Quantitative assessment of aberrant *P16^{INK4a}* methylation in ovarian cancer: a meta-analysis

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ABSTRACT

Epigenetic alteration of *P16^{INK4a}* is conventionally thought to induce the initiation of carcinoma. However, the role of *P16^{INK4a}* methylation in ovarian cancer still remains controversial. We therefore performed a meta-analysis to further elucidate the relationship between *P16^{INK4a}* methylation and ovarian cancer. A total of 24 studies, including 20 on risk, 10 on clinicopathological features, and 3 on prognosis, were included in our meta-analysis. Our results indicated that the frequency of *P16^{INK4a}* methylation in cancer tissues was significantly higher than normal tissues and low malignant potential tumor tissues (OR = 5.01, 95% CI = 1.55-16.14; OR = 1.88, 95% CI = 1.10-3.19, respectively), but similar to benign tissues (OR = 1.18, 95% CI = 0.52-2.65). Furthermore, *P16^{INK4a}* methylation was not strongly correlated with age, clinical stage, tumor differentiation or histological subtype in patients with ovarian cancer. Additionally, survival analysis showed that patients with *P16^{INK4a}* methylation had a shorter progression-free survival in univariate and multivariate Cox regression models (HR = 1.68, 95% CI = 1.26-2.24; HR = 1.55, 95% CI = 1.15-2.08; respectively). *P16^{INK4a}* methylation was also linked to an unfavorable overall survival, although the correlation was not statistically significant. In conclusion, the present meta-analysis suggests that *P16^{INK4a}* methylation may be useful in distinguishing malignant cancer from healthy ovarian tissues, and it may be a potential predictive marker for prognosis in patients with ovarian cancer.

INTRODUCTION

Ovarian cancer is the fifth-leading cause of cancer related deaths in women, with an estimated 22,280 new cases and 14,240 deaths in the United States in 2016 [1]. Thereinto, approximately 70% is high-grade serous carcinomas [2]. Up to now, despite the effective treatments including radical resection, systemic chemotherapy, and targeted drugs for patients, the average 5-year survival is still only at 46% [1]. Ovarian cancer is a multifactorial disease caused by the interaction of genetic and epigenetic factors. DNA methylation, as the most prominent epigenetic alteration, could occur at CpG island in the promoter region, 5' or 3' untranslated regions, and even in gene body of tumor suppressor genes (TSGs). Hypermethylation in the proximal promoter region often contributes to the transcriptional down-regulation but methylation in exons is associated with active transcription [3, 4]. Recently, mounting evidences demonstrated that DNA methylation was involved in ovarian cancer [5–7]. Therefore, identifying the role of TSG methylation in patients with ovarian cancer is of value.

P16^{INK4a} (also known as *CDKN2A*), a classical TSG, is located on chromosome 9p21 and plays an important role in cell cycle regulation by decelerating cells progression from G1 to S phase [8, 9]. It has

become clear that the expression of *P16* is reduced by DNA methylation [10–12]. And $P16^{INK4a}$ inactivation upregulates retinoblastoma (RB) protein by stimulating the cyclin dependent kinases (CDKs) and RB pathway, which leads to dysfunction of cell proliferation and apoptosis, thereby further facilitating carcinogenesis [13]. Indeed, several types of cancer, including ovarian cancer, exhibit a methylation phenotype of $P16^{INK4a}$[14–16].

To date, even though abundant studies have been conducted to explore the role of $P16^{INK4a}$ methylation in ovarian cancer, the results are still inconclusive. Several studies reported that $P16^{INK4a}$ methylation was associated with an increasing trend in ovarian cancer [17–20]. While, other studies suggested that $P16^{INK4a}$ methylation was not related to the occurring of ovarian cancer [21–27]. Interestingly, even the conclusions in two published meta-analyses were inconsistent. Xiao *et al.* reported that aberrant methylation of $P16^{INK4a}$ promoter was significantly associated with ovarian carcinogenesis [28], while Jiang *et al.* suggested no association between $P16^{INK4a}$ promoter methylation and epithelial ovarian cancer [29].

Considering these conflicting conclusions on the role of methylated $P16^{INK4a}$ in ovarian cancer, we performed an adaptive synthesized analysis to quantitatively evaluate the occurrence frequency, clinicopathological features and potential prognostic significance of $P16^{INK4a}$ methylation in ovarian cancer. Moreover, we searched The Cancer Genome Atlas (TCGA) database, collecting hundreds of ovarian cancer samples with whole genome DNA methylation datasets to validate our meta-analysis.

RESULTS

Identification of relevant studies

The procedure of study selection is outlined in Figure 1. We identified 233 articles in the initial literature search. A total of 153 references remained after removing duplicates. After reading titles and abstracts, 84 records were identified for further full-text assessment, which further excluded 60 more articles. Finally, 24 studies from 1997 to 2015 were included in this meta-analysis [14, 17, 19-27, 30-42].

