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COL1A2 gene polymorphisms (*Pvu* II and *Rsa* I), serum calciotropic hormone levels, and dental fluorosis

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Abstract – *Objectives:* To investigate the relationship between dental fluorosis, polymorphisms in the *COL1A2* gene, and serum calciotropic hormone levels. *Methods:* We conducted a case-control study among children between 8 and 12 years of age with (n = 75) and without (n = 165) dental fluorosis in two counties in Henan Province, China. The *Pvu*II and *Rsa*I polymorphisms in the *COL1A2* gene were genotyped using the PCR-RFLP procedure. Calcitonin and osteocalcin levels in the serum were measured using radioimmunassays. *Results:* Children carrying the homozygous genotype PP of *COL1A2 Pvu*II had a significantly increased risk of dental fluorosis (OR = 4.85, 95% CI: 1.22–19.32) compared to children carrying the homozygous genotype pp in an endemic fluorosis village (EFV). However, the risk (OR = 1.07, 95% CI: 0.45–2.52) was not elevated when the control population was recruited from a non-endemic fluorosis village. Additionally, fluoride levels in urine and osteocalcin levels in serum were found to be significantly lower in controls from non-endemic villages compared to cases. However, the differences in fluoride and osteocalcin levels were not observed when cases were compared to a control population from endemic fluorosis villages. *Conclusions:* This study provides the first evidence of an association between polymorphisms in the *COL1A2* gene with dental fluorosis in high fluoride exposed populations. Future studies are needed to confirm the association.

Key words: calcitonin; *COL1A2* gene; dental fluorosis; fluoride; osteocalcin; polymorphisms; type I collagen

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Dental fluorosis is the disturbance of tooth formation caused by excessive ingestion of fluoride during tooth development. Excessive fluoride intake has also been shown to affect dentin and cementum mineralization throughout life, diminishing bone density and adversely impacting bone health (1). High fluoride exposure can result in debilitating bone deformities, an increased risk of fracture, and many other problems resulting from skeletal fluorosis. Previous research has shown that the degree of fluorosis in a population is directly related to the concentration of fluoride in drinking water (2). In China, fluoride levels as high as five times the WHO guideline (1 mg/l) have been measured in many parts of the country (3). This is concerning as an increased risk of fluorosis has been demonstrated even at low doses (about 0.03 mg/kg·bw) (4).

The prevalence of dental and skeletal fluorosis is not entirely clear. Although fluorosis in general is believed to affect millions of people around the world, the very mild or mild forms of dental fluorosis are observed most frequently. It is currently estimated that approximately 43 million people have dental fluorosis, and over 2 million people have skeletal fluorosis in China (5). This issue is, however, not limited to China. In fact, the

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Centers for Disease Control estimate that about 32% of American children have some form of dental fluorosis, with 2–4% of children having the moderate to severe stages (6). Because fluorosis is paradoxically related to an effort to prevent dental caries (by adding fluoride to drinking water), the identification of relatively more susceptible populations may be a means of mitigating some of the burden.

Collagen is a protein that strengthens and supports bones as well as many other tissues including cartilage, tendons, and skin. Two genes, *COL1A1* and *COL1A2* (collagen type I alpha 1 and collagen type 1 alpha 2) provide instructions for making components of collagen. Specifically, the *COL1A1* gene produces a component of type I collagen, called the pro-alpha1(I) chain; whereas the *COL1A2* gene produces the pro-alpha2 (I) chain. The chains combine to make a molecule of type I procollagen. The 'rope-like' procollagen molecules arrange themselves into long, thin fibrils that cross-link to one another in the spaces around cells. The cross-links result in the formation of very strong mature type I collagen fibers. Because of their important role in bone formation and bone architecture (7), polymorphisms in *COL1A1* and *COL1A2* may influence the occurrence of dental fluorosis (8). To date, mutations in the *COL1A2* gene in particular have been linked to a wide spectrum of diseases of bone, cartilage, and blood vessels (9). Caciotropic hormones, such as calcitonin and osteocalcin, are often employed as biomarkers of bone formation status and may also play a role in dental fluorosis.

We subsequently hypothesized that the variation in *COL1A2* genotypes and/or caciotropic hormone would be associated with dental fluorosis status. Although previous research has reported *COL1A2* allele frequencies in different populations (10–13), the differential effect on gene expression is currently unclear. We conducted a case-control study in Henan Province, China to investigate the *COL1A2* (*Pvu*II, rs414408 and *Rsa*I, rs4266) polymorphisms and the levels of calciotropic hormones in relation to dental fluorosis in Chinese children.

