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Original Article

Lack of association between *cyclin D1* A870G (rs9344) polymorphism and esophageal squamous cell carcinoma risk: case-control study and meta-analysis

Weifeng Tang[1*], Ping Yu[2*], Yafeng Wang[3*], Mingqiang Kang[4], Bin Sun[1], Jun Yin[1], Haiyong Gu[1]

[1]Department of Cardiothoracic Surgery, Affiliated People's Hospital of Jiangsu University, Zhenjiang, China; [2]Department of Pharmaceutics, China Pharmaceutical University, Nanjing, China; [3]Department of Cardiology, The People's Hospital of Xishuangbanna Dai Autonomous Prefecture, Jinghong, Yunnan Province, China; [4]Department of Thoracic Surgery, The Union Clinical Medical College of Fujian Medical University, Fuzhou, Fujian Province, China. [*]Equal contributors.

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Abstract: Studies examining the association between the *cyclin D1* (*CCND1*) A870G (rs9344 G>A) polymorphism and esophageal squamous cell carcinoma (ESCC) have yielded inconsistent results. Here, we conducted a hospital-based case-control study in a Chinese Han population to assess the association between the *CCND1* A870G polymorphism and ESCC. We then performed a meta-analysis to further investigate this association. We recruited 629 patients with ESCC and 686 cancer-free controls. Genotyping was performed with the polymerase chain reaction-ligase detection reactions (PCR-LDR) method. The meta-analysis was performed with the STATA 12.0 software. The case-control study showed no significant difference between the ESCC cases and controls in the allele frequencies or genotype distributions of the *CCND1* A870G polymorphism. To obtain a more precise estimate of this relationship, we performed a meta-analysis of seven case-control studies involving a total of 2080 ESCC cases and 2833 controls. The meta-analysis suggested that the *CCND1* A870G polymorphism is not associated with a risk of ESCC. A further subgroup analysis based on ethnicity also detected no association. This study suggests that the *CCND1* A870G polymorphism is not associated with the risk of ESCC.

Keywords: *Cyclin D1*, polymorphism, cancer susceptibility, meta-analysis, esophageal cancer

Introduction

It is estimated that 455,800 new cases of esophageal cancer (EC) and 400,200 deaths resulting from the disease occurred worldwide in 2012 [1]. In China, based on annual report on status of cancer estimates, 287,632 new cases of esophageal cancer (EC) and 208,473 deaths occurred in 2010 [2]. Each year, approximately half of the global cases of EC are diagnosed in China [3, 4]. The mortality rate for EC patients is very high with a five-year survival rate of 12.3% [5]. In the highest-risk geographic areas, such as China and Iran, esophageal squamous cell carcinoma (ESCC) is the most frequent subtype of EC, with an estimated 90% of cases shown histologically to be ESCC [6]. However, the etiology of EC is mixed. Evidence is mounting that EC is a multifactorial disease resulting from interactions between genetic and environmental factors [7-12]. Contributing environmental risk factors to EC are not completely understood, but are deemed to include low intake of vegetables and fruits, poor nutritional status, smoking, alcohol consumption and eating at high temperatures [13-15]. Recently, a mass of molecular epidemiological studies demonstrated that susceptibility to EC is affected by both genetic and environmental factors [16]. Thus, it is quite likely that there are a number of genetic mutations contributing to the remaining indistinct EC susceptibility, which have not yet been found.

The *cyclin D1* (*CCND1*) gene is located on chromosome 11q13. It encodes a subunit of the holoenzyme that phosphorylates the protein, subsequently affects the transcription of genes

Table 1. Distribution of selected demographic variables and risk factors in the 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 x^2 test and Student's *t* test; bold values are statistically significant ($P < 0.05$).

