**ABSTRACT**

**Purpose of the study:** Cytotoxic T-lymphocyte antigen-4 (CTLA-4) has been shown to play an important role in the development and progression of thyroid associated ophthalmopathy (TAO). A number of case-control studies focused on the association between CTLA-4 +49A/G polymorphism and risk for TAO. But the results were not always consistent. So we performed a meta-analysis to evaluate the precise association between this polymorphism and risk for TAO.

**Materials and methods:** All publications on the association between CTLA-4 +49A/G polymorphism and TAO were searched in the following electronic databases: PubMed, Embase, the Cochrane library and Chinese Biomedical Literature Database, with the last report up to May 2014. This meta-analysis was assessed by Review Manager 5.1.

**Results:** A total of 14 studies were involved in this meta-analysis, including 1128 cases and 2539 controls. Overall, we found a significant association between CTLA-4 +49A/G polymorphism and TAO (G versus A: OR = 1.64, 95% CI = 1.40–1.92, \( p < 0.00001 \); GG versus AG + AA: OR = 2.02, 95% CI = 1.59–2.57, \( p < 0.00001 \); GG + AG versus AA: OR = 2.01, 95% CI = 1.66–2.43, \( p < 0.00001 \); GG versus AA: OR = 2.74, 95% CI = 1.83–4.10, \( p < 0.00001 \); AG versus AA: OR = 1.75, 95% CI = 1.42–2.15, \( p < 0.00001 \)). The results were not materially altered after the studies which did not fulfill Hardy–Weinberg equilibrium were excluded. Significant association was also detected in both Caucasian and Asian populations in subgroup analysis divided by different ethnicity.

**Conclusion:** Our meta-analysis supports the association between the CTLA-4 +49A/G polymorphism and TAO.

**Keywords:** Cytotoxic T-lymphocyte antigen-4, polymorphism, thyroid-associated ophthalmopathy

**INTRODUCTION**

Graves’ disease (GD) is an autoimmune thyroid disease (AITD) that affects ∼1% of the general population and is characterized by T-cell and B-cell reactivity to the TSH receptor (TSHR).1,2 The symptoms of GD include hyperthyroidism, diffuse goiter, thyroid-associated ophthalmopathy (TAO) and Graves’ dermopathy.2 It has been estimated that 25–50% of GD patients have clinical signs of TAO, with the vast majority having relatively mild disease.3,4 In its severe form, which occurs in 3–5% of patients with eye signs, ophthalmopathy is a potentially sight-threatening disorder requiring active medical or surgical interventions.

TAO is considered to be a chronic, autoimmune inflammatory disorder that impacts all orbital tissue sections and results in various eye features, including lid retraction (NOSPECS class I), soft tissue

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inflammation (class II), proptosis (class III), extraocular muscle dysfunction (class IV), corneal involvement (class V) and sight loss (class VI).4,5 As with all autoimmune disorders, it is thought that GD is caused by the interaction of genetic, epigenetic and environmental factors in a stochastic manner. The role of genetic factors in the development of TAO is currently unknown.6 Since the infiltration of retrolubar tissues by T-cells is likely to play a key role in the pathogenesis of orbital inflammation in TAO,7 one can naturally think of the cytotoxic-T lymphocyte antigen-4 (CTLA-4) gene as a potential candidate.

CTLA-4 was initially described as a classical type I glycoprotein on the surface of activated T-cells.8 CTLA-4 is a member of the Ig gene superfamily and along with its homologue, CD28, which is a B7 binding protein.9,10 The emerging opinion about the function of CTLA-4 is that it represents a negative regulator of T-cell activation. Thus, ligation of CTLA-4 on the T-cell surface initiates a cascade of biochemical events that attenuate an ongoing immune response.11 Theoretically, a polymorphism reducing CTLA-4 expression or function may cause autoimmune T-cell clonal proliferation and thus contribute to the pathogenesis of autoimmune diseases.7

Many research groups have investigated the relationship between polymorphisms of CTLA-4 gene and TAO, most of which focused on the +49A/G polymorphism in exon 1.12–32 However, the results from these studies are conflicting and inconclusive. There are several possible explanations for this discordance, such as small sample size, ethnic background, uncorrected multiple hypothesis testing and publication bias. Meta-analysis is a statistical procedure for combining the results of several studies to produce a single estimate of the major effect with enhanced precision. The aim of the present study is to perform a comprehensive meta-analysis to evaluate the association between the +49A/G polymorphism of CTLA-4 gene and TAO.

