Original Article

Dietary carotenoid intake and dental fluorosis in relation to SOD2 (rs 11968525) polymorphisms in Guizhou, China

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Background and Objectives: Genetic and dietary factors are important contributors to the development of dental fluorosis (DF). This study investigated the association between DF and dietary carotenoids, and explored whether the association was modified by polymorphisms of the antioxidant enzyme superoxide dismutase 2 (SOD2 rs11968525) in Guizhou, China. **Methods and Study Design:** A cross-sectional study with a total of 899 adults aged 18-75 years were enrolled in the study. Face-to-face interviews were conducted to assess dietary habits using a validated 75 item food frequency questionnaire (FFQ). Sociodemographic and lifestyle information, and blood and urine samples were also collected. Genotypes were evaluated using TaqMan single nucleotide polymorphism (SNP) Genotyping Assay. **Results:** There were significant dose-dependent inverse associations of the prevalence of DF with intake of α-carotene, β-carotene, lutein/zeaxanthin, lycopene and total carotenoids (*p*-trend ranged from <0.001–0.004). The odds ratios (ORs) and 95% confidence intervals (CIs) of DF comparing the highest against lowest quartile were 0.56 (0.35, 0.92) for α-carotene, 0.53 (0.35, 0.81) for β-carotene, 0.44 (0.27, 0.74) for lycopene, 0.35 (0.21, 0.58) for lutein/zeaxanthin in combination and 0.42 (0.25, 0.69) for total carotenoids (all *p*-trend<0.005). Intake of β-cryptoxanthin was not found to be related to DF. The inverse association of DF with dietary intake of α-carotene and β-carotene was more evident in individuals with the AG+AA genotype (*p*-interaction<0.05). **Conclusions:** Higher dietary carotenoids were associated with a lower occurrence of DF, polymorphisms in SOD2 (rs 11968525) modified the associations between dietary intake of carotene and DF. These findings provide evidence for precision prevention of fluorosis.

Key Words: dietary carotenoids, dental fluorosis, superoxide dismutase, cross-sectional study, gene-environment interaction

INTRODUCTION

Dental fluorosis (DF) is caused by excessive fluoride intake, which leads to structural changes in the development of tooth enamel. It is known to occur in several parts of the world, including China, India, Africa and South America and over 70 million people may be affected. It has been estimated that over 26 million people in China suffer from DF due to elevated concentration of fluoride in drinking-water, and 16.5 million cases of DF are estimated to result from exposure to coal burning pollution.[1] It was reported that the most severe coal burning endemic fluorosis cases in China were located in Guizhou province. Based on the latest statistics, there were 8.79 million cases of DF in Guizhou Province by the end of 2018.[2] In addition to endemic fluorosis, there are also other health problems associated with domestic coal burning in Guizhou Province, such as arsenosis (Figure 1). Local governments have taken many effective measures to control endemic fluorosis, such as the use of clean fuels and ventilated stoves. The prevalence of endemic fluorosis has decreased significantly. However, the prevalence of DF in some regions is still high, such as in Zhijin and Qianxi counties.[3] Therefore, it is necessary to further explore other preventive solutions.

Oxidative stress is considered to play a critical role in the pathogenesis of DF.[4] Excessive fluoride induces oxidative stress, which leads to the impairment of ameloblast cells, responsible for dental enamel formation.[5] The use of antioxidant-rich food ingredients as an antidote to fluoride toxicity has been previously suggested.[6] Given their

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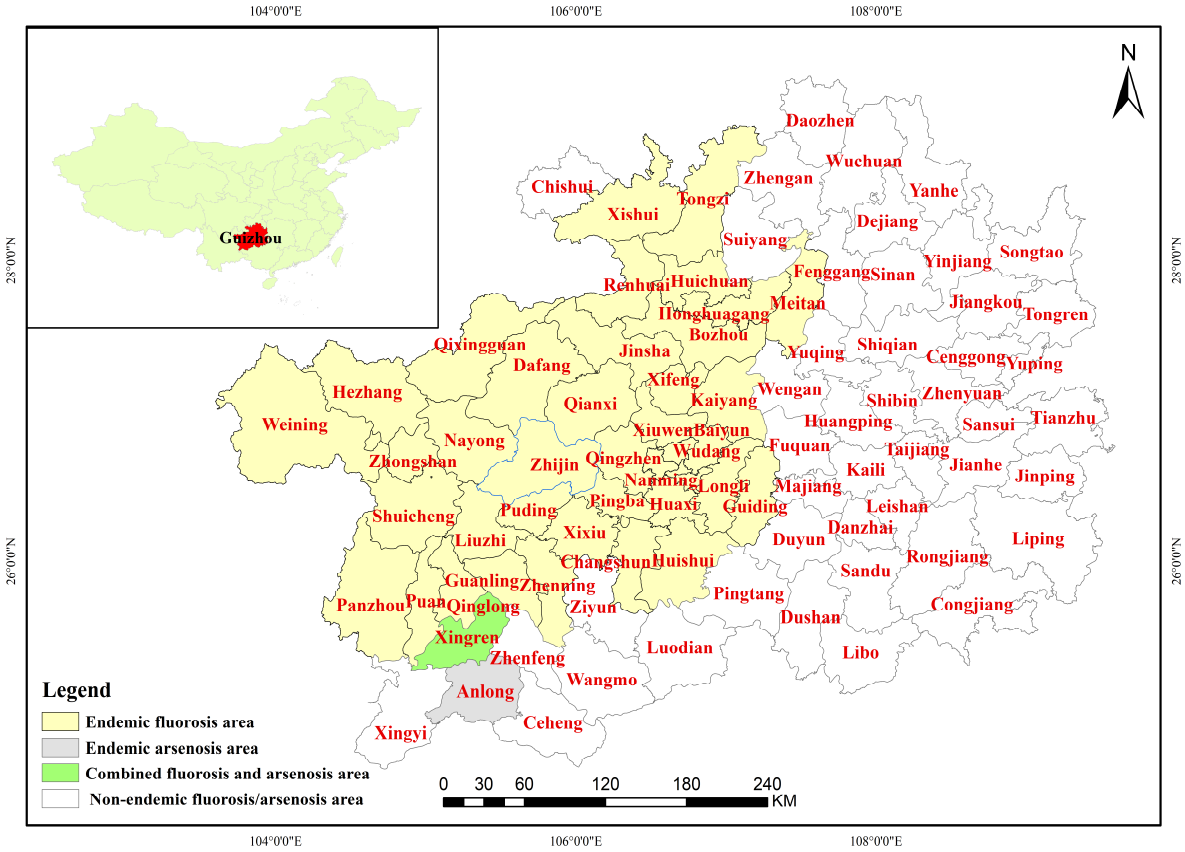

