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Original Article

Association between *Cytotoxic T-lymphocyte antigen 4* (*CTLA-4*) +49 G>A (rs231775) polymorphism and esophageal cancer: from a case-control study to a meta-analysis

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Abstract: The aim of this study was to evaluate the association between *CTLA-4* +49 G>A polymorphism and esophageal cancer (EC) susceptibility in a hospital based case-control study and a subsequent meta-analysis. We implemented genotyping analyses for *CTLA-4* +49 G>A polymorphism with 629 esophageal squamous cell carcinoma cases and 686 controls in a Chinese Han population. Polymerase chain reaction ligase detection reaction (PCR-LDR) method was used to identify genotypes of *CTLA-4* +49 G>A polymorphism. We first assessed the association between *CTLA-4* +49 G>A polymorphism and EC risk in a hospital based case-control study, and then performed a comprehensive meta-analysis to derive a more precise estimation. Our results demonstrated that *CTLA-4* +49 G>A polymorphism was not associated with EC risk. This case-control study and further meta-analysis, failed to identify the association between *CTLA-4* +49 G>A polymorphism and EC risk. And additional, further well designed studies with large sample sizes and detailed gene-environment data are required.

Keywords: *CTLA4*, polymorphism, esophageal cancer, susceptibility, meta-analysis

Introduction

Esophageal cancer (EC) is the sixth most common cancer with an estimated 482,300 new cases and more than eighty percent death rate occurred worldwide in 2008 [1]. In 2009, the incidence rate of EC was 22.14 per 10,000 in China [1, 2]. Every year, there are about 250,000 new EC cases diagnosed in China, accounting for half of the global cases [3]. The death rate for EC patients is very high and the 5 years survival rate accounts only 12.3% [4]. In the highest risk area, such as Iran and China, the most frequent subtype of EC is esophageal squamous cell carcinoma (ESCC) and counts more than 90% [5]. However, the etiology of EC is still indistinct. Accumulating evidence suggests genetic components, environmental factors, and gene-environment interactions play vital roles in EC development and progression [6-10]. Recently, several studies have focused on the role of the immune system to explore the etiology of EC [11, 12].

Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is one of the most important members of the immunoglobulin superfamily. CTLA-4, a vital restraining regulator of T-cell proliferation and activation, induces Fas-independent apoptosis of activated T cells [11, 13]. It suggests that CTLA-4 plays an important role in carcinogenesis. *CTLA-4* gene is located on chromosome 2q33 and is composed of four exons that possess several vital single nucleotide polymorphisms (SNPs), such as the +49 G>A, -318 C>T, +6230 G>A (CT60), and -1722 T>C, etc. [11, 14]. A meta-analysis conducted by Zhang *et al.* demonstrated that *CTLA-4* +49 G>A polymor-

Table 1. Distribution of selected demographic variables and risk factors in ESCC cases and controls

Variable	Cases (n=629)		Controls (n=686)		*P*[a]
	n	%	n	%	
Age (years) mean ± SD	62.85 (±8.13)		62.58 (±7.89)		0.541
Age (years)					0.155
<63	310	49.28	365	53.21	
≥63	319	50.72	321	46.79	
Sex					0.185
Male	444	70.59	461	67.20	
Female	185	29.41	225	32.80	
Tobacco use					**<0.001**
Never	355	56.44	499	72.74	
Ever	274	43.56	187	27.26	
Alcohol use					<0.001
Never	428	68.04	526	76.68	
Ever	201	31.96	160	23.32	

[a]Two-sided χ^2 test and student t test; Bold values are statistically significant (P<0.05).

