REVIEW

Quantitative assessment of aberrant $P16^{INK4a}$ methylation in ovarian cancer: a meta-analysis based on literature and TCGA datasets

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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. Therefore, we performed a meta-analysis to further elucidate the relationship between $P16^{INK4a}$ promoter 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 (odds ratio [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, $P16^{INK4a}$ promoter 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 $P16^{INK4a}$ promoter methylation had a shorter progression-free survival in univariate and multivariate Cox regression models (hazard ratio =1.68, 95% CI=1.26–2.24; hazard ratio =1.55, 95% CI=1.15–2.08; respectively). In The Cancer Genome Atlas datasets, the methylation levels of seven out of nine CpG sites were significantly increased in the ovarian tumor tissues compared with the normal tissues. In conclusion, the present meta-analysis suggests that $P16^{INK4a}$ promoter 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.

Keywords: ovarian cancer, $P16^{INK4a}$ promoter methylation, TCGA datasets, meta-analysis

Introduction

Ovarian cancer is the fifth leading cause of cancer-related deaths in women. According to the GLOBOCAN 2012 database, the incidences of ovarian cancer are 9.1 per 1,00,100 in developed countries and 5.0 per 1,00,000 in developing countries.[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%.[3] Ovarian cancer is a multifactorial disease caused by the interaction of genetic and epigenetic factors.[4,5] DNA methylation, as the most common 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 downregulation but methylation in exons is associated with active transcription.[6,7] Recently, mounting evidences demonstrated that DNA methylation was involved in ovarian cancer.[8–10] Therefore, identifying the role of TSG methylation in patients with ovarian cancer is of value.

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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.[11,12] It has become clear that the expression of *P16* is reduced by DNA methylation.[13–15] Also, *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.[16] Indeed, several types of cancer, including ovarian cancer, exhibit a methylation phenotype of *P16^{INK4a}*.[17–19]

To date, even though abundant studies have been conducted to explore the role of *P16^{INK4a}* promoter methylation in ovarian cancer, the results are still inconclusive. Several studies reported that *P16^{INK4a}* promoter methylation was associated with an increasing trend in ovarian cancer,[20–23] while, other studies suggested that *P16^{INK4a}* promoter methylation was not related to the occurrence of ovarian cancer.[24–30] Interestingly, even the conclusions in two published meta-analyses were inconsistent. Xiao et al reported that aberrant methylation of *P16^{INK4a}* was significantly associated with ovarian carcinogenesis,[31] while Jiang et al suggested no association between *P16^{INK4a}* methylation and epithelial ovarian cancer.[32]

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}* promoter 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.

Materials and methods
Search strategy and selection criteria
PubMed, Embase, Web of Science, and China National Knowledge Infrastructure were searched up to April 12, 2017, 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% CI, or study offering hazard ratio (HR) and 95% CI directly; 3) sample types limited to tissues; and 4) studies with full-text articles. It is 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 Newcastle Ottawa Scale (NOS) scores greater than or equal to five 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 was 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.[33] The NOS criteria are based on three aspects: 1) subject selection: 0–4; 2) comparability of subject: 0–2; 3) clinical outcome: 0–3.

Statistical analysis
Statistical analysis was conducted with Review Manager 5.2 (Cochrane Collaboration, Oxford, UK) and the Stata 12.0 (Stata Corporation, College Station, TX, USA). 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, and 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, sensitivity analysis was performed to investigate the influence of individual study. Publication bias was evaluated by funnel plots and Begg's test, and $P<0.05$ was considered statistically significant. It is

worth mentioning that, for some trials containing no events in both case and control arms, as no information supplied about the likely magnitude of the effect, we excluded such trials when synthesizing data.[34]

TCGA datasets extraction and analysis

We collected DNA methylation datasets of 582 ovarian cancer cases and 12 ovarian normal tissues from TCGA ("TCGA-ovary [OV]" project) program.[35] 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: beta value = M/(M+U).[36] The difference of DNA methylation level of CpG sites between ovarian tumor tissues and normal ovarian tissues in TCGA

database was analyzed by Student's *t*-test on the means. *P16^INK4a* gene expression value (fragments per kilobase of transcript per million mapped reads) 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 was evaluated. Data analysis was performed using R software (R i386 3.4.0). *P*-values were adjusted via Bonferroni correction.

