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Complete List of Authors:	Yang, dan; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Liu, Yang; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Chu, Yanru; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Yang, Qing; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Jiang, Wei; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Chen, Fuxun; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Li, Dandan; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Qin, Ming; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081

	Sun, Dianjun; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Yang, Yanmei; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Gao, Yanhui; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081
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Title page

Vitamin D receptor gene FokI polymorphism may have a protective effect on skeletal fluorosis of the brick-tea type fluorosis.

Dan Yang,[1,2] Yang Liu,[1,2] Yanru Chu,[1] Qing Yang,[1] Wei Jiang,[1] Fuxun Chen,[1] Dandan Li,[1] Ming Qin,[1] Dianjun Sun,[1] Yanmei Yang[1,*] and Yanhui Gao[1,*]

[1]Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin, China

[2]Equal contributions

***Corresponding author:** Yanhui Gao, E-mail address: gaoyh411@163.com;

***Corresponding author:** Yanmei Yang, E-mail address: yangym0916@163.com

Address of corresponding author: Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 150081, Heilongjiang Province, China.

Telephone: +86(0)451 8750 3097 Fax: +86(0)451 8667 6184

Key Words: Brick-tea type fluorosis; VDR-FokI polymorphism; Skeletal fluorosis.

Vitamin D receptor gene FokI polymorphism may have a protective effect on skeletal fluorosis of the brick-tea type fluorosis.

ABSTRACT

Background: Brick-tea type fluorosis is a public health concern in the north-west area of China. The VDR-FokI polymorphism is considered to be a regulator of bone metabolism and calcium resorption. However, the association of VDR-FokI polymorphism with the risk of brick-tea type fluorosis has not been reported.

Materials and Methods: A cross-sectional case-control study was conducted in three provinces (Inner Mongolia, Qinghai, Sinkiang), China. The fluoride contents of Brick-tea water or urinary were tested by the standard of GB 1996-2005 or WS/T89-2006 (China), respectively. The skeletal fluorosis was diagnosed by the standard of WS/192-2008(China). The VDR-FokI polymorphism was detected by Sequenom MassARRAY system.

Result: Compared to the carriers with CC genotype, the participants with CT/TT genotype had a significantly decreased risk of skeletal fluorosis (OR=0.761 [95%CI, 0.580-0.997]), after adjustment of risk factors. This protective of CT/TT genotype in VDR-FokI appeared to be more pronounced in participants with 3.2mg/L~ UF (OR=0.514 [95%CI, 0.306-0.865]). When investigated among ethnic groups, the protective effect of the CT/TT was limited in the Mongolian participants (OR=0.525 [95%CI, 0.278-0.991]). Moreover, the interaction of VDR-FokI with risk factors was only found in Mongolian participants: the protective effect of the CT/TT was limited

to participants with 7.0mg/day~ IF (OR=0.085 [95%CI, 0.009-0.851], or participants with 3.2mg/L~ UF (OR=0.103 [95%CI, 0.017-0.633]), or participants aged 46 to 65 (OR=0.404 [95%CI, 0.177-0.922].

Conclusion: Our data suggest that the CT/TT genotype of VDR-FokI may be a protective factor for the brick-tea type skeletal fluorosis and this effect is pronounced in Mongolian participants.

Key Words: Brick-tea type fluorosis; VDR-FokI polymorphism; Skeletal fluorosis.

Strengths and limitations of this study:

1、 It is the first time to report the association between FokI polymorphism and skeletal fluososis overall or by ethnicity. Improved understanding of such gene-environment interactions would not only contribute to knowledge on the skeletal fluorosis, but also help in putting forward corresponding preventive measures.

2、 We find the CT/TT genotype of VDR-FokI plays a protective role in the brick-tea type fluorosis.

3、 We find the interaction between VDR-FokI and urinary fluoride in which reduced the risk of skeletal fluorosis was only present in participants with 3.2 mg/L~.

4、 We noticed that a more than 20% reduction in risk of skeletal fluorosis related with CT/TT genotype in VDR-FokI in all participants was largely attributable to the reduced risk in Mongolian participants.

5、 Study has suggested that the VDR-FokI polymorphism may influence the bone metabolism through the dietary calcium. Unfortunately, in this study we did not have the data on the dietary of calcium.

INTRODUCTION

Fluoride is a natural component of the biosphere and ingestion of excess fluoride can lead to fluorosis.[1, 2] Skeletal fluorosis and dental fluorosis are the main clinical outcomes of fluorosis.[3] Fluorosis affects millions of people around the world, and there are no established treatments for fluorosis patients, so it has becoming a major public health issue throughout the world.[4-6] According the sources of fluoride to human body, fluorosis can be divided into three: drinking-water type, burning-coal type and brick-tea type. The brick-tea type of fluorosis is caused by the habitual consumption of brick-tea in large quantities.[7] In China, the prevalence rate and severity of both the water-type and coal-burning type of fluorosis trended to decline, because of a series of preventive measures, however, brick-tea type of fluorosis is still a severe public health issue, because it is impossible to alter the habitual consumption of brick-tea.

The pathogenesis of skeletal fluorosis, a chronic metabolic bone disease caused by excessive accumulation of fluoride in the bones, is a complex process, bone metabolism strengthen is the most prominent characteristic of it. So, besides the level and duration of fluoride exposure, the diversity of clinical manifestations of skeletal fluorosis correlates directly with the complexity of bone metabolism.[8] Bone metabolism is characterized by two opposite but finely coupled processes, bone formation and resorption. Osteoblasts are bone-forming cells that are responsible for the formation of new bone, while osteoclasts are mononucleated cells that are responsible for bone resorption. When bone formation and resorption are well

balanced, normal bone mass is maintained. In contrast, excessive resorption by osteoclasts contributes to osteoporosis, whereas the excessive formation by osteoblasts may result in osteopetrosis.[9] So, the number and activity of osteoblasts and osteoclasts, which is regulated by a number of genes, determine whether bone metabolism is balanced, or whether bone mass is increased or decreased. Therefore, individual differences in response to the disease of bone abnormal metabolism largely depend on genetic susceptibility.[10] Previous study reported that polymorphism of myeloperoxidase gene[11] is related to burning-coal type fluorosis, which suggested that gene polymorphism may play an important role in the pathogenesis of skeletal fluorosis.

Among potential genes relevant to bone metabolism,[10] vitamin D receptor (VDR) gene is considered to be an important candidate gene.[12] Morrison first reported that VDR gene polymorphism is associated with bone mineral density and it can predict the bone density.[13] VDR gene is located on chromosome 12 and contains 9 exons.[14, 15] Four VDR gene restriction endonuclease sites are known: FokI (rs2228570), BsmI (rs1544410), ApaI (rs7975232), and TaqI (rs731236).[13, 15, 16] Different VDR gene polymorphism plays different roles in regulating the bone metabolism.[17-20] It was reported that FokI polymorphism was related to bone mineral density and calcium absorption.[21-23] However, the association of FokI polymorphism with the risk of skeletal fluorosis has not been reported.

Our prior study has demonstrated ethnic difference in prevalence rate of the brick-tea type fluorosis and that glutathione S-transferases (GST) rs1695

polymorphism may play an important role in the pathogenesis of brick-tea fluorosis.[24] However, there are no studies reported the effect of VDR-FokI on skeletal fluorosis of the brick-tea type fluorosis. Therefore, in this study, we conducted a cross-sectional case-control study in the brick-tea fluorosis area of China to investigate the association between FokI polymorphism and skeletal fluorosis overall or by ethnicity.

MATERIALS AND METHODS

Subjects and data collection

On the brick-tea type fluorosis survey data, we undertook a cross-sectional case-control study in sixteen villages from the Inner Mongolia, Qinghai and Sinkiang province, People's Republic of China, from July to August in 2012. In this study, overall participants were older than 16 years old, born and grew up in their village. Each participant received the questionnaire survey and clinical examination. The clinical examination included physical examination, medical history and X-ray diagnosis (Beijing Longsafe Imaging Technology Co., Beijing City, China). The content of the questionnaire included general information of respondents (name, sex, age, education, economic income, the history of bone related disease, etc), and fluoride exposure (the amount of drinking brick-tea water per day, the consumption of brick-tea per year). In addition, we also collect everyone's blood, urine and brick-tea water samples. All participants signed informed consent, and this research was approved by Harbin Medical University Ethics Committee.

Diagnose of skeletal fluorosis

The skeletal fluorosis was diagnosed according to the Chinese diagnostic criteria of endemic skeletal fluorosis (WS192-2008, china). As described in previous report,[24] an individual's skeletal fluorosis was divided into three categories: mild, moderate or severe.

Determination of fluoride concentration

All urine and brick-tea water samples were stored at -20℃ until use. Urinary fluoride content was determined by the fluoride ion electrode method based on The China's Urinary Fluoride Detection-Fluoride Electrode Method WS/T89-1996. The concentration of brick-tea water was also determined by the fluoride ion electrode method with the standard of GB19965-2005 (China).

Genotyping

All blood samples were stored at -80℃ until use. The genomic DNA was extracted from the blood samples using the DNA extraction kit (Axygen Biosciences, Union City, USA). The DNA concentration was tested by TU1901 Spectrophotometry (Purkinje General Company, Beijing City, China). When the DNA concentration was greater than 20μg/ml, preserve the extracted DNA was preserved at -80℃. The genotyping sequencing from the extract were performed by the shanghai Fenglin Clinical Laboratory Company (http://www.fenglinlab.com/index.asp) using the

Sequenom MassARRAY system (Sequenom, Inc., San Diego, CA, USA). The primer sequence of the FokI: forward-5'-ACGTTGGATGTGGCCTGCTTGCTGTTCTTA-3', reverse-5'-ACGTTGGATGACGTTCCGGTCAAAGTCTCC-3', extended-5'-GTGCTGGCCGCCATTGCCTCC-3'. For the genotyping sequencing quality control, blinded blood duplicate was used.

