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ORIGINAL RESEARCH

CTLA4 tagging polymorphisms and risk of colorectal cancer: a case–control study involving 2,306 subjects

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Background: *CTLA4* is a candidate gene which has been implicated in the development of colorectal cancer (CRC).

Patients and Methods: To determine the important role of *CTLA-4* polymorphisms on risk of CRC, we genotyped four *CTLA-4* tagging polymorphisms and calculated crude/adjusted ORs with their 95% CIs. We recruited 1,003 sporadic CRC cases and 1,303 controls.

Results: The findings suggested that CTLA-4 rs231775 G>A polymorphism increased the risk of CRC (homozygote model: adjusted OR=1.40, 95% CI=1.05–1.87, P=0.022; dominant model: adjusted OR=1.19, 95% CI=1.00–1.41, P=0.047; and recessive model: adjusted OR=1.38, 95% CI=1.05–1.82, P=0.021). In a stratified analysis by site of tumor, this association was also found in colon cancer. We also found that *CTLA-4* rs231775 GA/AA genotypes might be associated with an increased risk of CRC in Zhenjiang cohort. In addition, we found the CTLA-4 rs16840252 C>T polymorphism was associated with the risk of colon cancer. Haplotype comparison analysis showed that *CTLA-4* $G_{rs3087243}C_{rs16840252}C_{rs733618}A_{rs231775}$, $G_{rs3087243}C_{rs16840252}T_{rs733618}A_{rs231775}$, and other haplotypes increased the risk of CRC (P<0.001, <0.001, and 0.002, respectively).

Conclusion: This study evidences an association of *CTLA-4* tagging polymorphisms and haplotypes with CRC risk. Additional well-designed studies with large sample sizes are required to confirm our findings.

Keywords: polymorphism, immune, *CTLA-4*, tagging, colorectal cancer, susceptibility

Introduction

Colorectal cancer (CRC) is one of the most common malignancies and is becoming the fifth leading cause of cancer death in China.[1] It is reported that the incidence rate and CRC-related mortality are increasing rapidly worldwide.[2,3] These may be attributed to certain lifestyles and environmental factors, including physically inactive, overweight, smoking, and drinking. Recently, Katsidzira et al[4] reported that diabetes mellitus, previous schistosomiasis, approximation to a western lifestyle, and family history were the predominant associations with CRC. Besides these unhealthy lifestyle and environmental risk factors, genetic factors may also affect the development of CRC. A previous study demonstrated that genetic risk factors may contribute to ~35% etiology of CRC cases.[5] Up to now, the inherited factor of CRC remains controversial. Recently, a number of investigations have been devoted to exploring the potential molecular mechanism of CRC carcinogenesis, and inherited factors have been considered to play a vital role in the occurrence and development of CRC.[6,7] Investigations

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of these conceivable inherited factors may enrich our view on the etiology of CRC.

The *cytotoxic T-lymphocyte antigen-4* (*CTLA-4*) gene, also named as a cluster of differentiation 152 (*CD152*), is one of the costimulatory molecule genes involved in immune response. CTLA-4 is transiently expressed on some activated T cells.[8] The expression of CTLA-4 inhibits cytokine production of T cells and then provides a negative signal to T cells.[9,10] The structure of CTLA-4 shares some homologies with CD28 and binds to B7.1 and B7.2 ligands competitively. However, CTLA-4 has a higher binding affinity with B7 molecules compared with CD28. Through interaction of CTLA-4 with B7 molecules, T-cell proliferation, activation, and cytokine production can be inhibited.[11–13] The *CTLA-4* gene is located in chromosome 2q33, which belongs to several immune regulatory gene regions. Since CTLA-4 acts as a vital regulatory factor for some immune responses, any genetic variation in *CTLA-4* gene may influence normal immune function and then alter the risk of cancer. Hence, exploring the impact of these genetic variations in *CTLA-4* gene could determine their relationship with cancer susceptibility. CTLA-4 is polymorphic and contains more than 100 single-nucleotide polymorphisms (SNPs). Among them, some SNPs (eg, rs3087243 G>A, rs16840252 C>T, rs4553808 T>C, rs5742909 C>T, rs733618 T>C, and rs231775 G>A polymorphisms) in *CTLA-4* gene were extensively studied and were reported to be correlated with risk of human malignancy.[14,15] Some case–control studies explored the relationship between *CTLA-4* polymorphisms and CRC;[16,17] however, the sample sizes were limited and the results remained conflicting.

The evasion of immune surveillance and the production of immunosuppressive cytokines are two of the most common immune defects identified to be correlated with CRC. *CTLA-4* is a candidate gene which has been implicated in immune response.[18] Moreover, due to the emerging role of CTLA-4 as an immune checkpoint molecule, anti-CTLA-4 antibody has been tested recently in the treatment of CRC patients.[19] Previous case–control studies, conducted in diverse population to assess the relationship of CRC with *CTLA-4* polymorphisms, have generated conflicting findings. Hence, we undertook a study to determine whether *CTLA-4* variations could cause a predisposition toward CRC. In this study, we analyzed the tagging SNPs of *CTLA-4* (rs3087243 G>A, rs16840252 C>T, rs733618 T>C, and rs231775 G>A polymorphisms) and identified whether these SNPs confer susceptibility to CRC in an Eastern Chinese Han population.

