RESEARCH COMMUNICATION

Functional SNPs in Human C20orf54 Gene Influence Susceptibility to Esophageal Squamous Cell Carcinoma

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Abstract

Objectives: C20orf54, also known as a human riboflavin transporter 2 (RFT2), encodes an open reading frame protein RFT2 newly identified to play an important role in esophageal carcinogenesis by modulating riboflavin uptake. Missense cSNPs on exon 3,1172 C>A (T391M) and 1246A>G (I416V) have been suggested to modulate protein expression. The aim of present study was to explore the association of C20orf54 functional SNPs with susceptibility to esophageal squamous cell carcinoma (ESCC) in a northern Chinese population. Methods: 240 patients with ESCC and 198 healthy individuals without overt cancer were chosen as our experimental subjects. Information about family address, sex, age, BMI, smoking and drinking habits and family history of cancer were collected. Blood samples were taken from all subjects and tumor tissues were freshly sampled from resected specimens. After DNA was extracted and amplified, the C20orf54 SNPs were sequenced by ABI 3730XL in BGI China. Freqencies were then calculated and associated with the collected suspicous risk factors. Results: Drinking status, a family history of ESCC, blood type and BMI were found to have great influence on the risk of developing ESCC. Overall genotype frequencies of the RFT2 SNP 1172 C>A (rs3746803) and 1246A>G (rs3746802) in ESCC patients are significantly different from that in healthy controls (x2=13.10, P=0.001 and x2=7.97, P=0.019, respectively). For RFT2 rs3746803, C/T+T/T genotype did not show a relationship with the risk of ESCC (the age and gender adjusted OR=0.66, 95% CI=0.41-1.05) when using C/C genotype as the reference. For RFT2 rs3746802, the A/G +G/G genotype demonstrated a significantly decreased risk to the development of ESCC (the age and sex adjusted OR=0.53, 95% CI=0.34-0.84) with A/A as the reference. Conclusions: The present study suggests that the C20orf54 functional SNPs might be associated with a risk of ESCC development.

Keywords: *C20orf54* - esophageal squamous cell carcinoma - single nucleotide polymorphisms - China

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Introduction

ESCC (esophageal squamous cell carcinoma) is one of the most common reasons for cancer-related death in the world. China is one of the highest incidence areas, especially in Taihang Mountain (Ke, 2002). The long-term effect of ESCC therapy is still far from optimism. Therefore, identification of high risk people or early cases by genetic and molecular marker screening remains crucial for ESCC prevention.

In 2010, a GWAS (genome wide association study) analysis was performed by using technology of Illumina Human 610-Quad BeadChips among 1,089 patients with ESCC and 1,763 control cases of Chinese Han descent and 849 xinjiang Hazakh-uygur people. Firstly, we found one esophageal cancer susceptibility gene c20orf54, which locates in 20p13 and includes 2716 bps (NM_033409.3) and encodes riboflavin transporter 2 protein (RFT2) that was newly identified to play an important role in esophageal carcinogenesis by modulating riboflavin uptake (Siassi and Ghadirian, 2005; He et al., 2009). Riboflavin deficiency has been suggested to be one of the risk factors to ESCC (Siassi and Ghadirian, 2005) and riboflavin supplementation has already been reported to reduce this cancer risk (He et al, 2009). However, the relationship between C20orf54 gene and susceptibility to ESCC has not been reported so far. The aim of present study was to explore the C20orf54 functional SNP (Single Nucleotide Polymorphism) with the susceptibility to ESCC in a northern Chinese population.

Materials and Methods

Participants

Present study included 240 ESCC patients and 198 healthy individuals without overt cancer. The cases were

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outpatients for endoscopic examination or inpatients for tumor resection in Heping Hospital of Changzhi Medical College between 2008 and 2011. Histological type of tumor was detected on the basis of biopsies or resected specimens in the Department of Pathology of the same hospital. Among the 252 healthy subjects, 44 (17.4%) were excluded due to refuse to join the epidemiological study of ESCC or refuse to donate their blood samples. All the subjects with and without ESCC were related Han nationality from Chang Zhi city or the surrounding regions. Information about family address, sex, age, BMI (Body Mass Index), smoking and drinking habits and family history were obtained from all subjects by face-to-face or telephone interviews. Individual who smokes/ smoked/ have smoked 5 cigarettes per day or more for at least 2 years is defined as smoker. Individuals who have at least one first degree relative or two second degree relatives with esophageal cancer are defined as having family history. The study protocol was approved by the Medical Ethics and Human Clinical Trial Committee of Changzhi Medical College and informed consent was provided after the explanation from all participants.

