Mitogen/Extracellular Signal-Regulated Kinase Kinase-5 Promoter Region Polymorphisms Affect the Risk of Sporadic Colorectal Cancer in a Southern Chinese Population

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Mitogen/extracellular signal-regulated kinase kinase-5 (MEK5), which belongs to a network of mitogen-activated protein kinase pathways, play a pivotal role in carcinogenesis. The purpose of this study was to investigate whether variants in the *MEK5* gene promoter were involved in susceptibility of individuals to sporadic colorectal cancer (CRC). In the present hospital-based case-control study of 737 patients with sporadic CRC and 703 healthy control subjects in a southern Chinese population, the two polymorphisms of *MEK5* promoter (i.e., rs7172582C>T and rs3743354T>C) were genotyped by TaqMan assay. There were significant differences between cases and controls in the genotype and allele distribution of the *MEK5* gene rs3743354T>C polymorphism. The rs3743354 CC genotype was associated with a significantly decreased risk of CRC when compared with the TT genotype (adjusted odds ratios [ORs]=0.43; 95% confidence interval [CI], 0.24–0.77). Compared to the T allele, a significant correlation was detected between the presence of the C allele and decreased risk of CRC (adjusted OR=0.79; 95% CI, 0.61–0.94). The decreased risk of CRC associated with rs3743354 variant genotypes (i.e., CT+CC) was found in the smoker subgroup (adjusted OR=0.63; 95% CI=0.45–0.88). Further, environmental factors, including smoking and drinking, interacted with rs3743354C variant genotypes to reduce CRC risk. Western blot analysis showed that the levels of MEK5 protein in sporadic CRC neoplastic tissues and adjacent normal colorectal epithelium tissues were lower in the carriers of rs3743354 CC genotypes than that in those with rs3743354 TT genotypes or those with rs3743354 TC genotypes. However, no significant association was found between the rs7172582C>T polymorphism and risk of CRC. These data indicate that the rs3743354 polymorphism in the *MEK5* promoter may affect the risk of developing CRC.

Introduction

COLORRECTAL CANCER (CRC) IS a common malignant disease and constitutes the second most common cause of all cancer-related mortality in the Western world (Potter, 1999; Jemal *et al.*, 2007; Coleman *et al.*, 2008). Although the incidence of CRC in China is lower than that in the West, it has increased in recent years and has become a substantial cancer burden in China, particularly in the more developed provinces such as Guangdong (Li *et al.*, 2007; Xu *et al.*, 2010; Zhao *et al.*, 2010). The carcinogenesis of CRC is often a multistep process and possibly a consequence of a complex interaction between multiple factors, both endogenous and

environmental (Ahmed, 2006). The published environmental risk factors of developing CRC include smoking (Tsong *et al.*, 2007; Nisa *et al.*, 2010), drinking (Tsong *et al.*, 2007), body mass index (BMI) (Moghaddam *et al.*, 2007), and dietary factors. These are stressors to the cells and in turn activate some important signal transduction cascades including mitogen-activated protein kinase (MAPK) pathways, leading to apoptosis, inflammation, and tumorigenesis.

Mitogen/extracellular signal-regulated kinase kinase-5 (MEK5), also known as MAP2K5, belongs to a network of MAPK pathways. MEK5 in turn specifically phosphorylates and activates extracellular signal-regulated kinase-5 (ERK5) (Zhou *et al.*, 1995), which directly phosphorylates and

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activates several transcription factors including c-Myc, Sap-1, c-Fos, Fra-1, and myocyte enhancer factor family members (Kato *et al.*, 1997; English *et al.*, 1998), eliciting a variety of biological responses to extracellular signals that include cytokines, growth factors, and various stress stimuli (Roberts *et al.*, 2009). As the only kinase upstream of ERK5, MEK5 has two splice variants MEK5 α and MEK5 β . The expression of the MEK5 β protein is greater than that of MEK5 α in terminally differentiated tissues, whereas MEK5 α expression is greater in mitotically active tissues such as the liver. MEK5 α directly stimulates ERK5 activity, whereas MEK5 β plays a kinase-dead dominant-negative role that suppresses ERK5 signaling (Seyfried *et al.*, 2005). A growing number of studies showed that overexpression of MEK5 α is associated with tumorigenesis and malignancies (Cameron *et al.*, 2004; Song *et al.*, 2004; Ghosh *et al.*, 2005) and the expression ratio of MEK5 α to MEK5 β is higher in cancer cell lines, whereas overexpression of MEK5 β inhibits serum-induced DNA synthesis (Cameron *et al.*, 2004). Therefore, alternative splicing of MEK5 α and MEK5 β may play a pivotal role in ERK5 activation and subsequent carcinogenesis. Further, it is thought that MEK5 is directly involved in the progress of malignancies and tumorigenesis (Song *et al.*, 2004). There are many studies suggesting that MEK5 plays a critical role in cancer occurrence and progression, such as prostate cancer (Mehta *et al.*, 2003; McCracken *et al.*, 2008), breast cancer (Li *et al.*, 2008), hepatocellular cancer (Yan *et al.*, 2009), and lung cancer (Winn *et al.*, 2006). However, the association between MEK5 and CRC remains unclear.