Patients and methods

Study population and patient selection

The study population consisted of 2,306 subjects (1,003 diagnosed as sporadic CRC and 1,303 controls) between October 2014 and August 2017. Among them, 757 CRC patients and 680 controls were enrolled from the Affiliated Union Hospital of Fujian Medical University (Fuzhou, China), and 246 CRC patients and 623 controls were enrolled from the Affiliated People's Hospital of Jiangsu University (Zhenjiang, China). CRC was confirmed via pathology. The age of CRC cases ranged from 21 to 90 years old (mean age at diagnosis was 61.10±12.17 years). The major exclusion criteria were autoimmune disorders, hereditary nonpolyposis CRC, and history of another malignancy. CRC cases who had received neoadjuvant chemoradiotherapy were also excluded. The age of controls ranged from 21 to 87 years old (mean age at sampling was 61.40±9.61 years). In this study, the cancer-free controls included 1,303 healthy volunteers who participated in a routine examination in hospitals mentioned above. The primary information of the participants was collected by a pre-structured questionnaire. The definitions of "ever smokers" and "ever drinkers" are described in our previous study.[20] In addition, according to the criterion for overweight and obesity, a body mass index (BMI) of 24 was used as the cutoff point in Chinese adults.[21,22] Each participant was informed about the present study and signed a standard informed consent form. The Ethical Committee of Fujian Medical University and Jiangsu University approved the protocols of the study (No KY-2013–11 and No 2012-00-18, respectively).

Data collection

All participants were personally questioned by two experienced doctors. The questionnaire included the primary information about demographics (eg, age, sex), smoking, drinking, height, and weight (Table 1). The clinical and pathological information of CRC cases was collected from their medical records.

Selection of tagging SNPs

The tagging SNPs across the entire region of *CTLA-4* gene (16.2 kbp spanning from 203862788–203878960 in chromosome 2 [upstream and downstream of gene extending 5 kb, respectively]) were selected from the Chinese Han in Beijing cohort via the HapMap Project (http://hapmap.ncbi.nlm.nih.gov/index.html.en) and analyzed with Haploview 4.2 software using a pairwise linkage disequilibrium (LD) r^2 threshold

Table 1 Distribution of selected demographic variables and risk factors in CRC cases and controls

Variable	Cases (n=1,003)		Controls (n=1,303)		*P*-value[a]
	n	%	n	%	
Age (years), M (±SD)	61.10 (±12.17)		61.40 (±9.61)		0.496
Age (years)					0.605
<61	451	44.97	600	46.05	
≥61	552	55.03	703	53.95	
Sex					0.867
Male	620	61.81	801	61.47	
Female	383	38.19	502	38.53	
Smoking status					*0.002*
Never	744	74.18	1,038	79.66	
Ever	259	25.82	265	20.34	
Alcohol use					*<0.001*
Never	829	82.65	1,167	89.56	
Ever	174	17.35	136	10.44	
BMI (kg/m^2)					*<0.001*
<24	670	66.80	688	52.80	
≥24	333	33.20	615	47.20	
Primary site of tumor					
Colon cancer	431	42.97			
Rectum cancer	572	57.03			
Degree of differentiation					
Poorly differentiated	124	12.36			
Moderately differentiated	832	82.95			
Well differentiated	47	4.69			
Lymph node status					
Positive	518	51.65			
Negative	485	48.35			
AJCC TMN stage					
0–I	167	16.65			
II	290	28.91			
III	420	41.87			
IV	126	12.56			

Notes: Italic values are statistically significant ($P<0.05$). [a]Two-sided chi-squared test and Student's *t*-test.
Abbreviations: CRC, colorectal cancer; SD, standard deviation; BMI, body mass index; AJCC, American Joint Committee on Cancer.

of 0.8 between SNPs (with a minimum LD of $r^2>0.8$). SNPs with a Hardy–Weinberg equilibrium (HWE) $P\geq0.05$, minor allele frequency (MAF) ≥0.05, and call rate ≥95% in the CHB cohort were included.[23] The detailed information of the selected four SNPs is summarized in Table 2.

DNA extraction and genotyping

Ethylenediamine tetraacetic acid (EDTA)-anticoagulated intravenous blood was collected after an overnight fast. The genomic DNA was isolated using the Promega DNA Blood Mini Kit (Promega, Madison, WI, USA).

Table 2 Primary information for *CTLA-4* polymorphisms

Genotyped SNPs	*CTLA-4* rs3087243 G>A	*CTLA-4* rs231775 G>A	*CTLA-4* rs16840252 C>T	*CTLA-4* rs733618 T>C
Chromosome	2	2	2	2
Function	nearGene-3	Missense	nearGene-5	nearGene-5
Location	Intron 3+6,230	Exon 1+49	Promoter 1147	Promoter 1722
Chr Pos (Genome Build 38)	203874196	203867991	203866796	203866221
MAF for Chinese in database	0.183	0.314	0.122	0.390
MAF in our controls (n=1,303)	0.189	0.305	0.117	0.412
P-value for HWE test in our controls	0.411	0.430	0.065	0.335
Genotyping method	SNPscan	SNPscan	SNPscan	SNPscan
% Genotyping value	98.87%	98.79%	98.87%	98.87%

Abbreviations: SNP, single-nucleotide polymorphism; MAF, minor allele frequency; HWE, Hardy–Weinberg equilibrium.

