Functional Genetic Variations in Cytotoxic T-Lymphocyte Antigen 4 and Susceptibility to Multiple Types of Cancer

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Abstract

Antitumor T lymphocytes play a pivotal role in immunosurveillance of malignancy. The CTL antigen 4 (CTLA-4) is a vital negative regulator of T-cell activation and proliferation. This study examined whether genetic polymorphisms in CTLA-4 are associated with cancer susceptibility. A two-stage investigation using haplotype-tagging single nucleotide polymorphism approach and multiple independent case-control analyses was performed to assess the association between CTLA-4 genotypes and cancer risk. Functional relevance of the polymorphisms was examined by biochemical assays. We found that the 49G>A polymorphism in the CTLA-4 leading sequence caused 17Ala to 17Thr amino acid substitution is associated with increased susceptibility to multiple cancers, including lung, breast, esophagus, and gastric cardia cancers. Genotyping in 5,832 individuals with cancer and 5,831 control subjects in northern and southern Chinese populations showed that the CTLA-4 49AA genotype had an odds ratio of 1.72 (95% confidence interval, 1.50–2.10; P = 3.4 × 10–7) for developing cancer compared with the 49GG genotype. Biochemical analyses showed that CTLA-4–17Thr had higher capability to bind B7.1 and stronger inhibitory effect on T-cell activation compared with CTLA-4–17Ala. T cells carrying the 49AA genotype had significantly lower activation and proliferation rates compared with T cells carrying the 49GG genotype upon stimulation. These results are consistent with our hypothesis and indicate that genetic polymorphisms influencing T-cell activation modify cancer susceptibility.

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Introduction

Cytotoxic T-lymphocyte antigen 4 (CTLA-4 and also known as CD152), a member of the immunoglobulin superfamily, is a costimulatory molecule expressed by activated T cells (1, 2). CTLA-4 is similar to another T-cell costimulatory molecule (CD28; 18, 20, 21), a costimulatory molecule expressed by activated T cells (1, 2). However, while the B7 family of costimulatory/coinhibitory molecules is involved in T-cell activation and proliferation, CTLA-4 acts as a negative regulator to reduce T-cell function and proliferation through multiple mechanisms such as reducing both interleukin (IL)-2 and IL-2 receptor productions and arresting T cells at the G1 phase in cell cycle (5). On the other hand, it has been shown that blockade of CTLA-4 function by antibodies enhances T-cell activation (6), and mice deficient in CTLA-4 gene develop lymphoproliferative disease (7). In addition to inhibiting T-cell activation and proliferation, CTLA-4 may also induce FAS-independent apoptosis of activated T cells (8).

Antitumor T lymphocytes play a pivotal role in immune surveillance of cancer cells (9). It has been shown that tumor-transplanted mice injected with antibodies that block CTLA-4 activity rejected several different types of tumors and had long-lasting antitumor immunity (10). Several studies have reported that antibodies that block the activity of this key immunoregulatory molecule cause tumor shrinkage in patients with metastatic melanoma when administered along with a cancer vaccine (11–13). Preclinical and clinical trials have also shown that CTLA-4 blockade may be a promising immunotherapeutic approach to treat patients with other advanced cancers (14–16). These findings suggest that CTLA-4 may play an important role in cancer development and progression.

The CTLA-4 gene is composed of four exons that encode separate functional domains: leader sequence, extracellular domain, transmembrane domain, and cytoplasmic domain (17). Several important polymorphic sites have been reported and haplotype block structure has been identified in this gene locus (18, 19). Extensive studies have been conducted to address the association between polymorphisms within CTLA-4 and autoimmune disease, including Hashimoto thyroiditis (20), Graves’ disease (18, 20, 21), systemic lupus erythematosus (22, 23), and type 1 diabetes mellitus (18, 24–26), resulting from inflammatory destruction of insulin producing β cells of the pancreas caused by overproliferated T lymphocytes. However, little is known about the relationship between genetic polymorphisms in CTLA-4 and cancer susceptibility. Moreover, the biological function of the polymorphisms associated with risk of these cancers is not elucidated yet.

We hypothesized that polymorphisms in CTLA-4, which enhance the CTLA-4 pathway and thus interfere with T-cell proliferation and/or function, might be a genetic susceptibility factor for common human cancers. To test this hypothesis, we have investigated the association between CTLA-4 genotype and risk for the development of multiple types of cancer in two Chinese populations using tagging single nucleotide polymorphisms (SNP) and select candidate functional SNPs. By genotyping a large size of samples of cancer patients and controls, we have identified 49G>A SNP in the leading sequence of CTLA-4 as a potential cancer
susceptibility variant. Functional analyses reveal that this SNP enhances the interaction between CTLA-4 and B7.1, resulting in stronger CTLA-4–triggered inhibition of T-cell activation and proliferation.

