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Fluoride-Induced Oxidative and Inflammatory Stress in Osteosarcoma Cells: Does It Affect Bone Development Pathway?

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Abstract Oxidative stress is reported to negatively affect osteoblast cells. Present study reports oxidative and inflammatory signatures in fluoride-exposed human osteosarcoma (HOS) cells, and their possible association with the genes involved in osteoblastic differentiation and bone development pathways. HOS cells were challenged with sublethal concentration (8 mg/L) of sodium fluoride for 30 days and analyzed for transcriptomic expression. In total, 2632 transcripts associated with several biological processes were found to be differentially expressed. Specifically, genes involved in oxidative stress, inflammation, osteoblastic differentiation, and bone development pathways were found to be significantly altered. Variation in expression of key genes involved in the abovementioned pathways was validated through qPCR. Expression of serum amyloid A1 protein, a key regulator of stress and inflammatory pathways, was validated through western blot analysis. This study provides evidence that chronic oxidative and inflammatory stress may be associated with the fluoride-induced impediment in osteoblast differentiation and bone development.

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Introduction

Though fluoride is considered as an essential trace element, chronic exposure to fluoride is known to cause toxic effects. Recent reports illustrate that fluoride can alter various functional pathways in the biological system even at low concentrations [1]. Chronic exposure to fluoride has been reported to cause dental and skeletal fluorosis [2]. Around 62 million people in India are suffering from dental and skeletal fluorosis [3].

Once entered into the body, fluoride readily distributes through the bloodstream, especially in the calcium-rich areas. This in turn impedes the signal transduction pathways involved in bone remodeling processes [4]. Recent studies report fluoride-induced alterations in RANKL/RANK/OPG cascade and its impact on the osteoclast/osteoblast differentiation and bone mineralization [4, 5]. However, the effect of fluoride on other functional cellular networks is not well established.

Accumulating evidence suggests possible association between oxidative/inflammatory stress and bone loss [6]. Most chronic inflammatory diseases, such as rheumatoid arthritis, Crohn's disease, asthma, chronic obstructive pulmonary disease, alveolitis, nephritis, vasculitis, myositis, and inflammatory neuropathy, have common characteristic of bone loss. Subclinical inflammation has also been suggested to impede bone mineralization process, leading to increased fracture risk [7]. These studies substantiate bone loss in chronic inflammatory diseases. However, the molecular mechanism involved in inflammatory stress-mediated bone loss has not been addressed. Identification of signatures of oxidative/ inflammatory stress and alterations in osteoblast differentiation/bone development pathways can reveal the possible mechanism involved in stress-mediated bone loss in chronic inflammatory diseases.

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At present, there are no reports on the correlation of genes involved in the oxidative/inflammatory stress with osteoblast differentiation/bone development pathways in fluoride-exposed osteosarcoma cells. Our earlier study reported epigenetic signatures (miR-124 and miR-155) of fluoride exposure in osteosarcoma cells and their possible role in the development of fluorosis [8]. Here, we report the genetic signatures of oxidative/inflammatory stress and osteoblast differentiation/bone development pathways and their possible association upon fluoride exposure in osteosarcoma cells.

Material and Methods

Chemicals and Cell Culture

Minimum essential medium (MEM), fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were purchased from Gibco-BRL (USA). Sodium fluoride (NaF) was obtained from Sigma-Aldrich (USA). The stock solution of NaF (25,000 mg/L) was prepared in milli-Q water. The human osteosarcoma (HOS) cell line was purchased from the National Centre for Cell Science (NCCS) Pune, India. The cells were cultured in MEM supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, and 10 % heat-inactivated FBS. The cultures were maintained in a humidified incubator (37 °C, 5 % CO_2).

NaF Treatment

LC_{50} of NaF in HOS cells (40 mg/L) has been reported earlier from our laboratory [8]. In current study, HOS cells were seeded in 75 cm^2 culture flask at the density of 50,000 cells/ml. The cells were exposed to sublethal concentration (8 mg/L) of NaF for 30 days. Appropriate controls were also maintained. During exposure, cells were subcultured every 3 days, and 8 mg/L of NaF was replenished. At the end of exposure period, cells were harvested into TRIzol reagent (Invitrogen, USA).