METHODS

We attempted to identify, review and include case-control studies assessing the association of TAO and the +49A/G polymorphism of CTLA-4 gene. This meta-analysis was performed according to a predetermined protocol described in the following paragraph, using standard systematic review techniques, as outlined by the Cochrane Handbook for Systematic Reviews of Interventions and PRISMA Statement.33,34

Search Strategy

We searched several databases including PubMed, Embase, the Cochrane library and Chinese Biomedical Literature Database (CBM), through May 2014 for all publications on the association between CTLA-4 +49A/G polymorphism and TAO. The search combined terms related to “cytotoxic T-lymphocyte associated antigen OR cytotoxic T-lymphocyte antigen OR CTLA-4” concatenated with “gene OR polymorphism OR variant” AND “thyroid associated ophthalmopathy OR thyroid associated orbitopathy OR thyroid eye disease OR Graves’ ophthalmopathy OR TAO OR TED OR GO”. No language restrictions were applied. Only the studies with complete data on comparison of frequency of CTLA-4 +49A/G gene polymorphism between TAO patients with control donors were selected. The original search was performed in January 2014. Regular searches every 2 months were established on PubMed and Embase databases to capture new studies until May 2014, and updated searches on the Cochrane databases regularly. We also searched the reference lists of original reports, retrieved through the electronic searches, to identify studies not yet included in the computerized databases.

Studies Selection

Published and unpublished case-control studies fulfilling the following selection criteria were included in the present meta-analysis: (1) it was published up to May 2014; (2) it was a case-control study of the TAO +49A/G polymorphism and TAO. Exclusive criteria: no report about the genotype or allele frequency, or insufficient information for data extraction.

After completion of the searches, three review authors (HW, JWC and LSZ) working independently assessed the titles and abstracts of all obtained reports for a rough judgment of an article’s eligibility. The full-text copies of possibly and definitely relevant studies were obtained and assessed by the three authors independently according to the definitions in the criteria, which were checked by RLW. Only studies meeting these criteria were assessed for methodological quality. When there were multiple studies from the same population, only the largest study was included, and data that could not be obtained from this publication were obtained from others.

Data Extraction

Three investigators (HW, JWC and LSZ) independently extracted data and reached consensus on the following characteristics of the selected studies: the first author’s name, year of publication, source of publication, ethnicity, number of cases and controls, and available allele and genotype frequencies information. If original data was unavailable in articles,
a request for original data was sent to the corresponding author.

**Statistical Analysis**

The meta-analysis was performed by using Review manager 5.1 (The Cochrane Collaboration, Oxford, UK), with the method described by DerSimonian and Laird. The following genotype contrasts were evaluated: homozygotes GG versus a combination of AG and AA [GG versus (AG + AA), recessive model], a combination of GG and AG versus AA [(GG + AG) versus AA, dominant model], GG versus AA and AG versus AA (codominant model). Contrast of G allelic frequency versus A allelic frequency (G versus A) was also evaluated. We also conducted subgroup analysis by ethnicity. The distribution of genotypes in the control group of each study was checked for the Hardy–Weinberg equilibrium (HWE). Studies with controls not in HWE were subjected to a sensitivity analysis (i.e. repeatedly performed the meta-analysis via excluding the study with the controls not in HWE).

Heterogeneity among studies was examined with $I^2$ statistic interpreted as the proportion of total variation contributed by between-study variation. The data from individual studies were pooled by use of the random-effect model with the DerSimonian-Laird method, which considers within-study and between-study variation. The potential publication bias was estimated using Egger’s linear regression test and visual inspection of the funnel plot. All $p$ values were two-tailed.