DNA extraction

Five milliliters of venous blood from each individual was added into Vacutainer tubes containing EDTA and stored at 4°C. Genomic DNA was extracted within one week after being collected. Purification of genomic DNA from whole blood was done by using the Maxwell® 16 System. High concentration (>100ng/μl) genomic DNA was directly used for PCR reaction after a short centrifugation.

Selection of C20orf54 SNPs

C20orf54 gene has 9 missense cSNP (coding SNP) sites in 54 exons (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi Gene Id=113278). We selected 7 of 9 cSNP sites as our objects. Experimental results showed that there were 4 missense cSNP sites (mRNA POS 860, 1139, 1172, 1246) in ESCC patients, especially in 1139, 1172 and 1246 sites, three missense cSNP sites with higher frequency, and present essay will discuss the missense cSNP sites in 1172and 1246.

Genotyping

C20orf54 gene was genotyped by PCR method. PCR primers used to amplify RFT2 gene were 5'-GGAGTTCCCAGAGCTTTGGTGTCCG-3' (forward primer) and 5'-CATAGGACAGGCAGGAGTAGGT-3' (reverse primer). PCR reaction were conducted in a 20μl tube containing 100ng DNA template, 2.0μl of 10×PCR buffer, 1.5mmol MgCl2, 1U Taq DNA-polymerase (Sangon, Shanghai, China), 200 μmol dNTPs and 200 nmol each primer. PCR cycling conditions were 5min at 95°C followed by 35 cycles at 95°Cfor 1min, 65°C for 45s, 72°C for 1min and 72°C for 10min. A 5μl PCR product was obtained and subjected to separating on a 2% agarose gel stained with ethidium bromide. After electrophoresis, the products were sequenced by ABI (Applied Biosystems) 3730XL in BGI (Beijing Genomics Institute) China. The frequency of C20orf54 SNPs were then calculated and associated with the collected suspicous risk factors.

Table 1. Demographic Characteristics of ESCC Patients and Healthy Controls

	Controls n (%)	ESCC n (%)	P -value
Sex			
Male	130(65.7)	159(66.3)	0.92
Female	68(34.3)	81(33.8)	
Age (years)			
<55	76(38.4)	84(35.0)	0.46
≥55	122(61.6)	156(65.0)	
Mean age(SD)	56.9±9.3	58.4±7.9	0.07
Smoking status			
Ex- or current smokers	99(50.0)	134(55.8)	
Non-smokers	99(50.0)	106(44.2)	0.22
Drinking status			
Ex- or current drinker	73(36.9)	112(46.7)	
Non- drinker	125(63.1)	128(42.4)	0.04*
Family history of ESCC			
Positive	36(18.2)	66(27.5)	0.01**
Negative	162(81.8)	174(72.5)	
Blood type			
A	42 (21.2)	71 (29.6)	0.02*
B	71 (35.9)	63 (26.3)	
O	74 (37.4)	81 (33.8)	
AB	11 (5.6)	25 (10.4)	
BMI			
<18.5	21(10.6)	50 (20.8)	0.00**
18.5-24	112 (56.6)	156 (65.0)	
>24	65 (32.8)	34 (14.2)	

*P<0.05; **P<0.01

Statistical analysis

Parametric data were expressed as mean ± standard deviation while categorical data were presented as number (percentage). Hardy-Weinberg equilibrium (HWE) was performed to compare the genotype frequencies of observed and expected using the free online software dedicated for HWE test (http://www.kursus.kvl.dk/shares/vetgen/_Popgen/genetik/applets/kitest.htm). Comparision of C20orf54 genotype distribution in the study groups was performed by means of two-sided contingency tables using the chi-square test. The odds ratio (OR) and its 95% confidence interval (95%CI) were calculated using an unconditional logistic regression model and adjusted for age and sex. A probability level of less than 5% was considered significant for all statistical analysis. Statistical analysis was performed using the SPSS18.0 software (SPSS Inc., Chicago, IL, USA).