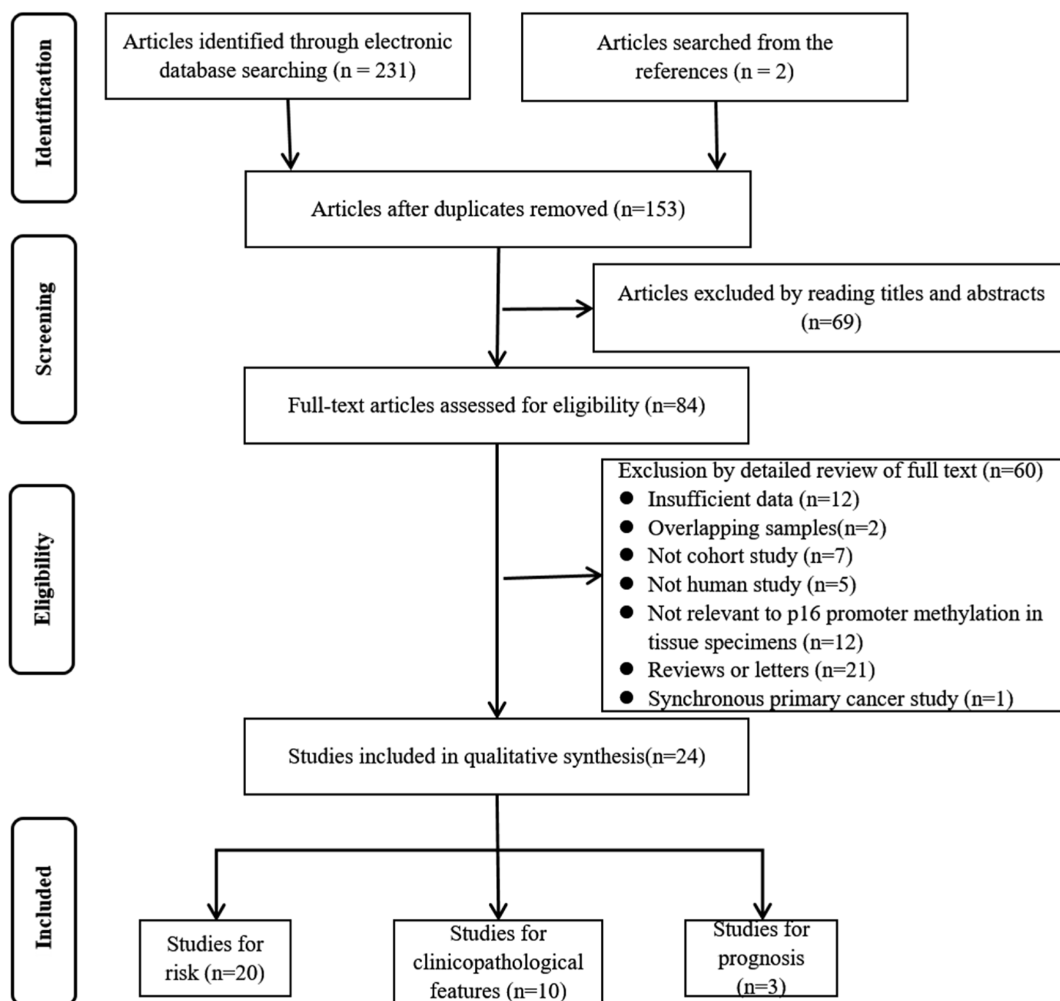

Figure 1: Flow diagram of study selection.

Table 1: Characteristics of studies included for the association between $P16^{INK4a}$ methylation and ovarian cancer risk

First author	Year	Country	Geographical location	Sample size*	Case number				Age(year)	Sample type	Method	Methylation site	NOS score
					C(M/n)	LMP(M/n)	B(M/n)	N(M/n)					
Moselhy	2015	Saudi Arabia	Asia	Small	14/18	-	12/32	-	52.3±12.1	FFPET	MSP	Promoter	7
Bhagat	2014	India	Asia	Large	58/134	5/23	11/26	0/15	49.55±9.72	FFT	qMSP	Promoter	7
Ozdemir	2012	Turkey#	Asia	Large	1/75	-	-	0/75	-	Tissue	MS-MLPA	Promoter	7
Ho	2012	Taiwan	Asia	Small	1/47	-	6/29	-	50(32-66)	FFT	MS-MLPA	Promoter	7
Culľbová M	2011	Slovakia	Europe	Small	5/13	0/2	5/19	-	54.8(34-74)	FFT	MSP	Promoter	6
Abou-zeid	2011	Egypt$	Africa	Large	21/52	-	9/43	4/40	60(49-74)	FFT	qMSP	Promoter	7
Gu	2009	China	Asia	Large	8/87	-	13/42	-	51(21-69)	Tissue	MethyLight	Promoter	7
Shen	2008	China	Asia	Large	13/63	-	-	0/30	52.8(33-76)	FFT	MSP	Promoter	6
Wu	2007	Norway	Europe	Large	0/52	0/2	0/2	-	-	FFT	MSP	Promoter	6
Tam	2007	Hong Kong	Asia	Large	17/89	1/16	1/19	4/16	53.1±1.4	FFT	MSP	Promoter	7
Wiley	2006	Italy	Europe	Large	89/215	4/19	-	-	57.7±11.4	FFT	MSP	Promoter	7
Li	2006	China	Asia	Small	6/18	-	-	0/10	-	Tissue	MSP	Promoter	5
Makarla	2005	USA	America	Small	7/23	5/23	3/23	0/16	51.5(20-86)	FFT	MSP	Promoter	7
Liu	2005	USA	America	Large	13/52	-	-	15/40	61.5±9.4	FFT	MSP	Promoter	5
Dhillon	2004	India	Asia	Small	10/25	-	-	1/75	-	Tissue	MSP	Promoter	7
Rathi	2002	USA	America	Small	5/49	-	-	0/16	56(40-79)	FFT	MSP	Promoter	7
Strathdee	2001	UK	Europe	Large	0/93	-	-	0/18	-	FFT	MSP	Promoter	6
Brown	2001	UK	Europe	Small	0/30	0/13	0/14	-	-	FFT	MSP	Promoter	5
McCluskey	1999	USA	America	Small	21/37	11/15	14/20	-	-	FFT	MSP	Promoter	6
Shih	1997	Australia	Oceania	Small	0/45	0/3	0/2	-	-	Tissue	Southern analysis	Promoter	5

Abbreviations: C: cancer tissues; LMP: low malignant potential or borderline tumor tissues; B: benign tissues; N: normal tissues; M: methylated; n: number of patients; FFPET: formalin fixed and paraffin embedded tissues; FFT: fast frozen tissues; MSP: methylation-specific PCR; qMSP: real-time quantitative MSP; MS-MLPA: methylation-specific multiplex ligation-dependent probe amplification.

*We defined n<50 as small size and ≥50 as large size. n, the number of patients in case group.

Turkey is a transcontinental Eurasian country and is usually assigned to Asia internationally.

$ Egypt is a transcontinental country spanning the northeast corner of Africa and southwest corner of Asia, usually assigned to Africa internationally.

Baseline characteristics of included studies

Out of the 24 studies, 11 studies were conducted in Asia, 7 in Europe, 4 in America, 1 in Africa and 1 in Oceania. The detection methods of methylation in 20 studies were methylation-specific PCR (MSP) and real-time quantitative MSP (qMSP), while methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) was used in 2 studies, MethyLight was used in 1 study, and Southern analysis was used in 1 study. Among the 24 articles, 20 studies [14, 17, 19-27, 30-33, 35, 38-40, 42] addressed the risk of $P16^{INK4a}$ methylation in ovarian cancer, 10 studies [17, 22, 25, 26, 31, 34, 36, 37, 40, 41] covered clinicopathological features, and 3 studies [17, 35, 36] discussed prognosis. To explore the relationship between $P16^{INK4a}$ methylation and ovarian cancer risk, three groups, i.e., normal tissues, benign tissues and low malignant potential or borderline tumor (LMP) tissues, were compared. The Newcastle Ottawa Scale (NOS) scores of all case-control studies were greater than or equal to 5. The basic characteristics of all included studies are summarized in Tables 1 and 2.