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
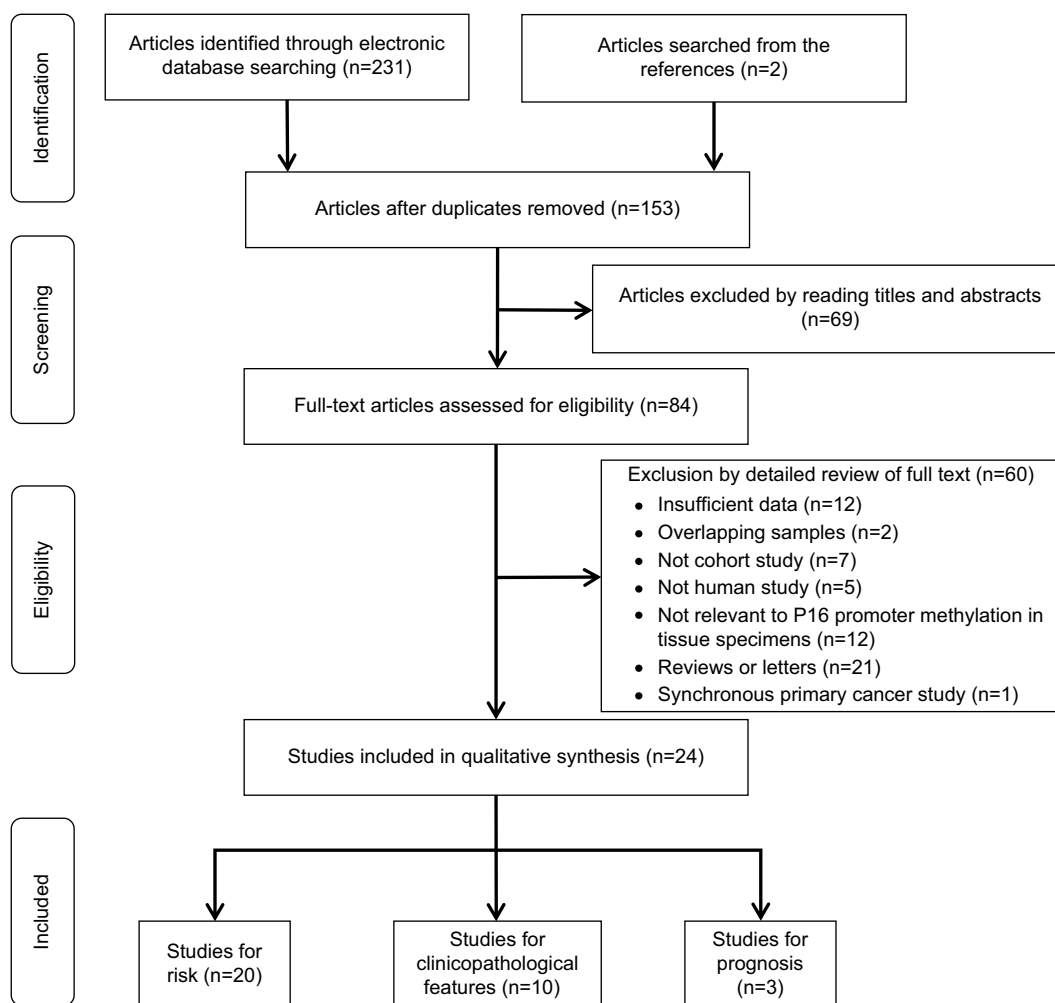

Figure 1 Flow diagram of study selection.

articles. Finally, 24 studies from 1997 to 2015 were included in this meta-analysis.[17,20,22–30,37–49]

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, while methylation-specific multiplex ligation-dependent probe amplification was used in two studies, MethyLight was used in one study, and Southern analysis was used in one study. Among the 24 articles, 20 studies[17,20,22–30,37–40,42,45–47,49] addressed the risk of $P16^{INK4a}$ promoter methylation in ovarian cancer, 10 studies[20,25,28,29,38,41,43,44,47,48] covered clinicopathological features, and 3 studies[20,42,43] discussed prognosis. To explore the relationship between $P16^{INK4a}$ promoter methylation and ovarian cancer risk, three groups, that is, normal tissues, benign tissues, and low malignant potential or borderline tumor tissues (LMP), were compared. The NOS scores

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

Authors	Year	Country	Geographical location	Sample size[a]	Case number				Age (years)[d]	Sample type	Method	Methylation site	NOS score
					C (M/n)	LMP (M/n)	B (M/n)	NT (M/n)					
Moselhy et al[17]	2015	Saudi Arabia	Asia	Small	14/18	NA	12/32	NA	52.3±12.1	FFPET	MSP	Promoter	7
Bhagat et al[20]	2014	India	Asia	Large	58/134	5/23	11/26	0/15	49.55±9.72	FFT	qMSP	Promoter	7
Ozdemir et al[24]	2012	Turkey[b]	Asia	Large	1/75	NA	NA	0/75	NA	Tissue	MS-MLPA	Promoter	7
Ho et al[37]	2012	Taiwan	Asia	Small	1/47	NA	6/29	NA	50(32–66)	FFT	MS-MLPA	Promoter	7
Cul'bová et al[38]	2011	Slovakia	Europe	Small	5/13	0/2	5/19	NA	54.8 (34–74)	FFT	MSP	Promoter	6
Abou-Zeid et al[22]	2011	Egypt[c]	Africa	Large	21/52	NA	9/43	4/40	60(49–74)	FFT	qMSP	Promoter	7
Gu et al[39]	2009	China	Asia	Large	8/87	NA	13/42	NA	51(21–69)	Tissue	MethyLight	Promoter	7
Shen et al[25]	2008	China	Asia	Large	13/63	NA	NA	0/30	52.8 (33–76)	FFT	MSP	Promoter	6
Wu et al[40]	2007	Norway	Europe	Large	0/52	0/2	0/2	NA	NA	FFT	MSP	Promoter	6
Tam et al[26]	2007	Hong Kong	Asia	Large	17/89	1/16	1/19	4/16	53.1±1.4	FFT	MSP	Promoter	7
Wiley et al[42]	2006	Italy	Europe	Large	89/215	4/19	NA	NA	57.7±11.4	FFT	MSP	Promoter	7
Li et al[27]	2006	China	Asia	Small	6/18	NA	NA	0/10	NA	Tissue	MSP	Promoter	5
Makarla et al[29]	2005	USA	America	Small	7/23	5/23	3/23	0/16	51.5 (20–86)	FFT	MSP	Promoter	7
Liu et al[28]	2005	USA	America	Large	13/52	NA	NA	15/40	61.5±9.4	FFT	MSP	Promoter	5
Dhillon et al[23]	2004	India	Asia	Small	10/25	NA	NA	1/75	NA	Tissue	MSP	Promoter	7
Rathi et al[30]	2002	USA	America	Small	5/49	NA	NA	0/16	56(40–79)	FFT	MSP	Promoter	7
Strathdee et al[46]	2001	UK	Europe	Large	0/93	NA	NA	0/18	NA	FFT	MSP	Promoter	6
Brown et al[45]	2001	UK	Europe	Small	0/30	0/13	0/14	NA	NA	FFT	MSP	Promoter	5
McCluskey et al[47]	1999	USA	America	Small	21/37	11/15	14/20	NA	NA	FFT	MSP	Promoter	6
Shih et al[49]	1997	Australia	Oceania	Small	0/45	0/3	0/2	NA	NA	Tissue	Southern analysis	Promoter	5

Notes: [a]We defined n<50 as small size and ≥50 as large size. [b]Turkey is a transcontinental Eurasian country and is usually assigned to Asia internationally. [c]Egypt is a transcontinental country spanning the northeast corner of Africa and southwest corner of Asia, usually assigned to Africa internationally. [d]Age data are presented as mean ± SD or median (IQR).

