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The Relationship between *Alu* I Polymorphisms in the Calcitonin Receptor Gene and Fluorosis Endemic to Chongqing, China

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Key Words
Endemic fluorosis · Calcitonin receptor gene · *Alu* I polymorphism · Fluoride

Abstract
Objective: This study explored the association between an *Alu* I polymorphism at position 1,377 of the calcitonin receptor *(CTR)* gene and endemic fluorosis. ***Subjects and Methods:*** A case-control study of 321 participants was conducted in regions with high fluorosis rates (Wushan and Fengjie counties) and those without high fluorosis rates (Yubei Qu county; termed nonfluorosis areas) in Chongqing, China. The participants were divided into three groups: the fluorosis group (FG) from areas with high fluoride exposure (121), the nonfluorosis group (NFG) from areas with high fluoride exposure (130), and a control group (CG) from areas with no excessive fluoride exposure (70). An *Alu* I polymorphism in the *CTR* gene was genotyped using polymerase chain reaction-restriction fragment length polymorphism analysis. ***Results:*** The genotype distributions within each group were as follows: CC 60.33% (73/121), CT 30.58% (37/121) and TT 9.09% (11/121) for the FG; CC 74.62% (97/130), CT 21.54% (28/130) and TT 3.85% (5/130) for the NFG, and CC 68.57% (48/70), CT 31.43% (22/70) and TT 0% (0/70) for the CG. Significant differences in *Alu* I genotypes were observed among the groups ($\chi^2 = 12.317$, $\upsilon = 4$, $p = 0.015$). Allele frequencies of *CTR* genotypes differed significantly among the groups ($\chi^2 = 8.859$, $\upsilon = 2$, $p = 0.012$): C 75.62% (183/242) and T 24.38% (59/242) for the FG, C 85.38% (222/260) and T 14.62% (38/260) for the NFG, and C 84.29% (118/140) and T 15.71% (22/140) for the CG. ***Conclusion:*** An association between fluorosis and the *Alu* I polymorphism in the *CTR* gene was observed in fluoride-exposed populations.

Introduction

Fluorosis is a chronic disease caused by regular consumption of an amount of fluoride exceeding the safety threshold, resulting in major health problems in humans. Fluorosis is prevalent in many countries around the world [1], and a significant positive relationship between fluoride intake in drinking water (Chinese national standard of 1.2 mg/l [2]) and the prevalence of fluorosis has been demonstrated [3]. Excessive fluoride intake can cause damage predominantly in teeth and bones, resulting in dental fluorosis and skeletal fluorosis, respectively [4]. In recent years, the incidence of fluorosis in endemic fluorosis areas in China has tended to decline; currently, about 43 million people have dental fluorosis and 3 million have

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skeletal fluorosis in China [4]. Fluorosis is still a major public health problem and an increasing disease burden for many countries. In Chongqing, a typical coal-burning region of China with a high incidence of endemic fluorosis, data from 2002 to 2004 showed that the prevalence of dental fluorosis was 45.86%, with moderate and severe fluorosis observed in individuals living in the remote mountainous areas (e.g. the prevalence of dental fluorosis in Pengshui has been reported to be as high as 80% [5]).

However, no studies have assessed the relationship between calcitonin receptor *(CTR)* gene polymorphisms and fluorosis. Therefore, in this manuscript, we present a case-control study conducted in the Chongqing region to investigate the association between *CTR* gene (*Alu* I) polymorphisms and dental fluorosis.

Materials and Methods

This study was approved by the Medical Research Ethics Committees, Faculty of Medicine, Chongqing Medical University. Informed consent for all subjects was obtained from participants or their parents or legal guardians.

Location and Subjects

The case group consisted of 100 children (8–12 years of age) with a confirmed diagnosis of dental fluorosis and 21 adult patients randomly selected from Wushan county with skeletal fluorosis, confirmed by clinical criteria and standard X-ray diagnosis [6, 7]. The children were selected using a stratified random method: 5 boys and 5 girls were selected randomly from each age group. Dental fluorosis was assessed in all permanent teeth using Dean's fluorosis index. The internal control group consisted of 8- to 12-year-old children without dental fluorosis. The children in the case and internal control groups were matched by age, gender and living conditions (according to region), with 1:1 pairwise matching. The external control group from nonepidemic areas was matched to members of the case group ('patients') by age and gender. One hundred patients (13 with very mild fluorosis and 87 with mild fluorosis, defined by Dean's fluorosis index), 100 internal control subjects and 50 external control subjects comprised the research set. All the children were diagnosed by two experienced dentists and two epidemiologists. Thirty internal control subjects (aged 43.0 ± 14.2 years) were matched with patients by age (±3 years), gender and living conditions, and 20 external control subjects (aged 42.0 ± 15.3 years) were matched with patients by age (±3 years) and gender. All subjects were diagnosed by one orthopedic surgeon, one radiologist and two epidemiologists. The study was conducted in regions with a high incidence of fluorosis (Wushan and Fengjie counties) and regions without a high incidence of fluorosis (Yubei Qu county) in Chongqing, China. In addition to the groups detailed above, the subjects (n = 321) were also divided into three further groups: the fluorosis group (FG) from areas with high fluoride exposure; the nonfluorosis group (NFG) from areas with high fluoride exposure, and the control group (CG) from areas with no excessive fluoride exposure.

