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CDKN2A METHYLATION - AN EPIGENETIC BIOMARKER FOR CERVICAL CANCER RISK: A META-ANALYSIS

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ABSTRACT

Background: The methylation of *CDKN2A* is reported to be associated with the tumorigenesis of the human cervix, the most common and high incidence cancer of female. **Objective:** The current study aimed to carry out a meta-analysis to evaluate the association between the *CDKN2A* gene methylation and cervical cancer, and its correlation could be used as an epigenetic biomarker for cervical cancer risk. **Materials and methods:** Relevant articles were identified by searching the MEDLINE database. The frequency and Odds ratio (OR) were applied to estimate the effect of *CDKN2A* methylation based on random-/fix-effects models. **Results:** A total of 16 studies, included 1,977 samples: 965 samples from cervical cancer patients, and 1,068 samples from non-cancerous samples, were enrolled in the meta-analysis. The overall frequency of *CDKN2A* methylation in the case-group was significantly higher than in the control group based on the random-effects model. Omitting the relative poor-quality studies, no heterogeneity among studies was recorded, the association increased (OR: 21.65; 95% CI = 12.47-37.58, fix-effects model). The association was also confirmed in all subgroup analyses. Additionally, the significant association was also found between the methylation of *CDKN2A* and pre-cancer risk: LSIL, HSIL (LSIL: OR = 6.15, 95% CI = 2.01-18.84; AD: HSIL: OR = 10.28, 95% CI = 3.53-29.91).
Conclusion: This meta-analysis provides scientific pieces of evidence to suggest that the *CDKN2A* methylation was the early epigenetic biomarker for the risk of cervical cancer.

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Introduction

Cancer has been a major threat to mankind, and it kills many people every year [1, 2]. It is the second leading cause of death in the world [3]. Cervical cancer is the most common cancer death for female which is closely related to the infection of human papillomavirus (HPV) [4, 5]. Besides the infection of high-risk HPV types, such as HPV 16, HPV 18, etc., the aberrant methylation plays an important role in cervical cancer progression [5-7]. The hypermethylation occurred in the tumor suppressor genes' promoter leads to gene inactivation, which inhibits the functions of the tumor suppressor gene [8]. *CDKN2A* (*Cyclin-dependent kinase inhibitor 2A*, also known as *p16$^{INK4\alpha}$*), located at 9p21, encodes p16$^{INK4\alpha}$ protein, functions as a specific inhibitor of cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase 6 (CDK6), hence, initiate the phosphorylation of the retinoblastoma tumor suppressor protein (RB), which directly regulates the cell cycle through the G1/S checkpoint [9-11]. The methylation of *the CDKN2A* gene promoter has been reported to be an essential epigenetic modification in human cervical cancer [5, 7, 10, 11]. Additionally, the identification of *CDKN2A* gene inactivation in tumor progression via the hypermethylation of its promoter, could be used as a prognostic and predictive biomarker of cancer, has been reported by many clinical case-control studies [5, 7, 10, 11]. However, due to the different sensitivities and intra/interassay coefficients of variation of methods, the reported frequency of *CDKN2A* gene promoter methylation, and its prognostic value is highly variable, and also remain controversial. Therefore, we performed the present study to carry out a systematic review and a meta-analysis, notably, the first systematic review and a meta-analysis, to summarize the previously published studies and to evaluate the methylation frequency of *CDKN2A* gene as prognostic, and predictive biomarker for cervical cancer risk.

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Materials and Methods

Search strategy and inclusion/exclusion criteria

The current meta-analysis was performed according to the guidelines of Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) [12]. Separation or combination of the following keywords: "cervical cancer", "hypermethylation", "$p16^{INK4a}$", "*CDKN2A*", "prognosis", "diagnosis", etc. were applied to collect related published articles in MEDLINE database (updated on December 2019). Additional studies were also identified via the references listed in the articles.

Studies were considered eligible only when they met all of the following inclusion criteria: i) The articles were limited to studies written in English; ii) case-control study designed; iii) provided that data about the frequency of *CDKN2A* methylation as well as the sample size in both case and control group. Exclusion criteria were as follows: i) The articles were written in other languages; ii) abstracts, case reports, letter to the editor or unpublished articles were eliminated; iii) studies were related to other tumors and not specific for cervical cancer; iv) studies lacked vital information for analysis.

