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Significantly association of diabetes mellitus with *CTLA-4* gene polymorphisms based on a meta-analysis of epidemiological evidence in Asians and non-Asians

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ABSTRACT. We evaluated association of polymorphisms in the *CTLA-4* gene with the risk of type 1 diabetes mellitus. Comprehensive meta-analysis was applied to case-control studies of the association between *CTLA-4* and type 1 diabetes mellitus to assess the joint evidence for the association, the influence of individual studies, and evidence for publication bias. We searched PubMed, Medline, Embase, Cochrane Library, and reference lists of relevant studies to February 2012, and made

email contact with authors. For the case-control studies, we found 1) support for an association between *CTLA-4* and type 1 diabetes mellitus, 2) no evidence that this association was accounted for by any one study, and 3) no evidence for publication bias. In all, although the association between *CTLA-4* polymorphisms and type 1 diabetes mellitus is weak, we suggest that it is real. Further studies are needed to clarify what variant of *CTLA-4* (or some related gene) accounts for this association.

Key words: Diabetes; *CTLA-4* gene; Meta-analysis; SNPs

INTRODUCTION

The *CTLA-4* (cytotoxic T-lymphocyte-associated antigen-4) gene is located on the long arm of chromosome 2q33. It consists of 4 exons and 3 introns. Exon 1 encodes a leader peptide sequence, exon 2 codes for an immunoglobulin domain, and exons 3 and 4 code for the hydrophobic transmembrane domain and the cytoplasmic domain, respectively. This gene encodes a receptor expressed by activated T cells. This receptor functions as a key negative regulator of T-cell activation. Based on function and experimental data, it has been suggested as a candidate gene for conferring susceptibility to autoimmune disease (Greenwald et al., 2002). In normal immune response, antigen recognition by Th cells is mediated through interaction between CD28, which is expressed on virtually all T cells, and B7 proteins on the surface of antigen-presenting cells. This binding between CD28 and B7 is essential for initiating the responses of naïve T cells. In some cases, T cells that encounter self-antigens may begin to express CTLA-4 molecules as a protective mechanism. CTLA-4 molecules have high affinity for B7 molecules and deliver inhibitory signals to T cells. CTLA-4 has a greater affinity for the B7 molecule than does CD28 and it downregulates T-cell function (Leung and Linsley, 1994). Therefore, it may play a crucial role in T-cell-mediated autoimmunity and thus in susceptibility to autoimmune diseases, including T1D.

T1D is the most prevalent form of diabetes in children and young adults and results from autoimmune CD4+ and CD8+ T-cell-directed destruction of insulin-producing pancreatic β-islet cells in genetically susceptible individuals, leading to irreversible hyperglycemia and related complications. Several genes have been associated with the risk of developing T1D, including IDDM12 located on chromosome 2q3; which encodes key lymphocyte co-receptors, including CTLA-4, CD28, and inducible costimulator (ICOS). All of these genes are in close linkage. Many molecular epidemiologic studies have evaluated the potential role of +49A/G (Donner et al., 1997; Van der Auwera et al., 1997; Krokowski et al., 1998; Djilali-Saiah et al., 1998; Awata et al., 1998; Yanagawa et al., 1999; Hayashi et al., 1999; Abe et al., 1999; Takara et al., 2000; Lee et al., 2000; Ihara et al., 2001; Kamoun et al., 2001; Osei-Hyiaman et al., 2001; McCormack et al., 2001; Kikuoka et al., 2001; Cosentino et al., 2002; Fajardy et al., 2002; Cinek et al., 2002; Ma et al., 2002; Klitz et al., 2002; Wood et al., 2002; Ongagna et al., 2002; Mochizuki et al., 2003; Bouqbis et al., 2003; Zalloua et al., 2004; Haller et al., 2004; Ide et al., 2004; Kawoura et al., 2005; Zhernakova et al., 2005; Ahmedov et al., 2006; Baniasadi et al., 2006; Ikegami et al., 2006; Caputo et al., 2007; Saleh et al., 2008; Douroudis et al., 2009; Balic et al., 2009; Jung et al., 2009; Korolija et al., 2009; Ferreira et al., 2009; Benmansour et al., 2010; Ei Wafai et al., 2011; Philip and Isabel, 2011; Mosaad et al., 2012) in susceptibility to type 1 diabetes (T1D). Given the amount of accumulated data, we considered it worthwhile to perform a quantitative synthesis of the evidence.

