Original Article

Association of Dietary Carotenoids Intake with Skeletal Fluorosis in the Coal-burning Fluorosis Area of Guizhou Province*

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Abstract

Objective To explore whether the intake of dietary carotenoids could protect against skeletal fluorosis in Guizhou province in which coal-burning fluorosis is endemic.

Methods A case-control study of 196 patients with skeletal fluorosis and 196 age and gender-matched controls was conducted in Zhijin, Guizhou Province. Face-to-face interviews were conducted to assess habitual dietary intake using a 75-item food frequency questionnaire and various covariates with structured questionnaires. Urinary fluoride was measured using an ion-selective electrode method. The genotype of superoxide dismutase 2 (SOD2) rs11968525 was detected by TaqMan method.

Results We observed significant dose-dependent inverse associations of skeletal fluorosis with intake of β-carotene, lutein/zeaxanthin, lycopene, and total carotenoids (*P*-trend = 0.002 to 0.018), whereas α-carotene and β-cryptoxanthin intakes were not found to be related to skeletal fluorosis, after adjustment for potential confounders. The adjusted *OR*s and 95% *CI* of skeletal fluorosis for the highest versus lowest quartile were 0.30 (0.10, 0.86) for β-carotene, 0.23 (0.08, 0.66) for lycopene, 0.26 (0.10, 0.75) for lutein/zeaxanthin and 0.34 (0.14, 0.74) for total carotenoids (all *P*-trend < 0.05). Stratified analyses showed that the protective effects of lutein/zeaxanthin and total carotenoids on skeletal fluorosis were more evident for individuals with the AG+AA genotypes of SOD2 (rs11968525).

Conclusion Increased intakes of β-carotene, lutein/zeaxanthin, lycopene, and total carotenoids are independently associated with a lower risk of coal-burning skeletal fluorosis. SOD2 (rs11968525) polymorphisms might modify the inverse associations between dietary carotenoids and skeletal fluorosis.

Key words: Case-control study; Dietary intake; Carotenoids; Skeletal fluorosis

Biomed Environ Sci, 2018; 31(6): 438-447 *doi: 10.3967/bes2018.057* *ISSN: 0895-3988*

www.besjournal.com (full text) *CN: 11-2816/Q*

INTRODUCTION

Endemic fluorosis, caused by exposure to high levels of fluorine in a person's immediate environment, affects mainly the bones and teeth. It is global in scope, occurs on all continents and affects millions of people[1]. The coal-burning fluorosis is the one of most popular endemic fluorosis in China[2]. It was reported that there were more than 16.1 million dental fluorosis

*This study was supported by the National Natural Science Foundation of China [grant number 81460497].

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patients and 1.8 million skeletal fluorosis patients due to coal-burning pollution[3]. The area most severely affected by coal-burning endemic fluorosis was located in China's Guizhou province. Nineteen million people live in areas in which coal-burning fluorosis is endemic and 0.95 million people suffering from skeletal fluorosis[4]. Endemic fluorosis, especially skeletal fluorosis, is harmful to health and reduces quality of life.

Skeletal fluorosis is a chronic metabolic bone disease characterized by pain in the joints and neck due to large amounts of fluoride accumulate in bone tissue. A variety of conditions are associated with skeletal fluorosis, such as osteosclerosis, osteomalacia, osteoporosis, and secondary hyperpa rathyroidism[5]. Several studies have suggested that oxidative stress plays an important role in the pathogenesis of endemic fluorosis[6,7]. Carotenoids such as α-carotene, β-carotene, β-cryptoxanthin, lycopene, and lutein/zeaxanthin are among the most common pigments of the human diet and have strong antioxidant activity[8]. Several animal and *in vitro* studies[9,10] demonstrated that carotenoids could minimise the toxic effects of fluoride and provide benefits to bone metabolism against oxidative stress[11-13]. Moreover, a prospective study demonstrated that greater carotenoid intake was correlated with a lower loss in bone mineral density (BMD) at 4 y in the trochanters in men and in the lumbar spine in women[14], and a meta-analysis reported that higher intake of dietary total carotenoids or β-carotene might be associated with a low risk of hip fracture[15]. These findings indicated that carotenoids might have a protective role against skeletal fluorosis. However, to our knowledge, no studies have explored the association between the intake of dietary carotenoids and the development of skeletal fluorosis.

