MiR-1a-3p inhibits apoptosis in fluoride-exposed LS8 cells by targeting Map3k1

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Abstract

Dental fluorosis is a chemical disease that is common. At present, it is not clear how fluorosis happens at the molecular level. We have used miRNA-seq to look at the differences between miRNAs in the cell line of ameloblasts LS8 cells that had been treated with 3.2 mmol/L NaF. and did GO and KEGG pathway enrichment analysis. We found that the levels of miR-1a-3p were significantly lower in mouse LS8 cells treated with 3.2 mmol/L NaF, and the genes that miR-1a-3p targets were significantly enriched in the MAPK pathway. The LS8 cells were divided into four groups: the control, NaF, NaF + miR-1a-3p mimics and NaF + miR-1a-3p mimics NC groups. The morphology of the cells were observed by an inverted microscope. We detected the proliferation activity of LS8 cells by Cell Counting Kit-8 (CCK-8). Using the real-time quantitative polymerase chain reaction (RT-qPCR), we detected the transcription levels of miR-1a-3p and Map3k1. The expressions of Bax, Bcl-2, Map3k1, p38MAPK, ERK1/2, p-p38MAPK and p-ERK1/2 were measured by Western Blot. After doing a bioinformatics analysis, we used a luciferase reporter assay (LRA) to validate the target of miR-1a-3p. Our results indicated that miR-1a-3p could inhibit the apoptosis while increasing proliferation in fluoride-exposed LS8 cells. In general, miR-1a-3p may directly inhibit Map3k1, reduce MAPK signal pathway activation, and promote phosphorylation. Thus, Our findings revealed that miR-1a-3p's interaction with its target gene Map3k1 and MAPK signal pathway might decrease LS8 cells’ apoptosis treated with 3.2 mmol/L NaF.

1. Introduction

Endemic fluorosis is a chemical disease that affects people all over the world. It is also one of the most serious diseases that happen naturally in China. Dental fluorosis is a precursor and specific indicator of chronic fluorosis in the oral cavity, and it has had a significant impact around the world. When dental fluorosis happens, it not only changes how a person looks, but it can also affect their mental health in different ways. According to the results of the fourth oral epidemiological survey, 13.4% of 12-year-olds in China have dental fluorosis[1]. At present, it is clear why tooth fluorosis happens, but the molecular mechanism of dental fluorosis is not completely clear. Due to abnormal post-transcriptional cascades that cause bone and teeth deformities are unknown, there is no prevention and treatment of dental fluorosis. LS8 cells are the key cells in the development of enamel. A large number of studies have shown that excessive fluoride can damage LS8 cells and cause dental fluorosis[2-3]. It has been known for a long time that the MAPK pathway is an important one that affects the formation of enamel, matrix secretion and cusp formation[4]. Odontoblast differentiation is also affected by MAPK signal pathway[5]. Fluoride can mediate the expressions of downstream genes through MAPK signal pathway to participate in the formation of dental fluorosis[6-8].

microRNA (miRNA) is an endogenous (18-25nt) non-protein coding RNA molecule that regulates post-transcriptional gene expression by pairing with the 3’UTR of the target mRNA. Many pathophysiological processes are regulated by miRNAs[9]. Studies have shown that miRNAs play an important role in enamel development by regulating epithelial cell differentiation, enamel mineralization and extracellular matrix receptor interaction. In recent years, epigenetic studies on fluorosis have emerged. Some studies have
reported that miRNAs affect epigenetic modification by regulating target genes, and miRNAs and fluoride are related closely. Excessive fluoride exposure may significantly disrupt the expression pattern of miRNA[10–12]. So far, the important role of miRNA in fluorosis and tooth development has been widely recognized. Therefore, our team thought that the process of ameloblast apoptosis caused by excessive fluoride might involve the different ways that miRNAs are expressed. The experimental results showed that excessive sodium fluoride promoted the apoptosis of LS8 cells and inhibited their proliferation, and the effect increased with concentration and time. In this study, we analyzed the previous sequencing data to find the differentially expressed miRNAs of LS8 cells into apoptosis by 3.2 mmol/L NaF and explore the mechanism of regulating fluoride-stained LS8 cells’ apoptosis.