Table 2. Primary information for the *CCND1* A870G (rs9344) polymorphism

Genotyped SNPs	*CCND1* A870G (rs9344)
Chromosome	11
Gene official symbol	*CCND1* (*cyclin D1*)
Function	Cds-synon
Chr pos (Genome Build 36.3)	69172091
Regulome DB Score[a]	3a
MAF[b] for Chinese in database	0.427
MAF in our controls (n = 686)	0.470
P value for HWE[c] test in our controls	0.388
Genotyping method[d]	LDR
% Genotyping value	98.48%

[a]http://www.regulomedb.org/; [b]MAF: minor allele frequency; [c]HWE: Hardy-Weinberg equilibrium; [d]LDR: ligation detection reaction; Bold values are statistically significant ($P < 0.05$).

and promotes progression from G1 to S phase of the cell cycle during cell differentiation [17]. Thus, it is regarded as a key regulatory protein in cell proliferation and differentiation. The accumulating evidence highlights that overexpression of *CCND1* were observed in many malignances, which might be correlated with metastases and the poor prognoses [18-20].

In the last decade, the *cyclin D1* (*CCND1*) A870G polymorphism has been widely investigated, and several studies have tested the hypothesis that genetic variants of *CCND1* A870G are associated with a risk of ESCC [21-25]. Two meta-analyses indicated that the *CCND1* A870G polymorphism might be a risk factor for the development of EC, especially in Asian populations [26, 27]. However, results of other previous studies suggested that the *CCND1* G870A polymorphism were not associated with the risk of ESCC [28, 29], so the issue remains unclear. Although a single study could be underpowered, an analysis of the accumulated data from all ESCC epidemiological studies could provide evidence of an association between *CCND1* A870G polymorphism and the risk of ESCC. Therefore, to further investigate this potential relationship, we conducted a hospital-based case-control study followed by a comprehensive meta-analysis to establish a more detailed understanding of this relationship.

Materials and methods

Subjects

In total, 629 consecutive ESCC patients and 686 cancer-free controls were recruited from the Affiliated People's Hospital of Jiangsu University and the Affiliated Hospital of Jiangsu University (Zhenjiang City, Jiangsu Province, China), between October 2008 and December 2010 as described previously [8, 9, 30]. All subjects were from the city of Zhenjiang and its surrounding regions. The controls were well matched with ESCC cases on average age (±5 years), gender and ethnicity described previously [8, 9, 31]. The control individuals were selected from the two hospitals for cure of fracture. The selection process was described in previous molecular epidemiological study [9, 30, 32]. This investigation was approved by the Ethics Committee of Jiangsu University (Zhenjiang, China). A structured questionnaire was used to extract detailed information on the

Table 3. Logistic regression analysis of the association between the *CCND1* A870G, 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	%				
AA	192	31.0	184	27.2	1.00		1.00	
AG	308	49.8	348	51.5	0.85 (0.66-1.09)	0.204	0.85 (0.66-1.10)	0.213
GG	119	19.2	144	21.3	0.79 (0.58-1.09)	0.148	0.80 (0.58-1.10)	0.164
GG vs. AG vs. AA						0.289		
AG+GG	427	69.0	492	72.8	0.83 (0.65-1.06)	0.133	0.83 (0.65-1.06)	0.144
AA+AG	500	80.8	532	78.7	1.00		1.00	
GG	119	19.2	144	21.3	0.88 (0.67-1.15)	0.355	0.88 (0.67-1.17)	0.380
A allele	692	55.9	716	53.0	1.00		-	
G allele	546	44.1	636	47.0	0.89 (0.76-1.04)	0.134	-	-

[a]Adjusted for age, sex, smoking and drinking status; bold values are statistically significant ($P < 0.05$). Genotyping for *CCND1* A870G was successful in 619 (98.4%) ESCC cases, and 676 (98.5%) controls.