To deal with the ambiguities raised by inconsistent results among molecular genetic studies and to examine the putative association between *CTLA-4* and T1D, we applied meta-analysis to all available case-control association studies.

MATERIAL AND METHODS

Identification of eligible studies

A total of 43 published studies between *CTLA-4* and T1D were identified according to our inclusion criteria, involving 8021 cases and 9570 controls (Nistico et al., 1996; Donner et al., 1997; Van der Auwera et al., 1997; Krokowski et al., 1998; Djilali-Saiah et al., 1998; Awata et al., 1998; Yanagawa et al., 1999; Hayashi et al., 1999; Abe et al., 1999; Takara et al., 2000; Lee et al., 2000; Ihara et al., 2001; Kamoun et al., 2001; Osei-Hyiaman et al., 2001; McCormack et al., 2001; Kikuoka et al., 2001; Cosentino et al., 2002; Fajardy et al., 2002; Cinek et al., 2002; Ma et al., 2002; Klitz et al., 2002; Wood et al., 2002; Ongagna et al., 2002; Mochizuki et al., 2003; Bouqbis et al., 2003; Zalloua et al., 2004; Haller et al., 2004; Ide et al., 2004; Ahmedov et al., 2006; Baniasadi et al., 2006; Ikegami et al., 2006; Saleh et al., 2008; Douroudis et al., 2009; Balic et al., 2009; Jung et al., 2009; Korolija et al., 2009; Ferreira et al., 2009; Benmansour el al., 2010; Ei Wafai et al., 2011; Philip and Isabel, 2011; Mosaad et al., 2012). The main characteristics of these studies are described in Table 1. The +49A/G group was subdivided into 4 subgroups and analyzed (6 studies in Africans, 19 studies in Asians, 3 studies in Americans and 15 studies in Europeans). Sources included MEDLINE and EMBASE (search last updated in February 2012). The search strategy was based on combinations of the terms "CTLA-4", "cytotoxic T-lymphocyte-associated antigen-4", "CD152", and "diabetes". Reference lists in retrieved articles were also screened.

Nonfamilial case-control studies were eligible if the researchers had determined the distribution of genotypes for any of these polymorphisms in T1D cases and disease-free controls. We excluded studies with family-based designs in which the analysis was based on linkage considerations.

Data extraction

The following information was independently extracted from the identified studies by two participants in the meta-analysis: first author, journal, year of publication, study design, ethnicity of the study population, clinical characteristics, genotyping method, the number of cases and controls or odds ratio (OR) and 95% confidence interval (CI), country in which the study was conducted and confirmation of diagnosis. The results were compared and any disagreement was discussed and resolved by consensus.

Quality evaluation

All the studies included satisfied all the following criteria: they 1) were association studies between any of the three polymorphisms in the CTLA-4 gene and T1D; 2) used disease-free subjects as controls; 3) provided genotype or allele distribution in both case and control groups; 4) were independent studies and the subject groups investigated did not overlap with each other; 5) were published in peer-reviewed journals and were indexed by PubMed or cited by articles indexed by PubMed. Authors were contacted where clarification was required.

Table 1. Characteristics of the studies included.

