CTLA-4 +49A>G Polymorphism Is Associated with Advanced Non-Small Cell Lung Cancer Prognosis

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Key Words

Cytotoxic T lymphocyte antigen 4 • Immune response • Polymorphism • Non-small cell lung cancer • Survival • Prognosis

Abstract

Background: Cytotoxic T lymphocyte antigen 4 (CTLA-4) is a potent immunoregulatory molecule that suppresses antitumor response by downregulating T cell activation. The most studied +49A>G polymorphism of the CTLA-4 gene has been associated with several autoimmune diseases. However, little is known about the association between this functional polymorphism of CTLA-4 and cancer prognosis.

Objective: To investigate the association between CTLA-4 +49A>G polymorphism and prognosis of advanced non-small cell lung cancer (NSCLC) patients in a Chinese population. **Methods:** The CTLA-4 +49A>G polymorphism was detected by polymerase chain reaction-restriction fragment length polymorphism in 338 advanced NSCLC patients. **Results:** The frequencies of CTLA-4 +49 GG, GA and AA in advanced NSCLC patients were 44.4%, 42.0% and 13.6%, respectively. No significant association was observed between CTLA-4 +49A>G polymorphism and clinicopathologic features of advanced NSCLC including gender, histopathologi-

cal type, clinical stage and tumor markers. Patients with the AA genotype had a survival time of 9.8 months, significantly shorter than those with the GG genotype (12.5 months) or the GA genotype (12.0 months) ($p < 0.001$; log-rank test). Multivariate Cox analysis further revealed that the CTLA-4 +49AA genotype is an independent adverse prognostic indicator for NSCLC patients. **Conclusion:** Our data suggest that the polymorphism of CTLA-4 +49A>G is a prognostic predictor for advanced NSCLC.

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Introduction

Lung cancer has the highest morbidity and mortality rates among malignant cancers worldwide. Approximately 2.2 million lung cancer cases and 1.6 million lung cancer deaths occur each year in China [1]. Non-small cell lung cancer (NSCLC) accounts for about 80% of primary lung cancers, and around two thirds of NSCLC patients are diagnosed in advanced stage [2]. Despite chemotherapy and radiotherapy, prognosis of advanced

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NSCLC patients remains very poor, with the 1-, 3- and 5-year survival rates for stage III NSCLC being 54, 24 and 13%, respectively [2]. While factors such as age, clinical stage and treatment methods have been suggested as potential prognosis factors [3, 4], it remains difficult to predict the survival of advanced NSCLC patients.

Alterations in the immune system have been proposed to be linked with the incidence and clinical course of malignant diseases [5]. The reduction in the reactivity of T lymphocytes has a major impact on cancer development, progression and prognosis. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4; also known as CD152) is mainly expressed on activated T cells. With a structure similar to CD28, CTLA-4 is the second receptor of the co-stimulating factors B7-1 (CD80) and B7-2 (CD86). While the CD28-ligand interaction plays a critical role in maintaining the T cell response initiated through the engagement of the T cell antigen receptor, the CTLA-4-ligand interaction has an inhibitory effect on T cell activation and might contribute to peripheral tolerance [6, 7]. Indeed, mice deficient in CTLA-4 develop a lymphoproliferative disorder with multiorgan lymphocytic infiltration [8]. Additionally, CTLA-4 blockade leads to the enhancement of the immune response [9], rejection of tumors [10] or even cure of tumors in mice when in combination with tumor vaccines [11]. Moreover, clinical studies have shown that CTLA-4 blockade promotes cancer regression in patients with metastatic melanoma [12, 13].

