IDENTIFICATION OF PATHOGENESIS-RELATED MICRORNA PROFILES IN SKELETAL FLUOROSIS

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ABSTRACT: Skeletal fluorosis is a chronic metabolic bone disease with adverse effects on human health. However, its pathogenesis remains unclear. In this study, we carried out a high-throughput profiling of human serum by using a miRNA array to primarily explore the role of miRNAs in skeletal fluorosis. The results showed the expression levels of 31 and 85 miRNAs were differentially regulated between the case group versus (i) a control group and (ii) a high-fluoride group, respectively. Three miRNAs (miR-200c-3p, miR-3185, and miR-1231) were successfully identified by PCR. Bioinformatic analyses revealed that the target genes of the differentiated miRNAs were highly enriched in genes promoting transcription. Pathway analysis of miRNAs by KEGG revealed the MAPK signaling pathway, pathways in cancer, the PI3K-Akt signaling pathway, proteoglycans in cancer, and the endocytosis pathway to be regulated by the differentially expressed miRNAs. miR-200c-3p and miR-1231 were closely associated with skeletal fluorosis. Thus, this study is the first to identify the miRNA profile of skeletal fluorosis in the population. The unique miRNA expression data obtained in this study provide a new insight into the molecular mechanisms and biomarkers of skeletal fluorosis.

Keywords: Gene microarray; MicroRNA; Skeletal fluorosis.

INTRODUCTION

Endemic fluorosis has been reported in many parts of the world and has a significant adverse impact on public health and well-being. Skeletal fluorosis is the most serious form of endemic fluorosis. The mild and moderate types of skeletal fluorosis manifest with chronic joint pain, stiffness of the joints, osteosclerosis of cancellous bone, and calcification of ligaments. Severe skeletal fluorosis shows limited movement of the joints, skeletal deformities, and intense calcification of ligaments.[1] A study on the disease burden of skeletal fluorosis estimated that DALYs per 1000 population for about 20 years, reached very high level in China.[2] There is not as yet any specified medication for the treatment of skeletal fluorosis because its pathogenesis remains unclear.

Researchers have found interesting results with studying the role of genes and proteins in the pathogenesis of skeletal fluorosis. Studies have shown that fluoride can regulate the expression of bone metabolism-related genes and proteins *in vivo* and *in vitro*.[3] However, the regulation mechanism of fluoride on these genes and proteins is largely unknown. Studies have demonstrated that miRNAs play a crucial role in regulating mRNA transcription and protein expression in cell differentiation and growth.[4] Some of the miRNAs have been found to be closely related with bone

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metabolism through the regulation of osteocyte differentiation, growth, and biological activity.[5] The effects of fluoride on miRNA profiling have been observed in animal fluorosis models and in cell lines, and a dysregulation of miRNA profiling has been found between the fluoride treatment groups and the control group.s[6-8] However, this is the maiden study of miRNA profiling of patients with skeletal fluorosis.

In this study, by comparing the miRNA expression patterns between skeletal fluorosis subjects and controls with a different fluoride exposure, we sought to identify some skeletal fluorosis-related serum miRNAs, which could be a potential targeted gene for fluorosis therapy and provide a new insight into the pathogenesis of skeletal fluorosis.

METHODS AND MATERIALS

Study subjects and design: This study was conducted in five villages (Hongguang, Xiaoshan, Fushan, Wanfa, and Leye) affected by drinking water-born endemic fluorosis in Zhao Dong County, Heilongjiang Province, People's Republic of China. The subjects enrolled in this cross-sectional study were older than 16 years, and born and bred in the named villages. Skeletal fluorosis was diagnosed using the national diagnostic standard for endemic skeletal fluorosis (WS192-2008). The subjects were investigated using a questionnaire which was designed to obtain the name, address, sex, age, nationality, past medical history, and the volume of drinking water consumed daily. A face-to-face interview was performed by well-trained staff. Every subject received a clinical examination which included a physical examination and an X-ray diagnosis (Beijing Long safe Imaging Technology Co., Beijing City, China). In addition, drinking water, blood, and urine samples were collected for each participant.

In the screening study of miRNA arrays, 3 subjects were selected from the case group, a control group with exposure to a drinking water fluoride level ≤1.2 mg/L, and a high fluoride group with exposure to a drinking water fluoride level >4 mg/L. The patients in the case group were of the mild type of skeletal fluorosis. There were 1 male and 2 females in each group. The average ages within the three groups were 51.67±6.03, 49.00±3.00, and 52.00±6.56 yr, respectively.