Figure 1. Distribution areas of coal-burning endemic fluorosis and arsenosis in Guizhou Province.

antioxidant and anti-inflammatory properties, dietary carotenoids have been examined as possible mediators of DF.[7,8] Several animal and in vitro studies have demonstrated that carotenoids could minimise the toxic effects of fluoride and provide benefits to bone metabolism, by preventing oxidative stress.[8-10] Furthermore, a case control study reported dietary carotenoids might play a protective role against skeletal fluorosis.[11] These studies indicate that dietary carotenoids may have favourable effects on DF. However, to our knowledge, no epidemiological studies have reported the association between dietary carotenoids and DF.

Genetic variation in the enzyme superoxide dismutase 2 (SOD2) changes enzyme function and may have a significant effect on susceptibility to oxidative stress related diseases.[12,13] Previous studies reported that coal burning fluorosis was associated with decreased gene expression and activity of SOD and SOD2 (rs11968525) polymorphisms may impact susceptibility to skeletal fluorosis.[4,11] Genetic and dietary factors are important contributors to the development of DF. However, no studies have examined the interaction between dietary carotenoids and SOD2 polymorphisms on DF.

Therefore, the present study investigated the association of DF with dietary intake of carotenoids, and explored the interaction between dietary intake of carotenoids and SOD2 (rs11968525) polymorphisms in a cross-sectional study conducted in Guizhou province, China

METHODS

Study participants

This population-based cross-sectional study was performed in Zhijin County, Guizhou province, which is one of the most severe fluorosis areas, but not known for arsenosis (Figure 1). In July and August of 2015, 1101 participants between 18–75 years old were recruited through village doctors and the Centre for Disease Control and Prevention. We excluded 109 participants who had a previously confirmed diagnosis of coronary heart disease, stroke, cancer, gout or kidney disease. We also excluded 93 participants with missing or incomplete dietary data. Thus, the analyses finally involved 899 participants. However, we obtained 723 urine samples and 755 blood samples from participants, which was partially due to data missing and subjects' failure to follow the rules. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1995 Helsinki declaration and its later amendments or comparable ethical standards. All study participants signed informed consent forms prior to the interview and the Medical Ethics Committee of Zunyi Medical University approved the project (No. 2014-1-003).

Data collection

A structured questionnaire was used to investigate participants' dietary behaviours and confounding factors by trained interviewers. All eligible participants provided detailed information on sociodemographic characteristics (e.g., age, gender, marital status and household income), lifestyle habits (e.g., smoking, tea drinking, alcohol intake), personal medical history of selected diseases (e.g., hypertension, renal diseases, diabetes, stroke) and use of an improved stove (improving the kitchen stove to ex-

clude the fluoride out of the room to decrease the pollution of the air indoor), fuel type of cooking, and roasted food. Participants, who smoked at least five packs of cigarettes a year, were defined as smokers. Those, who drank alcohol at least once a week continuously for at least 6 months, were alcohol drinkers. Tea drinkers were defined as participants who drank tea at least twice a week. All eligible participants were required to respond to the same questionnaire.

Dietary assessment

Dietary consumption was evaluated by FFQ during a face-to-face interview. All participants were required to report their consumption frequency (never, per year, per month, per week, or per day) and one quantitative response (average amounts) as applicable. Food photographs were provided as visual aids to assess portion sizes. Each item was converted to the daily intake of grams per day, and the average daily intake of total energy and individual nutrient content were computed based on the Chinese Food Composition Database.[14] Intake of carotenoids, including α-carotene, β-carotene, lycopene, β-cryptoxanthin and lutein/zeaxanthin, was estimated according to the US Department of Agriculture database.[15] The validity and reproducibility of the FFQ have been assessed previously.[16,17]

Laboratory assay

A 10-mL urine sample was collected from each participant. Urinary fluoride concentration was analysed by a standardized method (WS/T 89-2015, China). Further descriptions of the detection have previously been presented.[11,18] Briefly, we mixed 1 mL of urine with 24 mL of deionized water, one tablet of fluoride adjustment buffer was added to each 25-mL mixture. The concentration of urine fluoride in the mixture was measured by a HQ40d Portable Meter and the concentration of fluorine in the urine of each patient was calculated, adjusting to each corresponding proportion. The coefficient of variation for the urine fluoride concentrations was 2.29%. The urine fluoride concentrations for all participants were categorized into normal or abnormal concentrations of less than or greater than 1.6 mg/L, respectively, according to national criteria (WS/T 256-2005).[19]

Real-time polymerase chain reaction genotyping

Peripheral venous 5mL blood samples were collected from each participant and were stored with EDTA anticoagulant at – 70℃ until further analysis. Genomic DNA was extracted using a whole blood DNA extraction kit (Transgen, Beijing, China) and stored at –80℃ for genotyping. The Polymerase chain reaction (PCR) TaqMan probe and primers were designed and synthesized by ABI PE Applied Biosystems. DNA samples were genotyped using TaqMan single nucleotide polymorphism (SNP) Genotyping Assay on a Lightcycler® 480 platform (Roche, Applied Biosystems). The reaction mixture used consisted of 1 μL template DNA, 0.25 μL of Universal PCR Master Mix and 0.25 μL of probe primer mix. PCR amplification conditions were an initial heating at 95 °C for 4 min, followed by 40 cycles of 95 °C for 7 s and 60 °C for 40s. We repeated the genotyping for 10% of samples at random.