**Materials and Methods**

**Study subjects.** All subjects in this study were unrelated Han Chinese and they were derived from Northern Chinese population or Southern Chinese population. In Northern Chinese population, patients with lung cancer (\(n = 1,163\)), breast cancer (\(n = 1,060\)), esophageal cancer (\(n = 1,010\)), and gastric cardia cancer (\(n = 530\)) were recruited at the Cancer Hospital, Chinese Academy of Medical Sciences. These patients were from Beijing city and surrounding provinces and therefore may represent well all cases in this region of Northern China. All eligible patients were recruited between January 1997 and July 2003, with response rates of 87% to 94%. Controls (\(n = 1,132\)) were randomly selected from a pool of 20,000 individuals living in the same region during the same period as patients were collected, which is believed to well-represent the Chinese population in this region. The participation response rate for controls was 83%. Controls were selected based on physical examinations and frequency matched to each set of cancer patients on age (± 5 y), sex, and residential area (urban or countryside). The most parts of these case-control sets have been also published previously (28, 29). The distributions of selected characteristics by case-control status are shown in Table 1. For functional assays, blood samples were obtained from 37 healthy volunteers (19 males and 18 females) working in our institution ages 19 to 49 years old. At recruitment, informed consent was obtained from each subject and this study was approved by the Institutional Review Board of the Chinese Academy of Medical Sciences Cancer Institute and the Nanjing Medical University.

**SNP selection and genotyping.** Candidate SNPs consisted of haplotype-tagging SNPs (htSNP) and SNPs that have been proposed to be of functional significance (18, 30, 31). htSNPs were chosen from genotyped SNPs reported in literature (18, 19) and in Han Chinese population of the HapMap Project (Phase I database) with Haplovie 3.22 software on a block-by-block basis using a method described previously (32) with the sample size inflation factor \(R^2_0 \geq 0.8\). Coding-region SNPs were directly selected from National Center for Biotechnology Information public SNP database on the basis of their frequency in Asians. SNPs in the CTLA-4 promoter region and coding region were identified by directly sequencing PCR products of genomic DNAs from 40 individuals. SNPs in stage I association analysis were genotyped by the MassARRAY system (Sequenom) as described (27). For quality control, the positive and negative (no DNA) samples were included on every 96-well assay plate. To reduce genotyping cost, we developed a PCR-RFLP approach to determine lung cancer-associated CTLA-4 49G>A

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**Table 1. Distribution of selected characteristics by case-control status in multiple types of cancer association analysis**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Lung cancer ((n = 1,163))</th>
<th>Controls ((n = 1,132))</th>
<th>Esophageal cancer ((n = 1,010))</th>
<th>Controls ((n = 1,008))</th>
<th>Gastric cardia cancer ((n = 530))</th>
<th>Controls ((n = 530))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>814 (70.0)*</td>
<td>781 (69.0)</td>
<td>798 (79.0)</td>
<td>806 (80.0)</td>
<td>371 (70.0)</td>
<td>360 (68.0)</td>
</tr>
<tr>
<td>Female</td>
<td>349 (30.0)</td>
<td>351 (31.0)</td>
<td>212 (21.0)</td>
<td>202 (20.0)</td>
<td>159 (30.0)</td>
<td>170 (32.0)</td>
</tr>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤40</td>
<td>93 (8.0)</td>
<td>91 (8.0)</td>
<td>101 (10.0)</td>
<td>101 (10.0)</td>
<td>53 (10.0)</td>
<td>74 (14.0)</td>
</tr>
<tr>
<td>41–60</td>
<td>605 (52.0)</td>
<td>600 (53.0)</td>
<td>475 (47.0)</td>
<td>484 (48.0)</td>
<td>286 (54.0)</td>
<td>270 (51.0)</td>
</tr>
<tr>
<td>&gt;60</td>
<td>465 (40.0)</td>
<td>441 (39.0)</td>
<td>434 (43.0)</td>
<td>423 (42.0)</td>
<td>191 (36.0)</td>
<td>186 (35.0)</td>
</tr>
<tr>
<td>Smoking status †</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsmokers</td>
<td>395 (34.0)</td>
<td>555 (49.0)</td>
<td>394 (39.0)</td>
<td>464 (46.0)</td>
<td>148 (28.0)</td>
<td>207 (39.0)</td>
</tr>
<tr>
<td>Smokers</td>
<td>768 (66.0)</td>
<td>577 (51.0)</td>
<td>616 (61.0)</td>
<td>544 (54.0)</td>
<td>382 (72.0)</td>
<td>323 (61.0)</td>
</tr>
<tr>
<td>Smoking level †</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>≤30 pack-years</td>
<td>284 (37.0)</td>
<td>404 (70.0)</td>
<td>312 (50.7)</td>
<td>370 (68.0)</td>
<td>183 (48.0)</td>
<td>197 (61.0)</td>
</tr>
<tr>
<td>&gt;30 pack-years</td>
<td>484 (63.0)</td>
<td>173 (30.0)</td>
<td>304 (49.3)</td>
<td>174 (32.0)</td>
<td>199 (52.0)</td>
<td>126 (39.0)</td>
</tr>
</tbody>
</table>