Data extraction

The eligibility of each study, the relevant data from the eligible studies were independently retrieved by two authors. Disagreements were resolved through discussion within the third author or our research team. The following information, including the Author's last name, year of publication, the country where the study was performed, sample type, experimental methods to assess *CDKN2A* methylation, and some cases and controlled subjects, were extracted from each study.

Statistical analysis

All data were statistically analyzed using the MedCalc® software by MedCalc Software Ltd. (https://www.medcalc.org/). The frequency of *CDKN2A* methylation was calculated in both the case and control group. The strength of the association between *CDKN2A* methylation and CC was evaluated by odds ratio (OR) with 95% confidence intervals (95%CI). In the present study, the heterogeneity among the included studies was estimated by the Cochran Q test and I^2 statistics [13]. The cut-off point: $p = 0.05$ for the Q test and I^2 were used to test the heterogeneity between studies [14, 15]. The scale of I^2 value is classified as following: $I^2 < 25\%$: no heterogeneity, $25\% \leq I^2 \leq 50\%$: moderate heterogeneity, and $I^2 > 50\%$: strong heterogeneity.21,22 The random-effects model was applied if the heterogeneity among studies existed ($p < 0.05$ for Q test, $I^2 > 50\%$). In the case of no between-study heterogeneity, a fixed-effects model was applied to compute the pooled ORs. To determine the presence of publication bias, the symmetry of the funnel plots in which ORs were plotted against their corresponding standard errors were assessed by the Begg's funnel plot and Egger's test ($p < 0.05$ indicates statistically significant [16, 17].

Results

The characteristics of eligible studies

After exclusion of studies that not met the inclusion criteria, finally, 16 studies, published from 2001 to 2017, included 1,977 samples: 965 samples from cervical cancer patients, and 1,068 samples from non-cancerous samples, were enrolled in the meta-analysis. The characteristics of the included studies of *CDKN2A* methylation and risk of cervical cancer were summarized in Table 1.

Table 1. The characteristics of studies included in the meta-analysis of *CDKN2A* methylation and risk of cervical cancer

Author, Reference	Year	Region	Case		Control		Method	Source of	
			N	P	N	P		Case	Control
Dong et al. [18]	2001	Korea	53	16	24	0	MSP	B	B
Gustafon et al. [19]	2004	United States	28	10	11	1	MSP	B	B
Yang et al. [20]	2004	China	85	24	100	0	MSP	B	B
Yang et al. [20]	2004	China	40	4	30	0	MSP	P	P
Feng et al.	2005	United States	169	5	131	5	MSP	B	B
Lin et al. [21]	2005	Korea	67	37	20	0	MSP	B	B
Jeong et al. [22]	2006	Korea	78	45	24	2	MSP	B	B
Attaleb et al. [23]	2009	Morocco	22	13	20	0	MSP	B	S
Furtado et al. [24]	2010	Brazil	24	15	20	4	MSP	B	S
Kim et al. [25]	2010	Korea	69	9	41	1	MSP	S	S
Huang et al. [26]	2011	Taiwan	20	13	11	1	MSP	B	S
Spathis et al. [27]	2011	Greece	12	4	41	5	MSP	S	S
Jha et al. [10]	2012	India	125	45	100	1	MSP	B	B
Carestiato [11]	2013	Brazil	29	27	28	3	MSP	B	B
Banzai et al. [28]	2014	Japan	53	9	24	2	MSP	B	B

Li et al. [29]	2017	Taiwan	13	2	395	52	MSP	C	C
Wang et al. [30]	2017	China	78	61	48	[23]	MSP	B	B

Note: B: Cervical cancer biopsy tissue; P: Plasma; S: Cervical swab; C: Cervical craping; MSP: Methylation-specific PCR.

The number of patients in the included studies ranged from 12 to 169 (mean: 60.30). A total of 16 studies were from Asian countries: Korea, China, Taiwan, Japan, India (counting for 62.50%); American countries: Brazil, United States (counting for 25.00%), European country: Greece (counting for 6.25%) and African country: Morocco (counting for 6.25%). The source of cervical cancer samples encompassed cervical cancer tissue (counting for 76.47%), cervical swab (counting for 11.77%), plasma (counting for 5.88%), cervical craping (counting for 5.88%). Regarding the method of evaluation of the *CDKN2A* gene methylation status, the methylation-specific PCR (MSP) was used in all studies (counting for 100.00%)

Meta-analysis: The frequency of *CDKN2A* promoter methylation, and the association between CDKN2A gene methylation and cervical cancer

