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Original Article

A functional polymorphism in promoter of *CD3G* as potential biomarker for colorectal carcinoma risk in Chinese adults

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Abstract: Colorectal carcinoma (CRC) represents the most common malignancy of the intestine with a worldwide increasing incidence. Although the risk factors for CRC are well identified, the molecular mechanism for CRC was still to be revealed. Evidence demonstrated that genetic factors were involved in colorectal carcinogenesis. Recently, the functional polymorphism CD3G rs150376137 was reported to correlate with hepatocellular carcinoma risk. However, its role in CRC risk was not clear. In the current study, a hospital-based case-control study including 523 CRC cases and 617 healthy controls was implemented to evaluate the association between rs150376137 and CRC risk. As genotyping data showed, when compared with the reference (del/del), the heterozygote and homozygote ins/ins of rs150376137 were associated with a significantly increased risk of CRC after controlling for other confounders such as age, sex, drinking status, smoking status and BMI (adjusted OR=1.33, 95% C.I. 1.01-1.72, P=0.049; OR=1.69, 95% C.I.1.18-2.67, P=0.017, respectively). It had similar trends in other three genetic models. Moreover, further stratification analysis showed that the differences between cases and controls were more salient in smokers (P<0.05). Further luciferase-based transient transfection assays revealed that rs150376137 can affect promoter activity of CD3G (P<0.01). Our data suggested that common genetic polymorphisms in CD3G may influence HCC risk in Chinese population. The replication of our studies in other ethnicities and further functional studies are required for fully understanding the roles of CD3Gpolymorphisms in predisposition for CRC.

Keywords: Colorectal carcinoma, CD3G, promoter, rs150376137, smoking

Introduction

Colorectal carcinoma (CRC) represents the most common primary malignancy of the intestine with a worldwide high incidence [1, 2]. Although present five-year survival rate showed increase, CRC patients with distant metastases are still threaten by marked reduce in survival rate [3]. CRC carcinogenesis is widely recognized as a multistep and complex process, numerous genes such as APC, c-myc, MDM2, P53 played pivotal role in its incidence and progression [4]. However, the exact underlying mechanisms of CRC remain elusive. As was known to us, high-fat diet, obesity, smoking, alcohol consumption and genetic factor involved in the process of carcinogenesis of CRC [4]. Increasing epidemiological investigations also provided evidences that genetic factor is one of important variants for modulating CRC susceptibility in various populations [5-8].

From a decade ago, rapid progress has been made in discovery of the genetic predisposition to CRC, the implementation of genome wide association studies (GWAS) which are currently routinely applied to identification of causative polymorphisms in the large population [9].

Table 1. Demographic characteristics of study subjects

Characteristics	Case		Control		*P*-value
	N (=523)	Frequencies (%)	N (=617)	Frequencies (%)	
Age (mean ± SD)	60.3 ± 8.4		59.6±9.7		0.29[a]
BMI (kg/m^2) Sex	26.5 ± 5.2		25.8±4.9		0.21[a]
Male	383	73.2	462	74.5	0.24[b]
Female	141	26.8	145	23.5	
Smoking Status[†]					
Smokers	235	44.9	294	47.6	0.36[b]
Nonsmokers	288	55.1	323	52.4	
Drinking status[‡]					
Drinker	220	42.1	272	44.1	0.49[b]
Nondrinker	303	57.9	345	55.9	
Family history of CRC					
Yes	52	9.9	NA	NA	
No	471	90.1	NA	NA	
Tumor stages					
I	52	9.9	NA	NA	
II	144	27.5	NA	NA	
III	215	41.1	NA	NA	
IV	112	21.4	NA	NA	

[a]Two-sided two-sample t-test between cases and controls. [b]χ^2 test for differences between cases and controls. [†]Individuals who smoked more than one cigarette per day for more than one year were classified as smokers. [‡]Participants were considered alcohol drinkers if they drank at least once per week. NA = not applicable, SD = standard deviation.

Nevertheless, understanding of genetic basis of susceptibility to CRC was still on the way and early screening of CRC is necessary because of insufficient reliable candidate biomarkers.

Our research group focused on causative insertion/deletion polymorphism discovery, and reported a functional polymorphism within CD3G correlates with hepatocellular carcinoma susceptibility in recent years [10]. CD3G was involved in TCR-CD3 complex formation and immune response [11], and its mutation often resulted in disorder such as inflammation and cancer [10, 12]. We then assumed that the polymorphism within CD3G promoter correlates with CRC susceptibility. The aim of our current study was to investigate whether rs150376137 was associated with the risk of CRC in a Chinese population and to assess the possible functional significance of the polymorphism.