Subjects and methods

Location and population

A case-control study was conducted in four villages of two counties (Kaifeng and Tongxu) in Henan Province, China in 2005–2006. Both counties include one endemic fluorosis village (EFV) and one non-endemic fluorosis village (NEFV). EFV was defined as a village with fluoride levels exceeding 2.0 mg/l in drinking water. NEFV was defined as a village with fluoride levels of less than 1.0 mg/l in drinking water. Both EFV and NEFV have the similar levels of calcium in the diet and the similar levels of calcium and magnesium in drinking water. A total of 423 children who were aged between 8 and 12 years and who were born and raised in the four villages were identified. Among them, 126 children who had received drug treatment in the form of bisphosphonates, calcitonin, fluoride, or hormone replacement therapy and/or who had hip fractures were excluded. A total of 240 children participated in this study with participation rate of 81%. All participants were examined for dental fluorosis using the Dean's Method (14). According to specific clinical diagnostic criteria of the development of fluorotic enamel opacities, dental fluorosis was categorized as 0 (normal), 1 (questionable), 2 (very mild), 3 (mild), 4 (moderate), and 5 (severe). Children who were diagnosed as grade 0 or 1 were classified as controls, whereas those who were diagnosed as grade 2, 3, 4, or 5 dental fluorosis were classified as cases. A total of 75 dental fluorosis cases, 69 controls from EFV and 96 controls from NEFV. Each child provided about 6 ml of fasting blood and 10 ml of instant urine samples. All procedures were approved by the Institutional Review Board at Zhengzhou University, China.

Detection of urine fluoride

The fluoride levels in the samples of urine from the two areas were detected by fluoride ion selective electrode (Shanghai Exactitude Instrument Company, China). Participants with a fluoride concentration exceeding 1.5 mg/l in the urine were classified as highly exposed.

Calciotropic hormones measurements

Serum was stored in a −20°C freezer and had no thaws prior to assay for calcitonin (CT) and osteocalcin (OC) levels using commercial radioimmunassays (CT: Center of Science and Technology Explore, General Hospital of PLA, Beijing, China; OC: Chemclin Biotech, Beijing, China). The intra- and inter-assay variation was <10% for these assays. Serum calcium was measured using flame atomic absorption spectrometry (Hitachi Z-5000, Japan) with recoveries in the range of 98.0–110.2%.

Genotyping

Blood (3 ml) was drawn by certified phlebotomists and stored at 4°C until DNA extraction. DNA was

extracted using whole blood genomic DNA miniprep kits (Axygen Biosciences, Union City, USA). DNA was genotyped at the following markers: the *Pvu*II RFLP (in exon 25) and *Rsa*I RFLP (in intron 38) inside the COL1A2 gene. Genotyping procedures for these two markers were modified from previous published protocols (12, 13). For the *Pvu*II RFLP, the forward primer (5-gga aat atc ggc ccc gct gga aaa-3), and the reverse primer (5-gtc cag cca atc caa tgt tgc c-3) were used in polymerase chain reaction (PCR) to produce a 584-base pair (bp) DNA fragment. For the *Rsa*I RFLP, the forward primer (5-ctg ctg gaa gtc gtg gtg at-3), and the reverse primer (5-cac cag gga aac cag tca ta-3) were used in polymerase chain reaction (PCR) to produce a 852-base pair (bp) DNA fragment.

The PCR amplification was conducted in reaction mixtures each containing 35.5 μl of ddH2O, 5 μl of 10 PCR buffer, 1.5 mM of $MgCl_2$, deoxynucleoside triphosphate (dNTP; 200 μM each), 2.5 U of Taq Polymerase (TaKaRa, Japan), 0.4 μM each of the two primers for each marker, and 300 ng of genomic DNA. The PCR was performed on T-Gradient thermocyclers (GeneAmp PCR System; Whatman biometra, Goettingen, Germany). After PCR amplification, 7 μl of the respective PCR products were removed and digested with 10 U of *Pvu*II (MBI, Vilnius, Lithuania) or 10 U of *Rsa*I (MBI, Lithuania) at 37°C for 3 h. Then the samples were electrophoresed in 1.8% Metaphor agarose gels in 1x TAE buffer and 0.3 μg/ml of ethidium bromide. Gels were then visualized on a transilluminator under UV light and photographed. The absence and presence of the *Pvu*II and *Rsa*I restriction sites of the *COL1A2* gene were designated as *P* and *p* alleles, *R* and *r* alleles respectively.