In current meta-analysis, considering the significant heterogeneity between studies (Case: Q = 344.51, p < 0,0001, I^2 = 95.36%, 95% CI for I^2 = 93.79-96.53; Control: Q = 68.15, p < 0,0001, I^2 = 76.52%, 95% CI for I^2 = 62.62-85.26), the random-effects model was applied to explore the frequency of *CDKN2A* gene methylation in cervical cancer (case-group) and non-cancerous samples (control-group) (Fig. 1, Fig. 2). According to Fig. 1, and Fig. 2, the frequency of *CDKN2A* gene methylation in case and control group were 39.57% (95% CI = 25.64-54.42) and 5.80% (95% CI = 2.94-9.52), respectively. The meta-analysis result also indicated that the frequency of *CDKN2A* gene methylation in the case-group was significantly higher than the control group (p < 0.0001).

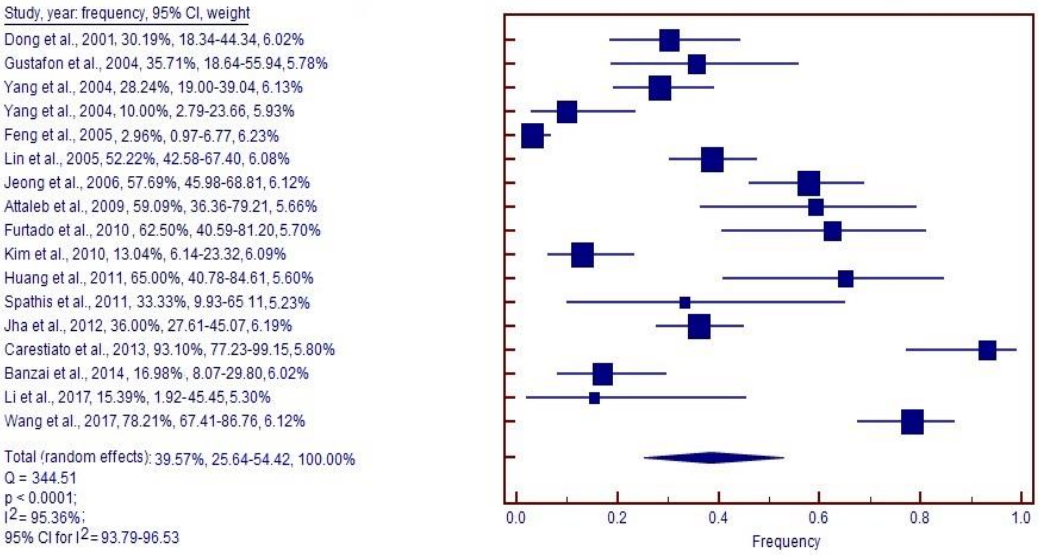

Figure 1. Forest plot of the frequency of *CDKN2A* gene methylation detected in cervical cancer samples

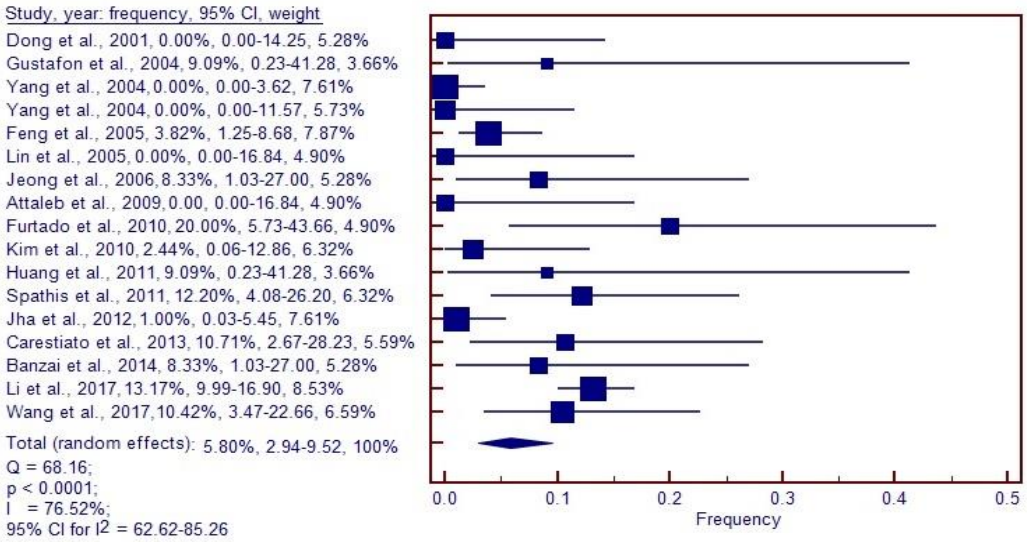

Figure 2. Forest plot of the frequency of *CDKN2A* gene methylation detected in non-cancerous samples

