ORIGINAL RESEARCH

Evaluation of the Changes in microRNA Expression Profiles in Rat Primary Osteoblastic Cells Stimulated with Cotinine

Fengjuan Zhou, MD; Runhe Liu, MM; Lingke Huang, MM; Zhiqun Tang, MD; Hongkun Wu, MD

ABSTRACT

Objective • Smoking stands as a significant factor contributing to aberrations in bone metabolism, while microRNAs are intricately linked to the regulation of bone metabolism. This study aimed to identify cotinine-responsive microRNAs (miRNAs) and downstream regulatory pathways of their target genes involved in the regulation of osteoblastic cells, providing a foundation for new treatments targeting miRNAs for the bone metabolism imbalance induced by smoking.

Methods • Primary osteoblastic cells of Sprague-Dawley rats were cultured through a modified enzymatic digestion method from the cranial bone of neonatal rats and stimulated with a high concentration of cotinine (1000 ng/mL) for 7 days. Then, miRNA gene chip technology was utilized to detect the changes in miRNA expression profiles in cotinine-stimulated osteoblastic cells, and differential expression profiles of cotinine-responsive miRNAs in osteoblastic cells were identified. Real-time polymerase chain reaction was used to detect the levels of significantly differentially expressed miRNAs in rat osteoblastic cells. Gene ontology (GO) and Kyoto encyclopedia of Genes and Genomes (KEGG) pathway analyses were utilized to predict target genes of these miRNAs to reveal the potential biological functions and pathways.

Results • We identified 6 statistically differentially expressed miRNAs in the miRNA microarray analysis, of which 3 were upregulated and 3 were downregulated. We chose bone metabolism-related miRNAs as the miRNAs of interest. Quantitative real-time polymerase chain reaction was used to detect the expression levels of the differentially expressed miRNAs, and only miR-210 was significantly upregulated (3.34-fold), consistent with the microarray data. GO and KEGG pathway analyses of predicted miR-210 target genes revealed that miR-210 might participate in numerous signaling pathways, such as the RAS, Rap, PI3K-Akt, and calcium signaling pathways.

Conclusion • We found that the strongly upregulated miR-210 may play an important regulatory role in osteoblast cells' biological behavior and bone formation function. The GO analysis results showed that miR-210 mainly involved protein binding, transporter activity, growth factor binding, and ion channel activity. According to the results of the KEGG analysis, miR-210 might negatively regulate the PI3K-Akt signaling pathway, thus affecting the proliferation of osteoblastic cells. These findings suggest that miR-210 may be a potential target for regulating the imbalance of bone metabolism caused by smoking, offering a new direction for clinical treatment of patients with bone metabolism-related diseases. (*Altern Ther Health Med.* 2024;30(10):262-267).

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INTRODUCTION

Tobacco smoking is one of the risk factors for bone resorption. Nicotine, the main toxic component of tobacco, is closely associated with bone metabolic imbalance and has a short half-life of approximately 30 min.[1] Nicotine undergoes rapid metabolism to cotinine, with a plasma half-life ranging from 10 to 30 hours. Moreover, cotinine levels remain almost stable.[2] Because of its longer half-life, cotinine has been used as a reliable and sensitive biochemical marker of tobacco in different experiments to determine the extent of tobacco smoke exposure.[3] Kim et al.[4] reported that urinary cotinine levels had a significant dose-dependent effect on bone mineral density of the femur neck, total femur, and lumbar spine among postmenopausal females. A cross-sectional investigation

