RESEARCH ARTICLE

Association between *RASSF1A* Promoter Hypermethylation and Oncogenic HPV Infection Status in Invasive Cervical Cancer: a Meta-analysis

Jin-Yun Li[1,2], Tao Huang[1,2], Cheng Zhang[1], Dan-Jie Jiang[1], Qing-Xiao Hong[1], Hui-Hui Ji[1], Meng Ye[2]*, Shi-Wei Duan[1]*

Abstract

Cervical carcinoma is the main cause of cancer-related mortality in women and is correlated with more than 15 risk cofactors, including infection of cervical cells with high-risk types of HPV (hrHPV). Indeed, both aberrant methylation of the *RASSF1A* promoter and hrHPV infection are often observed in cervical carcinomas. The purpose of our meta-analysis was to evaluate the role of *RASSF1A* promoter methylation and hrHPV infection in cervical cancer. Our meta-analysis involved 895 cervical cancer patients and 454 control patients from 15 studies. Our results suggested that *RASSF1A* promoter hypermethylation increased the risk of cervical cancer (OR=9.77, 95%CI=[3.06, 31.26], *P*=0.0001, I^2=78%). By grouping cases according to cancer subtypes, we found that HPV infection was higher in cervical squamous cell carcinomas (SCCs) than in cervical adenocarcinomas/ adenosquamous cancers (ACs/ASCs) (OR=4.00, 95%CI=[1.41, 11.30], *P*=0.009, I^2=55%). Interestingly, HPV infection tended to occur in cervical cancers with relatively low levels of *RASSF1A* promoter methylation (OR=0.59, 95%CI=[0.36, 0.99], *P*=0.05, I^2=0%). Our study provides evidence of a possible interaction between HPV infection and *RASSF1A* promoter methylation in the development of cervical cancers.

Keywords: Cervical cancer - RASSF1A methylation - human papilloma virus - HPV infection - meta-analysis

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Introduction

Cervical cancers remain the third leading cause of genital system cancer-related mortality in women, despite the widespread application of screening methods for the prevention and early detection of cervical cancer. Numerous epidemiological studies have proved that genital HPV infections, as the central etiological factor, were highly correlated with cervical cancer and were present in almost 90% of cervical carcinomas (Bosch et al., 1995). Among all of the HPV subtypes, of which more than 100 have been identified, only the high-risk HPVs (including types 16, 18, 33, 58 and 59) were strongly associated with the progression of cervical cancers (Munoz et al., 2003). However, a majority of the women infected with HPV do not develop cervical cancer, and 90% of HPV infections usually resolve on their own within 2 years. Therefore, other complex genetic and epigenetic alterations might cooperate to drive the development of cervical cancers.

In addition to cancer-related genetic mutations (Zhu et al., 2014), epigenetic alterations without changes in gene sequences, such as promoter hypermethylation, are relatively common events in many human cancers (Bird, 1986; Jiang et al., 2014a; Jiang et al., 2014b). Several tumor suppressor genes (TSGs) have been found to be frequently inactivated by promoter hypermethylation and linked with the pathogenesis and progression of cervical cancers (Cheung et al., 2004; Jha et al., 2012). *RASSF1A* (Ras association domain family 1 isoform) is one of the RASSF (Ras association domain family) members, which play an extremely important role in various cellular processes such as the modulation of apoptosis, migration and regulation of adhesion (van der Weyden and Adams, 2007; Richter et al., 2009). Aberrant methylation of the *RASSF1A* promoter was often observed in cervical carcinomas (Yu et al., 2003; Maliukova et al., 2004).

Meta-analyses provide the advantage of combining data from multiple studies and establishing relationships across studies, allowing one to analyze larger sample numbers and draw relatively more reliable conclusions(Lipsey and Wilson, 2001). In light of previous studies, we performed a meta-analysis of the studies monitoring the *RASSF1A* promoter methylation status and HPV infection status in cervical cancer and attempted to establish the role of these risk cofactors in cervical cancer development.