Data analysis

Differences in gender and high exposure fluoride status were analyzed using the chi-square test. Differences in urine and serum measurements among case and control groups were examined using the analysis of variance (ANOVA) method. Odds ratios (ORs) and 95% confidence intervals (CI) were calculated using multivariate logistic regression analysis. Potential confounding variables, such as age and gender were adjusted for in the regression analysis. Adjustment for urine fluoride level did not result in a material change of the observed results. Therefore, the urine fluoride level was not included in the final model. The chi-square test was used to test for departures from the Hardy-Weinberg equilibrium among controls. All of the SNP genotype frequencies in control subjects were consistent with the Hardy-Weinberg equilibrium (*Pvu*II $P = 0.816$, *Rsa*I $P = 0.314$). The pairwise D′ values estimated using HaploView (15) indicated that the two *COL1A2* SNPs are not in strong linkage disequilibrium in this study (D′ = 0.58). All tests of significance were two-sided. A *P*-value of < 0.05 was considered statistically significant. All analyses were performed using the SAS Software, version 8.02 (SAS Institute Inc., 1996).

Results

There were 75 subjects who were diagnosed with dental fluorosis. All of the participants with dental fluorosis lived in EFV (Table 1). While the prevalence rate of high fluoride exposure in dental fluorosis cases was 88%, its prevalence in EFV controls and NEFV controls was found to be 80% and 11%, respectively. The dental fluorosis cases were older than the controls from EFV ($P = 0.01$), but the age distribution was similar between the cases and controls from NEFV. The gender distribution among cases did not significantly differ from that of controls.

We compared levels of urine fluoride, serum calcium, and serum calcitonin and osteocalcin between cases and controls in EFV and NEFV (Table 2). No statistically significant differences in urine fluoride, serum calcium, serum calcitonin

Table 1. Distributions of select variables in dental fluorosis cases and controls

	Cases ($n = 75$)	Controls in EFV ($n = 69$)	Controls in NEFV ($n = 96$)
Age* (years)	10.01 ± 1.33	9.45 ± 1.31	9.74 ± 1.28
P-value		0.01	0.18
Gender (*n*)			
Boys	33	41	52
Girls	42	28	44
P-value		0.06	0.19
High-loaded fluoride status (*n*)			
Yes	66	55	11
No	9	14	85
P-value		0.17	<0.01
Dean's scoring			
0 Normal	0	57	92
1 Questionable	0	12	4
2 Very mild	11	0	0
3 Mild	26	0	0
4 Moderate	24	0	0
5 Severe	14	0	0

*Values are means ± SD.

Table 2. Urine and serum measurements in relation to dental fluorosis

Groups	*n*	Urine fluoride (mg/l)	Serum Ca (μg/l)	Calcitonin (ng/ml)	Osteocalcin (U/l)
Cases	75	2.47 ± 0.80	2.44 ± 0.43	339.24 ± 452.76	6.55 ± 2.65
Controls in EFV	69	2.33 ± 0.75	2.54 ± 0.51	302.85 ± 345.11	6.51 ± 3.15
Controls in NEFV	96	0.83 ± 0.46*	2.44 ± 0.50	394.58 ± 565.79	5.36 ± 2.95*

*$P < 0.05$.

Table 3. Association between dental fluorosis and COL1A2 polymorphisms

		EFV controls		NEFV controls	
Polymorphisms	Cases *N* = 75	*N* = 69	OR (95% CI)	*N* = 96	OR (95% CI)
*Pvu*II					
pp	37	30	1.00	42	1.00
Pp	24	36	0.68 (0.32–1.42)	39	0.77 (0.39–1.53)
PP	14	3	4.85 (1.22–19.32)	15	1.07 (0.45–2.52)
*Rsa*I					
rr	38	32	1.00	44	1.00
Rr	27	32	0.76 (0.37–1.58)	44	0.75 (0.39–1.44)
RR	10	5	2.24 (0.66–7.68)	8	1.49 (0.53–4.19)

Adjusted for age and gender.

and osteocalcin concentrations were found between cases and controls from EFV. The levels of urine fluoride and serum osteocalcin were significantly lower in controls from NEFV compared to cases (*P*-values <0.01 and 0.02 respectively).