Microarray Set Up

RNA was extracted from control and exposed cells using TRIzol reagent. RNA was quantified with the NanoDrop instrument (ND8000, Isogen Life Science), and its integrity was checked using Bioanalyzer 2100 (Agilent, USA). RNA displaying good integrity was used for microarray and quantitative PCR (qPCR) analysis. RNA was labeled with biotin according to the Affymetrix protocol and hybridized to Affymetrix GeneChip PrimeView human gene expression array (Affymetrix Inc., USA) for 16 h at 45 °C. The GeneChip array was washed and stained (streptavidin-phycoerythrin) in the Affymetrix Fluidics Station 400 followed by scanning on Affymetrix GeneChip Scanner 3000. Experiment was carried out in triplicate.

Microarray Data Analysis

Microarray data were normalized first, using robust multiarray averaging method. To eliminate background noise, genes with <20 percentile signal intensities were eliminated from the analysis. Differentially expressed gene was defined as a significant difference in transcript levels between exposed and control samples, where the following three statistical parameters were met: (1) fold change of ≥1.5 or ≤1.5; (2) $p < 0.05$ (analysis of variance (ANOVA)); and (3) a false discovery rate corrected q value <0.05. Microarray data analysis was performed using Partek Genome Suite and Affymetrix Expression Console Software. Microarray data has been submitted to NCBI Gene Expression Omnibus repository and is accessible under accession no. GSE70719. Functional annotation of differentially expressed genes was carried out using the Database for Annotation, Visualization, and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/).

Quantitative Polymerase Chain Reaction

To confirm the microarray result, qPCR analysis was performed on ABI 7300 thermal cycler using SYBR green master mix (Invitrogen, USA). The list of PCR primer of the selected genes used for verification of microarray result is given in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as the housekeeping gene. The amplification program consisted of 1 cycle at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 58–60 °C for 10 s, and 72 °C for 30 s. Relative fold change between control and exposed samples was calculated using $2^{-\Delta\Delta Ct}$ method, and statistical significance was calculated using unpaired t test. The experiment was carried out in biological triplicates, and each replicate was analyzed in technical duplicates.

Western Blotting

Expression pattern of serum amyloid A1 (SAA1) was determined by western blot analysis. β-actin expression was used as the housekeeping control for normalization. Cells were exposed to 8 mg/L of NaF for 30 days. Then whole cell protein was extracted from both control and exposed cells using protein lysis buffer as described earlier [9]. Equal amount of total protein (150 μg) from each sample was electrophoresed on 10 % SDS-PAGE gel and transferred onto nitrocellulose membrane. Membranes were blocked with 5 % nonfat milk and then hybridized with mouse anti-β-actin and anti-SAA1 monoclonal antibodies (Santa Cruz Biotechnology,

Table 1 List of qPCR primers

Gene	Forward primer	Reverse primer	Annealing temperature (°C)	Product size (bp)
RARRES3	ATGGCAAGTCCCGCTGTAAA	CCCCACAGCTGCTTCTAACA	58	169
SAA1	TCAGCAGCCGAAGCTTCTTT	TCCCCTTTTGGCAGCATCAT	58	152
IFITM1	GACAGGAAGATGGTTGGCGA	AGTGCAAAGGTTGCAGGCTA	58	216
APOL1	AATGCAAACATTTTATTAGGGGGA	CCACACTCTCCAGTTCCCAT	58	187
CCL2	GATCTCAGTGCAGAGGCTCG	TGGGTTGTGGAGTGAGTGTT	58	194
ANXA8	ACAGGAGATGCCCAAAGCC	TCCCAGGGAGATGAAGAGACA	60	182
SMAD5	CGTCAATGGCCAGCTTGTTT	CCTGCAGGCGTCCATCTAAA	58	228
WWTR1	AGGCGATGAATCAGCCTCTG	AGCGCATTGGGCATACTCAT	58	205
TRAM2	CTTTCCGCAGGAGGACGAAA	GTAGTGCACGGTCTCACTGT	58	200
SATB2	AGAGCCAACCAACTCTTCCG	GCATGGCCCTCAGGTTTACT	58	203
HIF1A	TTTCCTCAGTCGACACAGCC	TCCACCTCTTTTGGCAAGCA	59	217
CLTC	TACTGAGGCATTAGGGAGGCA	AAAAACCTCGGTGTCACTTGG	58	198
OPG	CTGGAACCCCAGAGCGAAAT	GCCTCCTCACACAGGGTAAC	58	217
RANKL	TCGTTGGATCACAGCACATCA	TATGGGAACCAGATGGGATGTC	58	141
RUNX2	GGCAGGCACAGTCTTCCC	GGCCCAGTTCTGAAGCACC	58	174
IC (GAPDH)	GTCAAGGCTGAGAACGGGAA	ATGACGAACATGGGGGCATC	58–60	221