*Number in parentheses are percentage.
† Two-sided \(\chi^2\) test between cases and controls, \(P = 2.4 \times 10^{-13}, 0.0014, 0.001\), and 0.017 for lung cancer, esophageal cancer, gastric cardia cancer, and breast cancer in Northern Chinese population, and \(P = 3.5 \times 10^{-19}\) and 0.02 for lung cancer and breast cancer in Southern Chinese population, respectively.
‡ Two-sided \(\chi^2\) test between cases and controls, \(P = 3.7 \times 10^{-35}, 2.0 \times 10^{-9}, 0.0005,\) and 0.498 for lung cancer, esophageal cancer, gastric cardia cancer, and breast cancer in Northern Chinese population, and \(P = 5.3 \times 10^{-18}\) and 0.145 for lung cancer and breast cancer in Southern Chinese population, respectively.

http://www.hapmap.org
polymorphism. The PCR primers used for amplifying DNA fragment containing the CTLA-4 49G-A site were 5'-aaggtcgtcaagctttg-3' and 5'-ctgctgaaacaaatgaaaccc-3', which produced a 152-bp fragment. We introduced a mismatch nucleotide by changing the last nucleotide C to T in the forward primer to create an endonuclease Eco91I (MBI Fermentas) site. With the digestion of Eco91I, the major GG allele produces a single 152-bp band, whereas the minor AA allele produces 130-bp and 22-bp two bands. To validate the PCR-RFLP method, randomly selected 500 DNA samples were analyzed by both pyrosequencing assay and Eco91I restriction digestion assay (see Supplementary Methods), and the concurrence rate of these two methods was 99%, indicating that the PCR-RFLP method is reliable. Genotyping was performed independently in two laboratories (Chinese Academy of Medical Sciences and Nanjing Medical University) without knowledge of the case/control status. A 5% random sample was reciprocally tested by different persons in the two laboratories, and the reproducibility were 100%.

**Peripheral blood mononuclear cells isolation and culture.** Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood using Ficoll-Paque (Sigma), washed, and resuspended in RPMI 1640 containing 10% fetal bovine serum. We incubated PBMCs with or without 1 mg/mL recombinant 6-His tag CTLA-4 proteins or 25 μg/mL phytohemagglutinin (PHA; Sigma) for 6 h.

**Immunoprecipitation and immunoblotting.** Immunoprecipitation (IP) and immunoblotting (IB) were performed as previously described (33) with minor modifications. Briefly, PBMC lysates were prepared in lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% Tween 20, 0.2% Nonidet P-40, and 10% glycerol] supplemented with protease inhibitor (Roche), with the digestion of Eco91I site. With the digestion of Eco91I, the major GG allele produces a single 152-bp band, whereas the minor AA allele produces 130-bp and 22-bp two bands. To validate the PCR-RFLP method, randomly selected 500 DNA samples were analyzed by both pyrosequencing assay and Eco91I restriction digestion assay (see Supplementary Methods), and the concurrence rate of these two methods was 99%, indicating that the PCR-RFLP method is reliable. Genotyping was performed independently in two laboratories (Chinese Academy of Medical Sciences and Nanjing Medical University) without knowledge of the case/control status. A 5% random sample was reciprocally tested by different persons in the two laboratories, and the reproducibility were 100%.

**Flow cytometry analysis.** A FACSCaliber flow cytometry system and CellQuest software (BD Bioscience) were performed to detect the binding levels of CTLA-4 and B7.1 in vivo and T-cell activation status using the proportion of CD25+/CD4+ cells to lymphocytes as an index by using CTLA-4 (clone 48815), B7.1 (clone 2D10), CD4 (clone SK3; BD), and CD8 (clone 53.6; BD) expression on lymphocytes. The concentrations of CTLA-4 proteins were determined by Bichemiconic acid kit (Pierce).
sex, age, smoking status, age at menarche, and menopausal status, where it was appropriate. Kruskal-Wallis one-way ANOVA test was performed to analyze differences in the levels of CTLA-4 and B7.1 binding, the proportions of CD4+/CD25+ lymphocytes, the concentrations of IL-2 in cell culture medium, and the rates of MTT. Student’s t test was used to examine the differences of two recombinant CTLA-4 proteins in suppressing T lymphocyte activation. These statistical analyses were implemented in Statistic Analysis System software (version 9.0, SAS Institute). A P value of <0.05 was used as the criterion of statistical significance, and all statistical tests were two-sided tests.

Results

Identification of cancer susceptibility SNP in CTLA-4. Eleven candidate SNPs were genotyped in stage I case-control analysis consisting of 800 lung cancer patients and 800 controls to determine their allele and genotype frequencies (Table 2). A significant association with lung cancer was observed for the CTLA-4-17Ala (49GG genotype) showed a detected interaction.

