

Original Article

Genes associated with sodium fluoride-induced human osteoblast apoptosis

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Abstract: This study aims to explore the potential pathways and molecular characteristics of fluorine-induced osteoblast apoptosis. In vitro fluorine-induced model was established with an osteogenesis sarcoma cell line Saos-2. Then flow cytometry was used to determine the mitochondrial membrane potential at 24 h after the intervention. 84 apoptosis-related genes in the cells were determined using the functional polymerase chain reaction (PCR) chip and part of the differentially expressed genes was verified with immune blotting. When the stimulated concentration of sodium fluoride were 20 mg/L, 40 mg/L and 80 mg/L, the mitochondrial membrane potential of the osteoblast cells were 27.0%, 28.8% and 38.6%, respectively, significantly higher than that in the blank control group ($P < 0.05$). The PCR chip detection found 13 up-regulating genes and 15 down-regulating genes, among which the expression of Bim, Caspase 9, Caspase 14, B-cell lymphoma-2 (BCL2) and BAX increased with the doses of sodium fluoride, while the expression of Caspase 3 down-regulated in 5 mg/L sodium fluoride but up-regulated at the concentration of sodium fluoride more than 10 mg/L. Caspase 7 expression showed no obvious difference between the different concentration groups. However, Caspase 10 decreased with the increasing doses of sodium fluoride. Fluoride-induced osteoblast apoptosis may be through the mitochondrial pathway (including endoplasmic reticulum stress pathway) and death receptor pathway.

Keywords: Osteoblast, apoptosis, fluorosis, PCR chip

Introduction

Endemic fluorosis is caused by excessive amounts of fluorine ion in the environment or too much fluoride uptake [1-3]. Chronic intake of excess fluoride for a long time can cause extensive damages in multi-organs and systems, especially in bones and teeth, which can lead to skeletal fluorosis and dental fluorosis, respectively [4]. The pathogenesis of endemic fluorosis is complex and has not been fully elucidated yet. Currently, it is believed that apoptosis in endemic fluorosis is the basis of the generalized cell damages [5, 6]. High concentration of fluoride injures the skeletal system mainly by inhibiting the proliferation and the secretory function of osteoblast [7], but its mechanism is still unclear. The mechanism of fluoride-induced osteoblast apoptosis is also not clear. Human osteogenesis sarcoma cell line Saos-2 has the osteoblast activity and can be induced to dif-

ferentiate. Thus, it has been applied for establishing fluorosis cell model, which is a good tool for studying the effect of high fluoride on osteoblast [8-10]. In this study, osteoblast Saos-2 was used to systemically explore the effect of fluorine on the apoptosis signaling pathways of osteoblasts. Key signaling molecules in the fluoride-induced osteoblast apoptosis may be screened out to provide a theoretical basis for illustrating the effects of fluorine on the apoptosis-related signaling molecules within osteoblast. The results will help to find out the regulation of fluorosis so as to find out some intervention or treatment target molecules.

Methods

Cell culture and treatment

Osteogenesis sarcoma cell line Saos-2 (Shanghai Cell Bank, China) was cultured in DMEM

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medium (Hyclone, Thermo Scientific, Logan, UT, USA) containing 10% fetal bovine serum (FBS, Gibco® life technologies) and incubated in an incubator with 5% CO₂ and saturated humidity at 37°C. When the cells grew to 80% confluence, they were harvested by trypsinization for the next experiments.

Detection of mitochondrial membrane potential in osteoblasts

Mitochondrial membrane potential in osteoblasts was determined using JC-1 Mitochondrial Membrane Potential Assay kit (Cayman Chemical Company, USA) in combination with flow cytometry. CCCP (Sigma-Aldrich, USA) was used as positive control. It was dissolved in dimethyl sulphoxide (DMSO) to a concentration of 100 mM for stock solution and used at the working concentration of 50 µM. Briefly, 100 µL JC-1 staining solution was added into the cells treated with different concentration of sodium fluoride (NaF, Sangon Biotech Co., Ltd Shanghai, China) and incubated in the 5% CO₂ and 37°C circumstance for 15 min. Then the cells were washed twice with the assay buffer and harvested by conventional trypsinization. After washed twice with assay buffer and centrifuged at 400 g for 5 min, cells were resuspended in 500 µL assay buffer and detected on a flow cytometer (Beckman Coulter FC 500 MCL, German).