Statistical analysis

All statistical analyses were conducted using the SPSS version 19.0 (SPSS Inc., Chicago, IL). Pearson's chi-square test was used for test of the differences between fluorosis patients and control people. Odds ratios (OR) and corresponding 95% confidence intervals (CI) were calculated for skeletal fluorosis risk using logistic regression. Testing for deviation from Hardy-Weinberg equilibrium (HWE) was performed within the participants stratified by case and control using a chi-square test. Wald's test statistic was used for test the Gene-environment interactions. The significance level in this study was taken as $P<0.05$.

Ethical statement

The study was proved by the Harbin Medical University Ethics Committee (HMUIRB20120021). All of these participants singed informed consent, and we also obtained written informed consent from the guardians on behalf of the minors. For the brick-tea water samples collection, there were no specific permits required for the locations or activities association in this field study. The locations were not privately owned or protected in any way and this field study did not involve endangered or

protected species.

RESULTS

Participants characteristics.

There were 336 subjects who were diagnosed with skeletal fluorosis with the prevalence rate 26.2% (336/1284). Demographic of skeletal fluorosis cases and controls are presented in **Table 1**. Skeletal fluorosis cases were significantly older than controls (p<0.001). And there more male in the skeletal fluorosis cases than in the controls (p=0.015). Skeletal fluorosis cases were significantly more likely to have a more daily fluoride intake (IF) (p<0.001) and a high urine fluoride (UF) status (p<0.001). In addition, ethnical differences in skeletal fluorosis were observed (p<0.001): more Tibetan cases are the most in skeletal fluorosis cases, following are Kazakh cases and the least are Mongolian and Han cases.

Association of VDR-FokI polymorphism with skeletal fluorosis.

Testing for deviation from Hardy-Weinberg equilibrium (HWE) was performed within the control participants stratified by ethnicity using a chi-square test. All ethnical participants were found to be in Hard-Weinberg equilibrium for VDR-FokI. Because of low frequency of T allele, we divided the participants into two groups by the presence or absence of T allele, that is, CC and CT/TT groups **(Table2)**. Participants with CT/TT genotype had a significantly decreased risk of skeletal fluorosis (OR=0.717 [95%CI, 0.556-0.925]). After adjustment of the risk factors, the protective effect of CT/TT genotype remained statistically significant (OR=0.761

[95%CI, 0.580-0.997]). In addition, there was the suggestion of a difference in skeletal fluorosis risk by ethnicity in relation to VDR-FokI polymorphism. After adjustment of the risk factors, the protective effect of the CT/TT was limited in the Mongolian participants (OR=0.525 [95%CI, 0.278-0.991]). The smaller percentage of Mongolian participants in our analysis appear to be driving the overall more than 20% reduction in risk as our data do not show a similar reduction did not find shared by other three ethnical participants.

Stratification by IF.

We investigated the potential interactions between FokI-SNP and IF **(Table3)**. This interaction was only apparent for Mongolian participants but not other three ethnical participants. For Mongolian participants, a decrease risk of skeletal fluorosis among carriers with CT/TT genotype was limited to participants with 7.0mg/day~ IF (OR=0.085 [95%CI, 0.009-0.851] vs. OR=0.538 [95%CI, 0.216-1.337] in participants with ~3.5mg/day IF, vs. OR=0.671 [95%CI, 0.220-2.048] in participants with 3.5~7.0mg/day IF).

Stratification by UF.

We also observed the potential interactions between VDR-FokI SNP and UF. The risk of skeletal fluorosis varied according to UF for FokI-SNP **(Table 4)**. A decrease risk of skeletal fluorosis among the carriers with CT/TT genotype was limited to participants 3.2mg/L~ UF (OR=0.514 [95%CI, 0.306-0.865] vs. OR=0.701 [95%CI, 0.444-1.105] in participants with ~1.6mg/L UF, vs. OR=1.145 [95%CI, 0.713-1.837] in participants with 1.6~3.2mg/L UF). We further evaluated the interaction between

FokI-SNP and UF by ethnicity. This interaction was also only apparent for Mongolian participants but not other three ethnical participants. For Mongolian participants, a decrease risk of skeletal fluorosis among carriers with CT/TT genotype was limited to participants with 3.2mg/L~ UF (OR=0.103 [95%CI, 0.017-0.633] vs. OR=0.617 [95%CI, 0.245-1.555] in participants with ~1.6mg/L UF, vs. OR=0.772 [95%CI, 0.246-2.426] in participants with 1.6~3.2mg/L UF).

Stratification by Age.

We also investigated the interactions between FokI SNP and age **(Table 5)**. This interaction was also only apparent for Mongolian participants but not other three ethnical participants. For Mongolian participants, a decrease risk of skeletal fluorosis among carriers with CT/TT genotype was limited to participants aged 46 to 65 (OR=0.404 [95%CI, 0.177-0.922] vs. (OR=0.443 [95%CI, 0.127-1.548] in participants aged below 45 vs. OR=3.808 [95%CI, 0.156-93.179] in participants aged above 66).

DISCUSSION

Although high fluoride exposure is highly relevant, it is not the only factor influencing the susceptibility to fluorosis. Not everyone living in areas with naturally high fluoride levels in water suffers fluorosis.[5, 25] Three inbred strains of mice (A/J, SWR/J and 129P3/J) that showed different susceptibilities to dental fluorosis, displayed variation in bone response to fluoride exposure,[26-28] So, genetics might influence the bone response of individuals to fluoride exposure. In our previous study, we observed that the prevalence rate and severity in different ethnical participants was different, which suggested the possible contribution of a genetic differences in

fluorosis disparities.[24] In addition, the distribution of environment factors related with fluoride exposure, including age, IF and UF, differs by ethnicity. As a result, it is plausible that the interaction between gene and environment factors might explain, in part, the racial/ethnic differences in fluorosis prevalence.

Of the previous studies on genetic variation and fluorosis risk, the contribution or association of SNPs on dental fluorosis or skeletal fluorosis has been reported in several studies.[11, 24, 29-31] However, none studies have reported the association between VDR-FokI polymorphism and skeletal fluorosis of the brick-tea type fluorosis. Calcium could against the toxic effects of fluoride to a certain extent,[32] but fluoride ingestion reduces intestinal calcium absorption. VDR-FokI gene polymorphism has been reported to be associated with bone metabolism and calcium absorption.[21-23] Therefore, in the present study we focus on the role of VDR-FokI polymorphism in the skeletal fluorosis of the brick-tea type fluorosis. We noticed that a more than 20% reduction in risk of skeletal fluorosis related with CT/TT genotype in VDR-FokI in all participants was largely attributable to the reduced risk in Mongolian participants; moreover, this protective effect remained significant after adjustment of age, sex, IF and UF. These results suggested that the protective of CT/TT genotype against brick-tea type of fluorosis might be present among Mongolian participants, but not other three ethnical participants.

Factors, including age, sex, does and duration of fluoride intake, that influence the fluoride toxicity and the clinical presentation, are the major risk factors of fluorosis.[4, 8] So, we investigated the potential interactions between VDR-FokI SNP and known

risk factor overall or by ethnic. Stratified our analysis by UF, we found that this protective of CT/TT genotype of VDR-FokI against brick-tea type of fluorosis appeared to be more pronounced in participants with 3.2mg/L~ UF, and this was also attributable to the reduced risk in Mongolian participants. Stratified our analysis by IF or age, we did not observed this protective of CT/TT genotype of VDR-FokI against brick-tea type of fluorosis. Despite this, we further evaluated this interaction by race. This protective effect of CT/TT was detected only in Mongolian participants with 7.0mg/day~ IF or aged 46 to 65. However, these findings of the VDR-FokI SNP analysis stratified by risk factors need to be interpreted with caution because the data became sparse when several factors were investigated simultaneously.

In the present study, this protective effect of CT/TT genotype of VDR-FokI was limited in Mongolian participants with 7.0mg/day~ IF, or with 3.2 mg/L~ UF. UF reflected the fluoride accumulation in the human body. So we can conclude that this protective effect was pronounced in high fluoride exposure condition. It might result from the limitation of the sample size, because large sample is required to get difference while the prevalence rate of fluorosis is low under the low exposure to fluoride.

The amount of fluoride accumulation in the body increased with age,[33-35] so the prevalence rate and severity of skeletal fluorosis is also increased with age. In our data, this protective effect of CT/TT genotype was limited in Mongolian participants aged 46~65. For younger people, the fluoride load in body is low because of short time periods of fluoride exposure, so the prevalence rate of fluorosis is small. Thus, it is

necessary to investigate this interaction in a large sample population. For the older people (over 66 years old), the protective effect of CT/TT genotype might be compromised by the toxicity of high fluoride load in body.

A study in Indian girls indicated after supplementation of calcium, the carriers of TT genotype had significant increase in bone mass as compared to CC genotype,[20] which suggested that the VDR-FokI polymorphism may influence the bone metabolism through the dietary calcium. Unfortunately, we did not have the data on the dietary of calcium. So, it is need to investigate the interaction of VDR-FokI SNP with dietary calcium in fluorosis.

CONCLUSIONS

In summary, we find the CT/TT genotype of VDR-FokI plays a protective role in the brick-tea type fluorosis and the interaction between VDR-FokI and UF in which reduced the risk of skeletal fluorosis was only present in participants with 3.2 mg/L~ UF. And we also indicated this protective was apparent in Mongolian participants. Improved understanding of such gene-environment interactions would not only contribute to knowledge on the skeletal fluorosis of brick-tea type fluorosis, but also help in putting forward corresponding preventive measures. Therefore, larger studies are necessary to study the role of genes and/or polymorphic site influencing bone mass in the pathogenesis of fluorosis.

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Data sharing statement: No additional data available.

