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ORIGINAL ARTICLE

Interactive effect of fluoride burden with calcitonin receptor gene polymorphisms on the risk of F bone injury

Jun Tu · Kejian Liu · Yu'e Song · Yuzeng Zhang · Caiyan Cui · Cuirong Lu

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Abstract
Purpose This study aims to examine the interactive effect of fluoride burden with calcitonin receptor (CTR) gene polymorphisms on the risk of fluoride (F) bone injury and provide the basis for determination of F bone injury risk factors.
Methods In this case–control study, a total of 119 cases and 126 controls were enrolled from 2 aluminum plants in Hubei province. F burden (UF) was measured by F ion-selective electrode method. The CTR gene polymorphisms were determined using the polymerase chain reaction—restriction fragment length polymorphism (PCR–RFLP) method. Logistic regression analysis was used to estimate multivariate-adjusted odds ratios, 95% confidence intervals (CI).
Results The odds of developing F bone injury for participants in the moderate F burden group versus the mild F burden group were 4.1 (95% CI: 1.9, 8.7); the heavy F burden group versus the mild F burden group were 14.1 (95% CI: 6.5, 30.6). The odds of developing F bone injury for participants with the TC & TT genotypes versus the CC genotype were 2.6 (95% CI: 1.4, 4.7). The interactions between TC & TT genotypes and moderate, heavy F burden were significant (OR = 14.4; OR = 40.3).
Conclusion The interactive effect of F burden and CTR genotype was significant, which increased the F bone injury risk.

J. Tu · K. Liu (✉) · Y. Song · Y. Zhang · C. Cui · C. Lu
Department of Occupational and Environmental Health, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Hubei, Wuhan 430030, People's Republic of China
e-mail: 172929473@qq.com

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Introduction

Fluoride (F) is an important factor in bone mineralization. F stimulates the osteogenetic process, resulting in an increase in bone mass (Baylink et al. 1983). However, long-term F exposure leads to high F burden, which can finally result in F bone injury (Ma et al. 2009). F bone injury is very common where there is industrial exposure to fluoride from dust or fumes (Zhang. 2007). F bone injury is especially prevalent in parts of China, India, and Africa and affects millions of people worldwide (Yang et al. 2003; Choubisa et al. 2001; Malde et al. 2003). F bone injury often results in osteosclerosis of the skeleton with significant long-term difficulties, including impaired neck and lumbar mobility, aching of the axial skeleton, kyphosis, and painful lower extremities, ultimately causing crippling and incapacitation (Tamer et al. 2007).

However, in the workers with the same F burden, only a few of them got F bone injury. There are individual differences during the occurrence of F bone injury, which may be caused by polymorphism of the bone metabolism–related genes. One of the hormones involved in bone metabolism is calcitonin (CT), a polypeptide hormone secreted by parafollicular cells of the thyroid gland (Bussolati and Pearse 1967), able to inhibit osteoclastic bone resorption and to stimulate urinary calcium excretion (Bijvoet et al. 1971). The human CT receptor (CTR) was cloned from an ovarian carcinoma cell line (BIN-67) (Gorn et al. 1992) and recognized to be a prototypic member of a distinct family of G-protein-coupled receptors with seven spanning domains (Segre and Goldring 1993). Recent studies showed

the significant associations with CTR gene polymorphism for osteoporosis (Drews et al. 2005). However, both the associations with CTR gene polymorphism for F bone injury and the interactive effect of F burden with genotype are not clear.

In order to investigate the potential interactive effect of F burden and CTR genotype on F bone injury, we conducted this case–control study in Hubei province. To the best of our knowledge, this study was the first case–control study to investigate the synergistic effects of CTR gene polymorphisms and F burden on F bone injury risk.

Materials and methods

Selection of case and control

The Molecular Epidemiology Study of Fluoride Bone Injury started in January 2007 and is an ongoing project. The project is sponsored by the China National Nature Science Foundation and involves one research institute and two aluminum plants, including the Occupational Health Institute of Tongji Medical College in Wuhan China, the Danjiang aluminum plant in Danjiang city of Hubei province, and the Bailian aluminum plant in Huanggang city of Hubei province. All fluoride bone injury cases and healthy controls were recruited from the two aluminum plants, who have been working in their present position for 5 years or longer. Due to the lack of female workers, all participants in our study were male.