Table 2: Characteristics of studies included for the association between *P16^{INK4a}* methylation and clinicopathological features of ovarian cancer

First author	Year	Country	Geographical location	No. of patients	Age (year)	Stage		Grade		Histological subtype	
						I~II (M/n)	III~IV (M/n)	1~2 (M/n)	3 (M/n)	Serous (M/n)	Non-serous (M/n)
Bhagat	2014	India	Asia	134	49.55±9.72	19/41	39/93	18/45	40/89	32/76	26/58
Cuľbová M	2011	Slovakia	Europe	13	54.8(34-74)	-	-	-	-	2/6	3/7
Shen	2008	China	Asia	63	52.8(33-76)	4/22	9/41	6/36	7/27	7/34	6/29
Yang	2006	Hong Kong	Asia	49	48.8(26-79)	4/24	5/25	6/22	3/25	3/17	6/32
Makarla	2005	USA	America	23	51.5(20-86)	-	-	-	-	2/9	5/14
Liu	2005	USA	America	52	61.5±9.4	-	-	10/41	3/11	-	-
Katsaros	2004	Italy	Europe	249	57(19-82)	22/68	68/152	26/75	64/141	40/86	50/141
Hashiguchi	2001	Japan	Asia	46	-	4/21	2/20	7/33	0/13	2/14	5/32
McCluskey	1999	USA	America	29	-	-	-	-	-	1/14	1/15
Milde-langosch	1998	Germany	Europe	44	-	-	-	-	-	3/11	13/33

Abbreviations: M: methylated; n: number of patients.

Quantitative data synthesis

Association between *P16^{INK4a}* methylation and ovarian cancer risk

A total of 1,217 ovarian cancers, 116 LMP cancers, 271 benign patients and 351 normal controls were quantitatively synthesized in this analysis. Results indicated that the frequency of *P16^{INK4a}* methylation in cancer tissues was significantly elevated than that in normal tissues (odds ratio (OR) = 5.01, 95% confidence interval (CI) = 1.55-16.14) and LMP tissues (OR = 1.88, 95% CI = 1.10-3.19), but similar to benign tissues (OR = 1.18, 95% CI = 0.52-2.65) (Figure 2). Further analyses showed that the frequencies of *P16^{INK4a}* methylation in benign tissues and LMP tissues were not higher than that in normal tissues (OR = 2.28, 95% CI = 0.37-14.09; OR = 2.28, 95% CI = 0.15-34.73, respectively) (Figure 3).

With large heterogeneity, meta-regression and subgroup analyses were conducted by the publication year, geographical location, method and case sample size in the comparison of cancer tissues *vs.* normal tissues. Meta-regression found that case sample size was significantly correlated with the inter-study heterogeneity ($P = 0.041$) while other covariates was not (Table 3). Furthermore, as shown in Table 3, subgroup analyses revealed that the OR was 5.69 (95% CI = 0.42-76.14) for the publication year ≤2005 and 4.71 (95% CI = 1.30-17.07) for >2005 under the random effects model. For geographical location, the OR was 7.85 (95% CI = 1.33-46.32) in Asia, 2.31 (95% CI = 0.24-22.01) in America and 6.10 (95% CI = 1.89-19.69) in Africa under random effects model. For test method, the OR for MSP was 4.49 (95% CI = 0.97-20.64) under random effects model and 8.11 (95% CI = 2.93-22.40) for other methods under fixed effects model. In addition, the OR was 15.75 (95% CI = 4.05-61.34) for sample size <50 in fixed effects model and 2.21 (95% CI = 1.33, 3.67) for that ≥ 50 in random effects model.

Association between *P16^{INK4a}* methylation and clinicopathological features in patients with ovarian cancer

10 studies comprising 680 samples were enrolled to assess whether or not the abnormal *P16^{INK4a}* methylation was associated with ovarian cancer clinicopathological characteristics. As displayed in Figure 4, no statistically significant correlation was found between *P16^{INK4a}* methylation and age of patients (≥60 *vs.* <60: OR = 1.39, 95% CI = 0.66-2.92), clinical stage (III~IV *vs.* I~II: OR = 1.21, 95% CI = 0.81-1.82), grade (3 *vs.* 1~2: OR = 1.20, 95% CI = 0.82-1.1.75) as well as histological subtype (serous *vs.* non-serous: OR = 1.09, 95% CI = 0.76-1.55).

Prognostic value of *P16^{INK4a}* methylation in patients with ovarian cancer

Only two studies [35, 36] containing 464 patients evaluated the *P16^{INK4a}* methylation on progression-free survival (PFS), and three studies [17, 35, 36] containing 600 patients on overall survival (OS). The combined results revealed *P16^{INK4a}* methylation was significantly

associated with a poor PFS by univariate Cox proportional hazards regression model (hazard ratio (HR) = 1.68, 95% CI = 1.26-2.24) (Figure 5A). After considering potential confounders by adjusting for age at diagnosis or surgery, disease stage, histological grade and residual tumor size, the pooled HR was 1.55 (1.15-2.08) (Figure 5B). Survival analysis also showed that *P16*^{INK4a} methylation reduced OS in univariate and multivariate Cox regression models (HR = 1.28, 95% CI = 0.97-1.68; HR = 1.16, 95% CI = 0.87-1.55, respectively) (Figure 5C and 5D), but the differences were not statistically significant.

Sensitivity analysis and publication bias

As presented in Figure 6 (6A, 6B, and 6C), no single study significantly affected the pooled ORs in the sensitivity analysis, indicating our analysis was relatively stable and credible. Funnel plots and Begg's test were used to evaluate the publication bias. The funnel plots were largely symmetric suggesting there were no publication biases in the meta-analysis of *P16*^{INK4a} methylation and ovarian cancer risk, which was confirmed by the Begg's test (Figure 6D, 6E, and 6F).