Contributor statement

1、 Analysis or interpretation of data; Writing articles: Yang, Dan and Liu, Yang

2、 Collected data: Chu, Yanru、Yang, Qing、Jiang, Wei and Chen, Fuxun

3、 Interpretation of data: Li, Dandan and Qin, Ming

4、 Science advisors; The conception or design of the work: Gao, Yanhui、Yang, Yanmei and Sun, Dianjun

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464 **Table 1. Characteristics of skeletal fluorosis cases and controls (N=1284).**

	Cases N(%)	Controls N(%)	*p*
Age			＜0.001
～45	59(17.6)	331(34.9)	
46～65	203(60.4)	499(52.6)	
66～	74(22.0)	118(12.4)	
Sex			0.015
Male	156(46.4)	368(38.8)	
Female	180(53.6)	580(61.2)	
Ethnicity			＜0.001
Tibetan	123(36.6)	185(19.5)	
Kazakh	98(29.2)	192(20.3)	
Mongolian	58(17.3)	203(21.4)	
Han	57(17.0)	368(38.8)	
IF			＜0.001
~3.5mg/day	91(27.1)	309(32.6)	
3.5~7.0mg/day	131(39.0)	446(47.0)	
7.0~mg/day	114(33.9)	193(20.4)	
UF			＜0.001
~1.6mg/L	107(31.8)	494(52.1)	
1.6~3.2mg/L	119(35.4)	266(28.1)	
3.2mg/L	110(32.7)	188(19.8)	

465 Percentages are adjusted for sampling weights and may not sum to 1 due to rounding;

466 *P* value difference by case status.

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Table 2. Risk of skeletal fluorosis associated with VDR-FokI polymorphic genotypes in study participants overall and stratified by ethnicity.

	Case N(%)	Control N(%)	crude OR(95%CI)	adjusted OR(95%CI)*
All participants				
CC	143(42.6)	329(34.7)	1.0(ref)	1.0(ref)
CT+TT	193(57.4)	619(65.3)	**0.717(0.556,0.925)**	**0.761(0.580,0.997)**
Tibetan				
CC	44(35.8)	62(33.5)	1.0(ref)	1.0(ref)
CT+TT	79(64.2)	123(66.5)	0.905(0.561,1.461)	0.947(0.562,1.598)
Kazakh				
CC	50(51.0)	85(44.3)	1.0(ref)	1.0(ref)
CT+TT	48(49.0)	107(55.7)	0.763(0.468,1.242)	0.729(0.439,1.210)
Mongolian				
CC	31(53.4)	70(34.5)	1.0(ref)	1.0(ref)
CT+TT	27(46.6)	133(65.5)	**0.458(0.254,0.828)**	**0.525(0.278,0.991)**
Han				
CC	18(31.6)	112(30.4)	1.0(ref)	1.0(ref)
CT+TT	39(68.4)	256(69.6)	0.948(0.520,1.729)	0.945(0.503,1.774)

*Adjusted for age, sex, IF and UF.

Table 3. Risk of skeletal fluorosis associated with VDR-FokI polymorphic genotypes in study participants, stratified by IF levels.

	~3.5mg/day			3.5~7.0mg/day			7.0mg/day~		
	Case N(%)	Control N(%)	OR(95%CI)*	Case N(%)	Control N(%)	OR(95%CI)*	Case N(%)	Control N(%)	OR(95%CI)*
All participants									
CC	40(44.0)	106(34.3)	1.0(ref)	49(37.4)	156(35.0)	1.0(ref)	54(47.4)	67(34.7)	1.0(ref)
CT+TT	51(56.0)	203(65.7)	0.630(0.377,1.053)	82(62.6)	290(65.0)	1.039(0.667,1.619)	60(52.6)	126(65.3)	0.626(0.376,1.045)
Tibetan									
CC	12(44.4)	13(31.7)	1.0(ref)	7(21.2)	25(32.1)	1.0(ref)	25(39.7)	24(36.4)	1.0(ref)
CT+TT	15(55.6)	28(68.3)	0.509(0.158,1.637)	26(78.8)	53(67.9)	1.447(0.504,4.157)	38(60.3)	42(63.6)	0.951(0.446,2.026)
Kazakh									
CC	9(39.1)	21(46.7)	1.0(ref)	21(56.8)	31(54.4)	1.0(ref)	20(52.6)	33(36.7)	1.0(ref)
CT+TT	14(60.9)	24(53.3)	1.364(0.440,4.228)	16(43.2)	26(45.6)	0.785(0.318,1.941)	18(47.4)	57(63.3)	0.646(0.286,1.459)
Mongolian									
CC	13(46.4)	26(31.7)	1.0(ref)	9(50.0)	37(36.6)	1.0(ref)	9(75.0)	7(35.0)	1.0(ref)
CT+TT	15(53.6)	56(68.3)	0.538(0.216,1.337)	9(50.0)	64(63.4)	0.671(0.220,2.048)	3(25.0)	13(65.0)	**0.085(0.009,0.851)**
Han									
CC	6(46.2)	46(32.6)	1.0(ref)	12(27.9)	63(30.0)	1.0(ref)	0(0)	3(17.6)	1.0(ref)
CT+TT	7(53.8)	95(67.4)	0.380(0.096,1.508)	31(72.1)	147(70.0)	1.236(0.586,2.605)	1(100.0)	14(82.4)	—

*Adjusted for age, sex and UF.

Table 4. Risk of skeletal fluorosis associated with VDR-FokI polymorphic genotypes in study participants, stratified by UF levels.

	~1.6mg/L			1.6~3.2mg/L			3.2mg/L~		
	Case N(%)	Control N(%)	OR(95%CI)*	Case N(%)	Control N(%)	OR(95%CI)*	Case N(%)	Control N(%)	OR(95%CI)*
All participants									
CC	44(41.1)	153(31.0)	1.0(ref)	46(38.7)	114(42.9)	1.0(ref)	53(48.2)	62(33.0)	1.0(ref)
CT+TT	63(58.9)	341(69.0)	0.701(0.444,1.105)	73(61.3)	152(57.1)	1.145(0.713,1.837)	57(51.8)	126(67.0)	**0.514(0.306,0.865)**
Tibetan									
CC	9(42.9)	18(30.5)	1.0(ref)	17(28.8)	27(39.1)	1.0(ref)	18(41.9)	17(29.8)	1.0(ref)
CT+TT	12(57.1)	41(69.5)	0.799(0.249,2.565)	42(71.2)	42(60.9)	1.502(0.667,3.382)	25(58.1)	40(70.2)	0.598(0.237,1.511)
Kazakh									
CC	10(55.6)	9(37.5)	1.0(ref)	16(50.0)	38(52.8)	1.0(ref)	24(50.0)	38(39.6)	1.0(ref)
CT+TT	8(44.4)	15(62.5)	0.531(0.133,2.125)	16(50.0)	34(47.2)	1.015(0.423,2.437)	24(50.0)	58(60.4)	0.681(0.321,1.442)
Mongolian									
CC	10(40.0)	36(29.3)	1.0(ref)	10(55.6)	30(47.6)	1.0(ref)	11(73.3)	4(23.5)	1.0(ref)
CT+TT	15(60.0)	87(70.7)	0.617(0.245,1.555)	8(44.4)	33(52.4)	0.772(0.246,2.426)	4(26.7)	13(76.5)	**0.103(0.017,0.633)**
Han									
CC	15(34.9)	90(31.3)	1.0(ref)	3(30.0)	19(30.6)	1.0(ref)	0(0)	3(16.7)	1.0(ref)
CT+TT	28(65.1)	198(68.8)	0.833(0.411,1.690)	7(70.0)	43(69.4)	1.753(0.349,8.813)	4(100.0)	15(83.3)	—

*Adjusted for age, sex and IF.

Table 5. Risk of skeletal fluorosis associated with VDR-FokI polymorphic genotypes in study participants, stratified by age.

	~45			46~65			66~		
	Case N(%)	Control N(%)	OR(95%CI)*	Case N(%)	Control N(%)	OR(95%CI)*	Case N(%)	Control N(%)	OR(95%CI)*
All participants									
CC	28(47.5)	107(32.3)	1.0(ref)	87(42.9)	176(35.3)	1.0(ref)	28(37.8)	46(39.0)	1.0(ref)
CT+TT	31(52.5)	224(67.7)	0.625(0.347,1.125)	116(57.1)	323(64.7)	0.789(0.555,1.121)	46(62.2)	72(61.0)	0.825(0.423,1.608)
Tibetan									
CC	7(35.0)	27(34.6)	1.0(ref)	23(34.8)	31(35.6)	1.0(ref)	14(37.8)	4(20.0)	1.0(ref)
CT+TT	13(65.0)	51(65.4)	1.183(0.383,3.645)	43(65.2)	56(64.4)	1.081(0.535,2.182)	23(62.2)	16(80.0)	0.308(0.072,1.313)
Kazakh									
CC	13(65.0)	29(43.9)	1.0(ref)	30(47.6)	44(45.8)	1.0(ref)	7(46.7)	12(40.0)	1.0(ref)
CT+TT	7(35.0)	37(56.1)	0.466(0.160,1.357)	33(52.4)	52(54.2)	0.884(0.461,1.696)	8(53.3)	18(60.0)	1.074(0.219,5.276)
Mongolian									
CC	6(42.9)	27(29.7)	1.0(ref)	23(60.5)	35(35.4)	1.0(ref)	2(33.3)	8(61.5)	1.0(ref)
CT+TT	8(57.1)	64(70.3)	0.443(0.127,1.548)	15(39.5)	64(64.6)	**0.404(0.177,0.922)**	4(66.7)	5(38.5)	3.808(0.156,93.179)
Han									
CC	2(40.0)	24(25.0)	1.0(ref)	11(30.6)	66(30.4)	1.0(ref)	5(31.3)	22(40.0)	1.0(ref)
CT+TT	3(60.0)	72(75.0)	0.456(0.054,3.849)	25(69.4)	151(69.6)	0.881(0.398,1.952)	11(68.8)	33(60.0)	1.476(0.428,5.095)

*Adjusted for sex, IF and UF.