Table 2. Primary information for CTLA4 rs231775 G>A polymorphism

Genotyped SNPs	CTLA4 rs231775 G>A
Chromosome	2
Function	missense
Chr Pos (Genome Build 36.3)	204440959
Regulome DB Score[a]	No Data
Splicing (ESE or ESS)	Y
nsSNP	Y
MAF[b] for Chinese in database	0.314
MAF in our controls (n=686)	0.310
P value for HWE[c] test in our controls	0.284
Genotyping method[d]	LDR
% Genotyping value	96.43%

[a]http://www.regulomedb.org/; [b]MAF: minor allele frequency; [c]HWE: Hardy-Weinberg equilibrium; [d]LDR: ligation detection reaction.

phism may be a risk factor for cancer, whereas in that analysis, only one case-control study conducted on EC [15]. However, up to now, three investigations [11, 12, 16] focused on the association of the *CTLA-4* +49 G>A polymorphism with EC risk, and a definitive conclusion remained elusive. To further investigate this potential relationship, we first assessed the association between *CTLA-4* +49 G>A polymorphism and ESCC risk in a hospital based case-control study, and then conducted a comprehensive meta-analysis to derive a more precise estimation.

Materials and methods

Subjects

A total of 629 esophageal squamous cell carcinoma (ESCC) patients and 686 cancer-free controls were consecutively recruited from the Affiliated People's Hospital of Jiangsu University and Affiliated Hospital of Jiangsu University (Jiangsu Province, China) between October 2008 and December 2010. All patients were confirmed by postoperative pathologic means. In this study, the patients who had a history of cancer or autoimmune diseases before, or had undergone radiotherapy or chemotherapy were excluded. Ethnicity (Chinese), frequency of sex, and average age (±5 years) of the controls were well matched to patients. At recruitment, this investigation was approved by the Institutional Review Board of Jiangsu University (Zhenjiang, China) and each subject signed the written informed consent. Experienced doctors were assigned to administer a structured questionnaire and information of all subjects, such as demographic data (e.g. age, gender) and risk factors (including tobacco use and alcohol consumption), were collected. After completed the in-person interview, each individual donated 2-ml peripheral venous blood. The "smokers" criterion was subjects who smoked more than one cigarette per day over one year, and the "alcohol drinkers" criterion was those who consumed ≥3 alcoholic drinks a week for >6 months.

DNA extraction, SNP selection, and genotyping

Ethylenediamine tetra-acetic acid (EDTA)-anticoagulated peripheral venous blood samples were collected by using Vacutainers (BD Franklin Lakes NJ, USA). The QIAamp DNA Blood Mini Kit (Qiagen, Berlin, Germany) was used to isolate genomic DNA from peripheral blood lymphocytes and DNA samples were frozen at -80°C. Genotypes of *CTLA-4* +49 G>A site were

Table 3. Logistic regression analyses of associations between CTLA4 rs231775 G>A, polymorphism and risk of ESCC

Genotype	Cases (n=629)		Controls (n=686)		Crude OR (95% CI)	*P*	Adjusted OR[a] (95% CI)	*P*
	n	%	n	%				
CTLA4 rs231775 G>A								
GG	307	50.83	310	46.69	1.00		1.00	
GA	254	42.05	296	44.58	0.87 (0.69-1.09)	0.223	0.85 (0.68-1.08)	0.189
AA	43	7.12	58	8.73	0.75 (0.49-1.15)	0.182	0.70 (0.45-1.07)	0.100
GA+AA	297	49.17	354	53.31	0.85 (0.68-1.06)	0.141	0.83 (0.66-1.04)	0.100
GG+GA	561	92.88	606	91.27	1.00		1.00	
AA	43	7.12	58	8.73	0.80 (0.53-1.21)	0.289	0.75 (0.49-1.14)	0.177
G allele	868	71.85	916	68.98	1.00			
A allele	340	28.15	412	31.02	0.87 (0.73-1.03)	0.114		

[a]Adjusted for age, sex, smoking and drinking status.

analyzed by using polymerase chain reaction ligase detection reaction (PCR-LDR) method [17, 18]. Shanghai Biowing Applied Biotechnology Company provided the technical support. For quality control, one hundred and sixty samples were randomly selected and reciprocally tested with high DNA quality, and the reproducibility rate of was 100%.

Statistical analysis

Chi-square test (x^2) was performed to test the differences in the distributions of demographic characteristics, selected variables and genotypes between patients and controls. The association of *CTLA-4* +49 G>A genotypes with the risk of ESCC was evaluated by odds ratios (ORs) and corresponding 95% confidence intervals (CIs) using logistic regression analyses for crude ORs and adjusted ORs when it was appropriate. An internet-based Hardy-Weinberg equilibrium (HWE) calculator (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) was used to measure the deviation from the HWE among the controls. Statistical analysis was implemented by SAS 9.1.3 software (SAS Institute, Cary, NC). Statistical significance was defined as $P<0.05$ with two-tailed for all statistical analyses.