2. Materials and methods

2.1. Cell source

The cell line of mouse ameloblasts LS8 was donated by Professor Malcolme Snead of University of Southern California and Professor Xiaohong Duan of the fourth military Medical University.

2.2. Bioinformatics analysis of differentially expressed miRNAs in LS8 cells treated with 3.2 mmol/L for 24 h

Bioinformatics analysis of LS8 cells treated with 3.2 mmol/L NaF for 24h showed that some miRNAs were expressed differently than others. This information came from the research group's previous miRNA sequencing data. Through screening, we got the differentially expressed miRNAs of LS8 cells treated with high concentration of fluoride. A total of 2 groups: 0 mmol/L and 3.2 mmol/L groups. The screening criteria for differential miRNAs are: $|\log_2 FC| \geq 1.5$, Q value $< 0.05$. Target genes were predicted and screened by "multimiR package" in R language (sum $\geq 4$). Using the R language "clusterprofiler 4.0 package", the GO and KEGG pathway enrichment analysis of each miRNA predicted differential target gene was performed.

2.3. Cell culture

LS8 cells were cultured using D-MEM(Livning,China) high glucose medium with 10% fetal bovine (Livning,China) serum and 1% penicillin-streptomycin(Livning,China) mixture, cultured cells in a 5% CO$_2$ incubator at 37 °C. In the same way, the cells were divided into two groups: 0 mmol/L and 3.2 mmol/L groups. LS8 cells were inoculated into a 10-mm-diameter Petri dish at a rate of $1 \times 10^4$/mL. The 0 mmol/L group did not add NaF, and other culture conditions were the same as above. 24 h later, the morphology of cells was observed under an inverted microscope and photographed.

2.4. Transfection

MiR-1a-3p mimics(5’-CGCACGCCTGGAATGTAAGAGTATG-3’) and miR-1a-3p mimics NC were purchased from Ribobio (Guangzhou,China). LS8 cells were inoculated into 6-well plates with $1 \times 10^4$ /mL. The experiment was divided into control group, NaF group, NaF + miR-1a-3p mimics and NaF +
mimics NC groups. When the cell growth density reached 30–50%, the cells were transfected according to the instructions in the transfection kit. After 24 h of growing in the incubator, the cells were treated 3.2 mmol/L of NaF in a medium for 24 h.

2.5. RNA isolation and qPCR

The Total RNA Extraction Kit (Solarbio, China) was used to perform RNA extractions. For miRNA research, RNAs were reversed into cDNA by miRNA First Strand cDNA Synthesis (Sangon, China). qPCR was performed on the Detection system with Universal SYBR® Green Master Mix (Roche Light Cycler 96). β-actin and U6 were used as internal references. The results of cell lines were computed by the $2^{-\Delta\Delta Ct}$ method.

2.6. Cell proliferation

LS8 cells of each group was inoculated at a density of $1 \times 10^4$ cells per well on 96-well plates. When the cell growth density was about 30%-50%, the cells were transfected for 24 h, then treated with 3.2 mmol/L NaF for 24 h. The CCK-8 detection kit's instructions were followed, and the absorbance of each well was measured using the enzyme-linked immunosorbent assay at 450nm.

2.7. Western blot

The Bax, Bcl-2 and β-actin antibody were purchased from meilunbio (Dalian, China); the p38MAPK, p-p38 MAPK, ERK1/2, p-ERK1/2 antibody were purchased from Ambart. The proteins were then exposed and analyzed with ImageJ software.

2.8. Luciferase reporter assay

The wild-type and mutant plasmids were constructed by Ribobio. The m-Map3k1-WT and m-Map3k1-MUT were the wild-type and mutant 3'UTR recombinant plasmids. The plasmids were co-transferred into LS8 cells. After 48 h of induction, the double luciferase report kit was used to measure fluorescence and normalize the final intensity.