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Complete List of Authors:	Yang, dan; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Liu, Yang; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Chu, Yanru; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Yang, Qing; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Jiang, Wei; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Chen, Fuxun; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Li, Dandan; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Qin, Ming; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081

	Sun, Dianjun; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Yang, Yanmei; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Gao, Yanhui; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081
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Title page

Vitamin D receptor gene FokI polymorphism may have a protective effect on skeletal fluorosis of the brick-tea type fluorosis.

Dan Yang,[1,2] Yang Liu,[1,2] Yanru Chu,[1] Qing Yang,[1] Wei Jiang,[1] Fuxun Chen,[1] Dandan Li,[1] Ming Qin,[1] Dianjun Sun,[1] Yanmei Yang[1,*] and Yanhui Gao[1,*]

[1]Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin, China

[2]Equal contributions

***Corresponding author:** Yanhui Gao, E-mail address: gaoyh411@163.com;

***Corresponding author:** Yanmei Yang, E-mail address: yangym0916@163.com

Address of corresponding author: Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 150081, Heilongjiang Province, China.

Telephone: +86(0)451 8750 3097 Fax: +86(0)451 8667 6184

Key Words: Brick-tea type fluorosis; VDR-FokI polymorphism; Skeletal fluorosis.

Vitamin D receptor gene FokI polymorphism may have a protective effect on skeletal fluorosis of the brick-tea type fluorosis.

ABSTRACT

Background: Brick-tea type fluorosis is a public health concern in the north-west area of China. The VDR-FokI polymorphism is considered to be a regulator of bone metabolism and calcium resorption. However, the association of VDR-FokI polymorphism with the risk of brick-tea type fluorosis has not been reported.

Materials and Methods: A cross-sectional case-control study was conducted in three provinces (Inner Mongolia, Qinghai, Sinkiang), China. The fluoride contents of Brick-tea water or urinary were tested by the standard of GB 1996-2005 or WS/T89-2006 (China), respectively. The skeletal fluorosis was diagnosed by the standard of WS/192-2008(China). The VDR-FokI polymorphism was detected by Sequenom MassARRAY system.

Result: Compared to the carriers with CC genotype, the participants with CT/TT genotype had a significantly decreased risk of skeletal fluorosis (OR=0.761 [95%CI, 0.580-0.997]), after adjustment of risk factors. When investigated among ethnic groups, the protective effect of the CT/TT was limited in the Mongolian participants (OR=0.525 [95%CI, 0.278-0.991]). Moreover, the interaction of VDR-FokI with risk factors was only found in Mongolian participants: the protective effect of the CT/TT was limited to participants with 7.0 mg/day~ IF (OR=0.085 [95%CI, 0.009-0.851], or participants with 3.2 mg/L~ UF (OR=0.103 [95%CI, 0.017-0.633]), or participants

aged 46 to 65 (OR=0.404 [95%CI, 0.177-0.922].

Conclusion: Our data suggest that the CT/TT genotype of VDR-FokI may be a protective factor for the brick-tea type skeletal fluorosis and this effect is pronounced in Mongolian participants.

Key Words: Brick-tea type fluorosis; VDR-FokI polymorphism; Skeletal fluorosis.

Strengths and limitation of this study:

1. It is the first time to report the association between FokI polymorphism and skeletal fluososis overall or by ethnicity. Improved understanding of such gene-environment interactions would not only contribute to knowledge on the skeletal fluorosis, but also help in putting forward corresponding preventive measures.
2. We find the CT/TT genotype of VDR-FokI plays a protective role in the skeletal fluososis of brick-tea type fluorosis.
3. For Mongolian participants, this protective were apparent in participants with 3.2 mg/L~ UF, with 7.0 mg/day~ ITF or aged 46 to 65.
4. Our sample size of Tibetan and Han paticipants was inadequate, so the true association of VDR-FokI polymorphism with skeletal fluorosis in these two ethicities is worth to further study.
5. The VDR-FokI polymorphism may influence the bone metabolism through the dietary calcium, but we did not have the data on the dietary of calcium.
6. We did not have the data on the serum vitamin D.

7. Besides FokI, others SNPs, such as BsmI et al. also play an important role in bone metabolism. So it is required to study the association between these SNPs and skeletal fluorosis.

INTRODUCTION

Fluorosis is caused by long-term exposure to excessive amounts of fluoride. [1 2] Skeletal fluorosis is serious clinical outcomes of fluorosis, which causes pain and damage to bones and joints, or even crippling.[3] Fluorosis affects millions of people around the world, and there are no established treatments for fluorosis patients, so it has becoming a major public health issue throughout the world.[4-6] According the sources of fluoride to human body, fluorosis can be divided into three: drinking-water type, burning-coal type and brick-tea type. The brick-tea type of fluorosis is caused by the habitual consumption of brick-tea in large quantities.[7] In China, the prevalence rate and severity of both the water-type and coal-burning type of fluorosis trended to decline, however, brick-tea type of fluorosis is still a severe public health issue, because it is impossible to alter the habitual consumption of brick-tea.

Skeletal fluorosis is a chronic metabolic bone disease caused by excessive accumulation of fluoride in the bones, and bone metabolism strengthen is the most prominent characteristic. So, besides the level and duration of fluoride exposure, the diversity of clinical manifestations of skeletal fluorosis correlates directly with the complexity of bone metabolism.[8] Bone metabolism is characterized by two opposite but finely coupled processes, bone formation and resorption. It is known that individual differences in response to the disease of bone abnormal metabolism largely depend on genetic susceptibility.[9] Previous study reported that polymorphism of myeloperoxidase gene[10] is related to burning-coal type fluorosis, which suggested that gene polymorphism may play an important role in the pathogenesis of skeletal

fluorosis.

Among potential genes relevant to bone metabolism, [9] vitamin D receptor (VDR) gene is considered to be an important candidate gene.[11] VDR gene, located on chromosome 12 (12q12-14), plays an important role in bone metabolism, including intestinal calcium absorption.[12 13] It is reported that fluoride modulates the VDR mRNA expression. [14] Since Morrison first reported that VDR gene polymorphism is associated with bone mineral density and it can predict the bone density,[15] several VDR gene restriction endonuclease sites, such as FokI (rs2228570), BsmI (rs1544410), ApaI (rs7975232), and TaqI (rs731236) have been used in population-based study.[13 15 16] Different VDR gene polymorphism plays different roles in regulating the bone metabolism.[17-20] FokI polymorphism, a C→T transition found within the translation initiation site in exon 2, results in long and short variants ofVDR due to creating an upstream initiation codon (ACG→ATG).[21] FokI polymorphism was related to bone mineral density and calcium absorption. [21-23] Some reports suggest that the short receptor protein (C allele), initiated from the second ATG site, may plays a more active role in the VDR responsive gene expression because it interacts more efficiently with the transcriptional factor IIB compared to the long receptor protein (T allele), initiated from the first ATG site.[24] The association of FokI polymorphism with bone mineral density have been reported, [21-23] however, the association of FokI polymorphism with the risk of skeletal fluorosis has not been reported.

Our prior study has demonstrated ethnic difference in prevalence rate of the

brick-tea type fluorosis and that glutathione S-transferases (GST) rs1695 polymorphism may play an important role in the pathogenesis of brick-tea fluorosis.[25] However, there are no studies reported the effect of VDR-FokI on skeletal fluorosis of the brick-tea type fluorosis. Therefore, in this study, we conducted a cross-sectional case-control study in the brick-tea fluorosis area of China to investigate the association between FokI polymorphism and skeletal fluorosis overall or by ethnicity.

MATERIALS AND METHODS

Subjects and data collection

On the brick-tea type fluorosis survey data, we undertook a cross-sectional case-control study in sixteen villages from the Inner Mongolia, Qinghai and Sinkiang province, People's Republic of China, from July to August in 2012. In this study, overall participants were older than 16 years old, born and grew up in their village. Each participant received the questionnaire survey and clinical examination. The clinical examination included physical examination, medical history and X-ray diagnosis (Beijing Longsafe Imaging Technology Co., Beijing City, China). The content of the questionnaire included general information of respondents (name, sex, age, education, economic income, the history of bone related disease, etc), and fluoride exposure (the amount of drinking brick-tea water per day, the consumption of brick-tea per year). In addition, we also collect everyone's blood, urine and brick-tea water samples. All participants signed informed consent, and this research was

approved by Harbin Medical University Ethics Committee.

Diagnose of skeletal fluorosis

The skeletal fluorosis was diagnosed according to the Chinese diagnostic criteria of endemic skeletal fluorosis (WS192-2008, China). As described in previous report,[25] the diagnose of skeletal fluorosis was based on the sign of X ray, included osteoporosis, osteomalacia, sclerosis, ossification of soft tissue and joint degeneration in the forearm, shank, and pelvic, and could be classified into three categories: mild, moderate or severe.

Determination of fluoride concentration

All urine and brick-tea water samples were stored at -20℃ until use. Urinary fluoride content was determined by the fluoride ion electrode method based on The China's Urinary Fluoride Detection-Fluoride Electrode Method WS/T89-1996. The concentration of brick-tea water was also determined by the fluoride ion electrode method with the standard of GB19965-2005 (China).