Table 2. Associations between candidate SNPs in CTLA-4 and risk of lung cancer

<table>
<thead>
<tr>
<th>Identity</th>
<th>Location</th>
<th>Position*</th>
<th>Subjects</th>
<th>N</th>
<th>Common genotype (%)</th>
<th>Heterozygous genotype (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs231806 (G&gt;C)</td>
<td>Upstream</td>
<td>204911892</td>
<td>Cases</td>
<td>795</td>
<td>50.9</td>
<td>40.3</td>
</tr>
<tr>
<td>rs733618 (A&gt;G)</td>
<td>Promoter -1722</td>
<td>204933487</td>
<td>Cases</td>
<td>765</td>
<td>94.0</td>
<td>5.6</td>
</tr>
<tr>
<td>rs4553808 (T&gt;C)</td>
<td>Promoter -1661</td>
<td>204933548</td>
<td>Cases</td>
<td>800</td>
<td>94.6</td>
<td>5.0</td>
</tr>
<tr>
<td>rs5742909 (C&gt;T)</td>
<td>Promoter -318</td>
<td>204934890</td>
<td>Cases</td>
<td>800</td>
<td>70.3</td>
<td>26.5</td>
</tr>
<tr>
<td>rs231775 (G&gt;A)</td>
<td>Exon 1 +49</td>
<td>204935257</td>
<td>Cases</td>
<td>800</td>
<td>43.6</td>
<td>45.4</td>
</tr>
<tr>
<td>rs3087243 (G&gt;A)</td>
<td>Intron 3 +6230</td>
<td>204941462</td>
<td>Cases</td>
<td>800</td>
<td>67.6</td>
<td>29.9</td>
</tr>
<tr>
<td>rs7565213 (G&gt;A)</td>
<td>3′untranslated region</td>
<td>204945952</td>
<td>Controls</td>
<td>791</td>
<td>64.2</td>
<td>30.3</td>
</tr>
<tr>
<td>rs960792 (T&gt;C)</td>
<td>Down stream</td>
<td>204951793</td>
<td>Cases</td>
<td>786</td>
<td>62.2</td>
<td>33.5</td>
</tr>
<tr>
<td>rs1365965 (G&gt;A)</td>
<td>Down stream</td>
<td>204954413</td>
<td>Cases</td>
<td>798</td>
<td>59.0</td>
<td>35.0</td>
</tr>
<tr>
<td>rs2033171 (T&gt;C)</td>
<td>Down stream</td>
<td>204990699</td>
<td>Cases</td>
<td>794</td>
<td>53.2</td>
<td>39.3</td>
</tr>
<tr>
<td>rs1978595 (C&gt;T)</td>
<td>Down stream</td>
<td>204994072</td>
<td>Cases</td>
<td>800</td>
<td>57.5</td>
<td>36.5</td>
</tr>
</tbody>
</table>

*Position in NCBI build 34.
†Data were calculated by unconditional logistic regression, adjusted for age, sex, and smoking.
‡Tests for trend of odds were two sided and were based on likelihood ratio tests assuming a multiplicative model.

Differential binding capability of CTLA-4 variants to B7.1. It has been well-known that the inhibitory effect of CTLA-4 on T lymphocyte proliferation is through competing with CD28 to bind B7 on APCs. We therefore examined whether the CTLA-4-17Ala and CTLA-4-17Thr variants have differential binding capability toward B7.1 molecule. Equal cell lysates from PBMCs carrying the CTLA-4 49GG or 49AA genotype were immunoprecipitated with either anti-CTLA-4 or anti-B7.1 antibody in reciprocal Co-IP assays. Western blot analysis of whole cell lysates showed that the expression levels of B7.1 in PBMCs treated with or without PHA were similar, but the levels of CTLA-4 were significantly higher in PBMCs treated with PHA than in PBMCs treated without PHA (Fig. 1A). It was found that a similar amount of protein was specifically immunoprecipitated by anti-B7.1 antibody (Fig. 1B, top, lanes 1, 3, 5, and 7) but not by anti–CTLA-4 antibody (Fig. 1B, bottom, lanes 1, 3, 5, and 7). Interaction between different CTLA-4 variants and B7.1 were both robust when PBMCs were stimulated with PHA (Fig. 1B, bottom, lanes 3 and 7). However, in PBMCs without PHA stimulation, only CTLA-4-17Thr (49AA genotype) but not CTLA-4-17Ala (49GG genotype) showed a detected interaction with B7.1 (Fig. 1B, bottom, lane 5 versus 3). When similar amount of CTLA-4 in different cell lysates were applied in Co-IP assays (Fig. 1C, top), it was observed in reciprocal experiments that the amount of B7.1 bound to CTLA-4-17Thr (49AA genotype) was considerably higher than that bound to CTLA-4-17Ala (49GG genotype) in both PHA-stimulated or PHA-untreated PBMCs (Fig. 1C, right).
considerably decreased proportion of CD25+/CD4+ cells (mean ± SD) in PBMCs treated with CTLA-4 compared with that in PBMCs carrying the 49AA genotype was significantly lower than that in PBMCs carrying the 49GG genotype [median, 4.37%; range, 3.52–5.22% versus 6.46% (range, 5.04–7.88%); n = 22]; P = 0.004; Fig. 3A]. Analysis of IL-2 concentrations in cell culture medium showed that PBMCs carrying the 49AA genotype produced significantly lower IL-2 levels compared with PBMCs carrying the 49GG genotype [median, 844.0 pg/mL (range, 609.0–1,000; n = 4) versus 1,368.0 pg/mL (range, 708.5–3,331; n = 22); P = 0.004; Fig. 3B].