Single-nucleotide polymorphisms (SNPs, i.e., small genetic changes of single-base nucleotides in DNA) vary among individuals, and this variance is a susceptibility in cancer (Lo *et al.*, 2007; Li *et al.*, 2009), particularly when these SNPs are located within regulating regions (Pan *et al.*, 2010). Genetic variations in the promoter region may affect its functions. In addition, some environmental factors can affect gene activity by acting on the promoter region. We previously demonstrated that the polymorphism rs3826392 (-1304T>G) in the promoter of the mitogen-activated protein kinase kinase 4 (MKK4) gene, which also belongs to a network of MAPK pathways, is associated with decreased risk of sporadic CRC. Further, the gene-environmental interaction analysis showed that the age and alcohol drinking status interacted with rs3826392 G variant genotypes to reduce cancer risk (Wei *et al.*, 2009). Several SNPs in the promoter of the MEK5 gene have been described. We investigated whether the genetic variations within the promoter region of the MEK5 gene, and their possible interactions with environmental factors, play a role in the risk of sporadic CRC.

The aims of this case-control study were to clarify the impact of two common MEK5 SNPs in the promoter region on CRC risk in a Chinese population and the gene-environmental interactions between potential confounders and to investigate these SNPs. Moreover, we analyzed the correlation between a significant MEK5 promoter region SNP and protein expression levels in sporadic CRC neoplastic and adjacent normal colorectal epithelium tissues.

Materials and Methods

Study subjects and sample collection

This study population included 737 sporadic CRC patients and 703 cancer-free controls. All enrolled subjects were un-

related Chinese individuals and were from Guangzhou City and surrounding regions in South China. All patients were recruited between July 2002 and March 2009 at the Sixth Affiliated Hospital (Gastrointestinal and Anal Hospital) of Sun Yat-Sen University (Guangzhou, China), Sun Yat-Sen University Cancer Center (Guangzhou, China), the First Affiliated Hospital of Sun Yat-Sen University (Guangzhou, China), the Affiliated Tumor Hospital of Guangzhou Medical College (Guangzhou, China), Guangdong Provincial People's Hospital (Guangzhou, China) and Panyu People's Hospital (Guangzhou, China). Cases suffering from hereditary nonpolyposis CRC, familial adenomatous polyposis, or inflammatory bowel disease and other related malignancies were excluded. All patients were untreated sporadic CRC cases and were histopathologically diagnosed and recruited with a response rate of about 95% in our study. Cancer-free controls were randomly selected from about 12,000 individuals who participated in the healthy checkup programs in the community health stations conducted in Guangzhou City during the same time period when the cases were recruited, with a response rate of about 85%. These control subjects were frequency-matched to the cases on age (65 years) and sex. Each participant first gave an informed consent and was scheduled for an interview, in which a structured questionnaire was administered by trained interviewers to collect data on smoking status, alcohol use, and other factors including sex, age, family history of cancer, and BMI. Smoking status, alcohol use, and family history of cancer were defined as previously described (Wei *et al.*, 2009). The BMI was categorized by the cutoff points suggested by the Cooperative Meta-Analysis Group of Working Group on Obesity in China (Bei-Fan, 2002). Subjects whose BMI was ≤ 23.9 kg/m² were categorized as being underweight or normal body weight, subjects whose BMI was from 24.0 to 27.9 kg/m² were defined as being overweight, and those with BMI ≥ 28.0 kg/m² were categorized as obese. Subjects who have any first- or second-degree relative (or both) with a history of cancer were defined as "Yes" and the remaining subjects as "No." Each subject was asked to donate 5 mL of peripheral venous blood after giving informed consent. The study was approved by the institutional review boards of Sun Yat-Sen University.

SNP selection

Based on the GenBank dbSNP database (www.ncbi.nlm.nih.gov), all reported SNPs in the MEK5 gene with a minor allele frequency (MAF) of >0.05 in Asian descendents were included. We identified three common SNPs (i.e., with an MAF of $\geq 5\%$) for the MEK5 promoter, namely rs7172582C>T, rs3743353C>T, and rs3743354T>C. The linkage disequilibriums (LDs) of the three SNPs were unknown. To validate these polymorphisms in Chinese population, we amplified the MEK5 promoter region (from -1200 to +126 nt) in 40 normal Chinese (control subjects of our study) by polymerase chain reaction (PCR). The amplified fragments were sequenced by Invitrogen with an Applied Biosystems ABI 3730 DNA analyzer (Fig. S2). The results were similar to the data from GenBank dbSNP database, and the results showed that rs3743354 and rs3743353 were in strong LD ($D' = 0.880$, $\gamma^2 = 0.852$), whereas rs3743354 and rs7172582 were in weak LD ($D' = 0.440$, $\gamma^2 = 0.268$). Therefore, we genotyped rs7172582 and rs3743354 in the subjects of our study.