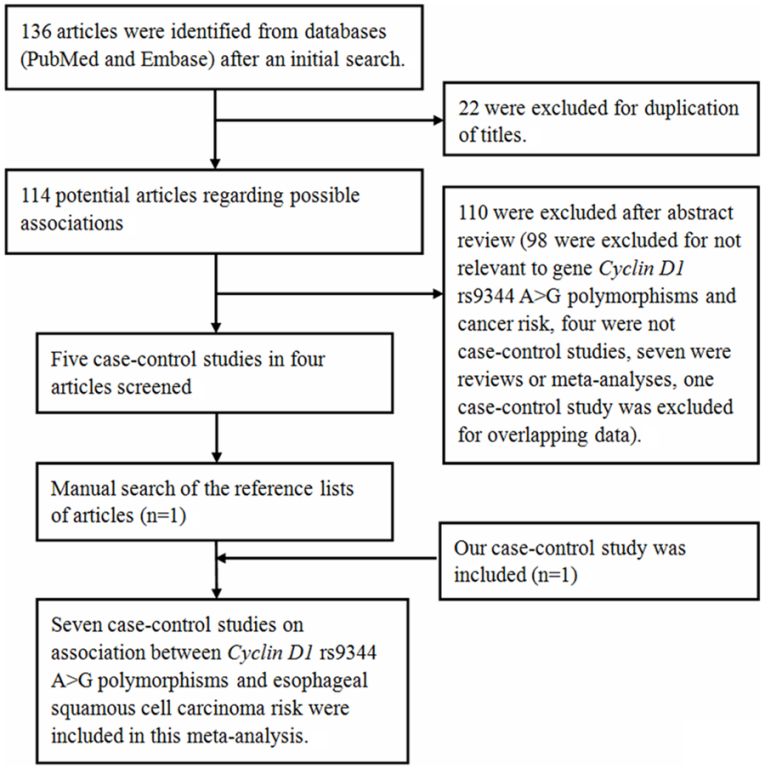

Figure 1. Flow diagram of articles selection process for *CCND1* A870G polymorphism and esophageal cancer risk meta-analysis.

demographics and risk factors of the study subjects and the results are given in **Table 1**. Each subject completed an in-person interview and gave his/her written informed consent.

DNA extraction and genotyping

Peripheral blood (2 ml) was collected from each subject. DNA was extracted from the peripheral blood lymphocytes with the QIAamp DNA Blood Mini Kit (Qiagen, Berlin, Germany) following the manufacturer's protocol. *CCND1* A870G polymorphism was genotyped using the polymerase chain reaction-ligation detection reaction (PCR-LDR) method [30, 33]. As a measure of quality control, 160 DNA assays were randomly selected and tested reciprocally, with 100% concordance.

Statistical analysis

The x^2 test was used to assess the differences in the categorical variables including genotypes, demographic characteristics, tobacco use, and alcohol use between the ESCC cases and the controls. Deviation from Hardy-Weinberg equilibrium (HWE) in the controls was assessed with an internet-based calculator (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). The association between the *CCND1* A870G polymorphism and the risk of ESCC was estimated with logistic regression for crude odds ratios (ORs) and adjusted ORs, where appropriate. All statistical analyses were conducted with the SAS 9.1.3 software (SAS Institute, Cary, NC). A $P < 0.05$ (two-sided probability) was considered as the criterion for statistical significance.

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

Study	Year	Country	Ethnicity	No. of cases/controls	Histological type of EC	Genotype method
Djansugurova et al. [24]	2013	Kazakhstan	Caucasians	115/100	ESCC	Direct sequencing
Hussain et al. [21]	2011	India	Asians	151/151	ESCC	PCR-RFLP
Akbari et al. [25]	2009	Iran	Caucasians	195/250	ESCC	MALDI-TOF MS
Akbari et al. [25]	2009	Iran	Caucasians	549/1118	ESCC	MALDI-TOF MS
Zhang et al. [22]	2003	China	Asians	120/183	ESCC	PCR-SSCP
Yu et al. [23]	2003	China	Asians	321/345	ESCC	PCR-RFLP
Our study	2013	China	Asians	629/686	ESCC	LDR-PCR

PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism; MALDI-TOF MS: Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry; ESCC: esophageal squamous cell carcinoma; LDR-PCR: ligation detection reaction polymerase chain reaction; PCR-SSCP: polymerase chain reaction single-stranded conformation polymorphism.