Effects of CTLA-4 49G>A genotypes on T-cell proliferation and IL-2 production. Having the results of differential effects of recombinant CTLA-4 proteins on T-cell activation in vitro, we further investigated whether the CTLA-4 49G>A genotypes have effect on T-cell proliferation and IL-2 production ex vivo. PBMCs isolated from 37 healthy individuals carrying different CTLA-4 49G>A genotype were incubated with or without PHA, and the proportions of CD25+/CD4+ cells to CD3+ cells and levels of IL-2 in the cell culture supernatants were determined, respectively. In addition, MTT assays were performed to detect T-cell proliferation after stimulation with PHA. We observed a significant difference in the activation of T cells after stimulation with PHA as a function of CTLA-4 genotype, with the median proportion of CD25+/CD4+ cells in PBMCs carrying the CTLA-4 49AA genotype being significantly lower than that in PBMCs carrying the CTLA-4 49GG genotype [6.46% (range, 5.62–7.11; n = 4) versus 10.72% (range, 8.44–20.34; n = 22); P = 0.023; Fig. 3C]. Analysis of IL-2 concentrations in cell culture medium showed that PBMCs carrying the CTLA-4 49AA genotype produced significantly lower IL-2 levels compared with PBMCs carrying the CTLA-4 49GG genotype [median, 844.0 pg/mL (range, 609.0–1,000; n = 4) versus 1,302.0 pg/mL (range, 708.5–3,331; n = 22); P = 0.004; Fig. 3B].

Table 2. Associations between candidate SNPs in CTLA-4 and risk of lung cancer (Cont’d)

<table>
<thead>
<tr>
<th>Rare genotype (%)</th>
<th>OR ↑ (95% CI) for heterozygote</th>
<th>P</th>
<th>OR ↑ (95% CI) for rare genotype</th>
<th>P</th>
<th>Prand ↑</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.8</td>
<td>1.06 (0.86–1.31)</td>
<td>0.586</td>
<td>1.02 (0.70–1.47)</td>
<td>0.934</td>
<td>0.720</td>
</tr>
<tr>
<td>8.9</td>
<td>1.17 (0.73–1.90)</td>
<td>0.493</td>
<td>3.17 (0.30–79.34)</td>
<td>0.291</td>
<td>0.291</td>
</tr>
<tr>
<td>0.4</td>
<td>1.14 (0.70–1.86)</td>
<td>0.577</td>
<td>3.00 (0.28–74.86)</td>
<td>0.318</td>
<td>0.357</td>
</tr>
<tr>
<td>0.1</td>
<td>1.09 (0.88–1.42)</td>
<td>0.613</td>
<td>1.10 (0.84–1.36)</td>
<td>0.588</td>
<td>0.292</td>
</tr>
<tr>
<td>2.4</td>
<td>1.22 (0.99–1.51)</td>
<td>0.054</td>
<td>2.09 (1.41–3.10)</td>
<td>0.0001</td>
<td>0.0002</td>
</tr>
<tr>
<td>6.1</td>
<td>1.09 (0.87–1.37)</td>
<td>0.425</td>
<td>0.93 (0.48–1.80)</td>
<td>0.820</td>
<td>0.602</td>
</tr>
<tr>
<td>2.7</td>
<td>1.05 (0.84–1.31)</td>
<td>0.648</td>
<td>0.94 (0.58–1.51)</td>
<td>0.782</td>
<td>0.896</td>
</tr>
<tr>
<td>5.1</td>
<td>0.91 (0.73–1.13)</td>
<td>0.369</td>
<td>0.68 (0.42–1.10)</td>
<td>0.100</td>
<td>0.101</td>
</tr>
<tr>
<td>6.0</td>
<td>0.98 (0.79–1.22)</td>
<td>0.884</td>
<td>0.93 (0.65–1.33)</td>
<td>0.688</td>
<td>0.716</td>
</tr>
<tr>
<td>10.1</td>
<td>1.02 (0.82–1.27)</td>
<td>0.837</td>
<td>0.85 (0.58–1.25)</td>
<td>0.388</td>
<td>0.629</td>
</tr>
<tr>
<td>7.5</td>
<td>0.91 (0.73–1.12)</td>
<td>0.346</td>
<td>0.85 (0.55–1.31)</td>
<td>0.441</td>
<td>0.270</td>
</tr>
<tr>
<td>6.7</td>
<td>0.91 (0.73–1.12)</td>
<td>0.346</td>
<td>0.85 (0.55–1.31)</td>
<td>0.441</td>
<td>0.270</td>
</tr>
</tbody>
</table>

Bottom, lanes 6 and 8 versus lanes 2 and 4). Similar results were obtained from other two independent Co-IP assays (data not shown). These findings indicate that amino acid substitution of 17Ala with 17Thr significantly enhances the interaction between CTLA-4 protein and B7.1 molecule.