Skeletal fluorosis is highly variable in its clinical severity among individuals living in the same town, which indicated genetic influenced in fluorosis[16]. A number of studies have shown the association between genetic polymorphisms in candidate genes and the skeletal fluorosis[17]. Since oxidative stress plays an crucial role in the pathogenesis of fluorosis[6,7]. Superoxide dismutase 2 (SOD2) gene encodes a free radical scavenging enzyme, which removes superoxidate and catalyzes the production of hydrogen peroxide. Evidence showed that coal-burning fluorosis was associated with reduced activity in superoxide dismutase enzymes and gene expression[18,19]. Moreover, Deng et al. identified eight single nucleotide polymorphisms (SNPs) at SOD2 gene locus that were suggestively associated with bone health and strongest association signals was observed at SNP rs11968525 in Chinese population[20]. Thus SOD2 (rs11968525) polymorphisms might be potentially associated with skeletal fluorosis. It is well known skeletal fluorosis involves combined effects of numerous genetic, environmental, and behavioral risk factorS[21,22]. However, no studies reported the interaction of dietary carotenoids and the SOD2 polymorphisms on skeletal fluorosis.

We therefore conducted a 1:1 case-control study to investigat the relationships between dietary intake of the five dietary carotenoids mentioned above and risk of skeletal fluorosis, as well as explore the interaction of dietary carotenoids and SOD2 (rs11968525) polymorphisms on skeletal fluorosis.

SUBJECTS AND METHODS

Study Subjects

We performed a 1:1 matched case-control study in Zhijin County, Guizhou province, in which coal-burning fluorosis is endemic in July 2015. Skeletal fluorosis is a chronic metabolic bone disease which affects those living in areas with endemic fluoride poisoning, whose cause is the excess intake of fluoride, and whose major signs and symptoms include pain in the joints of the neck, back, and four limbs, dysfunction in body movement, and abnormalities in bone and joint[23]. It was identified according to the Chinese Diagnostic Criteria of Endemic (WS192-2008, China)[23]. Patients were enrolled in Zhijin County Centers for Disease Control and Prevention. Inclusion criteria for the cases were patients with skeletal fluorosis of either sex who voluntarily accepted to participate in the study and resident in the Zhijin County for over 10 years. We used a convenience sampling to enroll cases. Exclusion criteria for the cases were presence of cancer, coronary heart disease, stroke, gout, renal, liver diseases and dental fluorosis, and those with substantial self-reported changes in dietary habits over the previous 5 years. Eligible control subjects were recruited from healthy individuals in the same community with living for at least 10 years as the cases. Control subjects were individually matched to the cases by sex and age (± 3 years). The control's exclusion criteria were previous diagnosis of cancer, coronary heart disease, stroke, gout, renal, liver

diseases, and dental fluorosis. A total of 196 cases and 196 controls were included in this study. Three hundred sixty urine sample and three hundred thirty-four blood sample were collected.

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 1,964 Helsinki declaration and its later amendments or comparable ethical standards. All study participants signed informed-consent forms before their enrolment, and the Medical Ethics Committee of Zunyi Medical University approved the study (No. 2014-1-003).

Data Collection

A personal interview was conducted by trained interviewers who administered a structured questionnaire. The following information was collected: (1) socio-demographic characteristics (e.g., age, gender, marital status, household income); (2) lifestyle habits (e.g., smoking, passive smoking, alcohol drinking, tea drinking, use of an improved stove (improving the kitchen stove to exclude the fluoride out of the room to decrease the pollution of the air indoors), fuel type, roasted corn or roasted chili intake, calcium intake); (3) Usual dietary intake in the year before the interview was investigated with a food-frequency questionnaire (FFQ); (4) relevant disease (hypertension, diabetes, gout, heart-related diseases, stroke, etc.). Individuals who drank tea at least twice weekly were considered tea drinkers. Participants who smoked at least five packs of cigarettes a year were defined as smokers and those who drank alcohol at least once a week continuously for at least 6 month were considered alcohol drinkers. Passive smokers were defined as people present in a room in which others smoked at least one cigarette for 5 min per day. All eligible participants were required to respond to the same questionnaire.

Dietary Assessment

Dietary intake was gained from administration of a 75-item FFQ during a face-to-face interview. The information included the intake frequency (never, per year, per month, per week, or per day). To help the participants quantify the amount, photographs of foods and portion sizes were provided as visual aids. Food intakes were converted into daily intake in grams per day. Energy and nutrient intakes were computed based on the China food composition database[24]. Carotenoid intake, including α-carotene, β-carotene, β-cryptoxanthin, lycopene, and lutein/zeaxanthin, was estimated from information in the US Department of Agriculture database[25]. The validity and reproducibility of the FFQ have been assessed previously[26-28].

Laboratory Assay

A 10-mL urine sample was collected from each participant. Urinary fluoride was measured using an ion-selective electrode method in the HQ40d Portable Meter with IntelliCAL Probe (Hach, USA). After mixing 1 mL of urine with 24 mL of deionized water, one pillow of fluoride adjustment buffer powder was added to each 25-mL mixture. The concentration of urine fluoride in the mixture was then measured and the concentration of fluorine in the urine of the subjects calculated according to the corresponding proportion. According to WS/T 256-2005 requirements, a normal urinary fluoride concentration is 1.6 mg/L or less[29]. The coefficient of variation for the urinary fluoride was 2.29%.