2.9. Statistical analysis

All measurement data were expressed as mean ± S.E.M. Difference between groups was analyzed with the t-test and one-way analysis of variance for multiple comparisons. $P < 0.05$ presented a statistically significant difference.

3. Results

3.1. Bioinformatics analysis of differential expressions of miRNAs in LS8 cells treated with 3.2 mmol/L NaF

Our previously miRNA sequencing data from BGI, and the differentially expressed miRNAs of 3.2 mmol/L and 0 mmol/L NaF treatment groups were analyzed to fluoride-induced enamel cell apoptosis miRNA.
The 28 differential miRNAs were screened from 1177 miRNAs. Figure 1 showed that mmu-miR-1a-3p was significantly down-regulated.

The red dots represent differential MiRNAs that are up-regulated while the blue represent down-regulated ones

**3.2. GO and KEGG enrichment analysis**

GO and KEGG pathway enrichment were performed differential miRNA form target genes. It was found that the target genes of miR-1a-3p significantly enriched in biological processes such as apoptosis, gene expression, protein synthesis and RNA synthesis. Further pathway enrichment analysis of predicted target genes showed that the differential miRNAs were enriched to 32 pathways, of which MAPK pathway enrichment was the most prominent (Fig. 2).

**3.3. Morphological changes and miR-1a-3p expression of fluoride-stained LS8 cells**

The normal LS8 cells were fusiform or triangular, and it was in good shapes with tight connections (Fig. 3A). When treated with 3.2 mmol/L NaF for 24 h, LS8 cells shrunk, apoptotic cells increased, and cells were successfully stained with fluoride (Fig. 3B). RT-qPCR showed that the expression of miR-1a-3p in 3.2 mmol/L NaF group was significantly lower than the 0 mmol/L group \((P < 0.05)\). The results were consistent with the sequencing data (Fig. 3C).

**3.4. Effects of miR-1a-3p on morphology, proliferation and apoptosis of fluoride-exposed LS8 cells**

After transfection of miR-1a-3p mimics into LS8 cells, the expression of miR-1a-3p increased significantly \((P < 0.01)\). Compared with the control group, the floating cells in NaF, NaF + miR-1a-3p mimics and NaF + miR-1a-3p mimics NC groups significantly increased and shrank in different degrees after fluoride exposure. After miR-1a-3p mimics transfection, LS8 cells proliferated well but changed from long fusiform to short fusiform. The cells could still form a grid and had no morphological changes (Fig. 4C). After fluoride treatment, the NaF, NaF + miR-1a-3p mimics, and NaF + miR-1a-3p mimics NC groups had decreased significantly cell proliferation activity than the control group, while the cell proliferation activity of NaF + miR-1a-3p mimics group was significantly higher than NaF group. NaF + mimics NC and control groups had similar cell viability (Fig. 4B). The expression of Bax increased, Bcl-2 decreased, and Bax/Bcl-2 increased in NaF group compared to control group, but there was no significant difference between NaF + miR-1a-3p mimics NC and NaF groups. Compared with NaF group, overexpression of miR-1a-3p inhibited the apoptosis-inducing effect of excessive fluoride on LS8 by decreasing the expression of proteins Bax, Bcl-2 and Bax/Bcl-2 in NaF + miR-1a-3p mimics group (Fig. 4D). To sum up, compared with NaF group, overexpression of miR-1a-3p can inhibit the apoptosis and promote the proliferation of fluoride-stained LS8.
3.4 miR-1a-3p targeting \textit{Map3k1} regulates MAPK signal pathway