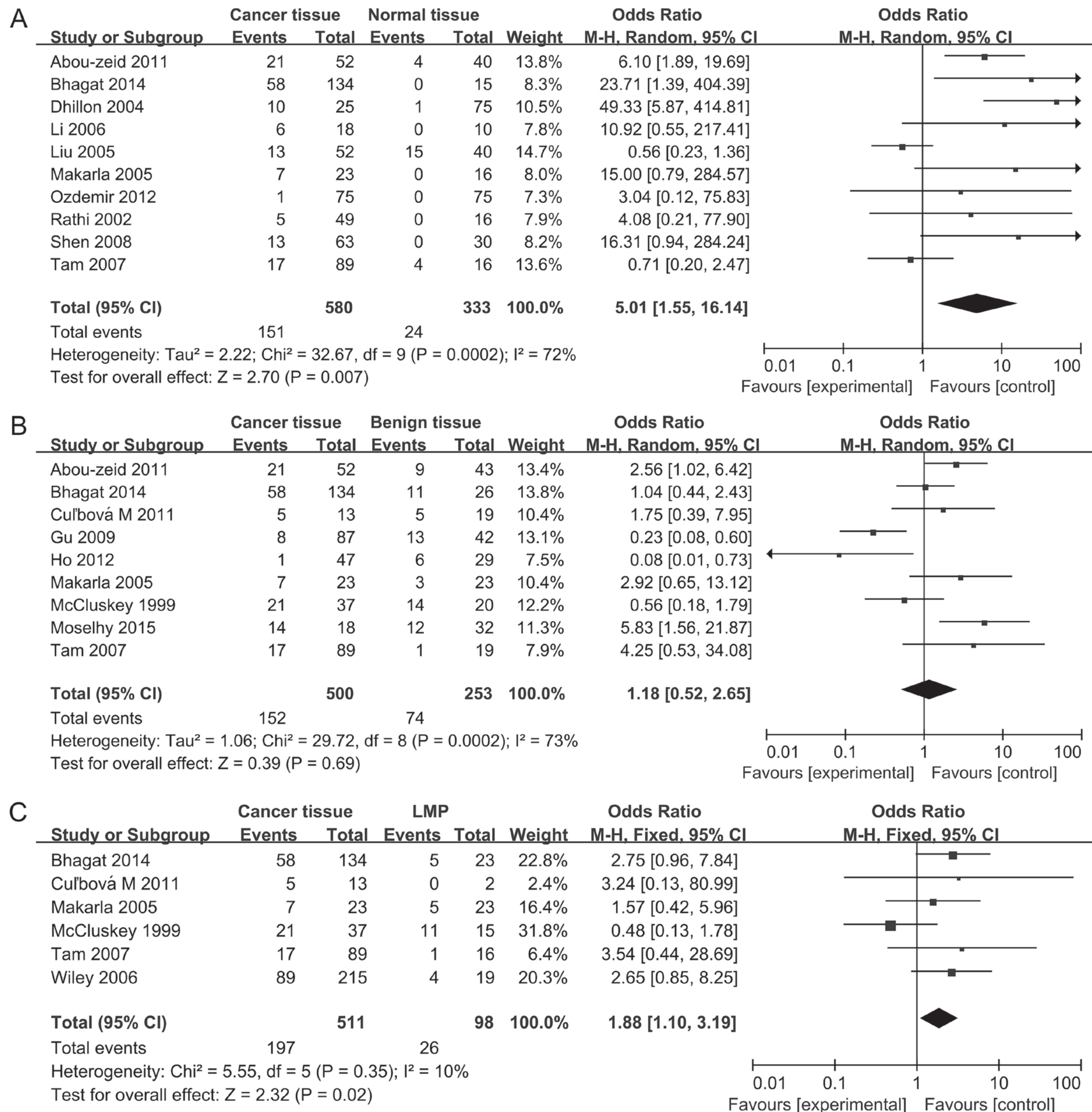

Figure 2: Forest plots for the association between *P16*^{INK4a} methylation and ovarian cancer risk. **(A)** Cancer tissues *vs*. normal tissues; **(B)** Cancer tissues *vs*. benign tissues; **(C)** Cancer tissues *vs*. LMP tissues.

Table 3: Meta-regression and subgroup analyses of *P16^{INK4a}* methylation in comparison of cancer tissues *vs*. normal tissues

Stratified analysis	No. of studies	Pooled OR (95% CI)		Meta-regression	Heterogeneity	
		Random	Fixed	*P*-value	I^2 (%)	*P*-value
Publication year				0.376		
≤2005	4	5.69 (0.42, 76.14)	2.17 (1.17, 4.05)		84%	0.0003
>2005	6	4.71 (1.30, 17.07)	4.65 (2.32, 9.30)		56%	0.05
Geographical location				0.161		
Asia	6	7.85 (1.33, 46.32)	5.87 (2.70, 12.78)		70%	0.005
America	3	2.31 (0.24, 22.01)	1.15 (0.56, 2.37)		68%	0.05
Africa	1	6.10 (1.89, 19.69)	6.10 (1.89, 19.69)		-	-
Method				0.651		
MSP	7	4.49 (0.97, 20.64)	2.33 (1.38, 3.92)		77%	0.0003
others	3	6.79 (2.43, 18.94)	8.11 (2.93, 22.40)		0%	0.57
Case sample size				0.041		
<50	4	17.21 (4.54, 65.28)	15.75 (4.05, 61.34)		0%	0.58
≥50	6	2.21 (1.33, 3.67)	2.74 (0.71, 10.53)		75%	0.001

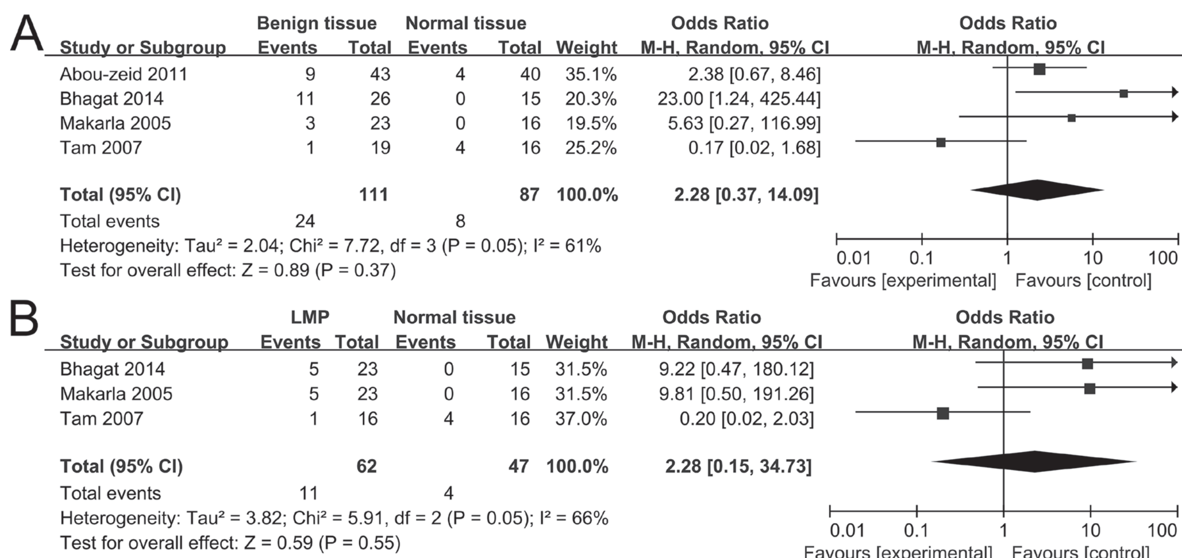

Figure 3: Forest plots for the association between *P16^{INK4a}* methylation and ovarian diseases. **(A)** Benign tissues *vs*. normal tissues; **(B)** LMP tissues *vs*. normal tissues.