Real-time Polymerase Chain Reaction Genotyping

Venous blood samples (5 mL) were collected from each participant using EDTA anticoagulant and were stored at -70 °C until further analysis. DNA was extracted from the blood sample using a genomic DNA extraction kit (Transgen, Beijing, China). The Polymerase chain reaction (PCR) TaqMan probe and primers for superoxide dismutase 2 (SOD2) 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). A reaction mixture was used, which 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 as follows: an initial step of 95 °C for 4 min, followed by 40 cycles of 95 °C for 7 s and 60 °C for 40 s. We repeated the genotyping for 10% of samples at random.

Statistical Analysis

All data were entered twice into a computer using Epidata 3.1 and then corrected. To achieve an approximately normal distribution for statistical analysis, logarithmic transformation was applied to daily energy intake. Dietary nutrient intake data were adjusted for total energy intake by the regression residual method[30]. Dietary data are

expressed as median (P_{25}, P_{75}) for continuous variables because of skewed distributions and as frequencies with percentages for categorical variables. The Wilcoxon signed-rank or McNemar's test was used to test differences in the socio-demographic and nutrient intakes between the case and control subjects, as appropriate. Testing for deviation from Hardy-Weinberg equilibrium was performed within participants stratified by cases and controls using the χ^2 test. Energy-adjusted intakes of carotenoids were grouped into quartile 1 to quartile 4 (Q1-Q4) based on control subjects by gender and the cutoffs were then applied to the cases. The lowest quartile (Q1) served as the reference group. Univariate (crude) and multivariate (adjusted) conditional logistic regression models were used to assess the associations between the quartiles of intake and risk of skeletal fluorosis, and odds ratios (*OR*s) and 95% confidence intervals (*CI*s) were calculated. Three models were used to examine the relationships between carotenoids and skeletal fluorosis. Crude adjusted *OR*s (95% *CI*s) were obtained without further adjustment of covariates. Model 2 controlled for energy intake, marital status, household income, alcohol drinking, tea drinking, fuel type, improved stove use, roasting corn, and roasted chilli consumption. Model 3 further adjusted for calcium intake, rs11968525 genotype, and urinary fluoride level. To examine the linear trend, ordinal values of the quartile of each dietary carotenoid were entered as a continuous variable in the logistic regression analysis model. The criteria for entry and non-entry of these confounders were $P < 0.05$ and $P > 0.10$, respectively, using the forward stepwise method.

Stratified analyses were conducted according to rs11968525 genotype (GG/AG+TT), gender (women/men), smoking status (yes/no), education (≤ 6 years / > 6 years), use of mixed coal (yes/no), and use of improved stoves (yes/no). Multiplicative interactions were estimated by adding the interaction terms according to their likelihood. We used unconditional logistic regression models to assess the associations because these factors were not matched between case and control subjects. The potential interactions were also examined *via* multiplicative interaction terms in the multivariate unconditional logistic regression models. All *P* values were two-sided, and a *P* value of less than 0.05 was considered to indicate statistical significance. SPSS 18.0 software (SPSS Inc., Chicago, IL) was used for data analysis.

RESULTS

Distribution of Characteristics

The demographics, lifestyle characteristics, and selected fluorosis risk factors of the cases and controls are presented in Table 1. There were 196 case-control pairs, including 105 female pairs and 91 male pairs. The median age was 49.0 years in the skeletal fluorosis cases and 51.0 years in the controls. Compared with the controls, the cases with skeletal fluorosis were more likely to have lower levels of education and proportion of improved stove use, but higher levels of urinary fluoride and mixed coal use. Furthermore, cases with skeletal fluorosis had a lower intake of α-carotene, β-carotene, lutein/zeaxanthin, lycopene and total carotenoids (*P* for trend range < 0.005 to 0.001); only for β-cryptoxanthin were these results not significant (*P* = 0.373). The genotype frequency distribution of rs11968525 in control and case groups was consistent with HardyWeinberg equilibrium (χ^2 = 0.601, *P* = 0.438; χ^2 = 2.397, *P* = 0.122) and No significant differences were observed in genotype frequencies of rs11968525 between case and control groups (*P* = 0.591).