*[1]Zhejiang Provincial Key Laboratory of Pathophysiology, School of Medicine, [2]The Affiliated Hospital, Ningbo University, Ningbo, Zhejiang, China *For correspondence: yemeng@nbu.edu.cn, duanshiwei@nbu.edu.cn*

Materials and Methods

Identification of relevant studies

All relevant studies, updated until October 8, 2014, were systematically searched from the PubMed, China National Knowledge Infrastructure, and Wanfang databases using the keywords "*RASSF1A* methylation" and "HPV" or "human papilloma virus" in conjunction with "cervical cancer" or "cervical intraepithelial neoplasia" or "uterine cervix cancer". In addition, we conducted a manual search to identify other potential studies within the reference lists of the retrieved studies.

Inclusion and exclusion criteria

All selected studies met the following eligibility criteria: (1) the study should refer to the methylation status of *RASSF1A* or HPV infection in invasive cervical cancers; (2) the study should assess and present the details of the *RASSF1A* methylation status in cervical cancer tissues; and (3) the study should provide detailed information on the HPV infection status, such as the HPV subtype. Studies assessing the *RASSF1A* methylation status in cell lines or in cervical intraepithelial neoplasia rather than in invasive cervical cancers were excluded. We defined the case groups as invasive cervical carcinomas, and the control cases were the corresponding normal tissues or benign diseases. Furthermore, studies without detailed information on gene methylation and HPV infection data were removed from our analysis.

Data extraction

From the eligible studies, we extracted the first author's name, publication year, ethnicity of the study subjects, methylation assessment method, number of cervical cancers and corresponding normal control group, hrHPV infection status as hrHPV-positive and hrHPV-negative cervical cancers, histological type of the tumors and frequency of *RASSF1A* promoter methylation.

Statistical analysis

Using the Review Manager 5 software, we calculated the combined odds ratios (ORs) with corresponding 95% confidence intervals (95%CIs) to estimate the associations included in our meta-analysis. An I^2 metric analysis was used to assess heterogeneity among the included studies. When the I^2 metric value was more than 50%, we considered the involved studies to show an obvious level of heterogeneity that needed to be adjusted by applying a random-effect model. Otherwise, a fixed-effect model was used.

Results

A total of 677 studies were initially retrieved using the aforementioned keywords (Figure 1). After removing duplications, 590 studies remained for further filtration. After carefully scrutinizing the titles and abstracts of the remaining articles, we removed 278 irrelevant studies, 53 entries that were review articles, abstracts or inaccessible full-text manuscripts, 87 non-human studies, and 160 studies that were not case-controlled or lacked the *RASSF1A* methylation data. Along with an additional 3 studies that were manually selected from the reference lists included within the retrieved studies, we collected