RARRES3 retinoic acid receptor responder (tazarotene induced) 3, *SAA1* serum amyloid A1, *IFITM1* interferon-induced transmembrane protein 1, *APOL1* apolipoprotein L1, *CCL2* chemokine (C-C motif) ligand 2, *ANXA8* annexin A8, *SMAD5* SMAD family member 5, *WWTR1* WW domain containing transcription regulator 1, *TRAM2* translocation-associated membrane protein 2; *SATB2* SATB homeobox 2, *HIF1A* hypoxia-inducible factor 1, alpha subunit, *CLTC* clathrin, *Hc* heavy chain , *IC* internal control, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *OPG* osteoprotegerin, *RANKL* receptor activator of nuclear factor kappa-B ligand, *RUNX2* runt-related transcription factor 2

USA) at 1:200 and 1:100 dilutions, respectively. Immunodetection was performed using WesternDot 625 goat anti-mouse western blot kit according to manufacturer's protocol (Invitrogen, USA). The image was captured under UV light using Versa Doc instrument (Bio-Rad Laboratories, USA) and the relative quantification of bands was carried out using PDQuest software (Bio-Rad Laboratories, USA).
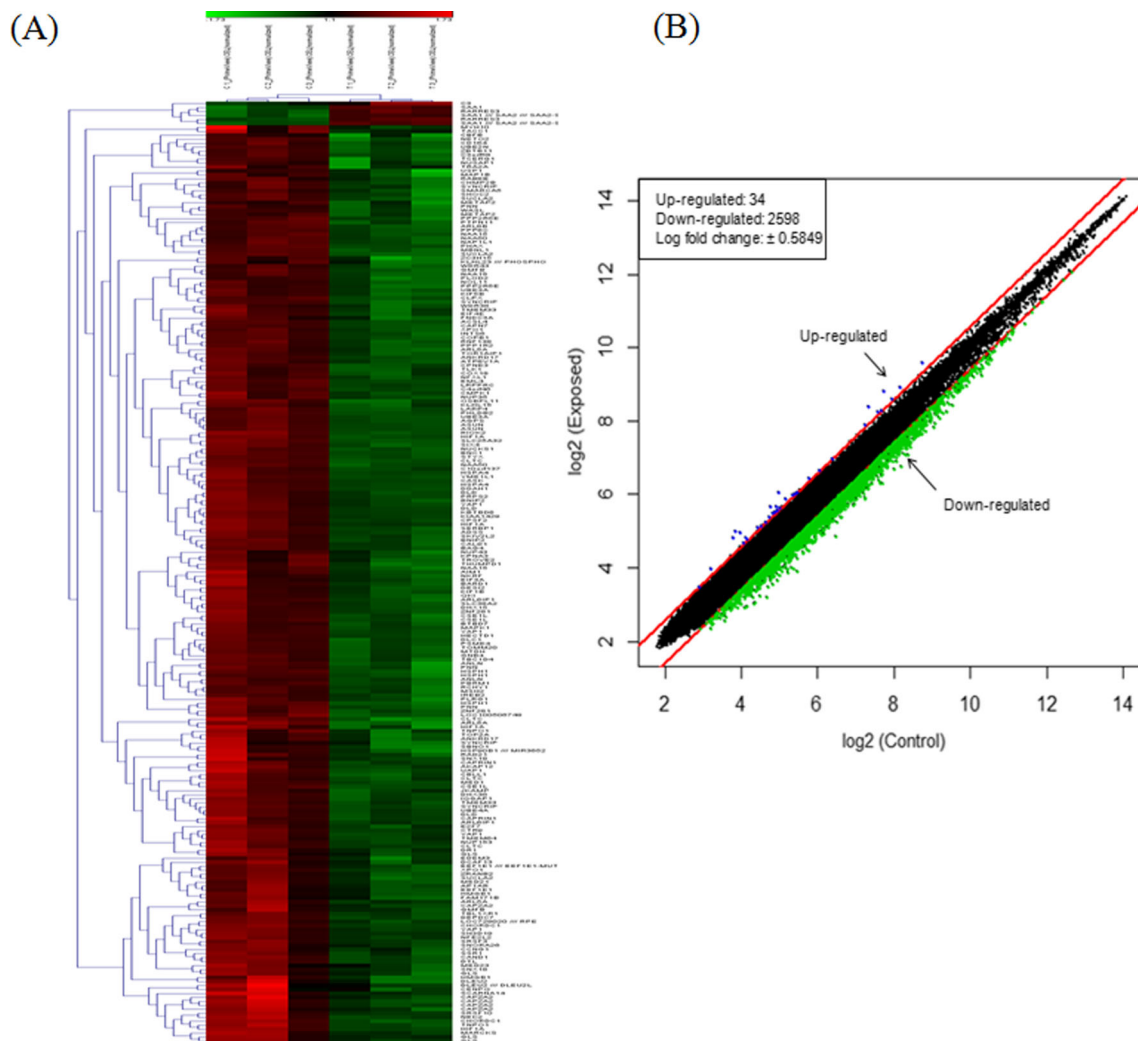
Results