Univariate and Multivariable Logistic Regression Analysis

The univariate logistic regression analyses showed that α-carotene, β-carotene, lutein/zeaxanthin, lycopene, and total carotenoids were inversely associated with skeletal fluorosis (*P* for trend range < 0.001 to 0.002) (Table 2). These associations were attenuated after adjusting for socio-demographic characteristics and potential confounding factors related to coal-burning fluorosis (Model 2). With further adjustments made for calcium intake, rs11968525 genotype and urinary fluoride level (Model 3), great intakes of β-carotene, lutein/zeaxanthin, lycopene, and total carotenoids were inversely associated with the risk of skeletal fluorosis (*P*-trend = 0.002-0.018), whereas α-carotene intakes were not found to be related to skeletal fluorosis (*P*-trend = 0.769). Comparisons of the first-quartile *OR*s (95% *CI*s) of skeletal fluorosis with the fourth quartiles of β-carotene, lutein/ zeaxanthin, lycopene, and total carotenoids were 0.30 (0.10, 0.86), 0.33 (0.13, 0.84), 0.26 (0.10, 0.75), 0.23 (0.08, 0.66), and 0.34 (0.14, 0.74), respectively (Table 2). No associations between the skeletal fluorosis and the intake of β-cryptoxanthin (*P*-trend = 0.407) were observed (Table 2).

Table 1. Characteristics of the Case and Control in Coal-burning Fluorosis Area in Guizhou, China, in 2015*

Variables	Median (P_{25}, P_{75})		*P*-value
	Case (*n* = 196)	Control (*n* = 196)	
Age, years	49.0 (44.0, 60.0)	51.0 (43.2, 60.0)	0.703
Total energy, kcal/d	2676.4 (2002.0, 3487.7)	2587.7 (1998.0, 3213.7)	0.219
Calcium intake, mg/d	457.9 (326.3, 706.6)	482.5 (344.0, 723.6)	0.274
Roasted chili consumption, g/d	6.03 (2.43, 17.2)	5.79 (2.41, 12.5)	0.271
Urinary fluoride, mg/L	1.58 (1.21, 2.31)	1.23 (0.85, 1.73)	< 0.001
Carotenoids, μg/d			
α-carotene	223.5 (112.5, 413.0)	295.5 (136.8, 636.9)	0.001
β-carotene	2478.1 (1388.6, 3783.8)	3186.2 (1999.4, 5051.1)	< 0.001
β-cryptoxanthin	241.0 (103.5, 596.2)	210.1 (118.6, 449.3)	0.373
lycopene	296.5 (116.4, 761.6)	559.2 (246.0, 1112.0)	< 0.001
Lutein/zeaxanthin	1887.3 (1109.1, 3264.1)	2817.3 (1749.5, 4319.2)	< 0.001
Total carotenoids†	5408.5 (3269.2, 9234.4)	7531.6 (4854.5, 11825.7)	< 0.001
Marital status, *n* (%)			0.541
married or cohabitation	161 (82.1)	169 (86.2)	
divorce or widowed	27 (13.8)	21 (10.7)	
unmarried	8 (4.1)	6 (3.1)	
Education level, *n* (%)			< 0.001
primary school or below	177 (90.3)	144 (73.5)	
secondary school	17 (8.7)	40 (20.4)	
high school or above	2 (1.0)	12 (6.1)	
Fuel type, *n* (%)			0.008
raw coal	104 (53.1)	114 (58.2)	
mixed coal	56 (28.6)	31 (15.8)	
firewood	5 (2.6)	14 (7.1)	
electricity	31 (15.8)	37 (18.9)	
Smoker‡, *n* (%)	79 (40.3)	77 (39.3)	0.880
Alcohol drinker§, *n* (%)	61 (31.1)	65 (33.2)	0.665
Tea drinker‖, *n* (%)	72 (36.7)	74 (37.8)	0.834
Roasting food, *n* (%)	109 (55.6)	95 (48.5)	0.142
Improved stove use¶, *n* (%)	143 (73.0)	169 (86.2)	0.002
Vitamin supplement, *n* (%)	3 (1.5)	6 (3.1)	0.312
SOD rs11968525, *n* (%)			0.591
GG	114 (65.1)	108 (67.9)	
AG+AA	61 (34.9)	51 (32.1)	

Note. *Continuous and categorical variables were described by means median (P_{25}, P_{75}) or numbers and percentages, and evaluated by the Wilcoxon signed-rank or McNemar's test, respectively, to compare the categorical and continuous variables of case and control group. †Total carotenoids indicate the sum of α-carotene, β-carotene, β-cryptoxanthin, lycopene, and lutein/zeaxanthin. ‡Smokers were defined as having smoked at least one cigarette daily for at least six consecutive months. §Alcohol drinking was defined as having had wine (beer, white wine, and red wine) at least once per week for at least six consecutive months. ‖Tea drinkers were defined having drank tea at least twice weekly. ¶Improving the kitchen stove to exclude the fluoride out of the room to decrease the pollution of the air indoors.