Methylation level of $P16^{INK4a}$ measured by TCGA program

To further explore the methylation level of $P16^{INK4a}$ in ovarian tumor tissues, we extracted DNA methylation data of $P16^{INK4a}$ CpG sites measured with Illumina HumanMethylation27 BeadChip from TCGA program. As shown in Table 4, the beta value of 582 ovarian tumor tissues and 12 normal ovarian tissues were extracted for analysis. Obviously, the methylation levels of 7 out of 9 CpG sites were significantly increased in the ovarian tumor tissues compared with the normal tissues (cg03079681, cg07752420, cg09099744, cg10895543, cg11653709, cg12840719, and cg26673943). Among these regions, methylation level of probe cg26673943 region (located at the promoter region of $P16^{INK4a}$) was negatively associated with $P16^{INK4a}$ expression in ovarian cancer patients (adjusted P value < 0.000001). However, methylation levels of the rest 6 probes, which located at non-promoter

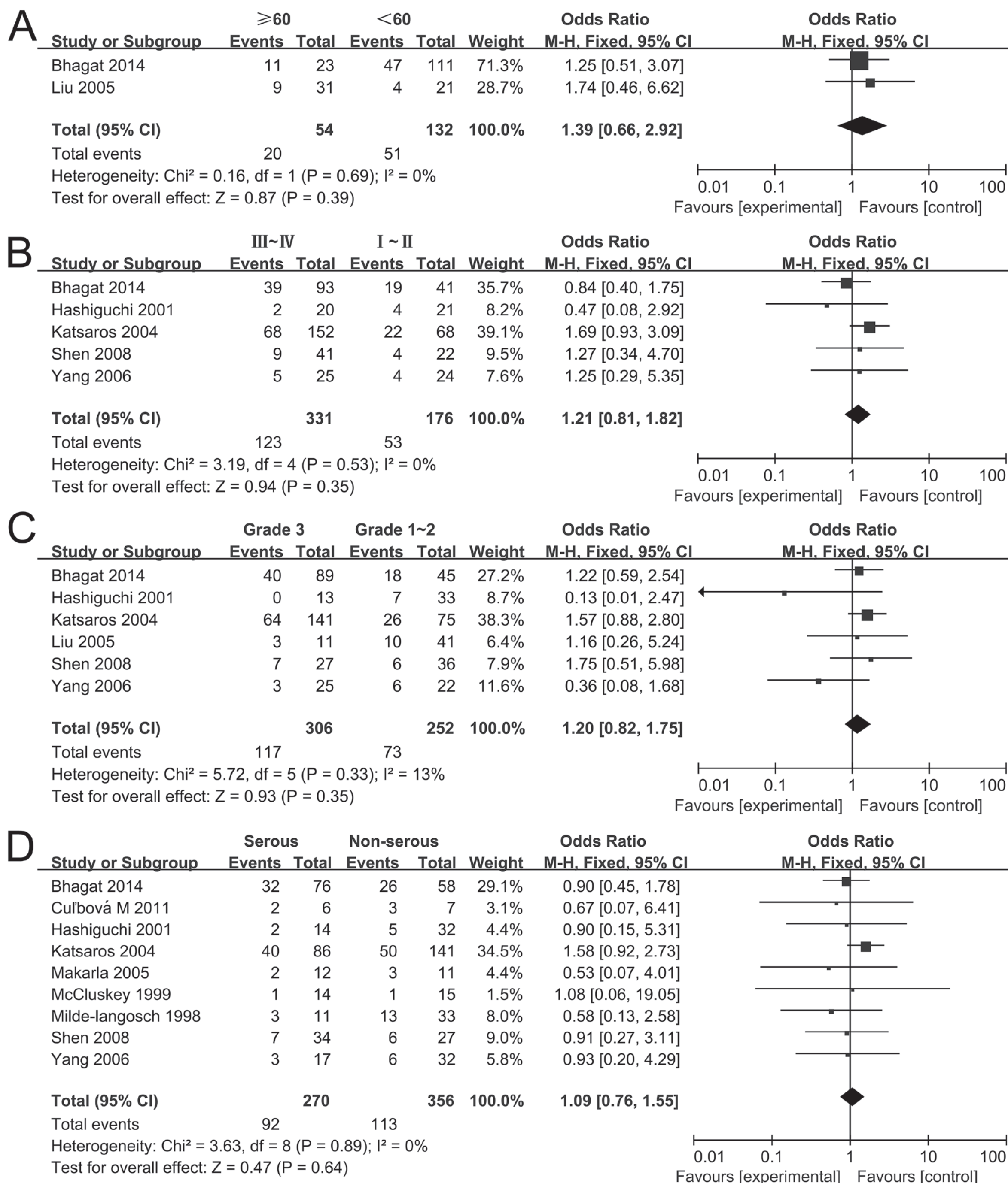

Figure 4: Forest plots for the association between $P16^{INK4a}$ methylation and clinicopathological features in ovarian cancer. (A) Age; **(B)** Clinical stage; **(C)** Tumor grade; **(D)** Histological subtype.

region tended to positively associate with $P16^{INK4a}$ gene expression. Additionally, we found that methylation level of probe cg13479669 region was lower in tumor tissues compared with normal tissues, and negatively associated with $P16^{INK4a}$ gene expression in tumor tissues. These results suggest that hypermethylation of $P16^{INK4a}$ might be correlated with ovarian carcinogenesis and development. Nevertheless, it seems that the methylation at promoter region or non-promoter region has contrary effects on $P16^{INK4a}$ gene expression.

DISCUSSION

Ovarian cancer is one of the leading causes of cancer-related death in women [43]. Identification of early disease indicators for diagnosis and prognosis is of clinical value. $P16^{INK4a}$, which resembles classic TSGs such as *P53*, is an important negative regulator of cell growth and proliferation [13]. It has been synthetically evaluated for aberrant $P16^{INK4a}$ methylation in numerous cancers [44–47], including ovarian cancer [28, 29]. Considering the conflicting conclusions in two meta-analyses, and the lack of comprehensive assessment on the role of methylated $P16^{INK4a}$ in ovarian cancer, we performed an adaptive synthesized analysis to investigate the relationships between $P16^{INK4a}$ methylation and ovarian cancer risk, as well as clinicopathological features and prognostic value in ovarian cancer. Meanwhile, we searched TCGA data to validate our meta-analysis.