The genotyping of the *CTLA-4* rs3087243 G>A, rs16840252 C>T, rs733618 T>C, and rs231775 G>A polymorphisms were performed by a custom-by-design 48-Plex SNPscan Kit (Genesky Biotechnologies Inc., Shanghai, China) as described in previous studies.[24,25] This 48-Plex SNPscan Kit was based on double ligation and multiplex fluorescence PCR.[26] For quality control, 92 (4%) samples were randomly selected and were tested again by the same genotyping method. The accordance ratio was 100%.

Statistical analysis

Statistical analysis was performed using SAS version 9.4 software package for Windows (SAS Institute, Cary, NC, USA), and a $P<0.05$ (two-tailed) was considered for level of significance. The continuous variables were expressed as mean±SD. We used the Student's *t*-test to check the differences for normally distributed continuous variables between CRC cases and controls. We used the chi-squared test to determine the differences in demographic variables, risk factors (smoking, BMI, and drinking), and the frequencies of genotype between CRC cases and controls. An internet-based calculator program (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) was harnessed to examine the deviation of HWE.[14] Multivariate logistic regression was used to analyze the associations between *CTLA-4* rs3087243 G>A, rs16840252 C>T, rs733618 T>C, and rs231775 G>A SNPs and risk of CRC. The relationships between *CTLA-4* rs3087243 G>A, rs16840252 C>T, rs733618 T>C, and rs231775 G>A polymorphisms genotypes and risk of CRC were assessed by crude/adjusted ORs and the corresponding 95% CIs when appropriate. The relationships were assessed in additive, homozygote, dominant, and recessive models. We used a Bonferroni correction to adjust for multiple testing.[27,28]

Results

Demographic characteristics

In our study, 1,003 CRC patients were included. Of them, 620 were males (61.81%) and 383 were females (38.19%). The mean age and SD were 61.10±12.17 years. The primary tumor site was the colon in 431 (42.97%) patients and the rectum in 572 (57.03%) patients. For the control group, we recruited 1,303 non-cancer controls, 801 males (61.47%) and 502 females (38.53%). Their age mean±SD was 61.40±9.61 years. All participants were Chinese Han population. The differences of age and sex between CRC and control groups were not statistically significant ($P\geq0.05$) (Table 1). As summarized in Table 1, significant differences were found on alcohol consumption, smoking status, and BMI between the cases and the controls ($P=0.002$, <0.001, and <0.001, respectively). The primary information for CTLA-4 rs733618 T>C, rs3087243 G>A, rs16840252 C>T, and rs231775 G>A SNPs is shown in Table 2. For these SNPs, the successful ratio was more than 98.50%. In controls, MAF of CTLA-4 tagging SNPs was very close to the MAF data for Chinese (Table 2). Table 2 shows the genotype frequencies of CTLA-4 tagging SNPs polymorphisms were all in HWE.

Association of CTLA-4 rs3087243 G>A, rs16840252 C>T, rs733618 T>C, and rs231775 G>A polymorphisms with CRC in overall analysis

The frequencies of *CTLA-4* rs733618 TT, TC, and CC genotypes were 35.31%, 47.35%, and 17.35% in the CRC group and 35.23%, 47.15%, and 17.62% in the control group, respectively (Table 3). The frequencies of *CTLA-4* rs16840252 CC, CT, and TT genotypes were 75.71%, 22.76%, and 1.53% in the CRC group and 77.38%, 21.77%, and 0.85% in controls, respectively (Table 3). There was no statistically significant difference in genotype distribution of *CTLA-4* rs16840252 C>T and rs733618 T>C polymorphisms among CRC patients and controls. The frequencies of *CTLA-4* rs3087243 GG, GA, and AA genotypes were 65.00%, 30.20%, and 4.80% in the CRC group and 65.38%, 31.38%, and 3.23% in controls, respectively (Table 3). The *CTLA-4* rs3087243 AA genotype was associated with a borderline statistically increased risk of CRC, compared with *CTLA-4* rs3087243 GG/GA genotypes (crude OR=1.51, 95% CI=0.99–2.31, $P=0.058$). When adjusted for age, sex, BMI, smoking, and drinking, a borderline statistically increased risk of CRC was also found (crude OR=1.52, 95% CI=0.99–2.34, $P=0.058$; Table 4).