Diagnosis of dental fluorosis

The participants were seated in a chair, before certified doctors examined them using a periodontal probe and mirror during the day in natural light according to the Chinese Diagnostic Criteria of Dental Fluorosis (WS/T208-2011, China). DF was categorized as 0 (normal), 1 (questionable), 2 (very mild), 3 (mild), 4 (moderate) and 5 (severe).[20] Subjects who scored 0-1 were placed in the non-DF group, and scores of 2-5 were placed in the DF group.

Statistical analysis

The data was tabulated as mean and SDs for continuous variables, or proportions for categorical variables. To achieve an approximately normal distribution for statistical analysis, a logarithmic transformation and a square root transformation were applied to energy intake and dietary nutrient intake, respectively. Dietary nutrient intake was adjusted for total energy intake using the regression residual method.[21] The differences between groups were compared using t-tests for continuous variables and chi squared (χ^2) tests for categorical variables. Subjects were categorized into quartiles (Q1–Q4) by dietary carotenoid intake. Unconditional logistic regression models were used to calculate ORs and 95% CIs for the association of dietary carotenoids and occurrence of DF, using the lowest quartile as the reference group. Multivariate logistic regression analysis was used to adjust for sociodemographic characteristics, including gender, age, marital status, household income, smoking status, tea intake, alcohol intake and energy intake (Model 1). Subsequent models were further adjusted for roasted chili consumption, use of an improved stove, fuel type, dietary calcium intake and roasted corn intake (Model 2). Tests were performed by entering the ordinal values of the quartile of dietary carotenoid as continuous variables in the models.

We further conducted stratified analyses by gender, smoking status, abnormal concentration of urinary fluoride (yes/no) and genotype frequencies of SOD2 (rs 11968525) to assess whether these factors modified the associations of dietary carotenoids and DF. Multiplicative interactions were estimated by adding the interaction terms according to their likelihood. All p values were two-sided and statistical significance was defined as $p<0.05$. SPSS 17.0 software (SPSS Inc., Chicago, IL) was used for data analysis.

RESULTS

The participants' characteristics are presented in Table 1. This cross-sectional study involved 899 participants: 599 had DF and 300 did not. The prevalence of DF was estimated to be 66.6% in this study. Overall, the DF patients had a lower household income, proportion of improved stoves use, while they had higher concentration of urinary fluoride, proportion of female, had habits of burning of coal mixed with loess, and had higher rates of divorce. Furthermore, compared with the subjects without DF, participants with DF had a lower intake of α-carotene, β-carotene, lutein/zeaxanthin, lycopene and total carote-

Table 1. characteristics of the study population

Variables	DF		Non-DF		*p*-value
	% or Mean±SD	n	Mean±SD	n	
Age (years)	50.3±12.3	599	47.8±14.4	300	0.011
Total energy (1000 kcal/day)	2.74±0.98	599	2.70±0.98	300	0.542
Calcium intake (mg/day)	517±287	599	555±321	300	0.068
Roasted chili consumption (g/day)	13.3±17.5	599	12.7±17.3	300	0.621
Urinary fluoride (mg/L)	1.71±1.00	497	1.37±1.10	226	<0.001
α-carotene (mg/d)	0.47±0.97	599	0.68±1.34	300	0.017
β-carotene (mg/d)	3.16±2.45	599	4.24±3.83	300	<0.001
Lutein/zeaxanthin (mg/d)	2.79±2.46	599	4.14±4.58	300	<0.001
β-cryptoxanthin (mg/d)	0.43±0.48	599	0.41±0.50	300	0.531
Lycopene (mg/d)	0.72±0.92	599	0.97±1.33	300	0.004
Total carotenoids† (mg/d)	7.58±5.84	599	10.4±9.72	300	<0.001
male	48.2	599	72.3	300	<0.001
Marital status		599		300	0.033
married or cohabitation	84.3		83.7		
divorce	11.7		8.70		
unmarried	4.00		7.70		
SOD2 (rs 11968525)		509		264	0.201
GG	61.9		66.7		
AG+AA	38.1		33.3		
Household income (Yuan/month/ person)		599		300	0.001
<500	10.0		3.70		
500～2000	40.6		40.0		
2000～6000	39.6		40.3		
≥6000	9.80		16.0		
Fuel type		599		300	0.002
raw coal	59.9		59.0		
mixed coal	23.0		18.3		
firewood	2.00		6.70		
other	15.0		16.0		
Smoking	49.2	599	36.0	300	0.047
Alcohol drinking	31.4	599	30.0	300	0.659
Tea drinking	38.6	599	36.7	300	0.581
Roasted corn	60.6	599	48.7	300	0.001
Use of an improved stove‡	69.8	599	86.3	300	<0.001

DF: dental fluorosis.
†Total carotenoids indicate the sum of α-carotene, β-carotene, β-cryptoxanthin, lycopene and lutein/zeaxanthin.
‡Improving the kitchen stove to exclude the fluoride out of the room to decrease the pollution of the air indoor.

noids, except for β-cryptoxanthin (all *p*-values ranged from <0.001–0.017). Carrots, gourds, tomatoes and spinach were the main sources of α-carotene, β-cryptoxanthin, lycopene and lutein/zeaxanthin, respectively. β-carotene and total carotenoids were predominantly consumed from lettuce and Chinese cabbage, respectively (Figure 2). There were no significant differences in genotype frequencies of rs 11968525 between DF and non-DF patients.