Microarray Analysis

Microarray analysis (Fig. 1a) showed differential expression of genes in HOS cells upon exposure to sublethal concentration (8 mg/L) of NaF. Bivariate scatter plot (Fig. 1b) revealed the dispersion of gene expression levels in control and exposed cells. Genes crowded near the diagonal (black dots) indicate equal expression in exposed and control cells. However, genes beyond the diagonal on each side represent differential expression due to fluoride exposure. An optimal cutoff value of 1.5 fold change in gene expression was chosen which generated a false discovery rate (FDR) of 0.23. In total, 2632 genes were found to be differentially expressed in exposed cells compared to the control. Blue dots in Fig. 1b above the upper threshold represent upregulated genes ($n = 34$), whereas green dots below the threshold line indicate downregulated genes ($n = 2598$).

Functional Annotation of Differentially Expressed Genes

DAVID analysis revealed the categorization of differentially expressed genes based on biological processes, molecular functions, and cellular components (Table S1). Figure 2 entails differentially expressed genes grouped into different biological processes. Genes involved in oxidative stress response (10 genes), inflammatory stress response (6 genes), regulation of cell proliferation (6 genes), homeostasis (6 genes), neurological system process (3 genes), and 9 unclustered genes were found to be upregulated (Fig. 2a, Table S1). On the other hand, majority of downregulated genes were found to be involved in cell cycle process (154 genes), protein localization (151 genes), proteolysis (133 genes), RNA processing (110 genes), chromosome organization including histone modification (76 genes), regulation of cell cycle (49 genes), DNA repair (45 genes), negative regulation of apoptosis (38 genes), bone development (10 genes), and regulation of osteoblast differentiation (5 genes) (Fig. 2b, Table S1). SAA1, the gene associated with inflammation, oxidative stress, and immune response showed the highest upregulation (2.12-fold). CLTC,

(A)
(B)