Genotyping

Genomic DNA was extracted from peripheral blood using a QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's protocol. DNA purity and concentrations were determined by measurement of spectrophotometric absorbance at 260 and 280 nm. The corresponding primers and probes were designed using Primer Express 1.5 software (Applied Biosystems) and were purchased from Shanghai GeneCore BioTechnologies Co (Table S1). All samples were genotyped using an allelic discrimination assay on the Applied Biosystems 7500 FAST Real Time PCR instrument with TaqMan® probes. Real-time PCR was performed using 10 µL of TaqMan 2× universal master mix (Applied Biosystems), 2 µL of primer-probe (470 nM and 100 µM, respectively), 7 µL of DNase- and RNase-free water (Sigma), and 1 µL of sample DNA, in a total volume of 20 µL per single tube reaction. DNase-free water was used as a nontemplate control and DNA of the known MEK5 genotype (appropriate to the SNP of interest) was used as a positive control in each assay run. Assay conditions were as follows: 2 min at 50°C, 10 min at 95°C, and 55 cycles of 95°C for 15 s and 60°C for 1 min. First, the SNP assay was set up using sequence detection systems (SDS), version 2.1, software (Applied Biosystems) as an absolute quantification assay, and then the plate was read using the allelic discrimination settings. Eventually, postassay analysis was performed using the SDS software (Fig. S1). Ten percent of the samples were randomly repeated for quality control, and the concordance rate was 100%. The PCR products of every genotype were purified and confirmed by direct sequencing.

Western blot analysis

To analyze the correlation between observed significant SNP and MEK5 protein expression levels *in vivo*, fresh tissue samples of CRC and the adjacent normal colorectal epithelium were collected at the Sixth Affiliated Hospital of Sun Yat-Sen University. All tumor samples were histologically confirmed.

The frozen tissues were homogenized in T-PER tissue protein extraction (Thermo) lysis buffer, which contained a protease inhibitor. The mixture was centrifuged at 12,000 g for 15 min at 4°C and the supernatant was obtained. Sixty micrograms of a protein extract were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto Polyvinylidene fluoride (PVDF) filters (Millipore Company). The membranes were blocked at 4°C overnight with blocking buffer (pH 7.6) containing 5% nonfat dry milk and incubated with rabbit anti-human monoclonal antibody against MEK5 (1:2000) purchased from BD Transduction Laboratories™. Immunoreactive proteins were stained using a chemiluminescence detection system. Membranes were then washed with stripping solution for 1 h and treated as described earlier but with mouse β-actin antibody (ProteinTech Group) as an internal control. The MEK5 protein expression levels were normalized to that of β-actin by calculating the relative expression levels.

Statistical analysis

Baseline sociodemographic characteristics between cases and controls were analyzed using the two-sided chi-square

TABLE 1. FREQUENCY DISTRIBUTIONS OF SELECTED VARIABLES IN COLORECTAL CANCER PATIENTS AND CANCER-FREE CONTROLS

Variables	CRC(n = 737)		Control(n = 703)	
	N	%	N	%
Age (years)				
≤ 49	165	22.4	174	24.8
50-60	200	27.1	218	31.0
≥ 61	372	50.5	311	44.2
Sex				
Male	449	60.9	442	62.9
Female	288	39.1	261	37.1
Smoking status				
Current	280	38.0	215	30.6
Former	80	10.9	111	15.8
Never	377	51.1	377	53.6
Drinking status				
Current	308	41.8	133	18.9
Former	35	4.7	35	5.0
Never	394	53.5	535	76.1
Family history of cancer				
Yes	80	10.9	61	8.7
No	657	89.1	642	91.3
BMI (kg/m ²)				
≤ 23.9	447	60.7	454	64.6
24.0-27.9	225	30.5	195	27.7
≥ 28.0	65	8.8	54	7.7
Site				
Colon	341	46.3		
Rectum	396	53.7		
Duke stage				
A	131	17.8		
B	226	30.7		
C	245	33.2		
D	135	18.3		

tests. The expected frequency of control genotypes was checked by the Hardy-Weinberg equilibrium test, which was tested by a goodness-of-fit chi-square test. LD between MEK5 polymorphisms was assessed by the 2LD program and the PROC ALLELE statistical procedure in SAS/Genetics (SAS Institute, Inc.) software. Odds ratio (OR) and 95% confidence intervals (CIs) were calculated to assess the relative risk conferred by a particular allele and genotype using an unconditional logistic regression model, with and without adjustment for age, sex, smoking status, alcohol drinking status, BMI, and family history of cancer. Logistic regression modeling was also used for the trend test. For the stratification analysis, we assessed the main effect of MEK5 in each subgroup and the interaction among the MEK5 polymorphisms and selected variables on cancer risk. A multiplicative interaction was indicated when $OR_{11} > OR_{10} \times OR_{01}$, in which OR_{11} = the OR when both factors were present, OR_{01} = the OR when only factor 1 was present, and OR_{10} = the OR when only factor 2 was present. Interaction terms between variables were modeled using standard unconditional logistic regression to assess evidence for departure from a multiplicative model (Wang *et al.*, 2005). All statistical analyses were two-sided and $p < 0.05$ was considered statistically significant.

Results

Characteristics of the study population

The distribution of demographic characteristics of the 737 sporadic CRC cases and 703 matched controls is presented in Table 1. The proportion of current smokers and current drinkers in CRC cases was higher than that in the controls (current smokers: 38.0% vs. 30.6%; current drinkers: 41.8% vs. 18.9%), suggesting the matching was not adequate enough. Therefore, these variables were further adjusted for in the multivariate logistic regression model, and the adjusted variables were used in the later stratification and gene-environment interaction analysis.