Materials and methods

Study populations

The case-control study was performed on genomic DNA extracted from peripheral blood of newly diagnosed incident CRC cases together with controls matched for sex and age after obtaining informed consent. All subjects recruited were unrelated ethnic Han Chinese. The case series comprised 523 CRC patients diagnosed, hospitalized and treated in the Affiliated Hospital of Xuzhou Medical University from 2012 January to 2015 December. A total of 617 controls were cancer-free individuals selected from a community nutritional survey that was conducted in the same regions during the same period as recruitment of cancer patients. Briefly, the diagnosis of these patients was confirmed by a pathological examination combined with positive imaging (Magnetic resonance imaging and/or computerized tomography). Tumor stages were determined according to a modified American Joint Committee on Cancer (AJCC) and international union against cancer (UICC) standard. The subjects who smoked more than two cigarettes per day for more than one year were classified as smokers. Others were defined as non-smokers. Subjects were considered as alcohol drinkers, if they drank at least once per week. The design of the study was approved by the Ethical Committee of Xuzhou Medical University.

DNA extraction and genotyping

A Chelex method was used for extracting genomic DNA of human peripheral blood samples [13]. DNA fragments with the polymorphism rs150376137 were amplified under previously reported protocol [10]. The PCR products were analyzed by 7% non-denaturing polyacrylamide gel electrophoresis (PAGE) and visualized by silver staining [14]. The allelic dis-

Table 2. Associations between rs150376137 genotype and CRC susceptibility

Genetic Model	Genotype	Cases n (%)		Control n (%)		OR (95% c.i.)[a]	P
Co-dominant model	Del/Del	129	24.7	195	31.6	1.00 (Reference)	
	Del/Ins	272	52.0	312	50.6	1.33 (1.01-1.72)	0.049
	Ins/Ins	122	23.3	110	17.8	1.69 (1.18-2.67)	0.017
Dominant model	Del/Del	129	24.7	195	31.6	1.00 (Reference)	
	Del/Ins + Ins/Ins	394	75.3	422	68.4	1.42(1.07-1.81)	0.009
Recessive model	Del/Del + Del/Ins	401	76.7	507	82.2	1.00 (Reference)	
	Ins/Ins	122	23.3	110	17.8	1.41 (1.05-1.85)	0.022
Additive model[b]	Del Allele	530	50.7	702	56.9	1.00 (Reference)	
	Ins allele	516	49.3	532	43.1	1.29 (1.11-1.53)	0.003
	P_{trend}						0.003

[a]Adjusted for proper confounders when appropriate. [b]Assuming an additive effect of the del allele.

crimination of rs150376137 was determined by the numbers and the positions of the band on the gels. The 4-bp deletion allele yielded a 132-bp band and the insertion allele yields a 136-bp band. To validate the genotyping method, we analyzed 30 randomly selected DNA samples by both direct sequencing and PCR method; the concurrence rate of these two methods was 100%, suggesting that the PCR method is reliable. Genotyping was performed without knowledge of the case or control status. A 20% random sample was tested in duplicate by different researchers, and the reproducibility was 100%.

Construction of reporter plasmids and luciferase assays

The construction of reporter plasmids was applied as previous report [10]. HCT116 and LoVo cells were maintained in DMEM with high glucose (Gibico) supplemented with 10% heat-inactivated fetal bovine serum (Gibico) and 50 μg/mL streptomycin (Gibico) at a 37°C incubator supplemented with 5% CO_2. Cells were seeded at 1×10^5 cells per well in 24-well plates (BD Biosciences). Sixteen hours after the plating, cells were transfected by Lipofectamine 2000 according to manufacturer's suggestion. In each well, 500 ng constructed pGL3-basic vector and 50 ng pRL-TK vector (Promega) were co-transfected into cells. The un-constructed pGL3-basic vector was added as negative control. After transfection for 24 hours, cells were harvested by the addition of 100 μl passive lysis buffer. Firefly luciferase activities in cell lysate were measured with the Dual Luciferase assay system (Promega) in TD-20/20 luminometer (Turner Biosystems) and were normalized with the Renilla luciferase activities. Six replicates for each group and the experiments were repeated at least three times.