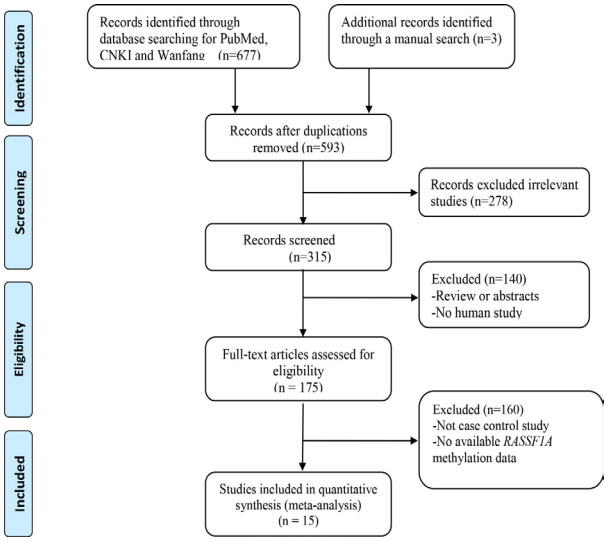
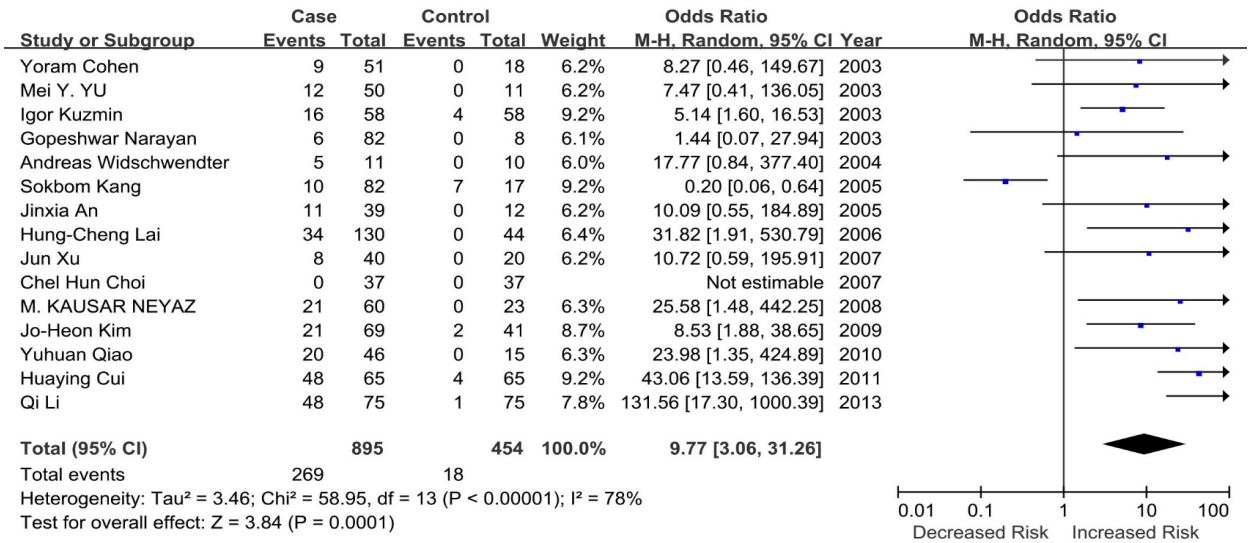


Figure 1. Flow Diagram Representing the Stepwise Selection of Relevant Studies



Study or Subgroup	Case Events	Case Total	Control Events	Control Total	Weight	Odds Ratio M-H, Random, 95% CI	Year
Yoram Cohen	9	51	0	18	6.2%	8.27 [0.46, 149.67]	2003
Mei Y. YU	12	50	0	11	6.2%	7.47 [0.41, 136.05]	2003
Igor Kuzmin	16	58	4	58	9.2%	5.14 [1.60, 16.53]	2003
Gopeshwar Narayan	6	82	0	8	6.1%	1.44 [0.07, 27.94]	2003
Andreas Widschwendter	5	11	0	10	6.0%	17.77 [0.84, 377.40]	2004
Sokbom Kang	10	82	7	17	9.2%	0.20 [0.06, 0.64]	2005
Jinxia An	11	39	0	12	6.2%	10.09 [0.55, 184.89]	2005
Hung-Cheng Lai	34	130	0	44	6.4%	31.82 [1.91, 530.79]	2006
Jun Xu	8	40	0	20	6.2%	10.72 [0.59, 195.91]	2007
Chel Hun Choi	0	37	0	37		Not estimable	2007
M. KAUSAR NEYAZ	21	60	0	23	6.3%	25.58 [1.48, 442.25]	2008
Jo-Heon Kim	21	69	2	41	8.7%	8.53 [1.88, 38.65]	2009
Yuhuan Qiao	20	46	0	15	6.3%	23.98 [1.35, 424.89]	2010
Huaying Cui	48	65	4	65	9.2%	43.06 [13.59, 136.39]	2011
Qi Li	48	75	1	75	7.8%	131.56 [17.30, 1000.39]	2013
Total (95% CI)		**895**		**454**	**100.0%**	**9.77 [3.06, 31.26]**	
Total events	269		18				

Heterogeneity: Tau² = 3.46; Chi² = 58.95, df = 13 (P < 0.00001); I² = 78%
Test for overall effect: Z = 3.84 (P = 0.0001)

Figure 2. Meta-analysis of *RASSF1A* Promoter Methylation Status in Cervical Cancers

a total of 15 studies, representing 895 cervical cancer cases and 454 corresponding controls, for inclusion in our subsequent meta-analyses (Figure 1).