Meta-analysis

The meta-analysis is reported on the basis of the Preferred Reporting Items for Meta-analyses (PRISMA) guideline (Table S1. PRISMA checklist) [19].

Embase, PubMed and CBM (Chinese BioMedical Disc), as well as CNKI (China National Knowledge Infrastructure) database were searched up to September 23th, 2014 for publications investigating the association of *CTLA-4* +49 G>A polymorphism with EC. The combination terms were 'esophageal' or 'esophagus' and 'cancer' or 'tumor' or 'carcinoma' or 'neoplasm' or 'malignance' and 'Cytotoxic T-lymphocyte antigen 4' or 'CTLA-4' or 'CD152', annexed with 'SNP' or 'mutation', 'variant' or 'polymorphism'. Additionally, the publication language was restricted to English and Chinese, and all studies carried out in human subjects were identified. The results of electronic retrieval were supplemented by manual search of all bibliographies listed in these studies or published reviews. The major included criteria were: (a) designed as case-control study, (b) evaluated the *CTLA-4* +49 G>A polymorphism and EC risk, (c) provided genotype counts of *CTLA-4* +49 G>A polymorphism between cases and controls. The major excluded criteria included the following: (a) not case-control study, (b) review publications and (c) overlapping data.

In this meta-analysis, the crude OR with the corresponding 95% CI was used to evaluate the strength of association between *CTLA-4* +49 G>A polymorphism and EC risk. The Z-test and *P*-value (two-tailed) was used to measure the significance of pooled OR, and statistical significance was defined as $P<0.05$ (two-tailed). Heterogeneity among studies was evaluated by a Chi-square-based I^2 test. If $I^2>50\%$ or $P<0.10$, the pooled ORs were calculated by the random-effects model (the DerSimonian-Laird method) [20], otherwise the fixed-effects model was performed (the Mantel-Haenszel method) [21]. The funnel plot and Egger's test were implemented to evaluate publication bias, which