Interaction Analysis

The results of the above calculations show whether the potential risk factors modified the association between carotenoids and skeletal fluorosis (Table 3). Stratified analyses showed that there were inverse associations of total carotenoids and lutein/zeaxanthin with skeletal fluorosis significant in subjects with the SOD2 (rs11968525) AG+AA genotype (*P*-trend < 0.05), but this association was not significant in subjects with GG genotypes (*P*-trend > 0.05). There were significant interactions between dietary intake of total carotenoids and lutein/zeaxanthin and rs11968525 polymorphisms (*P*-interactions < 0.05), and marginal interactions between dietary intake of β-carotene and rs11968525

Table 2. Odds Ratios (95% *CI*s) of Skeletal Fluorosis for Quartiles of Dietary Carotenoids Intake in Adults of Coal-burning Fluorosis Area in Guizhou, China, in 2015

Items	Quartiles of Dietary Carotenoids Intake				*P*-trend
	Quartile 1[§]	Quartile 2	Quartile 3	Quartile 4	
α-carotene					
Median, μg/d	68.1	211.0	404.6	1035.3	
Case/control	61/ 49	61/49	50/49	24/49	
Model 1[#]	1	0.96 (0.54, 1.72)	0.69 (0.40, 1.21)	0.31 (0.15, 0.63)**	0.002
Model 2[‡]	1	0.94 (0.50, 1.79)	0.82 (0.45, 1.49)	0.33 (0.17, 0.71)**	0.011
Model 3[‖]	1	0.75 (0.32, 1.77)	0.89 (0.41, 1.94)	0.83 (0.30, 2.28)	0.769
β-carotene					
Median, μg/d	1287.1	2535.5	3788.2	7535.5	
Case/control	77/ 49	50/49	43/49	26/49	
Model 1[#]	1	0.59 (0.33, 1.06)	0.45 (0.25, 0.81)**	0.26 (0.13, 0.52)**	< 0.001
Model 2[‡]	1	0.47 (0.25, 0.90)*	0.44 (0.23, 0.85)*	0.25 (0.12, 0.53)**	0.001
Model 3[‖]	1	0.50 (0.20, 1.24)	0.47 (0.19, 1.12)	0.30 (0.10, 0.86)*	0.018
β-cryptoxanthin					
Median, μg/d	76.2	158.9	313.9	834.1	
Case/control	56/49	35/49	36/49	69/49	
Model 1[#]	1	0.58 (0.33, 1.04)	0.63 (0.34, 1.13)	1.19 (0.69, 2.06)	0.430
Model 2[‡]	1	0.63 (0.34, 1.18)	0.56 (0.29, 1.09)	1.25 (0.69, 2.29)	0.474
Model 3[‖]	1	0.97 (0.40, 2.47)	0.42 (0.16, 1.09)	0.84 (0.36, 1.95)	0.407
Lutein/zeaxanthin					
Median, μg/d	1103.1	2170.3	3379.2	6420.3	
Case/control	88/49	44/49	35/49	29/49	
Model 1[#]	1	0.43 (0.23, 0.78)**	0.30 (0.16, 0.59)**	0.23 (0.11, 0.47)**	< 0.001
Model 2[‡]	1	0.34 (0.17, 0.67)**	0.25 (0.12, 0.53)**	0.28 (0.13, 0.60)**	< 0.001
Model 3[‖]	1	0.25 (0.10, 0.65)**	0.18 (0.06, 0.54)**	0.26 (0.10, 0.75)**	0.002
Lycopene					
Median, μg/d	113.1	379.8	790.3	1962.9	
Case/control	85/49	43/49	40/49	28/49	
Model 1[#]	1	0.43 (0.24, 0.78)**	0.33 (0.17, 0.63)**	0.24 (0.12, 0.48)**	< 0.001
Model 2[‡]	1	0.42 (0.21, 0.85)*	0.33 (0.16, 0.67)**	0.30 (0.14, 0.67)**	< 0.001
Model 3[‖]	1	0.37 (0.42, 1.09)	0.35 (0.14, 0.94)*	0.23 (0.08, 0.66)**	0.006
Total carotenoids					
Median, μg/d	3067.3	6175.7	9421.1	16613.3	
Case/control	86/48	43/49	36/49	30/48	
Model 1[#]	1	0.46 (0.24, 0.88)*	0.36 (0.19, 0.67)**	0.24 (0.12, 0.48)**	< 0.001
Model 2[‡]	1	0.47 (0.24, 0.93)*	0.35 (0.18, 0.70)*	0.36 (0.19, 0.68)**	0.001
Model 3[‖]	1	0.43 (0.17, 1.04)	0.23 (0.10, 0.53)*	0.34 (0.14, 0.74)*	0.002