The *RASSF1A* promoter methylation frequency ranged from 0% to 73.85% (median=26.15%) in cervical cancer tissues and from 0% to 41.18% (median=0%) in the paired normal tissues. Our meta-analysis included 15 studies with an overall number of 895 cases and 454 controls and showed a statistically significant correlation between *RASSF1A* promoter hypermethylation and cervical cancer (OR=9.77, 95%CI=(3.06, 31.26), P=0.0001, I^2=78%, Figure 2). A further grouping of the cases within the meta-analysis by specific cervical cancer subtypes showed that aberrant *RASSF1A* promoter hypermethylation tended to be more common in ACs or ASCs (median=24.37%) than in SCCs (median=12.26%), although the difference did not reach statistical significance (OR=0.48, 95%CI=(0.15, 1.53), P=0.21, I^2=80%, Figure 3).

As the critical etiological factor of cervical cancer, oncogenic HPV infection could be detected in most of the invasive cervical carcinomas. Among the 6 studies that included the HPV infection information, the frequency of oncogenic HPV infection ranged from 80% to 100% in the invasive SCCs (median=80.95%) and from 40% to 100%

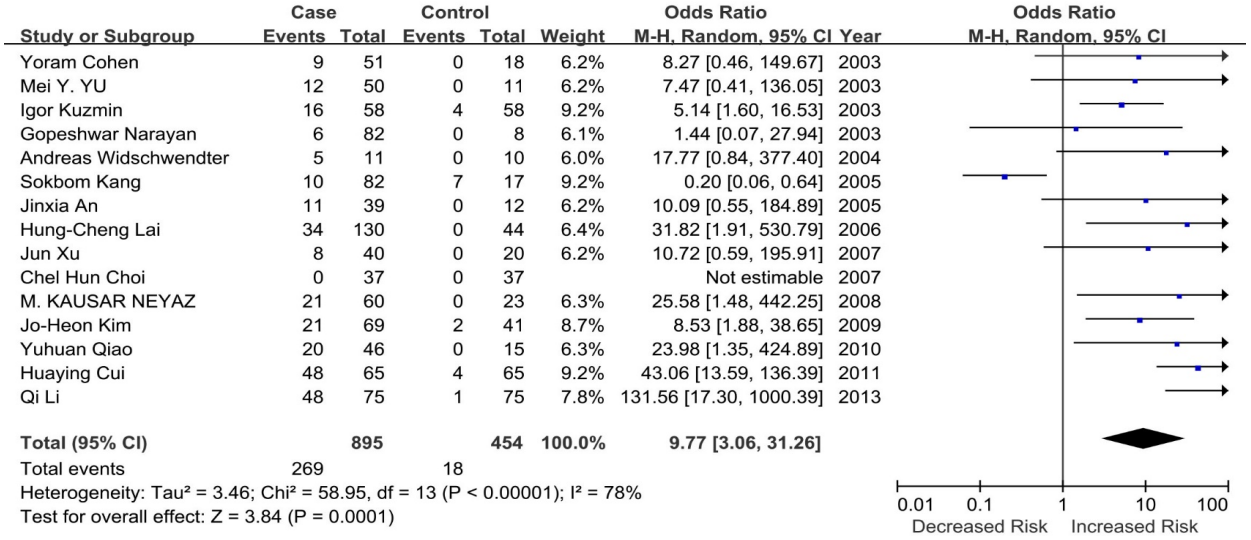


Figure 3. Meta-analysis of *RASSF1A* Promoter Methylation Status in Different Histological Subtypes of Cervical Cancer

