

BMJ Open

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

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2016-011980
Article Type:	Research
Date Submitted by the Author:	19-Mar-2016
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Primary Subject Heading:	Epidemiology
Secondary Subject Heading:	Epidemiology, Global health
Keywords:	EPIDEMIOLOGY, GENETICS, Bone diseases < ORTHOPAEDIC & TRAUMA SURGERY

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1 **Title page**

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

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

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4 23 **Vitamin D receptor gene FokI polymorphism may have a protective effect on**
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6 24 **skeletal fluorosis of the brick-tea type fluorosis.**
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11 **ABSTRACT**

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14 27 **Background:** Brick-tea type fluorosis is a public health concern in the north-west
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16 28 area of China. The VDR-FokI polymorphism is considered to be a regulator of bone
17
18 29 metabolism and calcium resorption. However, the association of VDR-FokI
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20 30 polymorphism with the risk of brick-tea type fluorosis has not been reported.

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24 31 **Materials and Methods:** A cross-sectional case-control study was conducted in three
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26 32 provinces (Inner Mongolia, Qinghai, Sinkiang), China. The fluoride contents of
27
28 33 Brick-tea water or urinary were tested by the standard of GB 1996-2005 or
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30 34 WS/T89-2006 (China), respectively. The skeletal fluorosis was diagnosed by the
31
32 35 standard of WS/192-2008(China). The VDR-FokI polymorphism was detected by
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34 36 Sequenom MassARRAY system.

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37 37 **Result:** Compared to the carriers with CC genotype, the participants with CT/TT
38
39 38 genotype had a significantly decreased risk of skeletal fluorosis (OR=0.761 [95%CI,
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41 39 0.580-0.997]), after adjustment of risk factors. This protective of CT/TT genotype in
42
43 40 VDR-FokI appeared to be more pronounced in participants with 3.2mg/L~ UF
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45 41 (OR=0.514 [95%CI, 0.306-0.865]). When investigated among ethnic groups, the
46
47 42 protective effect of the CT/TT was limited in the Mongolian participants (OR=0.525
48
49 43 [95%CI, 0.278-0.991]). Moreover, the interaction of VDR-FokI with risk factors was
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51 44 only found in Mongolian participants: the protective effect of the CT/TT was limited
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4 45 to participants with 7.0mg/day~ IF (OR=0.085 [95%CI, 0.009-0.851], or participants
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6 46 with 3.2mg/L~ UF (OR=0.103 [95%CI, 0.017-0.633]), or participants aged 46 to 65
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8 47 (OR=0.404 [95%CI, 0.177-0.922].

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11 48 **Conclusion:** Our data suggest that the CT/TT genotype of VDR-FokI may be a
12
13 49 protective factor for the brick-tea type skeletal fluorosis and this effect is pronounced
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15 50 in Mongolian participants.
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21 52 **Key Words:** Brick-tea type fluorosis; VDR-FokI polymorphism; Skeletal fluorosis.
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26 54 **Strengths and limitations of this study:**
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29 55 1、 It is the first time to report the association between FokI polymorphism and
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31 56 skeletal fluorosis overall or by ethnicity. Improved understanding of such
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33 57 gene-environment interactions would not only contribute to knowledge on the
34
35 58 skeletal fluorosis, but also help in putting forward corresponding preventive
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37 59 measures.
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39 60 2、 We find the CT/TT genotype of VDR-FokI plays a protective role in the brick-tea
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41 61 type fluorosis.
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43 62 3、 We find the interaction between VDR-FokI and urinary fluoride in which reduced
44
45 63 the risk of skeletal fluorosis was only present in participants with 3.2 mg/L~.
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47 64 4、 We noticed that a more than 20% reduction in risk of skeletal fluorosis related
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49 65 with CT/TT genotype in VDR-FokI in all participants was largely attributable to
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51 66 the reduced risk in Mongolian participants.
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4 67 5、 Study has suggested that the VDR-FokI polymorphism may influence the bone
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6 68 metabolism through the dietary calcium. Unfortunately, in this study we did not
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9 69 have the data on the dietary of calcium.
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89 INTRODUCTION

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

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

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4 111 balanced, normal bone mass is maintained. In contrast, excessive resorption by
5
6 112 osteoclasts contributes to osteoporosis, whereas the excessive formation by
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9 113 osteoblasts may result in osteopetrosis.[9] So, the number and activity of osteoblasts
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11 114 and osteoclasts, which is regulated by a number of genes, determine whether bone
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13 115 metabolism is balanced, or whether bone mass is increased or decreased. Therefore,
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15 116 individual differences in response to the disease of bone abnormal metabolism largely
16
17 117 depend on genetic susceptibility.[10] Previous study reported that polymorphism of
18
19 118 myeloperoxidase gene[11] is related to burning-coal type fluorosis, which suggested
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21 119 that gene polymorphism may play an important role in the pathogenesis of skeletal
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23 120 fluorosis.

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29 121 Among potential genes relevant to bone metabolism,[10] vitamin D receptor (VDR)
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31 122 gene is considered to be an important candidate gene.[12] Morrison first reported that
32
33 123 VDR gene polymorphism is associated with bone mineral density and it can predict
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35 124 the bone density.[13] VDR gene is located on chromosome 12 and contains 9
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37 125 exons.[14, 15] Four VDR gene restriction endonuclease sites are known: FokI
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39 126 (rs2228570), BsmI (rs1544410), ApaI (rs7975232), and TaqI (rs731236).[13, 15, 16]
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41 127 Different VDR gene polymorphism plays different roles in regulating the bone
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43 128 metabolism.[17-20] It was reported that FokI polymorphism was related to bone
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45 129 mineral density and calcium absorption.[21-23] However, the association of FokI
46
47 130 polymorphism with the risk of skeletal fluorosis has not been reported.

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49 131 Our prior study has demonstrated ethnic difference in prevalence rate of the
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51 132 brick-tea type fluorosis and that glutathione S-transferases (GST) rs1695
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4 133 polymorphism may play an important role in the pathogenesis of brick-tea
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6 134 fluorosis.[24] However, there are no studies reported the effect of VDR-FokI on
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9 135 skeletal fluorosis of the brick-tea type fluorosis. Therefore, in this study, we
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11 136 conducted a cross-sectional case-control study in the brick-tea fluorosis area of China
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14 137 to investigate the association between FokI polymorphism and skeletal fluorosis
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16 138 overall or by ethnicity.
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20 140 **MATERIALS AND METHODS**