Abbreviations: B, benign tissues; BL, borderline; C, cancer tissues; FFPET, formalin fixed and paraffin embedded tissues; FFT, fast frozen tissues; LMP, low malignant potential or borderline tumor tissues; M, methylated; MS-MLPA, methylation-specific multiplex ligation-dependent probe amplification; MSP, methylation-specific PCR; n, number of patients in the group; NA, not available; NT, normal tissues; NOS, Newcastle Ottawa Scale; qMSP, real-time quantitative MSP.

of all case–control studies were ≥5. The basic characteristics of all included studies are summarized in Tables 1 and 2.

Quantitative data synthesis

Association between *P16^INK4a* promoter 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* promoter methylation in cancer tissues was significantly elevated than that in normal tissues (OR=5.01, 95% 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* promoter methylation in benign tissues and LMP tissues were not higher than those 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 were 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* promoter methylation and clinicopathological features in patients with ovarian cancer

Ten studies comprising 680 samples were enrolled to assess whether or not the abnormal *P16^INK4a* promoter methylation was associated with ovarian cancer clinicopathological characteristics. As displayed in Figure 4, no statistically significant correlation was found between *P16^INK4a* promoter 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* promoter methylation in patients with ovarian cancer

Only two studies[42,43] containing 464 patients evaluated the *P16^INK4a* promoter methylation on progression-free survival (PFS) and three studies[20,42,43] containing 600 patients on overall survival (OS). The combined results revealed *P16^INK4a* promoter methylation was significantly associated with a poor PFS by univariate Cox proportional hazards regression model (HR=1.68, 95% CI=1.26–2.24; Figure 5A). After considering potential confounders by adjusting for age at diagnosis

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

Authors	Year	Country	Geographical location	Number of patients	Age (years)[a]	Tumor stage		Tumor 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 et al[20]	2014	India	Asia	134	49.55±9.72	19/41	39/93	18/45	40/89	32/76	26/58
Cuľbová et al[38]	2011	Slovakia	Europe	13	54.8 (34–74)	NA	NA	NA	NA	2/6	3/7
Shen et al[25]	2008	China	Asia	63	52.8 (33–76)	4/22	9/41	6/36	7/27	7/34	6/29
Yang et al[41]	2006	Hong Kong	Asia	49	48.8 (26–79)	4/24	5/25	6/22	3/25	3/17	6/32
Makarla et al[29]	2005	USA	America	23	51.5 (20–86)	NA	NA	NA	NA	2/9	5/14
Liu et al[28]	2005	USA	America	52	61.5±9.4	NA	NA	10/41	3/11	NA	NA
Katsaros et al[43]	2004	Italy	Europe	249	57(19–82)	22/68	68/152	26/75	64/141	40/86	50/141
Hashiguchi et al[44]	2001	Japan	Asia	46	NA	4/21	2/20	7/33	0/13	2/14	5/32
McCluskey et al[47]	1999	USA	America	29	NA	NA	NA	NA	NA	1/14	1/15
Milde-Langosch et al[48]	1998	Germany	Europe	44	NA	NA	NA	NA	NA	3/11	13/33