The frequencies of *CTLA-4* rs231775 GG, GA, and AA genotypes were 42.59%, 45.25%, and 12.16% in the CRC group and 47.77%, 43.31%, and 8.85% in the control group, respectively (Table 3). When compared with the *CTLA-4* rs231775 GG genotype, *CTLA-4* rs231775 AA and GA/AA genotypes significantly increased the risk of CRC (homozygote model: crude OR=1.47, 95% CI=1.10–1.95, $P=0.008$; and dominant model: crude OR=1.23, 95% CI=1.05–1.46, $P=0.014$). When compared with *CTLA-4* rs231775 GG/GA genotypes, the *CTLA-4* rs231775 AA genotype also increased the risk of CRC (crude OR=1.43, 95% CI=1.09–1.87, $P=0.011$). When adjusting for age, sex, BMI, smoking, and drinking, the results were not essentially changed (homozygote model: adjusted OR=1.40,

Table 3 The frequencies of *CTLA-4* rs3087243 G>A, rs16840252 C>T, rs733618 T>C, and rs231775 G>A polymorphisms in CRC patients and controls

Genotype	CRC cases (n=1,003)		Colon cancer (n=431)		Rectum cancer (n=572)		Controls (n=1,303)	
	n	%	n	%	n	%	n	%
rs3087243 G>A								
GG	637	65.00	271	64.07	366	65.71	850	65.38
GA	296	30.20	132	31.21	164	29.44	408	31.38
AA	47	4.80	20	4.73	27	4.85	42	3.23
A allele	390	19.90	172	20.33	218	19.57	492	18.92
rs16840252 C>T								
CC	742	75.71	319	75.41	423	75.94	1,006	77.38
CT	223	22.76	94	22.22	129	23.16	283	21.77
TT	15	1.53	10	2.36	5	0.90	11	0.85
T allele	253	12.91	114	13.48	139	12.48	305	11.73
rs733618 T>C								
TT	346	35.31	140	33.10	206	36.98	458	35.23
TC	464	47.35	216	51.06	248	44.52	613	47.15
CC	170	17.35	67	15.84	103	18.49	229	17.62
C allele	804	41.02	350	41.37	454	40.75	1,071	41.19
rs231775 G>A								
GG	417	42.59	178	42.18	239	42.91	621	47.77
GA	443	45.25	187	44.31	256	45.96	563	43.31
AA	119	12.16	57	13.51	62	11.13	115	8.85
A allele	681	34.78	301	35.66	380	34.11	793	30.50

Abbreviation: CRC, colorectal cancer.

95% CI=1.05–1.87, *P*=0.022; dominant model: adjusted OR=1.19, 95% CI=1.00–1.41, *P*=0.047; and recessive model: adjusted OR=1.38, 95% CI= 1.05–1.82, *P*=0.021; Table 4).

Association of CTLA-4 rs3087243 G>A, rs16840252 C>T, rs733618 T>C, and rs231775 G>A polymorphisms with CRC in a stratification group by primary site of tumor

To determine whether the effect of *CTLA-4* tagging SNPs was modified by the primary site of tumor, we performed a stratified analysis. For *CTLA-4* rs16840252 C>T, stratified analysis revealed this polymorphism was associated with an increased risk of colon cancer (homozygote model: adjusted OR=2.51, 95% CI=1.04–6.03, *P*=0.040 and recessive model: adjusted OR=2.54, 95% CI=1.06–6.09, *P*=0.037; Table 4). For the *CTLA-4* rs231775 G>A polymorphism, we found that *CTLA-4* rs231775 AA genotypes might be associated with an increased risk of colon cancer (homozygote model: adjusted OR=1.61, 95% CI=1.12–2.30, *P*=0.010 and recessive model: adjusted OR=1.59, 95% CI=1.13–2.23, *P*=0.009; Table 4). The results of other genetic comparisons are summarized in Table 4.

Association of CTLA-4 rs3087243 G>A, rs16840252 C>T, rs733618 T>C, and rs231775 G>A polymorphisms with CRC in a stratification group by geographical area

In this study, CRC patients and controls were enrolled from two different geographical areas (Fuzhou and Zhenjiang, China). We performed a stratified analysis according to geographical area. Compared with *CTLA-4* rs231775 GG, we found *CTLA-4* rs231775 GA/AA genotypes might be associated with an increased risk of CRC in the Zhenjiang cohort (adjusted OR=1.38, 95% CI=1.01–1.88, *P*=0.041; Table 5). In addition, the stratified analysis revealed the *CTLA-4* rs231775 G>A polymorphism also had a tendency of increased risk to CRC in the Fuzhou cohort (recessive model: adjusted OR=1.40, 95% CI=0.99–1.98, *P*=0.061; Table 5).

