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

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Introduction
Colorectal cancer (CRC) is one of the most common malignancies and is becoming the fifth leading cause of cancer death in China. It is reported that the incidence rate and CRC-related mortality are increasing rapidly worldwide. These may be attributed to certain lifestyles and environmental factors, including physically inactive, overweight, smoking, and drinking. Recently, Katsidzira et al 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. 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.
of these conceivable inherited factors may enrich our view on the etiology of CRC.

The cytotoxic T-lymphocyte antigen-4 (CTLA-4) gene, also known 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. The expression of CTLA-4 inhibits cytokine production of T cells and then provides a negative signal to T cells. 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.

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. Some case–control studies explored the relationship between CTLA-4 polymorphisms and CRC, 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. 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. 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 neo-adjuvant 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. 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. 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) r² threshold.
of 0.8 between SNPs (with a minimum LD of $r^2 > 0.8$). SNPs with a Hardy–Weinberg equilibrium (HWE) $P \geq 0.05$, minor allele frequency (MAF) $\geq 0.05$, and call rate $\geq 95\%$ in the CHB cohort were included. 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 1 Distribution of selected demographic variables and risk factors in CRC cases and controls

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases (n=1,003)</th>
<th>Controls (n=1,303)</th>
<th>P-value $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), M ($\pm$SD)</td>
<td>61.10 ($\pm$12.17)</td>
<td>61.40 ($\pm$9.61)</td>
<td>0.496</td>
</tr>
<tr>
<td>Age (years)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>$&lt;$61</td>
<td>451</td>
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<td>0.605</td>
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<tr>
<td>$\geq$61</td>
<td>552</td>
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</tr>
<tr>
<td>Sex</td>
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</tr>
<tr>
<td>Male</td>
<td>620</td>
<td>801</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>383</td>
<td>502</td>
<td></td>
</tr>
<tr>
<td>Smoking status</td>
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</tr>
<tr>
<td>Never</td>
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<td>1,038</td>
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<td>Ever</td>
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<td>265</td>
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<td>Alcohol use</td>
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</tr>
<tr>
<td>Ever</td>
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<td>BMI ($\mathrm{kg/m^2}$)</td>
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<td>$&lt;$24</td>
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<td>615</td>
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<td>Primary site of tumor</td>
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<tr>
<td>Colon cancer</td>
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</tr>
<tr>
<td>Rectal cancer</td>
<td>572</td>
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<tr>
<td>Degree of differentiation</td>
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<td></td>
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<tr>
<td>Poorly differentiated</td>
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<td>12.36</td>
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</tr>
<tr>
<td>Moderately differentiated</td>
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<tr>
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<td>0–I</td>
<td>167</td>
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<tr>
<td>II</td>
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</tr>
<tr>
<td>III</td>
<td>420</td>
<td>41.87</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>126</td>
<td>12.56</td>
<td></td>
</tr>
</tbody>
</table>

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.

### Table 2 Primary information for CTLA-4 polymorphisms

<table>
<thead>
<tr>
<th>Genotyped SNPs</th>
<th>CTLA-4 rs3087243 C&gt;T</th>
<th>CTLA-4 rs231775 G&gt;A</th>
<th>CTLA-4 rs16840252 C&gt;T</th>
<th>CTLA-4 rs733618 T&gt;C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosome</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Function</td>
<td>nearGene-3</td>
<td>Missense</td>
<td>nearGene-5</td>
<td>nearGene-5</td>
</tr>
<tr>
<td>Location</td>
<td>Intron 3-6,230</td>
<td>Exon 1+49</td>
<td>Promoter 1147</td>
<td>Promoter 1722</td>
</tr>
<tr>
<td>Chr Pos (Genome Build 38)</td>
<td>203874196</td>
<td>203867991</td>
<td>203866786</td>
<td>203866221</td>
</tr>
<tr>
<td>MAF for Chinese in database</td>
<td>0.183</td>
<td>0.314</td>
<td>0.122</td>
<td>0.390</td>
</tr>
<tr>
<td>MAF in our controls (n=1,303)</td>
<td>0.189</td>
<td>0.305</td>
<td>0.117</td>
<td>0.412</td>
</tr>
<tr>
<td>P-value for HWE test in our controls</td>
<td>0.411</td>
<td>0.430</td>
<td>0.065</td>
<td>0.335</td>
</tr>
<tr>
<td>Genotyping method</td>
<td>SNPScan</td>
<td>SNPScan</td>
<td>SNPScan</td>
<td>SNPScan</td>
</tr>
<tr>
<td>% Genotyping value</td>
<td>98.87%</td>
<td>98.79%</td>
<td>98.87%</td>
<td>98.87%</td>
</tr>
</tbody>
</table>

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=0.05) (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 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,
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\(_{rs3087243}^A\) G\(_{rs16840252}^C\) G\(_{rs733618}^C\) A\(_{rs231775}^T\).