MEK5 genotype distribution and risk of sporadic CRC

Both the SNPs (rs7172582 and rs3743354) were successfully genotyped. The genotype and allele distributions of the rs7172582 and rs3743354 polymorphisms between the cases and controls are presented in Table 2. The genotype distributions of the two SNPs among the participants in the control sample did not deviate from the Hardy-Weinberg equilibrium. The LD analyses showed that the linkage among the two SNP loci in controls were relatively weak ($D' = 0.532$, $\gamma^2 = 0.206$), indicating every locus may have an independent effect on risk of sporadic CRC.

As revealed in Table 3, the frequency of the rs3743354 genotypes in the CRC patients (TT 64.5%, TC 32.8%, CC 2.7%) was significantly different from that in the control groups (TT 59.9%, TC 34.3%, CC 5.8%). Using the rs3743354 TT genotype as a reference, rs3743354 CC homozygotes have a significant 0.57-fold decreased risk of sporadic CRC (adjusted 95% CI, 0.24–0.77; $p = 0.004$). Compared to the T allele, a significant correlation was detected between the presence of the C allele and a decreased risk of developing CRC (OR = 0.79; 95%

CI, 0.61–0.94; $p = 0.011$). In addition, although our results for rs3743354 C variant genotypes (i.e., rs3743354 TC + CC) and the decreased risk of CRC did not reach statistical significance, the observed trend (adjusted OR = 0.83; 95% CI, 0.66–1.03; $p = 0.095$) indicates a somewhat protective effect. For the other SNP rs7172582, the genotype and allele frequencies did not significantly differ between the cases and the controls.

Stratification analysis of MEK5 genotypes and risk of sporadic CRC

A stratification analysis of the associations was conducted between the two SNPs' variant genotypes and the risk of sporadic CRC in subgroups by age, sex, smoking and drinking status, family history of cancer, and BMI. As shown in Table 3, a protected risk of the rs3743354 C variant genotypes associated with CRC achieved statistical significance in the subgroups of smokers (adjusted OR = 0.63; 95% CI, 0.45–0.88; $p = 0.007$). In the gene-environment interaction analysis, interactions between rs3743354 variant genotypes and smoking and drinking (p interaction = 0.006 and 0.001; Bonferroni correction, p interaction = 0.012 and 0.002) were observed, suggesting a possibility of more than multiplicative effects between rs3743354 C variant genotypes and smoking or alcohol drinking status. For the rs7172582 variant genotypes (i.e., rs7172582 CT + TT), however, no significant association with the risk of CRC was found in the stratification analysis.

Association of the rs3743354 polymorphism with MEK5 protein expression

To further analyze the association between rs3743354 polymorphism and the level of MEK5 protein expression, we collected 40 fresh tissue samples of CRC and the adjacent normal colorectal epithelium from the untreated sporadic CRC patients and found only one patient carrying the

TABLE 2. DISTRIBUTION OF GENOTYPES AND ALLELES IN MITOGEN/EXTRACELLULAR SIGNAL-REGULATED KINASE KINASE-5 PROMOTER AND RESULTS OF LOGISTIC REGRESSION ANALYSIS FOR ASSOCIATIONS WITH RISK OF SPORADIC COLORECTAL CANCER

Genotypes/alleles	CRC n (%)	Control ^a n (%)	p^b	Crude OR (95% CI)	Adjusted OR (95% CI) ^c
Total no. of subjects	737	703			
Total no. of alleles	1474	1406			
rs7172582			0.183		
CC	236 (32.0)	257 (36.6)		1.00 (ref.)	1.00 (ref.)
CT	374 (50.8)	337 (47.9)	0.106	1.21 (0.96–1.52)	1.22 (0.96–1.56)
TT	127 (17.2)	109 (15.5)	0.133	1.27 (0.93–1.73)	1.20 (0.87–1.67)
Trend test p -value				0.122	0.110
CT + TT	501 (68.0)	446 (63.4)	0.070	1.22 (0.98–1.52)	1.22 (0.97–1.53)
T allele	0.426	0.398	0.088		
rs3743354			0.008		
TT	475 (64.5)	421 (59.9)		1.00 (ref.)	1.00 (ref.)
CT	242 (32.8)	241 (34.3)	0.302	0.89 (0.71–1.11)	0.89 (0.71–1.13)
CC	20 (2.7)	41 (5.8)	0.003	0.43 (0.25–0.75)	0.43 (0.24–0.77)
Trend test p -value				0.223	0.257
CT + CC	262 (35.5)	282 (40.1)	0.074	0.82 (0.67–1.02)	0.83 (0.66–1.03)
C allele	0.191	0.230	0.011		0.79 (0.61–0.94)

^aThe observed genotype frequency among the control subjects was in agreement with the Hardy-Weinberg equilibrium ($p^2 + 2pq + q^2 = 1$) ($\chi^2 = 0.007$, $p = 0.932$ for rs7172582; $\chi^2 = 0.690$, $p = 0.406$ for rs3743354).

^bTwo-sided χ^2 test for the distribution of either genotype or allele frequency.

^cORs were adjusted in a logistic regression model that included age, sex, smoking status, drinking status, family history of cancer, and BMI. CRC, colorectal cancer; CI, confidence interval; OR, odds ratio.