Differential effects of recombinant CTLA-4 variants on T-cell activation. We next examined whether CTLA-4-17Ala and CTLA-4-17Thr variants have differential inhibitory effects on T-cell activation in vitro. To do this, two plasmid constructs containing the full-length CTLA-4 cDNA with G or A at the polymorphic site 49 were respectively expressed in E. coli to produce soluble CTLA-4-17Ala and CTLA-4-17Thr. The inhibitory effects of these CTLA-4 variants on T-cell activation were determined by flow cytometry analyses of lymphocytes stimulated with PHA. Flow cytometry analyses of cultured lymphocytes stained with both anti-CTLA-4 and anti-B7.1 antibodies showed that there was no significant difference in the amount of CTLA-4/B7.1 cells (mean ± SD) in lymphocytes incubated with recombinant CTLA-4-17Ala or CTLA-4-17Thr [1.29% ± 0.14% (n = 6) versus 1.12% ± 0.20% (n = 6); P = 0.120]. The proportion of CD25+/CD4+ cells to CD3+ cells in PHA-stimulated PBMCs in the presence or absence of the recombinant CTLA-4 proteins was then determined by flow cytometry as an index of T lymphocyte activation (Fig. 2A). We found that although both recombinant CTLA-4 proteins significantly inhibited T lymphocyte activation, which was shown by considerably decreased proportion of CD25+/CD4+ cells (mean ± SD) in PBMCs treated with CTLA-4 compared with that in PBMCs treated without CTLA-4, the inhibitory effect of CTLA-4-17Thr was greater than that of CTLA-4-17Ala (1.34% ± 0.42% versus 2.77% ± 0.82%; P = 0.003; Fig. 2B).
polymorphism has significant effect on the T-cell proliferation and activation process.

**Association of CTLA-4 49A>G genotype with multiple types of cancer.** Functional significance of the CTLA-4 49G>A SNP prompted us to examine the associations of this SNP and other two well-studied SNPs (−318C>T and 6230G>A) with risk of human cancer. To do this, six independent case-control analyses were performed by two independent laboratories at Chinese Academy of
Medical Sciences and Nanjing Medical University in Northern (Beijing and surrounding provinces) and Southern (Jiangsu Province) Chinese populations. In northern Chinese population, we genotyped 1,163 lung cancer patients, 1,010 esophageal squamous cell carcinoma patients, 530 gastric cancer patients, 1,060 breast cancer patients, and 3,740 frequency-matched controls, and in Southern Chinese population, we genotyped 1,032 lung cancer patients, 1,037 breast cancer patients, and 2,091 frequency-matched controls. The detailed baseline characteristics of patients and controls are shown in Table 2. The genotype distributions of all three CTLA-4 polymorphisms in controls were in agreement with those expected under HWE (P = 0.909, 0.748, and 0.444 in Northern population; and P = 0.152, 0.346, and 0.619 in Southern population, respectively). In Northern Chinese population, the frequency of the CTLA-4 49AA genotype was significantly higher in patients with lung cancer (P = 4.2 × 10⁻⁵), esophageal cancer (P = 7.5 × 10⁻⁴), gastric cancer (P = 0.026), and breast cancer (P = 4.5 × 10⁻³) compared with the corresponding controls. The odds of carrying the CTLA-4 49GA or 49AA genotype in cancer patient groups were 1.18 to 1.26 or 1.79 to 2.09, respectively, compared with the CTLA-4 49GG genotype. The association was confirmed in the Southern population where the odds of carrying the CTLA-4 49AA genotype in lung cancer and breast cancer patient groups were 1.46 (95% CI, 1.08–1.97; P = 0.013) and 1.56 (95% CI, 1.12–2.17; P = 0.009), respectively, compared with the CTLA-4 49GG genotype, whereas the heterozygous CTLA-4 GA genotype seemed not to be associated with increased risk. The ORs of the CTLA-4 49GA and CTLA-4 49AA genotype for overall cancer patients were 1.19 (95% CI, 1.09–1.28; P = 7.9 × 10⁻⁴) and 1.72 (95% CI, 1.50–2.10; P = 3.4 × 10⁻³), with a P_strong < 0.0001 (Table 3). Adjustment for age, sex, smoking status, age at menarche, and menopausal status did not significantly change the respective ORs. However, no significant association between CTLA-4 –318C>T or 6230G>A SNP and risk of the investigated cancers was observed (data not shown), which is consistent with the results of stage I association analysis.