Statistical analysis

Hardy-Weinberg equilibrium was assessed using a goodness-of-fit χ^2 test for biallelic markers. The adjusted odds ratios (ORs) with their 95% confidence intervals (C.I.) of the association between polymorphism and CRC risk were estimated by multiple logistic regression models after controlling for sex, age, smoking status, drinking status, tumor stage and BMI. In all cases, homozygosis for the most common allele (i.e. del/del) was used as the reference category. Student's t test was used to examine the differences in luciferase reporter gene expression. The statistical analyses were implemented in SPSS software (version 18.0). $P<0.05$ was used as the criterion of statistical significance.

Results

The statistical analysis of demographic characteristics of the 523 CRC patients and 617 controls were summarized in **Table 1**. There were no statistically significant differences between cases and controls in terms of the frequency distribution of sex, age, smoking and drinking status. Beyond expected, BMI was not shown as a significant risk factor for CRC in recruited subjects. Genotype distributions had no deviation from Hardy-Weinberg equilibrium in control or case group (P=0.357 and P=0.458 respectively).

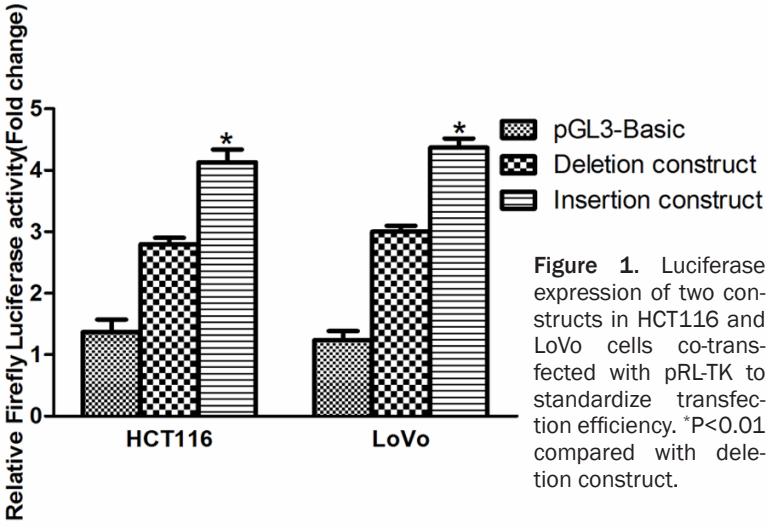

Figure 1. Luciferase expression of two constructs in HCT116 and LoVo cells co-transfected with pRL-TK to standardize transfection efficiency. *P<0.01 compared with deletion construct.

Our results showed that rs150376137 was significantly associated with CRC susceptibility, at both the allele and genotype levels (**Table 2**). In the co-dominant model, compared with the reference (del/del), the heterozygote and homozygote ins/ins of rs150376137 were associated with a significantly increased risk of CRC after controlling for other covariates such as age, sex, drinking status, smoking status and BMI (adjusted OR=1.33, 95% C.I. 1.01-1.72, P=0.049; OR=1.69, 95% C.I.1.18-2.67, P=0.017, respectively). In the dominant model (ins/ins+ins/del vs del/del), significant associations were also observed between cases and controls (adjusted OR=1.42, 95% C.I. 1.07-1.81, P=0.009). In the additive model, each additional copy of insertion allele was associated with a 29% increased risk of developing cancer (OR=1.29, 95% C.I. 1.11-1.53, P=0.003).

To further investigate whether rs150376137 would potentially influence the transcriptional activity of CD3G, two luciferase reporter gene constructs were generated by PCR, and they were used to transiently transfect CRC cell lines. As shown in **Figure 1**, we found that CD3G promoter containing insertion allele drove a 1.46~1.51 fold increased reporter expression compared with the deletion allele containing counterpart in HCT116 and LoVo cells.

We did additional stratified analysis based on risk factors, and found that only in smoking status it betrayed salient difference in cases and controls (shown in **Table 3**). In smokers' part, when compared with the reference (del/del), the heterozygote and homozygote ins/ins of rs150376137 were associated with a significantly increased risk of CRC after controlling for other covariates such as age, sex, drinking status, smoking status and BMI (adjusted OR=1.65, 95% C.I. 1.10-2.46, P=0.02; OR=2.24, 95% C.I.1.38-3.42, P=0.001, respectively). Trends in other three genetic models were similar with that in codominant model. While in nonsmokers, it had no significant difference in any genetic model (P>0.05). Other risk factors including BMI, drinking status and tumor stage had no statistically difference (data not shown).