Table 5. Distribution of *CCND1* A870G polymorphisms genotype and allele among esophageal squamous cell carcinoma patients and controls

		Case			Control			Case		Control		HWE,	Quality
Study	Year	AA	AG	GG	AA	AG	GG	A (%)	G (%)	A (%)	G (%)	*P* value	scores
Djansugurova et al.	2013	44	49	22	18	54	28	137 (59.57)	93 (40.43)	90 (45.00)	110 (55.00)	0.363	6.5
Hussain et al.	2011	32	99	20	23	72	56	163 (53.97)	139 (46.03)	118 (39.07)	184 (60.93)	0.986	7.5
Akbari et al.	2009	53	83	59	79	116	55	189 (48.46)	201 (51.54)	274 (54.80)	226 (45.20)	0.316	6.5
Akbari et al.	2009	158	276	115	355	550	213	592 (53.92)	506 (46.08)	1260 (56.35)	976 (43.65)	0.999	7.5
Zhang et al.	2003	35	74	11	43	102	38	144 (60.00)	96 (40.00)	188 (51.37)	178 (48.63)	0.118	7.0
Yu et al.	2003	96	157	68	110	177	58	349 (54.36)	293 (45.64)	397 (57.54)	293 (42.46)	0.354	6.0
Our study	2013	192	308	119	184	348	144	692 (55.90)	546 (44.10)	716 (52.96)	636 (47.04)	0.388	8.0

HWE: Hardy-Weinberg equilibrium.

Meta-analysis

We searched the Embase and PubMed for publications that investigated the association between the *CCND1* A870G polymorphism and EC risk. The last search update was on September 26, 2014. The combination terms were 'esophageal' or 'esophagus' or 'cancer' or 'neoplasm' or 'carcinoma' or 'tumor' and '*cyclin D1*' or '*CCND1*', annexed with 'polymorphism' or 'SNP' or 'mutation' or 'variant'. In addition, the search was limited to English and Chinese language publications, and all studies carried out in human subjects were included. Additional publications were identified with a manual search of all references listed in these studies and reviews. Studies were eligible for the meta-analysis if they met the major inclusion criteria: (1) designed as a case-control study; (2) evaluated the *CCND1* A870G polymorphism and ESCC risk; (3) provide genotype counts of the *CCND1* A870G polymorphism for the ESCC cases and controls; and (4) the control genotype distributions were consistent with HWE. The exclusion criteria were: (1) not designed as a case-control study, (2) a review, and (3) overlapping data.

The information was carefully and independently extracted by three reviewers (W. Tang, P. Yu and Y. Wang), and any differences were resolved after further discussion among all the reviewers. The following data were extracted: (1) first author, year of publication; (2) country, ethnicity of study subjects; (3) number of cases and controls, genotype method, allele and genotype frequencies; and (4) HWE in the controls. For literatures that did not provide sufficient data, the reviewers attempted to obtain these information by correspondence with the authors. If these could not be obtained, the publications were excluded.

The quality of studies included in our study was assessed by three reviewers (W. Tang, P. Yu and Y. Wang) according to the "methodological quality assessment scale" described previously [34, 35]. If the quality scores ≥ 6, studies were categorized as "high quality", and others were defined as "low quality".

Table 6. Summary of results of the meta-analysis from different comparative genetic models in the subgroup analysis