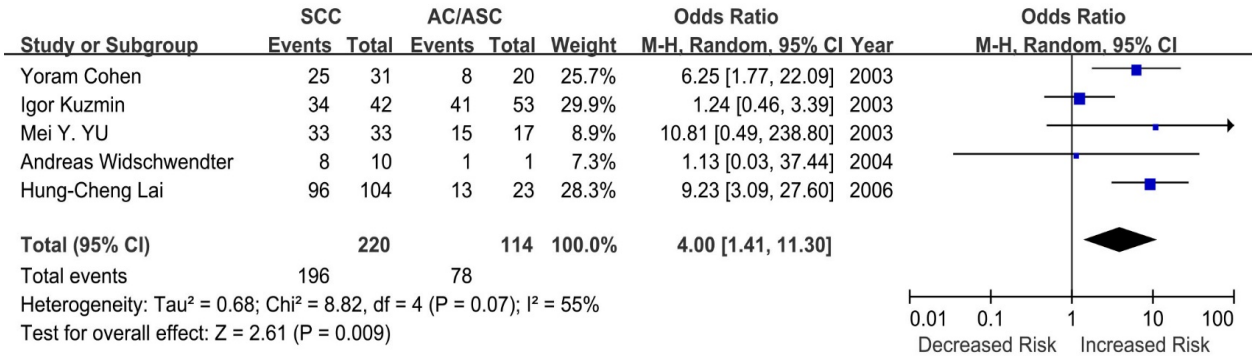


Figure 4. Subgroup Meta-analysis Segregated by HPV Infection Status in Different Histological Subtypes of Cervical Cancer

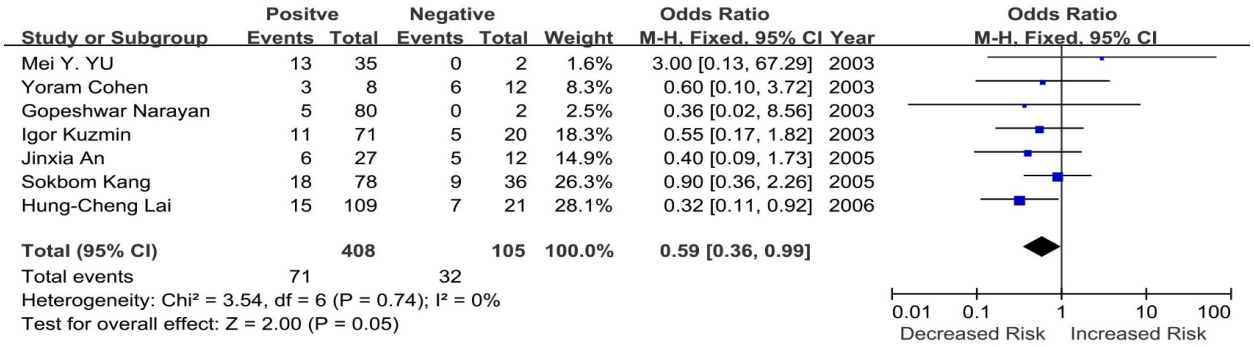


Figure 5. Meta-analysis of *RASSF1A* Promoter Methylation and HPV Infection Status

Table 1. Comparison of the *RASSF1A* Promoter Methylation Frequency in Invasive Cervical Carcinomas

Author	Year	Methylation frequency		Overall OR(95% CI)a	*P* value
		Case	Control		
MeiY.YU	2003	24%	0%	9.77 [3.06, 31.26]	0.0001
Igor Kuzmin	2003	27.6%	6.9%		
Gopeshwar Narayan	2003	7.32%	0%		
Yoram Cohen	2003	17.7%	0%		
Andreas Widschwendter	2004	45.5%	0%		
Jinxia An	2005	28.2%	0%		
Sokbom Kang	2005	12.2%	41.2%		
Hung-Cheng Lai	2006	26.2%	0%		
Jun Xu	2007	20%	0%		
Chel Hun Choi	2007	0%	0%		
M. KAUSAR NEYAZ	2008	35%	0%		
Jo-Heon Kim	2009	30.4%	4.88%		
Yuhuan Qiao	2010	0%	0%		
Huaying Cui	2011	73.9%	6.15%		
Qi Li	2013	64%	1.33%		
Mean		26.2%	0%		