Note: [a]Age data are presented as mean ± SD or median (IQR).
Abbreviations: M, methylated; n, number of patients in the group; NA, not available.
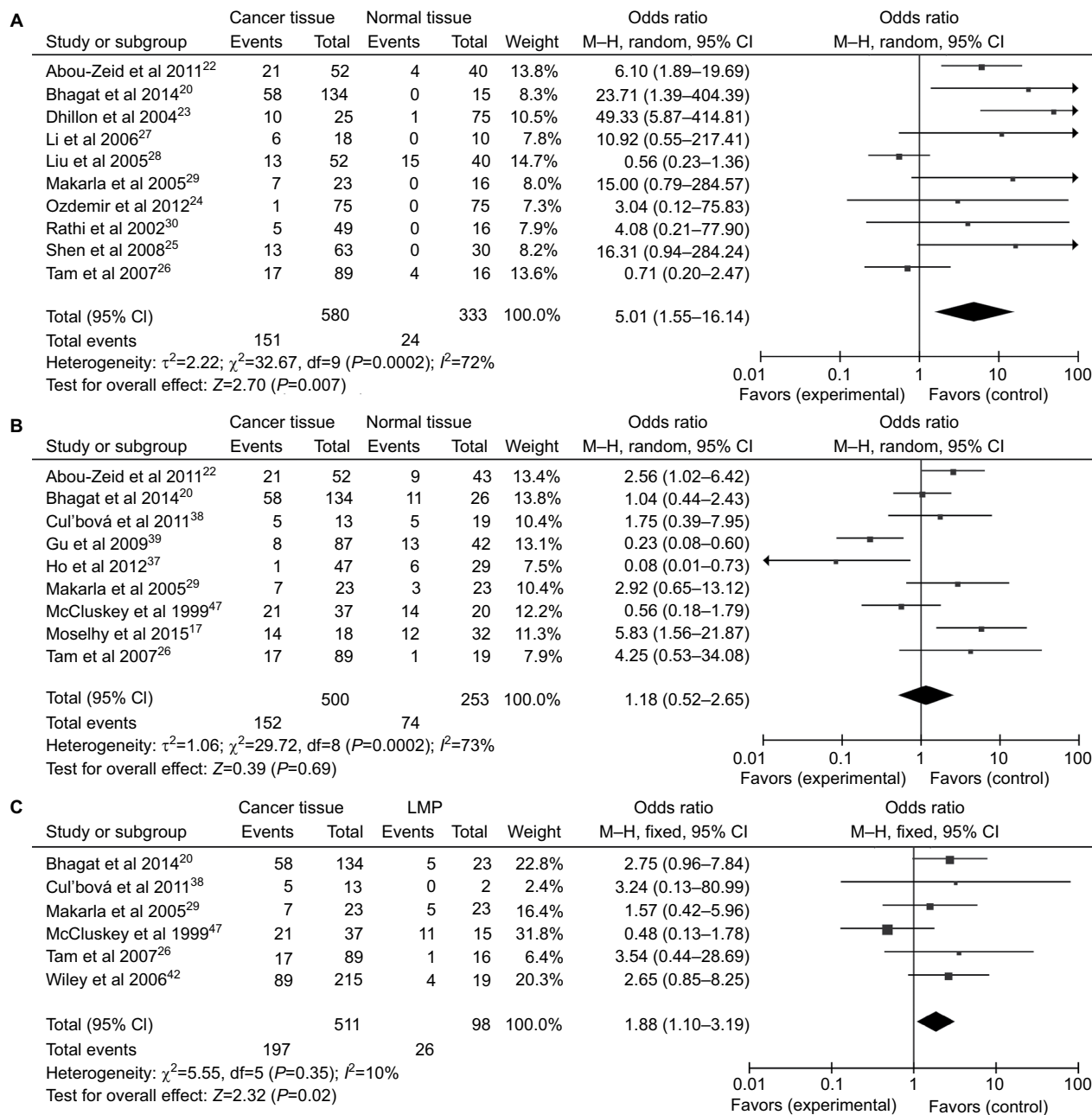
A

Study or subgroup	Cancer tissue Events	Total	Normal tissue Events	Total	Weight	Odds ratio M–H, random, 95% CI
Abou-Zeid et al 2011[22]	21	52	4	40	13.8%	6.10 (1.89–19.69)
Bhagat et al 2014[20]	58	134	0	15	8.3%	23.71 (1.39–404.39)
Dhillon et al 2004[23]	10	25	1	75	10.5%	49.33 (5.87–414.81)
Li et al 2006[27]	6	18	0	10	7.8%	10.92 (0.55–217.41)
Liu et al 2005[28]	13	52	15	40	14.7%	0.56 (0.23–1.36)
Makarla et al 2005[29]	7	23	0	16	8.0%	15.00 (0.79–284.57)
Ozdemir et al 2012[24]	1	75	0	75	7.3%	3.04 (0.12–75.83)
Rathi et al 2002[30]	5	49	0	16	7.9%	4.08 (0.21–77.90)
Shen et al 2008[25]	13	63	0	30	8.2%	16.31 (0.94–284.24)
Tam et al 2007[26]	17	89	4	16	13.6%	0.71 (0.20–2.47)
Total (95% CI)		580		333	100.0%	5.01 (1.55–16.14)
Total events	151		24			

Heterogeneity: $\tau^2=2.22$; $\chi^2=32.67$, df=9 (P=0.0002); $I^2=72\%$
Test for overall effect: Z=2.70 (P=0.007)

B

Study or subgroup	Cancer tissue Events	Total	Normal tissue Events	Total	Weight	Odds ratio M–H, random, 95% CI
Abou-Zeid et al 2011[22]	21	52	9	43	13.4%	2.56 (1.02–6.42)
Bhagat et al 2014[20]	58	134	11	26	13.8%	1.04 (0.44–2.43)
Cul'bová et al 2011[38]	5	13	5	19	10.4%	1.75 (0.39–7.95)
Gu et al 2009[39]	8	87	13	42	13.1%	0.23 (0.08–0.60)
Ho et al 2012[37]	1	47	6	29	7.5%	0.08 (0.01–0.73)
Makarla et al 2005[29]	7	23	3	23	10.4%	2.92 (0.65–13.12)
McCluskey et al 1999[47]	21	37	14	20	12.2%	0.56 (0.18–1.79)
Moselhy et al 2015[17]	14	18	12	32	11.3%	5.83 (1.56–21.87)
Tam et al 2007[26]	17	89	1	19	7.9%	4.25 (0.53–34.08)
Total (95% CI)		500		253	100.0%	1.18 (0.52–2.65)
Total events	152		74			

Heterogeneity: $\tau^2=1.06$; $\chi^2=29.72$, df=8 (P=0.0002); $I^2=73\%$
Test for overall effect: Z=0.39 (P=0.69)

C

Study or subgroup	Cancer tissue Events	Total	LMP Events	Total	Weight	Odds ratio M–H, fixed, 95% CI
Bhagat et al 2014[20]	58	134	5	23	22.8%	2.75 (0.96–7.84)
Cul'bová et al 2011[38]	5	13	0	2	2.4%	3.24 (0.13–80.99)
Makarla et al 2005[29]	7	23	5	23	16.4%	1.57 (0.42–5.96)
McCluskey et al 1999[47]	21	37	11	15	31.8%	0.48 (0.13–1.78)
Tam et al 2007[26]	17	89	1	16	6.4%	3.54 (0.44–28.69)
Wiley et al 2006[42]	89	215	4	19	20.3%	2.65 (0.85–8.25)
Total (95% CI)		511		98	100.0%	1.88 (1.10–3.19)
Total events	197		26			

Heterogeneity: $\chi^2=5.55$, df=5 (P=0.35); $I^2=10\%$
Test for overall effect: Z=2.32 (P=0.02)

Figure 2 Forest plots for the association between $P16^{INK4a}$ methylation and ovarian cancer risk.
Notes: (**A**) Cancer tissues vs normal tissues; (**B**) cancer tissues vs benign tissues; (**C**) cancer tissues vs LMP tissues.
Abbreviations: LMP, low malignant potential or borderline tumor tissues; M–H, Mantel–Haenszel.