Genotyping

All blood samples were stored at -80℃ until use. The genomic DNA was extracted from the blood samples using the DNA extraction kit (Axygen Biosciences, Union City, USA). The DNA concentration was tested by TU1901 Spectrophotometry (Purkinje General Company, Beijing City, China). When the DNA concentration was greater than 20μg/ml, preserve the extracted DNA was preserved at -80℃. The genotyping sequencing from the extract were performed by the shanghai Fenglin Clinical Laboratory Company (http://www.fenglinlab.com/index.asp) using the

Sequenom MassARRAY system (Sequenom, Inc., San Diego, CA, USA). The primer sequence of the FokI: forward-5'-ACGTTGGATGTGGCCTGCTTGCTGTTCTTA-3', reverse-5'-ACGTTGGATGACGTTCCGGTCAAAGTCTCC-3', extended-5'-GTGCTGGCCGCCATTGCCTCC-3'. For the genotyping sequencing quality control, blinded blood duplicate was used.

Statistical analysis

Most statistical analyses were conducted using the SPSS version 19.0 (SPSS Inc., Chicago, IL). Post-hoc power of the study was estimated using G*Power software (version 3.1). Pearson's chi-square test was used for test of the differences between fluorosis patients and control people. Odds ratios (OR) and corresponding 95% confidence intervals (CI) were calculated for skeletal fluorosis risk using logistic regression. Testing for deviation from Hardy-Weinberg equilibrium (HWE) was performed within the participants stratified by case and control using a chi-square test. Wald's test statistic was used for test the Gene-environment interactions. The significance level in this study was taken as $P<0.05$.

Ethical statement

The study was proved by the Harbin Medical University Ethics Committee (HMUIRB20120021). All of these participants singed informed consent, and we also obtained written informed consent from the guardians on behalf of the minors. For the brick-tea water samples collection, no specific permits required. The locations were not privately owned or protected in any way and this field study did not involve endangered or protected species.

RESULTS

Participants characteristics.

In total, 1284 subjects participated in this study. There were 336 subjects who were diagnosed with skeletal fluorosis with the prevalence rate 26.2% (336/1284). Demographic of skeletal fluorosis cases and controls are presented in **Table 1**. Skeletal fluorosis cases were significantly older than controls (p<0.001). And there more male in the skeletal fluorosis cases than in the controls (p=0.015). Skeletal fluorosis cases were significantly more likely to have a more daily intake of tea fluoride (ITF) (p<0.001) and a high urine fluoride (UF) status (p<0.001). In addition, ethnical differences in skeletal fluorosis were observed (p<0.001): more Tibetan cases are the most in skeletal fluorosis cases, following are Kazakh cases and the least are Mongolian and Han cases.

Association of VDR-FokI polymorphism with skeletal fluorosis.

Testing for deviation from Hardy-Weinberg equilibrium (HWE) was performed within the control participants stratified by ethnicity using a chi-square test. All ethnical participants were found to be in Hard-Weinberg equilibrium for VDR-FokI. In our study, VDR-FokI polymorphism followes a dominant model of inheritance, so we divided the participants into two groups by the presence or absence of T allele, that is, CC and CT/TT groups **(Table2)**. Participants with CT/TT genotype had a significantly decreased risk of skeletal fluorosis (OR=0.717 [95%CI, 0.556-0.925]). After adjustment of the risk factors, the protective effect of CT/TT genotype remained

statistically significant (OR=0.761 [95%CI, 0.580-0.997]). In addition, there was the suggestion of a difference in skeletal fluorosis risk by ethnicity in relation to VDR-FokI polymorphism. After adjustment of the risk factors, the protective effect of the CT/TT was limited in the Mongolian participants (OR=0.525 [95%CI, 0.278-0.991]). The smaller percentage of Mongolian participants in our analysis appear to be driving the overall more than 20% reduction in risk as our data do not show a similar reduction did not find shared by other three ethnical participants.

Given non-significant nature of association of VDR-FokI polymorphism in Tibetan, Kazakh and Han paticipants, it is necessary to estimate if our study has adequate power to detect the true association if present in these populations. We estimated the power of our study sing G Power software (version 3.1). We obtained the power of 98.9% and 99.4% at p = 0.05 for overall and Mongolian participants, respectively. But the power for Tibetan, Kazakh and Han paticipants is 11.7%, 64.5% and 8.1% respectively. This shows that our sample size of Tibetan, Han paticipants was inadequate and the study was insufficiently-powered to detect the true association of VDR-FokI polymorphism with skeletal fluorosis, if existent in these ethinic participants.

Stratification by potential risk factor.

We investigated the potential interactions between FokI-SNP and potential risk factor in Mongolian participants **(Table3)**. For Mongolian participants, a decrease risk of skeletal fluorosis among carriers with CT/TT genotype was limited to participants with 7.0 mg/day~ ITF (OR=0.085 [95%CI, 0.009-0.851] vs. OR=0.538 [95%CI,

0.216-1.337] in participants with ~3.5 mg/day ITF, vs. OR=0.671 [95%CI, 0.220-2.048] in participants with 3.5~7.0 mg/day ITF). We also observed that a decrease risk of skeletal fluorosis among carriers with CT/TT genotype was limited to participants with 3.2 mg/L~ UF (OR=0.103 [95%CI, 0.017-0.633] vs. OR=0.617 [95%CI, 0.245-1.555] in participants with ~1.6 mg/L UF, vs. OR=0.772 [95%CI, 0.246-2.426] in participants with 1.6~3.2 mg/L UF). In addition, we also noticed that a decrease risk of skeletal fluorosis among carriers with CT/TT genotype was limited to participants aged 46 to 65 (OR=0.404 [95%CI, 0.177-0.922] vs. (OR=0.443 [95%CI, 0.127-1.548] in participants aged below 45 vs. OR=3.808 [95%CI, 0.156-93.179] in participants aged above 66).

DISCUSSION

Although high fluoride exposure is highly relevant with skeletal fluorosis, it is not the only factor influencing the susceptibility to fluorosis. Not everyone living in areas with naturally high fluoride levels in water suffers fluorosis.[5 26] Three inbred strains of mice (A/J, SWR/J and 129P3/J) that showed different susceptibilities to dental fluorosis, displayed variation in bone response to fluoride exposure.[27-29] So, genetics might influence the bone response of individuals to fluoride exposure. In our previous study, we observed that the prevalence rate and severity in different ethnical participants was different, which suggested the possible contribution of a genetic differences in fluorosis disparities.[25] In addition, the distribution of environment factors related with fluoride exposure, including age, ITF and UF, differs by ethnicity. As a result, it is plausible that the interaction between gene and environment factors

might explain, in part, the racial/ethnic differences in fluorosis prevalence.

Of the previous studies on genetic variation and fluorosis risk, the contribution or association of SNPs on dental fluorosis or skeletal fluorosis has been reported in several studies.[10 25 30-32] However, none studies have reported the association between VDR-FokI polymorphism and skeletal fluorosis of the brick-tea type fluorosis. Calcium could alleviate the toxic effects of fluoride to a certain extent,[33] but fluoride ingestion reduces intestinal calcium absorption. Animal study indicated that excess fluoride had an inhibitory effect on duodenal VDR gene transcription, and thereby hindered the calcium absorption process.[34] VDR-FokI gene polymorphism has been reported to be associated with bone metabolism and calcium absorption.[21-23] Moreover, evidence indicated that the TT and CT forms of the VDR-FokI polymorphsim are associated with a decreased VDR efficiency, compared with the TT genotype.[24] Therefore, in the present study we focus on the role of VDR-FokI polymorphism in the skeletal fluorosis of the brick-tea type fluorosis. We noticed that a more than 20% reduction in risk of skeletal fluorosis related with CT/TT genotype in VDR-FokI in all participants was largely attributable to the reduced risk in Mongolian participants; moreover, this protective effect remained significant after adjustment of age, sex, ITF and UF. These results suggested that the protective of CT/TT genotype against the skeletal fluorosis of brick-tea type of fluorosis might be present among Mongolian participants.

Factors, including age, sex, dose and duration of fluoride intake, that influence the fluoride toxicity and the clinical presentation, are the major risk factors of fluorosis.[4

8] So, we investigated the potential interactions between VDR-FokI SNP and known risk factor in Mongolian participants. We found that this protective role of CT/TT genotype of VDR-FokI against brick-tea type of fluorosis appeared to be more pronounced in participants with 3.2 mg/L~ UF, with 7.0 mg/day~ ITF or aged 46 to 65. So our results suggested that this protective effect was pronounced in high fluoride exposure condition. However, these findings of the VDR-FokI SNP analysis stratified by risk factors need to be interpreted with caution, because the data became sparse when several factors were investigated simultaneously. The amount of fluoride accumulation in the body increased with age,[35-37] so the prevalence rate and severity of skeletal fluorosis is also increased with age. In our data, this protective effect of CT/TT genotype was limited in Mongolian participants aged 46~65. For younger people, the fluoride load in body is low because of short time periods of fluoride exposure, so the prevalence rate of fluorosis is small. Thus, it is necessary to investigate this interaction in a large sample population. For the older people (over 66 years old), the protective effect of CT/TT genotype might be compromised by the toxicity of high fluoride load in body.

There are several potential limitations in this study. First, in power analysis, we found that our sample size of Tibetan and Han paticipants was inadequate, so the true association of VDR-FokI polymorphism with skeletal fluorosis in these two ethicities is worth to further study. Second, a study in Indian girls indicated after supplementation of calcium, the carriers of TT genotype had significant increase in bone mass as compared to CC genotype,[20] which suggested that the VDR-FokI

polymorphism may influence the bone metabolism through the dietary calcium. Unfortunately, we did not have the data on the dietary of calcium. So, it is need to investigate the interaction of VDR-FokI SNP with dietary calcium in fluorosis. Third, we did not have the data on the serum vitamin D. Fouth, VDR contains numerous SNPs. Besides FokI, others SNPs, such as BsmI et al. also play an important role in bone metabolism. So it is required to study the association between these SNPs and skeletal fluorosis.