In the validation study, 15 cases, 15 controls, and 15 subjects from the high fluoride group were recruited. The case group was made up of 13 patients with mild type skeletal fluorosis and 2 patients with moderate type skeletal fluorosis. There were 7 males and 8 females in each group. The average ages within the three groups were 57.00±7.16, 57.40±8.14, and 56.40±6.85 yr, respectively.

The inclusion criterion for the case group was skeletal fluorosis. Exclusion criteria were bone diseases, hypertension, atherosclerosis, heart disease, and diabetes. All groups were age and gender matched, and presented a similar medical history. In addition, the levels of urinary fluoride in the case group and in the high fluoride group were similar.

Diagnosis of skeletal fluorosis: A radiograph of the forearm, the humerus in the arm, and the pelvis of each participant was used to evaluate the presence of skeletal fluorosis. Skeletal fluorosis was diagnosed and classified according to the Diagnostic

Criteria for Endemic Skeletal Fluorosis (WS192-2008, China) as previously described.[1, 9]

Fluoride analysis: The drinking water sample and the urine sample were stored until analysed at –20ºC. The fluoride content of the drinking water was detected by a F-ion selective electrode (Yingke Crystal Materials Company) using a China national standard (GB 5750.5-2006, China). The urine fluoride was also assessed by using the standard (WS/T 89–2015, China).

RNA preparation and miRNAs microarray: The serum miRNAs were extracted with miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). After assessing the RNA's quality and quantity, the miRNA microarray analysis (Affymetrix microRNA 4.0 Array, Santa Clara, CA, USA) was performed according to the manufacturer's instructions. Briefly, 1 μg of total RNA was labeled with Biotin using the FlashTag Biotin HSR RNA Labeling Kit (Genisphere, Hatfield, PA, USA) and then hybridized overnight with the array, which was washed, stained, and read by an GeneChip Scanner 3000 7G (Affymetrix). Differentially expressed genes were identified by fold-change analyses. The threshold value for significance used to define up-regulation or down-regulation of the miRNAs was a fold change >2, with the value of $p< 0.05$.

Validation by Quantitative Real-time PCR (qRT-PCR): The miRNAs were extracted from serum using a miRcute miRNA isolation and extraction kit (Tiangen Biotech Co., Ltd, Beijing, China). After assessing the RNA's quality and quantity, cDNA was synthesized from 0.2 μg of miRNA with a TaqMan miRNA RT reagent Kit (Haigene, Harbin, China). Quantitative PCR was performed using a TaqMan miRNA PCR kit (Haigene, Harbin, China) on an ABI7500 Fast real-time PCR system (ABI, USA). Table 1 shows the Taqman probe sequences used in the PCR reactions. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method normalizing to miR-16 expression for each sample.[10] PCR product specificity was measured by gel electrophoresis and melting curve. The experiment was carried out 3 times to give triplicate samples.

Table 1. Taqman probe sequence of miRNAs

MiRNA	Accession No.	Probe sequence
miR-16	MI0000070	UAGCAGCACGUAAAUAUUGGCG
miR-200c-3p	ENSG00000160691	UAAUACUGCCGGGUAAUGAUGGA
miR-1231	MI0006321	GUGUCUGGGCGGACAGCUGC
miR-3185	MI0014227	AGAAGAAGGCGGUCGGUCUGCGG

Bioinformation analysis of miRNAs microarray: CEL-files of the raw data were first exported by Affymetrix Gene Chip Command Console Software Version 4.0 (Affymetrix) and then uploaded to the website of Gminix-Cloud Biotechnology Information (GCBI, http://www.gcbi.com.cn/gclib/html/index, Genminix Informatics Co., Ltd., Shanghai, China) for further analysis, including different analysis for

miRNA profiles, prediction of miRNAs target genes, GO (gene ontology) enrichment analysis, and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis.

Statistical analysis: Continuous data were represented as mean±SD or median (P25, P75). ANOVA or Kruskal-Wallis test (two-tailed) was used to determine the statistical significance of the quantitative data. Chi-square test was used for the statistical analysis of constituent ratio. $p<0.05$ was considered statistically significant. All statistical analyses were performed with STATA (STATA, College Station, TX, Version 12.0).

RESULTS

A total of 302 subjects were investigated as candidates for the miRNA arrays study. Thirty-two subjects were diagnosed with skeletal fluorosis giving a prevalence of skeletal fluorosis of 10.6%. Twenty-seven cases had mild skeletal fluorosis, with 8 males and 19 females. Four cases had moderate skeletal fluorosis (2 males and 2 females) and there was one case of a male with severe skeletal fluorosis. The number, sex proportion, age, and urinary fluoride of the case group and the different fluoride exposure groups are shown in Table 2.