Western Blot was used to detect the expression of MAPK pathway-related proteins p38MAPK, ERK1/2 and their phosphorylated forms in each group (figure.5A-B). The results showed that NaF group had higher MAPK signal pathway proteins p38MAPK and ERK1/2 than the control group, but lower p-p38MAPK and p-ERK1/2 expressions. In order to further explore the target genes of MAPK signal pathway that are regulated by miR-1a-3p. Furthermore, we found the MAPK signal pathway has 9 differential target genes controlled by miR-1a-3p (Table 1). The target gene \textit{Map3k1} of miR-1a-3p is located in the central node of MAPK pathway (figure.5C), which is involved in cell proliferation and apoptosis. Therefore, we picked it as the object of study. Through the target gene prediction analysis, it was found that there was a predicted binding site (Fig. 5E) in the seed region sequence of miR-1a-3p and \textit{Map3k1}'s 3'UTR (Fig. 5E), and the \textit{Map3k1} 3'UTR seed region sequence was highly conserved across species (Fig. 5D). The fluorescence activity of m-Map3k1-WT and m-Map3k1-MUT was analyzed after cotransfection with mmu-miR-1a-3p mimics and mmu-miR-1a-3p mimics NC into LS8 cells. Luciferase reporter assay demonstrated that m-Map3k1-WT had lower fluorescence expression than m-Map3k1-MUT ($P < 0.01$). Then the mRNA and protein expressions of Map3k1 were detected in each group. The results showed that overexpression of miR-1a-3p inhibited the transcriptional and translational levels of Map3k1 in fluoride-stained LS8 (Fig. 5G-H). Accordingly, miR-1a-3p can directly target Map3k1 to regulate MAPK pathway, inhibiting the apoptosis of LS8 at high fluoride concentration.

<table>
<thead>
<tr>
<th>gene</th>
<th>Position in the UTR</th>
<th>seed match</th>
</tr>
</thead>
<tbody>
<tr>
<td>Map3k1</td>
<td>1593–1599</td>
<td>7mer-m8</td>
</tr>
<tr>
<td>Bdnf</td>
<td>213–219</td>
<td>7mer-m8</td>
</tr>
<tr>
<td>Rasa1</td>
<td>118–125</td>
<td>8mer</td>
</tr>
<tr>
<td>Igf1</td>
<td>186–193</td>
<td>8mer</td>
</tr>
<tr>
<td>Nfatc3</td>
<td>2232–2238</td>
<td>7mer-m8</td>
</tr>
<tr>
<td>Atf2</td>
<td>1326–1333</td>
<td>8mer</td>
</tr>
<tr>
<td>Met</td>
<td>537–543</td>
<td>7mer-m8</td>
</tr>
<tr>
<td>Rap1b</td>
<td>66–72</td>
<td>7mer-1A</td>
</tr>
<tr>
<td>Braf</td>
<td>6565–6571</td>
<td>7mer-m8</td>
</tr>
</tbody>
</table>

Position in the UTR: indicates the binding site of miRNA and target gene seed match: 8mer > 7mer-m8 > 7mer-1A > 6mer.
4. Discussion

At present, the mechanism of dental fluorosis is complicated, and several investigations are underway. The transcriptome alterations in LS8 cells before and after fluoride exposure have not been reported. This study analyzed the effects of high fluoride on LS8 cells miRNA expression using miRNA-seq. It was found that miR-1a-3p was significantly down-regulated in fluoride-exposed LS8, and its target genes significantly enriched MAPK signal pathway, which was verified by RT-qPCR. In vitro, miR-1a-3p promoted the proliferation and inhibited the apoptosis of fluoride-stained LS8.

MiR-1 is encoded by two precursor miRNA, miR-1-1 and miR-1-2, and then processed into mature miR-1 by Dicer enzyme[13]. MiR-1 can regulate the proliferation, differentiation and apoptosis of many kinds of cells. In research, the miR-1 family is composed of miR-1-5p and miR-1-3p, and miR-1-like refers to miR-1-3p. The down-regulation of miR-1-3p leads to a variety of functional disorders in the body. The overexpression of miR-1-3p can reduce the expression of SFRP1, thus promotes bone formation and mineral density, and prevents osteoporosis. Nakamura and other studies found that the expression of miRNA-1 in mouse tooth germs lasted until 16.5 days and gradually decreased on the 1st and 3rd day after birth, and decreasing the proliferation rate of tooth epithelial cells. This indicates that miR-1 is essential to tooth germ development[14].