21 141 **Subjects and data collection**

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25 142 On the brick-tea type fluorosis survey data, we undertook a cross-sectional
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28 143 case-control study in sixteen villages from the Inner Mongolia, Qinghai and Sinkiang
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31 144 province, People's Republic of China, from July to August in 2012. In this study,
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33 145 overall participants were older than 16 years old, born and grew up in their village.
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36 146 Each participant received the questionnaire survey and clinical examination. The
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38 147 clinical examination included physical examination, medical history and X-ray
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41 148 diagnosis (Beijing Longsafe Imaging Technology Co., Beijing City, China). The
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43 149 content of the questionnaire included general information of respondents (name, sex,
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46 150 age, education, economic income, the history of bone related disease, etc), and
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49 151 fluoride exposure (the amount of drinking brick-tea water per day, the consumption of
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51 152 brick-tea per year). In addition, we also collect everyone's blood, urine and brick-tea
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54 153 water samples. All participants signed informed consent, and this research was
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56 154 approved by Harbin Medical University Ethics Committee.
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6 **Diagnose of skeletal fluorosis**
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9 157 The skeletal fluorosis was diagnosed according to the Chinese diagnostic criteria of
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11 158 endemic skeletal fluorosis (WS192-2008, china). As described in previous report,[24]
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14 159 an individual's skeletal fluorosis was divided into three categories: mild, moderate or
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16 160 severe.
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19 16120
21 **Determination of fluoride concentration**
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24 163 All urine and brick-tea water samples were stored at -20°C until use. Urinary
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26 164 fluoride content was determined by the fluoride ion electrode method based on The
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28 165 China's Urinary Fluoride Detection-Fluoride Electrode Method WS/T89-1996. The
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30 166 concentration of brick-tea water was also determined by the fluoride ion electrode
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32 167 method with the standard of GB19965-2005 (China).
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39 **Genotyping**
4041 170 All blood samples were stored at -80°C until use. The genomic DNA was extracted
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43 171 from the blood samples using the DNA extraction kit (Axygen Biosciences, Union
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45 172 City, USA). The DNA concentration was tested by TU1901 Spectrophotometry
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47 173 (Purkinje General Company, Beijing City, China). When the DNA concentration was
48
49 174 greater than 20µg/ml, preserve the extracted DNA was preserved at -80°C. The
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51 175 genotyping sequencing from the extract were performed by the shanghai Fenglin
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55 176 Clinical Laboratory Company (<http://www.fenglinlab.com/index.asp>) using the
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4 177 Sequenom MassARRAY system (Sequenom, Inc., San Diego, CA, USA). The primer
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6 178 sequence of the FokI: forward-5'-ACGTTGGATGTGGCCTGCTTGCTGTTCTTA-3',
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9 179 reverse-5'-ACGTTGGATGACGTTCCGGTCAAAGTCTCC-3',
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11 180 extended-5'-GTGCTGGCCGCCATTGCCTCC-3'. For the genotyping sequencing
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14 181 quality control, blinded blood duplicate was used.
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19 183 **Statistical analysis**

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21 184 All statistical analyses were conducted using the SPSS version 19.0 (SPSS Inc.,
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23 185 Chicago, IL). Pearson's chi-square test was used for test of the differences between
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25 186 fluorosis patients and control people. Odds ratios (OR) and corresponding 95%
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27 187 confidence intervals (CI) were calculated for skeletal fluorosis risk using logistic
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29 188 regression. Testing for deviation from Hardy-Weinberg equilibrium (HWE) was
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31 189 performed within the participants stratified by case and control using a chi-square test.
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33 190 Wald's test statistic was used for test the Gene-environment interactions. The
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35 191 significance level in this study was taken as $P < 0.05$.

36 192 **Ethical statement**

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38 193 The study was proved by the Harbin Medical University Ethics Committee
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40 194 (HMUIRB20120021). All of these participants signed informed consent, and we also
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42 195 obtained written informed consent from the guardians on behalf of the minors. For the
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44 196 brick-tea water samples collection, there were no specific permits required for the
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46 197 locations or activities association in this field study. The locations were not privately
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48 198 owned or protected in any way and this field study did not involve endangered or
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9 201 **RESULTS**

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11 202 *Participants characteristics.*

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14 203 There were 336 subjects who were diagnosed with skeletal fluorosis with the
15
16 204 prevalence rate 26.2% (336/1284). Demographic of skeletal fluorosis cases and
17
18 205 controls are presented in **Table 1**. Skeletal fluorosis cases were significantly older
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21 206 than controls ($p<0.001$). And there more male in the skeletal fluorosis cases than in
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23
24 207 the controls ($p=0.015$). Skeletal fluorosis cases were significantly more likely to have
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26 208 a more daily fluoride intake (IF) ($p<0.001$) and a high urine fluoride (UF) status
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28 209 ($p<0.001$). In addition, ethnical differences in skeletal fluorosis were observed
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31 210 ($p<0.001$): more Tibetan cases are the most in skeletal fluorosis cases, following are
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34 211 Kazakh cases and the least are Mongolian and Han cases.

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36 212 *Association of VDR-FokI polymorphism with skeletal fluorosis.*

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39 213 Testing for deviation from Hardy-Weinberg equilibrium (HWE) was performed
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41 214 within the control participants stratified by ethnicity using a chi-square test. All
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44 215 ethnical participants were found to be in Hard-Weinberg equilibrium for VDR-FokI.
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46 216 Because of low frequency of T allele, we divided the participants into two groups by
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48
49 217 the presence or absence of T allele, that is, CC and CT/TT groups (**Table2**).
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51 218 Participants with CT/TT genotype had a significantly decreased risk of skeletal
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54 219 fluorosis (OR=0.717 [95%CI, 0.556-0.925]). After adjustment of the risk factors, the
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56 220 protective effect of CT/TT genotype remained statistically significant (OR=0.761
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4 221 [95%CI, 0.580-0.997]). In addition, there was the suggestion of a difference in
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6 222 skeletal fluorosis risk by ethnicity in relation to VDR-FokI polymorphism. After
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9 223 adjustment of the risk factors, the protective effect of the CT/TT was limited in the
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11 224 Mongolian participants (OR=0.525 [95%CI, 0.278-0.991]). The smaller percentage of
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13 225 Mongolian participants in our analysis appear to be driving the overall more than 20%
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15 226 reduction in risk as our data do not show a similar reduction did not find shared by
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18 227 other three ethnical participants.

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21 228 *Stratification by IF.*

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24 229 We investigated the potential interactions between FokI-SNP and IF (**Table3**). This
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26 230 interaction was only apparent for Mongolian participants but not other three ethnical
27
28 231 participants. For Mongolian participants, a decrease risk of skeletal fluorosis among
29
30 232 carriers with CT/TT genotype was limited to participants with 7.0mg/day~ IF
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32 233 (OR=0.085 [95%CI, 0.009-0.851] vs. OR=0.538 [95%CI, 0.216-1.337] in participants
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34 234 with ~3.5mg/day IF, vs. OR=0.671 [95%CI, 0.220-2.048] in participants with
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36 235 3.5~7.0mg/day IF).

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39 236 *Stratification by UF.*

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42 237 We also observed the potential interactions between VDR-FokI SNP and UF. The
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44 238 risk of skeletal fluorosis varied according to UF for FokI-SNP (**Table 4**). A decrease
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46 239 risk of skeletal fluorosis among the carriers with CT/TT genotype was limited to
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48 240 participants 3.2mg/L~ UF (OR=0.514 [95%CI, 0.306-0.865] vs. OR=0.701 [95%CI,
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50 241 0.444-1.105] in participants with ~1.6mg/L UF, vs. OR=1.145 [95%CI, 0.713-1.837]
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52 242 in participants with 1.6~3.2mg/L UF). We further evaluated the interaction between
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4 243 FokI-SNP and UF by ethnicity. This interaction was also only apparent for Mongolian
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6 244 participants but not other three ethnical participants. For Mongolian participants, a
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9 245 decrease risk of skeletal fluorosis among carriers with CT/TT genotype was limited to
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11 246 participants with 3.2mg/L~ UF (OR=0.103 [95%CI, 0.017-0.633] vs. OR=0.617
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13 247 [95%CI, 0.245-1.555] in participants with ~1.6mg/L UF, vs. OR=0.772 [95%CI,
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15 248 0.246-2.426] in participants with 1.6~3.2mg/L UF).

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19 249 *Stratification by Age.*

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21 250 We also investigated the interactions between FokI SNP and age (**Table 5**). This
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24 251 interaction was also only apparent for Mongolian participants but not other three
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26 252 ethnical participants. For Mongolian participants, a decrease risk of skeletal fluorosis
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29 253 among carriers with CT/TT genotype was limited to participants aged 46 to 65
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31 254 (OR=0.404 [95%CI, 0.177-0.922] vs. (OR=0.443 [95%CI, 0.127-1.548] in
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33 255 participants aged below 45 vs. OR=3.808 [95%CI, 0.156-93.179] in participants aged
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35 256 above 66).