Detection of apoptosis-related genes by RT2 polymerase chain reaction (PCR) ARRAY

Cells treated with 0, 20, 40 mg/L NaF for 24 h were harvested for total RNA extraction using RNeasy Kit (Qiagen, USA) according to the manufacturer's instruction. 400 ng RNA was subjected to synthesize the first chain cDNA using RT2 First Strand Kit (Qiagen) following the instruction supported in the kit. Then quantitative real-time PCR was carried out with RT2 SYBR Green qPCR Mastermix (Qiagen) and PCR ARRAY plates (PAHS-012Z, Qiagen) and run on a fluorescence quantitative PCR machine (Eppendorf Mastercycler ep realplex 2, German). The Ct values of each gene in each PCR chip were calculated. Each experiment was run in triplicate. Quality control was performed by the repeatability, amplification efficiency and removal of genome pollution. The relative expression of each gene was calculated by 2^{-ΔΔCt} method. The difference higher than 2 fold was regarded as significantly different gene. The genes with the difference higher than 1.9 fold and raised or lowered continuously in a concentration-de-

pendent manner from 20 mg/L to 40 mg/L NaF were also enrolled as candidate genes.

Protein expression detected by western blot

Total proteins were extracted from the cells treated with different concentration of NaF and quantified using BCA method. 30 µg total protein of each sample was separated by SDS-PAGE and transferred to NC membrane and then incubated with primary antibodies to BIM, Caspase-3 (CASP3), CASP9, CASP7, CASP10, CASP14, BAX, B-cell lymphoma-2 (BCL-2) and β-actin (dilution 1:1000, Abcam, USA) at 4°C overnight. Then the membrane was incubated with HRP-labeled goat anti-rabbit or goat anti-mouse secondary antibody (Wuhan Boster Bio-engineering Co., Ltd, China) at room temperature for 1 h. The specific bands on the membrane were detected using ECL chemiluminescence reagent (Sangon Biotech (Shanghai, China) Co., Ltd) and exposed to X-ray film. After development and fixing, the specific bands could be read on the film. The expression of each protein was normalized with β-actin.

Results

Mitochondrial membrane potential in Saos-2 cells

After treated with different concentration of NaF for 24 h, cells were used to detect the mitochondrial membrane potential with JC-1 method. When the mitochondrial membrane potential was low, JC-1 presented as monomer and could not gather in the mitochondrial matrix. It displayed green fluorescent and located at the lower right quadrant. As the osteoblast cells treated with 5 mg/L, 10 mg/L, 20 mg/L, 40 mg/L and 80 mg/L NaF, the mitochondrial membrane potential were 15.8%, 12.8%, 27.0%, 28.8% and 38.6%, respectively (**Figure 1**). It was 10.0% in the blank control group and 51.6% in the positive control group (treated with CCCP). The mitochondrial membrane potential in the cells treated with 20 mg/L, 40 mg/L and 80 mg/L NaF were all prominently higher than that in the blank control group ($P < 0.05$).

Gene expression profiled by PCR chip

Quantity control showed that the average Ct value of the PCR Chip was 19.58, with standard deviation less than or equal to 0.1. The amplifi-