Moreover, the random-effects model was also applied to calculate the summary of the odds ratio (OR). The methylation of *CDKN2A* gene was significantly associated with an increased cervical cancer risk with a pooled OR of 10.49 (95% CI = 4.76-23.12), based on the random-effects model (Q = 49.30, p < 0.0001, I^2 = 67.54%, 95% CI for I^2 = 46.18-80.42) (Fig. 3). The funnel plot of pooled analysis, which was quite asymmetric, indicated that there was significant bias among the included studies, therefore there was a factor of influence on the current meta-analysis (Fig. 4). Combined with the sensitivity analysis, it found that study by Feng et al. (2005), Spathis et al. (2011), Li et al. (2017), Carestiato et al. (2013) and Banzai et al. (2014) was the relative poor-quality study. Therefore, those studies were omitted to evaluate the association between the methylation of the *CDKN2A* gene and cervical cancer through OR. As the result, when a study by Feng et al. (2005), Spathis et al. (2011), Li et al. (2017), Carestiato et al. (2013) and Banzai et al. (2014) was removed, the between heterogeneity decreased to I^2 = 0.00% (p = 0.59), indicating that no heterogeneity among enrolled studies was observed. Additionally, the association between *CDKN2A* methylation and risk of cervical cancer increased, which was indicated by the increased OR of 21.65 in a fix-effects model (95% CI = 12.47-37.58) (Fig. 5) (compared to previously calculated OR of 10.49). Moreover, the funnel plot of pooled analysis, which was quite symmetric, indicated that there was no significant bias among the included studies (Fig. 6).

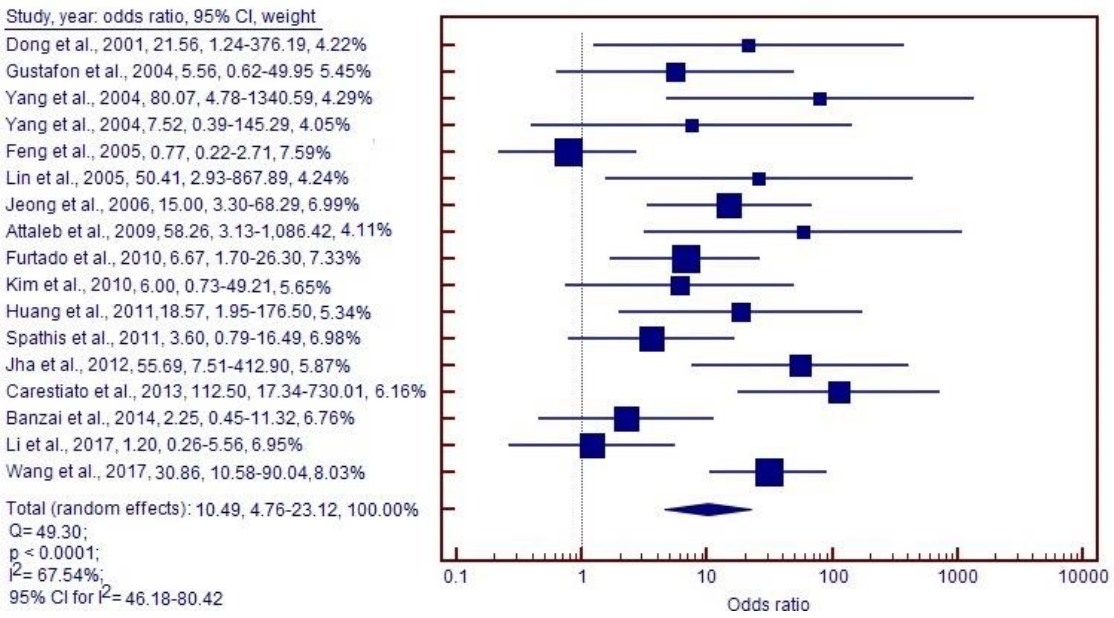

Figure 3. Forest plot of the association between the methylation of the *CDKN2A* gene and cervical cancer through OR based on the random-effects model.