Hundred and nineteen cases were enrolled in this study according to Chinese Diagnostic Criteria of Industrial Fluorosis, combined with F exposure history (more than 5 years) and the clinical symptoms (back and limb pain, neurasthenia syndrome, gastrointestinal symptoms, joint movement limitation, bone deformities, and nerve compression symptoms), ruled out the diseases that had similar X-ray changes (such as rheumatoid arthritis, osteopetrosis, bone metastases, and renal osteodystrophy). All healthy controls were recruited from persons who were undergoing occupational physical examinations in the aluminum plants. Controls were selected by frequency matching with cases on age, working history, smoking, and alcohol consumption. Written informed consent was obtained from each subject. The research was approved by the Institutional Ethical Boards of Tongji Medical College, Wuhan China, and had been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Blood sample, urine sample, and questionnaire information were collected from each participant. The structured questionnaires were administered in face-to-face interviews conducted by well-trained volunteers. The information requested in the questionnaire included standard demographic data, medicine history, lifestyle as well as occupational conditions. The database was established at the Occupational Health Institute of Tongji Medical College in Wuhan through a quality control and double-entry process.

After exclusion of people who had liver and/or kidney disease, or other illness that might affect the absorption and excretion of F in the F bone injury cases, a total of 119 cases were recruited. There were 126 frequency-matched controls who participated in this study. Controls in the present analysis were slightly younger than cases (2.0 years, on average).

Genotyping analyses

Blood samples were collected from a brachial vein within a few minutes after the end of the shift and immediately transferred into 3.7-ml borosilicate glass vials treated with edetic acid. Genomic DNA was extracted from the blood samples of all participants with a DNA extraction kit (AXYGEN, America). Polymorphisms in the CTR gene were determined by polymerase chain reaction (PCR) as previously described (Nakamura et al. 1997) using Taq DNA polymerase (Takara, Japan) and thermal cycler (PCR Primus 96 plus-MWG AG BIOTECH). Four ul of CTR-PCR product were digested with Alu (NEW England Biolabs, UK) according to manufacturer's instructions. Alu restriction digest yielded DNA fragments of 228-bp (CC), 228/120/108-bp (TC), and 120/108-bp (TT), which was visualized using a 3% agarose gel, stained with ethidium bromide.

Urinary fluoride analyses

Urine samples were collected as follows: just before the afternoon half-shift, the participants were asked to empty their bladders; then a sample of urine was collected at the end of the shift. Within a few minutes after voiding, urine was transferred into 40-ml borosilicate glass vials with air-tight silicone plugs. According to our procedure, only samples of the whole half-shift are considered acceptable, and samples are discarded if the worker had to urinate during the half-shift; in this study, no samples were lost for this reason. Vials were kept in the refrigerator until analysis. Ionic strength buffer solution was added to each sample, before fluoride concentrations were determined using an electronic meter (Orion 720A) and a fluoride-specific ion electrode (Orion 9609BN). Electrodes were calibrated with fresh, serially diluted standard solutions. All fluoride determinations were carried out in duplicate and the results averaged. According to The Normal Concentration of Chinese Population Urinary Fluoride, combined with the occupational exposure characteristics, the subjects were divided

into three groups (Mild F burden group: UF < 2 mg/l; Moderate F burden group: 2 ≤ UF ≤ 4 mg/l; and Heavy F burden group: UF > 4 mg/l).

Statistical analysis

Age, working history, and UF were divided into 3 levels. The χ^2 test was used to test for the statistical significance of different distributions of demographic characteristics and other risk factors between F bone injury cases and healthy controls. The χ^2 goodness-of-fit test was used to examine whether the CTR gene polymorphism was in Hardy–Weinberg equilibrium in the healthy controls.