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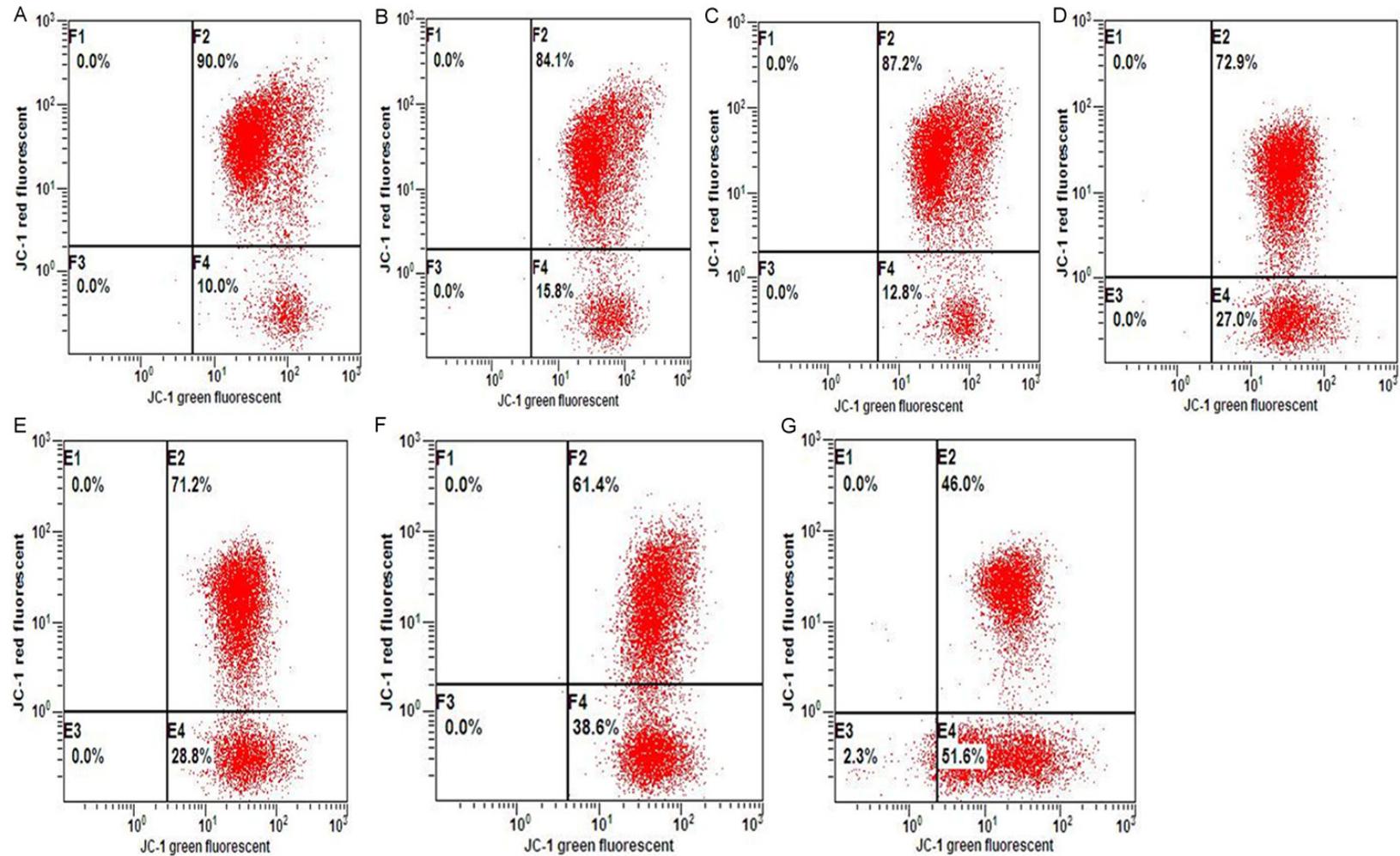


Figure 1. Mitochondrial membrane potential in Saos-2 cells treated with different concentration of NaF and CCCP, detected by flow cytometer. A. Blank control, treated with 0 mg/L NaF; B. Positive control, treated with CCCP; C to G. Cells treated with 5 mg/L, 10 mg/L, 20 mg/L, 40 mg/L and 80 mg/L NaF.

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Table 1. The expressions of apoptosis-related genes in the osteoblast Soas-2 treated by NaF

Gene groups	Genes	Difference fold of the gene expression in the 20 mg/L group	Difference fold of the gene expression in the 40 mg/L group
Death receptors	FADD, TNF, TNFRSF10B (DR5)	-2.2799, 1.2754, -2.4016	-1.9602, 1.904, -1.8869
DNA damage-related gene	TP73	-1.3794	-2.5955
Transmembrane signals	TNFRSF25 (DR3)	2.7435	3.093
Anti-apoptosis factors	BCL2, BCL2A1, BIRC3, BIRC6, FAS, IGF1R, NFKB1, RIPK2, TNF	1.9199, -110.968, -63.5139, 2.2752, -2.3522, 1.6888, -2.7492, -2.2564, 1.2754	2.4949, -46.2378, -34.3205, 2.1273, -1.683, 2.0907, -2.5856, -2.299, 1.904
Positive regulators on apoptosis	BIM, CASP10, CASP14, CASP8, CD70, FADD, LTA, RIPKA2, TNF, DR5, DR3, CD137 (TNFRSF9)	2.1825, 1.6483, 1.4349, 1.81, -8.3051, -2.2799, -2.3685, -2.2564, 1.2754, -2.4016, 2.7435, -3.5529	3.0610, 2.0335, 1.9779, 1.9305, -2.8699, -1.9602, -1.9945, -2.299, 1.904, -1.8869, 3.093, -3.0546
negative regulators on apoptosis	BCL2, BCL2A1, BIRC2, BIRC3, BIRC6, FAS, IGF1R, NAIP, TP73	1.9199, -110.968, -3.4558, -63.5139, 2.2752, -2.3522, 1.6888, 1.7363, -1.3794	2.4949, -46.2378, -2.6592, -34.3205, 2.1273, -1.683, 2.0907, 2.1421, -2.5955
Death domain protein	FADD, DR3, DR5	-2.2799, 2.7435, -2.4016	-1.9602, 3.093, -1.8869
Caspase family members	CASP10, CASP14, CASP5, CASP7, CASP8	1.6483, 1.4349, 1.325, 1.4349, 1.81	2.0335, 1.9779, 1.9779, 1.904, 1.9305
Caspase activator	APAF1, DR5	2.145, -2.4016	2.4099, -1.8869
Others	GADD45	-7.9608	-5.8604