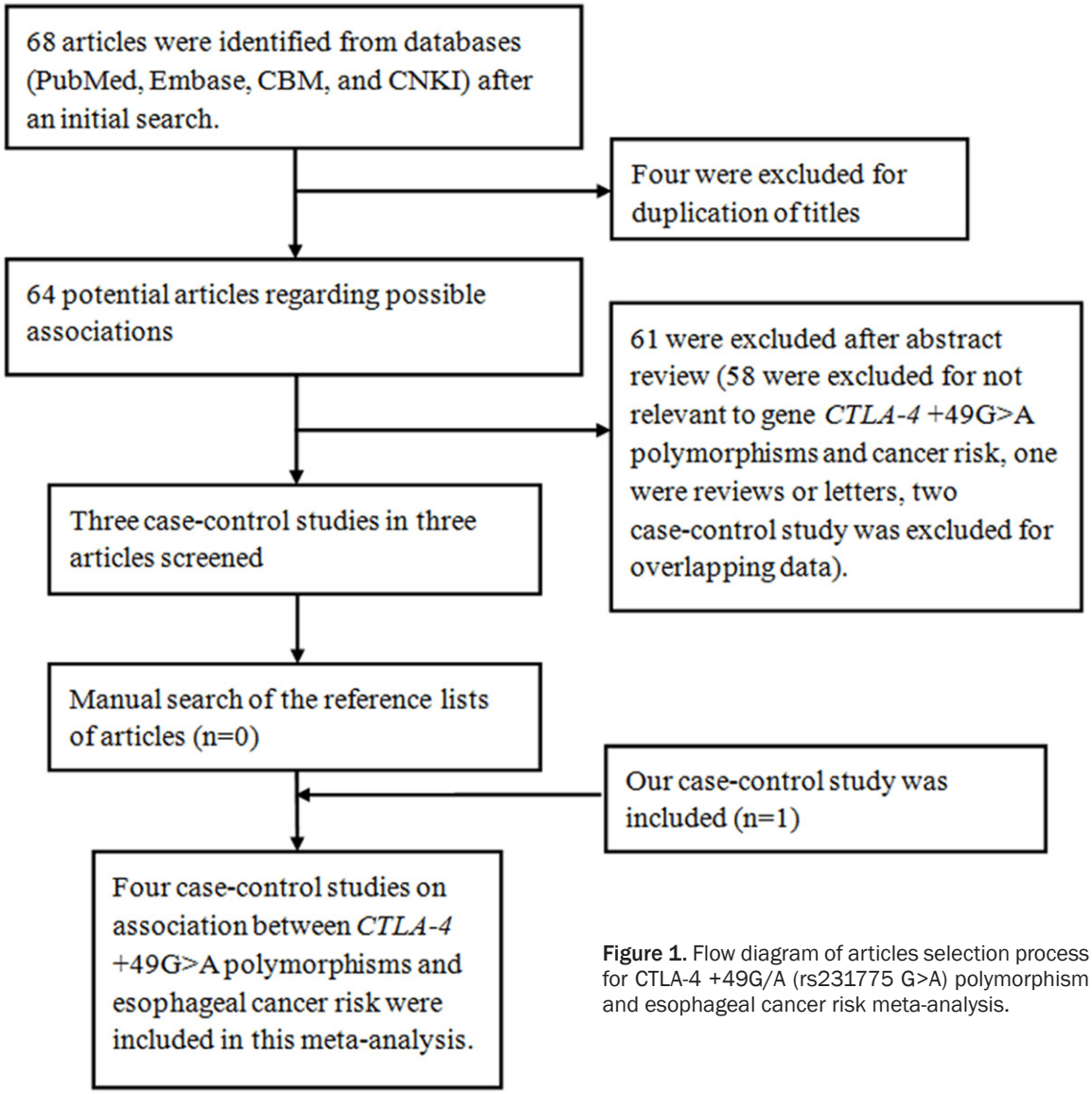

Figure 1. Flow diagram of articles selection process for CTLA-4 +49G/A (rs231775 G>A) polymorphism and esophageal cancer risk meta-analysis.

was measured by visual inspection of an asymmetric plot [22]. For the funnel plot, Egger's test and the I^2, statistical significance was considered at $P<0.1$. Sensitivity analysis was performed to determine whether any excluded studies affected the stability of our results. Galbraith radial plot was used to analyze the heterogeneity [23]. In current meta-analysis, all statistical analyses were performed by STATA software (version 12.0).

Results

Baseline characteristics

Characteristic of all subjects, such as the demographics and risk factors, are presented in **Table 1**. The terms of age and sex distributions were no significant differences between cases and controls ($P=0.155$ and $P=0.185$, respectively), which indicated that these factors were well matched. However, the results indicated that significant difference was found on drinking status and smoking rate between cases and controls ($P<0.001$). The primary information of *CTLA-4* +49 G>A polymorphism is included in **Table 2**. For this SNP, the genotyping success rate was 96.43% in all samples. The minor allele frequency (MAF) of controls was similar to data for Han populations in Chinese database (**Table 2**). The genotypic frequencies for *CTLA-4* +49 G>A polymorphism among controls were in HWE ($P=0.284$) (**Table 2**).

Table 4. Characteristics of populations and cancer types of the individual studies included in the meta-analysis

Study	Year	Ethnicity	Country	Sample size (case/control)	Histologic subtype	Genotype method	Case			Control			Case		Control		HWE
							GG	GA	AA	GG	GA	AA	G	A	G	A	
Cheng et al.	2011	Asians	China	205/205	ESCC	PCR-RFLP	54	105	46	90	79	36	213	197	259	151	no
Cai et al.	2011	Asians	China	125/250	ESCC	PCR-RFLP	30	68	27	70	133	47	128	122	273	227	Yes
Sun et al.	2008	Asians	China	1010/1008	ESCC	PCR-RFLP	448	434	128	529	406	73	1330	690	1464	552	Yes
Our study	2013	Asians	China	629/686	ESCC	PCR-LDR	307	254	43	310	296	58	868	340	916	412	Yes

ESCC, esophageal squamous cell carcinoma. PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism. PCR-LDR, polymerase chain reaction-ligase detection reaction. HWE, Hardy-Weinberg equilibrium.