SNP haplotypes

Using an expectation–maximization algorithm (SHESIS program; Bio-X Inc., Shanghai, China, http://analysis.bio-x.cn/myAnalysis.php),[29] we constructed seven haplotypes (Table 6). Haplotype comparison analysis suggested that CTLA4 $G_{rs3087243}C_{rs16840252}C_{rs733618}A_{rs231775}$,

Table 4 Overall and stratified analyses of *CTLA-4* rs3087243 G>A, rs16840252 C>T, rs733618 T>C, and rs231775 G>A polymorphisms with CRC by region

Genotype	CRC cases (n=1,003) vs controls (1,303)				Colon cancer (n=431) vs controls (1,303)				Rectum cancer (n=572) vs controls (1,303)			
	Crude OR (95% CI)	*P*-value	Adjusted OR[a] (95% CI)	*P*-value	Crude OR (95% CI)	*P*-value	Adjusted OR[a] (95% CI)	*P*-value	Crude OR (95% CI)	*P*-value	Adjusted OR[a] (95% CI)	*P*-value
rs3087243 G>A												
Additive model	0.94 (0.78–1.12)	0.485	0.89 (0.74–1.07)	0.223	0.99 (0.78–1.26)	0.929	0.95 (0.74–1.20)	0.654	0.90 (0.72–1.12)	0.343	0.86 (0.69–1.07)	0.182
Homozygote model	1.45 (0.94–2.22)	0.091	1.43 (0.93–2.21)	0.106	1.46 (0.84–2.52)	0.180	1.48 (0.85–2.59)	0.165	1.44 (0.87–2.37)	0.152	1.38 (0.83–2.28)	0.216
Dominant model	1.02 (0.86–1.21)	0.849	0.97 (0.81–1.16)	0.754	1.06 (0.84–1.33)	0.621	1.02 (0.81–1.29)	0.865	0.99 (0.80–1.22)	0.893	0.94 (0.76–1.17)	0.588
Recessive model	1.51 (0.99–2.31)	0.058	1.52 (0.99–2.34)	0.058	1.49 (0.86–2.56)	0.153	1.54 (0.89–2.67)	0.125	1.53 (0.93–2.50)	0.094	1.48 (0.90–2.45)	0.125
rs16840252 C>T												
Additive model	1.04 (0.85–1.27)	0.704	1.02 (0.84–1.25)	0.816	1.03 (0.79–1.34)	0.855	1.02 (0.78–1.33)	0.902	1.05 (0.83–1.33)	0.684	1.03 (0.81–1.31)	0.821
Homozygote model	1.80 (0.82–3.94)	0.142	1.72 (0.78–3.81)	0.182	2.80 (1.18–6.66)	0.020	2.51 (1.04–6.03)	0.040	1.05 (0.36–3.03)	0.932	0.96 (0.33–2.84)	0.943
Dominant model	1.10 (0.90–1.34)	0.349	1.08 (0.89–1.32)	0.441	1.12 (0.86–1.44)	0.404	1.10 (0.85–1.43)	0.468	1.08 (0.86–1.37)	0.499	1.06 (0.84–1.35)	0.632
Recessive model	1.82 (0.83–3.98)	0.133	1.72 (0.79–3.86)	0.170	2.84 (1.20–6.73)	0.018	2.54 (1.06–6.09)	0.037	1.06 (0.37–3.07)	0.911	0.98 (0.33–2.89)	0.965
rs733618 T>C												
Additive model	0.95 (0.79–1.14)	0.548	0.97 (0.81–1.17)	0.772	1.10 (0.86–1.40)	0.450	1.12 (0.88–1.43)	0.374	0.84 (0.68–1.05)	0.127	0.88 (0.71–1.10)	0.256
Homozygote model	0.93 (0.73–1.18)	0.540	0.97 (0.76–1.24)	0.797	0.91 (0.66–1.27)	0.581	0.95 (0.68–1.33)	0.771	0.94 (0.71–1.24)	0.658	0.99 (0.74–1.32)	0.920
Dominant model	1.00 (0.84–1.19)	0.970	1.03 (0.87–1.23)	0.729	1.10 (0.87–1.39)	0.423	1.13 (0.89–1.43)	0.307	0.93 (0.75–1.14)	0.470	0.97 (0.79–1.20)	0.771
Recessive model	0.98 (0.79–1.22)	0.868	1.01 (0.81–1.26)	0.938	0.88 (0.65–1.19)	0.401	0.91 (0.67–1.23)	0.543	1.06 (0.82–1.37)	0.652	1.09 (0.84–1.42)	0.526
rs231775 G>A												
Additive model	1.12 (0.94–1.33)	0.220	1.08 (0.90–1.28)	0.422	1.11 (0.88–1.40)	0.379	1.07 (0.85–1.36)	0.553	1.12 (0.91–1.38)	0.289	1.08 (0.87–1.33)	0.481
Homozygote model	1.47 (1.10–1.95)	0.008	1.40 (1.05–1.87)	0.022	1.66 (1.16–2.37)	0.006	1.61 (1.12–2.30)	0.010	1.33 (0.94–1.87)	0.105	1.22 (0.86–1.73)	0.256
Dominant model	1.23 (1.05–1.46)	0.014	1.19 (1.00–1.41)	0.047	1.26 (1.01–1.57)	0.044	1.21 (0.97–1.52)	0.091	1.22 (1.00–1.49)	0.053	1.17 (0.95–1.43)	0.138
Recessive model	1.43 (1.09–1.87)	0.011	1.38 (1.05–1.82)	0.021	1.61 (1.15–2.26)	0.006	1.59 (1.13–2.23)	0.009	1.29 (0.93–1.79)	0.126	1.21 (0.87–1.69)	0.260

Note: [a]Adjusted for age, sex, BMI, smoking status, and alcohol use in a logistic regression model.