Note. Abbreviations: *OR*, odds ratio; *CI*, confidence interval. [§]Quartile 1 was the reference quartile. [†]Total carotenoids indicate the sum of α-carotene, β-carotene, β-cryptoxanthin, lycopene, and lutein/zeaxanthin. [#]Model 1, crude adjusted *OR*s were obtained without further adjustment of covariates. [‡]Model 2, adjusted for energy intake, marital status, education level, smoking, alcohol drinking, tea drinking, fuel type, improved stove use, roasting food, roasted chilli consumption, [‖]Model 3, further adjusted calcium intake, rs11968525 genotype, and urinary fluoride level. *$P < 0.05$, **$P < 0.01$.

polymorphisms (*P*-interactions = 0.075). Stratified and interaction analyses demonstrated no significant interaction between the factors of gender, smoking, education, use of mixed coal and use of improved stoves, and dietary carotenoids in relation to the risk of skeletal fluorosis (*P* for trend range 0.101 to 0.963). (Supplementary Tables S1 to S6, available in www.besjournal.com).

DISCUSSION

This 1:1 matched case-control study investigated the association between dietary carotenoids and the risk for skeletal fluorosis in Zhijin, Guizhou province, in which coal-burning fluorosis is endemic. We observed a significant inverse association of the skeletal fluorosis with the dietary intake of β-carotene, lutein/zeaxanthin, lycopene and total carotenoids.

We are unaware of any other study that has reported a dose-dependent inverse association between carotenoids and the risk of skeletal fluorosis. However, some studies have indicated that carotenoid has protective effects on bone health. The Framingham Osteoporosis Study, a prospective study involving 8,800 subjects, observed the associations between lycopene intake and 4-y change in lumbar spine BMD for women (*P*-trend = 0.03) and in trochanter BMD for men[14]. That study also found that subjects in the highest intake group of total carotenoid and lycopene had a lower prevalence of hip fracture in older adults (*P*-trend: 0.01 to 0.02)[31]. Recently, cross-sectional analyses (*n* = 14,803) and longitudinal analyses (*n* = 25,439) of fracture cases were conducted on data from the European Prospective Investigation into Cancer and Nutrition-Norfolk cohort. The study demonstrated associations with bone density status and fracture risk

Table 3. Multivariate-adjusted *OR* (95% *CI*s) of the Skeletal Fluorosis for Each Quartile of Dietary Carotenoids Intake by Subgroups of rs11968525 Genotype in Adults of Coal-burning Fluorosis Area in Guizhou, China, in 2015

Items	Quartiles of Dietary Energy-adjusted Carotenoids Intake				P^a	P^b
	Quartile 1[§]	Quartile 2	Quartile 3	Quartile 4		
α-carotene						0.402
GG	1	0.82 (0.36, 1.83)	0.94 (0.40, 2.21)	0.69 (0.26, 1.87)	0.580	
AG+AA	1	1.14 (0.34, 3.85)	1.02 (0.30, 3.38)	0.50 (0.14, 1.79)	0.302	
β-carotene						0.075
GG	1	0.53 (0.23, 1.20)	0.92 (0.39, 2.16)	0.45 (0.17, 1.17)	0.229	
AG+AA	1	1.05 (030, 3.68)	0.30 (0.09, 0.98)	0.31 (0.08, 1.13)	0.010	
lutein/zeaxanthin						0.029
GG	1	0.48 (0.16, 1.09)	0.61 (0.26, 1.42)	0.51 (0.20, 1.29)	0.179	
AG+AA	1	0.51 (0.15, 1.73)	0.18 (0.05, 0.65)	0.16 (0.05, 0.52)	0.002	
β-cryptoxanthin						0.686
GG	1	0.76 (0.31, 1.85)	0.56 (0.20, 1.05)	1.18 (0.52, 2.71)	0.998	
AG+AA	1	0.70 (0.19, 2.60)	0.89 (0.24, 3.31)	1.88 (0.27, 2.97)	0.978	
Lycopene						0.289
GG	1	0.49 (0.21, 1.14)	0.41 (0.17, 0.95)	0.44 (0.18, 1.09)	0.043	
AG+AA	1	0.49 (0.15, 1.62)	0.34 (0.10, 1.14)	0.20 (0.05, 0.74)	0.004	
Total carotenoids[†]						0.028
GG	1	0.46 (0.20, 1.06)	0.82 (0.35, 1.94)	0.53 (0.21 1.35)	0.313	
AG+AA	1	0.72 (0.20, 2.67)	0.16 (0.05, 0.54)	0.19 (0.06, 0.68)	0.002	