Table 2. The results of gender, age, and urinary fluoride in the different fluoride exposure groups and cases. (No. = number)

Group	No. (n)	Male (n%)	Female (n%)	Age (mean ±SD)	Urinary fluoride (mg/L)[‖]
Cases	32	11 (34.4%)	21 (65.6%)	60.44 ±8.30[‡§]	3.55 (2.45, 5.90) *[†]
Controls (water fluoride≤1.2mg/L)	24	8 (33.3%)	16 (66.7%)	58.38 ±13.69	2.06 (1.61, 2.36)
1.2 mg/L<water fluoride≤2 mg/L	63	16 (25.4%)	47 (74.6%)	56.43 ±10.82	2.26 (1.66, 3.09)
2 mg/L<water fluoride≤4 mg/L	124	33 (26.6%)	91 (73.4%)	53.93 ±10.36	2.80 (2.06, 3.49)
High fluoride group (water fluoride>4 mg/L)	59	24 (40.7%)	35 (59.3%)	54.97 ±12.55	4.74 (2.57, 6.34) *[†‡]

[‖]Urinary fluoride was represented as median (P25, P75); *compared with ≤1.2 mg/L of water fluoride exposure, $p<0.05$; [†]compared with 1.2–2 mg/L of water fluoride exposure, $p<0.05$; [‡]compared with 2–4 mg/L of water fluoride exposure, $p<0.05$; [§]compared with >4 mg/L of water fluoride exposure, $p<0.05$.

Skeletal fluorosis miRNA profiles in serum: To identify the pathogenesis associated with the miRNAs of skeletal fluorosis, we independently chose 3 subjects from the cases, the controls, and the high fluoride group. Global miRNA profile analyses revealed, 31 miRNAs were significantly and differentially expressed between the cases and the controls. Of these, 21 miRNAs were up-regulated and 10 miRNAs were down-regulated. The top 10 of the differentially expressed miRNAs were sorted by the p value of significance (Table 3).

Table 3. Differentially expressed miRNAs between cases and controls.

Transcript ID	Cases	Controls	Change fold	p value	miRNA expression
miR-200c-3p	6.192643	1.785686	21.214175	0.001469	Up
miR-3185	7.771091	6.008617	3.392795	0.001888	Up
miR-7112-5p	4.45977	2.048765	5.318447	0.002168	Up
miR-1231	6.635748	4.133944	5.66393	0.002448	Up
miR-378a-3p	6.10493	4.610854	2.816836	0.002587	Up
miR-584-5p	2.88922	5.732619	–7.177087	0.004266	Down
miR-150-5p	2.982885	5.220921	–4.717546	0.004685	Down
miR-20b-5p	1.997102	3.981442	–3.956815	0.005245	Down
miR-126-3p	3.178232	5.916431	–6.672369	0.005385	Down
miR-15b-5p	2.02889	4.28711	–4.784009	0.005524	Down

Compared to the high fluoride group, the number of significant and differentially expressed miRNAs was 85 in the cases, all of which were up-regulated. Table 4 shows the top 10 differentially expressed miRNAs between the cases and the high fluoride group. In addition, 58 miRNAs were significantly expressed between the control group and the high fluoride group. Forty-four miRNAs were up-regulated and 14 miRNAs were down-regulated. The top 10 differentially expressed miRNAs are listed in Table 5. By comparing the differentially expressed miRNAs in Tables 3, 4, and 5, three miRNAs (miR-200c-3p, miR-3185, and miR-1231) in the cases were up-regulated compared with the control group and the high fluoride group.