Discussion

It has been established that immunodeficiency is associated with human cancer, but the mechanisms involved are poorly understood (34). We have recently identified a six-nucleotide deletion polymorphism in the promoter of the CASP8 gene that abolishes an Sp1 transcription factor binding site and is associated with lower caspase-8 activity and T lymphocyte apoptotic activity; this deletion variant is associated with reduced risk of multiple types of human cancer (27). These findings highlight the importance of antitumor T lymphocytes in immune surveillance of tumorigenesis. In the model of tumor-specific T-cell activation, accumulative evidence has documented that removal of CTLA-4-mediated inhibition leads to enhancement of antitumor responses (1, 6, 10, 12–17). However, whether genetic variations in CTLA-4 may influence T-cell activation in the immune response and thus affect susceptibility to cancer is largely unknown. In the present study, we show that susceptibility to certain common cancers is associated with genetic polymorphisms in CTLA-4 that presumably decrease the reactivity of T lymphocytes in antitumor immunity. By using haplotype tagging SNPs and select potentially functional SNPs, we identified the 49G>A SNP in the coding region of CTLA-4, which causes Ala to Thr amino acid change in the leader sequence of CTLA-4 protein, as a possible lung cancer susceptibility polymorphism. Functional assays revealed that the CTLA-4 encoded by the CTLA-4 49A allele has enhanced interaction with B7.1 molecule and enhanced inhibitory effect on T-cell activation and proliferation compared with that encoded by the CTLA-4 49G allele in vitro and in ex vivo model. More importantly, the CTLA-4 49A allele is associated with increased risks of many types of cancer, including breast, esophageal, and gastric cardiac cancer, indicating that this CTLA-4 variant might be a common genetic susceptibility factor for human cancer.

Figure 3. Differential levels of T lymphocyte activation and proliferation in PBMCs from healthy individuals carrying different CTLA-4 49 genotypes. A, data presented in box plot show a significantly lower T lymphocyte activation in 49AA genotype than in 49GG genotype when PHA were treated with PHA. B, IL-2 levels as a function of CTLA-4 49 genotype, showing that the AA genotype produced a significantly lower IL-2 than the GG genotype when PBMCs were treated with PHA. C, cell proliferation rate detected by MTT assay in PBMCs carrying different CTLA-4 49 genotypes, showing that T lymphocyte carrying the AA genotype had significantly low cell proliferation when stimulated with PHA. The line inside each box is the median, the upper and lower limits of the box are the 75th and 25th percentiles, respectively, and the vertical bars above and below the box indicate the maximum and minimum values, respectively. Solid circles, outlier values.
The importance of CTLA-4 in antitumor immunity has been well-recognized (1–10). Therefore, it is biologically reasonable that functional CTLA-4 polymorphisms might play a role in the development of cancer. In fact, studies have shown that CTLA-4 polymorphisms might be associated with susceptibility to lymphoma (35, 36), myeloma (37), breast cancer (38, 39), oral squamous cell carcinoma (40), and human papillomavirus-16–related cervical cancer (41). However, these previous studies genotyped only one or a few polymorphisms and recruited relatively small number of study subjects. The CTLA-4 49G>A SNP has been linked to elevated risk of breast cancer in an Iranian population (39), non–Hodgkin’s lymphoma in an European Caucasian population (37), and poor survival of oral squamous cell carcinoma in a Taiwanese population (40), which are in agreement with our results in Chinese populations. Despite of having aforementioned previous studies, it was not clear which polymorphism in CTLA-4 is the causative site.

By using more comprehensive strategy of htSNPs and potentially functional SNPs together, we provided evidence that the 49G>A variant might be the causative polymorphism because of its strong association with risk of multiple cancers and because of its functional consequence associated with T lymphocyte activation and proliferation phenotype. The CTLA-4 49G>A polymorphism displays frequencies that are dependent on ethnicity. According to the HapMap Project and Environmental Genome Project, the frequencies of the CTLA-4 49A allele are 0.793 and 0.675, respectively, in European Caucasians and Africans (sub-Saharan Africans and African Americans) but 0.331 in Asians. It would be interesting and important to conduct independent studies in other ethnic populations for comparison.

The biological function of the CTLA-4 49G>A SNP is not fully characterized. Two previous studies reported that the CTLA-4 49G>A polymorphism enhances the inhibitory effect of CTLA-4 on T-cell activation (21, 42), which are consistent with our results. It was shown that the 49G>A SNP influence cell-surface expression of cell-surface expression of

Table 3. Associations between CTLA-4 49G/A genotypes and multiple types of cancer