The CTLA-4 gene is located on chromosome 2q33, consisting of 4 exons that encode separate functional domains: leader sequence, extracellular domain, transmembrane domain and cytoplasmic domain [14, 15]. More than 100 single nucleotide polymorphisms (SNPs) have been identified in the CTLA-4 gene region [14]. A SNP located in exon 1, CTLA-4 +49A>G (rs 231775), is the most frequently studied and has been identified as a functional polymorphism of CTLA-4. Increased expression of CTLA-4 mRNA and protein has been shown in individuals with the CTLA-4 +49GG genotype [15]. It was reported that functional gene polymorphism is related to cancer susceptibility, outcome and drug sensitivity [16]. Several studies indicated that the CTLA-4 +49A>G polymorphisms were associated with the development of various cancers, such as colorectal cancer, breast cancer, gastric cancer, lymphoma, lung cancer and other cancers [17–29]; however, no studies have investigated the association between CTLA-4 +49A>G variant and cancer prognosis. Therefore we designed this study to evaluate the association between CTLA-4 +49A>G SNP and the prognosis of advanced NSCLC in a Chinese population.

Subjects and Methods

Study Subjects

A total of 338 patients (220 men and 118 women) with histologically or cytologically confirmed advanced NSCLC cancer registered between July 2005 and July 2009 at the Department of Oncology at the Shandong Cancer Hospital and Institute were included in this study. The patients were diagnosed as lung cancer with an unresectable primary tumor and/or metastases that were measurable or assessable by means of clinical examination, X-ray and computed tomography. Bone scan or computed tomography of the brain was performed in patients with suspected bone or brain metastases. The median age of the patients was 59.8 years (aged 27–80). 250 cases had smoked (pack-years ≥ 5) and 88 cases were never-smokers (pack-years < 5). According to the sixth edition of the International Union against Cancer TNM classification, there were 98 patients with stage IIIB disease and 240 patients with stage IV disease. For Eastern Cooperative Oncology Group (ECOG) score, 125 cases were 0 and 213 cases were 1–2. 108 patients had squamous cell carcinoma, 190 patients had adenocarcinoma and 40 patients had other carcinoma types. A total of 270 patients received conventional, platinum-based chemotherapy, 30 patients received radiotherapy, 23 patients received a combination of radiotherapy and chemotherapy, and 15 patients received treatment by other methods or no treatment (table 1). Serum CEA and CYFRA21-1 levels were measured by the Roche E170 electrochemiluminescence instrument (Roche). The average CEA level was 51.49 ng/ml (range 0.7–711.5) and CYFRA21-1 level was 12.86 ng/ml (range 0.8–380.6) in the patients. Patients were followed by telephone, outpatient interviews and letters. Follow-up time was from the initiation of diagnosis to July 2010. All subjects were unrelated Northern Chinese and written informed consent was obtained from each participant. The study was approved by the Review Board of Shandong Cancer Hospital.

Sample Collection and SNP Genotyping

Genomic DNA was extracted from 2 ml peripheral blood obtained from each participant using a Genomic DNA Extraction Kit (Fastagen) according to the manufacturer's protocol; a 152-bp region surrounding the CTLA-4 +49A>G SNP was amplified using primers 5'-AAGGCTCAGCTGAACCTGGT-3' and 5'-CTGCTGAAACAAATGAAACCC-3'. PCR amplification was performed in a 25- μ l reaction containing 50 ng genomic DNA, 1 $\times$ PCR mix buffer and 0.1 nmol of each primer. PCR products were digested with *Eco91I* (New England Biolabs) and separated on 10% polyacrylamide gels. The presence of the A allele allowed the digestion of the 152-bp amplicon into two products of 130 and 22 bp. PCR products containing the G allele remain undigested.

Statistical Analysis

Statistical analysis was performed using SPSS 13.0 software. A χ^2 test was used to assess the association between CTLA-4 49G>A SNP and categorical clinicopathologic parameters. Hardy-Weinberg equilibrium was tested with a goodness-of-fit χ^2 test with 1 degree of freedom to compare the observed genotype frequencies among the subjects with the expected genotype frequencies. A survival curve was drawn with the Kaplan-Meier method for each of the different genotypes. Comparisons were made with the log-rank test. Hazard ratios of death with 95% CI were estimated by

using the multivariate Cox model, with genotype, sex, age, smoke, ECOG score, histopathological type, treatment methods and stage of disease as covariates. $p < 0.05$ was considered as statistically significant.