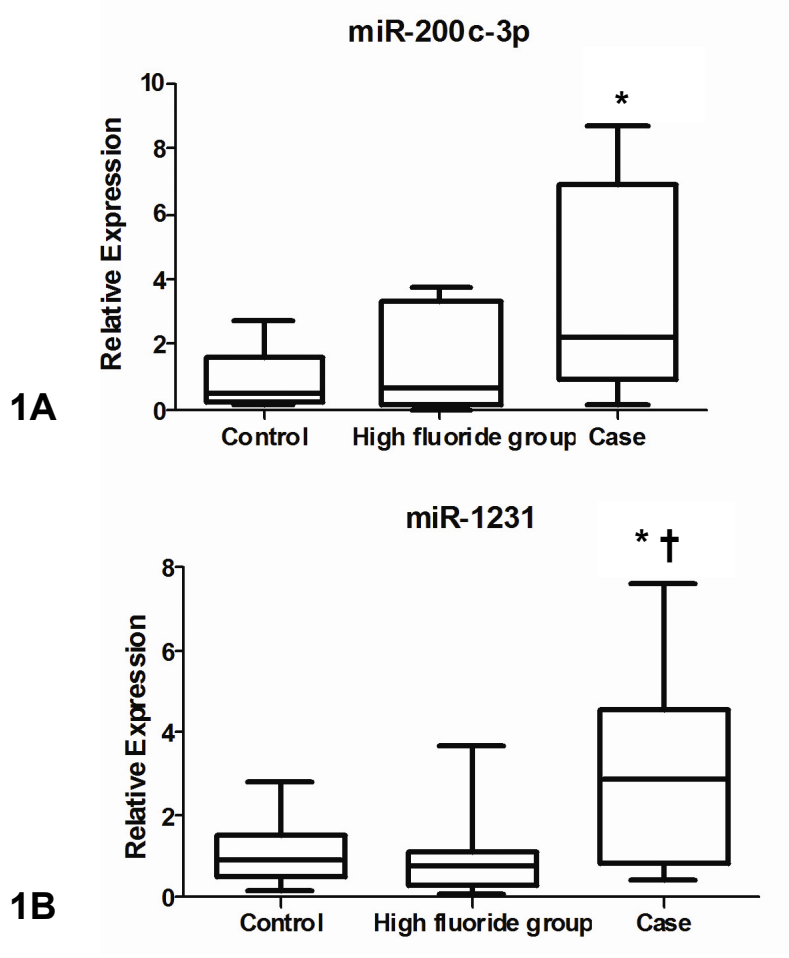
Table 4. Differentially expressed miRNAs between the case group and the high fluoride group.

Transcript ID	Cases	High fluoride group	Change fold	p value	miRNA expression
miR-200c-3p	6.192643	1.584963	24.380903	0.001458	Up
miR-7641	7.447138	2.625497	28.27865	0.001597	Up
miR-1231	6.635748	2.821773	14.064383	0.001736	Up
miR-4530	8.132647	4.992789	8.814371	0.002014	Up
miR-3185	7.771091	4.967602	6.981271	0.002153	Up
miR-6075	5.368999	2.74134	6.180225	0.002292	Up
miR-3937	5.516078	2.779355	6.665547	0.002431	Up
miR-4467	7.921077	5.619079	4.931401	0.002708	Up
miR-6800-5p	8.630873	6.017043	6.121266	0.002847	Up
miR-6068	6.204515	3.391975	7.025202	0.002986	Up

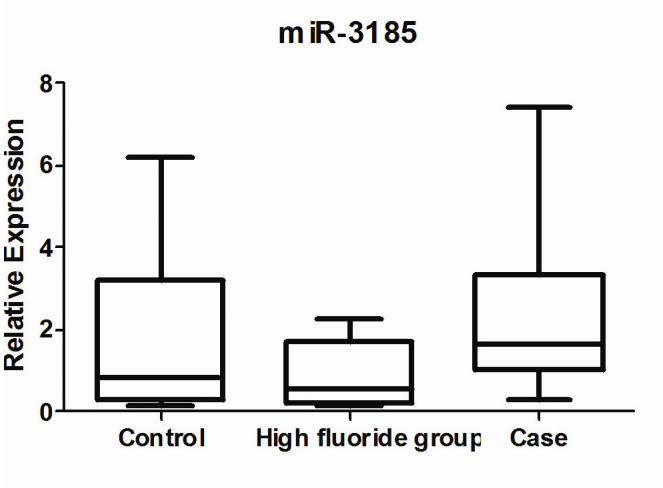
Table 5. Differentially expressed miRNAs between the control group and the high fluoride group.

Transcript ID	Control group	High fluoride group	Change fold	p value	miRNA expression
miR-106b-5p	2.326395	5.193692	7.296964	0.001464	Up
miR-652-3p	1.992205	5.071963	8.45473	0.001604	Up
miR-6068	3.391975	6.003144	6.109984	0.001743	Up
miR-30d-5p	2.495846	5.574966	8.450988	0.001883	Up
miR-6753-3p	5.53448	3.932219	–3.036188	0.002022	Down
miR-7160-5p	4.960903	3.452555	–2.844842	0.002162	Down
miR-6781-5p	3.858219	4.947547	2.127748	0.002301	Up
miR-6800-5p	6.017043	8.029502	4.034693	0.002441	Up
miR-22-3p	6.614089	7.949203	2.522954	0.00258	Up
miR-150-5p	2.190194	5.220921	8.172218	0.00272	Up