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38 257 **DISCUSSION**

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40 258 Although high fluoride exposure is highly relevant, it is not the only factor
41
42 259 influencing the susceptibility to fluorosis. Not everyone living in areas with naturally
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44 260 high fluoride levels in water suffers fluorosis.[5, 25] Three inbred strains of mice (A/J,
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46 261 SWR/J and 129P3/J) that showed different susceptibilities to dental fluorosis,
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48 262 displayed variation in bone response to fluoride exposure,[26-28] So, genetics might
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50 263 influence the bone response of individuals to fluoride exposure. In our previous study,
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53 264 we observed that the prevalence rate and severity in different ethnical participants was
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55 265 different, which suggested the possible contribution of a genetic differences in
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4 266 fluorosis disparities.[24] In addition, the distribution of environment factors related
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6 267 with fluoride exposure, including age, IF and UF, differs by ethnicity. As a result, it is
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9 268 plausible that the interaction between gene and environment factors might explain, in
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11 269 part, the racial/ethnic differences in fluorosis prevalence.

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14 270 Of the previous studies on genetic variation and fluorosis risk, the contribution or
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16 271 association of SNPs on dental fluorosis or skeletal fluorosis has been reported in
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18 272 several studies.[11, 24, 29-31] However, none studies have reported the association
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21 273 between VDR-FokI polymorphism and skeletal fluorosis of the brick-tea type
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23 274 fluorosis. Calcium could against the toxic effects of fluoride to a certain extent,[32]
24
25 275 but fluoride ingestion reduces intestinal calcium absorption. VDR-FokI gene
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28 276 polymorphism has been reported to be associated with bone metabolism and calcium
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30 277 absorption.[21-23] Therefore, in the present study we focus on the role of VDR-FokI
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33 278 polymorphism in the skeletal fluorosis of the brick-tea type fluorosis. We noticed that
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35 279 a more than 20% reduction in risk of skeletal fluorosis related with CT/TT genotype
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38 280 in VDR-FokI in all participants was largely attributable to the reduced risk in
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40 281 Mongolian participants; moreover, this protective effect remained significant after
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43 282 adjustment of age, sex, IF and UF. These results suggested that the protective of
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46 283 CT/TT genotype against brick-tea type of fluorosis might be present among
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49 284 Mongolian participants, but not other three ethnical participants.

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51 285 Factors, including age, sex, does and duration of fluoride intake, that influence the
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53 286 fluoride toxicity and the clinical presentation, are the major risk factors of fluorosis.[4,
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56 287 8] So, we investigated the potential interactions between VDR-FokI SNP and known
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4 288 risk factor overall or by ethnic. Stratified our analysis by UF, we found that this
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6 289 protective of CT/TT genotype of VDR-FokI against brick-tea type of fluorosis
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9 290 appeared to be more pronounced in participants with 3.2mg/L~ UF, and this was also
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11 291 attributable to the reduced risk in Mongolian participants. Stratified our analysis by IF
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14 292 or age, we did not observed this protective of CT/TT genotype of VDR-FokI against
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16 293 brick-tea type of fluorosis. Despite this, we further evaluated this interaction by race.
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19 294 This protective effect of CT/TT was detected only in Mongolian participants with
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21 295 7.0mg/day~ IF or aged 46 to 65. However, these findings of the VDR-FokI SNP
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23 296 analysis stratified by risk factors need to be interpreted with caution because the data
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26 297 became sparse when several factors were investigated simultaneously.

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29 298 In the present study, this protective effect of CT/TT genotype of VDR-FokI was
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31 299 limited in Mongolian participants with 7.0mg/day~ IF, or with 3.2 mg/L~ UF. UF
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34 300 reflected the fluoride accumulation in the human body. So we can conclude that this
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36 301 protective effect was pronounced in high fluoride exposure condition. It might result
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39 302 from the limitation of the sample size, because large sample is required to get
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41 303 difference while the prevalence rate of fluorosis is low under the low exposure to
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44 304 fluoride.

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46 305 The amount of fluoride accumulation in the body increased with age,[33-35] so the
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49 306 prevalence rate and severity of skeletal fluorosis is also increased with age. In our data,
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51 307 this protective effect of CT/TT genotype was limited in Mongolian participants aged
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54 308 46~65. For younger people, the fluoride load in body is low because of short time
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56 309 periods of fluoride exposure, so the prevalence rate of fluorosis is small. Thus, it is
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4 310 necessary to investigate this interaction in a large sample population. For the older
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6 311 people (over 66 years old), the protective effect of CT/TT genotype might be
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9 312 compromised by the toxicity of high fluoride load in body.

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11 313 A study in Indian girls indicated after supplementation of calcium, the carriers of
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13 314 TT genotype had significant increase in bone mass as compared to CC genotype,[20]
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15 315 which suggested that the VDR-FokI polymorphism may influence the bone
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17 316 metabolism through the dietary calcium. Unfortunately, we did not have the data on
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19 317 the dietary of calcium. So, it is need to investigate the interaction of VDR-FokI SNP
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21 318 with dietary calcium in fluorosis.
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29 320 **CONCLUSIONS**

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31 321 In summary, we find the CT/TT genotype of VDR-FokI plays a protective role in
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33 322 the brick-tea type fluorosis and the interaction between VDR-FokI and UF in which
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35 323 reduced the risk of skeletal fluorosis was only present in participants with 3.2 mg/L~
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37 324 UF. And we also indicated this protective was apparent in Mongolian participants.
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39 325 Improved understanding of such gene-environment interactions would not only
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41 326 contribute to knowledge on the skeletal fluorosis of brick-tea type fluorosis, but also
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43 327 help in putting forward corresponding preventive measures. Therefore, larger studies
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45 328 are necessary to study the role of genes and/or polymorphic site influencing bone
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47 329 mass in the pathogenesis of fluorosis.
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55 331 **ACKNOWLEDGMENTS**

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4 332 This study was supported by the National Natural Science Foundation of China
5
6 333 (No.81172605 and 30800956). The authors thank for all participates in this study and
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8
9 334 numerous members of the Center for Endemic Disease Control of Chinese Center for
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11 335 Disease Control and Prevention, Inner Mongolia institute for Endemic Disease
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13 336 Control, Qinghai institute for Endemic Disease Control Sinkiang institute for Endemic
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21 339 **Conflicts of interest:** None declared.
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26 341 **Data sharing statement:** No additional data available.
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31 343 **Contributor statement**
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Table 1. Characteristics of skeletal fluorosis cases and controls (N=1284).

	Cases N(%)	Controls N(%)	<i>p</i>
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)

477 *Adjusted for age, sex, IF and UF.

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487 **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)	—

488 *Adjusted for age, sex and UF.

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491 **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)	—

492 *Adjusted for age, sex and IF.

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495 **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)

496 *Adjusted for sex, IF and UF.

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

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2016-011980.R1
Article Type:	Research
Date Submitted by the Author:	03-Aug-2016
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Primary Subject Heading:	Epidemiology
Secondary Subject Heading:	Epidemiology, Global health
Keywords:	EPIDEMIOLOGY, GENETICS, Bone diseases < ORTHOPAEDIC & TRAUMA SURGERY

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1 **Title page**

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

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

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4 23 **Vitamin D receptor gene FokI polymorphism may have a protective effect on**
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6 24 **skeletal fluorosis of the brick-tea type fluorosis.**
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11 **ABSTRACT**

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14 27 **Background:** Brick-tea type fluorosis is a public health concern in the north-west
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16 28 area of China. The VDR-FokI polymorphism is considered to be a regulator of bone
17
18 29 metabolism and calcium resorption. However, the association of VDR-FokI
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20 30 polymorphism with the risk of brick-tea type fluorosis has not been reported.

