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Role of fluoride induced histone trimethylation in development of skeletal fluorosis

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ABSTRACT

Chronic exposure to fluoride has been associated with the development of skeletal fluorosis. Limited reports are available on fluoride induced histone modification. However, the role of histone modification in the pathogenesis of skeletal fluorosis is not investigated. In the present study, we have investigated the role of fluoride induced histone modification on fluorosis development using human osteosarcoma (HOS) cell line. The expression of histone methyltransferases (EHMT1 and EHZ2) and level of global histone trimethylation (H3K9 and H3K27) have been assessed and observed to be increased significantly after fluoride exposure (8 mg/L). EpiTect chromatin immunoprecipitation (CHIP) qPCR Array (Human TGFβ/BMP signaling pathway) was performed to assess the H3K9 trimethylation at promoter regions of pathway-specific genes. H3K9 ChIP PCR array analysis identified hyper H3K9 trimethylation in promoter regions of TGFBR2 and SMAD3. qPCR and STRING analysis was carried out to determine the repressive epigenetic effect of H3K9 trimethylation on expression pattern and functional association of identified genes. Identified genes (TGFBR2 and SMAD3) showed down-regulation which confirms the repressive epigenetic effect of promoter H3K9 hyper trimethylation. Expression of two other vital genes COL1A1 and MMP13 involved in TGFBR2-SMAD signaling pathway was also found to be down-regulated with a decrease in expression of TGFBR2 and SMAD3. STRING analysis revealed functional association and involvement of identified genes TGFBR2, SMAD3, COL1A1 and MMP13 in the collagen and cartilage development/morphogenesis, connective tissue formation, bio-mineral tissue development, endochondral bone formation, bone and skeletal morphogenesis. In conclusion, present investigation is a first attempt to link fluoride induced hyper H3K9 tri-methylation mediated repression of TGFBR2 and SMAD3 with the development of skeletal fluorosis.

1. Introduction

Fluoride is a trace element, and intake of small amount of fluoride (below 1 mg/L) helps in bone and tooth development. However, chronic exposure of fluoride above recommended limits (above 1.5 mg/L) leads to skeletal and dental deformities called fluorosis (Gandhi et al., 2017; Kanduti et al., 2016; Kebede et al., 2016; Daiwile et al., 2015). In India, millions of people are on the verge of developing fluorosis due to the intake of fluoride contaminated water and till date, no possible treatment is available against fluorosis (Gandhi et al., 2017; Daiwile et al., 2015). It has been observed that exposure of fluoride alters growth, extracellular matrix (ECM) formation, bone mineralization and skeletal development, thus leads to development of fluorosis (Chai et al., 2017; Chai et al., 2016; Zhang et al., 2014a,b; Zhang et al., 2011; Wang et al., 2009; Wang et al., 2004). TGFβ-SMAD signaling regulates expression of essential genes (MMP13, Collagen Type I, Collagen Type VII, Aggrecan and Biglycans) involved in the formation of ECM (Lu et al., 2012; Allori et al., 2008; Meinhard et al., 2004). Aberrant changes in the expression of TGFβ-SMAD signaling after fluoride exposure affects the expression of COL1A1, a vital isoform of collagen synthesis in bone, which provides mechanical strength and flexibility to bone (Nair et al., 2011). The genetic factors responsible for fluorosis are well characterized, but the epigenetic alteration in the pathogenesis of fluorosis is still an unexplored domain.