Validation of taqman quantitative PCR: Based on the above results, three miRNAs (miR-200c-3p, miR-3185, and miR-1231) were chosen for the PCR validation test in the serum of 15 cases, 15 controls, and 15 subjects in the high fluoride group. Figures 1A, 1B, and 1C, show the relative expression levels determined by quantitative PCR of each miRNA. As a result, the three selected miRNAs (miR-200c-3p, miR-3185, and miR-1231) were successfully detected in the serum. These three miRNAs were up-regulated in the cases. The miR-200c-3p expression showed an increasing trend in the control group, the high fluoride group, and the case group. The miR-200c-3p expression in the cases was significantly higher than that in the controls but was not significantly different from the values in the high fluoride group. A significant increase of the miR-1231 expression in was also observed in the cases compared to the control and high fluoride groups. There was no significant difference in miR-1231 expression between the control group and the high fluoride group.



Figures 1A and 1B. Validation of microRNA expression by real-time PCR. Relative expression levels of each miRNA after normalization to miR-16 (n = 15). 1A: relative expression of miR-200c-3p; and 1B: relative expression of miR-1231. The data are represented as median (P25, P75). *compared with controls: $p<0.05$; †compared with the high fluoride group: $p<0.05$.



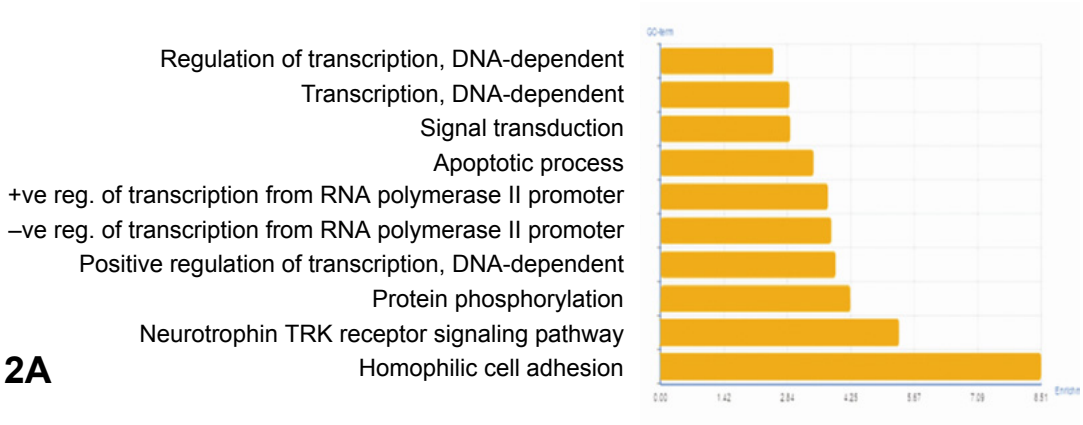
1C

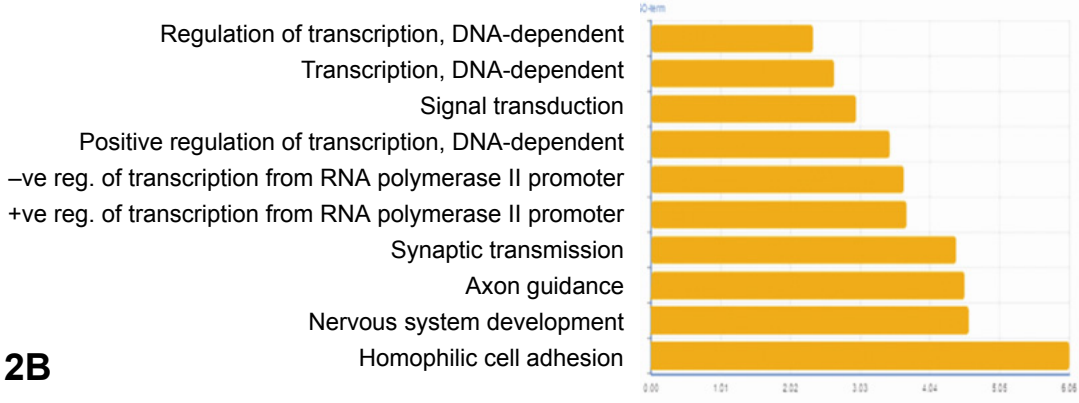
Figure 1C. Validation of microRNA expression by real-time PCR. Relative expression levels of each miRNA after normalization to miR-16 (n = 15). 1C: relative expression of miR-3185. The data are represented as median (P25, P75). *compared with controls: $p<0.05$; †compared with the high fluoride group: $p<0.05$.