Discussion

As far as we knew, this was the first molecular epidemiological study to investigate the associations of CD3G polymorphism with risk of CRC in Chinese Han ethnicity. The genotyping of 523 CRC patients and 617 healthy control individuals showed significant associations with the insertion/deletion variant within promoter of CD3G with CRC susceptibility. The difference was more salient between smokers and nonsmokers. Furthermore, in vitro analysis betrayed that the insertion variant allele significantly upregulated the transcription activity of the CD3G compared with the deletion allele.

CD3G formed T-cell receptor-CD3 complex when together with other CD3 subunits, it was involved in human immune response activity [11]. Based on current investigation, the insert allele enhanced expression of CD3G, and furtherly enhanced immune surveillance. Epithelial cells of intestine suffered long-period fecal accumulation were apt to somatic mutation when successful DNA repair after non-lethal cellular DNA damage, while few somatic mutation cells could escape from enhanced immune surveillance, thus chronic inflammation occurred in intestine especially in colon and rectum. Accumulating evidence has demonstrated that patients with chronic inflammation in bowels have an increased risk to develop CRC [15]. Data from primary in vitro analysis powerfully

Table 3. Stratified analysis on the association between rs150376137 genotype and CRC susceptibility based on smoking status

Comparison	Cases N (%)	Controls N (%)	OR (95% CI)[a]	*P*-value
Smokers	235	294		
Codominant Model				
Del/Del	54 (23.0)	103 (35.0)	1.00 (Reference)	
Ins/Del	120 (51.0)	139 (47.3)	1.65 (1.10-2.46)	0.02
Ins/Ins	61 (26.0)	52 (17.7)	2.24 (1.38-3.42)	0.001
Dominant Model				
Del/Del	54 (23.0)	103 (35.0)	1.00 (Reference)	
Ins/Del+Ins/Ins	181 (77.0)	191 (65.0)	1.81 (1.23-2.55)	0.002
Recessive Model				
Del/Del+Ins/Del	174 (74.0)	242 (82.3)	1.00 (Reference)	
Ins/Ins	61 (26.0)	52 (17.7)	1.63 (1.09-2.39)	0.02
Additive Model				
Del Allele	228 (48.5)	345 (58.7)	1.00 (Reference)	
Ins Allele	242 (51.5)	243 (41.3)	1.51 (1.18-1.89)	0.001
Nonsmokers	288	323		
Codominant Model				
Del/Del	75 (26.0)	92 (28.5)	1.00 (Reference)	
Ins/Del	152 (52.8)	173 (53.5)	1.08 (0.79-1.56)	0.69
Ins/Ins	61 (21.2)	58 (18.0)	1.29 (0.81-2.02)	0.29
Dominant Model				
Del/Del	75 (26.0)	92 (28.5)	1.00 (Reference)	
Ins/Del+Ins/Ins	213 (74.0)	231 (71.5)	1.14 (0.80-1.61)	0.50
Recessive Model				
Del/Del+Ins/Del	227 (88.8)	265 (82.0)	1.00 (Reference)	
Ins/Ins	61 (21.2)	58 (18.0)	1.23 (0.87-1.80)	0.32
Additive Model				
Del Allele	302 (52.4)	357 (55.3)	1.00 (Reference)	
Ins Allele	274 (47.6)	289 (44.7)	1.14 (0.95-1.39)	0.32

[a]Adjusted for proper confounders when appropriate.

supported our hypothesis. However, analysis of either stable transfection or tumor formation in nude mice was not found in previous reports, it was also the experimental support we hoped to find out in further functional assays.

Interestingly, the genotyping difference was more salient in smokers in our stratified analysis. It was well known that tobacco consumption was an immunosuppressant and often lead to lung cancer [16]. Evidence suggested that smoking contributed to incidence and mortality of CRC [17], but its impact with the polymorphism rs150376137 was still not revealed, and the underlying mechanism was to be elucidated.

Taken together, our data suggested the polymorphism rs150376137 could influence CRC risk in Chinese Han population.However,thisCRC-associated polymorphism showed none direct demonstration that rs150376137 is causative. Finally, the replication of our studies in other populations and further in vitro analysis are required for complete elucidation of the roles of CD3G polymorphisms in predisposition for CRC.

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Disclosure of conflict of interest

None.

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