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24 31 **Materials and Methods:** A cross-sectional case-control study was conducted in three
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26 32 provinces (Inner Mongolia, Qinghai, Sinkiang), China. The fluoride contents of
27
28 33 Brick-tea water or urinary were tested by the standard of GB 1996-2005 or
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30 34 WS/T89-2006 (China), respectively. The skeletal fluorosis was diagnosed by the
31
32 35 standard of WS/192-2008(China). The VDR-FokI polymorphism was detected by
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34 36 Sequenom MassARRAY system.

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36
37 37 **Result:** Compared to the carriers with CC genotype, the participants with CT/TT
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39 38 genotype had a significantly decreased risk of skeletal fluorosis (OR=0.761 [95%CI,
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41 39 0.580-0.997]), after adjustment of risk factors. When investigated among ethnic
42
43 40 groups, the protective effect of the CT/TT was limited in the Mongolian participants
44
45 41 (OR=0.525 [95%CI, 0.278-0.991]). Moreover, the interaction of VDR-FokI with risk
46
47 42 factors was only found in Mongolian participants: the protective effect of the CT/TT
48
49 43 was limited to participants with 7.0 mg/day~ IF (OR=0.085 [95%CI, 0.009-0.851], or
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51 44 participants with 3.2 mg/L~ UF (OR=0.103 [95%CI, 0.017-0.633]), or participants
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4 aged 46 to 65 (OR=0.404 [95%CI, 0.177-0.922].
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6 **Conclusion:** Our data suggest that the CT/TT genotype of VDR-FokI may be a
7
8 protective factor for the brick-tea type skeletal fluorosis and this effect is pronounced
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10 in Mongolian participants.
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16 **Key Words:** Brick-tea type fluorosis; VDR-FokI polymorphism; Skeletal fluorosis.
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21 **Strengths and limitation of this study:**
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24 1. It is the first time to report the association between FokI polymorphism and skeletal
25
26 fluososis overall or by ethnicity. Improved understanding of such gene-environment
27
28 interactions would not only contribute to knowledge on the skeletal fluorosis, but also
29
30 help in putting forward corresponding preventive measures.
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34 2. We find the CT/TT genotype of VDR-FokI plays a protective role in the skeletal
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36 fluososis of brick-tea type fluorosis.
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39 3. For Mongolian participants, this protective were apparent in participants with 3.2
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41 mg/L~ UF, with 7.0 mg/day~ ITF or aged 46 to 65.
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44 4. Our sample size of Tibetan and Han paticipants was inadequate, so the true
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46 association of VDR-FokI polymorphism with skeletal fluorosis in these two ethnicities
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48 is worth to further study.
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51 5. The VDR-FokI polymorphism may influence the bone metabolism through the
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53 dietary calcium, but we did not have the data on the dietary of calcium.
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57 6. We did not have the data on the serum vitamin D.
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67 7. Besides FokI, others SNPs, such as BsmI et al. also play an important role in bone
68 metabolism. So it is required to study the association between these SNPs and skeletal
69 fluorosis.

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70 INTRODUCTION

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

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

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4 92 fluorosis.
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6 93 Among potential genes relevant to bone metabolism, [9] vitamin D receptor (VDR)
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9 94 gene is considered to be an important candidate gene.[11] VDR gene, located on
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11 95 chromosome 12 (12q12-14), plays an important role in bone metabolism, including
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13 96 intestinal calcium absorption.[12 13] It is reported that fluoride modulates the VDR
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15 97 mRNA expression. [14] Since Morrison first reported that VDR gene polymorphism
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17 98 is associated with bone mineral density and it can predict the bone density,[15] several
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19 99 VDR gene restriction endonuclease sites, such as FokI (rs2228570), BsmI
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21 100 (rs1544410), ApaI (rs7975232), and TaqI (rs731236) have been used in
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23 101 population-based study.[13 15 16] Different VDR gene polymorphism plays different
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25 102 roles in regulating the bone metabolism.[17-20] FokI polymorphism, a C→T
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27 103 transition found within the translation initiation site in exon 2, results in long and
28
29 104 short variants of VDR due to creating an upstream initiation codon (ACG→ATG).[21]
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31 105 FokI polymorphism was related to bone mineral density and calcium absorption.
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33 106 [21-23] Some reports suggest that the short receptor protein (C allele), initiated from
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35 107 the second ATG site, may play a more active role in the VDR responsive gene
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37 108 expression because it interacts more efficiently with the transcriptional factor IIB
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39 109 compared to the long receptor protein (T allele), initiated from the first ATG site.[24]
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41 110 The association of FokI polymorphism with bone mineral density have been reported,
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43 111 [21-23] however, the association of FokI polymorphism with the risk of skeletal
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45 112 fluorosis has not been reported.

56 113 Our prior study has demonstrated ethnic difference in prevalence rate of the
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4 114 brick-tea type fluorosis and that glutathione S-transferases (GST) rs1695
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6 115 polymorphism may play an important role in the pathogenesis of brick-tea
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9 116 fluorosis.[25] However, there are no studies reported the effect of VDR-FokI on
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11 117 skeletal fluorosis of the brick-tea type fluorosis. Therefore, in this study, we
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13 118 conducted a cross-sectional case-control study in the brick-tea fluorosis area of China
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15 119 to investigate the association between FokI polymorphism and skeletal fluorosis
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17 120 overall or by ethnicity.
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23 122 **MATERIALS AND METHODS**

24 123 **Subjects and data collection**

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28 124 On the brick-tea type fluorosis survey data, we undertook a cross-sectional
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30 125 case-control study in sixteen villages from the Inner Mongolia, Qinghai and Sinkiang
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32 126 province, People's Republic of China, from July to August in 2012. In this study,
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34 127 overall participants were older than 16 years old, born and grew up in their village.
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37 128 Each participant received the questionnaire survey and clinical examination. The
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39 129 clinical examination included physical examination, medical history and X-ray
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41 130 diagnosis (Beijing Longsafe Imaging Technology Co., Beijing City, China). The
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43 131 content of the questionnaire included general information of respondents (name, sex,
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45 132 age, education, economic income, the history of bone related disease, etc), and
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47 133 fluoride exposure (the amount of drinking brick-tea water per day, the consumption of
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49 134 brick-tea per year). In addition, we also collect everyone's blood, urine and brick-tea
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51 135 water samples. All participants signed informed consent, and this research was
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4 136 approved by Harbin Medical University Ethics Committee.
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6 **137 Diagnose of skeletal fluorosis**
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9 138 The skeletal fluorosis was diagnosed according to the Chinese diagnostic criteria of
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11 139 endemic skeletal fluorosis (WS192-2008, China). As described in previous report,[25]
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13 140 the diagnose of skeletal fluorosis was based on the sign of X ray, included
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15 141 osteoporosis, osteomalacia, sclerosis, ossification of soft tissue and joint degeneration
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17 142 in the forearm, shank, and pelvic, and could be classified into three categories: mild,
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19 143 moderate or severe.
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23 **144 Determination of fluoride concentration**
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26 145 All urine and brick-tea water samples were stored at -20°C until use. Urinary
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28 146 fluoride content was determined by the fluoride ion electrode method based on The
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30 147 China's Urinary Fluoride Detection-Fluoride Electrode Method WS/T89-1996. The
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32 148 concentration of brick-tea water was also determined by the fluoride ion electrode
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34 149 method with the standard of GB19965-2005 (China).
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38 **150 Genotyping**
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41 151 All blood samples were stored at -80°C until use. The genomic DNA was extracted
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43 152 from the blood samples using the DNA extraction kit (Axygen Biosciences, Union
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45 153 City, USA). The DNA concentration was tested by TU1901 Spectrophotometry
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47 154 (Purkinje General Company, Beijing City, China). When the DNA concentration was
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49 155 greater than 20µg/ml, preserve the extracted DNA was preserved at -80°C. The
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51 156 genotyping sequencing from the extract were performed by the shanghai Fenglin
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53 157 Clinical Laboratory Company (<http://www.fenglinlab.com/index.asp>) using the
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4 158 Sequenom MassARRAY system (Sequenom, Inc., San Diego, CA, USA). The primer
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6 159 sequence of the FokI: forward-5'-ACGTTGGATGTGGCCTGCTTGCTGTTCTTA-3',
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9 160 reverse-5'-ACGTTGGATGACGTTCCGGTCAAAGTCTCC-3',
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11 161 extended-5'-GTGCTGGCCGCCATTGCCTCC-3'. For the genotyping sequencing
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14 162 quality control, blinded blood duplicate was used.