Fig. 1 **a** Heat map of the differentially expressed genes obtained from microarray result. The *color scale bar* in the top represents relative gene expression levels analogous to the color in the heat map. *Red* and *green* colors represent upregulated and downregulated genes, respectively. **b** Bivariate scatter plot demonstrating the distribution of upregulated and downregulated genes. 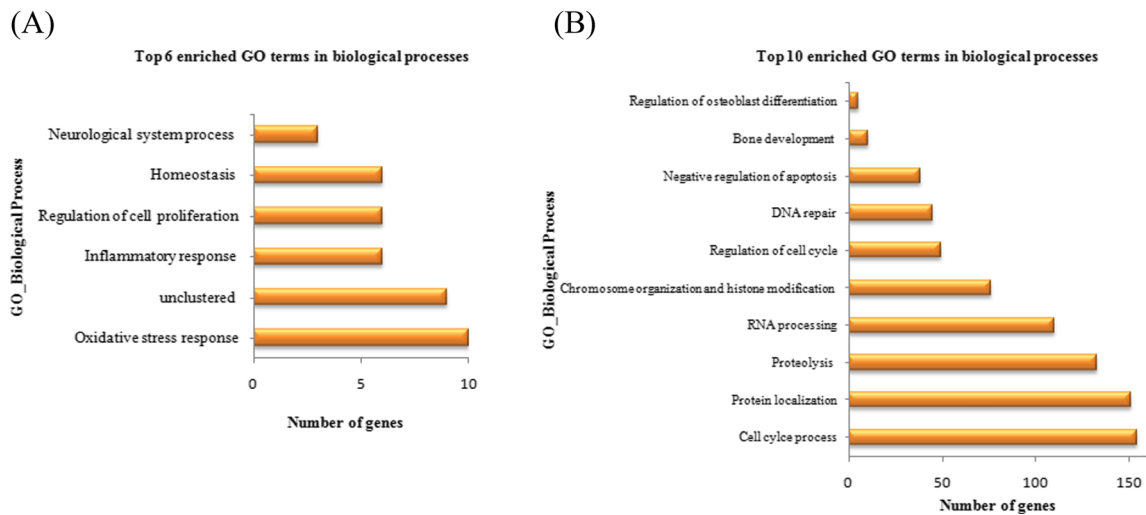*Blue* and *green dots* indicate differentially expressed genes with cutoff fold change ≥1.5. The *blue dots* above the upper threshold line and *green dots* below the lower threshold line represent upregulated and downregulated genes, respectively, in exposed sample compared to control sample. *Black dots* indicate no change in expression pattern

the gene associated with osteoblast differentiation, was found to be the most downregulated gene (3.06-fold).

Quantitative Polymerase Chain Reaction

Twelve candidate genes involved in oxidative/inflammatory stress and osteoblast differentiation/bone development pathways with fold change ≥1.5 in microarray analysis were chosen to verify the results using qPCR analysis (Fig. 3). Table 2 entails the list of candidate genes, functional categorization, and their expression profiles in microarray and qPCR analyses ($p < 0.05$). Genes involved in oxidative/inflammatory stress exhibited upregulation, whereas those involved in osteoblast differentiation/bone development pathways

showed downregulation in both the analyses. Thus, the qPCR results were in line with the results obtained from microarray analysis. However, the fold change values of few genes were significantly different between the two analyses. For example, ANXA8 gene was 1.5-fold upregulated in microarray analysis and 5.99-fold upregulated in qPCR analysis. In addition, the expression of key regulatory genes involved in osteoclastic differentiation and bone formation pathway was also analyzed using qPCR, as the alterations of these genes are well known due to fluoride exposure [8]. The targeted genes included osteoprotegerin (OPG), runt-related transcription factor 2(RUNX2), and receptor activator of nuclear factor kappa-B ligand (RANKL). Expression of OPG was found to be upregulated by 1.90-fold, whereas RANKL

(A)

Top 6 enriched GO terms in biological processes

(B)

Top 10 enriched GO terms in biological processes

Fig. 2 Functional annotation of **(a)** upregulated and **(b)** downregulated genes identified by microarray analysis using DAVID tool. X-axis represents total number of genes (including overlaps), whereas y-axis illustrates their involvement in respective biological process

and RUNX2 expressions were downregulated by 1.83- and 1.82-fold, respectively.

Western Blot Analysis of SAA1 Protein

To further confirm the results obtained by microarray analysis, expression of SAA1 protein was analyzed using western blot analysis. SAA1 has a key role in the regulation of stress and inflammatory response in osteoblast cells. Elevated level of SAA1 (2.0 fold) protein was observed in NaF-exposed cells as compared to control (Fig. 4). These results are in line with the data obtained in microarray (2.12-fold) and qPCR (twofold) analyses.