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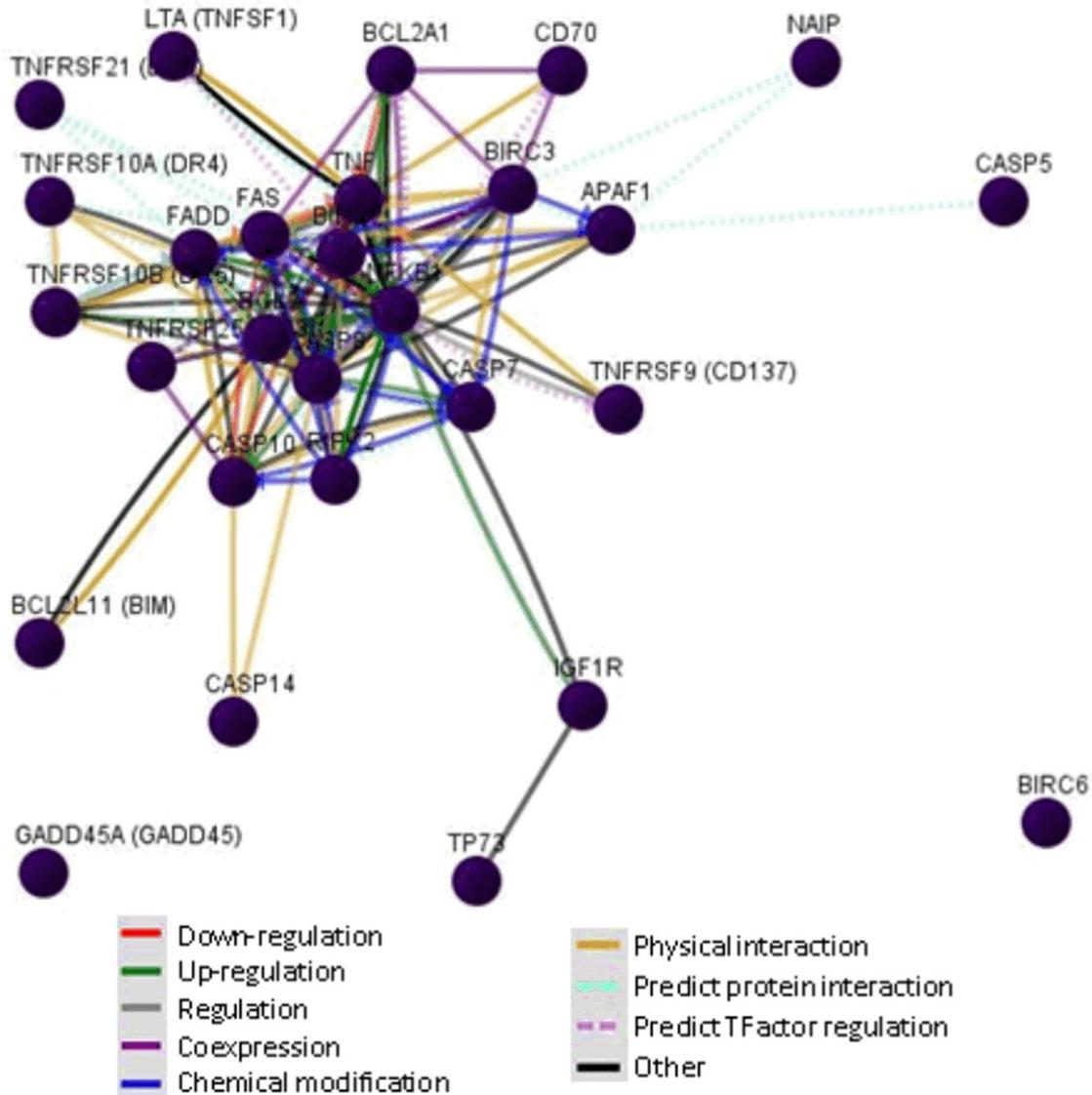