**RESULTS**

**Eligible Studies**

There were 302 papers relevant to the searching terms. The process of study selection is shown in Figure 1. A total of 21 studies examined the association between the CTLA-4 +49A/G polymorphism and TAO. Of these, seven were excluded (the data of three were unavailable, four were duplicate reports). Thus, 14 studies were included in the current meta-analysis.

Characteristics of studies included in the current meta-analysis are presented in Table 1. In the eligible studies, there were six studies of Caucasian, seven of Asian and one of Brazilian. The allele and the genotype frequencies of the CTLA-4 +49A/G polymorphism were extracted from 12 studies. But only allele frequency was extracted from one study while the authors of another study only compared the frequency of GG versus AA + AG. Therefore, examining the contrast of GG versus AA, AG versus AA and GG + AG versus AA, the meta-analysis was performed with 12 studies overall, and four Caucasian studies, seven Asian studies as well as one Brazilian study. Examining the contrast of GG versus AG + AA, A allele versus G allele, meta-analysis was performed with 13 studies overall, and five Caucasian studies, seven Asian studies as well as one Brazilian study.

The results of HWE test for the distribution of the genotype in control population are shown in Table 1. The distributions of the genotypes in controls were not in HWE in three studies, and unable to calculate in two studies.

**Statistics Summary**

In total, the meta-analysis included 1128 cases and 2539 controls (Table 1). Allele G was more frequent in cases while less frequent in controls. In both groups, the prevalence of the AG genotype was the highest. The prevalence of allele G was 53.6% and 40.2% for the cases and controls, respectively. For the cases, the prevalence of the GG genotype, the AG genotype and the AA genotype was 32.1%, 47.7% and 20.2%, respectively. For the controls, the prevalence of the GG genotype, the AG genotype and the AA genotype was 16.6%, 50.7% and 32.7%, respectively.

**Meta-Analysis**

The main results of this meta-analysis and the heterogeneity test are shown in Table 2, and the result of G versus A is also presented in Figure 2. Significant heterogeneity was found in several comparisons (Table 2).
Pooled Effects for the CTLa-4 +49A/G Polymorphism and TAO Risk in Overall Population

We first evaluated the association between the CTLa-4 +49A/G polymorphism and TAO in overall population. The association was investigated in 14 studies with a total of 1128 cases and 2539 controls. Significant heterogeneity was found in the contrast of G versus A and GG versus AA. Significant association was found between the CTLa-4 +49A/G polymorphism and TAO in all genetic models (G versus A: OR = 1.64, 95% CI = 1.40–1.92, p < 0.00001 [Figure 2]; GG versus AG + AA: OR = 2.02, 95% CI = 1.59–2.57, p < 0.00001 [Figure S1A]; GG + AG versus AA: OR = 2.01, 95% CI = 1.66–2.43, p < 0.00001 [Figure S1B]; GG versus AA: OR = 2.74, 95% CI = 1.83–4.10, p < 0.00001 [Figure S1C]; AG versus AA: OR = 1.75, 95% CI = 1.42–2.15, p < 0.00001 [Figure S1D]).

Pooled Effects for the CTLa-4 +49A/G Polymorphism and TAO Risk in HWE Population

There are a total of nine studies (692 cases and 1414 controls) fulfilling HWE, and the meta-analysis was performed. The pooled OR for the contrast of G versus A was 1.81 (95% CI = 1.55–2.11, p < 0.00001 [Figure S1A]; GG versus AG + AA: OR = 2.13, 95% CI = 1.72–2.63, p < 0.00001 [Figure S1B]; GG + AG versus AA: OR = 2.13, 95% CI = 1.72–2.63, p < 0.00001 [Figure S1C]; AG versus AA: OR = 1.80, 95% CI = 1.49–2.18, p < 0.00001 [Figure S1D]).
TABLE 2 Meta-analysis of the association of CTLA-4 +49A/G polymorphism and TAO.