Discussion

Chronic exposure to fluoride is known to cause dental and skeletal fluorosis. Many studies associate deleterious effects of fluoride to the disruption of bone development and bone remodeling pathway [5]. Similarly, growing evidence on bone loss in several inflammatory disorders suggests the possible effect of inflammatory stress on bone development. Current study attempts to link NaF-induced inflammatory response to disruption of bone development pathway. In this study, NaF exposure was found to induce significant alteration in the expression profiles of genes involved in oxidative/inflammatory stress (RARRES3, SAA1, IFITM1, APOL1, CCL2, ANXA8) and osteoblast differentiation/bone development pathways

(A)

(B)
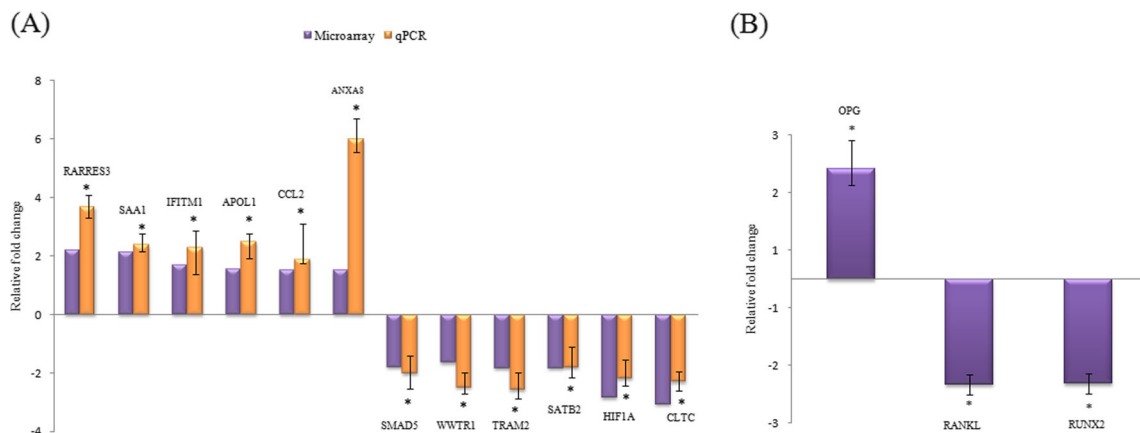
Fig. 3 *Bar graph* illustrating correlation of microarray data with qRT-PCR results. **a** Normalized ratio (Y-axis) of more than 1 indicates upregulation, whereas a ratio of less than 1 indicates downregulation. **b** Expression profiles of key regulatory genes (OPG, RANKL, and RUNX2) involved in bone development using qPCR. *Error bars* represent standard error ($n = 3$), and *asterisk* indicates statistical significance ($p < 0.05$)

Table 2 Validation of selected genes from microarray data by qPCR

| Gene ID[a]	Biological function	Fold change in qPCR	Fold change in microarray	Regulation
RARRES3	Inflammation and oxidative stress, negative regulation of cell proliferation, phospholipid metabolic process	5.99	1.50	Up
SAA1	Inflammation and oxidative stress, innate immune response	2.38	2.10	Up
IFITM1	Inflammation and oxidative stress, cytokine-mediated signaling pathway, regulation of immune response, ossification, response to virus	2.30	1.60	Up
APOL1	Inflammation and oxidative stress, cholesterol and lipid metabolism, innate immune response, chloride transport.	2.50	1.50	Up
CCL2	Inflammation and oxidative stress, chemotaxis	1.90	1.50	Up
ANXA8	Inflammation and oxidative stress, blood coagulation, actin filament binding, negative regulation of phospholipase A2 activity	5.99	1.50	Up
SMAD5	BMP signaling pathway, bone development	2.02	2.02	Down
WWTR1	Osteoblast differentiation, regulation of SMAD protein import into nucleus	2.48	2.00	Down
TRAM2	Collagen biosynthetic process, protein transport	2.57	2.20	Down
SATB2	Osteoblast development, neuron migration	1.81	2.50	Down
HIF1A	Maintenance of chondrocytes survival, cellular response to hypoxia, Notch signaling pathway, regulation of gene expression, signal transduction	2.19	2.80	Down
CLTC	Intracellular protein transport, osteoblast differentiation	2.30	2.00	Down
OPG	Negative regulation of bone resorption, cytokine activity, skeletal system development	1.90	NA	Up
RANKL	Bone resorption, cytokine activity, osteoclast proliferation, osteoclast differentiation, immune response, calcium ion homeostasis	1.83	NA	Down
RUNX2	BMP signaling pathway, osteoblast development, osteoblast differentiation, ossification	1.82	NA	Down