*[a]Odds ratio (OR) representing the gene methylation probability observed in cervical cancer patients compared with normal controls

Table 2. Comparison of the *RASSF1A* Promoter Methylation Frequency in Different Histological Subtypes of Cervical Carcinoma

Author	Year	Methylation frequency		Overall OR(95% CI)[b]	*P* value
		SCC	AC/ASC		
Mei Y. YU	2003	30.3%	11.8%	0.48 [0.15, 1.53]	0.21
Igor Kuzmin	2003	9.52%	22.7%		
Gopeshwar Narayan	2003	6.49%	20%		
Yoram Cohen	2003	0%	45%		
Hung-Cheng Lai	2006	15%	26.1%		
Sokbom Kang	2007	1.40%	23.5%		
Huaying Cui	2011	82%	46.7%		
Qi Li	2013	64.3%	68.4%		
Median		12.3%	24.8%		

*[b]Odds ratio (OR) represents the gene methylation probability observed in SCCs compared with ACs and/or ASCs

Table 3. Comparison of the HPV Infection Status in Different Histological Subtypes of Cervical Carcinoma

Author	Year	HPV infection frequency		Overall OR(95% CI)[C]	*P* value
		SCC	AC/ASC		
Mei Y. YU	2003	100%	88.2%	4.00[1.41, 11.30]	0.009
Igor Kuzmin	2003	81.0%	77.4%		
Yoram Cohen	2003	80.7%	40%		
Andreas Widschwendter	2004	80%	100%		
Hung-Cheng Lai	2006	92.3%	56.5%		
Median		81.0%	77.4%		

*[c]Odds ratio (OR) representing the HPV infection probability observed in SCCs compared to ACs and/or ASCs.

Table 4. Comparison of the *RASSF1A* Promoter Methylation Status in Cervical Cancers with or without Detectable hrHPVs DNA

Author	Year	HPV infection frequency		Overall OR(95% CI)[C]	*P* value
		SCC	AC/ASC		
Mei Y. YU	2003	37.1%	0%	0.59 [0.36, 0.99]	0.05
Igor Kuzmin	2003	15.5%	25%		
Gopeshwar Narayan	2003	6.25%	0%		
Yoram Cohen	2003	37.5%	50%		
Jinxia An	2005	22.2%	41.7%		
Hung-Cheng Lai	2006	13.8%	33.3%		
Sokbom Kang	2007	23.1%	25%		
Median		22.2%	25.0%		

*[d]Odds ratio (OR) representing the probability of RASSF1A promoter methylation observed in HPV-positive cervical cancers compared with HPV-negative cervical cancers

in the ACs or ASCs (median=77.36%). Subsequent meta-analysis suggested a significantly increased likelihood of hrHPV infection in SCCs compared with that of ACs or ASs (OR=4.00, 95%CI=(1.41, 11.30), P=0.009, I^2=55%, Figure 4).

Among the 7 studies that included both HPV infection and *RASSF1A* promoter methylation information, we found that *RASSF1A* hypermethylation ranged from 6.25% to 37.5% (median=22.22%) in hrHPV-positive cervical cancer samples and from 0% to 50% (median=25%) in hrHPV-negative samples. Interestingly, a further stratification within the meta-analysis, including 7 studies totalizing 408 hrHPV-positive cervical cancer patients and 105 hrHPV-negative patients, showed that the *RASSF1A* promoter hypermethylation rate was significantly lower in hrHPV-positive patients (OR=0.59, 95%CI=(0.36-0.99), P=0.05, I^2=0%, Figure 5), suggesting an interactive relationship between these cervical cancer risk factors.