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 $P16^{INK4a}$ promoter 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 D), but the differences were not statistically significant.

Sensitivity analysis and publication bias

As presented in Figure 6A–C, 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 $P16^{INK4a}$
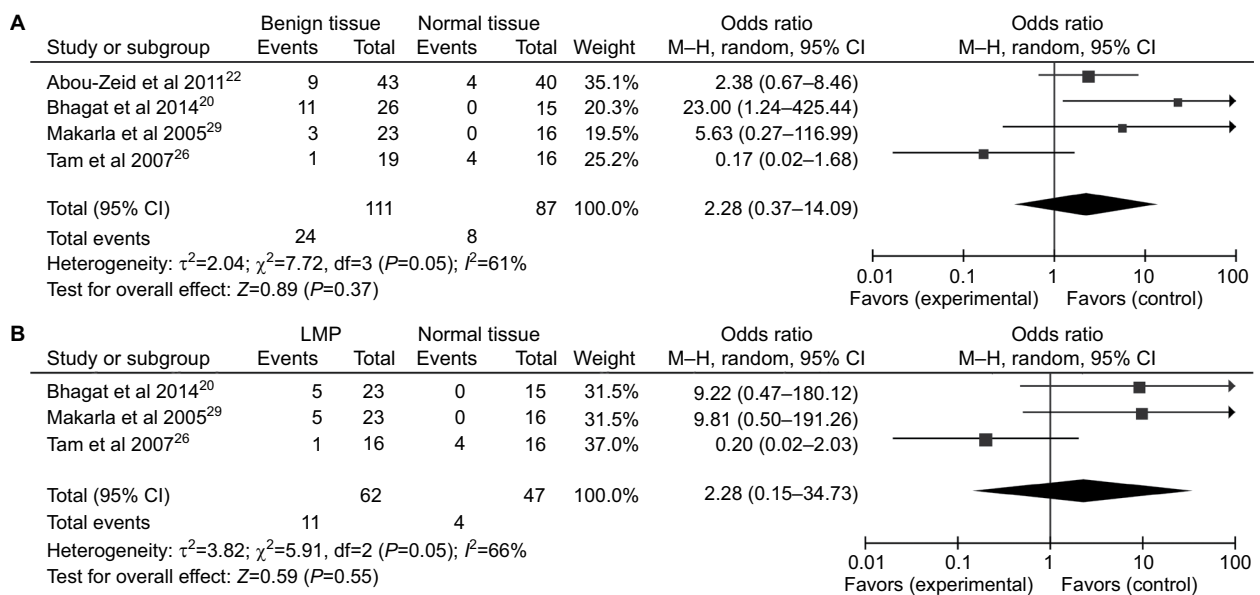
A

Study or subgroup	Benign tissue Events	Total	Normal tissue Events	Total	Weight	Odds ratio M–H, random, 95% CI	Odds ratio M–H, random, 95% CI
Abou-Zeid et al 2011[22]	9	43	4	40	35.1%	2.38 (0.67–8.46)	
Bhagat et al 2014[20]	11	26	0	15	20.3%	23.00 (1.24–425.44)	
Makarla et al 2005[29]	3	23	0	16	19.5%	5.63 (0.27–116.99)	
Tam et al 2007[26]	1	19	4	16	25.2%	0.17 (0.02–1.68)	
Total (95% CI)		111		87	100.0%	2.28 (0.37–14.09)	
Total events	24		8				

Heterogeneity: $\tau^2=2.04$; $\chi^2=7.72$, df=3 ($P=0.05$); $I^2=61\%$
Test for overall effect: $Z=0.89$ ($P=0.37$)

0.01 0.1 1 10 100
Favors (experimental)   Favors (control)

B

Study or subgroup	LMP Events	Total	Normal tissue Events	Total	Weight	Odds ratio M–H, random, 95% CI	Odds ratio M–H, random, 95% CI
Bhagat et al 2014[20]	5	23	0	15	31.5%	9.22 (0.47–180.12)	
Makarla et al 2005[29]	5	23	0	16	31.5%	9.81 (0.50–191.26)	
Tam et al 2007[26]	1	16	4	16	37.0%	0.20 (0.02–2.03)	
Total (95% CI)		62		47	100.0%	2.28 (0.15–34.73)	
Total events	11		4				

Heterogeneity: $\tau^2=3.82$; $\chi^2=5.91$, df=2 ($P=0.05$); $I^2=66\%$
Test for overall effect: $Z=0.59$ ($P=0.55$)

0.01 0.1 1 10 100
Favors (experimental)   Favors (control)

Figure 3 Forest plots for the association between *P16^{INK4a}* methylation and ovarian diseases.
Notes: (A) Benign tissues vs normal tissues; **(B)** LMP tissues vs normal tissues.
Abbreviations: LMP, low malignant potential or borderline tumor tissues; M–H, Mantel–Haenszel.

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

Stratified analysis	Number of studies	Pooled OR (95% CI) Random	Fixed	Meta-regression P-value	Heterogeneity 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

Abbreviations: MSP, methylation-specific PCR; OR, odds ratio.

promoter methylation and ovarian cancer risk, which was confirmed by the Begg's test (Figure 6D–F).