CONCLUSIONS

In summary, the CT/TT genotype of VDR-FokI might play a protective role in the brick-tea type fluorosis and this protective was apparent in Mongolian participants. For Mongolian participants, this protective were apparent in participants with 3.2 mg/L~ UF, with 7.0 mg/day~ ITF or aged 46 to 65. Improved understanding of such gene-environment interactions would not only contribute to knowledge on the skeletal fluorosis of brick-tea type fluorosis, but also help in putting forward corresponding preventive measures. Therefore, larger studies are necessary to study the role of genes and/or polymorphic site influencing bone mass in the pathogenesis of fluorosis.

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Contributor statement

1、 Analysis or interpretation of data; Writing articles: Yang, Dan and Liu, Yang

2、 Collected data: Chu, Yanru、Yang, Qing、Jiang, Wei and Chen, Fuxun

3、 Interpretation of data: Li, Dandan and Qin, Ming

4、 Science advisors; The conception or design of the work: Gao, Yanhui、Yang, Yanmei and Sun, Dianjun

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Table 1. Characteristics of skeletal fluorosis cases and controls (N=1284).

	Cases n (%)	Controls n (%)	*p*
Age			＜0.001
～45	59(17.6)	331(34.9)	
46～65	203(60.4)	499(52.6)	
66～	74(22.0)	118(12.4)	
Sex			0.015
Male	156(46.4)	368(38.8)	
Female	180(53.6)	580(61.2)	
Ethnicity			＜0.001
Tibetan	123(36.6)	185(19.5)	
Kazakh	98(29.2)	192(20.3)	
Mongolian	58(17.3)	203(21.4)	
Han	57(17.0)	368(38.8)	
ITF			＜0.001
~3.5 mg/day	91(27.1)	309(32.6)	
3.5~7.0 mg/day	131(39.0)	446(47.0)	
7.0~ mg/day	114(33.9)	193(20.4)	
UF			＜0.001
~1.6 mg/L	107(31.8)	494(52.1)	
1.6~3.2 mg/L	119(35.4)	266(28.1)	
3.2 mg/L	110(32.7)	188(19.8)	

Percentages are adjusted for sampling weights and may not sum to 1 due to rounding;

P value difference by case status. ITF: daily intake of tea fluoride; UF: urine fluoride.

Table 2. Risk of skeletal fluorosis associated with VDR-FokI polymorphic genotypes in study participants overall and stratified by ethnicity.

	Case n (%)	Control n (%)	crude OR(95%CI)	adjusted OR(95%CI)*
All participants				
CC	143(42.6)	329(34.7)	1.0(ref)	1.0(ref)
CT+TT	193(57.4)	619(65.3)	**0.717(0.556,0.925)**	**0.761(0.580,0.997)**
Tibetan				
CC	44(35.8)	62(33.5)	1.0(ref)	1.0(ref)
CT+TT	79(64.2)	123(66.5)	0.905(0.561,1.461)	0.947(0.562,1.598)
Kazakh				
CC	50(51.0)	85(44.3)	1.0(ref)	1.0(ref)
CT+TT	48(49.0)	107(55.7)	0.763(0.468,1.242)	0.729(0.439,1.210)
Mongolian				
CC	31(53.4)	70(34.5)	1.0(ref)	1.0(ref)
CT+TT	27(46.6)	133(65.5)	**0.458(0.254,0.828)**	**0.525(0.278,0.991)**
Han				
CC	18(31.6)	112(30.4)	1.0(ref)	1.0(ref)
CT+TT	39(68.4)	256(69.6)	0.948(0.520,1.729)	0.945(0.503,1.774)

*Adjusted for age, sex, ITF (daily intake of tea fluoride) and UF (urine fluoride).

Table 3. Association of VDR-FokI polymorphic genotypes with skeletal fluorosis in Mongolian subjects, stratified by potential risk factor levels.

	CC		CT+TT		OR (95%CI)*
	Case n(%)	Control n(%)	Case n(%)	Control n(%)	
ITF					
~3.5 mg/d	13 (46.4)	26 (31.7)	15 (53.6)	56(68.3)	0.538 (0.216,1.337) [a]
3.5-7.0 mg/d	9 (50.0)	37 (36.6)	9 (50.0)	64 (63.4)	0.671 (0.220,2.048) [a]
7.0~ mg/d	9 (75.0)	7 (35.0)	3 (25.0)	13 (65.0)	**0.085 (0.009,0.851)** [a]
UF					
~1.6 mg/L	10 (40.0)	36 (29.3)	15 (60.0)	87 (70.7)	0.617 (0.245,1.555) [b]
1.6~3.2 mg/L	10 (55.6)	30 (47.6)	8 (44.4)	33 (52.4)	0.772 (0.246,2.426) [b]
3.2~ mg/L	11 (73.3)	4 (23.5)	4 (26.7)	13 (76.5)	**0.103 (0.017,0.633)** [b]
Age					
~45	6 (42.9)	27 (29.7)	8 (57.1)	64(70.3)	0.443 (0.127,1.548) [c]
46~65	23 (60.5)	35 (35.4)	15 (39.5)	64(64.6)	**0.404 (0.177,0.922)** [c]
66~	2 (33.3)	8 (61.5)	4 (66.7)	5 (38.5)	3.808 (0.156,93.179) [c]

a. Adjusted for age, sex and UF (urine fluoride). b. Adjusted for age, sex and ITF (daily intake of tea fluoride). c. Adjusted for sex, ITF (daily intake of tea fluoride) and UF (urine fluoride).

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Complete List of Authors:	Yang, dan; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Liu, Yang; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Chu, Yanru; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Yang, Qing; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Jiang, Wei; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Chen, Fuxun; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Li, Dandan; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Qin, Ming; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081

	Sun, Dianjun; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Yang, Yanmei; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081 Gao, Yanhui; Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 1500 Harbin, Heilongjiang, CN 150081
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Title page

Association between vitamin D receptor gene FokI polymorphism and skeletal fluorosis of the brick-tea type fluorosis: a cross-sectional case-control study.

Dan Yang,[1,2,3] Yang Liu,[1,3] Yanru Chu,[1] Qing Yang,[1] Wei Jiang,[1] Fuxun Chen,[1] Dandan Li,[1]Ming Qin,[1] Dianjun Sun,[1] Yanmei Yang[1,*] and Yanhui Gao[1,*]

[1] Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin, China

[2] Chongqing Blood Center, Chongqing, China

[3] Equal contributions

***Corresponding author:** Yanhui Gao, E-mail address: gaoyh411@163.com;

***Corresponding author:** Yanmei Yang, E-mail address: yangym0916@163.com

Address of corresponding author: Baojian Road 194, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 150081, Heilongjiang Province, China.

Telephone: +86(0)451 8750 3097 Fax: +86(0)451 8667 6184

Key Words: Brick-tea type fluorosis; VDR-FokI polymorphism; Skeletal fluorosis.

Association between vitamin D receptor gene FokI polymorphism and skeletal fluorosis of the brick-tea type fluorosis: a cross-sectional case-control study.

ABSTRACT

Background: Brick-tea type fluorosis is a public health concern in the north-west area of China. The VDR-FokI polymorphism is considered to be a regulator of bone metabolism and calcium resorption. However, the association of VDR-FokI polymorphism with the risk of brick-tea type fluorosis has not been reported.

Materials and Methods: A cross-sectional case-control study was conducted in three provinces (Inner Mongolia, Qinghai, Sinkiang), China. The fluoride contents of Brick-tea water or urinary were tested by the standard of GB 1996-2005 or WS/T89-2006 (China), respectively. The skeletal fluorosis was diagnosed by the standard of WS/192-2008(China). The VDR-FokI polymorphism was detected by Sequenom MassARRAY system.

Result: Compared to the carriers with CC genotype, the participants with CT/TT genotype had a significantly decreased risk of skeletal fluorosis (OR=0.761 [95%CI, 0.580-0.997]), after adjustment of risk factors. When investigated among ethnic groups, the protective effect of the CT/TT was limited in the Mongolian participants (OR=0.525 [95%CI, 0.278-0.991]). Moreover, the interaction of VDR-FokI with risk factors was only found in Mongolian participants: the protective effect of the CT/TT was limited to participants with 7.0 mg/day~ IF (OR=0.085 [95%CI, 0.009-0.851], or participants with 3.2 mg/L~ UF (OR=0.103 [95%CI, 0.017-0.633]), or participants

aged 46 to 65 (OR=0.404 [95%CI, 0.177-0.922].

Conclusion: Our data suggest that the CT/TT genotype of VDR-FokI may be a protective factor for the brick-tea type skeletal fluorosis and this effect is pronounced in Mongolian participants.

Key Words: Brick-tea type fluorosis; VDR-FokI polymorphism; Skeletal fluorosis.

Strengths and limitation of this study:

1. This study includes Tibetan, Kazakh, Mongolian and Han participants.
2. Overall participants were older than 16 years old, born and grew up in the Inner Mongolia, Qinghai and Sinkiang province, People's Republic of China.
3. It is the first report describing the association between FokI polymorphism and skeletal fluososis overall or by ethnicity.
4. A limitation is that sample size of Tibetan and Han paticipants was inadequate.

INTRODUCTION

Fluorosis is caused by long-term exposure to excessive amounts of fluoride. [1 2] Skeletal fluorosis is serious clinical outcomes of fluorosis, which causes pain and damage to bones and joints, or even crippling.[3] Fluorosis affects millions of people around the world, and there are no established treatments for fluorosis patients, so it has becoming a major public health issue throughout the world.[4-6] According the sources of fluoride to human body, fluorosis can be divided into three: drinking-water type, burning-coal type and brick-tea type. The brick-tea type of fluorosis is caused by the habitual consumption of brick-tea in large quantities.[7] In China, the prevalence rate and severity of both the water-type and coal-burning type of fluorosis trended to decline, however, brick-tea type of fluorosis is still a severe public health issue, because it is impossible to alter the habitual consumption of brick-tea.