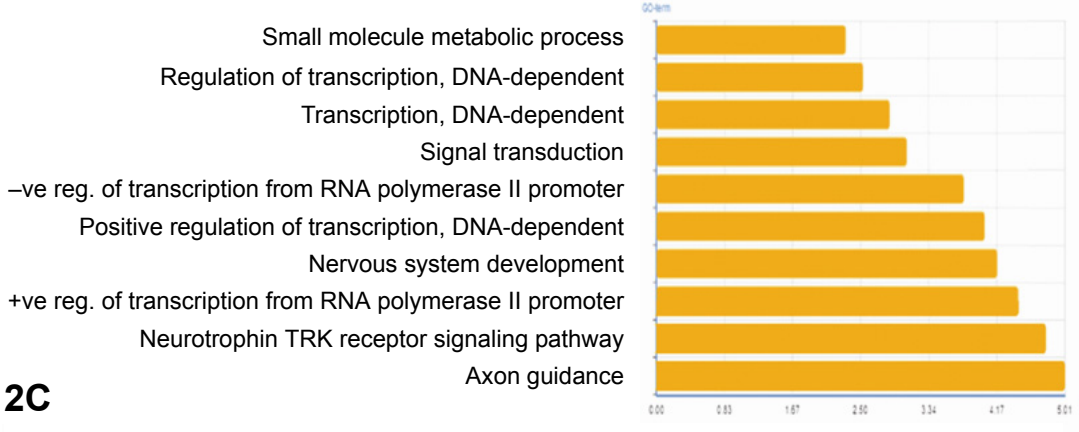
Although the miR-3185 expression of the cases was the highest amongst the 3 groups, no significant difference was observed compared to the control and high fluoride groups.

From the results above, the expression trend of the 3 selected miRNAs was similar in the miRNA arrays and the PCR validation test, which indicates that the differentially expressed miRNAs in the arrays test were reliable and could be used in further bioinformatic analyses.

GO and pathway analysis of the differentially expressed miRNAs: In order to gain insights into the functions of these skeletal fluorosis-related miRNAs, target genes of the differentially expressed miRNAs were predicted using TargetScan and miRDB. GO and KEGG pathway analysis were applied to their target pool. The number of target genes of the differentially expressed miRNAs were 4,013 between the case group and the control group, 7,742,between the case group and the high fluoride group, and 10,482 between control group and the high fluoride group. GO analysis revealed the target genes of the 3 groups had common enrichment GOs. Figures 2A, 2B, and 2C show that the high enrichment GOs targeted by the skeletal fluorosis-related miRNAs were transcription (DNA-dependent), positive regulation of transcription from RNA polymerase II promoter, signal transduction, positive regulation of transcription, DNA-dependent, negative regulation of transcription from RNA polymerase II promoter, and regulation of transcription (DNA-dependent).