Note. Abbreviations: *OR*, odds ratio; *CI*, confidence interval. Unconditional logistic regression analyses to *OR*s and 95% *CI*s. Covariates adjusted for age, gender, marital status, education level, smoking, alcohol drinking, tea drinking, total energy, calcium intake, roasted chili consumption, fuel type, roasting food, use of an improved oven, rs11968525 genotype and urinary fluoride level. [§]Quartile 1 was the reference quartile. [†]Total carotenoids indicate the sum of α-carotene, β-carotene, β-cryptoxanthin, lycopene, and lutein/zeaxanthin. [a]*P* value for linear trend, [b]*P* value for interaction.

exist for dietary intake of specific carotenoids and their plasma concentrations both in men and women[32]. A similar association was also observed in the Chinese population[33]. However, a recent meta-analysis reported that dietary β-carotene decreased the risk of hip fracture, whilst total carotenoids and lycopene showed only a marginal association with hip fracture risk; the pooled *OR*s were 0.72 (0.51 to 1.01) for total carotenoids and 0.84 (0.69 to 1.01) for lycopene[15]. The marginal effect might be attributed to the inclusion of fewer studies of lycopene and total carotenoids (four) than those of β-carotene (eight). A protective role of carotenoids against bone loss is supported by the results of two recent studies that showed direct effects of lycopene on osteoclast and osteoblast function[34,35]. Although no study has explored the association between dietary carotenoids and skeletal fluorosis, these results may suggest that carotenoids can protect the hip bone geometry and BMD, which may decrease the risk for skeletal fluorosis.

Carotenoids may decrease the risk of skeletal fluorosis *via* multiple mechanisms. Some studies have suggested that the beneficial effect of lycopene on bone might be *via* its antioxidant activity against oxidative stress. Oxidative stress has been shown to play a possible aetiological role *via* NF-κB ligand (RANKL) expression and signalling mechanism, which increases osteoclast genesis and stimulates osteoclastic differentiation in bone loss[36-39]. Some studies have demonstrated that oxidative damage is the major effect of fluoride toxicity[11,40]. Beyond their antioxidant function, carotenoids can also play important roles in bone health through other mechanisms. In vitro and animal studies have shown that the carotenoid family may exert dual anti-catabolic and pro-anabolic activities in bone by counteracting the same NF-κB signal transduction pathway, inhibiting osteoclast differentiation and promoting osteoblast mineralization[41]. These studies therefore suggest a possible role for beneficial effects of lycopene in bone formation and may point to potential implications with respect to its use in the chemoprevention of skeletal fluorosis.

This population-based case-control study provides evidence that genetic variation in SOD2 gene can modify the association of carotenoid intake on skeletal fluorosis. SOD2 gene encodes an antioxidant enzyme (SOD) with high activity in the catalytic dismutation of superoxide radical anion[42]. Deng et al. demonstrated that the SNPs of the SOD2 gene *via* regulating SOD2 mRNA transcription have been suggested to be associated with bone health in the Chinese population[20]. We observed that higher dietary β-carotene and lutein/zeaxanthin were particularly beneficial among subject with GG+AA genotype, which indicated that the SOD2 (rs11968525) polymorphisms modulated the effects of β-carotene and lutein/zeaxanthin on skeletal fluorosis risk. It was reported that differential relationships by dietary nutrient intake in studies related to genes involved in oxidative stress[43]. High concentrations of fluoride inhibited cell proliferation and the activity of antioxidant enzymes, which might be the main mechanism causing fluorosis[44-46]. Suzuki et al. demonstrated that fluoride exposure generated reactive oxygen species, with fluoride-treated mouse enamel having significantly higher quantitative fluorescence compared to the untreated controls[46]. The combined antioxidant effect of carotenoid and SOD might more beneficial to prevent oxidative damage induced by fluoride. However, the underlying biological mechanisms remain to be elucidated.

Some limitations to this study deserve consideration. First, the case-control study design prevents conclusions concerning causality, although we minimized possible reverse causation by excluding participants with essential changes in dietary habits over the past 5 years. Second, as a convenient sampling scheme was conducted, the inference of the results would be limited. The volunteers in this study may have more healthier lifestyles and less man who went out to work for earning, however, we observed the gender, smoking, education, use of mixed coal and use of improved stoves did not modify the effects of carotenoids on skeletal fluorosis. Third, the dietary carotenoid intake was calculated from US Department of Agriculture databases. There is wide variability in the carotenoid content of foods, depending on climate, soil, harvesting conditions and storage conditions, which may have led to attenuated estimates of effect size. In addition, our analysis was based on a single measurement of dietary intake, and the dietary questionnaires were not specifically designed to estimate carotenoid intake. Thus, both the dietary assessment and the carotenoid database may have skewed any true association. However, the FFQ used to assess carotenoids in our study has been validated[26,27]. Fourth, the sample size was relatively small in the

present study and caroteonids were divided into four groups according to the quartile of control subjects, which may result in wide confidence intervals and limit the precision of our risk estimates. Fifth, although we detected the urinary fluoride of subjects to reflect the accumulated body levels of fluoride, the measurements are highly variable with daily intake and bone turnover[47,48]. Finally, we did not collect the time of improve stove use, however, the subjects of case and control were from the same town, where the time of improved stove use was similar. Moreover, stratified and interaction analyses showed that improved stove use would not modify the effect of carotenoids on skeletal fluorosis.