Univariate logistic regression analyses showed that lutein/zeaxanthin, α-carotene, β-carotene, lycopene and total carotenoids were inversely associated with DF (all *p*-trend ranged from <0.001–0.004), except for β-cryptoxanthin (*p*-trend=0.086). Similar associations were observed after adjusting for age, gender, marital status, household income, smoking, alcohol intake, tea intake and total energy intake, with all *p*-trend ranging from <0.001–0.006, except for β-cryptoxanthin. Similar associations were still observed after further adjustment for fluorosis-related confounding factors, such as dietary calcium intake, roasted chili consumption, fuel type, roasted corn intake and use of an improved stove. The ORs (95% CIs) of DF when comparing the highest versus lowest quartile were 0.56 (95% CI: 0.35–0.92) for α-carotene, 0.53 (95% CI: 0.35–0.81) for β-carotene, 0.35 (95% CI: 0.21–0.58) for lutein and zeaxanthin in combination, 0.44 (95% CI: 0.27–0.74) for lycopene, and 0.42 (95% CI: 0.25–0.69) for total carotenoids (all *p*-trend<0.005), except β-cryptoxanthin (Table 2).

The results of the above calculations showed potential risk factors modified the association between carotenoids and DF. Stratified analyses showed that the favourable association between α-carotene and DF remained significant in subjects with the SOD2 (rs 11968525) AG+AA genotype (*p*-trend<0.005), however this association was not significant in GG genotype carriers (*p*-trend=0.729). The inverse association between dietary β-carotene and DF risk was more evident in AG+AA genotype carriers. There were interactions between dietary intake of carotene and rs 11968525 polymorphisms (both *p* for interactions<0.05). There were no statistically significant interactions observed between the rs 11968525 polymorphisms and other dietary carotenoids, such as β-cryptoxanthin, lycopene, and lutein/zeaxanthin (Table 3). There were no interactions between the presence of DF and the factors of smoking, urinary fluoride concentration,

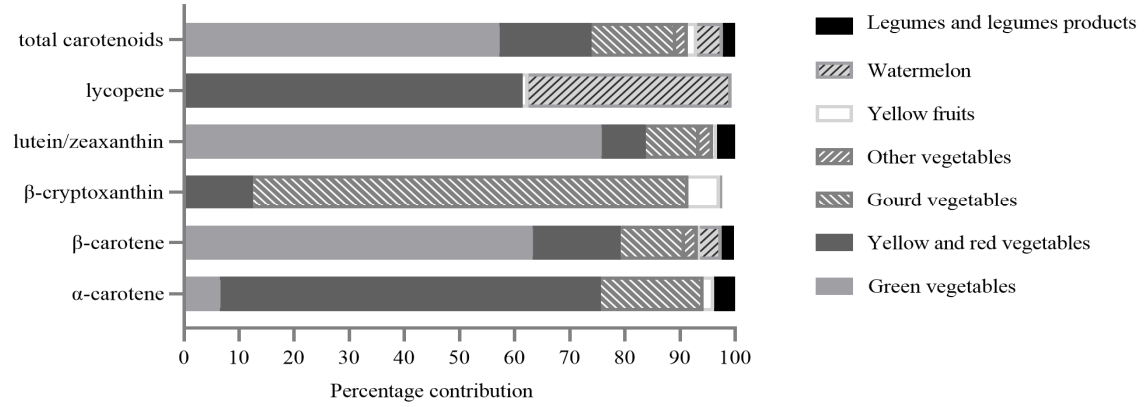

Figure 2. Foods contributing to a greater than 2 % of intake for carotenoids in the participants.