NA Not analyzed in microarray analysis

[a] Full gene names are given in Table 1

(SMAD5, WWTR1, TRAM2, SATB2, HIF1A, CLTC, RUNX2, RANKL, OPG) (Fig. 3).

IFITM1 is an inflammatory gene, reported to be involved in cytokine signaling in immunological and inflammatory responses. Overexpression of IFITM1, as observed in current study, can alter specific cytokine signaling pathways required for the expression of RANKL. RANKL, in turn, is involved in osteoclastic differentiation and bone formation pathway. Such deregulation in cytokine activities has been correlated with the diseases potentially accounting for bone loss [10]. Pro-inflammatory cytokines have also been reported to regulate the expression of key players (RUNX transcription factors and OPG/RANKL system) involved in osteoclastogenesis pathway [11]. Our earlier study [8] reported epigenetic modification of RUNX2, RANKL (downregulation), and OPG (upregulation) in NaF-exposed HOS cells. To revisit the results obtained in our earlier study, the expression of these three genes was analyzed using qPCR. Increased expression of OPG and decreased expression of RUNX2 and RANKL (Fig. 3b, Table 2) in current study supported the earlier findings.

Elevated levels of CCL2 (chemokine (C-C Motif) ligand 2, as observed in this study) are known to cause diseases related to bone development like rheumatoid arthritis and atherosclerosis [12]. Kim et al. reported that CCL2 can regulate osteoclast differentiation [13] and correlated elevated level of CCL2 in osteoarthritic patients with the osteoblast or osteoclast activities [14]. Elevated expression of CCL2 has also been established as a biomarker of inflammation [15].

SAA1 is a pro-inflammatory apolipoprotein principally expressed in adipocytes. SAA1 levels in plasma were found to be increased by thousand fold within few hours upon acute inflammatory stimuli [16]. Its presence has been implicated in
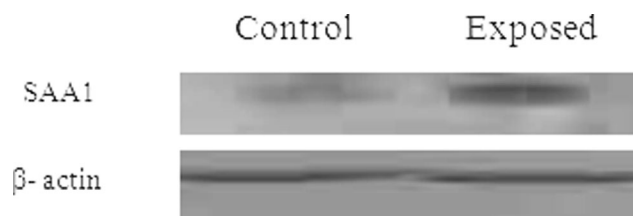
Fig. 4 Western blot analysis of SAA1 protein expression in control and NaF-exposed HOS cells. β-actin was used as the housekeeping control

diseases related to bone metabolism (atherosclerosis and rheumatoid arthritis) [17]. Several studies indicate that SAA1 can modulate the expression of pro-inflammatory cytokines [18]. These cytokines can further alter osteoclastic differentiation and bone metabolism. SAA1 can also trigger lipolysis in adipose tissues leading to insulin resistance, which in turn, can affect the osteocalcin production and thereby bone formation and remodeling processes [19]. Upregulation of SAA1 in fluoride-exposed HOS cells suggests its possible effects on inflammation-mediated bone loss.

Apolipoprotein L1 (ApoL1) has been implicated in the development of diseases related to bone development (atherosclerosis), inflammatory disorders, and obesity [20]. Duchateau et al. suggested the probable involvement of ApoL1 in lipolysis and its interference with the HDL metabolism [21]. Abovementioned findings suggest a vital role of inflammatory processes in fat accumulation thereby leading to the disruption in bone metabolic processes (OPG/RANKL system).