Abbreviations: CRC, colorectal cancer; BMI, body mass index; OR, odds ratio; CI, confidence interval.

Table 5 Logistic regression analyses of associations between *CTLA-4* polymorphisms and risk of CRC in two cohorts

Genotype	Zhenjiang cohort						Fuzhou cohort					
	Cases (n=246)		Controls (n=623)		Adjusted OR[a] (95% CI)	*P*-value	Cases (n=757)		Controls (n=680)		Adjusted OR[a] (95% CI)	*P*-value
	n	%	n	%			n	%	n	%		
rs231775 G>A												
GG	100	42.37	319	51.37	1.00		317	42.66	302	44.54	1.00	
GA	110	46.61	251	40.42	1.21 (0.88–1.66)	0.241	333	44.82	312	46.02	0.93 (0.74–1.17)	0.526
AA	26	11.02	51	8.21	1.50 (0.88–2.55)	0.135	93	12.52	64	9.44	1.32 (0.92–1.90)	0.138
GA + AA	136	57.63	302	48.63	1.38 (1.01–1.88)	0.041	426	57.34	376	55.46	1.03 (0.83–1.29)	0.765
GG + GA	210	88.98	570	91.79	1.00		650	87.48	614	90.56	1.00	
AA	26	11.02	51	8.21	1.45 (0.87–2.41)	0.155	93	12.52	64	9.44	1.40 (0.99–1.98)	0.061
A allele	162	34.32	353	28.42			519	34.93	440	32.45		
rs16840252 C>T												
CC	175	74.15	478	76.97	1.00		567	76.21	528	77.76	1.00	
CT	59	25.00	137	22.06	1.08 (0.76–1.55)	0.656	164	22.04	146	21.50	1.05 (0.81–1.36)	0.721
TT	2	0.85	6	0.97	0.81 (0.16–4.12)	0.800	13	1.75	5	0.74	2.13 (0.73–6.21)	0.164
CT + TT	61	25.85	143	23.03	1.13 (0.80–1.61)	0.484	177	23.79	151	22.24	1.12 (0.86–1.44)	0.408
CC + CT	234	99.15	615	99.03	1.00		731	98.25	674	99.26	1.00	
TT	2	0.85	6	0.97	0.83 (0.16–4.22)	0.825	13	1.75	5	0.74	2.14 (0.74–6.23)	0.162
T allele	63	13.35	149	12.00			190	12.77	156	11.49		
rs3087243 G>A												
GG	175	74.15	433	69.73	1.00		462	62.10	417	61.41	1.00	
GA	54	22.88	170	27.38	0.75 (0.52–1.07)	0.108	242	32.53	238	35.05	0.83 (0.66–1.04)	0.108
AA	7	2.97	18	2.90	0.88 (0.36–2.18)	0.787	40	5.38	24	3.53	1.39 (0.81–2.39)	0.234
GA + AA	61	25.85	188	30.27	0.80 (0.57–1.13)	0.208	282	37.90	262	38.59	0.90 (0.72–1.13)	0.368
GG + GA	229	97.03	603	97.10	1.00		704	94.62	655	96.47	1.00	
AA	7	2.97	18	2.90	0.99 (0.40–2.42)	0.974	40	5.38	24	3.53	1.51 (0.89–2.59)	0.130
A allele	68	14.41	206	16.59			322	23.71	286	21.06		
rs733618 T>C												
TT	70	29.66	224	36.07	1.00		276	37.10	234	34.46	1.00	
TC	123	52.12	296	47.67	1.17 (0.84–1.64)	0.349	341	45.83	317	46.69	0.89 (0.70–1.13)	0.341
CC	43	18.22	101	16.26	1.27 (0.81–1.99)	0.293	127	17.07	128	18.85	0.83 (0.61–1.14)	0.247
TC + CC	166	70.34	397	63.93	1.36 (0.97–1.88)	0.071	468	62.90	445	65.54	0.92 (0.73–1.15)	0.460
TT + TC	193	81.78	520	83.74	1.00		617	82.93	551	81.15	1.00	
CC	43	18.22	101	16.26	1.21 (0.81–1.81)	0.348	127	17.07	128	18.85	0.91 (0.68–1.20)	0.497
C allele	209	44.28	498	40.10			595	39.99	573	42.19		

Note: [a]Adjusted for age, sex, BMI, smoking status, and alcohol use.
Abbreviations: CRC, colorectal cancer; OR, odds ratio; CI, confidence interval; BMI, body mass index.

$G_{rs3087243}C_{rs16840252}T_{rs733618}A_{rs231775}$, and other haplotypes significantly increased the risk of CRC ($P<0.001$, <0.001, and 0.002, respectively, Table 6).