Discussion

Previous studies have shown that the frequency of *RASSF1A* promoter methylation was significantly higher in cervical cancer tissues compared with the corresponding adjacent normal tissue or with benign cervical disease tissue. In parallel, the *RASSF1A* expression was silenced or down-regulated in many cancer cells, including cervical cancer cells (Cohen et al., 2003; Brown et al., 2014; Feng et al., 2014), which suggested that promoter hypermethylation might lead to a reduction of *RASSF1A* expression.

Our meta-analysis revealed that *RASSF1A* promoter hypermethylation was highly associated with cervical cancer risk. An additional subgroup analysis according to different tumor histological parameters demonstrated that *RASSF1A* promoter hypermethylation tended to occur more frequently in ACs/ASCs than in SCCs. The lack of statistical significance in the subgrouped meta-analysis suggested a lack of power in the comparison or that other factors could affect the development of SCC and AC/ASC.

Persistent infection by certain oncogenic HPV types was well established as the underlying cause of most premalignant and malignant epithelial lesions of the cervix (Castellsagué, 2008). The most frequent viral DNA types observed in invasive cervical carcinomas were HPVs 16, 18, 45 and 31, which would account for almost 100% of the cases (Bosch et al., 1995; Walboomers et al., 1999). Interestingly, methylated *RASSF1A* promoter and oncogenic HPVs DNA could be detected simultaneously in ACs or ASCs, whereas in SCCs, the absence of *RASSF1A* promoter methylation coupled with the high presence of HPVs DNA (Cohen et al., 2003; Kuzmin et al., 2003; Yu et al., 2003; Lai et al., 2007). In the current meta-analysis, we combined 7 related studies and identified a significant inverse correlation between *RASSF1A* promoter hypermethylation and the presence of oncogenic HPVs, implying that these two interactive factors might potentially determine the development of cervical cancer subtypes.

Persistent HPV infections introduce the expression of E6 and E7 papillomavirus proteins resulting in the inactivation or deregulation of *p53* and *pRb* tumor suppressor genes and leading to the immortalization of primary human epithelial cells that eventually develop into cancer (Scheffner et al., 1990). The RASSF1A protein promotes apoptosis and arrests the cells in the G1 phase of the cell cycle by inhibiting the accumulation of native cyclin D1 and by engaging the Rb family-dependent cell cycle checkpoint (Shivakumar et al., 2002; Agathanggelou et al., 2005). The E7 papillomavirus protein can bypass Rb family-dependent cell cycle regulation by directly inhibiting the Rb proteins interactions (Farthing and Vousden, 1994), while the RASSF1A protein participates with the Rb family to regulate cell cycle arrest. Moreover, cancer cells expressing the E7 papillomavirus protein were resistant to *RASSF1A*-induced cell cycle arrest, suggesting an interactive relationship between *RASSF1A* and HPV infection. These findings might explain the interesting phenomenon identified by our results regarding the interaction between these risk factors. However, further studies are needed to clarify the interactive mechanisms by which the RASSF1A protein and HPV contribute to the development of specific cervical cancer subtypes.

Although our meta-analysis was performed with careful screening of numerous relevant studies, several limitations must be considered. Above all, conference abstracts and inaccessible full-text articles were excluded from our meta-analysis because we were unable to retrieve the relevant data for the meta-analysis. Moreover, only the literature available in the English or Chinese languages was selected, neglecting results reported in other languages, which introduces a bias in the literature selection. In addition, only 15 studies were included in our analysis. Future studies with a larger sample size are required to confirm our findings.

In summary, our meta-analysis suggested that abnormal *RASSF1A* promoter methylation and HPV infection, identified as two seemingly separate etiological factors in cervical carcinomas, might actually act competitively by involving similar cellular signal transduction pathways. However, the precise mechanistic connection between HPV infection and *RASSF1A* inactivation remains to be determined.

Acknowledgement

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