TABLE 3. STRATIFICATION ANALYSIS OF THE RS3743354 GENOTYPES BY SELECTED VARIABLES IN SPORADIC CRC PATIENTS AND CONTROLS

Variables	CRC(n = 737)		Control(n = 703)		Crude OR(95%CI)	Adjusted OR(95%CI) ^a	P ^b
	TT n (%)	TC+CC n (%)	TT n (%)	TC+CC n (%)	TC+CC vs TT	TC+CC vs TT	
Age (years)							0.261
≤60	233(63.8)	132(36.2)	234(59.7)	158(40.3)	0.84(0.63-1.13)	0.85(0.63-1.15)	
≥61	242(65.1)	130(34.9)	187(60.1)	124(39.9)	0.81(0.59-1.11)	0.78(0.56-1.08)	
Sex							0.359
Male	295(65.7)	154(34.3)	264(59.7)	178(40.3)	0.77(0.59-1.02)	0.76(0.57-1.02)	
Female	180(62.5)	108(37.5)	157(60.2)	104(39.8)	0.91(0.64-1.28)	0.90(0.64-1.27)	
Smoking status							0.006
Ever	242(67.2)	118(32.8)	190(58.3)	136(41.7)	0.68(0.50-0.93)	0.63(0.45-0.88)	
Never	233(61.8)	144(38.2)	231(61.3)	146(38.7)	0.98(0.73-1.31)	0.97(0.72-1.31)	
Drinking status							0.001
Ever	227(66.2)	116(33.8)	99(58.1)	69(41.9)	0.73(0.50-1.07)	0.69(0.47-1.02)	
Never	248(62.9)	146(37.1)	322(60.2)	213(39.8)	0.89(0.68-1.16)	0.87(0.66-1.15)	
Family history of cancer							0.336
Yes	47(58.8)	33(41.2)	38(62.3)	23(37.3)	1.16(0.59-2.30)	1.15(0.54-2.48)	
No	428(65.1)	229(34.9)	421(59.9)	282(40.1)	0.79(0.63-0.99)	0.80(0.63-1.00)	
BMI(kg/m ²)							0.139
≤23.9	288(64.4)	159(35.6)	278(61.2)	176(38.8)	0.87(0.67-1.14)	0.88(0.66-1.16)	
24.0-27.9	146(64.9)	79(35.1)	113(57.9)	82(32.1)	0.75(0.50-1.11)	0.74(0.49-1.13)	
≥28.0	41(63.1)	24(36.9)	30(55.6)	24(44.4)	0.73(0.25-1.53)	0.71(0.32-1.55)	

^a ORs were adjusted for age, sex, smoking status, alcohol use, family history of cancer and BMI in a logistic regression model.

^b P Value of test for the multiplicative interaction between MEK5 polymorphisms rs3743354T>C and selected variables on cancer risk were calculated using standard unconditional logistic regression models.

rs3743354 CC genotype. Therefore, we chose two patients carrying the rs3743354 TT genotype and two patients carrying the rs3743354 TC genotype randomly and the patient with the rs3743354 CC genotype and then detected the MEK5 protein levels of these tissue samples of these patients by western blot. We found that the levels of MEK5 protein were lower in the individual carrying the rs3743354 CC genotype than that in those with the rs3743354 TT or rs3743354 TC genotypes (Fig. 1).

Discussion

To our knowledge, this is the first study to investigate whether MEK5 gene polymorphisms are related to the occurrence of sporadic CRC in a Chinese population. We found that the polymorphism rs3743354T>C in the MEK5 promoter is associated with decreased risk of CRC, particularly in the smoker subgroup. For the other SNP rs7172582C>T, no evidence was found for any association between this SNP

and sporadic CRC risk. Further, we found that the MEK5 protein was downregulated *in vivo* in individuals who carried the rs3743354 CC allele, suggesting that rs3743354T→C substitution may decrease MEK5 protein expression in sporadic CRC tissues.

The signaling pathways that regulate cell proliferation, survival, and transformation, such as MAPK pathways, are of great importance in cancer biology. A number of studies demonstrated that the MAPK pathway was aberrantly activated in CRC (Shan *et al.*, 2009). The MEK5/ERK5 pathway is sequentially regulated by a series of upstream MAP kinase kinases (MEKs) in a signaling cascade. Upon activation, the MAPKs selectively phosphorylate cellular targets, leading to regulation of gene expression and biologic events such as proliferation, differentiation, and apoptosis (Ghosh *et al.*, 2005). There are reports that demonstrate that the MEK5/ERK5 pathway was involved in tumorigenesis and malignancies (Mehta *et al.*, 2003; Dudderidge *et al.*, 2007; Li *et al.*, 2008). Dudderidge *et al.* (2007) showed that the induction of

FIG. 1. The association of rs3743354 polymorphism and mitogen/extracellular signal-regulated kinase kinase-5 (MEK5) protein expression. T, neoplastic tissue of colorectal cancer (CRC); P, adjacent normal colorectal epithelium tissue of CRC.

MEK5/ERK5 signaling is associated with activation of the DNA replication licensing pathway in prostate cancer. As the key upstream BMK1 kinase, MEK5 has two naturally occurring splice variants, MEK5 α and MEK5 β . Song *et al.* (2004) observed that MEK5 α may be Stat3-regulated and play a critical role in oncogenesis mediated by aberrantly activated Stat3 signaling in breast carcinomas and malignancies. Ghosh *et al.* (2005) showed that exogenous expression of c-myc promoter-binding protein 1 induces prostate cancer cell death by downregulating the expression of MEK5 α and upregulating the level of MEK5 β . There are also some studies indicating that MEK5 plays an important role in the carcinogenesis of hepatocellular cancer (Yan *et al.*, 2009) and lung cancer (Winn *et al.*, 2006). However, few studies have investigated the role of MEK5 in sporadic CRC. Our finding reveals that the rs3743354 polymorphism in the MEK5 promoter region may be biologically important in the tumorigenesis of sporadic CRC.