Discussion

The individual's susceptibility to CRC may be diverse, even with the same environmental exposure. Host genetic predisposition may lead to these differences. In recent years, several case–control studies have been performed to test the hypothesis that some functional variants in *CTLA-4* and other immune checkpoint molecules such as *HLA-G* may influence the risk and the treatment of CRC.[30–36] Garziera et al[36] reported that HLA-G 3′UTR polymorphisms might significantly affect the development of CRC. However, the association between CRC susceptibility and *CTLA-4* polymorphisms remain conflicting. In addition, a comprehensive assessment was lacking. The aim of the present study was to identify the association between *CTLA-4* tagging polymorphisms (rs3087243 G>A, rs16840252 C>T, rs733618 T>C, and rs231775 G>A) and susceptibility of CRC in a case–control study. Genotyping of 1,003 CRC cases and 1,303 cancer-free controls was carried out in an Eastern Chinese Han population. Our findings demonstrated that *CTLA-4* rs231775 G>A polymorphism might be associated with the risk of CRC. In a stratified analysis by primary site of tumor, the association was also found in colon cancer. When a subgroup analysis was performed by cohort, we also found *CTLA-4* rs231775 GA/AA genotypes might be associated with an increased

Table 6 *CTLA-4* haplotype frequencies (%) in cases and controls and risk of CRC

Haplotypes	CRC cases (n=2,006)		Controls (n=2,606)		Crude OR (95% CI)	*P*-value
	n	%	n	%		
CTLA4 $G_{rs3087243}C_{rs16840252}C_{rs733618}G_{rs231775}$	765	39.11	1,069	41.15	1.00	
CTLA4 $G_{rs3087243}C_{rs16840252}T_{rs733618}G_{rs231775}$	490	25.05	726	27.94	0.94 (0.81–1.09)	0.437
CTLA4 $A_{rs3087243}C_{rs16840252}T_{rs733618}A_{rs231775}$	382	19.53	492	18.94	1.08 (0.92–1.28)	0.326
CTLA4 $G_{rs3087243}T_{rs16840252}T_{rs733618}A_{rs231775}$	237	12.12	294	11.32	1.13 (0.93–1.37)	0.230
CTLA4 $G_{rs3087243}C_{rs16840252}C_{rs733618}A_{rs231775}$	34	1.74	2	0.08	23.76 (5.69–99.21)	<0.001
CTLA4 $G_{rs3087243}C_{rs16840252}T_{rs733618}A_{rs231775}$	26	1.33	5	0.19	7.27 (2.78–19.01)	<0.001
Others	22	1.12	10	0.38	3.07 (1.45–6.53)	0.002

Abbreviations: CRC, colorectal cancer; OR, odds ratio; CI, confidence interval.

risk of CRC in the Zhenjiang cohort. Additionally, we found the *CTLA-4* rs16840252 C>T polymorphism was associated with a risk of colon cancer. Haplotype comparison analysis showed that *CTLA4* $G_{rs3087243}C_{rs16840252}C_{rs733618}A_{rs231775}$, $G_{rs3087243}C_{rs16840252}T_{rs733618}A_{rs231775}$, and other haplotypes increased the risk of CRC. Based on these primary findings, we found *CTLA-4* tagging polymorphisms and haplotypes might influence the susceptibility to developing CRC.

Several case–control studies focused on the association between *CTLA-4* rs16840252 C>T polymorphism and risk of cancer.[37,38] The observed results indicated that the *CTLA-4* rs16840252 C>T polymorphism might not confer a risk to cancer. However, *CTLA-4* rs16840252 C>T located in the promoter region of the *CTLA4* gene. HapMap data suggest that *CTLA-4* rs16840252 C>T and rs4553808 C>T (–1,661 C>T) are in complete LD.[37] Interestingly, Idris et al[39] also reported that strong LD was found between *CTLA-4* rs16840252 C>T and rs5742909 C>T (–318 C>T) across all LD structures in an Asian population. In the presence of these functional SNPs on the same LD block, it could be that the predisposing allele of *CTLA-4* rs4553808 C>T or rs5742909 C>T polymorphism is in LD with the protective allele of rs16840252 C>T. Ligers et al[40] found that individuals carrying thymine at position –318 of the CTLA4 promoter (*CTLA-4* rs5742909 C>T) showed significantly increased expression, both of CTLA-4 mRNA in non-stimulated cells and of cell-surface CTLA-4 after cellular stimulation. Recently, several meta-analyses indicated that *CTLA-4* rs4553808 T>C and rs5742909 C>T polymorphisms were associated with the risk of cancer, especially in Asians.[33,41,42] Since *CTLA-4* rs16840252 C>T, rs5742909 C>T, and rs4553808 T>C are in strong LD, the function of *CTLA-4* rs16840252 C>T could be influenced by *CTLA-4* rs4553808 C>T or rs5742909 C>T. To the best of our knowledge, this case–control study was the first investigation to assess the association between *CTLA-4* rs16840252 C>T genotype and CRC risk. Our findings indicated the *CTLA-4* rs16840252 C>T polymorphism represented a risk factor for colon cancer. Our findings are supported by those pool-analyses mentioned above.