For F bone injury, odds ratios and 95% confidence intervals were estimated for each risk factor, using unconditional logistic regression analysis with adjustment for potentially confounding variables, including age, working history, smoking, and alcohol consumption. We examined the modifying effect of F burden (mild, moderate, and heavy) on the association between F bone injury and CTR gene polymorphisms through stratification analysis. The interactions between CTR gene polymorphisms and fluoride burden were evaluated by including the product term in the unconditional logistic regression models. The estimated statistical power for the present analysis was 0.74 (2-sided $\alpha = 0.05$).

Then, we assessed the joint effects of CTR genotype and F burden on F bone injury risk by logistic regression after adjusting for age, working history, smoking, and alcohol consumption. All data analyses were performed using SPSS 16.0.

Results

As Table 1 shows, F bone injury cases were older (a mean age of 36.6 years in cases and 34.6 years in health controls) and had longer working history than controls (a mean working history of 15.9 years in cases and 14.5 years in healthy controls). The smoking status was similar in cases and controls. The prevalence of alcohol consumption was higher in cases (84.0%) than in controls (65.9%). For F burden, cases were more likely to have heavy burden (58.8%) than were controls (19.1%), meanwhile controls were more likely to have mild burden (46.0%) than were cases (10.1%). The polymorphism of CTR gene was in Hardy–Weinberg equilibrium in controls and in the overall sample ($P > 0.05$). We observed differences in the distribution of the genotypes in cases and controls, as the CC genotype was more represented in controls in comparison with cases, with a frequency of 82.5 versus 64.7%. Conversely, the TC and TT genotypes were more represented in cases with frequencies of 27.7 versus 14.3% and 7.6 versus 3.2%, respectively. As risks of the TT genotype with all together nine cases and four controls were difficult to estimate, genotypes TT and TC were considered together versus CC genotype.

Table 1 Distribution of basic informations, UF, and CTR gene polymorphisms among fluoride bone injury cases and healthy controls

Characteristic	Fluoride bone injury cases ($n = 119$)	Controls ($n = 126$)
Age [years, no. (%)]		
<30	15 (12.6)	18 (14.3)
30–39	66 (55.5)	92 (73.0)
≥40	38 (31.9)	16 (12.7)
Working history [years, no. (%)]		
<10	25 (21.0)	33 (36.2)
10–19	63 (52.9)	74 (58.7)
≥20	31 (26.1)	19 (15.1)
Smoking, no. (%)		
Yes	50 (42.0)	60 (47.6)
No	69 (58.0)	66 (52.4)
Alcohol consumption, no. (%)		
Yes	100 (84.0)	83 (65.9)
No	19 (16.0)	43 (34.1)
F burden (UF, mg/l)		
Mild (UF < 2)		
No. (%)	12 (10.1)	58 (46.0)
Mean ± SD	1.65 ± 0.27	1.48 ± 0.33
Range	1.31–1.97	0.79–1.97
Moderate (2 ≤ UF ≤ 4)		
No. (%)	37 (31.1)	44 (34.9)
Mean ± SD	2.92 ± 0.52	2.89 ± 0.50
Range	2.07–3.82	2.07–3.75
Heavy (UF > 4)		
No. (%)	70 (58.8)	24 (19.1)
Mean ± SD	9.35 ± 5.54	7.39 ± 2.84
Range	4.11–26.77	4.30–16.90
CTR gene polymorphism, no. (%)		
CC	77 (64.7)	104 (82.5)
TC	33 (27.7)	18 (14.3)
TT	9 (7.6)	4 (3.2)

UF urinary fluoride, *CTR* calcitonin receptor

As Table 2 shows, there were significant associations with F burden for F bone injury. The participants with moderate and heavy F burden had 4.1-fold (95% confidence interval (CI): 1.9, 8.7; $P < 0.001$) and 14.1-fold (95% CI: 6.5, 30.6; $P < 0.001$) higher odds of developing F bone injury than the ones with mild F burden after adjustment for age, working history, smoking, and alcohol consumption. There were significant associations with the TC&TT genotypes for F bone injury (odds ratio (OR) = 2.6, 95% CI: 1.4, 4.7; $P < 0.01$), as shown in Table 2.