The ERK(extracellular signal-regulated kinase,ERK), p38 and c-jun N-terminal kinases are highly conserved serine-threonine protein kinases in the MAPK family. ERK1/2, downstream of p38MAPK, is composed of ERK1 and ERK2, which perform the same function[15]. MAPK signaling pathway is activated by a variety of extracellular and intracellular stimuli, such as oxidative stress and endoplasmic reticulum stress, which regulates cell proliferation, differentiation, survival and death. The formation of tooth hard tissue and tooth morphology depend on P38MAPK signal pathway. While the ERK signal pathway regulates tooth development and tooth enamel production[16]. The Phosphorylated MAPK family inhibits apoptosis by increasing Bcl-2, an anti-apoptotic protein, and decreasing Bax, a pro-apoptotic protein. Under non-phosphorylation, Bax can form a heterodimer named Bax/Bcl-2 with Bcl-2, which makes Bcl-2 lose its function of the mitochondrial outer membrane integrity and induces cell apoptosis. Phosphorylation of ERK1/2 in dental epithelial cells plays an important role in enamel formation. Phosphorylated ERK1/2 is highly expressed in LS8 of mandibular molars and incisors[5]. However, excessive fluoride can inhibit enamel mineralization by decreasing the phosphorylation of p38MAPK and ERK1/2 in LS8. Similarly, JNK signal pathway is also one of MAPK signal pathways, NaF-induced apoptosis in LS8 cells is also closely related to the decreased JNK phosphorylation[17], and inhibiting the JNK signal pathway can reduce NaF-induced LS8 cell apoptosis[18]. The results show that fluoride exposure increases p38MAPK and ERK1/2 expression in LS8. The expression of phosphorylated protein p-p38MAPK and p-ERK1/2 decreased, while the expression of anti-apoptotic protein Bcl-2 decreased and the expression of pro-apoptotic protein Bax increased, indicating that excessive fluoride may increase the expression of MAPK signaling proteins p38MAPK and ERK1/2, reduce the expression of p-p38MAPK and p-ERK1/2, weaken phosphorylation and apoptosis inhibition, and promote ameloblast apoptosis, which is highly consistent with the above results. However, the overexpression of miR-1a-3p reversed this process by inhibiting the MAPK signal pathway protein p38MAPK and its downstream signal molecule ERK1/2, and increasing the expression of phosphorylated protein.
proteins in fluoride-stained LS8, thus promoting tBcl-2, inhibiting Bax, and increasing the proliferation activity.

The complementarity between miRNA targets and seed regions, the conservation of miRNA target sites across species, and the thermal stability of miRNA-mRNA double strands are used in computer software target prediction[19]. There is a 7nt matching region between the target gene and miRNA, so the seed region of microRNA is the 2nd-8th nucleotides of miRNA (7mer-m8) or the 2nd-7th (7mer-A1)[19].

The 7mer-m8 region of Map3k13'UTR, which is in the seed region, is where miR-1a-3p and Map3k1 connect. Secondly, the sequence and function of the miR-1 family are very similar across species, and the Map3k13'UTR gene is very reliable across species. The complementary free energy of miRNA/mRNA is thermodynamic stability. When microRNA binds to mRNA, after it has a higher affinity, the subsequent double strands have reduced free energy. To meet the above requirements, you need to think about where the binding site is. The binding site must be at least 15nt away from the base of the termination codon[20], and the binding site of miR-1a-3p and Map3k1 target gene is at 3'UTR 1593-1599bp. Meeting fully the above requirements. Map3k1, positioned above ERK1/2 and JNK in MAPK signal pathway, regulates many aspects of cell physiology. Like a fate switch, Map3k1 possesses anti- and pro-apoptotic functions. And, Map3k1 activates MAPK signaling to increase cell proliferation.

Map3k1 can also ubiquitinate c-Jun and ERK1/2, leading to their degradation [36]. In this study, excessive fluoride may promote the apoptosis of LS8 by promoting Map3k1 expression. These results indicate that miRNAs can affect cell proliferation by interacting with Map3k1. We found that the target of miR-1a-3p was in the 3'-UTR region of Map3k1 mRNA, which is written as "5 minutes m-Map3k1-WT ACAUCCMUCC 3".