Results

The demographic distribution of cancer patients and healthy controls was shown in Table 1. The mean age for ESCC patients was 58.4±7.9 years (ranged 41-81) and that of control was 56.9±9.3 years (ranged 40-77). There was no statistical difference between them (P=0.076). The sex distribution in ESCC patients (66.3% men) was comparable with that in healthy controls (65.8% men) (P=0.920) and the proportion smokers also was not significantly different from that in healthy controls (55.8% versus 50.0%, x2=1.48, P=0.223). However,

Table 2. Correlation Analysis of the Rs3746803 SNP with the Risk of the Development of ESCC

	C/C		C/T+T/T		OR(95% CI)[a]
	n	%	n	%	
Overall					
Control	150	75.8	48	24.2	1.00 (ref.)
ESCC	198	82.5	42	17.5	0.66 (0.41-1.05)
Smoker					
Control	76	76.8	23	23.2	1.00 (ref.)
ESCC	107	79.9	27	20.1	0.85 (0.45-1.61)
Non-smoker					
Control	74	74.7	25	25.3	1.00 (ref.)
ESCC	91	81.3	15	14.2	0.47 (0.23-0.97)
Drinker					
Control	56	76.7	17	23.3	1.00 (ref.)
ESCC	91	81.3	21	18.8	0.78 (0.38-1.62)
Non-drinker					
Control	94	75.2	31	24.8	1.00 (ref.)
ESCC	107	83.6	21	16.4	0.60 (0.32-1.12)
Positive family history					
Control	30	83.3	6	16.7	1.00 (ref.)
ESCC	53	80.3	13	19.7	1.09 (0.37-3.25)
Negative family history					
Control	120	74.1	42	25.9	1.00 (ref.)
ESCC	145	83.3	29	16.7	0.57 (0.34-0.98)
Blood type A					
Control	34	81.0	8	19.0	1.00 (ref.)
ESCC	62	87.3	9	12.7	0.62 (0.22-1.78)
Blood type B					
Control	59	83.1	12	16.9	1.00 (ref.)
ESCC	50	79.4	13	20.6	1.24 (0.52-2.98)
Blood type O					
Control	50	67.6	24	32.4	1.00 (ref.)
ESCC	66	81.5	15	18.5	0.47 (0.22-0.99)
Blood type AB					
Control	7	63.6	4	36.4	1.00 (ref.)
ESCC	20	75.0	5	20.0	0.44 (0.08-2.33)
BMI<18.5					
Control	15	71.4	6	28.6	1.00 (ref.)
ESCC	41	82.0	9	18.0	0.64 (0.18-2.29)
BMI 18.5-24					
Control	87	77.7	25	22.3	1.00 (ref.)
ESCC	130	83.3	26	16.7	0.69 (0.37-1.29)
BMI>24					
Control	48	73.8	17	26.2	1.00 (ref.)
ESCC	27	79.4	7	20.6	0.74 (0.27-2.01)

[a]The age and sex-adjusted odds ratio of the C/T+T/T genotyoe against the CC genotyoe

Table 3. Correlation Analysis of the Rs3746802 SNP with the Risk of the Development of ESCC