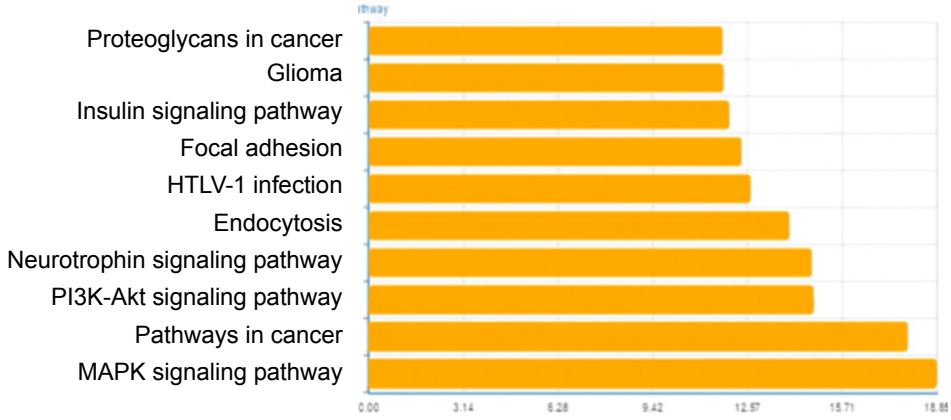




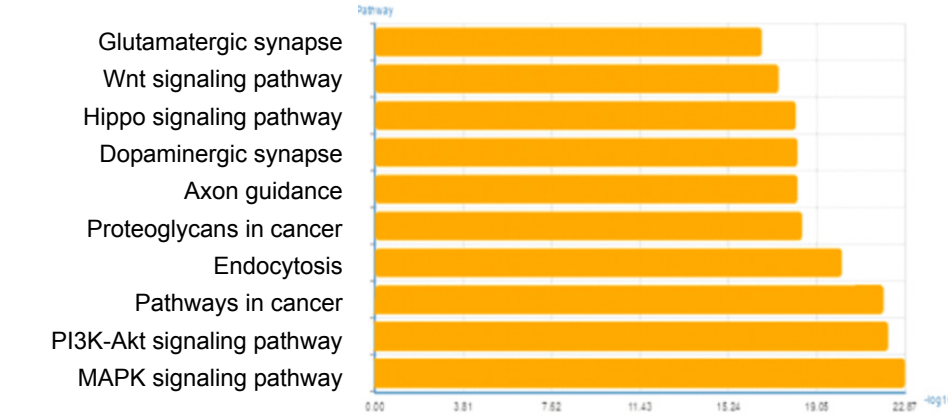


Figures 2A, 2B, and 2C. GO analysis expressed miRNAs. GO analysis based on differentially expressed miRNA target genes. The vertical axis is the GO category and the horizontal axis is the negative logarithm of p value (–Log p), that represents the significant level of GOs. 2A: GO analysis of 31differentially expressed miRNAs between case group and control group; 2B: GO analysis of 85differentially expressed miRNAs between case group and high fluoride group; 2C: GO analysis of 58differentially expressed miRNAs between control group and high fluoride group; +ve reg.= positive regulation; –ve reg.=negative regulation.

Another functional analysis of the miRNAs by KEGG revealed the pathways regulated by the differentially expressed miRNAs (Figures 3A, 3B, and 3C). MAPK signaling pathway, pathways in cancer, PI3K-Akt signaling pathway, proteoglycans in cancer, and endocytosis were the 5 common pathways in the 3 groups.



3A



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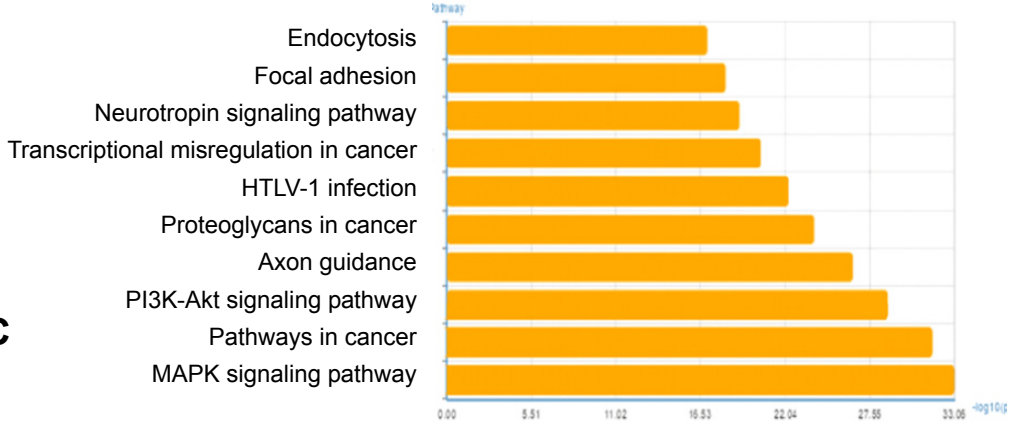

3C

Figures 3A, 3B, and 3C. KEGG pathway analysis of the differentially expressed miRNAs. Pathway analysis based on differentially expressed miRNA target genes. The vertical axis is the pathway category, and the horizontal axis is the negative logarithm of p value (–Log p), that represents the significance level of the pathways. 3A: KEGG pathway analysis of 31 differentially expressed miRNAs between the case group and the control group. 3B: KEGG pathway analysis of 85 differentially expressed miRNAs between the case group and the high fluoride group. 3C: KEGG pathway analysis of 58 differentially expressed miRNAs between the control group and the high fluoride group.

DISCUSSION

The clinical manifestations of skeletal fluorosis are joint pain, limitation of joint movement, and crippling deformities. Although fluorosis can be controlled and prevented by decreasing the fluoride intake, the clinical manifestations of patients with skeletal fluorosis are difficult to reverse. There is no specified and effective therapy available for skeletal fluorosis because its pathogenesis is still unclear.