Figure 2. The interacting network of apoptosis-related genes.

cation efficiency was equal. The Ct value of genome pollution was controlled higher than 35. Among the 84 genes detected by PCR chip, there were 13 genes up-regulated, including AP-APAF1, BCL2, BIM, BIRC6, DR3, CASP10, CASP14, CASP5, CASP7, CASP8, IGF1R, NAIP and TNF, and 15 genes down-regulated, including BCL2-A1, BIRC2, BIRC3, CD70, FADD, FAS, GADD45, TNFSF1, NFKB1, RIPK2, DR5 (TNFRSF10B), DR4 (TNFRSF10A), DR6 (TNFRSF21), CD137 (TNFRSF9) and TP73 (Table 1).

The interacting relationship between genes was analyzed on the website: <http://gncpro.sabiosciences.com/gncpro/gncpro.php>. GADD-45 and BIRC6 had no direct relationship with

other genes. TNF, BIRC2, BIRC3, Caspase 9, BCL2, FAS, FADD, RIPK2, Caspase 10, Caspase-7, Caspase 8, DR3 and DR5 located in the intensive regulatory center of the network, with a multiple regulatory function (Figure 2). They acted synergistically and influenced the apoptosis.

Apoptosis-related proteins in Soas-2 cells treated with NaF

After treated with different concentration of NaF for 24 h, Saos-2 cells were used for the extraction of total proteins and separated by SDS-PAGE gel electrophoresis. As detected by Western blot, the expression of BIM, Caspa-

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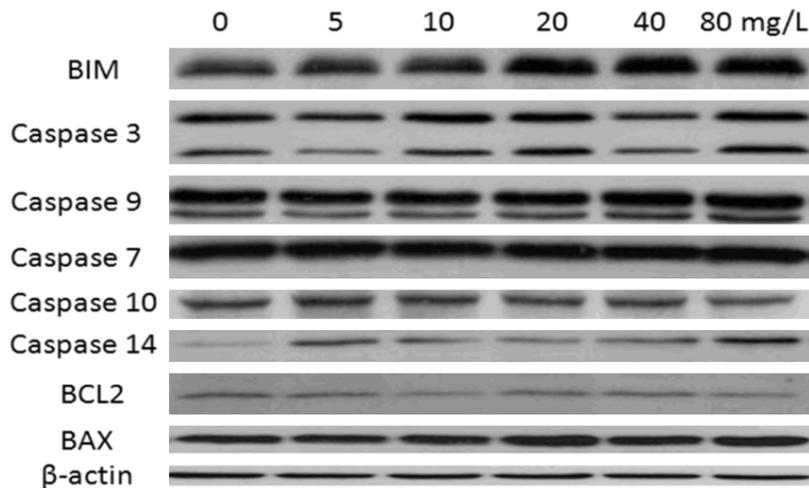


Figure 3. Apoptosis-related proteins in Saos-2 cells treated with different concentration of NaF.

se9, Caspase 14, BCL2 and BAX increased with the concentration of NaF, while Caspase 3 decreased in 5 mg/L group but enhanced in the groups treated with more than 10 mg/L NaF, as compared with those in the blank control group. Caspase 10 was down-regulated with the increasing concentration of NaF, but Caspase 7 showed no obvious difference among the groups (Figure 3).

Discussion

It is reported that the apoptosis caused by fluoride ion is correlated with the fluorosis-induced bone damage or osteoblast damage. Many mechanisms may be involved in apoptosis, such as mitochondrial membrane permeability, intracellular reactive oxygen species (ROS) and so on [11]. Damage of mitochondrial membrane potential ($\Delta\Psi_m$) can result in interruption of electron transport chain and stop the oxidative phosphorylation, which may increase the intracellular reactive oxygen species and thereby promoting cell damage [12]. A variety of factors can induce apoptosis in various cells. Decreasing mitochondrial transmembrane potential is believed to be the earliest event in the cascade process of apoptosis, which is earlier than chromatin condensed and DNA disruption. Once the mitochondrial membrane potential collapsed, the apoptosis is irreversible [13, 14].

Bcl-2 interacting mediator of cell death (Bim), a pro-apoptosis member in Bcl-2 family, is an important apoptosis-regulatory protein in the upstream of the mitochondrial apoptosis pa-

thway. The activation of Bim is in the upstream of mitochondria and caspase activation, thus it can be regarded as an initial event of apoptosis. In this study, high expression of Bim was found in osteoblast treated with NaF and it increased in a concentration-dependent manner, highly suggesting Bim plays an important role in osteoblast apoptosis and injury induced by high fluoride as a pro-apoptosis protein.