After co-transfection with miR-1a-3pmimics, the luciferase activity of m-Map3k1-WT was significantly lower than m-Map3k1-MUT. In vitro, miR-1a-3p supported Map3k1. And, the overexpression of miR-1a-3p increased the expressions of Map3k1 mRNA and protein. Based on double luciferase assay, miR-1a-3p directly negatively regulated the expression of Map3k1. On the other hand, ERK1/2 is downstream of p38MAPK, whereas Map3k1 protein is upstream. In this study, fluoride-induced LS8 down-regulated the expression of miR-1a-3p, which directly targeted Map3k1, activated downstream signal pathways p38MAPK and ERK1/2, and finally decreased the apoptosis and proliferation of LS8, while the overexpression of miR-1a-3p reversed this process.

This discovery not only reveals the new mechanism of post-translational gene regulation of Map3k1, but also broadens the biological function of miR-1a-3p by directly negatively regulating the MAPK signal pathway to Map3k1, which is important in the apoptosis of LS8 induced by excessive fluoride. However, the specific molecular mechanism of how the target gene Map3k1 of miR-1a-3p affects the apoptosis of fluoride-exposed LS8 is still being researched.

5. Conclusion
MiR-1a-3p can inhibit the apoptosis and promote the proliferation of fluoride-exposed LS8. The mechanism may be related to the direct targeting of miR-1a-3p to inhibit Map3k1, which inhibits the activity of MAPK signal pathway and promotes its phosphorus.

Declarations

CRediT authorship contribution statement

Ting Chen, Yu Gu: Conceptualization, Methodology, Visualization, Data curation, Formal analysis, Writing original draft. Guo-Hui Bai: Conceptualization, Methodology, Visualization, Funding acquisition. Bin Chen: Formal analysis, Resources, Data curation. Xia Liu: Visualization, Investigation. Qin Fan: Visualization, Investigation. Yuan Tian: Project administration, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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Figures
Figure 1

Differential miRNAs volcano map (R ggplot2 package)

The red dots represent differential MiRNAs that are up-regulated while the blue represent down-regulated ones.
Figure 2

Partially down-regulated (A) and up-regulated (B) differential miRNAs target gene prediction map. MiR-1a-3p target gene GO functional enrichment map (C) and KGEE pathway enrichment map (D).
Figure 3

Morphology of LS8 cells in normal group (A) and NaF group (B). The expression of :miR-1a-3p in LS8 cells decreased significantly (C). * compared with 0 mmol/L group, $P < 0.05$. 
Figure 4

The expressions of miR-1a-3p were significantly increased after miR-1a-3p mimics transfection into LS8 cells (A). MiR-1a-3p promoted the proliferation of fluoride-stained LS8 cells (B), induced the growth of fluoride-stained LS8 cells and slightly changed their morphology (C) and inhibited the apoptosis of fluoride-stained LS8 cells (D). * compared with the control group, \( P < 0.05 \); ** compared with the control group, \( P < 0.01 \); # compared with the NaF + miR-1a-3p mimics group, \( P < 0.05 \).
Figure 5

MiR-1a-3p targeting Map3k1 regulates MAPK signal pathway. MiR-1a-3p inhibits the expression of p38MAPK and ERK1/2 but promotes the expression of p-p38MAPK and p-ERK1/2 (A,B). Map3k1 is located at the central node of MAPK pathway (C). The sequence of Map3k1 3’UTR seed region among different species is highly conserved (D). Map3k1 is the direct target of miR-1a-3p (E), and miR-1a-3p can bind to the highly conserved target of Map3k13’ UTR region (1593-1599 5’-ACAUUCC-3’) (F). The expression of
Map3k1 in fluoride-exposed LS8 cells is inhibited by Gregory Homerimi miR-1a-3p(G,H).* compared with the control group, \( P < 0.05 \);# compared with the NaF+miR-1a-3p mimics group, \( P < 0.05 \).

**Figure 6**

MiR-1a-3p inhibits apoptosis in fluoride-exposed LS8 cells by targeting *Map3k1* via MAPK signal pathway.