Table 2 Odds ratios for fluoride bone injury according to fluoride burden and CTR gene polymorphisms (obtained by logistic regression)

Fluoride burden or genotype	Cases (n = 119)		Controls (n = 126)		OR	95% CI
	No.	%	No.	%		
Fluoride burden[a]						
Mild (UF < 2)	12	10.1	58	46.0	1.0	
Moderate (2 ≤ UF ≤ 4)	37	31.1	44	34.9	4.1**	1.9, 8.7
Heavy (UF > 4)	70	58.8	24	19.1	14.1**	6.5, 30.6
CTR gene polymorphism[b]						
CC	77	64.7	104	82.5	1.0	
TC + TT	42	35.3	22	17.5	2.6*	1.4, 4.7

UF urinary fluoride, *CTR* calcitonin receptor, *CI* confidence interval, *OR* odds ratio

* $P < 0.01$, ** $P < 0.001$

[a] Adjusted for age, working history, smoking and alcohol consumption

[b] Adjusted for age, working history, smoking, alcohol consumption and fluoride burden

Table 3 Odds ratios for fluoride bone injury according to fluoride burden and CTR gene polymorphisms (obtained by logistic regression with adjustment for age, working history, smoking and alcohol consumption)

Fluoride burden and genotype	Cases		Controls		OR	95% CI
	No.	%	No.	%		
Mild fluoride burden						
CC	8	66.7	46	79.3	1.0	
TC + TT	4	33.3	12	20.7	1.9	0.5, 7.5
Moderate fluoride burden						
CC	27	73.0	38	86.3	1.0	
TC + TT	10	37.0	6	13.7	2.3	0.7, 7.2
Heavy fluoride burden						
CC	42	60.0	20	83.3	1.0	
TC + TT	28	40.0	4	16.7	3.3*	1.0, 10.8

UF urinary fluoride, *CTR* calcitonin receptor, *CI* confidence interval, *OR* odds ratio

* $P < 0.05$

Table 4 Joint effects of fluoride burden and CTR gene polymorphisms on the odds of fluoride bone injury (obtained by logistic regression with adjustment for age, working history, smoking and alcohol consumption)

Fluoride burden and genotype	Fluoride bone injury cases (n = 119)	
	OR	95% CI
Mild fluoride burden		
CC	1.0	
TC + TT	1.9	0.5, 7.5
Moderate fluoride burden		
CC	4.1*	1.7, 10.0
TC + TT	14.4**	3.6, 57.2
Heavy fluoride burden		
CC	12.0**	4.8, 30.3
TC + TT	40.3**	11.1, 146.1

CI confidence interval, *OR* odds ratio

* $P < 0.01$, ** $P < 0.001$

Table 3 shows findings from stratification analyses of association between CTR gene polymorphisms and F bone injury risk, stratified by F burden. CTR genotype was not significantly associated with F bone injury among mild and moderate F burden groups. In heavy F burden group, however, the TC&TT genotypes were associated with a significantly increased risk of F bone injury (OR = 3.3, 95% CI: 1.0, 10.8; $P < 0.05$).

Table 4 presents the joint effects of the functional polymorphism of CTR gene and F burden on the risk of F bone injury. We found that participants with moderate (OR = 14.4, 95% CI: 3.6, 57.2; $P < 0.01$) and heavy (OR = 40.3, 95% CI: 11.1, 146.1; $P < 0.001$) F burden carrying the TC&TT genotypes (when compared with participants with mild F burden carrying the CC genotype) had significantly increased odds of developing F bone injury. The effects of F burden level on the association between CTR genotype and F bone injury risk are also shown in Table 4. Increasing odds of F bone injury with the TC&TT genotypes were observed in moderate and heavy F burden groups. These findings provided empirical evidence for synergistic effects of CTR genotype and F burden on F bone injury risk.