	A/A		A/G+G/G		OR(95% CI)[a]
	n	%	n	%	
Overall					
Control	143	72.2	55	27.8	1.00 (ref.)
ESCC	199	82.9	41	17.1	0.53 (0.34-0.84)
Smoker					
Control	72	72.7	27	27.3	1.00 (ref.)
ESCC	110	82.1	24	17.9	0.60 (0.32-1.12)
Non-smoker					
Control	71	71.7	28	28.3	1.00 (ref.)
ESCC	89	84.0	17	16.0	0.46 (0.23-0.92)
Drinker					
Control	51	69.9	22	30.1	1.00 (ref.)
ESCC	96	85.7	16	14.3	0.39 (0.19-0.82)
Non-drinker					
Control	92	73.6	33	26.4	1.00 (ref.)
ESCC	103	80.5	25	19.5	0.68 (0.37-1.24)
Positive family history					
Control	28	77.8	8	22.2	1.00 (ref.)
ESCC	57	86.4	9	13.6	0.50 (0.17-1.47)
Negative family history					
Control	115	71.0	47	29.0	1.00 (ref.)
ESCC	142	81.6	32	18.4	0.55 (0.33-0.92)
Blood type A					
Control	30	71.4	12	28.6	1.00 (ref.)
ESCC	64	90.1	7	9.9	0.27 (0.09-0.77)
Blood type B					
Control	57	80.3	14	19.7	1.00 (ref.)
ESCC	50	79.4	13	20.6	1.03 (0.44-2.41)
Blood type O					
Control	49	66.2	25	33.8	1.00 (ref.)
ESCC	65	80.2	16	19.8	0.48 (0.23-1.00)
Blood type AB					
Control	7	63.6	4	36.4	1.00 (ref.)
ESCC	20	80.0	5	20.0	0.34 (0.06-1.94)
BMI<18.5					
Control	15	71.4	6	28.6	1.00 (ref.)
ESCC	42	84.0	8	16.0	0.52 (0.14-1.92)
BMI 18.5-24					
Control	85	75.9	27	24.1	1.00 (ref.)
ESCC	130	83.3	26	16.7	0.64 (0.35-1.17)
BMI>24					
Control	43	66.2	22	33.8	1.00 (ref.)
ESCC	27	79.4	7	20.6	0.51 (0.19-1.37)

[a]The age and sex-adjusted odds ratio of the A/G+G/G genotyoe against the A/A genotyoe

drinkers were significantly more frequent among ESCC patients (46.7%) than among healthy controls (37.2%) (P=0.045). Therefore, drinking significantly increased the risk of ESCC with age and sex adjusted OR of 1.78 (95%CI=1.12-2.83). In addition, the frequency of a positive family history of UGIC (Upper Gastrointestinal Cancers) among ESCC patients (27.5%) was significantly higher than that in healthy controls (18.2%) (P=0.014). Therefore, a family history of UGIC obviously increased the risk of ESCC (age- and sex-adjusted OR=1.75, 95% CI= 1.10--2.79). The distribution of blood type in ESCC patients was significantly different from that in healthy controls (P=0.021), the proportion of blood type A, B, O and AB were 29.6%, 26.3%, 33.8% and 10.4% respectively in ESCC patients and 21.2%, 35.9%, 37.4% and 5.6% respectively in healthy controls. It can be deduced that blood type B obviously reduced the risk of ESCC with age and sex adjusted OR of 0.52 (95%CI=0.31-0.87). BMI in ESCC patients was significantly different from that in healthy controls (P=0.000).The percent of ESCC patients for BMI<18.5, 18.5-24 and >24 were 20.8%, 65.0% and 14.2% respectively, however 10.6%, 56.6% and 32.8% respectively in healthy controls, which indicated BMI<18.5 and 18.5-24 could influence ESCC incidence with age and sex adjusted OR of 4.52 (95%CI=2.34-8.72) and OR of 2.70 (95%CI=1.67-4.37).

Genotyping of RFT2 rs3746803 and rs3746802 SNP was successfully sequenced in all subjects. The results from the re-genotyped samples completely matched that from original ones (Figures 1, 2). The distribution of RFT2 rs3746803 (Figure 3) and rs3746802 (Figure 4) SNP was not correlated with gender and age both in ESCC patients

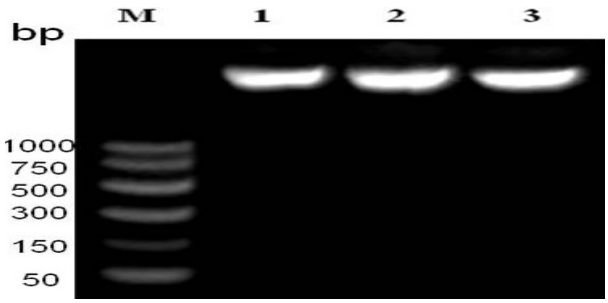

Figure 1. Electrophresis of Genomic DNA in ESCC Patients and Healthy Controls. M: DNA maker; Lane 1, 2, 3: genomic DNA; bp: base pair