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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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CRC cases (n=1,003)</th>
<th>Colon cancer (n=431)</th>
<th>Rectum cancer (n=572)</th>
<th>Controls (n=1,303)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>rs3087243</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G&gt;A</td>
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<tr>
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<td>GA</td>
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</tr>
<tr>
<td>AA</td>
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<td>4.73</td>
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<tr>
<td>A allele</td>
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<td>19.90</td>
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<td>20.33</td>
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</table>

Abbreviation: CRC, colorectal cancer.
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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CRC cases (n=1,003) vs controls (1,303)</th>
<th>Colon cancer (n=431) vs controls (1,303)</th>
<th>Rectum cancer (n=572) vs controls (1,303)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude OR (95% CI)</td>
<td>P-value</td>
<td>Adjusted OR* (95% CI)</td>
</tr>
<tr>
<td>rs3087243 G&gt;A</td>
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<td></td>
</tr>
<tr>
<td>Additive model</td>
<td>0.94 (0.78–1.12)</td>
<td>0.485</td>
<td>0.89 (0.74–1.07)</td>
</tr>
<tr>
<td>Homozygote model</td>
<td>1.45 (0.94–2.22)</td>
<td>0.091</td>
<td>1.43 (0.93–2.21)</td>
</tr>
<tr>
<td>Dominant model</td>
<td>1.02 (0.86–1.21)</td>
<td>0.849</td>
<td>0.97 (0.81–1.16)</td>
</tr>
<tr>
<td>Recessive model</td>
<td>1.51 (0.99–2.31)</td>
<td>0.058</td>
<td>1.52 (0.99–2.34)</td>
</tr>
<tr>
<td>rs16840252 C&gt;T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Additive model</td>
<td>1.04 (0.85–1.27)</td>
<td>0.704</td>
<td>1.02 (0.84–1.25)</td>
</tr>
<tr>
<td>Homozygote model</td>
<td>1.80 (0.82–3.94)</td>
<td>0.142</td>
<td>1.72 (0.78–3.81)</td>
</tr>
<tr>
<td>Dominant model</td>
<td>1.10 (0.90–1.34)</td>
<td>0.349</td>
<td>1.08 (0.89–1.32)</td>
</tr>
<tr>
<td>Recessive model</td>
<td>1.82 (0.83–3.98)</td>
<td>0.133</td>
<td>1.72 (0.79–3.86)</td>
</tr>
<tr>
<td>rs733618 T&gt;C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Additive model</td>
<td>0.95 (0.79–1.14)</td>
<td>0.548</td>
<td>0.97 (0.81–1.17)</td>
</tr>
<tr>
<td>Homozygote model</td>
<td>0.93 (0.73–1.18)</td>
<td>0.540</td>
<td>0.97 (0.76–1.24)</td>
</tr>
<tr>
<td>Dominant model</td>
<td>1.00 (0.84–1.19)</td>
<td>0.970</td>
<td>1.03 (0.87–1.23)</td>
</tr>
<tr>
<td>Recessive model</td>
<td>0.98 (0.79–1.22)</td>
<td>0.868</td>
<td>1.01 (0.81–1.26)</td>
</tr>
<tr>
<td>rs231775 G&gt;A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Additive model</td>
<td>1.12 (0.94–1.33)</td>
<td>0.220</td>
<td>1.09 (0.90–1.38)</td>
</tr>
<tr>
<td>Homozygote model</td>
<td>1.47 (1.10–1.95)</td>
<td>0.008</td>
<td>1.40 (1.05–1.87)</td>
</tr>
<tr>
<td>Dominant model</td>
<td>1.23 (1.05–1.46)</td>
<td>0.014</td>
<td>1.19 (1.00–1.41)</td>
</tr>
<tr>
<td>Recessive model</td>
<td>1.43 (1.09–1.87)</td>
<td>0.011</td>
<td>1.38 (1.05–1.82)</td>
</tr>
</tbody>
</table>