CONCLUSIONS

In summary, we found that dietary intakes of β-carotene, lutein/zeaxanthin, lycopene and total carotenoids were inversely associated with the risk of skeletal fluorosis in a Chinese coal-burning fluorosis area. The SOD2 (rs11968525) polymorphisms might modify this inverse association between dietary intake of carotenoids and skeletal fluorosis risk. These findings provided evidences for antioxidant approach of precision prevention of fluorosis. Due to the limitations of this study, there need to be prospective studies to confirm that a higher intake of dietary carotenoids is beneficial for the prevention of skeletal fluorosis.

ACKNOWLEDGEMENTS

The authors want to thank the study subjects for their willingness to participate and the students who participated in the recruitment of subjects and the interviews in this study. We also thank the Center of Disease Control and Prevention of Zhijin County for providing administrative support to our study.

CONFLICT OF INTEREST

No conflict of interest to declare.

AUTHORSHIP

LIU Jun conceived the idea, conducted the study, writing and revising the manuscript. YANG Sheng was responsible for data analysis, and wrote the first draft of the manuscript. LUO Ming Jiang and LUO Ya carried out the study of survey and determination. ZHAO Xun was responsible for organization and survey. ZHANG Yuan Mei was contributed to data administration and analysis. All authors participated in data interpretation, review and approved the final manuscript.

Received: January 7, 2018;
Accepted: May 21, 2018

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Supplemental Table S1. Multivariate-adjusted *OR* (95% *CI*s) of the Skeletal Fluorosis for Each Quartile of Dietary Carotenoids Intake by Subgroups of Gender in Adults of coal-burning Fluorosis Area in Guizhou, China, in 2015

Variables	Quartles of Dietary Carotenoids Intake				P^a	P^b
	Quartle 1[§]	Quartle 2	Quartle 3	Quartle 4		
α-carotene						0.922
woman	1	0.75 (0.31, 1.81)	0.52 (0.20, 1.34)	0.74 (0.25,2.17)	0.347	
man	1	1.12 (0.41, 3.05)	1.95 (0.69, 5.55)	0.38 (0.12, 1.23)	0.416	
β-carotene						0.238
woman	1	0.60 (0.25, 1.45)	0.70 (0.28, 1.78)	0.66 (0.24, 1.82)	0.380	
man	1	0.96 (0.33, 2.82)	0.65 (0.23, 1.83)	0.25 (0.08, 0.84)	0.023	
Lutein/zeaxanthin						0.232
woman	1	0.61 (0.24, 1.53)	0.49 (0.20, 1.22)	0.58 (0.21, 1.59)	0.148	
man	1	0.20 (0.07, 0.60	0.40 (0.13, 1.26)	0.14 (0.04, 0.45)	0.006	
β-cryptoxanthin						0.541
woman	1	0.52 (0.20, 1.37)	0.77 (0.29, 2.05)	0.73 (0.29, 1.83)	0.690	
man	1	1.40 (0.43, 4.56)	0.76 (0.34, 1.27)	1.60 (0.59, 4.32)	0.480	
Lycopene						0.419
woman	1	0.33 (0.13, 0.83)	0.35 (0.14, 0.89)	0.29 (0.11, 0.82)	0.006	
man	1	0.93 (0.32, 2.71)	0.54 (0.19, 1.55)	0.46 (0.16, 1.37)	0.102	
Total carotenoids[†]						0.312
woman	1	0.41 (0.16, 1.04)	0.58 (0.24, 1.44)	0.56 (0.20, 1.55)	0.316	
man	1	0.70 (0.24, 2.05)	0.54 (0.19, 1.57)	0.28 (0.09, 0.88)	0.027	

Note. Abbreviations: *OR*, odds ratio; *CI*, confidence interval. Unconditional logistic regression analyses to *OR*s and 95% *CI*s. Covariates adjusted for age, gender, marital status, education level, smoking, alcohol drinking, tea drinking, total energy, calcium intake, roasted chili consumption, fuel type, roasting food, use of an improved oven rs11968525 genotype and urinary fluoride level; [§]Quartle 1 was the reference quartle. [†]Total carotenoids indicate the sum of α-carotene, β-carotene, β-cryptoxanthin, lycopene and lutein/zeaxanthin. [a]*P* value for linear trend, [b]*P* value for interaction.