Discussion

The occurrence of F bone injury is a complex process that involves both exogenous and endogenous factors. Except for F burden, F bone injury is also associated with the genetic background (Cooper et al. 2006). F enters the body primarily through respiratory or digestive tract and

accumulates in bone and soft tissue via the blood circulation. By stimulating osteoblasts, F promotes bone formation in both cortical and trabecular bone, but its effect on trabecular bone is greater, occurs earlier, and leads to a more pronounced increase in spine density on x-ray films (Baylink et al. 1983) Most F bone injury patients' bone and soft tissue are involved. UF is a comprehensive index of the body F burden, bone F accumulation, and body F excretion. UF is stable enough to reflect the levels of F exposure and F intake and to evaluate the F burden and environmental F levels (Li et al. 2000). In the present study, the number of participants with moderate and heavy F burden in case group was significantly higher ($P < 0.001$), which indicated that F bone injury cases had a higher F burden than the controls. Compared with mild F burden group, both the moderate F burden (OR = 4.1) and the heavy F burden (OR = 14.1) group participants had a higher risk of F bone injury. The increased risk was due to the excessive F burden, which could lead to the accumulation of fluorapatite in bone. Fluorapatite is difficult to be absorbed, could destroy the dynamic balance of bone resorption and formation, and finally leads to bone metabolic disorders.

The CTR gene was located on human chromosome 7q21.3. And there were 11 polymorphic loci found in the scan of human CTR gene. Two of which were located in the gene coding region, one of them was with amino acid mutations. The C to T mutation in the nucleotide sequence 1377 leads to the leucine-to-proline mutation of the corresponding protein, so that there were three genotypes for the CTR gene [CC (pure proline type), TC (proline, leucine hybrid type), and TT (pure leucine type)] (Charopoulos et al. 2008). The association between the CTR genotype and the bone mineral density (BMD) has been described, which showed the inconsistent distribution of CTR genotype frequency between Asians (mainly CC type) and Caucasians (mainly TT type) (Masi et al. 1998; Nakamura et al. 2001). In our study, the results of the genotype analysis showed 181 participants possessed CC type, 51 participants possessed TC type and 13 participants possessed TT type, which was consistent with the CTR genotype frequency distribution in Asians (mainly CC type). In case group, a higher proportion of carrying TC&TT genotypes indicated that participants possessed the TC&TT (OR = 2.6) genotypes had a higher F bone injury risk. This may be related to the different BMD between TC&TT genotypes and CC genotypes.

Further analysis found that the interactions between moderate F burden and TC&TT genotypes in F bone injury was significant; the interaction made the higher risk of F bone injury than the single factor effect (OR = 14.4). Interactions between heavy F burden and the TC&TT genotypes in F bone injury were significant, which greatly increased the risk of F bone injury (OR = 40.3). It was clear that heavier F burden led to the stronger interactive effects between F burden and TC&TT genotypes.

The present study had several limitations. Firstly, one-time measurement of UF may not accurately reflect long-term F exposure. However, the subjects had been working for more than 5 years in their present positions, and the daily work environment was the same, post-shift UF could probably reflect the long-term exposure by one-time measurement on workday. Secondly, our cases were older, had longer working history and higher prevalence of alcohol consumption than controls, which may have resulted in biased odds ratio estimates. However, such a bias would have occurred only if younger controls with shorter working history and lower alcohol consumption were more likely to have a particular genotype, which is an unlikely scenario. Furthermore, we tried to control the confounding effects of age, working history, and alcohol consumption by including them in the logistic regression models in this study.

In summary, we found main effects of the TC&TT genotypes on the development of F bone injury, and there were significant interactive effects of F burden with TC&TT genotypes on F bone injury. Although F burden is the major cause of F bone injury, only a part of participants with F burden ever developed F bone injury. This may be due to some genetic factor(s) that may reduce the effect of F burden on the risk of F bone injury. The interactive effects of other genes and F burden on F bone injury risk are worth additional investigation in future studies. Meanwhile, the cases should be recommended to stop F exposure, enhance nutrition supplements, and receive symptomatic treatment. The others should be equipped with personal protective gears and accept periodic occupational health examinations. Monitoring and control of occupational hazards in the workplace need to be strengthened.

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

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