	No. (cases/controls)	G vs. A			GG vs. AA			GG+AG vs. AA			GG vs. AG+AA		
		OR (95% CI)	*P*	*P* (Q-test)	OR (95% CI)	*P*	*P* (Q-test)	OR (95% CI)	*P*	*P* (Q-test)	OR (95% CI)	*P*	*P* (Q-test)
Total	2080/2833	0.87 (0.71-1.08)	0.205	0.000	0.73 (0.47-1.14)	0.173	0.000	0.88 (0.69-1.12)	0.296	0.007	0.79 (0.54-1.16)	0.232	0.000
Ethnicity													
Asians	1221/1365	0.81 (0.62-1.07)	0.133	0.001	0.60 (0.31-1.15)	0.123	0.000	0.87 (0.73-1.04)	0.119	0.370	0.61 (0.32-1.18)	0.143	0.000
Caucasians	859/1468	0.96 (0.66-1.39)	0.817	0.001	0.93 (0.46-1.88)	0.832	0.003	0.85 (0.48-1.52)	0.592	0.002	1.08 (0.72-1.63)	0.694	0.060
Sample sizes													
< 600	581/684	0.71 (0.47-1.06)	0.097	0.000	0.45 (0.18-1.13)	0.089	0.000	0.71 (0.42-1.18)	0.183	0.003	0.53 (0.24-1.13)	0.100	0.000
≥ 600	1499/2149	1.07 (0.85-1.34)	0.548	0.030	1.16 (0.74-1.81)	0.528	0.032	0.97 (0.81-1.15)	0.711	0.178	1.18 (0.83-1.68)	0.361	0.053
Publication year													
> 2009	895/937	0.66 (0.46-0.96)	0.030	0.006	0.43 (0.19-0.95)	0.037	0.005	0.62 (0.38-1.01)	0.055	0.045	0.53 (0.25-1.13)	0.100	0.001
≤ 2009	1185/1896	1.06 (0.88-1.29)	0.543	0.037	1.09 (0.70-1.69)	0.696	0.017	1.10 (0.94-1.30)	0.223	0.457	1.07 (0.72-1.59)	0.754	0.010