Abbreviations: ChIP, chromatin immunoprecipitation; COL1A1, collagen type I alpha 1; ECM, extracellular matrix; EHMT1, euchromatic histone-lysine N-methyltransferase 1; EHZ2, enhancer of zeste homolog 2 (histone methyltransferases); HP1, heterochromatin protein 1; H3K27, histone H3 lysine 27; H3K9, histone H3 lysine 9; HMTs, histone methyltransferases; MMP13, matrix metallopeptidase 13; NaF, sodium fluoride; SMAD3, SMAD family member 3 TGFBR2 Transforming growth factor beta receptor II

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Modification of histone protein is one of the central epigenetic mechanisms. Histones undergo covalent modifications (methylation, acetylation, phosphorylation, ubiquitination, sumoylation, citrullination, and ADP-ribosylation) which alter the interaction between DNA and nuclear proteins (Chappell et al., 2016; Verma, 2015). These modifications in histone have been reported to regulate many diverse biological processes such as gene regulation, DNA repair, chromosome condensation, spermatogenesis (Chappell et al., 2016; Verma, 2015). Acetylation of ninth lysine (K) amino acid at H3 and trimethylation of H3 at K4 position were associated with activation of gene transcription, whereas trimethylation of H3K9 and H3K27 are associated with repression of gene transcription (Verma, 2015). Exposure to several environmental contaminants has been reported to alter histone protein. Modification of histone proteins by environmental contaminant plays an essential role in epigenetic activation or repression of genes, involved in pathogenesis in several diseases (Chappell et al., 2016; Tarale et al., 2016; Verma, 2015).

Modification of histone proteins due to fluoride exposure has been reported earlier. Yin et al. (2015) observed that administration of fluoride resulted in decreased levels of H3K9 and H3K18 acetylation in the mouse embryo, whereas Fu et al. (2014) reported that exposure of fluoride resulted in an increase in the level of H3K9 and H3K4 dimethylation, in early embryos of a mouse. These alterations in histone proteins result in impaired oocyte maturation and also have a detrimental effect on developing embryo of mouse (Yin et al., 2015; Fu et al., 2014). However available reports are unable to correlate fluoride induced histone modification with the pathogenesis of skeletal fluorosis. Identification of epigenetic alterations upon fluoride exposure can provide valuable information for better understanding of the mode of action of fluoride in the pathogenesis of fluorosis. Herein we report that exposure of fluoride resulted in significant increase in the global trimethylation of H3K9 and H3K27 with a subsequent increase in the expression profile of histone methyltransferase EHMT1 and EHZ2, which catalyses the transfer of methyl group at lysine position 9 and 27 of histone H3. TGFβ/BMP signaling ChIP PCR array was carried out in fluoride exposed cells to assess the status of H3K9 trimethylation at promoter region. Significant fold enrichment was observed in promoter regions of TGFBR2 and SMAD3. Gene expression analysis confirmed the repressive epigenetic effect of promoter H3K9 hyper trimethylation at transcription (mRNA) level of TGFBR2 and SMAD3. Expression of COL1A1 and MMP13 were also found to be downregulated with a decrease in expression of TGFBR2 and SMAD3. Hence for the first time, the present investigation suggests the possible involvement of H3K9 trimethylation mediated repression of TGFBR2 and SMAD3 genes on skeletal fluorosis development

2. Materials and methods

2.1. Chronic exposure of sodium fluoride (NaF)

Human osteosarcoma (HOS) cell line was procured from National Centre for Cell Science (NCCS) Pune, India and maintained in Minimum Essential Medium (MEM). In our previous publication, we have reported 40 mg/L as 24 h LC_{50} of sodium fluoride (NaF) for HOS cells (Daiwile et al., 2015). In this study, the HOS cells were chronically exposed to sub-lethal concentration of LC_{50} (8 mg/L) for the period of 30 days and respective controls were maintained. Briefly, 50,000 cells/ml were seeded in cell culture flask, followed by the treatment with NaF (8 mg/L). Cells were sub-cultured after every three days and concentration of NaF was replenished after every passage.