Results

CTLA-4 +49A>G Polymorphism Distribution in Advanced NSCLC Patients

In the studied cohort, the CTLA-4 +49A>G genotype frequencies in advanced NSCLC patients were detected with the following disposition: the frequencies of GG, GA and AA genotype were 44.4% (150/338), 42.0% (142/338) and 13.6% (46/338), respectively. The genotype distributions were in Hardy-Weinberg equilibrium. The distribution of CTLA-4 +49A>G genotypes and allele frequencies were analyzed with regard to clinicopathologic characteristics of advanced NSCLC patients. However, there were no significant differences between the presence of CTLA-4 +49A>G polymorphisms and gender, histopathological type, clinical stage or tumor markers (CEA and CYFRA21-1) in these cases (table 2).

CTLA-4 +49A>G Polymorphism and Survival of Advanced NSCLC

Of the 338 patients, 31 were lost to follow-up. Overall, the median follow-up time was 28.5 months (range 0.4–60) and the median survival time was 11.7 months. The median survival time was 12.5 months for patients with the GG genotype, 12.0 months for patients with the GA genotype and 9.8 months for patients with the AA genotype. Patients with the AA genotype had a significantly shorter survival time than those with the GG or GA genotypes ($p < 0.001$, log-rank test; fig. 1). When the survival curves were stratified according to different stages, treatment methods or age, the association between the CTLA-4 AA genotype and shorter NSCLC survival time was significant in patients with both stage IIIB and IV, patients who received both chemotherapy and radiotherapy, and both younger and older patients (data not shown). Multivariate Cox regression was used to analyze the effect of different risk factors (genotype, gender, age, smoke, ECOG score, histopathological type, treatment methods and clinical stage) on survival time. The results showed that CTLA-4 polymorphism and clinical stage were independent prognosis indicators for advanced NSCLC. The CTLA-4 49AA genotype was an independent adverse indicator for NSCLC prognosis (hazard ratio 1.6, 95% CI 1.3–1.9, $p = 0.001$; table 3).

Table 1. Characteristics of the subjects

Characteristics	Patients (n = 338)
Average age, years	59.8 (27–80)
Sex	
Male	220 (65.1)
Female	118 (34.9)
Smoker	
Yes	250 (74.0)
No	88 (26.0)
ECOG	
0	125 (37.0)
1–2	213 (63.0)
Histopathological type	
Squamous cell carcinoma	108 (31.9)
Adenocarcinoma	190 (56.2)
Other types	40 (11.9)
Clinical stage	
IIIB	98 (29.0)
IV	240 (71.0)
Treatment	
Chemotherapy	270 (79.9)
Radiotherapy	30 (8.9)
Chemoradiotherapy	23 (6.8)
Others	15 (4.4)

Figures in parentheses are ranges or percentages, as appropriate.

Fig. 1. Kaplan-Meier survival analysis for advanced NSCLC patients by CTLA-4 +49A>G genotypes. Comparison by log-rank test.

Table 2. Clinicopathological characteristics and CTLA-4 +49A>G polymorphism frequencies in advanced NSCLC patients

	Genotype				Allele		
	GG	GA	AA	p	G	A	p
Total	150 (44.4)	142 (42.0)	46 (13.6)		442 (65.4)	234 (34.6)	
Sex							
Male (n = 220)	100	90	30	0.841	290	150	0.735
Female (n = 118)	50	52	16		152	84	
Histopathological type							
Squamous cell carcinoma (n = 108)	46	46	16	0.635	138	78	0.616
Adenocarcinoma (n = 190)	84	80	26		248	132	
Other types (n = 40)	20	16	4		56	24	
Clinical stage							
IIIB (n = 98)	46	43	9	0.316	135	61	0.247
IV (n = 240)	104	99	37		307	173	
CEA, ng/ml							
≥10 (n = 266)	116	116	34	0.460	348	184	0.976
<10 (n = 72)	34	26	12		94	50	
Cyfra21-1, ng/ml							
≥10 (n = 257)	113	107	37	0.754	333	181	0.572
<10 (n = 81)	37	35	9		109	53	

Figures in parentheses are percentages, unless indicated otherwise.