	Study	Year	Country(ies)	Racial descent	Polymorphisms	Case	Control
1	Nistico et al.	1996	Belgium	European	+49A/G	483	529
2	Donner et al.	1997	Germany and Canada	European	+49A/G	293	325
3	Van der Auwera et al.	1997	Belgium	European	+49A/G	525	530
4	Krokowski et al.	1998	Poland	European	+49A/G	192	136
5	Djilali-Saiah et al.	1998	France	European	+49A/G	112	100
6	Awata et al.	1998	Japan	Asian	+49A/G	173	425
7	Yanagawa et al.	1999	Japan	Asian	+49A/G	110	200
8	Hayashi et al.	1999	Japan	Asian	+49A/G	117	141
9	Abe et al.	1999	Japan	Asian	+49A/G	111	445
10	Takara et al.	2000	Japan	Asian	+49A/G	74	107
11	Lee et al.	2000	China	Asian	+49A/G	253	91
12	Ihara et al.	2001	Japan	Asian	+49A/G	160	200
13	Kamoun Abid et al.	2001	Tunisia	North African	+49A/G	74	48
14	Osei-Hyiaman-1 et al.	2001	China	Asian	+49A/G	350	420
15	Osei-Hyiaman-2 et al.	2001	Ghana	African	+49A/G	182	201
16	McCormack et al.	2001	Northern Ireland	European	+49A/G	130	307
17	Kikuoka et al.	2001	Japan	Asian	+49A/G	125	200
18	Cosentino et al.	2002	Italy	European	+49A/G	80	85
19	Fajardy et al.	2002	France	European	+49A/G	134	273
20	Cinek et al.	2002	Czech Republic	European	+49A/G	305	289
21	Ma et al.	2002	China	Asian	+49A/G	31	36
22	Klitz et al.	2002	United States	Pacific Asian	+49A/G	90	94
23	Wood et al.	2002	Germany	European	+49A/G	176	220
24	Ongagna et al.	2002	France	European	+49A/G	62	84
25	Mochizuki et al.	2003	Japan	Asian	+49A/G	97	60
26	Bouqbis et al.	2003	Morocco	African	+49A/G	118	114
27	Zalloua et al.	2004	Lebanon	Middle Eastern	+49A/G	190	96
28	Haller et al.	2004	Estonia	European	+49A/G	69	158
29	Ide et al.	2004	Japan	Asian	+49A/G	116	114
30	BaniaSadi et al.	2006	India	North Indians	+49A/G,CT60A/G, -318C/T	130	180
31	Ahmedov et al.	2006	Azerbaijan	Asian	+49A/G	160	271
32	Ikegami et al.	2006	Japan	Asian	+49A/G,CT60A/G	769	723
33	Saleh et al.	2008	Egyptian	African	+49A/G	396	396
34	Douroudis-1 et al.	2009	Estonia	European	+49A/G,CT60A/G	170	230
35	Douroudis-2 et al.	2009	Finland	European	+49A/G,CT60A/G	404	725
36	Balic et al.	2009	Santiago	American	+49A/G,-318C/T	300	310
37	Jung et al.	2009	Korea	Asian	+49A/G,-318C/T	176	90
38	Korolija et al.	2009	Croatia	European	+49A/G	102	193
39	Ferreira et al.	2009	Brazil	American	+49A/G	49	48
40	Benmansour et al.	2010	Tunisia	African	+49A/G,CT60A/G, -318C/T	228	193
41	Ei Wafai et al.	2011	Lebanon	Asian	+49A/G	39	46
42	Philip and Isabel	2011	Southern India	Asian	+49A/G	53	53
43	Mosaad et al.	2012	Egyptian	African	+49A/G	104	78

Statistical analysis

The meta-analysis examined the overall association of alleles and genotypes and the risk of T1D for each polymorphism. The effect size was represented by OR with 95%CI. The Cochran Q statistic and I^2 test were used to assess heterogeneity in combined studies. Publication bias was checked using the Begg test, and the Egger test was used for funnel plot asymmetry. Both the random effect model and the fixed effect model were used to calculate pooled OR with Woolf 95%CI. P values of overall OR were generated using the Z test. Sensitivity analysis was conducted by removing each study and analyzing the others to ensure no single study was totally responsible for the overall results. The significance level was set at 0.05, and all P values were two-tailed. We used inverted funnel plots and the Begg-Mazumdar publication bias diagnostics (nonparametric s correlation coefficient) to evaluate whether the magnitudes of the observed associations were related to the variance of each study. The meta-analysis was performed using Comprehensive Meta Analysis software (Version 2.2.046, BIOSTAT, Englewood, NJ, USA).

RESULTS

The combined search yielded more than 200 references. After discarding overlapping references and those that clearly did not meet the criteria, 86 references were retained. These references were then filtered to ensure conformity with the inclusion criteria. Thirty-eight references were excluded because they were not studies referring to humans, and 5 references were excluded because they did not contain the +49A/G polymorphism. Finally, 43 studied the +49 A/G (rs231775) variant (Table 1).