We have previously argued that the polymorphism rs3826392 in the MKK4 gene promoter might modify the expression of the MKK4 protein and play a decrease role in risk of sporadic CRC, and the bioinformatics analysis showed that this may be due to altering the binding site of the transcriptional factor Nkx-2 (Wei *et al.*, 2009). In this research, for the three SNPs, we also performed bioinformatics analysis with the TFSEARCH program (www.cbrc.jp/research/db/TFSEARCH.html). The result revealed that the rs3743354 T allele, but not rs3743354 C, was a binding site for heat-shock factor (HSF). As a common transcriptional factor, HSF plays an oncogenic role in many tumors including prostate cancer (Tang *et al.*, 2005), thyroid cancer (Samadi *et al.*, 2009), and CRC (Hwang *et al.*, 2003; Akerfelt *et al.*, 2010). It has been reported that the HSF binding sites within the promoter region play a significant role in amplifying the transcription of heat shock-inducible tumor small protein gene *in vitro* and *in vivo* (Nakajima *et al.*, 2010). In this study, the MEK5 rs3743354 CC genotype was associated with downregulated expression of the MEK5 gene. Therefore, we hypothesized that rs3743354 T allele, which has the HSF binding site, may upregulate the expression of MEK5 (mainly MEK5 α) and eventually lead to the carcinogenesis of colorectal tissue. The rs3743354 C allele, which has no binding site for HSF or other transcriptional factors, may decrease the expression of MEK5 and play a protective role in CRC. This is consistent with our finding that the rs3743354 C variant allele was more frequent in controls than in sporadic CRC cases. This may be the biological plausibility for our finding of an association between MEK5 rs3743354 polymorphism and risk of CRC. However, for rs7172582C>T, we did not find any difference in binding factors, suggesting that the polymorphism may have no significant function in tumorigenesis. This is also consistent with our findings.

CRC is a very complex disease and is unlikely to be significantly affected by a single genetic or environmental factor. Studies examining the associations between a single genetic variant and CRC risk may provide limited information, and statistical power is difficult to achieve. Therefore, approaches to assess the interactions of multiple genetic variants and gene-environment are very important. The stratification analysis showed that the decreased risk of CRC associated with the rs3743354 C variant genotypes was more

pronounced in the subgroups of smokers and drinkers. Our data indicated that the protective effect of MEK5 rs3743354 genotype would mainly function under rigorous environmental and dietary influences. The interaction analysis showed that there were statistically significant interactions (or effect modifications) between MEK5 polymorphisms and smoking and drinking on cancer risk, indicating the gene-environment interaction in the etiology of sporadic CRC. This is coincident with the conclusion that occurrence of certain cancers is mediated by lots of relatively weak risk factors including genetic factors and environmental factors and the interactions among them (Ahmed, 2006; Torkamani *et al.*, 2008).

Several potential limitations of the present study require consideration. First, our study focused on a Chinese population, and thus it is unclear whether our findings can be generalized to other populations. Second, in this hospital-based case-control study, all cases were recruited from six hospitals, whereas controls were selected from the communities, and thus selection bias may have occurred. Third, this study may have suffered from an information bias: although the questionnaires were completed before the diagnosis in our six hospitals, some patients referred from other institutions might have known their diagnosis. Another limitation is that the sample size may not be large enough, as the ratio of rs3743354 CC genotype is just 5.8% in healthy control and 2.7% in the CRC group. We achieved 83.2% study power (two-sided test, $\alpha=0.05$) to detect an OR of 0.43 for the rs3743354 CC genotype compared with the rs3743354 TT genotype. Therefore, it appears that our finding that the rs3743354 CC genotypes associated with a decreased risk of sporadic CRC is unlikely to have been obtained by chance. Further, although we have recruited 40 fresh tissue samples of CRC and the adjacent normal colorectal epithelium, there was only one pair of tissues with rs3743354 CC genotype, and thus a larger study should be performed to conform this result.

In summary, the rs3743354T>C polymorphism was found to be associated with a moderate decreased risk of developing sporadic CRC in a population from southern China. Interactions between the rs3743354T>C polymorphism and smoking and drinking in the etiology of CRC were also found. However, further studies in different populations and with larger sample sizes, as well as well-designed mechanistic studies, are needed to confirm our finding.

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Author Contributions

J. Wang and J. Peng designed the study; D. Diao and L. Wang performed all the experiments and wrote the paper; D. Chen, Y. Wei, and J. Lu partly performed population

investigation, DNA extraction, and data management; J. Zhang performed the statistical analyses; and H. Liu revised the article.

Disclosure Statement

No competing financial interests exist.