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 values of 582 ovarian tumor tissues and 12 normal ovarian tissues were extracted for analysis. Obviously, the methylation levels of seven out of nine 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
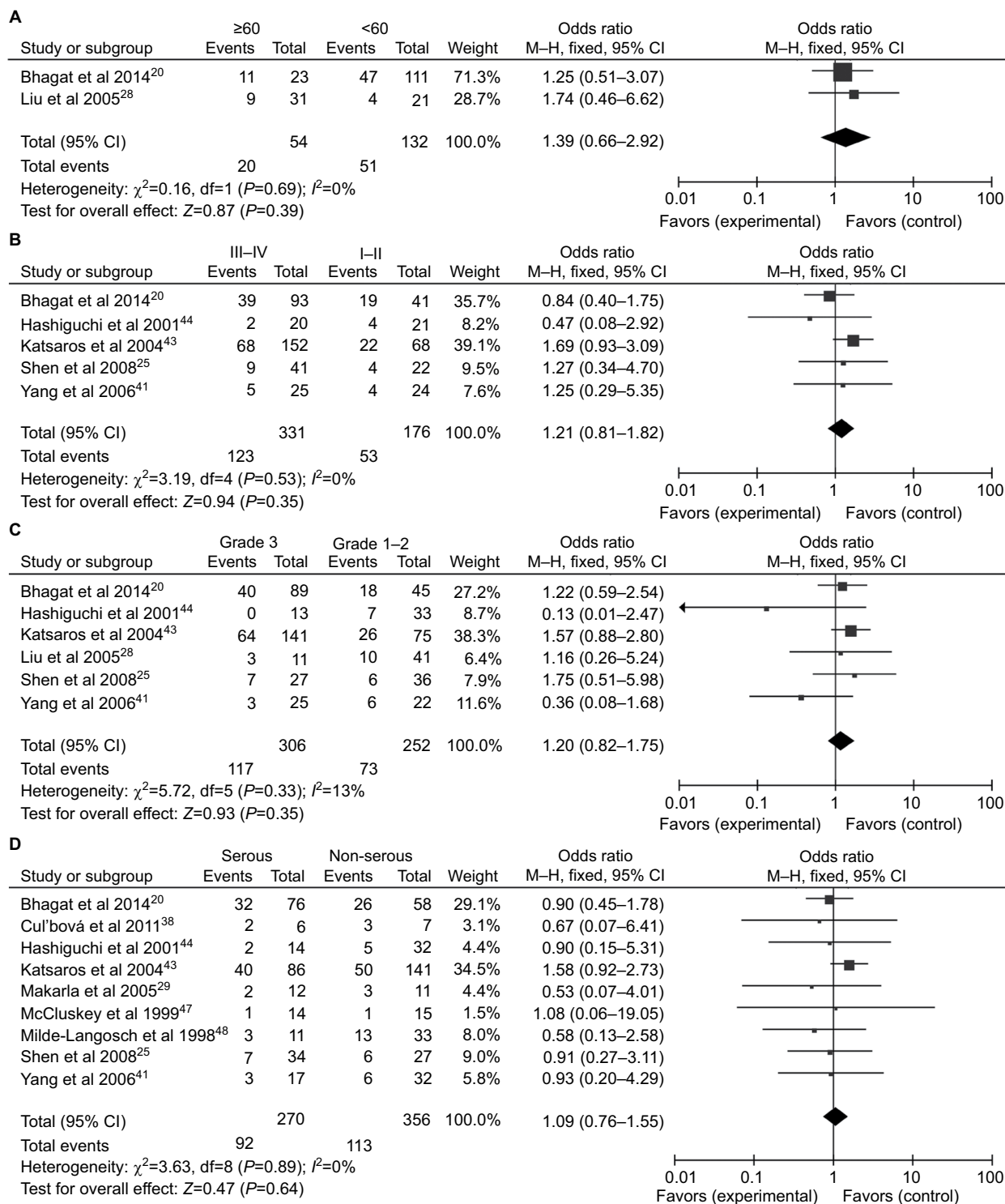
Figure 4 Forest plots for the association between *P16^INK4a* methylation and clinicopathological features in ovarian cancer.
Notes: (**A**) Age; (**B**) clinical stage; (**C**) tumor grade; (**D**) histological subtype.
Abbreviation: M–H, Mantel–Haenszel.