Allelic analysis

The eligible studies for analysis included 8021 cases and 9570 controls available for analysis of the +49A/G polymorphism (Table 1). The meta-analysis of all the studies on the +49A/G polymorphism was significantly associated with T1D [fixed: OR and 95%CI: 1.326 (1.254–1.402), P < 0.001; random: OR and 95%CI: 1.382 (1.257–1.519), P < 0.001] (Figure 1 and Figure S1).

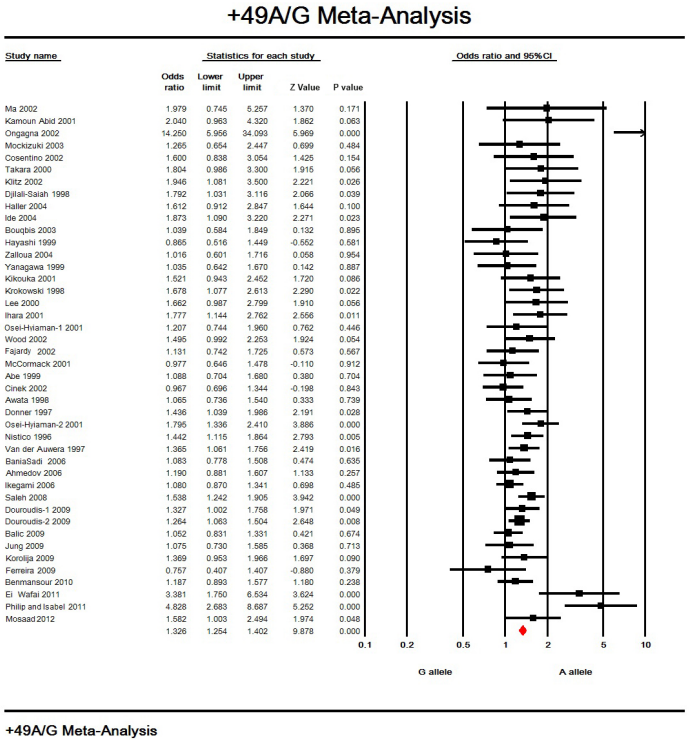

Figure 1. Meta-analysis of association studies of the +49A/G polymorphism and diabetes (fixed model). Pooled overall OR is shown. The OR of each study is marked with a black square. Pooled OR is indicated by a red diamond.

When divided into 4 subgroups (6 studies in Africans, 19 studies in Asians, 3 studies in Americans and 15 studies in Europeans), except for the American subgroup, the results also showed significant association. {Africans: [fixed/random: OR and 95%CI: 1.392 (1.205-1.608), P < 0.001]} (Figure 2A). {Asians: [fixed: OR and 95%CI: 1.355 (1.230-1.493), P < 0.001; random: OR and

95%CI: 1.457 (1.266-1.732), P < 0.001]} (Figure 2B, C). {Europeans: [fixed: OR and 95%CI: 1.352 (1.241-1.473), P < 0.001; random: OR and 95%CI: 1.424 (1.219-1.664), P < 0.001]} (Figure 2D, E). {Americans: [fixed/random: OR and 95%CI: 1.031 (0.859-1.239), P = 0.741]} (Figure 2F).