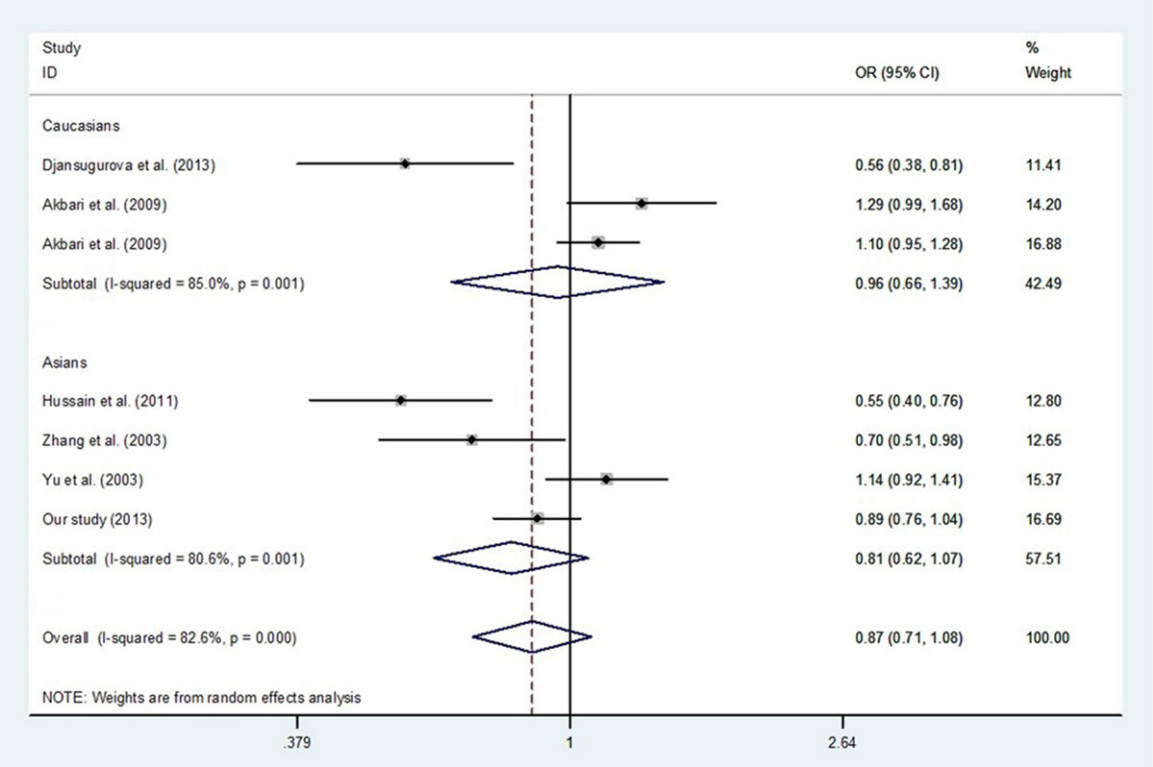

Figure 2. Meta-analysis with a random-effects model for the association between the risk of esophageal cancer and the *CCND1* A870G polymorphism (G vs. A).

Deviation from HWE in the controls was tested with an internet-based calculator (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). In this meta-analysis, the strength of the association between the *CCND1* A870G polymorphism and ESCC risk was evaluated with a crude OR and the corresponding 95% confidence intervals (95% CI). The significance of the pooled ORs was measured with the Z-test and *P*-value, and $P < 0.05$ (two-tailed) was considered the criterion for statistical significance. The heterogeneity across studies was estimated with a Q statistic test. If $P < 0.10$, the ORs were pooled with a random-effects model (the DerSimonian-Laird method) [36]; otherwise, a fixed-effects model was used (the Mantel-Haenszel method) [37]. Subgroup analyses were conducted to assess the specific effect of different factors. A funnel plot and Egger's test were used to assess potential publication bias. A one-way sensitivity analysis was conducted to evaluate the stability of the meta-analysis results. Further stratified analyses were used to detect the source of heterogeneity. In this meta-analysis, all statistical analyses were conducted with the STATA software (version 12.0) and all *P* values were two sided.

Results

Baseline characteristics

The demographics and risk factors of the subjects are given in **Table 1**. There was no significant difference between the ESCC cases and controls in term of their age or sex distribution, suggesting that the frequency matching was complete. However, there were significant differences between the ESCC cases and controls in their tobacco and alcohol consumption ($P < 0.001$). The primary data about the *CCND1* A870G polymorphism are shown in **Table 2**. The genotyping success rate was 98.48% for all samples. The observed genotypic frequencies in the control subjects indicated that there are no significant departures from HWE ($P = 0.388$; **Table 2**).

Single-locus analysis

The allele frequencies and genotype distributions of the *CCND1* A870G polymorphism are summarized in **Table 3**. In the controls, the frequencies of the AA, AG and GG genotype were 27.2%, 51.5% and 21.3%, respectively, which

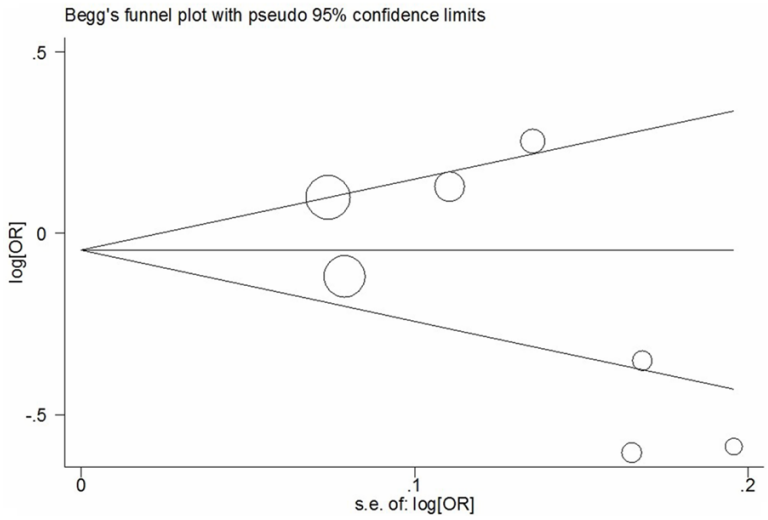

Figure 3. Begg's funnel plot of meta-analysis of between the *CCND1* A870G polymorphism and the risk of cancer (G vs. A).