in ovarian cancer patients (adjusted *P*-value <0.000001). However, methylation levels of the rest six probes, which are located at non-promoter region tended to be positively associated 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
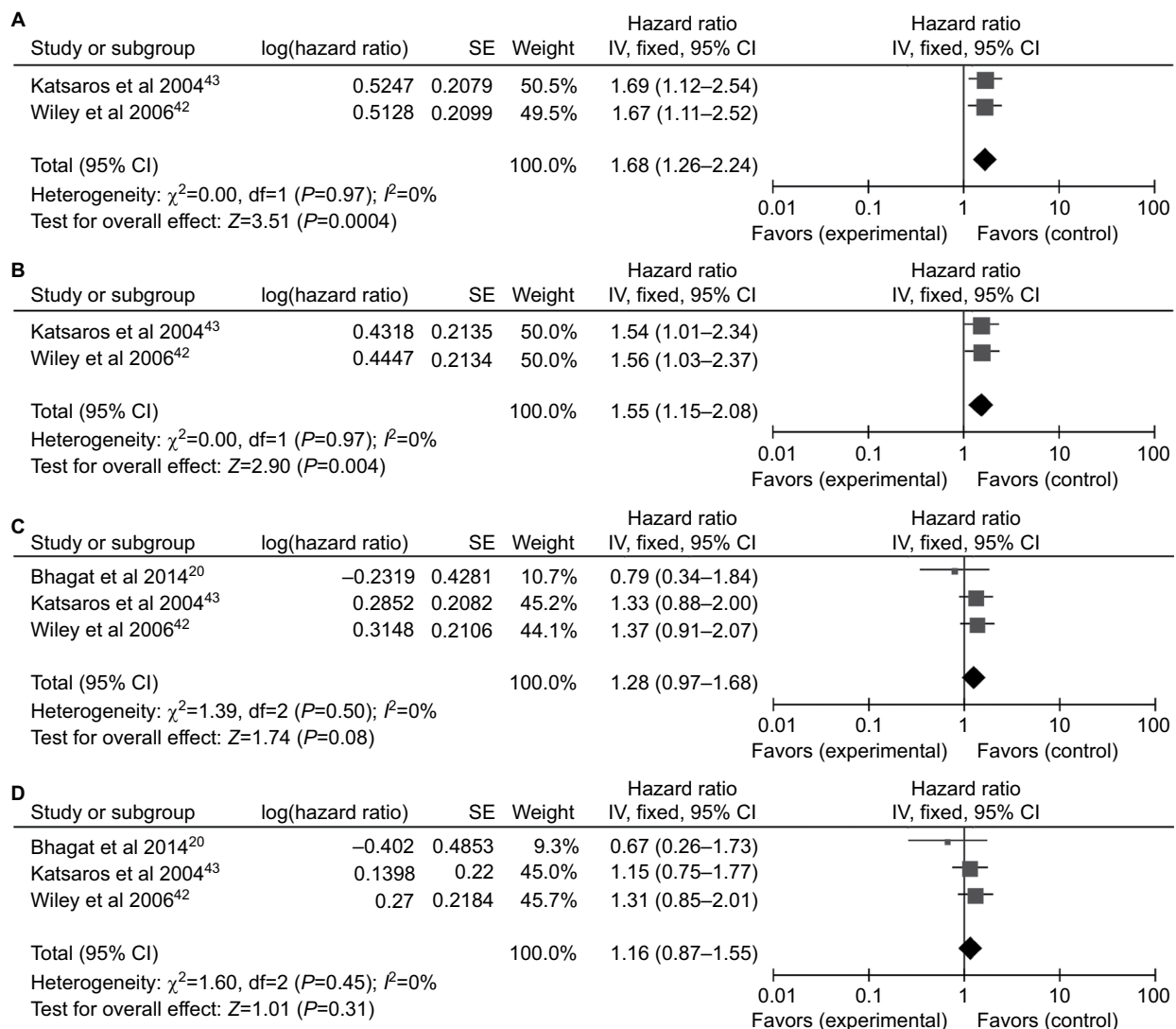
Figure 5 Forest plots for *P16^INK4a* methylation on survival analysis in univariate and multivariate Cox regression model.
Notes: (**A**) PFS in univariate Cox regression model; (**B**) PFS in multivariate Cox regression model; (**C**) OS in univariate Cox regression model; (**D**) OS in multivariate Cox regression model.
Abbreviations: OS, overall survival; PFS, progression-free survival; SE, standard error.

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 deaths in women.[50] 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.[16] It has been synthetically evaluated for aberrant *P16^INK4a* methylation in numerous cancers,[51–54] including ovarian cancer.[31,32] 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* promoter 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* promoter 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* promoter methylation was 2.28-fold higher in both benign tissues and LMP tissues (*P*>0.05), but the differences were not statistically significant. The reason for this phenomenon may be that the transformation of normal cells to cancer cells is a long-term, gradual, and
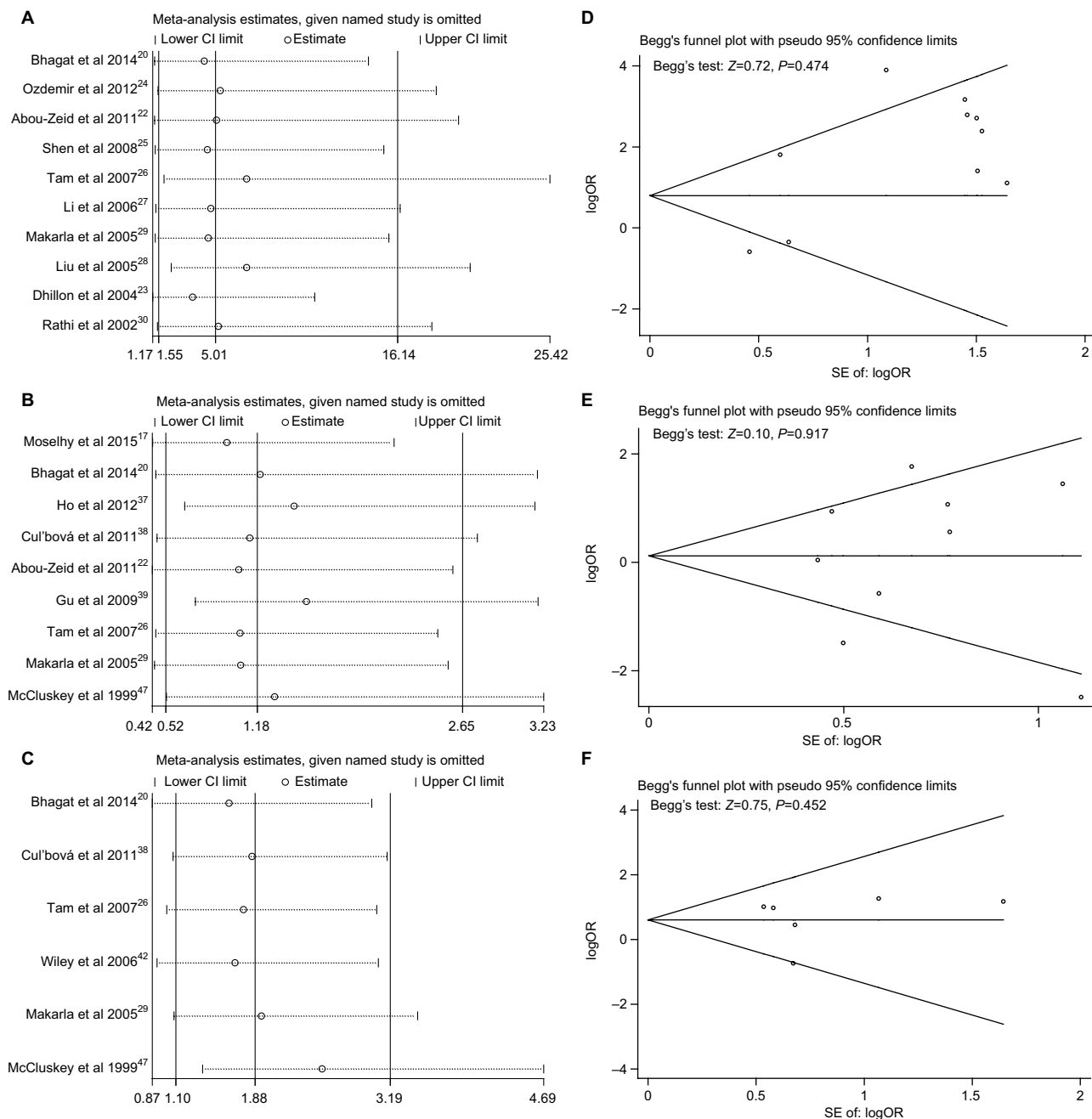
Figure 6 Sensitivity analyses and Begg's test for publication bias of $P16^{INK4a}$ methylation during the carcinogenesis of ovarian cancer.
Notes: (**A** and **D**) Cancer tissues vs normal tissues; (**B** and **E**) sensitivity analysis for the comparison of cancer tissues vs benign tissues; (**C** and **F**) sensitivity analysis for the comparison of cancer tissues vs LMP tissues.
Abbreviations: LMP, low malignant potential or borderline tumor tissues; SE, standard error.