Table 3. Cox regression analysis of variables in advanced NSCLC patients

Variable	p	Hazard ratio	95% CI	
			lower	upper
Genotype	0.001*	1.6	1.3	1.9
Sex	0.739	1.1	0.8	1.7
Age	0.495	1.2	0.7	2.1
Smoking status	0.465	0.9	0.5	1.6
ECOG score	0.726	1.3	0.8	2.6
Histopathological type	0.739	1.4	0.7	2.2
Treatment methods	0.223	1.3	0.8	2.2
Stage	0.032*	2.1	1.3	3.4

* p < 0.05.

Discussion

The development of malignant disease is frequently due to a failure of immune surveillance. Immune response to tumors involves the activation of T lymphocytes, antibodies, natural killer cells and macrophages. Immunologic tolerance and anergy allow tumors to

evade immune response. CTLA-4 emerges as an important negative regulator of T cell activation. Some studies suggest that anti-CTLA-4 monoclonal antibodies could prevent CTLA-4/B7.1 binding and allow T cell proliferation to promote antitumor immunity [30]. Genetic polymorphisms of CTLA-4 were shown to interfere with the transcriptional activity of this gene with the +49G allele leading to a lower CTLA-4 mRNA level [15]; therefore, we assume that the variants of the CTLA-4 gene may affect the development, progression and prognosis of lung cancer.

In the present study, we observed a significant association between the polymorphism of CTLA-4 +49A>G and prognosis of advanced NSCLC. Patients with the CTLA-4 +49AA genotype had significantly shorter survival time than those with the GG or GA genotype. Multivariate testing also identified the AA genotype as an independent adverse prognostic indicator. These results suggest that the CTLA-4 +49AA genotype could be used to predict advanced NSCLC prognosis.

The CTLA-4 +49A>G SNP causes a substitution of Ala for Thr at position 17 of the mature CTLA-4 protein, and increased expression of CTLA-4 mRNA and protein have been shown in individuals with the 49AA genotype [15]. Functional analysis has shown that the CTLA-4 pro-

tein encoded by the CTLA-4 +49A allele has enhanced interaction with B7.1 and an enhanced inhibitory effect on T cell activation and proliferation [21]. Furthermore, individuals homozygous for the CTLA-4 +49AA genotype had significantly higher expression of CTLA-4 on peripheral blood mononuclear cells than those with other genotypes, and had decreased mRNA and protein expression of interleukin-2, the primary T cell growth factor [31]. Thus, T cells from individuals homozygous for the CTLA-4 +49AA genotype would be expected to have an increased level of CTLA-4 which results in the inhibition of T cell activation. Our results shown in this study suggest that the CTLA-4 49AA polymorphism may affect CTLA-4 expression and attenuate T cell activation, which may contribute to poor prognosis of advanced NSCLC patients.

T cell-mediated immunity against tumor cells plays an important role in tumor rejection [32]. Therefore, increasing the T cell response is a fundamental strategy in tumor immunotherapy. It has been proposed that deficiency or inhibition of CTLA-4 induces or exacerbates autoimmunity while simultaneously enhancing tumor immunity and preventing the induction of immunologic tolerance. In a study of metastatic melanoma, approximately 15% of patients experienced objective tumor regression after the administration of anti-CTLA-4 mono-

clonal antibody [33]. Furthermore, patients with metastatic melanoma who were homozygous for the CTLA-4 +49A allele showed better response to the anti-CTLA-4 therapy than those with the CTLA-4 +49G allele, suggesting that CTLA-4 genotype may serve as a genetic marker for personalized anti-CTLA-4 therapy [34]. Our observations of an increased frequency of the CTLA-4 +49A allele among NSCLC patients and shorter survival time in those with the AA genotype further demonstrate the need for a specialized treatment for patients with the CTLA-4 +49AA genotype to enhance their antitumor response.

In conclusion, our findings suggest that the CTLA-4 +49A>G SNP may serve as a useful genetic marker in assessing the prognosis in advanced NSCLC patients. A more comprehensive understanding of the genetic basis of the immune response to cancer cells will allow tailored therapies that boost the immune response, leading to improved management strategies for cancer.

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