References

- Ahmed, F.E. (2006). Gene-gene, gene-environment & multiple interactions in colorectal cancer. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* **24**, 1–101.
- Akerfelt, M., Vihervaara, A., Laiho, A., Conter, A., Christians, E.S., Sistonen, L., and Henriksson, E. (2010). Heat shock transcription factor 1 localizes to the sex chromatin during meiotic repression. *J Biol Chem* **285**, 34469–34476.
- Bei-Fan, Z. (2002). Predictive values of body mass index and waist circumference for risk factors of certain related diseases in Chinese adults: study on optimal cut-off points of body mass index and waist circumference in Chinese adults. *Asia Pac J Clin Nutr* **11 Suppl 8**, S685–S693.
- Cameron, S.J., Abe, J., Malik, S., Che, W., and Yang, J. (2004). Differential role of MEK5 α and MEK5 β in BMK1/ERK5 activation. *J Biol Chem* **279**, 1506–1512.
- Coleman, M.P., Quaresma, M., Berrino, F., Lutz, J.M., De Angelis, R., Capocaccia, R., Baili, P., Rachet, B., Gatta, G., Hakulinen, T., Micheli, A., Sant, M., Weir, H.K., Elwood, J.M., Tsukuma, H., Koifman, S., E Silva, G.A., Francisci, S., Sant-Quilani, M., Verdecchia, A., Storm, H.H., and Young, J.L. (2008). Cancer survival in five continents: a worldwide population-based study (CONCORD). *Lancet Oncol* **9**, 730–756.
- Dudderidge, T.J., McCracken, S.R., Loddio, M., Fanshawe, T.R., Kelly, J.D., Neal, D.E., Leung, H.Y., Williams, G.H., and Stoeber, K. (2007). Mitogenic growth signalling, DNA replication licensing, and survival are linked in prostate cancer. *Br J Cancer* **96**, 1384–1393.
- English, J.M., Pearson, G., Baer, R., and Cobb, M.H. (1998). Identification of substrates and regulators of the mitogen-activated protein kinase ERK5 using chimeric protein kinases. *J Biol Chem* **273**, 3854–3860.
- Ghosh, A.K., Steele, R., and Ray, R.B. (2005). c-myc Promoter-binding protein 1 (MBP-1) regulates prostate cancer cell growth by inhibiting MAPK pathway. *J Biol Chem* **280**, 14325–14330.
- Hwang, T.S., Han, H.S., Choi, H.K., Lee, Y.J., Kim, Y.J., Han, M.Y., and Park, Y.M. (2003). Differential, stage-dependent expression of Hsp70, Hsp110 and Bcl-2 in colorectal cancer. *J Gastroenterol Hepatol* **18**, 690–700.
- Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., and Thun, M.J. (2007). Cancer statistics, 2007. *CA Cancer J Clin* **57**, 43–66.
- Kato, Y., Kravchenko, V.V., Tapping, R.L., Han, J., Ulevitch, R.J., and Lee, J.D. (1997). BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C. *EMBO J* **16**, 7054–7066.
- Li, D., Suzuki, H., Liu, B., Morris, J., Liu, J., Okazaki, T., Li, Y., Chang, P., and Abbruzzese, J.L. (2009). DNA repair gene polymorphisms and risk of pancreatic cancer. *Clin Cancer Res* **15**, 740–746.
- Li, S., Wang, J., Lu, Y., and Fan, D. (2007). Screening and early diagnosis of colorectal cancer in China: a 12 year retrospect (1994–2006). *J Cancer Res Clin Oncol* **133**, 679–686.
- Li, Z., Li, J., Mo, B., Hu, C., Liu, H., Qi, H., Wang, X., and Xu, J. (2008). Genistein induces cell apoptosis in MDA-MB-231 breast cancer cells via the mitogen-activated protein kinase pathway. *Toxicol In Vitro* **22**, 1749–1753.
- Lo, Y.L., Yu, J.C., Chen, S.T., Hsu, G.C., Mau, Y.C., Yang, S.L., Wu, P.E., and Shen, C.Y. (2007). Breast cancer risk associated with genotypic polymorphism of the mitotic checkpoint genes: a multigenic study on cancer susceptibility. *Carcinogenesis* **28**, 1079–1086.
- McCracken, S.R., Ramsay, A., Heer, R., Mathers, M.E., Jenkins, B.L., Edwards, J., Robson, C.N., Marquez, R., Cohen, P., and Leung, H.Y. (2008). Aberrant expression of extracellular signal-regulated kinase 5 in human prostate cancer. *Oncogene* **27**, 2978–2988.
- Mehta, P.B., Jenkins, B.L., McCarthy, L., Thilak, L., Robson, C.N., Neal, D.E., and Leung, H.Y. (2003). MEK5 overexpression is associated with metastatic prostate cancer, and stimulates proliferation, MMP-9 expression and invasion. *Oncogene* **22**, 1381–1389.
- Moghaddam, A.A., Woodward, M., and Huxley, R. (2007). Obesity and risk of colorectal cancer: a meta-analysis of 31 studies with 70,000 events. *Cancer Epidemiol Biomarkers Prev* **16**, 2533–2547.
- Nakajima, H., Ishigaki, Y., Xia, Q.S., Ikeda, T., Yoshitake, Y., Yonekura, H., Nojima, T., Tanaka, T., Umehara, H., Tomosugi, N., Takata, T., Shimasaki, T., Nakaya, N., Sato, I., Kawakami, K., Koizumi, K., Minamoto, T., and Motoo, Y. (2010). Induction of HITS, a newly identified family with sequence similarity 107 protein (FAM107B), in cancer cells by heat shock stimulation. *Int J Oncol* **37**, 583–593.
- Nisa, H., Kono, S., Yin, G., Toyomura, K., Nagano, J., Mibu, R., Tanaka, M., Kakeji, Y., Maehara, Y., Okamura, T., Ikejiri, K., Futami, K., Maekawa, T., Yasunami, Y., Takenaka, K., Ichimiya, H., and Terasaka, R. (2010). Cigarette smoking, genetic polymorphisms and colorectal cancer risk: the Fukuoka Colorectal Cancer Study. *BMC Cancer* **10**, 274.
- Pan, X.M., Gao, L.B., Liang, W.B., Liu, Y., Zhu, Y., Tang, M., Li, Y.B., and Zhang, L. (2010). CD86 +1057 G/A polymorphism and the risk of colorectal cancer. *DNA Cell Biol* **29**, 381–386.
- Potter, J.D. (1999). Colorectal cancer: molecules and populations. *J Natl Cancer Inst* **91**, 916–932.
- Roberts, O.L., Holmes, K., Muller, J., Cross, D.A., and Cross, M.J. (2009). ERK5 and the regulation of endothelial cell function. *Biochem Soc Trans* **37**, 1254–1259.
- Samadi, A., Loo, P., Mukerji, R., O'Donnell, G., Tong, X., Timmermann, B.N., and Cohen, M.S. (2009). A novel HSP90 modulator with selective activity against thyroid cancers *in vitro*. *Surgery* **146**, 1196–1207.
- Seyfried, J., Wang, X., Kharebava, G., and Tournier, C. (2005). A novel mitogen-activated protein kinase docking site in the N terminus of MEK5 α organizes the components of the extracellular signal-regulated kinase 5 signaling pathway. *Mol Cell Biol* **25**, 9820–9828.
- Shan, J.Z., Xuan, Y.Y., Zheng, S., Dong, Q., and Zhang, S.Z. (2009). Ursolic acid inhibits proliferation and induces apoptosis of HT-29 colon cancer cells by inhibiting the EGFR/MAPK pathway. *J Zhejiang Univ Sci B* **10**, 668–674.
- Song, H., Jin, X., and Lin, J. (2004). Stat3 upregulates MEK5 expression in human breast cancer cells. *Oncogene* **23**, 8301–8309.
- Tang, D., Khaleque, M.A., Jones, E.L., Theriault, J.R., Li, C., Wong, W.H., Stevenson, M.A., and Calderwood, S.K. (2005). Expression of heat shock proteins and heat shock protein messenger ribonucleic acid in human prostate carcinoma *in vitro* and in tumors *in vivo*. *Cell Stress Chaperones* **10**, 46–58.