demonstrated an association between increased second-hand tobacco smoke exposure and decreased bone health, and in the study, urinary cotinine was also a reliable and valid circulating biomarker of nicotine exposure.[5] Another cross-sectional survey found that an increase in urine cotinine levels was associated with a decrease in femur, neck, and lumbar spine bone mineral density among males older than 30 years.[6] Hapidin et al.[7] reported that after administration of 7 mg/kg nicotine for 4 months, the levels of bone resorption cytokines and cotinine increased, negatively affecting the dynamic histomorphometric parameters of bone trabecula. The studies described above suggest that the cotinine level in body fluid may indirectly reflect bone mineral density. Torshabi[2] reported that high, cotininenicotine, and cotinine concentrations have adverse effects on the adhesion and proliferation of human gingival fibroblasts. Rehani et al.[8] demonstrated that cotinine can significantly change the nature of the inflammatory response of human innate immune cells to gram-negative bacteria. Bagaitkar et al.[9] reported that cotinine inhibited the pro-inflammatory response initiated by multiple cell surface Toll-like receptors in monocytic THP cells. Unfortunately, there is no evidence of a direct relationship between cotinine and bone metabolism. MicroRNAs (miRNAs, miRs) are key regulators that modulate the differentiation and function of cells by targeting multiple genes in the same or distinct signalling pathways.[10] Smoking-related miRNA expression profile changes in human airway epithelium and peripheral blood mononuclear cells have been reported.[11,12] studies have also shown that environmental smoke exposure can affect miRNAs in macaque eyes and plasma circulation.[13] However, it is unclear whether smoking can change the miRNA expression profiles in osteoblastic cells of rats and whether it has a direct relationship with bone metabolic balance. This study aimed to screen and identify cotinine-responsive miRNAs and their downstream regulatory genes related to osteoblast function to lay the foundation for new miRNA-targeting treatment of smoking-induced bone metabolism imbalance.

METHODS

Cell culture and characterization

We isolated osteoblastic cells from the calvaria of newborn male Sprague-Dawley rats. Fresh cranial bone tissue from neonatal rats was cut into small fragments (approximately or less than 1 mm) using sterile ophthalmic scissors. The fragments were digested with 1.0 mg/mL type I collagenase at 37°C for 90 minutes and then centrifuged at 1000 rpm for 10 minutes. The supernatant was removed, and the bone chips were placed into culture flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 mL/L fetal bovine serum. The culture was incubated at 37°C in an atmosphere of 95% O_2 and 5.0% CO_2. Half of the medium was changed after 24 hours and then fully changed every 3.0 days. When the cells reached 80-90% confluence, they were treated with 0.25% trypsin and serially passaged for further experiments. Cells in the test group were treated with a high cotinine concentration (1000 ng/mL) for 7 days, while those in the control group were cultured without intervention. The cells were identified by cell morphology, alkaline phosphatase (ALP) staining and alizarin red staining, and fourth-generation cells were used for further studies. The BCIP/NBT alkaline phosphatase chromogenic substrate kit is utilized to assess alkaline phosphatase activity in bone cells. Concurrently, Alizarin Red staining is applied for visualizing bone tissue sections, facilitating the analysis of bone cell behavior and bone structure.

Table 1. The primer sequences of the identified genes.

Gene	Bidirectional primer sequence	Length (bp)
U6	5'CGCTTCACGAATTTGCGTGTCAT3'	89
rno-miR-33	5' GTGCGTGTCGTGGAGTCG 3'	55
rno-miR-210	5' GTGCGTGTCGTGGAGTCG 3'	60
rno-miR-488	5' GTGCGTGTCGTGGAGTCG 3'	65

MiRNA microarray analysis

Three samples in each group were included in miRNA microarray analysis with the miRCURYTMLNA Array power labeling kit to screen the different miRNA expression profiles (Cat #208032-A, Exiqon). KangChen Bio-tech performed the entire analysis procedure. According to the predetermined criteria, miRNAs that were up or downregulated 1.5-fold were identified as key miRNAs associated with the cotinine response.

Verification of differentially expressed miRNAs by qRT-PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to validate the results of the miRNA array, which showed significant upregulation or downregulation in miRNA microarray analysis. The ViiA 7 Real-time PCR System (Applied Biosystems, USA) and 2X PCR master mix (Arraystar, USA) were used for PCR amplification and analysis according to the manufacturer's protocols. The primer sequences are listed in Table 1. U6 was chosen for the normalization of tissue miRNA expression. Comparative qPCR was performed in 3 replicates. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative changes in miRNA expression.

