a may be a new therapeutic target to repress cancer progression. Additionally, as myogenic researches of miR-133a have determined its several other targets [16–18], these results are consistent with current opinions that a single miRNA can target multiple mRNAs, named “targetome”, to modulate gene expression [29]. Hence, it is probable that we are still far from unveiling the last target of miR-133a, and some of these potential targets may be still unknown in osteosarcoma development. According to this presumption, interesting future works may be raised to identify the entire roles of miR-133a in cancer development.

Acknowledgments

We thank Prof. Zhengdong Cai and Dr. Yue Wang for their helpful discussion, and Ms Jianfang Chen and Lijqing Fu for excellent technical assistance.

Grant support

This project was supported by grants from the National Natural Science Foundation of China (81202122, 30973019, 81272942), the Key Biomedical Research Programs of Science and Technology Commission in Shanghai (1041195600, 10411960400), and the Natural Science Foundation of Science and Technology Commission in Shanghai (064119605).

Conflicts of interest statement

Nothing to report.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bone.2013.05.020.

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