Table 2. ORs (95% CIs) of DF for quartiles of dietary carotenoids intake

	Quartiles of dietary energy-adjusted carotenoids intake				*p*-trend
	Quartile 1[†]	Quartile 2	Quartile 3	Quartile 4	
α-carotene					
Median (mg/d)	0.070	0.183	0.343	0.849	
DF, n (%)	157 (26.2)	157 (26.2)	158 (26.4)	127 (21.2)	
Crude ORs	1	0.98 (0.67, 1.48)	1.00 (0.67, 1.51)	0.55 (0.38, 0.82)**	0.004
Model 1[‡]	1	1.03 (0.69, 1.56)	1.07 (0.71, 1.62)	0.55 (0.37, 0.83)**	0.006
Model 2[§]	1	1.08 (0.67, 1.76)	1.30 (0.79, 2.13)	0.56 (0.35, 0.92)**	0.002
β-carotene					
Median (mg/d)	1.084	2.127	3.416	6.092	
DF, n (%)	158 (26.4)	170 (28.4)	139 (23.2)	132 (22.0)	
Crude ORs	1	1.29 (0.85, 1.97)	0.68 (0.46, 1.00)	0.59 (0.40, 0.88)**	<0.001
Model 1[‡]	1	1.31 (0.86, 2.01)	0.65 (0.44, 0.97)*	0.59 (0.39, 0.89)**	<0.001
Model 2[§]	1	1.67 (0.98, 2.82)	0.55 (0.34, 0.89)*	0.53 (0.35, 0.81)**	0.001
Lutein/zeaxanthin					
Median (mg/d)	0.922	1.742	2.853	6.051	
DF, n (%)	173 (28.9)	156 (26.0)	143 (23.9)	127 (21.2)	
Crude ORs	1	0.67 (0.44, 1.02)	0.52 (0.34, 0.78)**	0.38 (0.25, 0.58)**	<0.001
Model 1[‡]	1	0.67 (0.44, 1.03)	0.49 (0.33, 0.77)**	0.37 (0.25, 0.57)**	<0.001
Model 2[§]	1	0.68 (0.41, 1.12)	0.45 (0.27, 0.75)**	0.35 (0.21, 0.58)**	<0.001
β-cryptoxanthin					
Median (mg/d)	0.074	0.172	0.391	0.895	
DF, n (%)	158 (26.4)	170 (28.4)	139 (23.2)	132 (22.0)	
Crude ORs	1	0.84 (0.57, 1.23)	0.93 (0.63, 1.37)	1.38 (0.92, 2.07)	0.086
Model 1[‡]	1	0.83 (0.56, 1.23)	0.98 (0.66, 1.46)	1.51 (0.98, 2.29)	0.055
Model 2[§]	1	0.99 (0.61, 1.60)	0.95 (0.59, 1.53)	1.39 (0.90, 2.15)	0.089
Lycopene					
Median (mg/d)	0.076	0.290	0.643	1.835	
DF, n (%)	172 (28.7)	154 (25.7)	143 (23.9)	130 (21.7)	
Crude ORs	1	0.65 (0.43, 0.99)*	0.53 (0.34, 0.79)**	0.41 (0.27, 0.62)**	<0.001
Model 1[‡]	1	0.66 (0.43, 1.01)	0.52 (0.34, 0.79)**	0.41 (0.27, 0.62)**	<0.001
Model 2[§]	1	0.84 (0.49, 1.42)	0.52 (0.31, 0.87)*	0.44 (0.27, 0.74)**	<0.001
Total carotenoids[¶]					
Median (mg/d)	2.640	4.962	8.321	15.030	
DF, n (%)	165 (27.5)	165 (27.5)	140 (23.4)	129 (21.5)	
Crude ORs	1	0.98 (0.65, 1.49)	0.59 (0.39, 0.88)*	0.48 (0.32, 0.72)**	<0.001
Model 1[‡]	1	1.02 (0.67, 1.56)	0.56 (0.37, 0.85)**	0.49 (0.32, 0.72)**	<0.001
Model 2[§]	1	0.95 (0.57, 1.60)	0.48 (0.29, 0.79)**	0.42 (0.25, 0.69)**	<0.001

DF: dental fluorosis; OR: odds ratio; CI: confidence interval.
Categorical variables described by numbers and percentages, and unconditional logistic regression analyses to ORs and 95% Cis.
[†]Quartile 1 was the reference quartile.
[‡]Model 1 adjusted for age, gender, marital status, household income, smoking, alcohol drinking, tea drinking, total energy.
[§]Model 2 further adjusted calcium intake, roasted chili consumption, fuel type, roasted corn, use of an improved stove.
[¶]See Table 1
*p<0.05, **p<0.01.

gender, and dietary carotenoid intake (p for interactions=0.156–0.959), with data shown in supplementary tables 1 - 3.

DISCUSSION

This population-based cross-sectional study provides evidence that higher dietary intake of α-carotene, β-carotene, lycopene, zeaxanthin/lutein, and total carotenoids had a

Table 3. ORs (95% CIs) of the DF for dietary carotenoids intake by subgroups of genotype

	Quartiles of dietary energy-adjusted carotenoids intake				$p^{\ddagger}$	$p^{\S}$
	Quartile 1†	Quartile 2	Quartile 3	Quartile 4		
α-carotene						0.023
GG	1	1.23 (0.69, 2.18)	1.33 (0.74, 2.41)	0.87 (0.47, 1.61)	0.729	
AG+AA	1	0.73 (0.29, 1.79)	0.88 (0.36, 2.18)	0.27 (0.11, 0.64)**	0.004	
β-carotene						0.024
GG	1	1.21 (0.66, 2.22)	0.81 (0.46, 1.43)	0.55 (0.31, 0.98)*	0.024	
AG+AA	1	1.37 (0.49, 3.75)	0.29 (0.12, 0.70)*	0.27 (0.11, 0.65)**	<0.001	
lutein/zeaxanthin						0.123
GG	1	0.69 (0.38, 1.25)	0.53 (0.29, 0.96)*	0.41 (0.23, 0.76)**	0.003	
AG+AA	1	0.34 (0.13, 0.87)*	0.43 (0.16, 1.16)	0.17 (0.07, 0.42)**	<0.001	
β-cryptoxanthin						0.781
GG	1	0.99 (0.56, 1.75)	0.84 (0.48, 1.49)	1.23 (0.67, 2.24)	0.669	
AG+AA	1	0.62 (0.27, 1.42)	0.99 (0.43, 2.27)	1.33 (0.56, 3.15)	0.291	
Lycopene						0.408
GG	1	0.89 (0.48, 1.66)	0.45 (0.24, 0.83)*	0.41 (0.22, 0.76)*	0.001	
AG+AA	1	0.56 (0.22, 1.41)	0.52 (0.21, 1.30)	0.25 (0.11, 0.61)**	0.002	
Total carotenoids†						0.078
GG	1	0.87 (0.48, 1.57)	0.59 (0.33, 1.05)	0.47 (0.26, 0.86)*	0.006	
AG+AA	1	1.05 (0.38, 2.89)	0.28 (0.11, 0.67)**	0.27 (0.11, 0.67)**	<0.001	

DF: dental fluorosis; OR: odds ratio; CI: confidence interval.
Unconditional logistic regression analyses to ORs and 95% CIs. Covariates adjusted for age, gender, marital status, household income, smoking, alcohol drinking, tea drinking, total energy, calcium intake, roasted chili consumption, fuel type, roasted corn, use of an improved stove.
†Quartile 1 was the reference quartile.
‡p value for linear trend.
§p value for interaction.
¶See Table 1.
*p<0.05, **p<0.01.

significant protective effect against DF induced by the burning of coal. Additionally, SOD2 genotypes modified the effects carotenes on DF.