Table 5. Summary of results of the meta-analysis from different comparative genetic models

Polymorphism	Genetic comparison	OR (95%CI)	*P*	Test of heterogeneity		Model
				p-Value	I^2	
CTLA4 rs231775 G>A	AA+GA vs. GG	1.31 (0.90-1.89)	0.160	0.000	85.1%	R
	AA vs. GA+GG	1.26 (0.85-1.88)	0.251	0.013	72.1%	R
	AA vs. GG	1.45 (0.85-2.47)	0.171	0.001	81.2%	R
	GA vs. GG	1.26 (0.90-1.77)	0.181	0.002	80.1%	R
	AA vs. GA	1.15 (0.82-1.60)	0.419	0.082	55.2%	R
	A vs. G	1.21 (0.92-1.59)	0.179	0.000	86.0%	R

R indicates random-effects model.

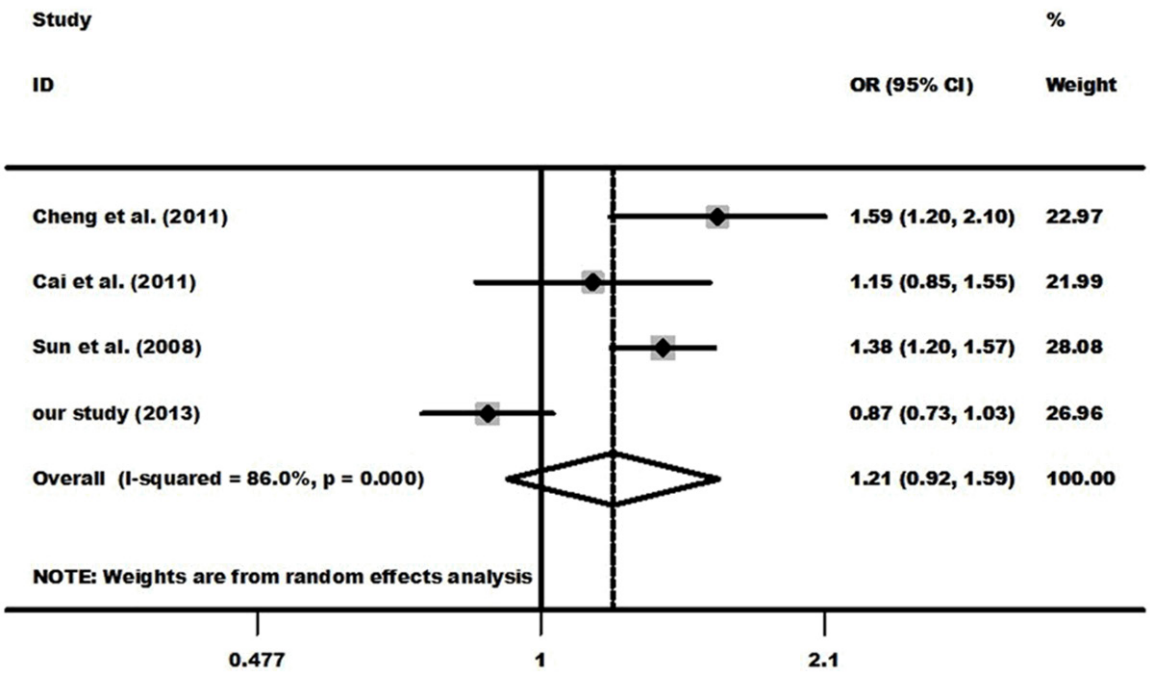

Figure 2. Meta-analysis with a random-effects model for the association between the risk of esophageal cancer and the *CTLA-4* +49 G>A polymorphism (A vs. G).