Dose-dependent relationship between cotinine and miRNA expression

We established four experimental groups and one control group: the cells of the experimental groups were treated with different concentrations of cotinine (1 ng/ mL, 10 ng/mL, 100 ng/mL, and 1000 ng/mL), and the cells in the control group were cultured without intervention. qRT-PCR was conducted to investigate the changes in miRNA expression in each group.

KEGG and GO enrichment analyses

The data on signaling pathways were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.kegg.jp/kegg/kegg2.html), and the threshold of significance was defined as $P < .05$ and FDR < 0.01. Gene Ontology (GO) analysis was performed using the GO database (http://www.geneontology.org), and P values

for all potential targets were calculated in all GO categories. The significance threshold was defined as $P < .05$ and FDR < .01. To further study the functions of the differentially expressed miRNAs, miRBase, miRanda, and miRDB were used to predict their potential targets and a network was established.

Statistical analysis

All values are expressed as the mean±standard deviation. One-way ANOVA and Statistic Package for Social Science (SPSS) 16.0 software (SPSS Inc., (Chicago, IL, USA) were used for comparative analysis. $P < .05$ was considered statistically significant.

RESULTS

Verification of primary osteoblastic cells

We successfully isolated primary osteoblastic cells from the cranial bone of neonatal rats using a modified enzymatic digestion method. Fibroblast-like adherent cells were observed around the bone chips after 24 hours, and approximately 6 days later, the cells reached 80–90% confluence. The cells were identified using the BCIP/NBT Alkaline Phosphatase Color Development Kit, and positively stained cells with blue-purple color were observed by inverted microscopy. Calcium nodules with an orange color were observed using alizarin red staining on the 21st day.

Expression profiles of cotinine-responsive miRNAs

Based on the miRNA microarray analysis data, we found 6 statistically differentially expressed miRNAs, of which 3 were upregulated (miR-33, miR-210, and miR-148a) and 3 were downregulated (miR-653, miR-410, and miR-488) (Figure 1). "Fold change" represents the multiple in expression level. They were upregulated 2.05-, 1.99- and 1.67-fold and downregulated 3.57-, 2.27- and 2.94-fold, respectively, with a significant difference (Table 2).

Verification of differentially expressed miRNAs by qRT-PCR

We chose the strongly downregulated miR-488, upregulated miR-33, and miR-210 as the miRNAs of interest. The qRT-PCR results showed that only miR-210 was significantly upregulated (3.34-fold), consistent with the microarray results (Figure 2).

Dose-dependent relationship between cotinine and miRNA expression

Through qRT-PCR, we found that cotinine could increase the expression of miR-210 in a dose-dependent manner. However, the expression of miR-210 was found to be significantly upregulated in the 1000 ng/mL group only (Table 3 and Figure 3).

KEGG and GO analyses of the differentially expressed miRNAs in cotinine-responsive osteoblasts

The significantly differentially expressed miR-210 target genes were analyzed using miRBase, miRanda and miRDB.

Figure 1. MiRNA microarray analysis: the top 3 upregulated miRNAs and top 3 downregulated miRNAs according to the miRNA microarray analysis.