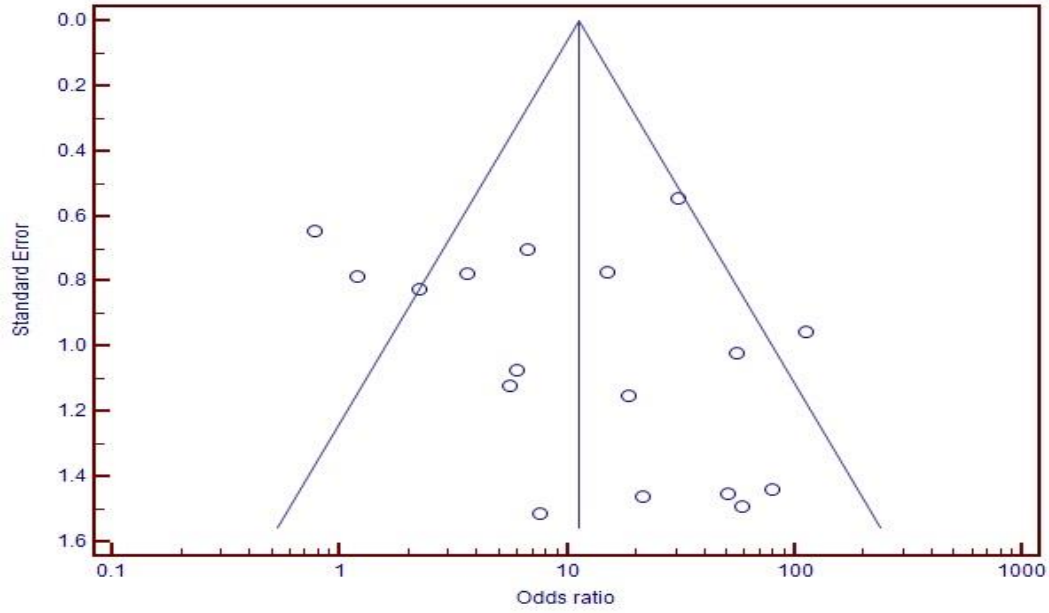

Figure 4. Funnel plot of *CDKN2A* methylation and cervical cancer risk based on the random-effects model

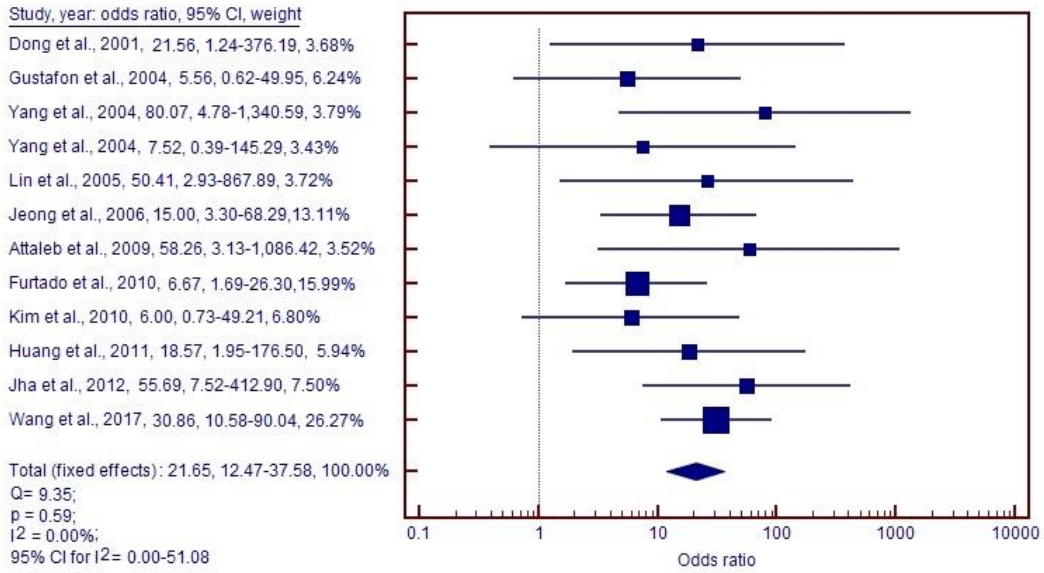

Figure 5. Forest plot of the association between the methylation of *the CDKN2A* gene and cervical cancer through OR based on the fix-effects model.