Skeletal fluorosis is a chronic metabolic bone disease caused by excessive accumulation of fluoride in the bones, and bone metabolism strengthen is the most prominent characteristic. So, besides the level and duration of fluoride exposure, the diversity of clinical manifestations of skeletal fluorosis correlates directly with the complexity of bone metabolism.[8] Bone metabolism is characterized by two opposite but finely coupled processes, bone formation and resorption. It is known that individual differences in response to the disease of bone abnormal metabolism largely depend on genetic susceptibility.[9] Previous study reported that polymorphism of myeloperoxidase gene[10] is related to burning-coal type fluorosis, which suggested that gene polymorphism may play an important role in the pathogenesis of skeletal

fluorosis.

Among potential genes relevant to bone metabolism, [9] vitamin D receptor (VDR) gene is considered to be an important candidate gene.[11] VDR gene, located on chromosome 12 (12q12-14), plays an important role in bone metabolism, including intestinal calcium absorption.[12 13] It is reported that fluoride modulates the VDR mRNA expression. [14] Since Morrison first reported that VDR gene polymorphism is associated with bone mineral density and it can predict the bone density,[15] several VDR gene restriction endonuclease sites, such as FokI (rs2228570), BsmI (rs1544410), ApaI (rs7975232), and TaqI (rs731236) have been used in population-based study.[13 15 16] Different VDR gene polymorphism plays different roles in regulating the bone metabolism.[17-20] FokI polymorphism, a C→T transition found within the translation initiation site in exon 2, results in long and short variants of VDR due to creating an upstream initiation codon (ACG→ATG).[21] FokI polymorphism was related to bone mineral density and calcium absorption. [21-23] Some reports suggest that the short receptor protein (C allele), initiated from the second ATG site, may plays a more active role in the VDR responsive gene expression because it interacts more efficiently with the transcriptional factor IIB compared to the long receptor protein (T allele), initiated from the first ATG site.[24] The association between FokI polymorphism and bone mineral density has been reported, [21-23] however, the association of FokI polymorphism with the risk of skeletal fluorosis has not been reported.

Our prior study has demonstrated ethnic difference in prevalence rate of the

brick-tea type fluorosis and that glutathione S-transferases (GST) rs1695 polymorphism may play an important role in the pathogenesis of brick-tea fluorosis.[25] However, there are no studies reported the effect of VDR-FokI on skeletal fluorosis of the brick-tea type fluorosis. Therefore, in this study, we conducted a cross-sectional case-control study in the brick-tea fluorosis area of China to investigate the association between FokI polymorphism and skeletal fluorosis overall or by ethnicity.

MATERIALS AND METHODS

Subjects and data collection

On the brick-tea type fluorosis survey data, we undertook a cross-sectional case-control study in sixteen villages from the Inner Mongolia, Qinghai and Sinkiang province, People's Republic of China, from July to August in 2012. In this study, overall participants were older than 16 years old, born and grew up in their village. Each participant received the questionnaire survey and clinical examination. The clinical examination included physical examination, medical history and X-ray diagnosis (Beijing Longsafe Imaging Technology Co., Beijing City, China). The content of the questionnaire included general information of respondents (name, sex, age, education, economic income, the history of bone related disease, etc), and fluoride exposure (the amount of drinking brick-tea water per day, the consumption of brick-tea per year). In addition, we also collect everyone's blood, urine and brick-tea water samples. All participants signed informed consent, and this research was

approved by Harbin Medical University Ethics Committee.

Diagnose of skeletal fluorosis

The skeletal fluorosis was diagnosed according to the Chinese diagnostic criteria of endemic skeletal fluorosis (WS192-2008, China). As described in previous report,[25] the diagnose of skeletal fluorosis was based on the sign of X ray, included osteoporosis, osteomalacia, sclerosis, ossification of soft tissue and joint degeneration in the forearm, shank, and pelvic, and could be classified into three categories: mild, moderate or severe.

Determination of fluoride concentration

All urine and brick-tea water samples were stored at -20℃ until use. Urinary fluoride content was determined by the fluoride ion electrode method based on The China's Urinary Fluoride Detection-Fluoride Electrode Method WS/T89-1996. The concentration of brick-tea water was also determined by the fluoride ion electrode method with the standard of GB19965-2005 (China).

Genotyping

All blood samples were stored at -80℃ until use. The genomic DNA was extracted from the blood samples using the DNA extraction kit (Axygen Biosciences, Union City, USA). The DNA concentration was tested by TU1901 Spectrophotometry (Purkinje General Company, Beijing City, China). When the DNA concentration was greater than 20μg/ml, preserve the extracted DNA was preserved at -80℃. The genotyping sequencing from the extract were performed by the shanghai Fenglin Clinical Laboratory Company (http://www.fenglinlab.com/index.asp) using the

Sequenom MassARRAY system (Sequenom, Inc., San Diego, CA, USA). The primer sequence of the FokI: forward-5'-ACGTTGGATGTGGCCTGCTTGCTGTTCTTA-3', reverse-5'-ACGTTGGATGACGTTCCGGTCAAAGTCTCC-3', extended-5'-GTGCTGGCCGCCATTGCCTCC-3'. For the genotyping sequencing quality control, blinded blood duplicate was used.

Statistical analysis

Most statistical analyses were conducted using the SPSS version 19.0 (SPSS Inc., Chicago, IL). Post-hoc power of the study was estimated using G*Power software (version 3.1). Pearson's chi-square test was used for test of the differences between fluorosis patients and control people. Odds ratios (OR) and corresponding 95% confidence intervals (CI) were calculated for skeletal fluorosis risk using logistic regression. Testing for deviation from Hardy-Weinberg equilibrium (HWE) was performed within the participants stratified by case and control using a chi-square test. Wald's test statistic was used for test the Gene-environment interactions. The significance level in this study was taken as $P<0.05$.

Ethical statement

The study was proved by the Harbin Medical University Ethics Committee (HMUIRB20120021). All of these participants singed informed consent, and we also obtained written informed consent from the guardians on behalf of the minors. For the brick-tea water samples collection, no specific permits required. The locations were not privately owned or protected in any way and this field study did not involve endangered or protected species.

RESULTS

Participants characteristics.

In total, 1284 subjects participated in this study. There were 336 subjects who were diagnosed with skeletal fluorosis with the prevalence rate 26.2% (336/1284). Demographic of skeletal fluorosis cases and controls are presented in **Table 1**. Skeletal fluorosis cases were significantly older than controls (p<0.001). And there more male in the skeletal fluorosis cases than in the controls (p=0.015). Skeletal fluorosis cases were significantly more likely to have a more daily intake of tea fluoride (ITF) (p<0.001) and a high urine fluoride (UF) status (p<0.001). In addition, ethnical differences in skeletal fluorosis were observed (p<0.001): more Tibetan cases are the most in skeletal fluorosis cases, following are Kazakh cases and the least are Mongolian and Han cases.

Association of VDR-FokI polymorphism with skeletal fluorosis.

Testing for deviation from Hardy-Weinberg equilibrium (HWE) was performed within the control participants stratified by ethnicity using a chi-square test. All ethnical participants were found to be in Hard-Weinberg equilibrium for VDR-FokI. In our study, VDR-FokI polymorphism followes a dominant model of inheritance, so we divided the participants into two groups by the presence or absence of T allele, that is, CC and CT/TT groups **(Table2)**. Participants with CT/TT genotype had a significantly decreased risk of skeletal fluorosis (OR=0.717 [95%CI, 0.556-0.925]). After adjustment of the risk factors, the protective effect of CT/TT genotype remained

statistically significant (OR=0.761 [95%CI, 0.580-0.997]). In addition, there was the suggestion of a difference in skeletal fluorosis risk by ethnicity in relation to VDR-FokI polymorphism. After adjustment of the risk factors, the protective effect of the CT/TT was limited in the Mongolian participants (OR=0.525 [95%CI, 0.278-0.991]). The smaller percentage of Mongolian participants in our analysis appear to be driving the overall more than 20% reduction in risk as our data do not show a similar reduction did not find shared by other three ethnical participants.

Given non-significant nature of association of VDR-FokI polymorphism in Tibetan, Kazakh and Han paticipants, it is necessary to estimate if our study has adequate power to detect the true association if present in these populations. We estimated the power of our study sing G Power software (version 3.1). We obtained the power of 98.9% and 99.4% at p = 0.05 for overall and Mongolian participants, respectively. But the power for Tibetan, Kazakh and Han paticipants is 11.7%, 64.5% and 8.1% respectively. This shows that our sample size of Tibetan, Han paticipants was inadequate and the study was insufficiently-powered to detect the true association of VDR-FokI polymorphism with skeletal fluorosis, if existent in these ethinic participants.

Stratification by potential risk factor.

We investigated the potential interactions between FokI-SNP and potential risk factor in Mongolian participants **(Table3)**. For Mongolian participants, a decrease risk of skeletal fluorosis among carriers with CT/TT genotype was limited to participants with 7.0 mg/day~ ITF (OR=0.085 [95%CI, 0.009-0.851] vs. OR=0.538 [95%CI,

0.216-1.337] in participants with ~3.5 mg/day ITF, vs. OR=0.671 [95%CI, 0.220-2.048] in participants with 3.5~7.0 mg/day ITF). We also observed that a decrease risk of skeletal fluorosis among carriers with CT/TT genotype was limited to participants with 3.2 mg/L~ UF (OR=0.103 [95%CI, 0.017-0.633] vs. OR=0.617 [95%CI, 0.245-1.555] in participants with ~1.6 mg/L UF, vs. OR=0.772 [95%CI, 0.246-2.426] in participants with 1.6~3.2 mg/L UF). In addition, we also noticed that a decrease risk of skeletal fluorosis among carriers with CT/TT genotype was limited to participants aged 46 to 65 (OR=0.404 [95%CI, 0.177-0.922] vs. (OR=0.443 [95%CI, 0.127-1.548] in participants aged below 45 vs. OR=3.808 [95%CI, 0.156-93.179] in participants aged above 66).