163 **Statistical analysis**

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

173 **Ethical statement**

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

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6 181 **RESULTS**

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9 182 *Participants characteristics.*

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11 183 In total, 1284 subjects participated in this study. There were 336 subjects who were
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13 184 diagnosed with skeletal fluorosis with the prevalence rate 26.2% (336/1284).

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16 185 Demographic of skeletal fluorosis cases and controls are presented in **Table 1**.

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18 186 Skeletal fluorosis cases were significantly older than controls ($p<0.001$). And there
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20 187 more male in the skeletal fluorosis cases than in the controls ($p=0.015$). Skeletal
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22 188 fluorosis cases were significantly more likely to have a more daily intake of tea
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24 189 fluoride (ITF) ($p<0.001$) and a high urine fluoride (UF) status ($p<0.001$). In addition,
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26 190 ethnic differences in skeletal fluorosis were observed ($p<0.001$): more Tibetan cases
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28 191 are the most in skeletal fluorosis cases, following are Kazakh cases and the least are
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30 192 Mongolian and Han cases.

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33 193 *Association of VDR-FokI polymorphism with skeletal fluorosis.*

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36 194 Testing for deviation from Hardy-Weinberg equilibrium (HWE) was performed
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38 195 within the control participants stratified by ethnicity using a chi-square test. All
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40 196 ethnic participants were found to be in Hardy-Weinberg equilibrium for VDR-FokI.
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42 197 In our study, VDR-FokI polymorphism follows a dominant model of inheritance, so
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44 198 we divided the participants into two groups by the presence or absence of T allele, that
45
46 199 is, CC and CT/TT groups (**Table2**). Participants with CT/TT genotype had a
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48 200 significantly decreased risk of skeletal fluorosis (OR=0.717 [95%CI, 0.556-0.925]).
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50 201 After adjustment of the risk factors, the protective effect of CT/TT genotype remained
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4 202 statistically significant (OR=0.761 [95%CI, 0.580-0.997]). In addition, there was the
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6 203 suggestion of a difference in skeletal fluorosis risk by ethnicity in relation to
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9 204 VDR-FokI polymorphism. After adjustment of the risk factors, the protective effect of
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11 205 the CT/TT was limited in the Mongolian participants (OR=0.525 [95%CI,
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13 206 0.278-0.991]). The smaller percentage of Mongolian participants in our analysis
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16 207 appear to be driving the overall more than 20% reduction in risk as our data do not
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19 208 show a similar reduction did not find shared by other three ethnical participants.

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21 209 Given non-significant nature of association of VDR-FokI polymorphism in Tibetan,
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24 210 Kazakh and Han participants, it is necessary to estimate if our study has adequate
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26 211 power to detect the true association if present in these populations. We estimated the
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29 212 power of our study using G Power software (version 3.1). We obtained the power of
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31 213 98.9% and 99.4% at $p = 0.05$ for overall and Mongolian participants, respectively. But
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34 214 the power for Tibetan, Kazakh and Han participants is 11.7%, 64.5% and 8.1%
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36 215 respectively. This shows that our sample size of Tibetan, Han participants was
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39 216 inadequate and the study was insufficiently-powered to detect the true association of
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41 217 VDR-FokI polymorphism with skeletal fluorosis, if existent in these ethnic
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44 218 participants.

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46 219 *Stratification by potential risk factor:*

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49 220 We investigated the potential interactions between FokI-SNP and potential risk
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51 221 factor in Mongolian participants (**Table3**). For Mongolian participants, a decrease risk
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54 222 of skeletal fluorosis among carriers with CT/TT genotype was limited to participants
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56 223 with 7.0 mg/day~ ITF (OR=0.085 [95%CI, 0.009-0.851] vs. OR=0.538 [95%CI,

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4 224 0.216-1.337] in participants with ~3.5 mg/day ITF, vs. OR=0.671 [95%CI,
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6 225 0.220-2.048] in participants with 3.5~7.0 mg/day ITF). We also observed that a
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9 226 decrease risk of skeletal fluorosis among carriers with CT/TT genotype was limited to
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11 227 participants with 3.2 mg/L~ UF (OR=0.103 [95%CI, 0.017-0.633] vs. OR=0.617
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13 228 [95%CI, 0.245-1.555] in participants with ~1.6 mg/L UF, vs. OR=0.772 [95%CI,
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15 229 0.246-2.426] in participants with 1.6~3.2 mg/L UF). In addition, we also noticed that
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19 230 a decrease risk of skeletal fluorosis among carriers with CT/TT genotype was limited
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21 231 to participants aged 46 to 65 (OR=0.404 [95%CI, 0.177-0.922] vs. (OR=0.443
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23 232 [95%CI, 0.127-1.548] in participants aged below 45 vs. OR=3.808 [95%CI,
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25 233 0.156-93.179] in participants aged above 66).

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235 **DISCUSSION**

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

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4 247 might explain, in part, the racial/ethnic differences in fluorosis prevalence.
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6 248 Of the previous studies on genetic variation and fluorosis risk, the contribution or
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8 249 association of SNPs on dental fluorosis or skeletal fluorosis has been reported in
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11 250 several studies.[10 25 30-32] However, none studies have reported the association
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14 251 between VDR-FokI polymorphism and skeletal fluorosis of the brick-tea type
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16 252 fluorosis. Calcium could alleviate the toxic effects of fluoride to a certain extent,[33]
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18 253 but fluoride ingestion reduces intestinal calcium absorption. Animal study indicated
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21 254 that excess fluoride had an inhibitory effect on duodenal VDR gene transcription, and
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24 255 thereby hindered the calcium absorption process.[34] VDR-FokI gene polymorphism
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26 256 has been reported to be associated with bone metabolism and calcium
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29 257 absorption.[21-23] Moreover, evidence indicated that the TT and CT forms of the
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31 258 VDR-FokI polymorphsim are associated with a decreased VDR efficiency, compared
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34 259 with the TT genotype.[24] Therefore, in the present study we focus on the role of
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36 260 VDR-FokI polymorphism in the skeletal fluorosis of the brick-tea type fluorosis. We
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39 261 noticed that a more than 20% reduction in risk of skeletal fluorosis related with
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41 262 CT/TT genotype in VDR-FokI in all participants was largely attributable to the
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44 263 reduced risk in Mongolian participants; moreover, this protective effect remained
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46 264 significant after adjustment of age, sex, ITF and UF. These results suggested that the
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49 265 protective of CT/TT genotype against the skeletal fluorosis of brick-tea type of
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51 266 fluorosis might be present among Mongolian participants.
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53 267 Factors, including age, sex, dose and duration of fluoride intake, that influence the
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56 268 fluoride toxicity and the clinical presentation, are the major risk factors of fluorosis.[4
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4 269 8] So, we investigated the potential interactions between VDR-FokI SNP and known
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6 270 risk factor in Mongolian participants. We found that this protective role of CT/TT
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9 271 genotype of VDR-FokI against brick-tea type of fluorosis appeared to be more
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11 272 pronounced in participants with 3.2 mg/L~ UF, with 7.0 mg/day~ ITF or aged 46 to
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14 273 65. So our results suggested that this protective effect was pronounced in high
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16 274 fluoride exposure condition. However, these findings of the VDR-FokI SNP analysis
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19 275 stratified by risk factors need to be interpreted with caution, because the data became
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21 276 sparse when several factors were investigated simultaneously. The amount of fluoride
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23 277 accumulation in the body increased with age,[35-37] so the prevalence rate and
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26 278 severity of skeletal fluorosis is also increased with age. In our data, this protective
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29 279 effect of CT/TT genotype was limited in Mongolian participants aged 46~65. For
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31 280 younger people, the fluoride load in body is low because of short time periods of
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34 281 fluoride exposure, so the prevalence rate of fluorosis is small. Thus, it is necessary to
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36 282 investigate this interaction in a large sample population. For the older people (over 66
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39 283 years old), the protective effect of CT/TT genotype might be compromised by the
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41 284 toxicity of high fluoride load in body.