<table>
<thead>
<tr>
<th>Cancer site</th>
<th>Genotype</th>
<th>Cases, n (%)</th>
<th>Controls, n (%)</th>
<th>OR* (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung (Beijing)</td>
<td>GG</td>
<td>509 (43.8)</td>
<td>563 (49.7)</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>519 (44.6)</td>
<td>488 (43.1)</td>
<td>1.18 (0.99–1.40)</td>
<td>0.068</td>
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<tr>
<td></td>
<td>AA</td>
<td>135 (11.6)</td>
<td>81 (7.2)</td>
<td>1.89 (1.39–2.57)</td>
<td>4.2 × 10⁻³</td>
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<tr>
<td>P_trend †</td>
<td>GG</td>
<td>468 (45.4)</td>
<td>493 (48.3)</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>439 (42.5)</td>
<td>438 (42.9)</td>
<td>1.07 (0.88–1.28)</td>
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<td>90 (8.8)</td>
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<td>474 (44.7)</td>
<td>559 (52.2)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>485 (45.8)</td>
<td>446 (41.7)</td>
<td>1.26 (1.04–1.51)</td>
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<td></td>
<td>AA</td>
<td>101 (9.5)</td>
<td>65 (6.1)</td>
<td>1.91 (1.33–2.74)</td>
<td>4.5 × 10⁻⁴</td>
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<td>482 (46.5)</td>
<td>546 (51.0)</td>
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<td>451 (42.2)</td>
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<tr>
<td></td>
<td>AA</td>
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<tr>
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<td>448 (44.4)</td>
<td>529 (52.5)</td>
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<tr>
<td></td>
<td>GA</td>
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<td>406 (40.3)</td>
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<td>AA</td>
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<td>235 (44.3)</td>
<td>282 (53.2)</td>
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<td>GA</td>
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<td>209 (39.4)</td>
<td>1.46 (1.07–1.98)</td>
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<td>2,972 (51.0)</td>
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<td></td>
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<td>2,400 (41.8)</td>
<td>1.19 (1.09–1.28)</td>
<td>7.9 × 10⁻⁴</td>
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<tr>
<td></td>
<td>AA</td>
<td>421 (7.2)</td>
<td>421 (7.2)</td>
<td>1.72 (1.50–2.10)</td>
<td>3.4 × 10⁻⁷</td>
</tr>
</tbody>
</table>

*Data were calculated by unconditional logistic regression, adjusted for age, sex, smoking status, age at menarche, and menopausal status, where it was appropriate. For all cancer together, the analyses were also adjusted for study.

†Number of subjects.

‡Tests for trend of odds were two-sided and based on likelihood ratio tests assuming a multiplicative model.
CTLA-4 (30, 31, 42); however, such an effect was not observed in other investigations (21, 43). In the present study, we showed that the CTLA-4 49G>A polymorphism enhances CTLA-4 protein to bind its ligand B7.1. Although we did not observe significant difference between exogenously expressed CTLA-4-17Ala and CTLA-4-17Thr in the reaction with B7.1 molecule on T-cell surface by flow cytometry analysis, we did find that the interaction between CTLA-4-17Thr and B7.1 is significantly stronger than that between CTLA-4-17Ala and B7.1 in cells by reciprocal Co-IP assays. On the basis of these findings, we proposed that the mechanism by which the CTLA-4 49G>A SNP alters CTLA-4 function might be mediated by amino acid substitution in the peptide leader sequence, which enhances the capability of variant CTLA-4 to communicate with B7.1 molecule. To deliver inhibitory signals and regulate immune response, CTLA-4 must interact with costimulatory B7 molecules on APCs (42–45) and, thus, the CTLA-4 variant having higher capability to bind B7 molecule would expect to have pronounced function than its counterpart. Our results in the present study are consistent with this notion. Quantitative analyses of CD25+/CD4+ cell number, IL-2 concentration, and lymphocyte proliferation in ex vivo model showed that the inhibitory effect of CTLA-4-17Thr on T-cell activation and proliferation was much stronger than that of CTLA-4-17Ala. Furthermore, PBMCs carrying the CTLA-4 49AA genotype showed significantly less activation of T regulatory cells (CD25+/CD4+) cells, less production of IL-2, and less T-cell proliferation compared with PBMCs carrying the CTLA-4 49GG genotype when stimulated with PHA. These findings are in agreement with previous observations showing that the CTLA-4 49AA genotype is correlated with decreased T-cell proliferation after stimulation with an allogenic cell line (42). Taken together, these findings indicate that subtle change of CTLA-4 function due to amino acid substitution-causing SNP may interfere with T-cell proliferation and/or activation via attenuating costimulation signaling. In addition, it has been shown that CTLA-4 crosslinking on the surface of activated T cells also leads to death of a substantial fraction of the cells (8). Because T lymphocytes play a very important role in immune surveillance of cancer cells (9, 46), it would be expected that individuals, who carry the CTLA-4 49A allele and thus have stronger negative regulation of T-cell proliferation and function, are at higher risk for developing cancer.

Our results of associations between the CTLA-4 49G>A polymorphism and susceptibility to cancer in the present study are obtained from multiple independent case-control analyses derived from Northern and Southern Han Chinese populations, and genotyping were performed in two independent laboratories. Having relative large sample sizes, significantly increased ORs with small P values, these results are unlikely to be attributable to selection bias or unknown confounding factors. The fact that genotype frequencies of all SNPs among controls fit HWE and are identical in the two populations further supports the randomness of our control selection. More notably, associations are biologically plausible and consistent with functional findings. Nevertheless, further studies of these and other cancers would be beneficial to confirm the results.

In conclusion, our study shows an association between the CTLA-4-49G>A SNP and susceptibility to multiple types of human cancer. This SNP causes amino acid substitution in the leading sequence of CTLA-4 molecule, resulting in intensified interaction with costimulatory receptor B7.1 and consequently reduced activation and proliferation of T lymphocytes among individuals carrying the variant CTLA-4 49A allele, which might be the underlying mechanism conferring cancer susceptibility. These findings together with our previous argument (27) further support the hypothesis that genetic polymorphisms influencing an individual’s immune status modify cancer susceptibility.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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