Table 1. Number of patients with specific *CTR* genotypes in each group and adherence to the Hardy-Weinberg equilibrium

Group	n	*CTR* genotype			χ^2	p
		CC	CT	TT		
FG	121	73	37	11	3.526	0.060
NFG	130	97	28	5	2.441	0.118
CG	70	48	22	0	2.433	0.119
Total	321	218	87	16	3.376	0.066

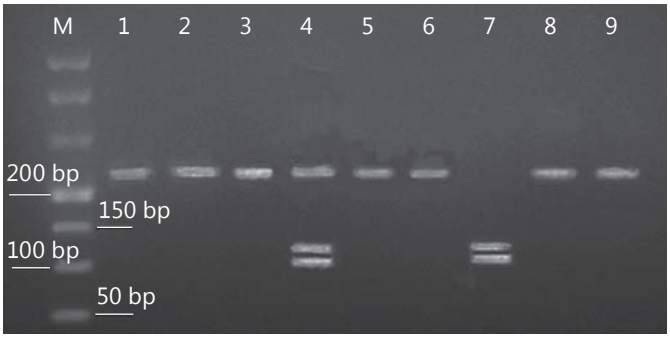

Fig. 1. PCR analysis yielded only a 228-bp band for the CC genotype (wild genotype), two fragments of 120 and 108 bp for the TT genotype (homozygote genotype), and 228, 120 and 108 bp for the CT genotype (heterozygote genotype). CC genotype: 1, 2, 3, 5, 6, 8, 9; TT genotype: 7; CT genotype: 4. M = DL500 marker.

DNA Extraction

Blood samples (5 ml) of all the participants were collected from the brachial vein, immediately transferred into EDTA-coated tubes and stored at –20°C. Genomic DNA was extracted from the blood samples of all participants with a DNA extraction kit (centrifugal column type; BioTeke Corp., Beijing, China).

Polymerase Chain Reaction

Polymerase chain reaction (PCR) analysis of the 1377C/T *CTR* gene polymorphism was carried out in a total volume of 50 μl, containing 25 μl of Premix Taq, 1 μl of each primer (10 μmol/l), 5 μl of genomic DNA, and sterilized ddH_2O to the final volume. Primers for the *CTR* gene polymorphism have been described previously [8], and the length of the amplicon was 228 bp. Mixing, instantaneous centrifugation and PCR amplification were performed using a programmable thermal cycler. The cycling conditions were as follows: 94°C for 5 min, 35 cycles at 94°C for 1 min, 57°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min. The PCR products were subjected to gel electrophoresis (110 V, 72 mA, 40 min) to check for the presence of a 228-bp band. Products were purified using the ethanol/sodium acetate method and digested overnight with *Alu* I at 37°C, according to a previously published protocol [8]. Reaction mixtures had a final volume of 20 μl, containing 2 μl of 10× NEB buffer, 10 μl of amplification product, 0.5 μl of the restriction enzyme *Alu* I, and 7.5 μl of sterilized ddH_2O.

Table 2. Distribution of *CTR* genotypes and allelic frequencies in the different groups (α = 0.05)

	FG (n = 121)	NFG (n = 130)	CG (n = 70)	Total (n = 321)	OR [95% CI]	χ^2	p
Genotype							
CC	73 (60.33)	97 (74.62)	48 (68.57)	218 (67.91)	0.517 [0.302 – 0.885][a], 0.679 [0.374 – 1.299][b]	5.851[a], 1.297[b]	0.021[a], 0.278[b]
CT	37 (30.58)	28 (21.54)	22 (31.43)	87 (27.10)	1.605 [0.908 – 2.836][a], 0.961 [0.509 – 1.815][b]	2.669[a], 0.015[b]	0.114[a], 1.000[b]
TT	11 (9.09)	5 (3.85)	0	16 (4.89)	2.5 [0.842 – 7.419][a], –	2.888[a], 6.573[b]	0.121[a], 0.008[b]
Allele							
C	183 (75.62)	222 (85.38)	118 (84.29)	523 (81.46)	0.531 [0.338 – 0.843][a], 0.578 [0.336 – 0.994][b]	7.666[a], 3.986[b]	0.007[a], 0.052[b]
T	59 (24.38)	38 (14.62)	22 (15.71)	119 (18.54)	1.884 [1.198 – 2.960][a], 1.729 [1.006 – 2.972][b]		

Data are presented as n with percentage in parentheses. [a] FG versus NFG. [b] FG versus CG.