Note: *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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Zhenjiang cohort</th>
<th>Fuzhou cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases (n=246)</td>
<td>Controls (n=623)</td>
</tr>
<tr>
<td>rs231775 G&gt;A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>100</td>
<td>319</td>
</tr>
<tr>
<td>GA</td>
<td>110</td>
<td>251</td>
</tr>
<tr>
<td>AA</td>
<td>26</td>
<td>51</td>
</tr>
<tr>
<td>GA + AA</td>
<td>136</td>
<td>302</td>
</tr>
<tr>
<td>GG + GA</td>
<td>210</td>
<td>570</td>
</tr>
<tr>
<td>AA</td>
<td>26</td>
<td>110</td>
</tr>
<tr>
<td>A allele</td>
<td>162</td>
<td>343</td>
</tr>
<tr>
<td>rs16840252 C&gt;T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>175</td>
<td>478</td>
</tr>
<tr>
<td>CT</td>
<td>59</td>
<td>137</td>
</tr>
<tr>
<td>TT</td>
<td>2</td>
<td>0.85</td>
</tr>
<tr>
<td>CT + TT</td>
<td>61</td>
<td>243</td>
</tr>
<tr>
<td>CC + CT</td>
<td>234</td>
<td>615</td>
</tr>
<tr>
<td>TT</td>
<td>2</td>
<td>0.85</td>
</tr>
<tr>
<td>T allele</td>
<td>63</td>
<td>133</td>
</tr>
<tr>
<td>rs3087243 G&gt;A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>175</td>
<td>433</td>
</tr>
<tr>
<td>GA</td>
<td>54</td>
<td>238</td>
</tr>
<tr>
<td>AA</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>GA + AA</td>
<td>61</td>
<td>262</td>
</tr>
<tr>
<td>GG + GA</td>
<td>297</td>
<td>653</td>
</tr>
<tr>
<td>AA</td>
<td>7</td>
<td>5.38</td>
</tr>
<tr>
<td>A allele</td>
<td>68</td>
<td>141</td>
</tr>
<tr>
<td>rs733618 T&gt;C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>70</td>
<td>224</td>
</tr>
<tr>
<td>TC</td>
<td>123</td>
<td>296</td>
</tr>
<tr>
<td>CC</td>
<td>43</td>
<td>182</td>
</tr>
<tr>
<td>TC + CC</td>
<td>166</td>
<td>397</td>
</tr>
<tr>
<td>TT + TC</td>
<td>193</td>
<td>520</td>
</tr>
<tr>
<td>CC</td>
<td>43</td>
<td>182</td>
</tr>
<tr>
<td>C allele</td>
<td>209</td>
<td>44.28</td>
</tr>
</tbody>
</table>

**Note:** *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. Garzia et al. 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
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<sub>rs3087243</sub>C<sub>rs16840252</sub>C<sub>rs733618</sub>A<sub>rs231775</sub> 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. 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. Interestingly, Idris et al<sup>19</sup> 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<sup>19</sup> 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.<sup>13,41,42</sup> 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.<sup>43,44</sup> 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.<sup>40,44</sup> 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.<sup>45</sup> 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.<sup>44</sup> Sun et al<sup>46</sup> 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.<sup>46</sup> The CTLA-4 rs231775 genotype was also considered as a genetic determinant in autoimmune Addison’s disease.<sup>47</sup> 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.<sup>32–35</sup> Three pooled-analysis studies also suggested that this polymorphism was associated with the development of CRC.<sup>15–17</sup> Although these findings tried to suggest an association between CTLA-4 rs231775 G>A

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

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>CRC cases (n=2,006)</th>
<th>Controls (n=2,006)</th>
<th>Crude OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL4A G&lt;sub&gt;rs3087243&lt;/sub&gt;C&lt;sub&gt;rs16840252&lt;/sub&gt;C&lt;sub&gt;rs733618&lt;/sub&gt;A&lt;sub&gt;rs231775&lt;/sub&gt;</td>
<td>765</td>
<td>1,069</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>490</td>
<td>726</td>
<td>0.84 (0.81–1.09)</td>
<td>0.437</td>
</tr>
<tr>
<td></td>
<td>382</td>
<td>492</td>
<td>1.08 (0.92–1.28)</td>
<td>0.326</td>
</tr>
<tr>
<td></td>
<td>237</td>
<td>294</td>
<td>1.13 (0.93–1.37)</td>
<td>0.230</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>2</td>
<td>23.76 (5.69–99.21)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>5</td>
<td>7.27 (2.78–19.01)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>10</td>
<td>3.07 (1.45–6.53)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

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

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

References


CTLA-4 tagging polymorphisms and colorectal cancer

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