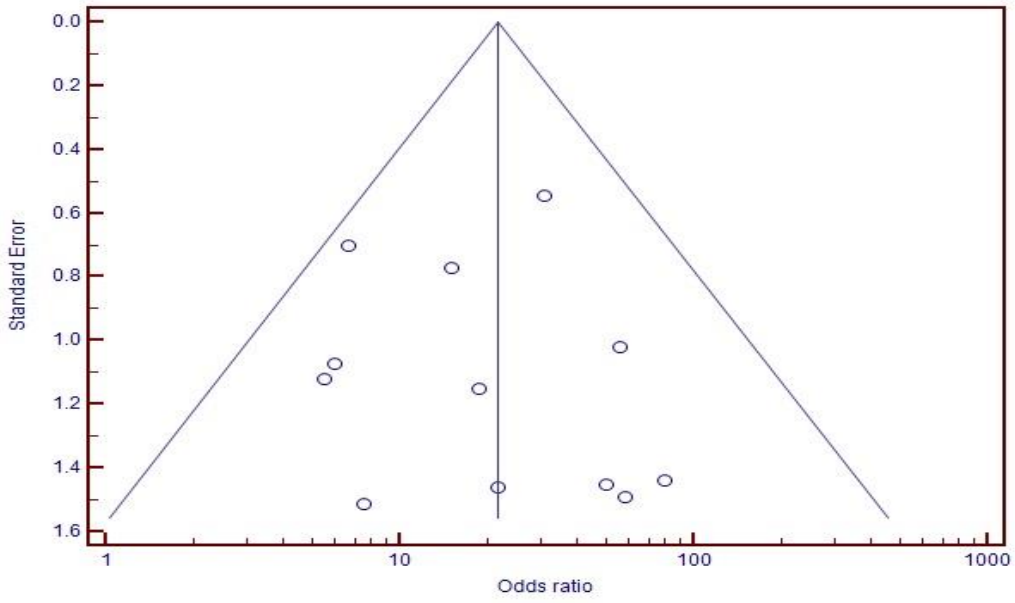

Figure 6. Funnel plot of *CDKN2A* methylation and cervical cancer risk based on the fix-effects model

Subgroup analysis was performed according to the region, source of samples for *CDKN2A* methylation evaluation, shown in Table 2. Subgroup analysis showed that the heterogeneity disappeared among a subgroup of the region, source of samples, and histological types of cancer (Fix-effects model, $p > 0.05$). Regarding the subgroup of the region, there was a significant association between *CDKN2A* methylation and cervical cancer among the group of Asia and the non-Asia group in a fix-effects model (Asia: OR = 26.45, 95% CI = 13.73-50.96; non-Asia: OR = 10.31, 95% CI = 3.64-29.23). Considering the source of samples, the significant association between *CDKN2A* methylation and cervical cancer was observed among the cervical cancer biopsy tissue group and non-biopsy group in the fix-effect model (biopsy tissue group: OR = 24.78, 95% CI = 13.83-44.39; non-biopsy group: OR = 6.48, 95% CI = 1.17-36.01). Concerning to the histological types of cervical cancer, two types, including SSC (squamous cell carcinoma), and AD (adenocarcinoma), were recorded in a study of Dong et al. (2001), Yang et al. (2004), Lin et al. (2005), and Jeong et al. (2006). Significant association between *CDKN2A* methylation and cervical cancer was found between SCC, AD and control group (SCC: OR = 29.23, 95% CI = 9.78-87.36; AD: OR = 35.74, 95% CI = 9.50-134.48). In other hands, concerning to the low-grade squamous intraepithelial lesion (LSIL: CIN-I) and high-grade squamous intraepithelial lesion (HSIL: CIN-II or CIN-III) cervical cytology samples, were recorded in a study of Gustafon et al. (2004), Lin et al. (2005), Kang et al. (2006) [31], Kim et al. (2010), and Huang et al. (2011). Methylation status of the *CDKN2A* gene was shown in 24.10% (20 of 83) of LSIL (CIN-I) samples and 34.52% (58 of 168) of HSIL (CIN-II or CIN-III), higher than in control group (counting for 3.41% (3 of 88)). Additionally, significant

association between *CDKN2A* methylation and cervical pre-cancer risk - LSIL, HSIL was found (LSIL: OR = 6.15, 95% CI = 2.01-18.84; AD: HSIL: OR = 10.28, 95% CI = 3.53-29.91).