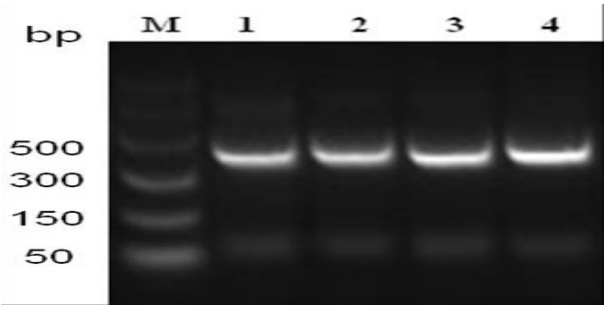

Figure 2. Electrophresis of PCR Product of Target DNA in ESCC and Controls. M: DNA maker; Lane 1, 2, 3, 4: PCR product of genomic DNA; bp: base pair

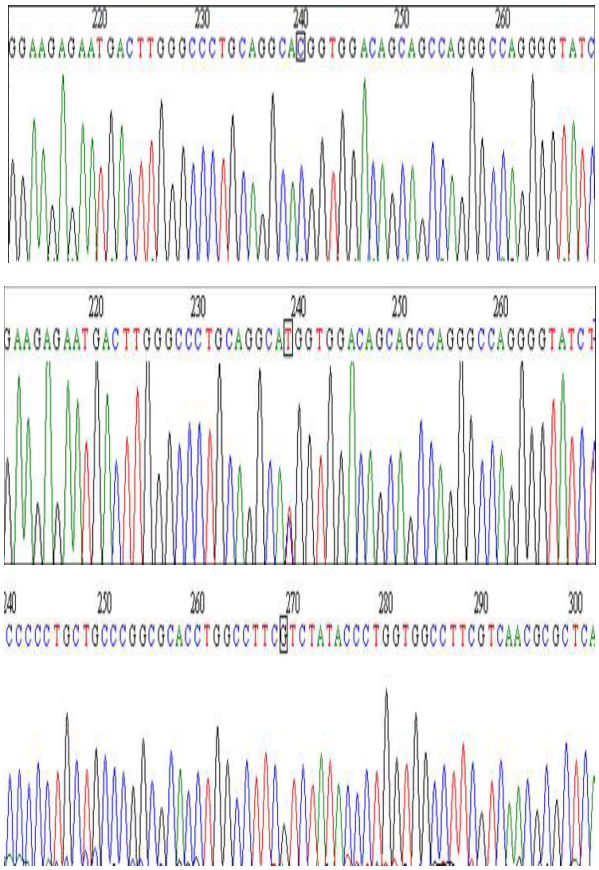

Figure 3. DNA Sequencing of rs3746803 SNP in ESCC Patients and Healthy Controls. A: genotype C/C; B: genotype C/T; C: genotype T/T