CTLA4 +49 G>A polymorphism and risk of ESCC

The genotype distributions of *CTLA-4* +49 G>A were presented in **Table 3**. In the single locus analysis, the genotype frequencies of *CTLA-4* +49 G>A were 50.83% (GG), 42.05% (GA) and 7.12% (AA) in cases, and 46.69% (GG), 44.58% (GA) and 8.73% (AA) in controls, and the difference was not statistically significant (*P*=0.270). When the *CTLA-4* +49 GG homozygote genotype was used as the reference group, the GA genotype was not associated with the risk of ESCC (GA vs. GG: OR 0.87, 95% CI 0.69-1.09, *P*=0.223). When the *CTLA-4* +49 GG homozygote genotype was used as the reference group, the AA genotype was not associated with the risk of ESCC (AA vs. GG: OR 0.75, 95% CI 0.49-1.15, *P*=0.182). In the recessive model, when the *CTLA-4* +49 GG/GA genotypes were used as the reference group, the AA homozygote genotype was not associated with the risk of ESCC (OR 0.80, 95% CI 0.53-1.21, *P*=0.289). In the dominant model, the *CTLA-4* +49 AA/GA variants were not associated with the risk of ESCC, compared with the *CTLA-4* +49 GG genotype (AA/GA vs. GG: OR 0.85, 95% CI 0.68-1.06, *P*=0.141). When the *CTLA-4* +49 G allele was used as the reference group, the A allele was not associated with the risk of ESCC (A vs.

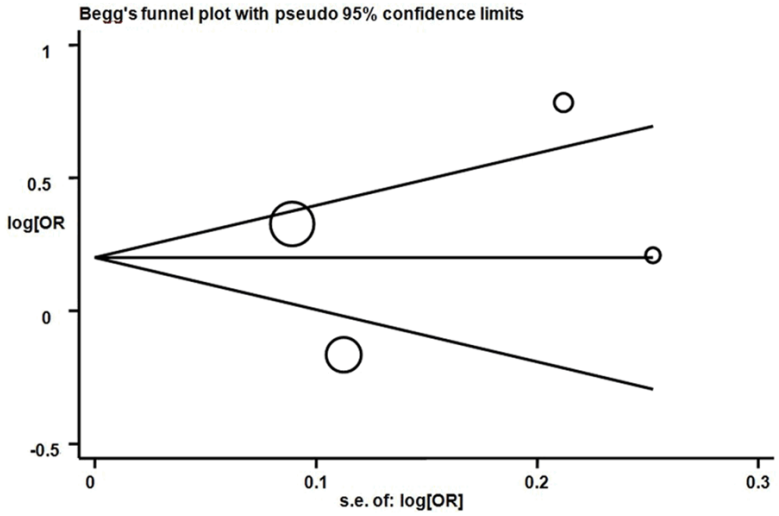

Figure 3. Begg's funnel plot of meta-analysis of between the *CTLA-4* +49 G>A polymorphism and the risk of cancer in the dominant model (random-effects model).

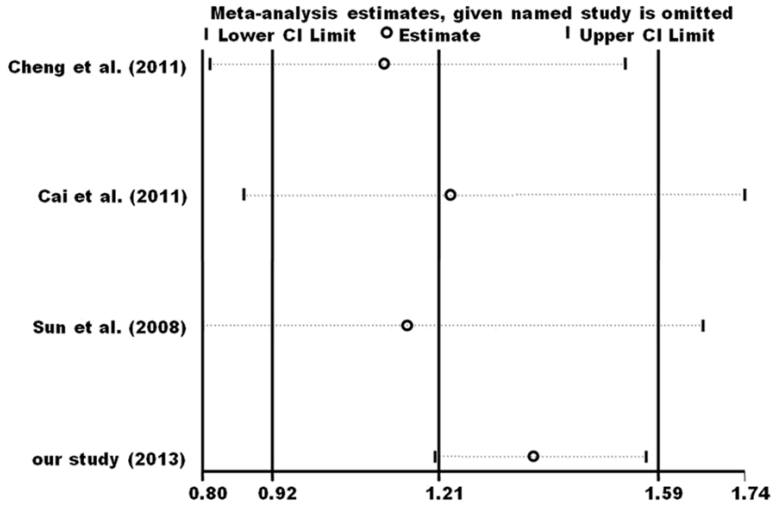

Figure 4. Sensitivity analysis of the influence of A vs. G in meta-analysis (random-effects estimates).