| Polymorphism                  | Study   | Case | Control | No. of studies | OR (95% CI) | Z    | p Value | \( \chi^2 \) | p Value | I² (%) |
|-------------------------------|---------|------|---------|----------------|-------------|------|---------|-------------|---------|-------|
| G versus A                    | Overall | 2148 | 4486    | 13             | 1.64 (1.40–1.92) | 6.07 | <0.00001 | 21.78       | 0.04    | 45    |
|                               | HWE     | 1384 | 2828    | 9              | 1.70 (1.43–2.03) | 5.93 | <0.00001 | 9.94        | 0.01    | 27    |
|                               | Caucasian | 1030 | 2596    | 5              | 1.55 (1.23–1.96) | 3.65 | 0.0003  | 9.52        | 0.05    | 58    |
|                               | Asian   | 1014 | 1732    | 7              | 1.81 (1.41–2.34) | 4.61 | <0.00001| 10.62       | 0.10    | 44    |
| GG versus (AG + AA)           | Overall | 1038 | 2246    | 13             | 2.02 (1.59–2.57) | 5.73 | <0.00001| 17.72       | 0.12    | 32    |
|                               | HWE     | 692  | 1414    | 9              | 1.98 (1.53–2.55) | 5.23 | <0.00001| 8.82        | 0.36    | 9     |
|                               | Caucasian | 479  | 1302    | 5              | 2.40 (1.80–3.18) | 6.03 | <0.00001| 3.18        | 0.53    | 0     |
|                               | Asian   | 507  | 866     | 7              | 1.92 (1.32–2.79) | 3.40 | <0.00001| 10.85       | 0.09    | 45    |
| (GG + AG) versus AA           | Overall | 984  | 1992    | 12             | 2.01 (1.66–2.43) | 7.11 | <0.00001| 9.48        | 0.58    | 0     |
|                               | HWE     | 692  | 1414    | 9              | 1.91 (1.52–2.39) | 5.57 | <0.00001| 6.00        | 0.65    | 0     |
|                               | Caucasian | 425  | 1048    | 4              | 1.94 (1.51–2.49) | 5.22 | <0.00001| 2.67        | 0.45    | 0     |
|                               | Asian   | 507  | 866     | 7              | 2.32 (1.66–3.23) | 4.95 | <0.00001| 4.87        | 0.56    | 0     |
| GG versus AA                  | Overall | 546  | 1073    | 12             | 2.74 (1.83–4.10) | 4.88 | <0.00001| 20.75       | 0.04    | 47    |
|                               | HWE     | 387  | 771     | 9              | 2.65 (1.91–3.67) | 5.86 | <0.00001| 7.24        | 0.51    | 0     |
|                               | Caucasian | 210  | 556     | 4              | 3.11 (2.14–4.52) | 5.96 | <0.00001| 3.33        | 0.34    | 10    |
|                               | Asian   | 309  | 501     | 7              | 3.05 (1.88–4.96) | 4.49 | <0.00001| 6.81        | 0.34    | 12    |
| AG versus AA                  | Overall | 637  | 1513    | 12             | 1.75 (1.42–2.15) | 5.32 | <0.00001| 9.19        | 0.60    | 0     |
|                               | HWE     | 448  | 1058    | 9              | 1.66 (1.30–2.12) | 4.05 | <0.00001| 7.05        | 0.53    | 0     |
|                               | Caucasian | 342  | 923     | 4              | 1.68 (1.30–2.19) | 3.89 | <0.0001  | 1.16        | 0.76    | 0     |
|                               | Asian   | 258  | 509     | 7              | 2.02 (1.33–3.08) | 3.28 | 0.0001  | 7.09        | 0.31    | 15    |

CTLA-4 = cytotoxic T-lymphocyte antigen-4; TAO = thyroid associated ophthalmopathy; HWE = Hardy-Weinberg equilibrium; OR = odds ratio; CI = confidence interval.

FIGURE 2 Meta-analysis of the association between the CTLA-4 +49A/G polymorphism and TAO (G versus A).
carried out in these studies. No significant heterogeneity was found. We detected a statistically significant association between the CTLA-4 +49A/G polymorphism and TAO in HWE population in all genetic models (G versus A: OR = 1.70, 95% CI = 1.43–2.03, \( p < 0.00001 \) [Figure S2A]; GG versus AG + AA: OR = 1.98, 95% CI = 1.53–2.55, \( p < 0.00001 \) [Figure S2B]; GG + AG versus AA: OR = 1.91, 95% CI = 1.52–2.39, \( p < 0.00001 \) [Figure S2C]; GG versus AA: OR = 2.65, 95% CI = 1.91–3.67, \( p < 0.00001 \) [Figure S2D]; AG versus AA: OR = 1.66, 95% CI = 1.30–2.12, \( p < 0.00001 \) [Figure S2E]).