DISCUSSION

Although high fluoride exposure is highly relevant with skeletal fluorosis, it is not the only factor influencing the susceptibility to fluorosis. Not everyone living in areas with naturally high fluoride levels in water suffers fluorosis.[5 26] Three inbred strains of mice (A/J, SWR/J and 129P3/J) that showed different susceptibilities to dental fluorosis, displayed variation in bone response to fluoride exposure.[27-29] So, genetics might influence the bone response of individuals to fluoride exposure. In our previous study, we observed that the prevalence rate and severity in different ethnical participants was different, which suggested the possible contribution of a genetic differences in fluorosis disparities.[25] In addition, the distribution of environment factors related with fluoride exposure, including age, ITF and UF, differs by ethnicity. As a result, it is plausible that the interaction between gene and environment factors

might explain, in part, the racial/ethnic differences in fluorosis prevalence.

Of the previous studies on genetic variation and fluorosis risk, the contribution or association of SNPs on dental fluorosis or skeletal fluorosis has been reported in several studies.[10 25 30-32] However, none studies have reported the association between VDR-FokI polymorphism and skeletal fluorosis of the brick-tea type fluorosis. Calcium could alleviate the toxic effects of fluoride to a certain extent,[33] but fluoride ingestion reduces intestinal calcium absorption. Animal study indicated that excess fluoride had an inhibitory effect on duodenal VDR gene transcription, and thereby hindered the calcium absorption process.[34] VDR-FokI gene polymorphism has been reported to be associated with bone metabolism and calcium absorption.[21-23] Moreover, evidence indicated that the TT and CT forms of the VDR-FokI polymorphsim are associated with a decreased VDR efficiency, compared with the TT genotype.[24] Therefore, in the present study we focus on the role of VDR-FokI polymorphism in the skeletal fluorosis of the brick-tea type fluorosis. We noticed that a more than 20% reduction in risk of skeletal fluorosis related with CT/TT genotype in VDR-FokI in all participants was largely attributable to the reduced risk in Mongolian participants; moreover, this protective effect remained significant after adjustment of age, sex, ITF and UF. These results suggested that the protective of CT/TT genotype against the skeletal fluorosis of brick-tea type of fluorosis might be present among Mongolian participants.

Factors, including age, sex, dose and duration of fluoride intake, that influence the fluoride toxicity and the clinical presentation, are the major risk factors of fluorosis.[4

8] So, we investigated the potential interactions between VDR-FokI SNP and known risk factor in Mongolian participants. We found that this protective role of CT/TT genotype of VDR-FokI against brick-tea type of fluorosis appeared to be more pronounced in participants with 3.2 mg/L~ UF, with 7.0 mg/day~ ITF or aged 46 to 65. So our results suggested that this protective effect was pronounced in high fluoride exposure condition. However, these findings of the VDR-FokI SNP analysis stratified by risk factors need to be interpreted with caution, because the data became sparse when several factors were investigated simultaneously. The amount of fluoride accumulation in the body increased with age,[35-37] so the prevalence rate and severity of skeletal fluorosis is also increased with age. In our data, this protective effect of CT/TT genotype was limited in Mongolian participants aged 46~65. For younger people, the fluoride load in body is low because of short time periods of fluoride exposure, so the prevalence rate of fluorosis is small. Thus, it is necessary to investigate this interaction in a large sample population. For the older people (over 66 years old), the protective effect of CT/TT genotype might be compromised by the toxicity of high fluoride load in body.

There are several potential limitations in this study. First, in power analysis, we found that our sample size of Tibetan and Han paticipants was inadequate, so the true association of VDR-FokI polymorphism with skeletal fluorosis in these two ethicities is worth to further study. Second, a study in Indian girls indicated after supplementation of calcium, the carriers of TT genotype had significant increase in bone mass as compared to CC genotype,[20] which suggested that the VDR-FokI

polymorphism may influence the bone metabolism through the dietary calcium. Unfortunately, we did not have the data on the dietary of calcium. So, it is need to investigate the interaction of VDR-FokI SNP with dietary calcium in fluorosis. Third, we did not have the data on the serum vitamin D. Fouth, VDR contains numerous SNPs. Besides FokI, others SNPs, such as BsmI et al. also play an important role in bone metabolism. So it is required to study the association between these SNPs and skeletal fluorosis.

CONCLUSIONS

In summary, the CT/TT genotype of VDR-FokI might play a protective role in the brick-tea type fluorosis and this protective was apparent in Mongolian participants. For Mongolian participants, this protective were apparent in participants with 3.2 mg/L~ UF, with 7.0 mg/day~ ITF or aged 46 to 65. Improved understanding of such gene-environment interactions would not only contribute to knowledge on the skeletal fluorosis of brick-tea type fluorosis, but also help in putting forward corresponding preventive measures. Therefore, larger studies are necessary to study the role of genes and/or polymorphic site influencing bone mass in the pathogenesis of fluorosis.

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Conflicts of interest: None declared.

Data sharing statement: No additional data available.

Contributor statement

Gao YH, Yang YM and Sun DJ participated in the design of the study. Gao YH, Yang YM, Yang D, Liu Y, Li DD and Qin M participated in the interpretation of the data and statistical analyses. Chu YR, Yang Q, Jiang W and Chen FX performed acquisition of data. The manuscript was drafted by Yang D and Liu Y, and was revised by Gao YH and Yang YM.

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448 **Table 1. Characteristics of skeletal fluorosis cases and controls (N=1284).**

	Cases n (%)	Controls n (%)	*p*
Age			＜0.001
～45	59(17.6)	331(34.9)	
46～65	203(60.4)	499(52.6)	
66～	74(22.0)	118(12.4)	
Sex			0.015
Male	156(46.4)	368(38.8)	
Female	180(53.6)	580(61.2)	
Ethnicity			＜0.001
Tibetan	123(36.6)	185(19.5)	
Kazakh	98(29.2)	192(20.3)	
Mongolian	58(17.3)	203(21.4)	
Han	57(17.0)	368(38.8)	
ITF			＜0.001
~3.5 mg/day	91(27.1)	309(32.6)	
3.5~7.0 mg/day	131(39.0)	446(47.0)	
7.0~ mg/day	114(33.9)	193(20.4)	
UF			＜0.001
~1.6 mg/L	107(31.8)	494(52.1)	
1.6~3.2 mg/L	119(35.4)	266(28.1)	
3.2 mg/L	110(32.7)	188(19.8)	

449 Percentages are adjusted for sampling weights and may not sum to 1 due to rounding;

450 *P* value difference by case status. ITF: daily intake of tea fluoride; UF: urine fluoride.

451

Table 2. Risk of skeletal fluorosis associated with VDR-FokI polymorphic genotypes in study participants overall and stratified by ethnicity.

	Case n (%)	Control n (%)	crude OR(95%CI)	adjusted OR(95%CI)*
All participants				
CC	143(42.6)	329(34.7)	1.0(ref)	1.0(ref)
CT+TT	193(57.4)	619(65.3)	**0.717(0.556,0.925)**	**0.761(0.580,0.997)**
Tibetan				
CC	44(35.8)	62(33.5)	1.0(ref)	1.0(ref)
CT+TT	79(64.2)	123(66.5)	0.905(0.561,1.461)	0.947(0.562,1.598)
Kazakh				
CC	50(51.0)	85(44.3)	1.0(ref)	1.0(ref)
CT+TT	48(49.0)	107(55.7)	0.763(0.468,1.242)	0.729(0.439,1.210)
Mongolian				
CC	31(53.4)	70(34.5)	1.0(ref)	1.0(ref)
CT+TT	27(46.6)	133(65.5)	**0.458(0.254,0.828)**	**0.525(0.278,0.991)**
Han				
CC	18(31.6)	112(30.4)	1.0(ref)	1.0(ref)
CT+TT	39(68.4)	256(69.6)	0.948(0.520,1.729)	0.945(0.503,1.774)

*Adjusted for age, sex, ITF (daily intake of tea fluoride) and UF (urine fluoride).

Table 3. Association of VDR-FokI polymorphic genotypes with skeletal fluorosis in

	CC		CT+TT		OR (95%CI)*
	Case n(%)	Control n(%)	Case n(%)	Control n(%)	
ITF					
~3.5 mg/d	13 (46.4)	26 (31.7)	15 (53.6)	56(68.3)	0.538 (0.216,1.337) [a]
3.5-7.0 mg/d	9 (50.0)	37 (36.6)	9 (50.0)	64 (63.4)	0.671 (0.220,2.048) [a]
7.0~ mg/d	9 (75.0)	7 (35.0)	3 (25.0)	13 (65.0)	**0.085 (0.009,0.851)** [a]
UF					
~1.6 mg/L	10 (40.0)	36 (29.3)	15 (60.0)	87 (70.7)	0.617 (0.245,1.555) [b]
1.6~3.2 mg/L	10 (55.6)	30 (47.6)	8 (44.4)	33 (52.4)	0.772 (0.246,2.426) [b]
3.2~ mg/L	11 (73.3)	4 (23.5)	4 (26.7)	13 (76.5)	**0.103 (0.017,0.633)** [b]
Age					
~45	6 (42.9)	27 (29.7)	8 (57.1)	64(70.3)	0.443 (0.127,1.548) [c]
46~65	23 (60.5)	35 (35.4)	15 (39.5)	64(64.6)	**0.404 (0.177,0.922)** [c]
66~	2 (33.3)	8 (61.5)	4 (66.7)	5 (38.5)	3.808 (0.156,93.179) [c]

Mongolian subjects, stratified by potential risk factor levels.

a. Adjusted for age, sex and UF (urine fluoride). b. Adjusted for age, sex and ITF (daily intake of tea fluoride). c. Adjusted for sex, ITF (daily intake of tea fluoride) and UF (urine fluoride).