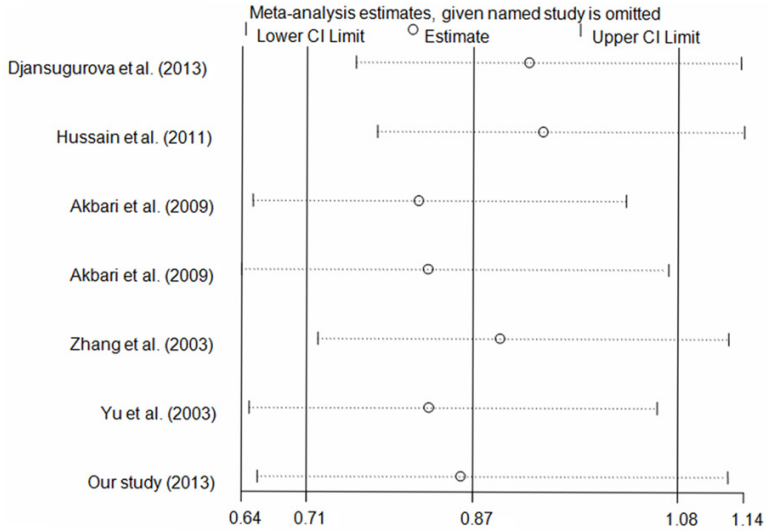

Figure 4. Sensitivity analysis of the influence of G vs. A genetic model (random-effects estimates).

did not differ significantly from those of the ESCC cases (31.0%, 49.8% and 19.2%, respectively; $P = 0.289$; **Table 3**). Logistic regression analyses were conducted and showed that the *CCND1* A870G single nucleotide polymorphism (SNP) was not associated with the risk of ESCC.

Article eligible for the meta-analysis

As shown in **Figure 1**, an extensive search yielded 136 potentially relevant publications. After additional filters were applied, six case-control studies and our study were eligible for the analysis.

Study characteristics

There were two subgroups in a publication conducted by Akbari *et al*. [25], we considered them separately. In total, six separate case-control studies and our study involving 2080 ESCC cases and 2833 controls were recruited for this meta-analysis [21-25]. Among them, four were Asian [21-23] and three were Caucasian studies [24, 25]. The relevant information for each study is listed in **Table 4**. The frequencies of the *CCND1* A870G polymorphism and allele among the cases and controls are listed in **Table 5**. Results of the quality scores are summarized in **Table 5**.

Meta-analysis results

When all eligible studies were combined, there was no significant difference in the allele frequencies or genotype distributions of the *CCND1* A870G polymorphism in the ESCC cases and controls, even when different ethnic groups were involved (**Table 6**; **Figure 2**).

Tests for publication bias, sensitivity analyses, and heterogeneity

Begg's funnel plots and Egger's tests were used to assess the publication bias, and the shape of the funnel was symmetrical in all genetic models (**Figure 3**). This suggested that our findings were robust (G vs. A: Begg's test $P = 0.133$, Egger's test $P = 0.165$; GG vs. AA: Begg's test $P = 0.368$, Egger's test $P = 0.109$; GG+AG vs. AA: Begg's test $P = 0.133$, Egger's test $P = 0.157$; GG vs. AG+AA: Begg's test $P = 0.368$, Egger's test $P = 0.166$).

We used a one-way sensitivity analysis to assess the influence of each study on the pooled ORs and CIs. The results demonstrated

that our findings were stable (**Figure 4**; data not shown).

As shown in **Table 6**, significant heterogeneity was observed among the studies in all the genetic comparison models. Because ethnicity, sample size and publication year can influence the results of a meta-analysis, we conducted subgroup analyses and the results are shown in **Table 6**. The results confirmed association between significant heterogeneity and Asian populations, studies published after 2009, and small samples (< 600 subjects) (**Table 6**).