### Pooled Effects for the CTLA-4 +49A/G Polymorphism and TAO Risk in Caucasian Population

The meta-analysis included six studies (566 cases and 1603 controls) in Caucasian. There was statistically significant between-study heterogeneity detected in the contrast of G versus A. Significant association was also found between the CTLA-4 +49A/G polymorphism and TAO in all genetic models (G versus A: OR = 1.55, 95% CI = 1.23–1.96, \( p = 0.0003 \); GG versus AG + AA: OR = 2.40, 95% CI = 1.80–3.18, \( p < 0.00001 \); GG + AG versus AA: OR = 1.94, 95% CI = 1.51–2.49, \( p < 0.00001 \); GG versus AA: OR = 3.11, 95% CI = 2.14–4.52, \( p < 0.00001 \); AG versus AA: OR = 1.68, 95% CI = 1.30–2.19, \( p = 0.0001 \)).

### Pooled Effects for the CTLA-4 +49A/G Polymorphism and TAO Risk in Asian Population

Seven studies (507 cases and 866 controls) in Asians were included in this meta-analysis. There was no significant between-study heterogeneity detected. Statistically significant association was established for the CTLA-4 +49A/G polymorphism in Asian population in all genetic models (G versus A: OR = 1.81, 95% CI = 1.41–2.34, \( p < 0.00001 \); GG versus AG + AA: OR = 1.92, 95% CI = 1.32–2.79, \( p < 0.00001 \); GG + AG versus AA: OR = 2.32, 95% CI = 1.66–3.23, \( p < 0.00001 \); GG versus AA: OR = 3.05, 95% CI = 1.88–4.96, \( p < 0.00001 \); AG versus AA: OR = 2.02, 95% CI = 1.33–3.08, \( p = 0.001 \)).

### Publication Bias

Publication bias was assessed by visual funnel plot inspection and Egger’s test. The funnel plot for A versus G was basically symmetric (Figure 3). As for Egger’s test, asymmetry was measured by the intercept \( a \), and the larger its deviation from zero, the more pronounced the asymmetry. The results of Egger’s linear regression test are shown in Table 3. It was shown that there was little evidence of bias for all comparisons.

### DISCUSSION

GD, the single most common cause of thyrotoxicosis, is an autoimmune disease of aberrant antibody production.\(^3\) Antibodies directed against TSHR target the endothelial surface of thyroid follicular cells where these receptors are most abundant.\(^3\) Although almost any organ may be involved in GD, it is classically described as a syndrome consisting of hyperthyroidism, goiter, orbitopathy and...
dermopathy.\textsuperscript{40} Orbital involvement, known as TAO, is not merely a byproduct of hyperthyroidism; rather, the same underlying immune processes involving TSHR antibodies are active in the orbits.\textsuperscript{41} TSHR antibody titers seem to be positively correlated with clinical features of TAO, whereas thyroid stimulating immunoglobulin (TSI) and thyroid peroxidase (TPO) antibody do not.\textsuperscript{42} Similar to other autoimmune conditions, TAO has a multifactorial etiology; genetic and environmental factors interact in concert and trigger a deviant process that presents self-antigens as non-self.\textsuperscript{43} Among the environmental factors, smoking has been considered as a main risk factor for TAO.\textsuperscript{44,45} Considering that TAO is an autoimmune condition, any imbalance between the production of pro- and anti-inflammatory cytokines can alter the downstream cascade of reactions and trigger the autoimmune response.\textsuperscript{46,47} Therefore, single nucleotide polymorphisms (SNPs) in the cytokine genes which influence the expression of the pro- and anti-inflammatory cytokines can protect or promote the development of TAO.\textsuperscript{46} Of all these SNPs, the \textit{CTLA-4} +49A/G polymorphism has been the focus of most studies on the association between genetic factors and TAO.\textsuperscript{12–32} The \textit{CTLA-4} gene encodes for a negative regulator of the T-cell immune response and seems to be an immunogenetic associate of several autoimmune diseases.\textsuperscript{48–51} The A/G polymorphism at position 49 in exon 1 results in an amino acid exchange (threonine/alanine) which may lead to decreased expression of \textit{CTLA-4}.