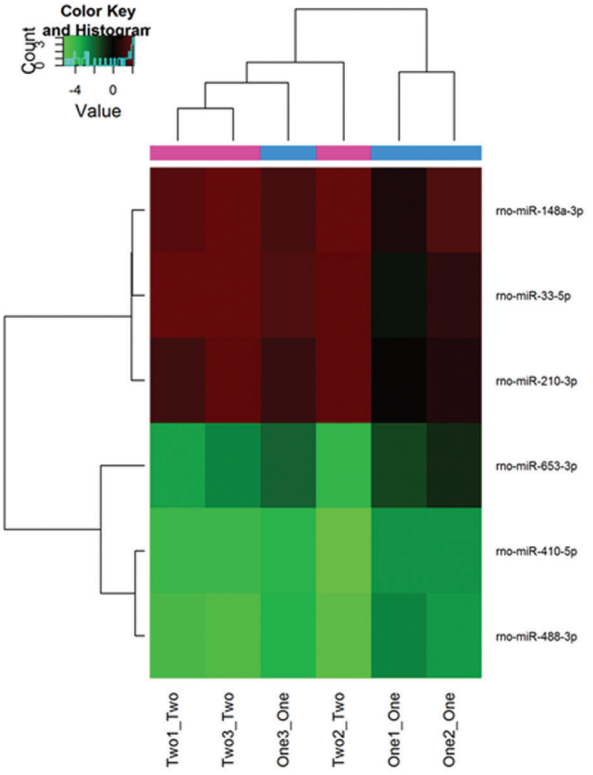
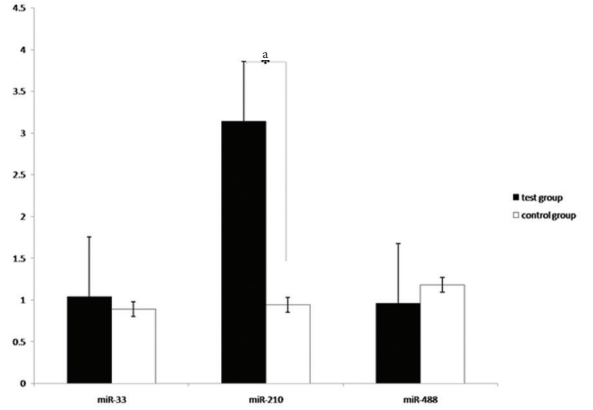


Table 2. Significantly up/downregulated ($P < .05$) miRNAs in osteoblastic cells of rats in the test groups compared with the control groups

miRNA	Fold change	*P* value
miR-33	2.05	.04
miR-210	1.99	.03
miR-148a	1.67	.04
miR-653	0.28	.03
miR-410	0.45	.04
miR-488	0.34	.03

Figure 2. The relative expression of miR-33, miR-210 and miR-488 in the test group (1000 ng/mL) and the control group.



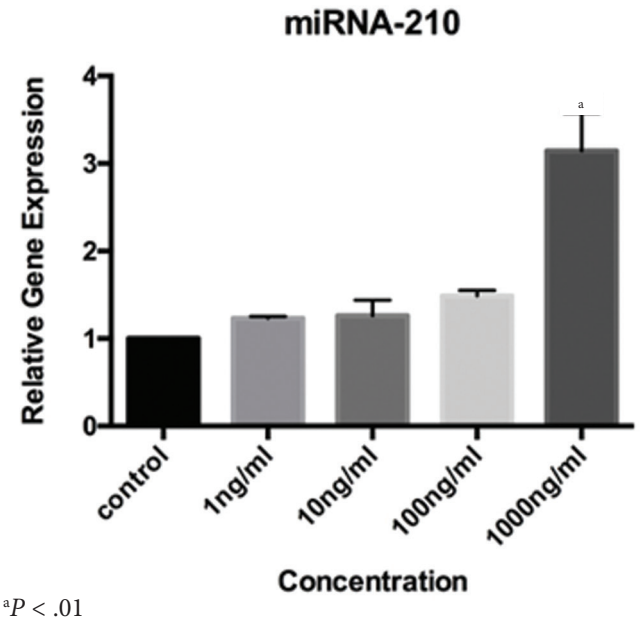
[a]$P < .05$

Table 3. The expression of miR-210 in the 1000 ng/mL group was significantly upregulated ($P < .01$) compared with that in the control group.

(I) concentration	(J) concentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	1 ng/mL	-0.22667	0.17259	1.000	-0.8448	0.3915
	10 ng/mL	-0.26000	0.17259	1.000	-0.8781	0.3581
	100 ng/mL	-0.48333	0.17259	0.188	-1.1015	0.1348
	1000 ng/mL	-2.14091[a]	0.17259	0.000	-2.7590	-1.5228

[a]The mean difference is significant at the .05 level.