Fluoride contamination in drinking water was not the major cause for DF in Guizhou Province. The real culprit was the unhealthy behaviours of the local residents, who usually roasted their foodstuffs using local coal or briquettes (a mixture of coal and clay) with high fluorine, resulting in the elevated fluorine in roasted foodstuffs, especially roasted pepper and corn.[22] The present study also found that the use of coal/clay briquettes and the intake of roasted corn were higher in the DF group.

Fluoride has a strong affinity for hard body tissues and chronic exposure to high fluoride leads to DF.[23] Oxidative damage is a major mechanism of action for fluoride.[4,7] Many studies have shown that carotenoids have beneficial properties, such as protection against free radicals.[24,25] Previous animal studies suggested that individual carotenoids could protect against fluorosis. Tian et al[26] observed that lycopene reduced urinary fluoride in rats exposed to burning coal. Li et al[8] also found that the lycopene treatment group had significantly less fluoride accumulation in teeth and a lower apoptosis rate of ameloblast cells compared with a rat group treated with NaF. Additionally, lycopene ameliorated the adverse effects of fluoride and improved liver, kidney and reproductive function as well as neurobehavior in rats.[27] Sharma et al[28] revealed the hemoprotective role of dietary lycopene in fluoride-treated mice. Moreover, Yasar et al[29] determined the concentrations and changes in vitamin A, vitamin C and vitamin E as well as calcium in 30 Morkaraman sheep with fluorosis and compared them against controls. They found that the sheep exposed to fluoride had lower blood concentrations of β-carotene. The beneficial effects of β-carotene on fluorosis has also been observed *in vitro*.[30] In addition, epidemiological studies provide evidence that dietary carotenoid intake improved bone health, demonstrating that carotenoids were associated with bone density and reduced fracture risk.[31,32] So beneficial effects of carotenoids on bone health may minimise the toxic effects of fluoride. Our previous case control study demonstrated that dietary intake of β-carotene, lycopene, lutein/zeaxanthin and total carotenoids were inversely associated with the risk of skeletal fluorosis.[11] The present cross-sectional study also observed higher dietary intakes of α-carotene, β-carotene, lycopene, zeaxanthin/lutein, and total carotenoids were lower prevalence of DF. Therefore, combined with previous *in vivo* and *in vitro* studies, we suggest that carotenoids have protective effects against DF. Fluorine also interacts with other elements such as arsenic and selenium.[33-35] We suggest to further explore the relationship between carotenoids and reduced fluorine toxicity and how it is influenced by other geochemical elements.

There are possible biological mechanisms accounting for the protective role of carotenoids. The increased generation of reactive oxygen species (ROS) and enhanced lipid peroxidation have been shown to be related to DF.[4,5] Carotenoids have antioxidant activities and are able to scavenge free radicals.[36] An *in vitro* study showed that β-carotene reduced lipid peroxidation in a rat microsomal system exposed to fluoride.[30] Mansour et al[7] found that lycopene ameliorated the adverse effects of fluoride by reducing elevated malondialdehyde and NO(x) concentrations and enhancing the endogenous antioxidant system, with increases in glutathione, SOD and total antioxidant

capacity. Lycopene prevented coal burning fluorosis-induced spermatogenic cell apoptosis through the suppression of oxidative stress-mediated JNK and ERK signaling pathways.[26] Furthermore, lycopene significantly combated NaF-induced apoptosis of ameloblasts and DF by attenuating oxidative stress and downregulating the caspase pathway.[8] Therefore, the beneficial effect of carotenoids on DF could be partially attributed to its role in the avoidance of fluoride-induced oxidative stress and enhancement of the cellular antioxidant defence system.

This study is the largest among published studies for DF assessing interactions between genotypes associated with nutrient intake. The SOD2 gene encodes a free radical scavenging enzyme (SOD), which is an antioxidant enzyme that catalyzes the dismutation of superoxide radical anions. Thus, this enzyme participates in various biological processes induced by oxidative stress.[37] Deng et al[38] found that the SNPs of the SOD2 gene regulated SOD2 mRNA transcription, influencing bone health in the Chinese population. Our data showed that higher dietary carotene was particularly beneficial among AG+AA genotype carriers, which indicated that the SOD2 polymorphisms modified the association of dietary carotene intake with DF risk. This result was consistent with our previous study, which reported SOD2 polymorphisms modified the effect of dietary carotene intake on skeletal fluorosis.[11] Fluoride increases oxidative stress by reducing the activity of antioxidant enzymes, which results in the accumulation of ROS.[39,40] Previous studies showed that high concentrations of fluoride inhibited cell proliferation and the activity of antioxidant enzymes, which may be the main mechanism causing DF.[41,42] It was reported that fluoride-treated mouse enamel had significantly higher quantitative fluorescence, due to fluoride exposure generating ROS.[43] These findings suggested a protective effect of carotenes against fluorosis risk that might be partially due to they prevented oxidative DNA damage. However, further study of the underlying biological mechanisms is needed.