Table 2. Summary of subgroup analysis in the meta-analysis of *CDKN2A* methylation and cervical cancer risk

Group	Case		Control		Model, OR, 95% CI (Fix-effects model)	Heterogeneity	
	N	P	N	P		I^2 (%)	p
Total	689	292	449	15	21.65, 12.47-37.58	0.00	0.59
Region							
Asia	615	254	398	10	26.45, 13.73-50.96	0.00	0.79
Non-Asia	74	38	51	5	10.31, 3.64-29.23	1.89	0.36
Source of sample							
Biopsy sample	580	279	378	14	24.78, 13.83-44.39	0.00	0.55
Non-biopsy	109	13	71	1	6.48, 1.17-36.01	0.00	0.90
The histological type of cancer							
SCC	205	91	168	2	29.23, 9.78-87.36	0.00	0.64
AD	78	31	168	2	35.74, 9.50-134.48	0.00	0.84
CIN grading (low-grade/high-grade cervical intraepithelial neoplasia)							
LSIL (CIN-I)	83	20	88	3	6.15, 2.01-18.84	0.00	0.89
HSIL (CIN-II or CINN-III)	168	58	88	3	10.28, 3.53-29.91	0.00	0.83

Sensitivity analysis and publication bias

After removing the relative poor-quality study, including Feng et al. (2005), Spathis et al. (2011), Li et al. (2017), Carestiato et al. (2013) and Banzai et al. (2014), the quite symmetric funnel plot, suggested there was no significant bias among included studies, was observed (Fig. 6). Additionally, aiming to evaluate the stability and reliability of the conclusions, the sensitivity analysis was performed according to the leave-one-out method by excluding one study. As results, the pooled OR was ranged from 18.34, (95% CI = 10.61-33.43) to 24.83, (95% CI = 13.49-45.72) under the fix-effects model within the I^2 = 0.00 (Table 3). Therefore, the results, and conclusion of the present meta-analysis, which was to evaluate the association between methylation of *CDKN2A* and cervical cancer risk, were stable and reliable.

Table 3. Sensitivity analysis of methylation of *CDKN2A* and cervical cancer risk by the fix-effects model

	OR, 95% CI	Heterogeneity	
		I^2, 95% CI	*p*
Omitting Dong et al., 2001	21.65, 12.36-37.93	0.00, 0.00-57.61	*0.50*
Omitting Gustafon et al., 2004	23.41, 13.21-41.50	0.00, 0.00-51.24	*0.62*
Omitting Yang et al., 2004	20.23, 11.39-35.93	0.00, 0.00-56.57	*0.56*
Omitting Lin et al., 2005	20.55, 11.71-36.07	0.00, 0.00-54.81	*0.55*
Omitting Jeong et al., 2006	22.72, 12.55-41.12	0.00, 0.00-57.41	*0.50*
Omitting Attaleb et al., 2009	19.97, 11.34-35.17	0.00, 0.00-51.57	*0.61*
Omitting Furtado et al., 2010	24.83, 13.49-45.72	0.00, 0.00-40.59	*0.76*
Omitting Kim et al., 2010	23.72, 13.39-42.03	0.00, 0.00-51.80	*0.61*
Omitting Huang et al., 2011	21.80, 12.35-38.49	0.00, 0.00-57.66	*0.50*
Omitting Jha et al., 2012	18.34, 10.61-33.43	0.00, 0.00-49.52	*0.64*
Omitting Wang et al., 2017	20.09, 10.76-37.50	0.00, 0.00-53.21	*0.58*

Discussion

Inactivation of TSG through the methylation of its promoter has been reported to play a crucial role in the carcinogenesis of the tumor [5, 8, 32]. *CDKN2A* gene, a TSG, could mediate cell cycle through the G1/S checkpoint by regulation of CDK4 and CDK6 levels [9-11]. Whereas inactivated *CDKN2A* gene by methylation, could lead to the pathogenesis and metastasis of the tumor, has been reported in human cervical cancer [5, 7, 10, 11, 33]. The present meta-analysis was performed base on the previous 16 studies, updated to present, included 1,977 samples: 965 samples from cervical cancer patients, and 1,068 samples from non-cancerous samples, which concerned about the methylation of *CDKN2A* gene, to evaluate the association between the methylation of *CDKN2A* gene and cervical cancer risk as well as evaluate the potential of *CDKN2A* methylation