Some studies have demonstrated that miRNAs play an important role in the development and differentiation of osteoblasts and osteoclasts.[11] In recent times, researchers have had their attention drawn to the relationship between miRNAs and fluorosis. An *in vitro* study of the miRNA array showed that, compared with the control group, 82 and 62 differentially expressed miRNAs were found in human osteosarcoma cells treated with 8 mg/L and 20 mg/L sodium fluoride (NaF), respectively.[6] The miRNA profile of MC3T3-E1 cells treated with 8 mg/L NaF have also been found to be dysregulated, with 45 miRNAs being up-regulated and 31 miRNAs being down-regulated.[8] The effect of fluoride on the miRNA profile has also been observed in an animal fluorosis model. The rats were fed with NaF at the concentrations of 25 mg/L and 100 mg/L in drinking water for 60 days. The miRNA profile of the rat sperm revealed that, compared with the control group, 31 miRNAs were significantly down-regulated in both of the two fluoride treatment groups.[7]

The results from these studies indicate that fluoride can induce a dysregulation of the miRNA profile in both cells and animals. However, the differentially expressed miRNAs of each screening study were different in these studies and there was no miRNA profile data from a field survey of a population. In the present study we have reported, for the first time, the miRNA profile in the serum of patients with skeletal fluorosis. In our microarray, 31 differentially expressed miRNAs were found between the cases and the controls, and 85 between the cases and the high fluoride groups. In addition, 3 miRNAs (miR-200c-3p, miR-1231 and miR-3185) were significantly up-regulated in the cases. The expressed trend of these 3 miRNAs was also validated by quantitative PCR. The high consistency of these 3 miRNAs between the miRNA microarray and the PCR indicated that our microarray produced reliable data which can be used in further bioinformatics analyses.

Dysregulation of molecular signaling pathways are involved in the process of fluoride-induced damage of osteoblasts and osteoclasts. It has been observed that RUNX2, Wnt, TGF-β, and other signaling pathways participate in the regulation of the bone turnover of skeletal fluorosis.[12-14] However, the regulatory mechanism of fluoride on molecular pathways is still not very clear. Previous studies revealed that miRNAs could regulate the activity of the Wnt and TGF-β signaling pathways.[15] Excessive fluoride increased the expression levels of miR-124 and miR-155 in osteosarcoma cells. The gene transcription of RUNX2 and RANKL, which are required for osteocyte differentiation, are regulated by these 2 miRNAs.[6] The miRNA microarray studies in cells and animals show that the MAPK, Wnt, TGF-β, VEGF, and Notch signaling pathways might be regulated by the differentially expressed miRNAs between the fluoride treatment groups and the control groups.[7,8] These molecular pathways are closely associated with the regulation of osteocyte differentiation and function.[16-19] In the present study, GO analysis suggested the high enrichment GOs targeted by the skeletal fluorosis-related miRNAs were transcription

(DNA-dependent), positive regulation of transcription from RNA polymerase II promoter, and signal transduction, etc. KEGG analysis indicated several pathways which might be regulated by the differentially expressed miRNAs. Some of these signaling pathways, such as the MAPK and PI3K-Akt pathways, have been demonstrated to participate in skeletal fluorosis.[20-22] This represents novel evidence for the modulator roles of miRNAs in skeletal fluorosis. Other pathways, such as pathways in cancer, proteoglycans in cancer, and the endocytosis signaling pathway may indicate new mechanisms of skeletal fluorosis, which will need to be clarified with further studies.

However, there are some limitations in this study: (i) due a restriction in the research funding, the serum samples of the subjects exposed to fluoride at 1.2–≤2 mg/L and 2–≤4 mg/L were not selected for the miRNA screening study; (ii) only three miRNAs of the differentially expressed miRNAs were validated by quantitative PCR, because the amount of serum from the subjects was insufficient to satisfy the test requirements for additional testing. The remaining differentially expressed miRNAs will be validated in a further study; (iii) larger studies are needed to confirm the results of our study; (iv) gain/loss-of-function and mimics/inhibitor biological studies should be performed to completely validate their functions.

CONCLUSIONS

In summary, this study is the first to identify the miRNAs profile of skeletal fluorosis in the population. A dysregulation of serum miRNAs expression was found in the patients with skeletal fluorosis. These differentiated miRNAs may be involved in gene transcription, such as DNA-mediated transcription, etc., and in the regulation of many molecular signaling pathways. The unique miRNAs expression data obtained in this study provide a new insight into the molecular mechanisms and biomarkers of skeletal fluorosis.

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CONFLICTS OF INTERAST

There are no conflicts of interest.

ETHICAL STATEMENT

The study was approved by the Ethical Review Board of Harbin Medical University. All participants signed informed consent, and written informed consent was obtained from guardians of minors. The locations were not privately owned or protected in any way, and this field study did not involve endangered or protected species. The methods were carried out in accordance with the approved guidelines.

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