Our meta-analysis demonstrated that $P16^{INK4a}$ methylation in cancer tissues was significantly higher than that in normal tissues ($P < 0.05$), but not much increased than that in benign tissues. Compared with normal tissues, the frequency of $P16^{INK4a}$ methylation was 2.28-fold higher in both benign tissues and LMP tissues ($P > 0.05$), but the differences were not statistically significant. Although not establishing a strong correlation between $P16^{INK4a}$ methylation and cancer progression,

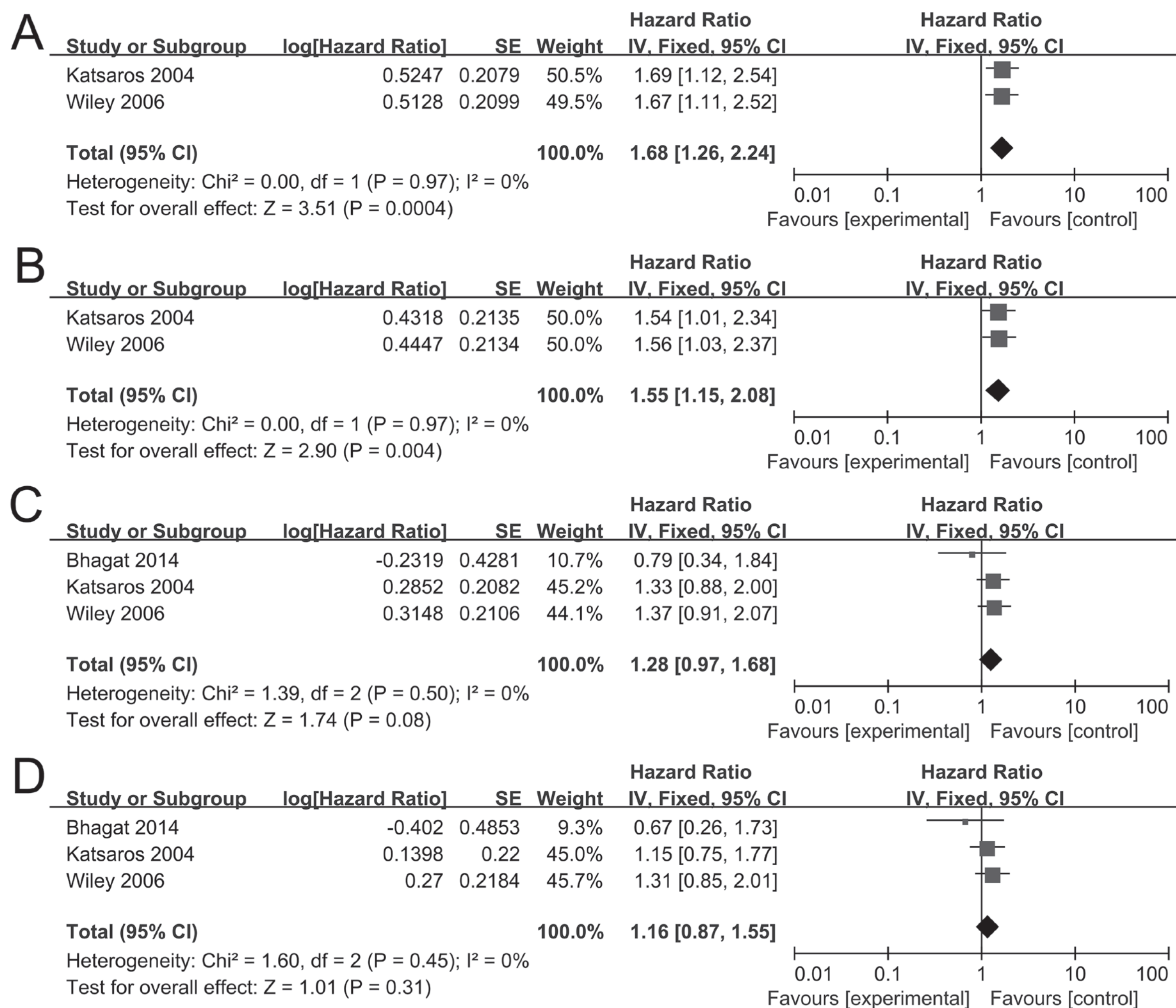

Figure 5: Forest plots for the evaluation of $P16^{INK4a}$ methylation on survival analysis in ovarian cancer. **(A)** PFS in univariate Cox regression model; **(B)** PFS in multivariate Cox regression model; **(C)** OS in univariate Cox regression model; **(D)** OS in multivariate Cox regression model.

the above results do suggest a possibility that epigenetic alteration of *P16^INK4a^* methylation might play a certain role in ovarian carcinogenesis and might be useful in distinguishing malignant tumor from healthy ovarian tissues. Considering the evident heterogeneity, we conducted subgroup analyses based on probable covariates in the comparison of cancer tissues *vs.* normal tissues. For geographical location, *P16^INK4a^* methylation is a risk factor in Asia and Africa, but not in America. The divergence may be underscored in a large part to a combination of differences in allele frequencies and complex epistasis or gene-environment interactions [48]. The similar findings appeared in the subgroup analyses of different methods and publication year. Kurdyukov *et al.* [49] suggested that it was essential to choose an appropriate method in a suitable region to answer a particular biological question in studies of DNA methylation. Additionally, the 95% CI was large in the group of small sample size while relatively small in the group of large sample size, implying the conclusion may not be reliable unless studies should be conducted using a sufficient number of samples. Previous studies also demonstrated that the methylation status in blood samples or fluids might be different from that in tissues [50, 51]. Thus, our results should be interpreted with caution because sample types were limited to tissues in studies included in this meta-analysis.

Previous studies indicated that *P16^INK4a^* methylation was associated with poorly differentiated tumors