as a biomarker for screening and diagnosis of cervical cancer. The overall frequency of *CDKN2A* gene promoter methylation in cervical cancer and control population was 39.57%, and 5.80%, respectively. The results of the meta-analysis indicated the individuals with *CDKN2A* gene methylation was significantly associated with cervical cancer (pooled OR = 10.49, 95% CI = 4.76-23.12), based on the random-effects model. A significant heterogeneity between studies was found by the Q-test in the meta-analysis (Q = 49.30, $p < 0.0001$, I^2 = 67.54%, 95% CI for I^2 = 46.18-80.42) (Fig. 3, Fig. 4). Therefore, the sensitivity analysis was performed to find out the influent studies. As the result, after omitting the relative poor-quality study by Feng et al. (2005), Spathis et al. (2011), Li et al. (2017), Carestiato et al. (2013) and Banzai et al. (2014), the heterogeneity of present meta-analysis was decreased to 0.00%, meant that there was no heterogeneity among the included studies. The OR was increased to 21.65 (95% CI = 12.47-37.58), which was double times to previously calculated OR of 10.49, indicated that the association between *CDKN2A* methylation and cervical cancer risk increased, suggested that the methylation of *CDKN2A* gene might play a crucial role in the pathogenesis of cervical cancer. The significant association between *CDKN2A* gene methylation and cervical cancer risk was found among all subgroups, including region, source of samples and histological types of cervical cancer. First of all, the MSP method was used in all studies, counting for 100%. It could be explained that MSP is the "gold standard method" of evaluation of methylation. The MSP shows a useful tool for the qualitative DNA methylation analysis within the ease of design and execution, sensitivity in the ability to detect small quantities of methylated DNA [34]. Moreover, in which MSP products are run on a gel, and the results are reported as methylated or unmethylated at the target DNA sequence [35]. Additionally, a significant association between methylation of *CDKN2A* and cervical cancer was found among the Asian region and the Non-Asia region (Asia: OR = 26.45, 95% CI = 13.73-50.96; Non-Asia: OR = 10.31, 95% CI = 3.64-29.23), which once again confirmed the nasopharyngeal cancer is native to the Asian region. With the histological types of cancer, the significant association between *CDKN2A* gene methylation and cervical cancer was found among the SCC, AD group and the control group in the fix-effects model (SCC: OR = 29.23, 95% CI = 9.78-87.36; AD: OR = 35.74, 95% CI = 9.50-134.48). Finally, the subgroup analysis by the source of cancer samples revealed a significant association in both subgroups: biopsy (OR = 24.78, 95% CI = 13.83-44.39) and non-biopsy (OR = 6.48, 95% CI = 1.17-36.01), and no heterogeneity was observed. It indicated that the type of biopsy was more suitable to apply to evaluate the methylation of *the CDKN2A* gene. Recently, the progressive increase in de-novo methylation has been focused extensively on precancerous cervical cancer lesions. LSIL (CIN-I) and HSIL (CIN-II or CIN-III) were considered as the suitable model for studying genetic and epigenetic changes in precancerous, preinvasive conditions to find out the early events to be served as the early genetic, epigenetic biomarker of cervical cancer risk. In a current meta-analysis study, epigenetic event – the methylation of *the CDKN2A* gene was found to exist in 24.10% of LSIL (CIN-I) samples and 34.52% of HSIL (CIN-II or CIN-III), higher than in control group. Additionally, significant association between *CDKN2A* methylation and LSIL, HSIL was found (LSIL: OR = 6.15, 95% CI = 2.01-18.84; AD: HSIL: OR = 10.28, 95% CI = 3.53-29.91). These results are consistent with those documented *CDKN2A* gene methylation to be the common and early epigenetic event in the progression of cervical tumorigenesis. So its epigenetic event might be used as the potential epigenetic biomarker for cervical cancer risk. However, the current meta-analysis exhibited some limitations due to the number of currently enrolled studies of 16 (modest), the data of non-English language studies may contribute to some bias, as well as the evaluation of the correlation between methylation of *CDKN2A* gene and clinicopathological features.

Conclusion

The usefulness of *CDKN2A* gene methylation as an epigenetic potential biomarker for diagnosis and early prognosis of cervical cancer was under intense investigation in a current meta-analysis study. Additionally, our findings underscore the correlation among *CDKN2A* gene methylation and all subgroups, including region, histological types of cancer, and source of samples. It is worth emphasizing that the methylation of the *CDKN2A* gene was recorded as the early epigenetic event in the progression of cervical tumorigenesis. It was provided by the observation of the association between the cervical pre-cancer: LSIL, HSIL, and *CDKN2A* methylation. These results show a strong, and significant correlation between *CDKN2A* gene methylation and risk of cervical cancer as well as pre-cancer by providing the scientific pieces of evidence through current meta-analysis

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