There are some limitations to the present study. First, we were unable to infer a causal relationship between carotenoids and the risk of DF in this cross-sectional study. However, we excluded participants who were known to have cancer, coronary heart disease, stroke, gout or kidney disease before the survey, which might change their dietary habits and nutritional factors. Second, fluorosis may overlap with related aberrant mineral exposures. Geochemical elements in the local environment were not detected in the environment so that we did not show Conceptual Diagram of exposure. However, we tried to determine whether dietary selenium and zinc mediated the relationship between carotenoids and DF using mediation analysis, and found no mediation effects (Supplementary Figure 1). Additionally, although we adjusted for many factors for the statistical analysis, residual confounding was still unavoidable due to some unmeasured environmental factors, such as arsenic, selenium. Third, food databases from the U.S. Department of Agriculture were used to quantify the intake of carotenoids, so wide variability in the carotenoid content of foods may attenuate estimates of effect size. In addition, inaccurate recall of participants might lead to misclassification of dietary intake, but differential reporting between DF and controls was unlikely, as there was little public awareness about dietary carotenoids and their potential effects on fluorosis. Fourth, dietary carotenoids were assessed by the subjective method of using a FFQ, which may have skewed any true association. However, previous studies have validated the FFQ to assess carotenoids in the diet.[15,16] Finally, we could not rule out the influence of coexisting bioactive compounds in plant foods that might positively modulate bone health.

Conclusion

In summary, higher dietary carotenoids were associated with a lower occurrence of DF. The SOD2 (rs 11968525) polymorphisms may modify this inverse association between dietary carotene and DF risk. We suggest that carotenoid-containing foods, such as fruits and vegetables, should be consumed to prevent DF in coal burning areas, especially for the SOD2 (rs 11968525) AG+AA genotype carriers. However, prospective studies are needed to clarify the favourable effect of dietary carotenoids on DF prevention.

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AUTHOR DISCLOSURES

The authors declare no competing interests.

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Supplementary table 1. Multivariate-adjusted ORs (95% CIs) of the DF for each quartile of dietary carotenoids intake by subgroups of gender

	Quartiles of dietary carotenoids intake					
	Quartile 1[†]	Quartile 2	Quartile 3	Quartile 4	p [a]	p [b]
α-carotene						0.959
male	1	1.05 (0.52, 2.15)	1.12 (0.59, 2.11)	0.68 (0.33,1.38)	0.606	
female	1	1.29 (0.65,2.57)	0.86 (0.44, 1.67)	0.40 (0.19, 0.84)*	0.012	
β-carotene						0.444
male	1	1.57 (0.69, 3.53)	0.91 (0.43, 1.89)	0.61 (0.29, 1.27)	0.062	
female	1	1.94 (0.94, 4.03)	0.42 (0.21, 0.81)*	0.61 (0.31, 1.23)	0.010	
lutein/zeaxanthin						0.699
male	1	0.67 (0.29, 1.54)	0.41 (0.18, 0.92)*	0.30 (0.14, 0.68)**	0.001	
female	1	0.78 (0.39, 1.54)	0.54 (0.27, 1.08)	0.39 (0.19, 0.80)*	0.006	
β-cryptoxanthin						0.293
male	1	0.85 (0.40, 1.81)	1.13 (0.54, 2.35)	1.99 (0.93, 4.29)	0.040	
female	1	0.94 (0.49, 1.81)	0.82 (0.43, 1.58)	1.28 (0.64, 2.57)	0.637	
lycopene						0.221
male	1	1.05 (0.44, 2.49)	0.51 (0.23, 1.16)	0.60 (0.27, 1.37)	0.074	
female	1	0.54 (0.27, 1.08)	0.54 (0.27, 1.09)	0.28 (0.14, 0.58)**	0.001	
total carotenoids[¶]						0.425
male	1	0.88 (0.38, 2.03)	0.50 (0.23, 1.08)	0.43 (0.19, 0.95)**	0.013	
female	1	1.08 (0.54, 2.16)	0.53 (0.27, 1.05)	0.39 (0.19, 0.79)*	0.002	

DF: dental fluorosis; OR: odds ratio; CI: confidence interval.
Unconditional logistic regression analyses to ORs and 95% CIs. Covariates adjusted for age, gender, marital status, household income, smoking, alcohol drinking, tea drinking, total energy, calcium intake, roasted chili consumption, fuel type, roasted corn, use of an improved stove.
[†]Quartile 1 was the reference quartile.
[‡]p value for linear trend.
[§]p value for interaction.
[¶] Total carotenoids indicate the sum of α-carotene, β-carotene, β-cryptoxanthin, lycopene and lutein/zeaxanthin.
*p<0.05, **p<0.01.

Supplementary table 2. Multivariate-adjusted ORs (95% CIs) of the DF for each quartile of dietary carotenoids intake by subgroups of smoking