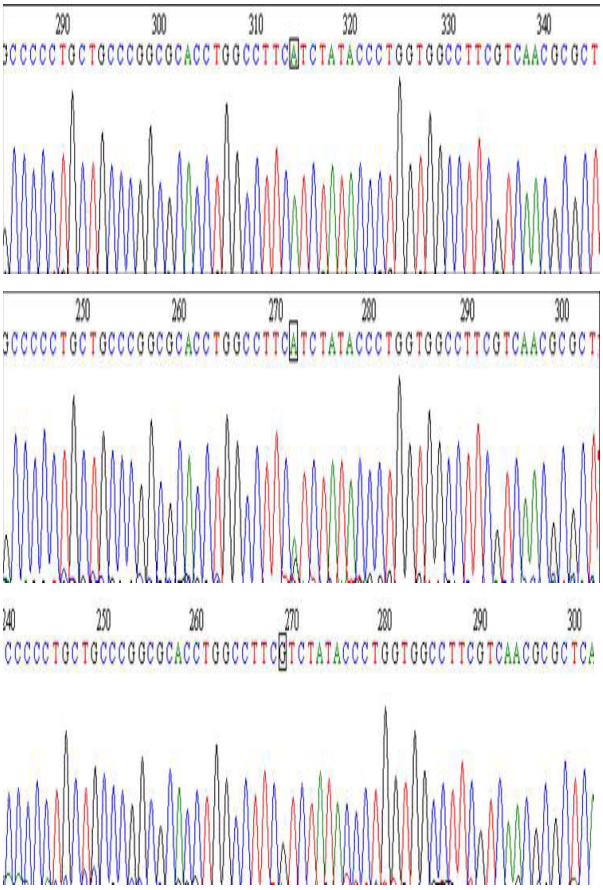

Figure 4. DNA Sequencing of rs3746802 SNP in ESCC Patients and Healthy Controls. A: genotype A/A; B: genotype A/G; C: genotype G/G

or healthy controls (data not shown). The genotype distribution among healthy controls did not significantly deviate from that expected for a Hardy--Weinberg equilibrium (x^2=3.77, P=0.523 and x^2=3.00, P=0.083). Considering that smoking status, drinking status, a family history of UGIC (upper gastrointestinal cancers), blood type and BMI index have been confirmed to modify risk of ESCC, it is essential to make stratification analysis in present study. As shown in Table 2, the overall genotype frequencies of the RFT2 rs3746803 and rs3746802 SNP in ESCC patients were significantly different compared with that in healthy controls (x^2=13.10, P=0.001 and x^2=7.97, P=0.019). For RFT2 rs3746803 gene, C/T +T/T genotype didn't show correlation with the risk of ESCC (age and gender adjusted OR=0.66, 95% CI=0.41-1.05) by using C/C genotype as the reference. Stratification analysis showed that in non-smokers, C/T+T/T genotype significantly reduced ESCC incidence compared with C/C genotype (age- and sex-adjusted OR=0.47, 95% CI=0.23--0.97). In the subjects with a negative family history of UGIC, C/T+T/T genotype significantly decreased the risk of developing ESCC compared with C/C genotype (age- and sex-adjusted OR=0.57, 95% CI=0.34--0.98). Conversely, in the subjects with a positive family history of UGIC, C/T and T/T genotype both increased the risk of development of ESCC, but without statistical significance. For the subjects with blood type O, C/T+T/T genotype obviously decreased the risk of ESCC compared with C/C genotype (age- and sex-adjusted OR=0.30 and 0.47, 95% CI=0.22-0.99).

For RFT2 rs3746802 gene, A/G +G/G genotype significantly reduced ESCC risk (the age and gender

adjusted OR=0.53, 95% CI=0.34-0.84) by using A/A genotype as the reference (Figure 7,8). Stratification analysis demonstrated that in non-smokers A/G +G/G genotype significantly decreased the risk of ESCC compared with A/A genotype (age- and sex-adjusted OR=0.46, 95% CI=0.23--0.92). For drinkers A/G +G/G genotype significantly also decreased the risk of ESCC compared with the A/A genotype (age- and sex-adjusted OR=0.39, 95% CI=0.19--0.82). In the subjects with a negative family history of UGIC, A/G+G/G genotype obviously decreased ESCC incidence compared with the A/A genotype (age- and sex-adjusted OR=0.55, 95% CI=0.33-- 0.92).

Discussion

The development of ESCC relates to a complex interactive process between multiple genetic susceptibilities and environmental exposure. Epidemiological and etiological studies have verified that environmental and genetic factors play crucial roles in esophageal carcinogenesis (Siassi and Ghadirian, 2005; Fujimura et al., 2010).

Tobacco smoking and alcohol consumption are the two main environmental factors for ESCC. Over 80% ESCC patients in developed countries (Lee et al., 2007; Islami and Kamangar F, 2008; Pandeya et al., 2008; Ishiguro et al., 2009; Pandeya et al., 2009) smoke and drink, especially in Europe and North America (Daniel et al., 1983; McCormick, 1989; Said, 2004). In high- prevalent areas, even a strong trend of familial aggregation of ESCC appears (Middleton, 1990; Tomei et al., 2001), indicating that genetic susceptibility in combination with environment exposure contributes to the high incidence rates of ESCC in these areas. However, smoking and drinking seem to be the minor risk factors for ESCC in the high-incidence areas of China (Said and Arianas, 1991; Said and Ma, 1994). High body mass index (BMI) has been suggested as a high risk factor for esophageal adenocarcinoma in western countries (Said et al., 2000; Fujimura et al., 2010). In current study, alcohol drinking, BMI and family history seems to exert effect on ESCC in China. This might suggest that C20orf54 expression may interact with the metabolic changes induced by smoking and drinking and influence the susceptibility to ESCC. Present study also found that Blood type B had significantly decreasing ESCC compared with blood type A.