multiphase process.[55] Although not establishing a strong correlation between $P16^{INK4a}$ promoter methylation and cancer progression, the above results do suggest a possibility that epigenetic alteration of $P16^{INK4a}$ promoter 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, $P16^{INK4a}$ promoter 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.[56] A review also outlined that some factors such as distinct physical appearance, behavior, and response to environ-

Table 4 Methylation of *P16^(INK4a)* CpG sites on Illumina HumanMethylation 27 BeadChip from TCGA datasets

Probe (Illumina HumanMethylation 27)	CpG island location (chromosome: DNA range)	Normal tissue beta value (mean, n=12)	Tumor tissue beta value (mean, n=582)	Adjusted *P*-value[a]	Pearson correlation coefficient	Adjusted *P*-value[b]
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

Notes: [a]*P*-value of *t*-test of the difference between normal tissue beta value and tumor tissue beta value. [b]*P*-value of Pearson's correlation between the tumor tissues beta value and CDKN2A expression (n=368).
Abbreviation: TCGA, The Cancer Genome Atlas.

mental agents and drugs between human populations could have contributed to the epigenetic variations.[57] Similar findings appeared in the subgroup analyses of different methods and publication year. Kurdyukov and Bullock[58] 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.[59,60] 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)* promoter methylation was associated with poorly differentiated tumors and was different in histological subtype in ovarian cancer.[22,43] However, we could not establish any significant correlations between *P16^(INK4a)* promoter 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[43] and Wiley et al[42] reported association of *P16^(INK4a)* promoter methylation with PFS and OS in ovarian cancer, while Bhagat et al[20] found no significant value in predicting prognosis. In the present study, we discovered that *P16^(INK4a)* promoter methylation represented a risk factor for PFS. For OS, patients with *P16^(INK4a)* promoter methylation also had a slightly elevated risk, though the differences are not statistically significant. This trend

was also observed in other types of cancer.[51,54] 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 was positively associated with *P16^(INK4a)* expression.

Compared with previous meta-analyses,[31,32] our meta-analysis had several improvements. First, the development of ovarian cancer is a multistep procedure involving normal tissues, benign disease, LMP or borderline tumor, and malignant tumor.[20] We compared malignant ovarian cancer with LMP tumors, benign disease, and normal samples to give more rigorously to the analysis. Second, 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)* promoter 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)* promoter methylation in ovarian cancer.

Limitations

Several potential limitations in our current study should be noted. First, the heterogeneity was still large after subgroup analyses in the assessment of the association between *P16^(INK4a)* promoter methylation and ovarian cancer risk, which may

affect the statistical power. Second, 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. Third, the total sample size was still relatively small for reliably assessing the prognostic value of $P16^{INK4a}$ promoter methylation in ovarian cancer. Fourth, 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 $P16^{INK4a}$ promoter methylation may depend on the histological subtype.[41,48,61] However, we are unable to extract sufficient data to analyze the association between $P16^{INK4a}$ promoter methylation and high-grade serous carcinomas because no detailed information of $P16^{INK4a}$ promoter methylation in high-grade serous carcinomas was provided in the eligible articles.

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

Conclusion

In conclusion, our meta-analysis suggests that aberrant methylation of $P16^{INK4a}$ promoter may be essential to the initiation of ovarian cancer and in distinguishing malignant from healthy ovarian tissues. Besides, $P16^{INK4a}$ promoter 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 $P16^{INK4a}$ promoter methylation in ovarian cancer.

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Disclosure

The authors report no conflicts of interest in this work.

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