G: OR 0.87, 95% CI 0.73-1.03, *P*=0.114) (**Table 3**). After adjusting for age, gender, smoking and drinking status, no statistically increased or decreased risk of ESCC was observed in all genetic models (**Table 3**).

Eligible articles for meta-analysis

The initial search yielded a total of 30 potentially relevant articles. After applying additional filters, three case-control studies and our study were eligible for inclusion. The detailed process is presented in **Figure 1**.

Study characteristics

In total, three previous studies plus our case-control study involving a total of 1969 EC cases and 2149 controls were recruited in this meta-analysis. As for subjects in these studies, all were Asians. Characteristics of all included study are presented in **Table 4**. The detailed distribution of the *CTLA-4* +49 G>A polymorphism and allele among cases and controls is presented in **Table 4**.

Meta-analysis results

After combining all recruited studies, a total of 1969 EC cases and 2149 controls from four investigations were included for meta-analysis of the association between *CTLA-4* +49 G>A polymorphism and EC risk and the results indicated that there was null association (**Table 5** and **Figure 2**). Among the four case-control studies, there was one case-control study deviated from HWE [12], after we excluded it and then obtained another result. However, this result was in accordance with the previous one (data not shown).

In this meta-analysis, Begg's Funnel plot and Egger's test was created to detect potential publication bias (**Figure 3**). The shape of funnel was symmetry in all genetic models, suggesting that there were no publication bias in this meta-analysis (A vs. G: Begg's test *P*=0.734, Egger's test *P*=0.970; AA vs. GG: Begg's test *P*=0.734, Egger's test *P*=0.750; GA vs. GG: Begg's test *P*=1.000, Egger's test *P*=0.596; AA vs. GA: Begg's test *P*=1.000, Egger's test *P*=0.255; AA+GA vs. GG: Begg's test *P*=1.000, Egger's test *P*=0.725; AA vs. GG+GA: Begg's test *P*=0.734, Egger's test *P*=0.416).

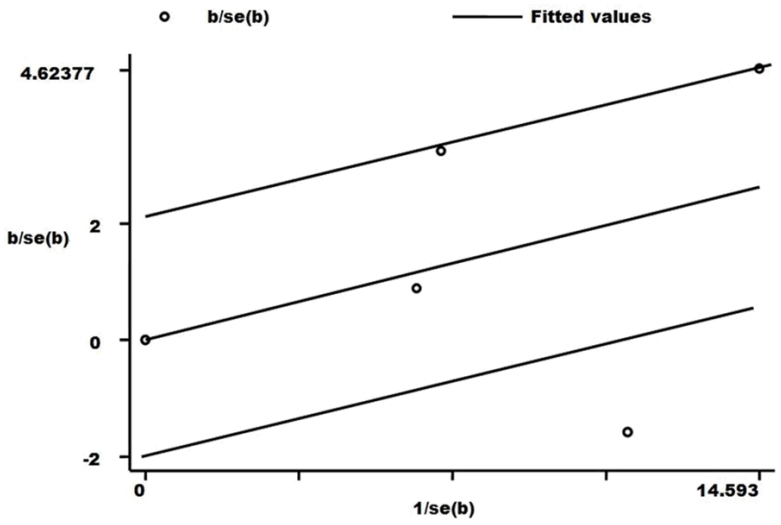

Figure 5. Galbraith radial plot of meta-analysis (A vs. G compare genetic model).

Sensitivity analyses were performed to evaluate the influence of each individual dataset on the pooled OR by omitting each dataset in turn. The results did not alter when any individual study was deleted, confirming the stability of our results (**Figure 4**).

The results indicated there were large heterogeneities among four case-control studies enrolled. As shown in **Table 5**, heterogeneity was significant in allele genetic model. Galbraith radial plot was used to analyze the source of heterogeneity (**Figure 5**). The result identified one outlier which might contribute to the major source of heterogeneity. From the forest plot in allele genetic model (**Figure 2**), we can identify that one study which conducted by Cheng *et al*. [12] was the main source of heterogeneity.