2.2. Histone isolation

Total histone proteins were isolated from the exposed and control cells using EpiQuik Total Histone Extraction kit (Epigentek, USA) following the manufacturer's protocol. In brief, the cells were centrifuged at 150*g*, 10^7 cells/ml for 5 min at 4 °C and cell pellet was re-suspended in Pre-Lysis Buffer, incubated for 10 min on ice and centrifuged at 1147 *g* for 5 min at 4 °C. The supernatant was discarded and the cell pellet was suspended in lysis buffer, incubated on ice for 30 min and centrifuged at 18514 *g* for 5 min at 4 °C. The supernatant was collected and the balance − DTT buffer was added to the supernatant, protein concentration was quantified using nanodrop ND-8000.

2.3. Histone quantification

Global change in the levels of H3K9, H3K27 tri-methylation and H3K9 acetylation following NaF exposure were determined using EpiQuikTM Global Trimethylation Histone (H3K9 and H3K27) Quantification Kit and EpiQuikTM Global Acetyl Histone H3K9 Quantification Kit as per manufacturer's instructions (Epigentek, USA). Briefly, 50 µl of C2 buffer was added to each well, which is followed by addition of 100 ng of isolated histone proteins to sample wells. Different concentrations of standard histone H3-K9, H3-K27 tri-methylation and H3K9 acetylation (1.5, 3, 6, 12, 25, 50, and 100 ng/µl) were added to standard wells. A blank was maintained and the plate was incubated for 2 h at room temperature. After incubation, the content of the plate was discarded and washed with 150 µl of C1 buffer. 50 µl of C3 buffer containing secondary antibody was added to each well and incubated for 60 min at room temperature on an orbital shaker. At the end of the incubation, the wells were washed with C1 buffer and 100 µl of C4 buffer was added to each well and incubated for 2 min at room temperature in the dark. The enzymatic reaction was stopped by addition of 50 µl of C5 buffer and absorbance was read at 450 nm. Levels of modified histone were calculated as% trimethylation and% acetylation.

2.4. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was performed using the EpiQuik™ Chromatin Immunoprecipitation Kit (Epigentek) as per manufacturer's protocol (Epigentek, USA). Briefly, 2×10^6 HOS cells were cross-linked by the addition of 9 ml fresh culture medium containing 1% formaldehyde and incubated for 10 min at room temperature on a rocking platform. After incubation 1 ml of 1.25 M glycine was added and cells were centrifuged at 150*g* for 5 min. The cell pellet was washed with 10 ml of PBS and resuspended in CP2A buffer, incubated for 10 min on ice and centrifuged at 3214*g* for 5 min. Cells were then suspended in CP3B lysis buffer containing a protease-inhibitor cocktail and incubated for 10 min on ice. Cells were sonicated for four pulses of 12 s each at 40% amplitude using Sonic Vibra cell (5 µl of Input DNA was kept separately and also processed for DNA isolation). The sonicated samples were immunoprecipitated with H3K9 trimethylation antibody (Epigentek, USA). Cross-linking between DNA and proteins was reversed by Proteinase K digestion of sample at 65 °C for 15 min followed by addition of CP6 buffer and heating at 65 °C for 90 min. Samples were cleaned using spin column and the DNA was eluted directly using CP8 elution buffer. The enrichment of isolated DNA was analyzed by EpiTect ChIP qPCR Array (Human TGFβ/BMP signaling pathway, Qiagen) with promoter-specific primers against 84 genes, according to manufacturer instructions. Data analysis was performed using ChIP qPCR Data Analysis Template (Qiagen).

2.5. Gene expression analysis

qPCR was carried out for histone methyltransferase gene expression, the primers used for histone methyltransferase EHMT1 and EZH2 were described earlier by Nagano et al. (2015); Grimaldi et al. (2011) (Supplementary Table 1). qPCR also carried to validate the expression of selected genes from ChIP qPCR Array. The genes selected were TGFBR2, SMAD3 and downstream signaling genes such as COL1A1 and MMP13 which are involved in the TGFBR2-SMAD signaling pathway. The primers used for SMAD3, TGFBR2, COL1A1 and MMP13 were