Table 3. Distribution of *CTR* genotypes and allelic frequencies in the different groups (α = 0.01)

	FG (n = 121)	NFG (n = 130)	CG (n = 70)	Total (n = 321)	OR [95% CI]	χ^2	p
Genotype							
CC	73 (60.33)	97 (74.62)	48 (68.57)	218 (67.91)	0.517 [0.302 – 0.885][a], 0.679 [0.374 – 1.299][b]	5.851[a], 1.297[b]	0.016[a], 0.255[b]
CT	37 (30.58)	28 (21.54)	22 (31.43)	87 (27.10)	1.605 [0.908 – 2.836][a], 0.961 [0.509 – 1.815][b]	2.669[a], 0.015[b]	0.102[a], 0.902[b]
TT	11 (9.09)	5 (3.85)	0	16 (4.89)	2.5 [0.842 – 7.419][a], –	2.888[a], 6.753[b]	0.089[a], 0.009[b]
Allele							
C	183 (75.62)	222 (85.38)	118 (84.29)	523 (81.46)	0.531 [0.338 – 0.843][a], 0.578 [0.336 – 0.994][b]	7.666[a], 3.986[b]	0.006[a], 0.046[b]
T	59 (24.38)	38 (14.62)	22 (15.71)	119 (18.54)	1.884 [1.198 – 2.960][a], 1.729 [1.006 – 2.972][b]		

Data are presented as n with percentage in parentheses. [a] FG versus NFG. [b] FG versus CG.

Data Analysis

All statistical analyses were performed using SPSS version 17.0. The gene-counting method was used to estimate allele frequencies. Deviation in genotype distribution from the expected Hardy-Weinberg equilibrium was estimated by the χ^2 test (α = 0.05). The significance of differences in allele frequencies between groups was also estimated by the χ^2 independence test (α = 0.01).

Results

The PCR analysis yielded only a 228-bp band for the CC genotype, two fragments of 120 and 108 bp for the TT genotype, and 228, 120 and 108 bp for the CT genotype (fig. 1). The distribution of genotypes for the *Alu* I restriction fragment length polymorphism (RFLP) did not deviate from the Hardy-Weinberg equilibrium (table 1; p > 0.05). The genotype and allele frequencies of the *Alu* I RFLP in the *CTR* gene are shown in table 2 (α = 0.05). The observed genotype frequencies of CC, CT and TT were 67.91, 27.1 and 4.98%, respectively, and the frequencies of the C and T alleles were 81 and 19%, respectively. There were statistically significant differences among the three groups with respect to both genotype distribution (χ^2 = 12.317, p = 0.015) and allele frequency (χ^2 = 8.859, p = 0.012). Moreover, statistically significant differences were observed between the case and control groups with respect to genotype distribution (FG vs. NFG: χ^2 = 6.57, p = 0.037; FG vs. CG: χ^2 = 6.849, p = 0.033; NFG vs. CG: χ^2 = 4.702, p = 0.095) and allele frequency (FG vs. NFG: χ^2 = 7.666, p = 0.006, OR = 0.531, 95% CI 0.338–0.834; FG vs. CG: χ^2 = 3.986, p = 0.046, OR = 0.578, 95% CI 0.336–0.994; NFG vs. CG: χ^2 = 0.086, p = 0.769, OR = 1.089, 95% CI 0.616–1.927).

The genotype and allele frequencies of the *Alu* I RFLP in the *CTR* gene are shown in table 3 (α = 0.01). The results are similar to data obtained for α = 0.05. The two groups (NFG and CG) exhibited the same allelic distribution (χ^2 = 0.086, p = 0.769, OR = 1.089, 95% CI 0.616–1.927). Statistically significant differences were observed between the FG and NFG or CG groups with respect to allele frequency distribution (χ^2 = 8.786, p = 0.003, OR = 0.547, 95% CI 0.366–0.818).

Discussion

The distribution sequence of the mixed *CTR* genotype – CC followed by CT and TT – of this study in Chinese subjects is consistent with the *CTR* genotype frequency distribution in Asians [9, 13], but different from that in Caucasians [9, 10]. Additionally, the allele frequencies, i.e. the presence of the C allele in 81.46% and the T allele in 18.54% of individuals, are similar to those reported for Guangzhou [11], Shanghai [12] and Beijing [13], but are clearly different from the frequencies observed in Italian populations (for which the T allele was the most frequent in 49.7%) [14, 15]. Thus, the present data support the observation that the distribution of *CTR* genotypes differs between Chinese and Caucasians.

Considering the distribution of genotypes in the FG, NFG and CG groups, the TT genotype was more frequent in the FG than in the NFG group (9.09 vs. 3.85%, respectively), probably because fluorosis may be influenced by the TT polymorphism of the *CTR* genotype. Furthermore, the *CTR* allele frequency distribution of the case and control groups was different. A possible explanation could be that carrying the C allele reduced the risk of fluorosis and, hence, this allele may have a protective effect against fluorosis. Thus, our data indicated that the T allele of the *Alu* I gene polymorphism is associated with an increased risk of fluorosis. Gene-gene or gene-environment interactions could lead to the varying genetic effects observed in different populations. Our results further confirmed that there were significant differences in the distribution of alleles among fluorosis patients and control subjects.

Conclusion

Our data supported the hypothesis that the *Alu* I polymorphism of the *CTR* gene may be one of the genetic components associated with fluorosis. Due to the modest sample size, it is essential to replicate these findings in different populations with larger sample sizes, and investigation of other related genes will be needed to clarify the relationship between this polymorphism and fluorosis.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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