Discussion

Recently, the association between *CTLA-4* +49 G>A polymorphism and EC risk have been investigated in several studies and a decisive answer is lacking. In this study, a hospital based case-control study in Chinese Han population, along with a meta-analysis on EC, attempted to derive a more precise evaluation and the results were remain non-significance. To the best of our knowledge, this is the first meta-analysis exploring the association of *CTLA-4* +49 G>A polymorphism with EC risk.

With a growing interest in the associations of genetic polymorphisms and EC risk, several studies have examined the hypothesis that *CTLA-4* +49 G>A polymorphism is relevant to the risk of EC; however, the results remain inconsistent and ambiguous. Considering the fact that most common SNPs usually make low cancer susceptibility, this meta-analysis recruits four case-control studies with relatively large sample sizes to get a more precise assessment. Two studies have reported positive signal of *CTLA-4* +49 G>A polymorphism with EC risk [11, 12]; the other individual study has reported negative signal [16]; however, as showed in the results of current meta-analysis among 4118 subjects, there were non-significance. Although there was one case-control study deviated from HWE [12], we excluded it or recruited it, this result was similar, suggesting our results were stable. The results should be interpreted with very caution. Considering only four case-control studies were conducted in EC and two studies were relatively small sample sizes, which might generate a fluctuated assessment or restrict the power to confirm a real influence. It was also possible that the real function of *CTLA-4* +49 G>A polymorphism was covered or diluted by other genetic or environment factors, and these vital factors should not be ignored. In the future, well designed investigations with large sample sizes should be carried out to verify these results.

Some merit should be addressed in current study. First, this is a case-control study with large sample sizes in detecting the association of *CTLA-4* +49 G>A polymorphism with EC risk and the meta-analysis is the first synthesis investigating the association Second, the results of our case-control study confirmed that of current meta-analysis. Third, in our case-control study, the genotype frequencies in the controls were in HWE, suggesting our results were less prone to selection bias, publication bias tests indicated that there was no bias in this meta-analysis.

Several limitations in this study should be acknowledged. First, in case-control study, all

subjects were recruited from two hospitals and might not fully represent the general Chinese populations. Second, only published studies in four databases were recruited in this meta-analysis, publication bias might have occurred. Third, large heterogeneity was observed in our meta-analysis, which means the results should be interpreted with very caution. Fourth, due to lack of uniform background information for recruited investigations, data were not further stratified by other factors (such as, age, gender, smoking, alcohol consumption, ethnicity and other lifestyle factors). Fifth, in this study, we only focused on *CTLA-4* +49 G>A polymorphism, and did not consider other polymorphisms in *CTLA-4* or other susceptibility genes.

In summary, this case-control study and subsequent meta-analysis failed to confirm the association between *CTLA-4* +49 G>A polymorphism and EC risk. Nevertheless, for practical reasons, further well designed studies with large sample sizes and detailed gene-environment data, should be performed to confirm or refute these results.

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Disclosure of conflict of interest

None.

Abbreviations

CI, confidence interval; OR, odds ratio; *CTLA4*, cytotoxic T-lymphocyte antigen 4; HWE, Hardy-Weinberg equilibrium; ESCC, esophageal squamous cell carcinoma; PCR-LDR, polymerase chain reaction ligase detection reaction; SNP, single nucleotide polymorphism.

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Table S1. PRISMA checklist, Checklist of items to include when reporting a meta-analysis

Section/topic	#	Checklist item	Reported on section #
Title			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	Title page
Abstract			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	Abstract page
Introduction			
Rationale	3	Describe the rationale for the review in the context of what is already known.	Introduction section
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	Introduction section
Methods			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	N/A
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	Materials and Methods section, Meta Analysis
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	Materials and Methods section, Meta-Analysis
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	Materials and Methods section, Meta-Analysis
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	Materials and Methods section, Meta-Analysis
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	Materials and Methods section, Meta-Analysis
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	Materials and Methods section, Meta-Analysis
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	Materials and Methods section, Meta-Analysis
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	Materials and Methods section, Meta-Analysis
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	Materials and Methods section, Meta-Analysis