- Torkamani, A., Topol, E.J., and Schork, N.J. (2008). Pathway analysis of seven common diseases assessed by genome-wide association. *Genomics* **92**, 265–272.
- Tsong, W.H., Koh, W.P., Yuan, J.M., Wang, R., Sun, C.L., and Yu, M.C. (2007). Cigarettes and alcohol in relation to colorectal cancer: the Singapore Chinese Health Study. *Br J Cancer* **96**, 821–827.
- Wang, L.E., Xiong, P., Strom, S.S., Goldberg, L.H., Lee, J.E., Ross, M.I., Mansfield, P.F., Gershenwald, J.E., Prieto, V.G., Cormier, J.N., Duvic, M., Clayman, G.L., Weber, R.S., Lippman, S.M., Amos, C.I., Spitz, M.R., and Wei, Q. (2005). *In vitro* sensitivity to ultraviolet B light and skin cancer risk: a case-control analysis. *J Natl Cancer Inst* **97**, 1822–1831.
- Wei, Y., Wang, L., Lan, P., Zhao, H., Pan, Z., Huang, J., Lu, J., and Wang, J. (2009). The association between –1304T>G polymorphism in the promoter of MKK4 gene and the risk of sporadic colorectal cancer in southern Chinese population. *Int J Cancer* **125**, 1876–1883.
- Winn, R.A., Van Scoyk, M., Hammond, M., Rodriguez, K., Crossno, J.J., Heasley, L.E., and Nemenoff, R.A. (2006). Antitumorigenic effect of Wnt 7a and Fzd 9 in non-small cell lung cancer cells is mediated through ERK-5-dependent activation of peroxisome proliferator-activated receptor gamma. *J Biol Chem* **281**, 26943–26950.
- Xu, A.G., Yu, Z.J., Jiang, B., Wang, X.Y., Zhong, X.H., Liu, J.H., Lou, Q.Y., and Gan, A.H. (2010). Colorectal cancer in Guangdong Province of China: a demographic and anatomic survey. *World J Gastroenterol* **16**, 960–965.
- Yan, F., Wang, X.M., and Pan, C. (2009). [Expression of ERK5 in multidrug-resistant hepatocellular carcinoma cell line]. *Nan Fang Yi Ke Da Xue Xue Bao* **29**, 483–486.
- Zhao, P., Dai, M., Chen, W., and Li, N. (2010). Cancer trends in China. *Jpn J Clin Oncol* **40**, 281–285.
- Zhou, G., Bao, Z.Q., and Dixon, J.E. (1995). Components of a new human protein kinase signal transduction pathway. *J Biol Chem* **270**, 12665–12669.
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