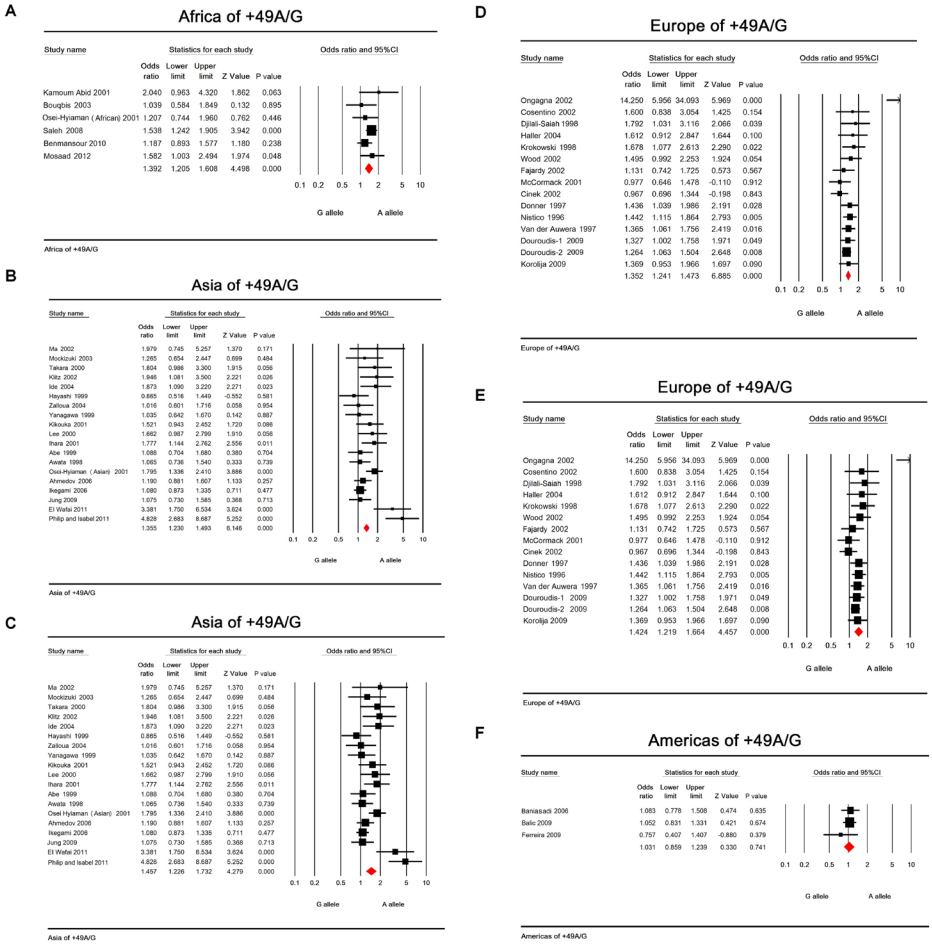

Figure 2. A. Meta-analysis of association studies of the +49A/G polymorphism of Africa population and diabetes (fixed/random models). Pooled overall OR is shown. The OR of each study is marked with a black square. Pooled OR is indicated by a red diamond. **B.** Meta-analysis of association studies of the +49A/G polymorphism of Asia population and diabetes (fixed model). Pooled overall OR is shown. The OR of each study is marked with a black square. Pooled OR is indicated by a red diamond. **C.** Meta-analysis of association studies of the +49A/G polymorphism of Asia population and diabetes (random model). Pooled overall OR is shown. The OR of each study is marked with a black square. Pooled OR is indicated by a red diamond. **D.** Meta-analysis of association studies of the +49A/G polymorphism of Europe population and diabetes (fixed model). Pooled overall OR is shown. The OR of each study is marked with a black square. Pooled OR is indicated by a red diamond. **E.** Meta-analysis of association studies of the +49A/G polymorphism of Europe population and diabetes (random model). Pooled overall OR is shown. The OR of each study is marked with a black square. Pooled OR is indicated by a red diamond. **F.** Meta-analysis of association studies of the +49A/G polymorphism of Americas population and diabetes (fixed/random models). Pooled overall OR is shown. The OR of each study is marked with a black square. Pooled OR is indicated by a red diamond.

A sensitivity analysis was carried out and the data are shown in Figure 3. The sensitivity analysis showed that when any one of the studies was removed, the heterogeneity of the population was not significantly changed, indicating that no heterogeneity existed in the population. There was no evidence that the magnitude of the overall OR estimates changed in the same direction over time. Also, the Egger funnel plots of publication bias analysis for the +49A/G (rs231775) polymorphism are shown. (Figure 4).

Genotypic analysis

For the genotype analysis of +49A/G, the result of GG versus (GA+AA) was significant {OR and 95%CI: 1.558 (1.432-1.696)}, indicating that the GG genotype was deleterious for people with T1D (Figure 5).