Figure 3. The relative expression of miR-210 in the 1 ng/mL, 10 ng/mL, 100 ng/mL, 1000 ng/mL and control groups



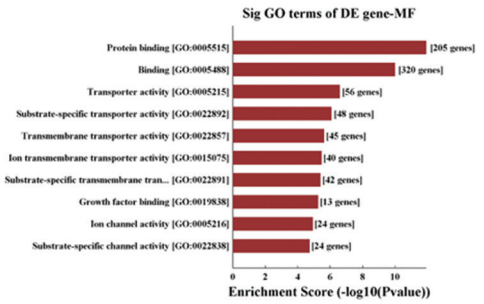
[a]$P < .01$

Table 4. KEGG pathway analysis of the predicted target genes of the validated significantly differentially expressed miRNAs.

Pathway ID	Definition	*P* value	n
rno04080	Neuroactive ligand-receptor interaction	.00	24
rno04014	Ras signaling pathway	.00	16
rno04015	Rap1 signaling pathway	.00	14
rno05033	Nicotine addiction	.01	5
rno05231	Choline metabolism in cancer	.01	8
rno00640	Propanoate metabolism	.01	4
rno04727	GABAergic synapse	.01	7
rno04151	PI3K-Akt signaling pathway	.02	17
rno04020	Calcium signaling pathway	.02	11
rno05218	Melanoma	.01	6
rno04142	Lysosome	.03	8
rno05323	Rheumatoid arthritis	.04	6
rno04966	Collecting duct acid secretion	.04	3
rno04060	Cytokine-cytokine receptor interaction	.05	11

According to the results, 939 miR-210 target genes were identified. GO category and KEGG pathway analyses were performed to investigate the biological functions of the predicted targets. The results showed that miR-210 might participate in numerous signaling pathways, such as the RAS, Rap, PI3K-Akt and calcium signaling pathways (Table 4). The predicted targets mainly involved protein binding, transporter activity, growth factor binding, and ion channel activity (Figure 4). These findings lay the groundwork for a more in-depth exploration and discussion regarding the implications for bone health and potential therapeutic approaches.

Figure 4. Significant GO molecular function terms of differentially expressed genes. The predicted targets were mainly involved in protein binding, transporter activity, growth factor binding, and ion channel activity.



DISCUSSION

miRNA, as a crucial endogenous post-transcriptional regulatory factor, plays a vital role in regulating bone metabolism by variously controlling the proliferation, differentiation, and other functions of bone marrow mesenchymal stem cells, osteoblasts, and osteoclasts. In the present study, we selected and identified, for the first time, cotinine-responsive miRNAs and their downstream regulatory pathways related to the function of rat osteoblasts. To imitate the microenvironment of bone tissue in old-aged heavy smokers, a high concentration of cotinine (1000 ng/mL) was selected for the long-term (7 days) treatment of primary osteoblasts in rats, and the concentration of cotinine was physiologically relevant to smokers (up to 1000 ng/mL, systemically) and those exposed to second-hand smoke (≤10 ng/mL, systemically).[9,14] According to the microarray analysis, 6 statistically differentially expressed miRNAs were identified in the miRNA microarray analysis, of which 3 were upregulated (miR-33, miR-210 and miR-148a) and 3 were downregulated (miR-653, miR-410, and miR-488).

The balance of bone metabolism is crucial for maintaining the stability and health of the skeletal structure. Any factor that disrupts this balance could potentially lead to bone-related conditions such as osteoporosis or fractures. We chose the bone metabolism-related miRNAs (miR-210, miR-33, and miR-488) as the miRNAs of interest. According to our literature search, the stem-loop of miR-210 is located in an intron of a long noncoding RNA, which is transcribed from AK123483 on chromosome 11p and functions in influencing cell growth arrest, supporting stem cell survival, regulating mitochondrial metabolism, stalling DNA repair, inducing angiogenesis, supporting cell differentiation, and playing an important role in bone marrow mesenchymal cells (MSCs).[15] This aligns with findings from our research. Mizuno et al.[16] reported that miR-210 acts as a positive regulator of osteoblastic differentiation by inhibiting the