The genetic polymorphism in *COL1A2 PvuII* but not *COL1A2 RsaI* was found to be associated with the risk of dental fluorosis (Table 3). Compared to children who carried homozygous p allele of *COL1A2 PvuII*, children who carried homozygous P allele had a significantly increased risk of dental fluorosis compared to controls from EFV (OR = 4.85, 95% CI: 1.22–19.32) but not in comparison to controls from NEFV (OR = 1.07, 95% CI: 0.45–2.52).

Discussion

Endemic fluorosis is a major public health concern in Henan province due to the excessive consumption of fluoride in drinking water. To date, studies of fluorosis mainly focused on two facets: bone deformities induced by the interaction of fluoride, calcium, magnesium and selenium, and nonbone toxicity including the change of some biochemical enzyme and hormones (11). The role of genetic susceptibility in relation to fluorosis, particularly dental fluorosis, has been unclear.

Previous studies have demonstrated an association between the *COL1A2* gene and bone development (16, 17). Type I collagen is an important fibrous protein that provides a major part of the tensile strength of bone and several other tissues. The mutations of *COL1A2* can result in the connective tissue disorder, osteogenesis imperfecta (OI), which is also known as brittle bone disease (18, 19). Studies have linked genetic variation of *COL1A2* gene and bone phenotypes (13, 17, 20). Willing et al. (17) reported a significant association between *COL1A2 Ras*I and intron12 *VNTR* genotypes and bone mineral density (BMD) and bone mineral content (BMC) in young children. Suuriniemi et al. (13) found that *COL1A2 Pvu*II polymorphism was associated with spine BMD/BMC in pre-pubertal girls. Deng et al. (20) for the first time reported a relationship between *COL1A2 Msp*I polymorphism and bone phenotypes in the Chinese population. It is well known there are some analogical constituents between bone and teeth. Furthermore, the pathogenesis of enamel fluorosis is the same as skeletal fluorosis, they both are related to a putative linkage among osteoblast activities, secreted structural matrix proteins and multiple proteases, and fluid composition, including calcium and fluoride ions. As such, we speculate that genetic polymorphisms in *COL1A2* gene, which are related to bone pathogenesis, may influence the dental formation of high-fluoride exposed children.

In the current study, children with homozygous P allele of *COL1A2 Pvu*II had about five times the risk of dental flouorosis compared to children with

homozygous p allele after adjusting for age and gender. The association between the *COL1A2 Pvu*II polymorphism and dental fluorosis was only observed for children who came from EFV. This may indicate that *COL1A2 Pvu*II polymorphism play a role in dental fluorosis in the presence of high levels of fluoride exposure. Although the mechanisms of the underlying relationship between *COL1A2 Pvu*II polymorphisms and dental fluorosis risk are not yet known, several studies have reported that individuals with the homozygous P genotype had higher risk of fracture (13) and lower BMD/BMC (16) compared to those with the homozygous p genotype. Although children with homozygous genotype PP had higher mean of Dean's score compared to children with the homozygous variant genotype pp ($P < 0.05$), the risk did not increase with severity of dental fluorosis. We speculate whether the high risk is driven by severe dental fluorosis. Because the *COL1A2 Pvu*II polymorphism does not cause an amino acid change, it is possible that this polymorphism may be in linkage disequilibrium with a causal mutation in the same gene or in genes nearby.

It has been reported that children with the *COL1A2 Rsa*I *RR* genotype had the lowest whole body BMC compared to those with either *Rr* or *rr* genotypes. Our study, however, did not find a relationship between *COL1A2 Rsa*I polymorphism and dental fluorosis.

Osteocalcin is the most abundant noncollagenous protein in bone produced exclusively by mature osteoblasts and is used as a marker for osteoblast specific activity. Although osteocalcin has been suggested to be involved in dental or skeletal de- and remineralizing processes, its relationship with bone development is currently unclear (21–24). On the other hand, fluoride is incorporated into bone during mineralization (25). Study showed that skeletal fluorosis patients had a greater number of osteoblasts than controls (26). The high serum levels of osteocalcin in children with high fluoride exposure observed in current study may reflect that the high number and/or high activity of osteoblasts in high exposed children.

In conclusion, this study provides first evidence that the *COL1A2 pvu*II polymorphism is associated with the risk of dental fluorosis in a high fluoride exposed population. This suggests that a new biomarker of dental fluorosis may be used to identify high-risk populations in areas with high levels of fluoride in the drinking water. Future studies are, however, needed to confirm this finding in a large population and to investigate the impact of this polymorphism in different study populations and to define ht-SNPs and haplotypes in the *COL1A2* gene.

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