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44 285 There are several potential limitations in this study. First, in power analysis, we
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46 286 found that our sample size of Tibetan and Han participants was inadequate, so the true
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48 287 association of VDR-FokI polymorphism with skeletal fluorosis in these two ethnicities
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51 288 is worth to further study. Second, a study in Indian girls indicated after
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54 289 supplementation of calcium, the carriers of TT genotype had significant increase in
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56 290 bone mass as compared to CC genotype,[20] which suggested that the VDR-FokI

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4 291 polymorphism may influence the bone metabolism through the dietary calcium.
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6 292 Unfortunately, we did not have the data on the dietary of calcium. So, it is need to
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9 293 investigate the interaction of VDR-FokI SNP with dietary calcium in fluorosis. Third,
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11 294 we did not have the data on the serum vitamin D. Fouth, VDR contains numerous
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13 295 SNPs. Besides FokI, others SNPs, such as BsmI et al. also play an important role in
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15 296 bone metabolism. So it is required to study the association between these SNPs and
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17 297 skeletal fluorosis.
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299 **CONCLUSIONS**

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

308

309 **ACKNOWLEDGMENTS**

310 This study was supported by the National Natural Science Foundation of China
311 (No.81172605 and 30800956). The authors thank for all participates in this study and
312 numerous members of the Center for Endemic Disease Control of Chinese Center for

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4 313 Disease Control and Prevention, Inner Mongolia institute for Endemic Disease
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6 314 Control, Qinghai institute for Endemic Disease Control Sinkiang institute for Endemic
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14 317 **Conflicts of interest:** None declared.

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19 319 **Data sharing statement:** No additional data available.

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24 321 **Contributor statement**

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53 333 **REFERENCES**

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

	Cases n (%)	Controls n (%)	<i>p</i>
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)	

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

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

459

460 **Table 2. Risk of skeletal fluorosis associated with VDR-FokI polymorphic**
 461 **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)

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

463

464 **Table 3. Association of VDR-FokI polymorphic genotypes with skeletal**
 465 **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

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

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

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2016-011980.R2
Article Type:	Research
Date Submitted by the Author:	11-Oct-2016
Complete List of Authors:	<p>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</p> <p>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</p> <p>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</p> <p>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</p> <p>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</p> <p>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</p> <p>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</p> <p>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</p>

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Primary Subject Heading:	Epidemiology
Secondary Subject Heading:	Epidemiology, Global health
Keywords:	EPIDEMIOLOGY, GENETICS, Bone diseases < ORTHOPAEDIC & TRAUMA SURGERY

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1 **Title page**

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

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

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4 23 **Association between vitamin D receptor gene FokI polymorphism and skeletal**
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6 24 **fluorosis of the brick-tea type fluorosis: a cross-sectional case-control study.**
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11 26 **ABSTRACT**
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14 27 **Background:** Brick-tea type fluorosis is a public health concern in the north-west
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16 28 area of China. The VDR-FokI polymorphism is considered to be a regulator of bone
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18 29 metabolism and calcium resorption. However, the association of VDR-FokI
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20 30 polymorphism with the risk of brick-tea type fluorosis has not been reported.
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24 31 **Materials and Methods:** A cross-sectional case-control study was conducted in three
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26 32 provinces (Inner Mongolia, Qinghai, Sinkiang), China. The fluoride contents of
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28 33 Brick-tea water or urinary were tested by the standard of GB 1996-2005 or
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30 34 WS/T89-2006 (China), respectively. The skeletal fluorosis was diagnosed by the
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32 35 standard of WS/192-2008(China). The VDR-FokI polymorphism was detected by
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34 36 Sequenom MassARRAY system.
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37 37 **Result:** Compared to the carriers with CC genotype, the participants with CT/TT
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39 38 genotype had a significantly decreased risk of skeletal fluorosis (OR=0.761 [95%CI,
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41 39 0.580-0.997]), after adjustment of risk factors. When investigated among ethnic
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43 40 groups, the protective effect of the CT/TT was limited in the Mongolian participants
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45 41 (OR=0.525 [95%CI, 0.278-0.991]). Moreover, the interaction of VDR-FokI with risk
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47 42 factors was only found in Mongolian participants: the protective effect of the CT/TT
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49 43 was limited to participants with 7.0 mg/day~ IF (OR=0.085 [95%CI, 0.009-0.851], or
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51 44 participants with 3.2 mg/L~ UF (OR=0.103 [95%CI, 0.017-0.633]), or participants
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4 aged 46 to 65 (OR=0.404 [95%CI, 0.177-0.922].

5
6 **Conclusion:** Our data suggest that the CT/TT genotype of VDR-FokI may be a
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9 protective factor for the brick-tea type skeletal fluorosis and this effect is pronounced
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11 in Mongolian participants.
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16 **Key Words:** Brick-tea type fluorosis; VDR-FokI polymorphism; Skeletal fluorosis.
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21 **Strengths and limitation of this study:**
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24 1. This study includes Tibetan, Kazakh, Mongolian and Han participants.
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26 2. Overall participants were older than 16 years old, born and grew up in the Inner
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28 Mongolia, Qinghai and Sinkiang province, People's Republic of China.
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30 3. It is the first report describing the association between FokI polymorphism and
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32 skeletal fluorosis overall or by ethnicity.
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34 4. A limitation is that sample size of Tibetan and Han participants was inadequate.
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60 INTRODUCTION

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

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

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4 82 fluorosis.

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6 83 Among potential genes relevant to bone metabolism, [9] vitamin D receptor (VDR)

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9 84 gene is considered to be an important candidate gene.[11] VDR gene, located on

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11 85 chromosome 12 (12q12-14), plays an important role in bone metabolism, including

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13 86 intestinal calcium absorption.[12 13] It is reported that fluoride modulates the VDR

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15 87 mRNA expression. [14] Since Morrison first reported that VDR gene polymorphism

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18 88 is associated with bone mineral density and it can predict the bone density,[15] several

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21 89 VDR gene restriction endonuclease sites, such as FokI (rs2228570), BsmI

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24 90 (rs1544410), ApaI (rs7975232), and TaqI (rs731236) have been used in

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27 91 population-based study.[13 15 16] Different VDR gene polymorphism plays different

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29 92 roles in regulating the bone metabolism.[17-20] FokI polymorphism, a C→T

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32 93 transition found within the translation initiation site in exon 2, results in long and

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34 94 short variants of VDR due to creating an upstream initiation codon (ACG→ATG).[21]

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37 95 FokI polymorphism was related to bone mineral density and calcium absorption.