TGF-β/activin signaling pathway by inhibiting AcvR1b. Alizadehe[17] showed that miR-210 could induce the proliferation of MSCs without any negative effect on their differentiation abilities. A previous study showed that miR-210 is strongly expressed in osteonecrosis (ON) and might play a role in ON pathogenesis.[18] Cai et al.[19] reported that upregulated miR-210 expression showed a strong correlation with tumor aggressive progression of pediatric osteosarcoma. MiR-33, located within the intronic sequences of the Srebp genes in many organisms, from Drosophila to humans, targets multiple genes that regulate cholesterol homeostasis.[20] Iwakiri[21] suggested that miR-33 could also be a key link between metabolism and proliferative responses in other organs where miR-33 has been identified. A study of Wang et al.[22] revealed that miR-33a was downregulated in gastric cancer tissues and cell lines, and overexpression of miR33a decreased CDK-6, CCND1, and PIM1 expression to inhibit gastric cancer cell proliferation by causing G1 phase arrest. Recently, a research group demonstrated that miR-488 contributes to the regulation of matrix metalloprotease-2 (MMP-2), while Song J and his colleagues speculated that miR-488 is involved in bone formation.[23,24] Song et al.[24] demonstrated that miR-488 regulates cell-to-extracellular matrix interactions through modulation of focal adhesion activity by MMP-2 during chondrogenesis of limb mesenchymal cells[_ENREF_2324]. Zhou et al.[25] suggested that miR-488 may predict the response to chemotherapy and as a therapeutic target in human osteosarcomas.

Interestingly, the qRT-PCR results showed that only miR-210 was significantly upregulated, consistent with the microarray results. GO category and KEGG pathway analyses showed that miR-210 might participate in many signaling pathways, such as the RAS, Rap, PI3K-Akt and calcium signaling pathways. The predicted targets mainly involved protein binding, transporter activity, growth factor binding, and ion channel activity. According to the literature review, the RAS, Rap, and PI3K-Akt signaling pathways are closely correlated with the proliferation and migration of cancer cells.[26-28] The process of bone metabolism is regulated by a complex network of cells and molecular pathways involving various cell types, including osteoblasts responsible for bone tissue formation, osteoclasts responsible for bone tissue resorption, and bone marrow mesenchymal stem cells, among other cell types. Despite our study highlighting the crucial role miR-210 in bone metabolism regulation, the specific regulatory mechanism of miR-210 still requires further investigation. Future research could focus on the collaborative or antagonistic roles of miR-210 and its target genes in the networks of relevant signaling pathways, as well as how they regulate the processes of osteoblast proliferation and aging. Of course, this study has certain limitations. Our research is primarily based on in vitro experiments, lacking direct validation of the in vivo effects of miR-210. The next step could involve experimental animal model studies, manipulating miR-210 with either overexpression or silencing, to observe its impact on bone health. Additionally, in clinical research, exploring the association between miR-210 and bone-related conditions such as osteoporosis and fractures among smokers, by analyzing patient samples or clinical cohort data, would further enhance our understanding of the physiological and pathological roles of miR-210 in bone health.

CONCLUSION

In this study, we identified cotinine-responsive miRNA that may be involved in primary rat osteoblastic cell function. We found that the strongly upregulated miR-210 may play an important regulatory role in osteoblast cells' biological behaviour and bone formation function. According to the results of the KEGG analysis, miR-210 might negatively regulate the PI3K-Akt signaling pathway, thus affecting the proliferation of osteoblastic cells. These findings suggest that miR-210 may be a potential target for regulating the imbalance of bone metabolism caused by smoking. Treatment intervention or modulation of miR-210 activity may potentially pave the way for novel therapeutic strategies concerning smoking-induced bone health issues. This study might also contribute to enhancing public awareness regarding the impact of smoking on bone health, advocating smoking cessation, and implementing measures to prevent or reduce the incidence of bone-related diseases.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The Animal Ethics Committee of Sichuan University Animal Center approved this study.

DECLARATION OF INTERESTS

There are no competing financial interests.

AUTHORS CONTRIBUTIONS

Fengjuan Zhou, Runhe Liuand Lingke Huang performed the experiments; Fengjuan Zhouand Runhe Liu and wrote the manuscript; Dan Liang prepared the figures; Zhiqun Tang prepared the tables; and Hongkun Wu supervised the entire project. All authors reviewed the manuscript.

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