Our result demonstrated elevated levels of inflammatory and stress genes (AnxA8 and RARRES3) in NaF-exposed HOS cells. AnxA8 is a calcium-dependent phospholipid-binding protein [22]. Lueck et al. demonstrated interactions of AnxA8 with Wnt proteins and suggested its role in the Wnt signaling pathway [23]. Wnt signaling plays a crucial role in bone development, differentiation of mesenchymal precursor cells into mature osteoblasts, and the development of skeleton in embryo [24]. Upregulation of AnxA8 in treated primary osteoblasts indicates its probable role in osteoblast differentiation. Similarly, RARRES3 has been reported to modulate acylation status of key proteins of Wnt/β-catenin signaling pathway [25]. β-catenin plays a vital role in osteoblast lineage differentiation [26].

Among the differentially expressed genes in this study, the involvement of RUNX2, RANKL, OPG, and BGLAP in osteoclast differentiation and bone development has already been well established [5, 8]. RUNX2 is a master transcriptional factor that regulates osteoblast differentiation and bone formation, whereas OPG/RANKL signaling regulates bone mass and strength [27]. OPG prevents excessive resorption of bone by binding to RANKL [27]. Many other genes reported to be associated with bone development and remodeling pathways were also found to be differentially expressed in this study (such as SATB2, HIF1A, SOX9, SMAD5, WWTR1, TWSG1, CBFβ, TRAM2, CLTC). SATB homeobox 2 (SATB2) has been reported as a potent transcription factor to augment osteoblastogensis and promote bone regeneration [28]. Knockout SATB2 mice were shown to exhibit defects in osteoblast differentiation and function [29]. Hypoxia-inducible factor 1A (HIF1A) is essential for the maintenance of chondrocytes survival. HIF1A can impede the apoptosis of chondrocytes and regulate extracellular matrix (ECM) synthesis [30]. Synthesis of ECM may be mediated by

transactivation of SOX9 (SRY (sex-determining region Y)-box 9), a key transcription factor for chondrocyte-specific genes such as collagen II [30]. In our study, downregulation of both HIF1A and SOX9 signifies that NaF may induce chondrocytes apoptosis and matrix disruption. Chondrocyte apoptosis may further lead to osteoarthritis [30]. SMAD family member 5 (SMAD5) can alter bone morphogenetic protein (BMP) signaling pathway, thereby affecting the bone development [31]. Hossain et al. reported that WWTR1 (WW domain containing transcription regulator 1) mediates bone development by regulating CBFA1 (core-binding factor alpha 1) expression [32]. Skeletal defects were reported in WWTR1 −/− mice [32]. Yoshida et al. suggested that CBFβ (core-binding factor beta subunit) plays an important role in RUNX2-dependent skeletal development by augmenting DNA binding of RUNX2 and RUNX2-dependent transcriptional activation [33]. A CBFβ-deficient embryo showed severe defects in skeletal development [34]. Similarly, the altered expression of TRAM2 and CLTC may impair osteoblast development and differentiation [35, 36].

These findings entail that fluoride exposure can induce inflammation and oxidative stress in osteosarcoma cells, which subsequently modulate osteoclast differentiation and bone development processes. The expression of genes involved in other biological pathways (cell cycle, cell proliferation, cell apoptosis, DNA repair, response to stress/stimulus, homeostasis, etc.) was also found to be significantly altered after fluoride exposure. Further studies are needed to understand their crosstalk with inflammatory/oxidative stress and bone development pathways.

Conclusion

Clinical findings suggest bone loss in many chronic inflammatory diseases. These findings imply possible crosstalks between inflammatory and bone development pathways. However, such crosstalks have not been established at molecular level. Identification of genetic signatures of inflammatory stress and bone development can facilitate the discovery of new therapeutic targets against bone-related diseases. This can reduce our dependence on conventional anti-inflammatory drugs (e.g., glucocorticoids) which have adverse effects on bone development. We report possible involvement of inflammatory signatures in modulating osteoblast differentiation and bone development pathways in fluoride-exposed HOS cells. Further validation of inflammatory signatures using knockout in vivo models can facilitate selection of therapeutically relevant leads against bone-related diseases.

Compliance with Ethical Standards

Conflict of Interest   The authors declare that they have no conflict of interest.

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