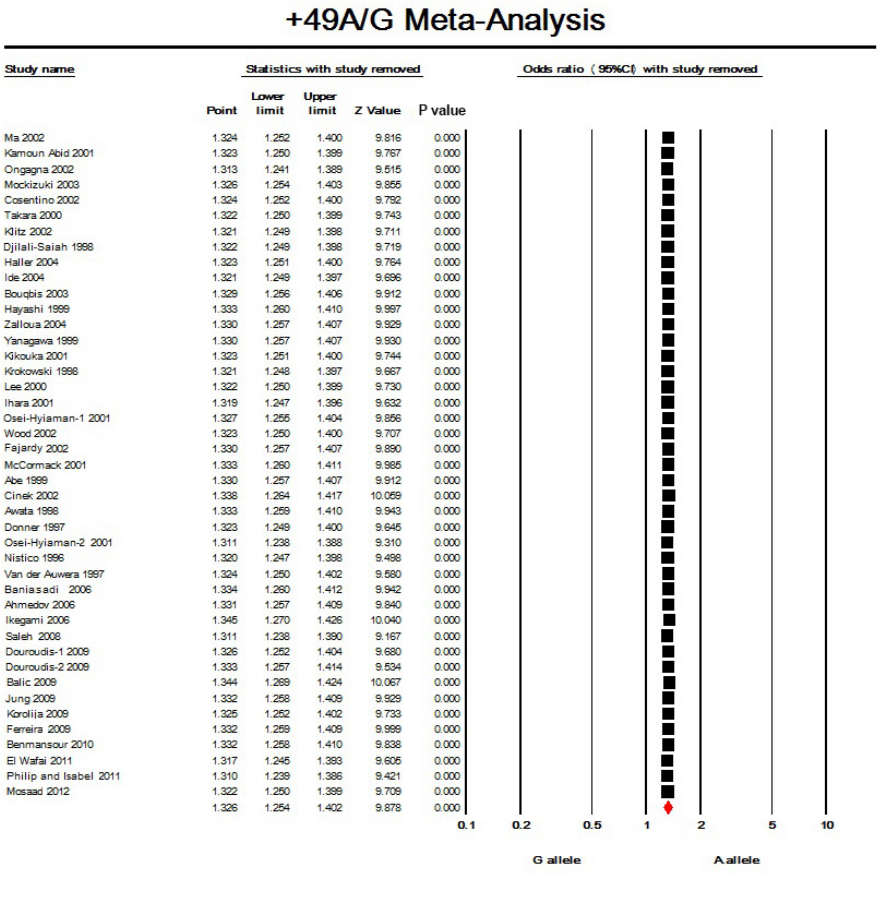

Figure 3. Sensitivity analysis of +49A/G. When any one of the studies was removed, the heterogeneity of the population remained unchanged.

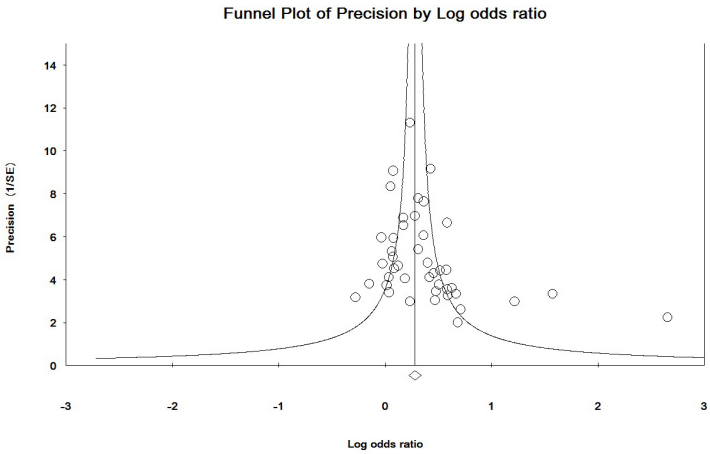

Figure 4. Egger's funnel plots of publication bias analysis for the +49A/G polymorphism. The larger the deviation from the funnel curve of each study, the more pronounced the asymmetry. Results from small studies scatter widely at the bottom of the graph, with the spread narrowing among larger studies.