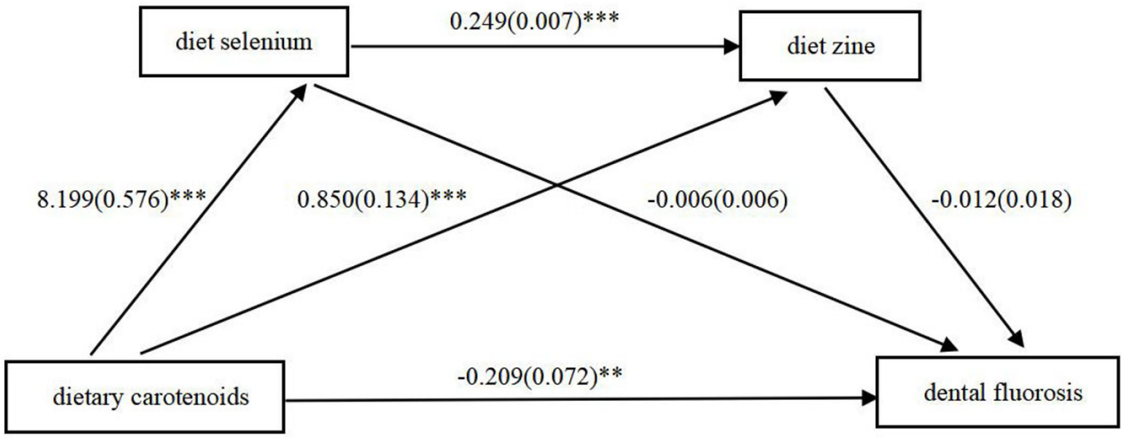
	Quartiles of dietary energy-adjusted carotenoids intake					
	Quartile 1†	Quartile 2	Quartile 3	Quartile 4	*p* ‡	*p* §
α-carotene						0.682
Yes	1	1.21 (0.55, 2.68)	1.96 (0.85, 4.51)	0.67 (0.31, 1.45)	0.248	
No	1	1.08 (0.58, 2.03)	1.03 (0.56, 1.93)	0.39 (0.19, 0.79)*	0.011	
β-carotene						0.432
Yes	1	0.88 (0.42, 1.86)	0.54 (0.27, 1.06)	0.45 (0.22, 0.89)*	0.009	
No	1	1.44 (0.82, 2.54)	0.71 (0.41, 1.22)	0.52 (0.30, 0.90)*	0.002	
lutein/zeaxanthin						0.762
Yes	1	0.53 (0.22, 1.27)	0.42 (0.18, 0.98)*	0.33 (0.14, 0.77)*	0.019	
No	1	0.89 (0.46, 1.71)	0.53 (0.27, 1.02)	0.36 (0.18, 0.69)**	0.001	
β-cryptoxanthin						0.156
Yes	1	0.90 (0.45, 1.79)	1.21 (0.62, 2.28)	2.05 (0.97, 4.12)	0.067	
No	1	0.84 (0.50, 1.41)	0.96 (0.56, 1.64)	1.19 (0.68, 2.07)	0.448	
lycopene						0.379
Yes	1	1.12 (0.46, 2.73)	0.58 (0.25, 1.33)	0.59 (0.25, 1.41)	0.203	
No	1	0.54 (0.27, 1.06)	0.49 (0.25, 0.96)*	0.33 (0.17, 0.65)**	0.001	
total carotenoids¶						0.883
Yes	1	0.62 (0.28, 1.37)	0.31 (0.15, 0.63)*	0.35 (0.17, 0.73)**	0.001	
No	1	1.11 (0.64, 1.92)	0.70 (0.40, 1.21)	0.46 (0.28, 0.80)**	0.001	

DF: dental fluorosis; OR: odds ratio; CI: confidence interval.
Unconditional logistic regression analyses to ORs and 95% CIs. Covariates adjusted for age, gender, marital status, household income, smoking, alcohol drinking, tea drinking, total energy, calcium intake, roasted chili consumption, fuel type, roasted corn, use of an improved stove.
†Quartile 1 was the reference quartile.
‡*p* value for linear trend.
§*p* value for interaction.
¶ See Supplementary table 1.
*$p<0.05$, **$p<0.01$.

Supplementary table 3. Multivariate-adjusted ORs (95% CIs) of the DF for each quartile of dietary carotenoids intake by subgroups of urine fluoride concentration

	Quartiles of dietary energy-adjusted carotenoids intake					
	Quartile 1†	Quartile 2	Quartile 3	Quartile 4	*p* ‡	*p* §
α-carotene						0.463
>1.6mg/L	1	1.13 (0.45, 2.82)	1.99 (0.77, 5.16)	0.53 (0.19, 1.42)	0.609	
≤1.6mg/L	1	1.19 (0.66, 2.15)	1.12 (0.62, 2.02)	0.49 (0.27, 0.91)*	0.020	
β-carotene						0.305
>1.6mg/L	1	2.02 (0.98, 9.70)	0.94 (0.39, 2.22)	0.89 (0.35, 2.29)	0.335	
≤1.6mg/L	1	1.20 (0.64, 2.25)	0.49 (0.28, 0.89)*	0.43 (0.24, 0.79)*	0.001	
lutein/zeaxanthin						0.284
>1.6mg/L	1	0.89 (0.33, 2.36)	0.72 (0.27, 1.88)	0.46 (0.17, 1.23)	0.097	
≤1.6mg/L	1	0.65 (0.35, 1.21)	0.41 (0.22, 0.75)*	0.28 (0.15, 0.52)**	<0.001	
β-cryptoxanthin						0.690
>1.6mg/L	1	0.87 (0.34, 2.24)	0.69 (0.29, 1.63)	1.75 (0.93, 7.13)	0.112	
≤1.6mg/L	1	0.87 (0.49, 1.56)	1.05 (0.59, 1.89)	1.31 (0.71, 2.39)	0.258	
lycopene						0.660
>1.6mg/L	1	0.72 (0.29, 1.82)	0.59 (0.23, 1.49)	0.59 (0.23, 1.52)	0.202	
≤1.6mg/L	1	0.72 (0.37, 1.40)	0.49 (0.26, 0.93)*	0.37 (0.19, 0.71)**	0.002	
total carotenoids¶						0.191
>1.6mg/L	1	1.18 (0.44, 3.11)	0.54 (0.21, 1.35)	0.82 (0.29, 2.29)	0.277	
≤1.6mg/L	1	0.94 (0.49, 1.76)	0.52 (0.29, 0.94)*	0.30 (0.16, 0.56)**	<0.001	

DF: dental fluorosis; OR: odds ratio; CI: confidence interval.
Unconditional logistic regression analyses to ORs and 95% CIs. For covariate adjustments see Supplementary Table 1.
†Quartile 1 was the reference quartile.
‡*p* value for linear trend.
§*p* value for interaction.
¶See Supplementary table 1.
*$p<0.05$, **$p<0.01$.



Supplementary figure 1. The mediation effects of diet selenium and diet zinc on the relationship between carotenoids and dental fluorosis.