So far, a large number of studies have been taken to assess the association between the \textit{CTLA-4} +49A/G polymorphism and TAO, however, the genetic evidence thus far for the association has been conflicting. This is largely accounted for by varying participant ethnicity, study design and in particular suboptimal power. To further provide insights into this debated subject, a meta-analysis is needed to achieve a more reliable and comprehensive conclusion.

As far as we know, Bednarczuk et al.\textsuperscript{51} and Han et al.\textsuperscript{21} performed a meta-analysis on the association between the \textit{CTLA-4} +49A/G polymorphism and TAO in 2007 and 2006, separately. In Bednarczuk’s meta-analysis, a total of 11 studies were included, with 851 cases and 1010 controls, and the controls were not healthy donors but GD patients without TAO. They found no association between \textit{CTLA-4} +49A/G and TAO in all genetic models. While in Han’s meta-analysis including 10 studies, there were 910 patients and 1245 controls in total, but only allele frequency (G versus A) was compared between the two groups. No significant association was confirmed, either. Now our current study included 14 studies involving 1128 cases and 2539 controls, an even larger sample than the former two meta-analysis and we compared all genetic models between the two groups. A significant association between the \textit{CTLA-4} +49A/G polymorphism and TAO was detected among overall populations. Moreover, the results were not materially altered after the studies which did not fulfill HWE were excluded. Since ethnic differences may attribute to these different results, we performed subgroup analysis in Caucasian and Asian population, respectively. The association was still significant in both subgroups.

Several specific details merit consideration in the current meta-analysis. First, the conclusion was based on a relatively small number of studies and participants. Second, many investigators generally preferred to publish the statistically significant results rather than negative findings. If the meta-analysis was based on such selectively published reports, pooled effect would be overestimated. Publication bias in this meta-analysis was assayed by funnel plot and Egger’s test. Although we did not detect publication bias in our meta-analysis, it must be recognized that publication bias is indeed difficult to exclude with certainty, particularly when, as in this case, the number of the eligible studies and the number of participants in individual studies are small. Finally, although we minimized the likelihood of bias by developing a detailed protocol before initiating the study, meta-analysis remains retrospective research that is subject to the methodological deficiencies of the included studies.

In conclusion, we found significant association between the \textit{CTLA-4} +49A/G polymorphism and TAO in overall population. Further research, with larger numbers of participants and fully confounding

### Table 3

| Comparisons | G versus A | GG versus (AG + AA) | (GG + AG) versus AA | GG versus AA | AG versus AA |
|-------------|------------|---------------------|---------------------|-------------|--------------|
| Overall     | 0.64 (–1.20~2.49) | 0.92 (–0.92~2.76) | 0.58 (–0.39~1.55) | 0.52 (–0.81~1.84) | 0.46 (–0.57~1.50) |
| HWE         | 1.99 (–8.65~12.62) | 0.90 (–12.78~14.57) | 7.65 (–5.77~21.08) | 5.30 (–9.83~20.42) | 2.46 (–8.06~12.97) |
| Caucasian   | −2.33 (–11.86~7.21) | −0.48 (–6.63~5.27) | 0.42 (–8.82~9.66) | −1.23 (–12.26~9.79) | 0.50 (–5.98~6.97) |
| Asian       | 1.31 (–0.14~2.76) | 1.35 (–2.99~3.00) | 0.68 (–0.36~1.72) | 0.98 (–0.12~2.08) | 0.56 (–1.03~2.16) |

HWE = Hardy-Weinberg equilibrium; NA = Not available.
*p > 0.05.
risk factors considered, such as age, sex, ethnicity and life style, is warrant to examine the possible effects of this polymorphism on TAO to confirm our conclusion.

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DECLARATION OF INTEREST

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The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online
Supplementary data for this article can be accessed at www.tandfonline.com/icey.