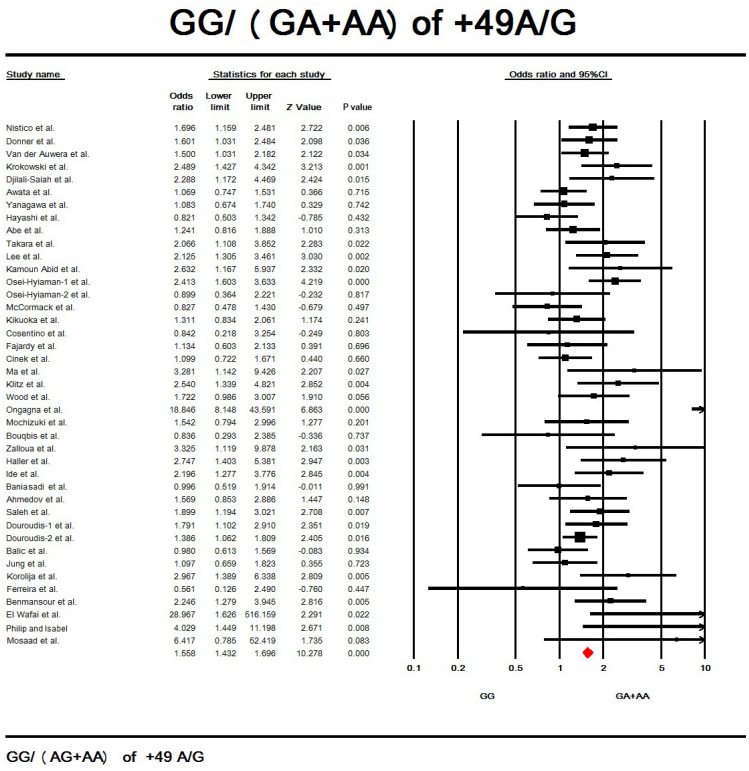

Figure 5. Meta-analysis of association studies of the +49A/G GG/(GA+AA) and diabetes. Pooled overall OR is shown. The OR of each study is marked with a black square. Pooled OR is indicated by a red diamond.

DISCUSSION

T1D is commonly considered to be an organ-specific autoimmune disorder with a multifactorial background, where onset is preceded by a period of autoimmune destruction of the insulin-producing pancreatic β-cells and with high levels of IFN-γ and TNF-β (Atkinson and Eisenbarth, 2001). In addition, it is a heterogeneous syndrome with considerable variability in age of onset, abruptness of onset, and autoantibody profile. However the pathogenesis of the development and progression of T1D is far from clear at present. Because of the various and serious lifelong complications of T1D, it is crucial to identify the etiologic factors in the pathogenesis of this disease. The major histocompatibility complex region explains approximately half of the genetic susceptibility to T1D, suggesting that additional determinants exist, and such determinants are suggested repeatedly by different genome scans (Polychronakos and Li, 2011).

A number of studies have indicated that variants of the *CTLA-4* gene may contribute to the disease. Fine mapping analyses have also suggested that peak linkage and association are present in the CTLA-4 region (+49A/G, -819 C/T, and (AT)n in the 3'UTR) (Marron et al., 1997). However, the results of genetic association studies have been confusing because of the difficulty in replicating significant associations. Different characteristics among studies such as ethnicities, diabetes mellitus type, and definition of case and control have introduced heterogeneity and made the results of association studies hard to interpret.

In this study, we performed a comprehensive meta-analysis aimed at identifying the origin of heterogeneity and assessing the overall effects of these variants on T1D. This comprehensive meta-analysis included data from 43 studies with approximately 17,591 T1D cases and controls. It revealed significant evidence of association between the *CTLA-4* gene and T1D. The +49A/G polymorphism showed both an overall association and subgroup associations, except for the American subgroup. The G allele in overall populations and subgroup populations, and the GG genotype showed a positive association with T1D. One explanation is that +49A/G may have a significant effect on the disease, and may be in LD with other causative mutations.

Given the limitations and potential biases in the study, the results of our meta-analysis should be treated with caution. Overall, we did not detect substantial publication bias. However, since we included only studies published in English, there may have been a language bias. In addition, most of the included studies were retrospective. Also, it was not possible to take into account some environmental factors such as lifestyle and diet.

In conclusion, the current comprehensive meta-analysis pooled larger sample sizes analyzing them both together and separately. The design of systematic methods and analytical approaches as well as heterogeneity tests and sensitivity analyses produced more significant results. These findings demonstrated the robustness of the association between both the allele and genotype of the +49A/G polymorphism of the CTLA-4 gene and T1D, an association which was significant in multiple studies. T1D is caused by the combined actions of many factors. Thus, for greater insight into its genetic components, more work is required to confirm the role of other genes that may have a small individual effect, and to identify new genetic risk factors. The large samples required will necessitate multi-site projects and meta-analyses on the basis of national and international collaboration.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

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