In this study, we used JC-1 staining method to detect the changes of mitochondrial membrane potential and found that mitochondrial membrane potential of Saos-2 cells decreased obviously with the increase of the stimulating doses of NaF, which has similar trend with Bim. Thus, it is presumed that Bim is activated by sodium fluoride and then causes apoptosis cascade. From the results, we did find that mitochondrial membrane potential has a role in the fluoride-induced osteoblast apoptosis. However, due to the low value, it also showed that mitochondrial membrane potential is not the only way to apoptosis, while death receptor pathway may also contribute to it. In addition to apoptosis, it may also be associated with autophagy [15].

It has been reported that high concentration of fluoride can inhibit the proliferation of renal cells and induce cell apoptosis by inhibiting the cells transforming from S phase to G2/M phase [16]. Yang et al. [17] found NaF can increase the mitochondrial membrane permeability of osteoblast and initiate the mitochondrial apoptotic signal transduction pathways through reducing the mRNA expression of Bcl-2 and raising Bax mRNA expression. Fluorine can significantly enhance the expression of the pro-apoptosis proteins Bax and Bak in human osteoblasts and inhibit the expression of anti-apoptotic protein Bcl-2. Fluorine can also significantly elevate the activity of caspase 3 [18]. Wei et al. [15] using fluoride to stimulate osteoblast MC-3T3 E1 and found that fluoride can remarkably inhibit the proliferation of osteoblast MC3T3 E1

in a time-dependent and dose-dependent manner and induce the apoptosis and autophagy. The previous researches mainly focused on the mitochondrial apoptotic pathways and single gene, which is not systemic for studying the mitochondrial apoptosis pathway and can't identify the specific pathway of fluoride-induced osteoblast apoptosis. In this study, the changes of the expression of caspase 3, caspase 9, BCL2 and Bax were consistent with the above results. However, in the PCR chip screening results, no significant change was found in the expression of BAK (-1.1559 to -1.0253), which may result from the cell lines used. In addition, the expressions of BID and BIK (-1.3624 to -1.2449, and 1.2277 to 1.8327, respectively) in the screening were meaningless. Caspase-9 is the initiating enzyme of mitochondrial apoptotic pathway. Caspase 3, 6 and 7 are the effective cysteine and aspartic acid proteases of apoptosis, which were the executors of the apoptosis [19, 20]. Therefore, how the apoptosis-related genes interact with the presence of fluoride and whether it has its own characteristics would be analyzed in this study using software, so as to present a potential interaction model.

Our results first discovered the expression of caspase 14 in osteoblast, whose expression increased with fluoride dose. High concentration of fluoride can inhibit the skin fibroblast cells differentiate to the osteoblast [21]. Caspase 14 is a member of the caspase family, which is mainly expressed in the epidermis, rarely in other tissues. It is involved in the terminal differentiation of the epidermal cells and the formation of the skin barrier [22]. Our results showed that high concentration of fluoride can induce the expression of caspase 14, which is obviously correlated with the osteoblast apoptosis. From **Figure 2** we could see that caspase 14 had a physical relationship with caspase 10 and caspase 8 and thereby being involved in the process of apoptosis. Thus, it is presumed the expression of caspase 14 in the fluoride-treated osteoblasts may be related to the terminal differentiation of osteoblast to bone cells. Whether caspase 14 can be used as a monitoring index for osteoblast fluorosis is yet to be studied.

Some genes such as FAS, JNK3, DR3 and DR5 are not expressed in Saos-2 cells, thus if they have some roles in the fluoride-induced osteo-

blast apoptosis had not been identified in this study. The apoptosis caused by endoplasmic reticulum stress has been confirmed in Saos-2 cells [23], but due to its approach is highly associated with mitochondrial apoptosis pathway, it is usually classified as mitochondrial pathway of apoptosis. The role of death receptor pathway in the fluoride-induced apoptosis is not clear, so further verification is needed yet.

In conclusion, this study confirmed Bim, caspase 3, caspase 9 and caspase 14 played a role in sodium fluoride-induced osteoblast apoptosis. It also suggested that these four proteins may be used as molecular targets or specific targeted therapy combined with other drugs in the clinical treatment for skeletal fluorosis, providing a new thought for the treatment of skeletal fluorosis.

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Disclosure of conflict of interest

None.

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