The *CTLA-4* rs231775 G>A polymorphism was the most frequently explored and was established as a functional SNP of the *CTLA-4* gene.[43,44] The *CTLA-4* rs231775 G>A (c.49 G>A) SNP causes p.17Ala >17 Thr change in the leading sequence of CTLA-4 receptor.[40,44] Previous studies have demonstrated that the *CTLA-4* rs231775 G allele has a lower mRNA efficiency and downregulates CTLA-4 protein more than the *CTLA-4* rs231775 A allele.[45] Therefore, individuals who carry the *CTLA-4* rs231775 AA genotype have lower T-cell proliferation and immune response than those with the *CTLA-4* rs231775 GG genotype.[44] Sun et al[43] also found that the p.17Ala >17 Thr substitution in CTLA-4 amino acid residue caused by the c.49 G>A SNP significantly increased the interaction of the CTLA-4 receptor with its ligand B7.1, and recombinant CTLA-4-17Thr had a higher inhibitory effect to T-cell proliferation and immune response compared with CTLA-4-17Ala. These primary studies suggested that p.17Ala>17 Thr change in CTLA-4 may lead to a significant effect of T-cell proliferation and activation. A previous study demonstrated that donor *CTLA-4* rs231775 genotype modulates the immune response to minor histocompatibility antigen mismatches.[46] The *CTLA-4* rs231775 genotype was also considered as a genetic determinant in autoimmune Addison's disease.[47] Recently, a number of case–control studies focused on the relationship between *CTLA-4* rs231775 G>A SNP and the risk of cancer, and results of subsequent meta-analyses evidenced that the *CTLA-4* rs231775 G>A polymorphism was a risk factor for multiple cancer, especially in Asian populations.[32–35] Three pooled-analysis studies also suggested that this polymorphism was associated with the development of CRC.[15–17] Although these findings tried to suggest an association between *CTLA-4* rs231775 G>A

polymorphism and CRC, the number of included studies and participants were limited. Thus, we conducted this case–control study with larger sample sizes to explore whether the *CTLA-4* rs231775 G>A polymorphism was a risk factor for CRC. As demonstrated in Table 4, we found that this polymorphism was associated with an increased risk of CRC. We also studied the association of *CTLA-4* rs231775 G>A polymorphism with CRC in different subgroups. Similar findings were also found when the Bonferroni correction was applied. The association was also significant in the colon cancer subgroup (AA vs GG: OR=1.61; 95% CI=1.12–2.30; *P*=0.010 and AA vs GG/GA: OR=1.59; 95% CI=1.13–2.23; *P*=0.009; Table 4). Results of the present study were in accordance with results of those meta-analyses and functional studies mentioned above.

CTLA-4 rs3087243 G>A, rs16840252 C>T, rs733618 T>C, and rs231775 G>A variants may not be inherited randomly. As summarized in Table 6, we found the frequency of $G_{rs3087243}C_{rs16840252}C_{rs733618}A_{rs231775}$, $G_{rs3087243}C_{rs16840252}T_{rs733618}A_{rs231775}$, and other haplotypes was significantly increased in CRC patients. We first reported the association of these *CTLA-4* haplotypes with CRC susceptibility. A previous study suggested that the *CTLA4* $G_{rs3087243}C_{rs16840252}C_{rs733618}A_{rs231775}$ haplotype significantly increased the risk of gastric cardia adenocarcinoma,[14] which was similar to our findings. However, these *CTLA4* haplotypes only influenced a very minor fraction (less than 2%) of the CRC patients.

Of note, we focused on the relationship of CTLA-4 tagging SNPs with CRC risk in an Eastern Chinese Han population. In addition, the sample size of our study was larger than before. Finally, the MAF in our controls was very similar to the data for Chinese in the database (Table 2).

Although there were some merits in our study, some limitations should also be addressed. First, this study was designed as a hospital-based investigation; the CRC patients and controls were recruited from hospitals in Eastern China and might not well represent the whole Eastern Chinese Han population. Second, the recruited CRC cases were moderate in stratified analyses. In the future, these findings should be verified in well-designed studies with a larger sample size. Third, because of the limited sample size of CRC patients and absence of a validation cohort, the power of the present study may be insufficient, especially in stratified analyses. Fourth, for insufficient samples, a replicated study was not conducted. Fifth, due to lack of other information, we did not carry out a further evaluation of potential interaction, such as dietary habit, family history, hormone level, intake of vitamins, other environmental factors, and lifestyles. In considering the complexity of CRC etiology, the gene–environment interaction should not be ignored. Finally, in our case–control study, we investigated four tagging SNPs in the *CTLA-4* gene and did not focus on other functional SNPs. In the future, a fine-mapping study is needed to further identify any potential association.

Conclusion

In summary, the findings of our case–control study evidence that *CTLA-4* rs16840252 C>T and rs231775 G>A SNPs are correlated with genetic susceptibility for development of CRC in an Eastern Chinese Han population. Additionally, this study first highlights that *CTLA-4* rs16840252 C>T polymorphism increases the susceptibility of CRC. Furthermore, findings are consistent with the biological functions of tagging SNPs in the *CTLA-4* gene and validate the hypothesis that *CTLA-4* tagging polymorphisms, which alter CTLA-4 mRNA and/or protein expression, may influence normal immune functions and lead to an increased risk of CRC.

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Disclosure

The authors report no conflicts of interest in this work.

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