Discussion

Studies investigating the association between the *CCND1* A870G polymorphism and the risk of ESCC have yielded inconclusive results. We conducted a case-control study in a Chinese Han population, together with a meta-analysis of several studies to determine the association between the *CCND1* A870G polymorphism and ESCC. To the best of our knowledge, this is so far the most comprehensive meta-analysis to investigate the association. The results of the case-control study showed no significant differences in the allele frequencies or genotype distributions of the *CCND1* A870G polymorphism between the ESCC cases and controls. The results of the meta-analysis suggested that the *CCND1* A870G polymorphism is not associated with a risk of ESCC, even in different populations.

CCND1 plays a crucial role in the transition from the G1 to S phase of the cell cycle during mitosis, and regulates cell proliferation and differentiation [38, 39]. The stabilized overexpression of *CCND1* can accelerate cell proliferation, disrupt the cell cycle, alter the capacity of cells to undergo DNA repair, which all promote tumorigenesis [40, 41]. With a growing interest in the association between the *CCND1* A870G and ESCC risk, several recent studies have tested the hypothesis that the *CCND1* A870G polymorphism is associated with a risk of ESCC [21-25], but the findings have been inconsistent. Because common SNPs are usually low-penetrance cancer susceptibility variants, our meta-analysis examined seven studies to comprehensively assess the association between the *CCND1* A870G genetic variant and ESCC risk. Several investigations have reported a negative correlation between the *CCND1* A870G polymorphism and ESCC [21, 22, 24]; whereas others have reported no link between the polymorphism and ESCC [23, 25]. Based on our meta-analysis of 4913 subjects (2080 ESCC cases and 2833 controls), the *CCND1* A870G polymorphism may not be associated with a risk of ESCC. However, it is possible that other genetic or environmental factors may have diluted or obscured the effects of the *CCND1* A870G polymorphism, and these important factors should not be ignored. Because only seven case-control studies, involving 4913 subjects were included in this meta-analysis with most studies designed for small samples, further investigations with larger samples and detailed individual data must be performed to validate or refute our findings.

Heterogeneity, publication bias and sensitivity analysis were assessed in this study. Significant heterogeneity was observed between the publications regarding the *CCND1* A870G polymorphism (**Table 6**). Potential sources of heterogeneity include ethnicity, publication year, sample size, and so on. When stratified analyses were conducted according to these potential biasing factors, heterogeneity was reduced or removed in some subgroups, confirming the effects of ethnicity, publication year, and sample size, even for the same polymorphism (**Table 6**). Begg's funnel plots and Egger's tests were used to detect publication bias, and indicated that there was no significant publication bias in the meta-analysis, suggesting that our results are robust. One-way sensitivity analyses indicated that our findings are stable (**Figure 4**).

The specific merits of the present study should be noted. First, the sample size in this case-control study is the largest used to investigate the association between the *CCND1* A870G polymorphism and ESCC risk, and the meta-analysis is the most comprehensive exploration of the association between *CCND1* A870G polymorphism and ESCC risk to date. Second, although our case-control study was hospital-based, the control genotype distributions were consistent with HWE.

Some limitations in interpreting the results should be acknowledged. In this study, the control subjects were recruited from two local hospitals, which might have led to an unavoidable selection bias. Moreover, all case-control studies included in the meta-analysis were of Asian

or Caucasian subjects; no data for other ethnicities (e.g., African) were available. Only published studies and our investigation were included, which might have increased the chance of publication bias. Furthermore, because individual data were limited in this review, we did not analyze other factors (e.g., age, sex, smoking, alcohol consumption, other lifestyle factors, or environmental factors). Finally, significant heterogeneity was observed among the publications.

In conclusion, this meta-analysis of seven case-control studies suggests that the *CCND1* A870G polymorphism is not involved in the pathogenesis of ESCC. Further investigations with larger samples and detailed individual information should be undertaken in future to validate or refute our results.

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Disclosure of conflict of interest

None.

Address correspondence to: Jun Yin and Haiyong Gu, Department of Cardiothoracic Surgery, Affiliated People's Hospital of Jiangsu University, Zhenjiang 212000, China. E-mail: yin912@126.com (JY); haiyong_gu@hotmail.com (HYG)

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