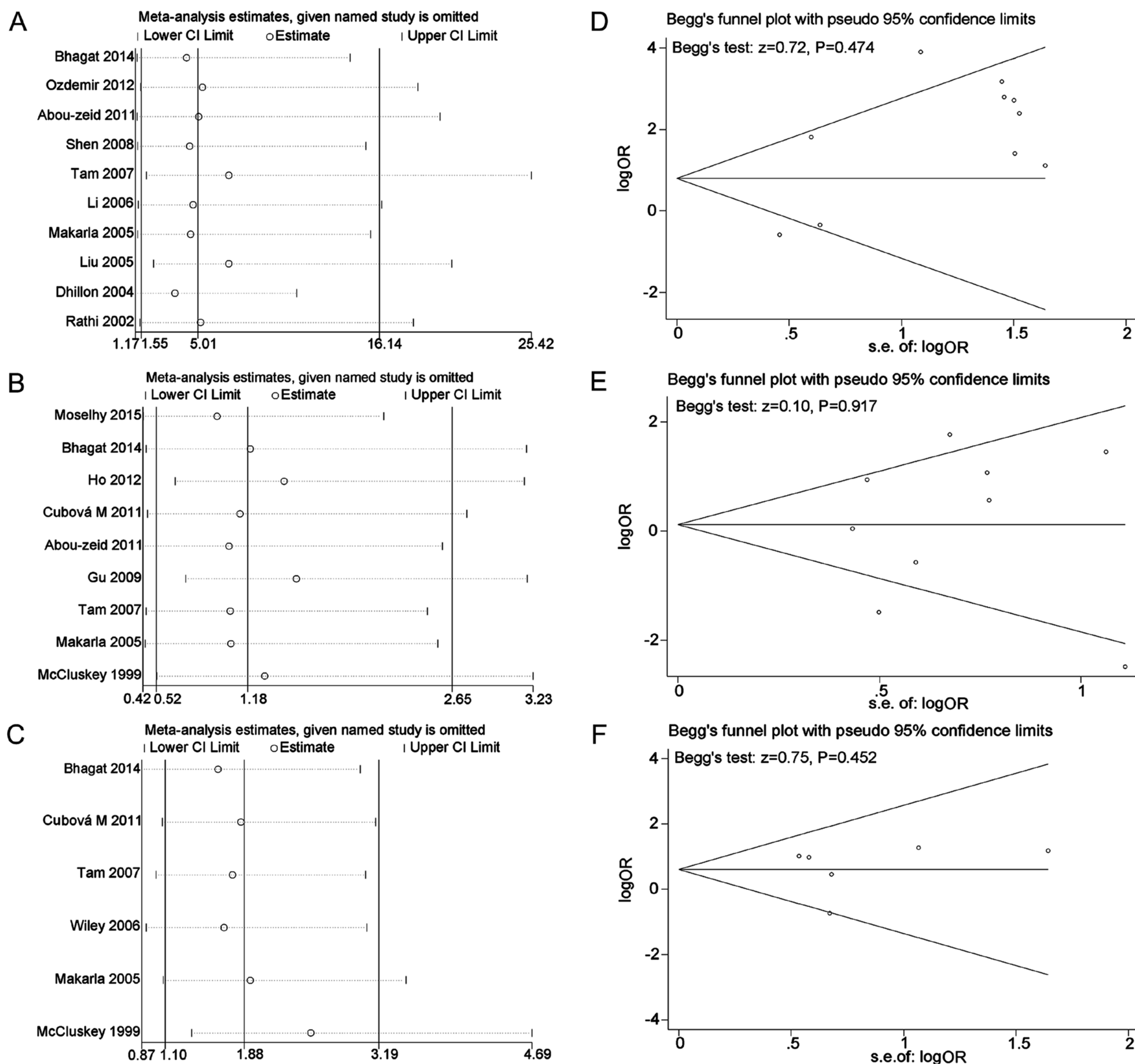

Figure 6: Sensitivity analyses and funnel plots for the publication bias of *P16^INK4a^* methylation during the carcinogenesis of ovarian cancer. **(A)** Sensitivity analysis for the comparison of cancer tissues *vs*. normal tissues; **(B)** Sensitivity analysis for the comparison of cancer tissues *vs*. benign tissues; **(C)** Sensitivity analysis for the comparison of cancer tissues *vs*. LMP tissues. **(D)** Funnel plot for the publication bias of cancer tissues *vs*. normal tissues; **(E)** Funnel plot for the publication bias of cancer tissues *vs*. benign tissues; **(F)** Funnel plot for the publication bias of cancer tissues *vs*. LMP tissues.

Table 4: Methylation of $P16^{INK4a}$ CpG sites on Illumina HumanMethylation 27 BeadChip from TCGA datasets

Probe (Illumina Human Methylation 27)	CpG island location (chromosome: DNA range)	Normal tissue Beta value (mean, n = 12)	Tumor tissue Beta value (mean, n = 582)	Adjusted P value#	Pearson Correlation Coefficient	Adjusted P value*
cg00718440	9: 21983444-21986348	0.016	0.016	0.960249	0.194104	0.001719
cg03079681	9: 21983444-21986348	0.015	0.026	< 0.000001	0.012972	1.0
cg07752420	9: 21958106-21958899	0.149	0.653	< 0.000001	0.569887	< 0.000001
cg09099744	9: 21958106-21958899	0.099	0.642	< 0.000001	0.630768	< 0.000001
cg10895543	9: 21958106-21958899	0.120	0.651	< 0.000001	0.624147	< 0.000001
cg11653709	9: 21958106-21958899	0.144	0.610	< 0.000001	0.555400	< 0.000001
cg12840719	9: 21958106-21958899	0.092	0.594	< 0.000001	0.627484	< 0.000001
cg13479669	9: 21983444-21986348	0.045	0.027	0.004226	-0.150891	0.0333435
cg26673943	9: 21983444-21986348	0.047	0.056	0.042428	-0.269361	< 0.000001

P value of t test of the difference between normal tissue Beta value and tumor tissue Beta value;
* *P* value of Pearson's correlation between the tumor tissues' Beta value and CDKN2A expression (n = 368).

and was different in histological subtype in ovarian cancer [19, 36]. However, we could not establish any significant correlations between $P16^{INK4a}$ methylation and clinicopathological features, including age, clinical stage, tumor differentiation or histological subtype in this study. Therefore, it might not be essential to predict the invasion and metastasis of ovarian cancer.

Katsaros *et al.* [36] and Wiley *et al.* [35] reported association of $P16^{INK4a}$ methylation with PFS and OS in ovarian cancer, while Bhagat *et al.* [17] found no significant value in predicting prognosis. In the present study, we discovered that $P16^{INK4a}$ methylation represented a risk factor for PFS. For OS, patients with $P16^{INK4a}$ methylation also had a slightly elevated risk, though the differences are not statistically significant. This trend was also observed in other types of cancer [44, 47]. However, its statistical confirmation requires large studies. The data from TCGA also indicated that methylation level of probe cg26673943 region (located at the promoter region of $P16^{INK4a}$) in the ovarian tumor tissues was higher than normal ovarian tissues. Increased methylation of CpG island at the promoter region was negatively associated with $P16^{INK4a}$ gene expression, while methylation of CpG islands at non-promoter regions were positively associated with $P16^{INK4a}$ gene expression.

Compared with previous meta-analyses [28, 29], our meta-analysis had several improvements. Firstly, the development of ovarian cancer is a multistep procedure involving normal tissues, benign disease, low malignant potential or borderline tumor and malignant tumor [17]. We compared malignant ovarian cancer with LMP tumors, benign disease, and normal samples to give more rigorously to the analysis. Secondly, with 1,217 malignant ovarian cancer patients, 116 LMP, 271 benign patients and 351 normal samples, the sample size in our study is much larger than that of all previous meta-analyses. Finally, we included the clinicopathological features and prognostic significance of $P16^{INK4a}$ methylation in ovarian cancer for more comprehensive understanding of the underlying pathogenesis of ovarian cancer. These strengths make our study a useful effort in seeking better understanding of the $P16^{INK4a}$ methylation in ovarian cancer.