ESCC has a striking geographic distribution worldwidely, with higher prevalence in some areas of China (Yamamoto et al., 2009), central Asia and southern Africa, where nutritional deficiency, intake of pickled vegetables, nitrosamine-rich or mycotoxin- contaminated foods and low socioeconomic status are likely to contribute to ESCC (McCormick, 1972; Ke, 2002; Powers, 2003; He et al., 2009). Previous investigation suggested that dietary staples, particularly foods lack of riboflavin, were closely related with esophageal cancer incidence, and vice versa (Van, 1981). The joint U.S.-China nutritional intervention study in Lin county found that the odds ratio for subjects in the treatment group (versus those in the placebo group) having esophageal dysplasia or cancer was 0.84 and riboflavin was a protective factor for esophageal cancer incidence through the way of protecting the integrity of esophageal epithelial (Van, 1981; Taylor et al., 1995). Riboflavin deficiency can increase the activity of nitrosamine metabolism activation enzyme, thereby promote metabolic activation of nitrosamines. In view of this, the researchers' study by implementation of riboflavin fortified nutrient salt interventions in high prevalent areas of esophageal cancer, which shows that riboflavin fortified nutrient salt can increase body level of riboflavin, ameliorate pathological changes of esophageal mucosa, delay and reverse processes of esophageal precancerous lesions, reduce the risks and of esophageal cancer incidence (Siassi and Ghadirian, 2005; He et al., 2009), but the effects of intervention had obviously individual differences.

Our previous GWAS study on ESCC successfully identified C20orf54 gene as one susceptibility loci to ESCC in Han Chinese people. Therefore, we speculate that C20orf54 gene variation may be the reason of dietary supplementation of riboflavin for ESCC but with significantly individual differences. Present study demonstrates that wild type of C20orf54 rs3746803 SNP CC and rs3746802 SNP AA was more common in ESCC cases, indicating that during evolution course, with mutation of other ESCC key susceptibility genes, the function of C20orf54 might be comparatively enhanced to strengthen the ability of riboflavin transportation for epithelial repair.

Present study also find that serous concentration of riboflavin is low in high incidence area people compared with that in low occurrence area, and even low in ESCC patients (Ji et al., 2011). Further IHC (Immunohistochemistry) study shows that the RFT2 staining is stronger in ESCC tissues than that in adjacent normal epithelium (unpublished data). We suppose that when rs3746803 SNP C->T and rs3746802 SNP A>G, the riboflavin transportation function of C20orf54 should be further strengthened to increase repairing ability of esophageal epithelial cells against cancer. However, if intake of food high in riboflavin is insufficient, enhanced function of C20orf54 might not work, and the ability of repairing could be correspondingly reduced. Furthermore, in the subjects having a positive family history of UGIC, C/T and T/T genotype both increase the risk of developing ESCC. Present study demonstrates that inherited susceptibility is also crucial for esophageal carcinogenesis, as suggested by familial aggregation (Chang-Claude et al., 1997; Shao et al., 1997), family history (FH) of cancer (Ghadirian., 1985; Tavani et al, 1994; Dhillon et al., 2001; Morita et al., 2002; Garavello et al., 2005; Akbari et al., 2006; Murtaza et al, 2006), race studies (Carter et al., 1992; Zhang et al., 2000), and gene association studies of participants (Kimura et al., 2005; Zhang et al, 2005 Larsson et al., 2006; Yang et al., 2007).

In conclusion, our preliminary study demonstrates that functional SNP in C20orf54 gene may modifiy susceptibility to sophageal squamous cell carcinoma.

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