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39 96 [21-23] Some reports suggest that the short receptor protein (C allele), initiated from

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42 97 the second ATG site, may play a more active role in the VDR responsive gene

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44 98 expression because it interacts more efficiently with the transcriptional factor IIB

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47 99 compared to the long receptor protein (T allele), initiated from the first ATG site.[24]

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49 100 The association between FokI polymorphism and bone mineral density has been

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52 101 reported, [21-23] however, the association of FokI polymorphism with the risk of

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54 102 skeletal fluorosis has not been reported.

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57 103 Our prior study has demonstrated ethnic difference in prevalence rate of the

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4 104 brick-tea type fluorosis and that glutathione S-transferases (GST) rs1695
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6 105 polymorphism may play an important role in the pathogenesis of brick-tea
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9 106 fluorosis.[25] However, there are no studies reported the effect of VDR-FokI on
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11 107 skeletal fluorosis of the brick-tea type fluorosis. Therefore, in this study, we
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13 108 conducted a cross-sectional case-control study in the brick-tea fluorosis area of China
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15 109 to investigate the association between FokI polymorphism and skeletal fluorosis
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17 110 overall or by ethnicity.
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23 112 **MATERIALS AND METHODS**

24 113 **Subjects and data collection**

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28 114 On the brick-tea type fluorosis survey data, we undertook a cross-sectional
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30 115 case-control study in sixteen villages from the Inner Mongolia, Qinghai and Sinkiang
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32 116 province, People's Republic of China, from July to August in 2012. In this study,
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34 117 overall participants were older than 16 years old, born and grew up in their village.
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37 118 Each participant received the questionnaire survey and clinical examination. The
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39 119 clinical examination included physical examination, medical history and X-ray
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41 120 diagnosis (Beijing Longsafe Imaging Technology Co., Beijing City, China). The
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43 121 content of the questionnaire included general information of respondents (name, sex,
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45 122 age, education, economic income, the history of bone related disease, etc), and
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47 123 fluoride exposure (the amount of drinking brick-tea water per day, the consumption of
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49 124 brick-tea per year). In addition, we also collect everyone's blood, urine and brick-tea
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51 125 water samples. All participants signed informed consent, and this research was
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126 approved by Harbin Medical University Ethics Committee.

127 **Diagnose of skeletal fluorosis**

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

134 **Determination of fluoride concentration**

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

140 **Genotyping**

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

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4 148 Sequenom MassARRAY system (Sequenom, Inc., San Diego, CA, USA). The primer
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6 149 sequence of the FokI: forward-5'-ACGTTGGATGTGGCCTGCTTGCTGTTCTTA-3',
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9 150 reverse-5'-ACGTTGGATGACGTTCCGGTCAAAGTCTCC-3',
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11 151 extended-5'-GTGCTGGCCGCCATTGCCTCC-3'. For the genotyping sequencing
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14 152 quality control, blinded blood duplicate was used.

153 **Statistical analysis**

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

163 **Ethical statement**

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

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6 171 **RESULTS**

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9 172 *Participants characteristics.*

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11 173 In total, 1284 subjects participated in this study. There were 336 subjects who were
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14 174 diagnosed with skeletal fluorosis with the prevalence rate 26.2% (336/1284).

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16 175 Demographic of skeletal fluorosis cases and controls are presented in **Table 1**.

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18 176 Skeletal fluorosis cases were significantly older than controls ($p<0.001$). And there
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21 177 more male in the skeletal fluorosis cases than in the controls ($p=0.015$). Skeletal
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24 178 fluorosis cases were significantly more likely to have a more daily intake of tea
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26 179 fluoride (ITF) ($p<0.001$) and a high urine fluoride (UF) status ($p<0.001$). In addition,
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29 180 ethnic differences in skeletal fluorosis were observed ($p<0.001$): more Tibetan cases
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31 181 are the most in skeletal fluorosis cases, following are Kazakh cases and the least are
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34 182 Mongolian and Han cases.

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36 183 *Association of VDR-FokI polymorphism with skeletal fluorosis.*

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39 184 Testing for deviation from Hardy-Weinberg equilibrium (HWE) was performed
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41 185 within the control participants stratified by ethnicity using a chi-square test. All
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44 186 ethnic participants were found to be in Hardy-Weinberg equilibrium for VDR-FokI.
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46 187 In our study, VDR-FokI polymorphism follows a dominant model of inheritance, so
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49 188 we divided the participants into two groups by the presence or absence of T allele, that
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51 189 is, CC and CT/TT groups (**Table2**). Participants with CT/TT genotype had a
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54 190 significantly decreased risk of skeletal fluorosis (OR=0.717 [95%CI, 0.556-0.925]).
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56 191 After adjustment of the risk factors, the protective effect of CT/TT genotype remained
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4 192 statistically significant (OR=0.761 [95%CI, 0.580-0.997]). In addition, there was the
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6 193 suggestion of a difference in skeletal fluorosis risk by ethnicity in relation to
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9 194 VDR-FokI polymorphism. After adjustment of the risk factors, the protective effect of
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11 195 the CT/TT was limited in the Mongolian participants (OR=0.525 [95%CI,
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13 196 0.278-0.991]). The smaller percentage of Mongolian participants in our analysis
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16 197 appear to be driving the overall more than 20% reduction in risk as our data do not
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19 198 show a similar reduction did not find shared by other three ethnical participants.

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21 199 Given non-significant nature of association of VDR-FokI polymorphism in Tibetan,
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23 200 Kazakh and Han participants, it is necessary to estimate if our study has adequate
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26 201 power to detect the true association if present in these populations. We estimated the
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29 202 power of our study using G Power software (version 3.1). We obtained the power of
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31 203 98.9% and 99.4% at $p = 0.05$ for overall and Mongolian participants, respectively. But
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34 204 the power for Tibetan, Kazakh and Han participants is 11.7%, 64.5% and 8.1%
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36 205 respectively. This shows that our sample size of Tibetan, Han participants was
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39 206 inadequate and the study was insufficiently-powered to detect the true association of
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41 207 VDR-FokI polymorphism with skeletal fluorosis, if existent in these ethnic
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44 208 participants.

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46 209 *Stratification by potential risk factor:*

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49 210 We investigated the potential interactions between FokI-SNP and potential risk
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51 211 factor in Mongolian participants (**Table3**). For Mongolian participants, a decrease risk
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54 212 of skeletal fluorosis among carriers with CT/TT genotype was limited to participants
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56 213 with 7.0 mg/day~ ITF (OR=0.085 [95%CI, 0.009-0.851] vs. OR=0.538 [95%CI,

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4 214 0.216-1.337] in participants with ~3.5 mg/day ITF, vs. OR=0.671 [95%CI,
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6 215 0.220-2.048] in participants with 3.5~7.0 mg/day ITF). We also observed that a
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9 216 decrease risk of skeletal fluorosis among carriers with CT/TT genotype was limited to
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11 217 participants with 3.2 mg/L~ UF (OR=0.103 [95%CI, 0.017-0.633] vs. OR=0.617
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13 218 [95%CI, 0.245-1.555] in participants with ~1.6 mg/L UF, vs. OR=0.772 [95%CI,
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15 219 0.246-2.426] in participants with 1.6~3.2 mg/L UF). In addition, we also noticed that
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18 220 a decrease risk of skeletal fluorosis among carriers with CT/TT genotype was limited
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21 221 to participants aged 46 to 65 (OR=0.404 [95%CI, 0.177-0.922] vs. (OR=0.443
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23 222 [95%CI, 0.127-1.548] in participants aged below 45 vs. OR=3.808 [95%CI,
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25 223 0.156-93.179] in participants aged above 66).