Several potential limitations in our current study should be also noted. Firstly, the heterogeneity was still large after subgroup analyses in the assessment of the association between $P16^{INK4a}$ methylation and ovarian cancer risk, which may affect the statistical power. Secondly, as a retrospective study, a potential unidentified confounding information and selection bias may exist in our meta-analysis. We could not eliminate the possibility of publication bias, where positive results are likely

published than negative results. Thirdly, the total sample size was still relatively small for reliably assessing the prognostic value of *P16*^{INK4a} methylation in ovarian cancer. Fourthly, none of the studies included in our meta-analysis defined the region considered as promoter or provided specific methylation sites. Therefore, we are unable to establish whether or not they focused on the same sequence of *P16*^{INK4a} gene. However, the impact of methylation on transcriptional potential depends on the density of the methylated CpG islands and their location relative to the transcription start site. This highlights the importance of a uniform and full-scale reporting of study designs and outcomes. Additionally, previous researches showed that the occurrence of *P16*^{INK4a} methylation may depend on the histological subtype [34, 41, 52]. However, we are unable to extract the sufficient data to analyze the association between *P16*^{INK4a} methylation and HGSC because no detailed information of P16^{INK4a} methylation in HGSC was provided in the eligible articles.

Although with certain limitations, our study is a comprehensive meta-analysis focusing on the correlation of aberrant *P16*^{INK4a} methylation with the initiation, development, and prognosis of ovarian cancer to provide new insight into the pathogenesis of ovarian cancer.

In conclusion, our meta-analysis suggests that aberrant methylation of *P16*^{INK4a} may be essential to the initiation of ovarian cancer and in distinguishing malignant from healthy ovarian tissues. Besides, *P16*^{INK4a} methylation is a potential predictive factor for poor prognosis in ovarian cancer. This study indicates the need for multicenter large-scale studies to confirm the role of *P16*^{INK4a} methylation in ovarian cancer.

MATERIALS AND METHODS

Search strategy and selection criteria

PubMed, EMBASE, Web of Science and China National Knowledge Infrastructure (CNKI) were searched up to October 12, 2016 by the following keywords and search items: (*P16* OR *P16*^{INK4a} OR *CDKN2A*) AND (methylation OR hypermethylation OR demethylation) AND (ovarian OR ovary) AND (cancer OR carcinoma OR neoplasm). The search was limited to human studies, without language restriction. Moreover, a manual search of the relevant references was implemented to identify the potentially additional articles.

The following criteria were used for screening eligible studies: (1) case-control studies evaluating the association between *P16*^{INK4a} promoter methylation and ovarian cancer risk, or case only studies evaluating the association of *P16*^{INK4a} promoter methylation with clinicopathological features or prognosis in ovarian cancer; (2) articles providing sufficient information for calculating an odds ratio (OR) and corresponding 95% confidence interval (95% CI), or study offering hazard ratio (HR) and 95% CI directly; (3) sample types limited to tissues; (4) studies with full text articles. It's worth noting that when multiple reports were published from a same study population, only the most recent or complete information was included in this meta-analysis. Meanwhile, studies with NOS scores greater than or equal to 5 were enrolled.

Data extraction and quality assessment

With a preformed unified form, data were extracted independently by two investigators and disagreements were resolved by discussion till consensus were achieved. The following information was extracted from studies: the first author's name, publication year, country, geographical location, sample size, age of patients in the case group, the frequencies of methylation in the case and control groups, methods for detecting methylation, methylation site, disease stage, tumor grade, histological subtype and effects on survival outcomes.

The quality of eligible case-control studies was assessed according to the NOS criteria [53]. The NOS criteria is based on three aspects: (1) subject selection: 0~4; (2) comparability of subject: 0~2; (3) clinical outcome: 0~3.

Statistical analysis

Our principal analysis was conducted using Review Manager 5.2 (Cochrane Collaboration, Oxford, UK). ORs with corresponding 95% CIs were calculated to estimate the association between *P16*^{INK4a} promoter methylation and ovarian cancer risk or clinicopathological features. Meanwhile, HRs and 95% CIs were used to assess the prognosis of *P16*^{INK4a} promoter methylation on ovarian cancer. Inter-study heterogeneity was estimated with the Cochran's Q statistic and I^2 tests. $P<0.05$ or $I^2>50\%$ indicated substantial heterogeneity, then the random effects model was applied. Otherwise, the fixed effects model was selected. We also explored sources of heterogeneity using meta-regression and subgroup analyses by publication year, geographical location, method and case sample size. Additionally, the Stata 12.0 (Stata Corporation, TX, USA) was performed to evaluate the sensitivity analysis and publication bias of our studies. Publication bias was evaluated by funnel plots and Begg's test, $P < 0.05$ was considered statistically significant. It's worth mentioning that, for some trials containing no events in both case and control arm, as no information supplied about likely magnitude of the effect, we excluded such trials when synthesizing data [54].

TCGA datasets extraction and analysis

We collected DNA methylation datasets of 582 ovarian cancer cases and 12 ovarian normal tissues from The Cancer Genome Atlas (TCGA, "TCGA-OV"

project) program [https://cancergenome.nih.gov/]. The methylation measurement was performed using Illumina HumanMethylation27 BeadChip. Beta value of each CpG site was extracted to assess the methylation level of *CDKN2A* gene. Beta value was calculated based on the intensities of the methylated (M) and unmethylated (U) bead types [55]: beta value = M/ (M+U). The difference of DNA methylation level of CpG sites between ovarian tumor tissues and normal ovarian tissues in the TCGA database were analyzed by t-test on the means. Plus, *P16*^{INK4a} gene expression value (fragments per kilobase of transcript per million mapped reads, FPKM) in ovarian tumor tissues (TCGA, "TCGA-OV" project) was also extracted. Pearson's product-moment correlation between *P16*^{INK4a} gene expression levels and methylation of its CpG islands were evaluated. Data analysis were performed using R software (R i386 3.4.0). *P* values were adjusted via Bonferroni correction.

Author contributions

P.X and F.W conceived and designed the study; P.X and X.G identified related studies as well as assessed the quality of included studies; P.X and Z.Z performed the statistical analysis and drafted the manuscript; Q.D, S.T, J.W and P.W revised the manuscript; M.Y revised and finalized the manuscript. All authors reviewed and approved the manuscript prior to submission.

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CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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