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30 225 **DISCUSSION**

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32 226 Although high fluoride exposure is highly relevant with skeletal fluorosis, it is not
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34 227 the only factor influencing the susceptibility to fluorosis. Not everyone living in areas
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36 228 with naturally high fluoride levels in water suffers fluorosis.[5 26] Three inbred
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38 229 strains of mice (A/J, SWR/J and 129P3/J) that showed different susceptibilities to
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40 230 dental fluorosis, displayed variation in bone response to fluoride exposure.[27-29] So,
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42 231 genetics might influence the bone response of individuals to fluoride exposure. In our
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44 232 previous study, we observed that the prevalence rate and severity in different ethnical
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46 233 participants was different, which suggested the possible contribution of a genetic
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48 234 differences in fluorosis disparities.[25] In addition, the distribution of environment
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50 235 factors related with fluoride exposure, including age, ITF and UF, differs by ethnicity.
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52 236 As a result, it is plausible that the interaction between gene and environment factors
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4 237 might explain, in part, the racial/ethnic differences in fluorosis prevalence.
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6 238 Of the previous studies on genetic variation and fluorosis risk, the contribution or
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8 239 association of SNPs on dental fluorosis or skeletal fluorosis has been reported in
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10 240 several studies.[10 25 30-32] However, none studies have reported the association
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12 241 between VDR-FokI polymorphism and skeletal fluorosis of the brick-tea type
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14 242 fluorosis. Calcium could alleviate the toxic effects of fluoride to a certain extent,[33]
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16 243 but fluoride ingestion reduces intestinal calcium absorption. Animal study indicated
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18 244 that excess fluoride had an inhibitory effect on duodenal VDR gene transcription, and
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20 245 thereby hindered the calcium absorption process.[34] VDR-FokI gene polymorphism
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22 246 has been reported to be associated with bone metabolism and calcium
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24 247 absorption.[21-23] Moreover, evidence indicated that the TT and CT forms of the
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26 248 VDR-FokI polymorphsim are associated with a decreased VDR efficiency, compared
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28 249 with the TT genotype.[24] Therefore, in the present study we focus on the role of
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30 250 VDR-FokI polymorphism in the skeletal fluorosis of the brick-tea type fluorosis. We
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32 251 noticed that a more than 20% reduction in risk of skeletal fluorosis related with
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34 252 CT/TT genotype in VDR-FokI in all participants was largely attributable to the
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36 253 reduced risk in Mongolian participants; moreover, this protective effect remained
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38 254 significant after adjustment of age, sex, ITF and UF. These results suggested that the
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40 255 protective of CT/TT genotype against the skeletal fluorosis of brick-tea type of
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42 256 fluorosis might be present among Mongolian participants.
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53 257 Factors, including age, sex, dose and duration of fluoride intake, that influence the
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55 258 fluoride toxicity and the clinical presentation, are the major risk factors of fluorosis.[4
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4 259 8] So, we investigated the potential interactions between VDR-FokI SNP and known
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6 260 risk factor in Mongolian participants. We found that this protective role of CT/TT
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9 261 genotype of VDR-FokI against brick-tea type of fluorosis appeared to be more
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11 262 pronounced in participants with 3.2 mg/L~ UF, with 7.0 mg/day~ ITF or aged 46 to
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14 263 65. So our results suggested that this protective effect was pronounced in high
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16 264 fluoride exposure condition. However, these findings of the VDR-FokI SNP analysis
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19 265 stratified by risk factors need to be interpreted with caution, because the data became
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21 266 sparse when several factors were investigated simultaneously. The amount of fluoride
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23 267 accumulation in the body increased with age,[35-37] so the prevalence rate and
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26 268 severity of skeletal fluorosis is also increased with age. In our data, this protective
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29 269 effect of CT/TT genotype was limited in Mongolian participants aged 46~65. For
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31 270 younger people, the fluoride load in body is low because of short time periods of
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34 271 fluoride exposure, so the prevalence rate of fluorosis is small. Thus, it is necessary to
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36 272 investigate this interaction in a large sample population. For the older people (over 66
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39 273 years old), the protective effect of CT/TT genotype might be compromised by the
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41 274 toxicity of high fluoride load in body.

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44 275 There are several potential limitations in this study. First, in power analysis, we
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46 276 found that our sample size of Tibetan and Han participants was inadequate, so the true
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48 277 association of VDR-FokI polymorphism with skeletal fluorosis in these two ethnicities
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51 278 is worth to further study. Second, a study in Indian girls indicated after
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54 279 supplementation of calcium, the carriers of TT genotype had significant increase in
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56 280 bone mass as compared to CC genotype,[20] which suggested that the VDR-FokI

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4 281 polymorphism may influence the bone metabolism through the dietary calcium.
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6 282 Unfortunately, we did not have the data on the dietary of calcium. So, it is need to
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9 283 investigate the interaction of VDR-FokI SNP with dietary calcium in fluorosis. Third,
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11 284 we did not have the data on the serum vitamin D. Fouth, VDR contains numerous
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13 285 SNPs. Besides FokI, others SNPs, such as BsmI et al. also play an important role in
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16 286 bone metabolism. So it is required to study the association between these SNPs and
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19 287 skeletal fluorosis.
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22 23 24 289 **CONCLUSIONS**

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26 290 In summary, the CT/TT genotype of VDR-FokI might play a protective role in the
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29 291 brick-tea type fluorosis and this protective was apparent in Mongolian participants.
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31 292 For Mongolian participants, this protective were apparent in participants with 3.2
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33 293 mg/L~ UF, with 7.0 mg/day~ ITF or aged 46 to 65. Improved understanding of such
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36 294 gene-environment interactions would not only contribute to knowledge on the skeletal
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39 295 fluorosis of brick-tea type fluorosis, but also help in putting forward corresponding
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41 296 preventive measures. Therefore, larger studies are necessary to study the role of genes
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44 297 and/or polymorphic site influencing bone mass in the pathogenesis of fluorosis.
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48 49 299 **ACKNOWLEDGMENTS**

50
51 300 This study was supported by the National Natural Science Foundation of China
52
53
54 301 (No.81172605 and 30800956). The authors thank for all participates in this study and
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56
57 302 numerous members of the Center for Endemic Disease Control of Chinese Center for
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4 303 Disease Control and Prevention, Inner Mongolia institute for Endemic Disease
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6 304 Control, Qinghai institute for Endemic Disease Control Sinkiang institute for Endemic
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9 305 Disease Control.

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14 307 **Conflicts of interest:** None declared.

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19 309 **Data sharing statement:** No additional data available.

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24 311 **Contributor statement**

25
26 312 Gao YH, Yang YM and Sun DJ participated in the design of the study. Gao YH, Yang

27
28 313 YM, Yang D, Liu Y, Li DD and Qin M participated in the interpretation of the data

29
30 314 and statistical analyses. Chu YR, Yang Q, Jiang W and Chen FX performed

31
32 315 acquisition of data. The manuscript was drafted by Yang D and Liu Y, and was revised

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34 316 by Gao YH and Yang YM.

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447

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

	Cases n (%)	Controls n (%)	<i>p</i>
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

452 **Table 2. Risk of skeletal fluorosis associated with VDR-FokI polymorphic**
 453 **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)

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

455

456 **Table 3. Association of VDR-FokI polymorphic genotypes with skeletal fluorosis**457 **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

458 **Mongolian subjects, stratified by potential risk factor levels.**

459 a. Adjusted for age, sex and UF (urine fluoride). b. Adjusted for age, sex and ITF

460 (daily intake of tea fluoride). c. Adjusted for sex, ITF (daily intake of tea fluoride) and

461 UF (urine fluoride).