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REVIEW

Retinoic acid receptor beta promoter methylation and risk of cervical cancer

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Abstract

Cervical cancer is one of the leading causes of death in women worldwide, particularly in developing countries. Human papillomavirus has been reported as one of the key etiologic factors in cervical carcinoma. Likewise, epigenetic aberrations have ability to regulate cancer pathogenesis and progression. Recent research suggested that methylation has been detected already at precancerous stages, which methylation markers may have significant value in cervical cancer screening. The retinoic acid receptor beta (*RARβ*) gene, a potential tumor suppressor gene, is usually expressed in normal epithelial tissue. Methylation of CpG islands in the promoter region of the *RARβ* gene has been found to be associated with the development of cervical cancer. To investigate whether *RARβ* methylation is a potential biomarker that predicts the progression of invasive cancer, we reviewed 14 previously published articles related to *RARβ* methylation. The majority of them demonstrated that the frequency of *RARβ* promoter methylation was significantly correlated with the severity of cervical epithelium abnormalities. However, methylation of a single gene may not represent the best approach for predicting disease prognosis. Analyzing combinations of aberrant methylation of multiple genes may increase the sensitivity, and thus this approach may serve as a better tool for predicting disease prognosis.

Key words: Methylation; Cervical cancer; Retinoic acid receptor beta; Human papillomavirus; Risk correlation; Promoter

Core tip: The frequency of retinoic acid receptor beta promoter methylation was significantly correlated with the severity of cervical epithelium abnormalities. However, a single gene may not represent the best approach for predicting disease prognosis. Thus, combinations of aberrant methylation of multiple genes may as a better tool for predicting disease.

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INTRODUCTION

Cervical cancer is the leading cause of death in women worldwide. The prevalence is high in women in low- to middle-income countries[1]. In 2012, approximately 522000 women globally were diagnosed with cervical cancer, and the mortality rate due to cervical cancer was reported to be 266,000 cases/year[2]. The highest incidence occurred in sub-Saharan Africa while in Asia, cervical cancer remains the third most common cancer (after breast and lung cancer), with an estimated 285000 new cases and 144000 deaths in 2012[3]. The age-standardized incidence rates (ASRs) of cervical cancer estimated by GLOBOSCAN in 2012 indicated that the ASR is higher in less developed compared to more developed regions[4]. In Thailand, the age group with the highest incidence is 45-70 years[5].

Several studies had found that cervical cancer is preceded by a pre-invasive stage, in which abnormal cells are confined to the cervical epithelium. The pre-invasive stage is also known as cervical intraepithelial neoplasia (CIN). The 2014 Bethesda System categorizes squamous epithelial cell abnormalities as atypical squamous cell of undetermined significance (AS-CUS); low-grade squamous intraepithelial lesion (LSIL), which was previously known as CIN Ⅰ; high-grade squamous intraepithelial lesion (HSIL), which was previously known as CIN Ⅱ and Ⅲ; or squamous cell carcinoma (SCC)[6]. SCC represents > 80% of cervical cancers, while adenocarcinoma (AC) accounts for the rest.

The standard method for screening for early-stage cervical neoplasia is cytological morphologic assessment of cervical scrapings. The sensitivity of the conventional Pap smear for identifying CIN Ⅱ+ is 55.2%, while the sensitivity of liquid-based cytology is 57.1%[7]. High-risk human papillomavirus (HPV) DNA testing in combination with the conventional Pap smear increases the sensitivity. Furthermore, biomarkers of oncogenic progression would improve the accuracy of cancer progression predictions. Epigenetic biomarkers may help to fulfil this role, and they have the additional benefit predicting the stage of cervical carcinogenesis progression[8].

GENOME OF HPV

HPV is a small, non-enveloped and circular double-stranded DNA virus with a genome of approximately 8 kb in length[9]. The HPV genome comprises eight protein-coding genes and a noncoding region that is referred to as the regulatory long control region[10]. Only one strand of the DNA carries the protein-coding sequence[11]. Regarding the protein-coding genes, the genes are designated as early (E) or late (L) to indicate when the proteins are expressed in the viral life cycle[12]. The eight protein-coding gene consist of E1, E2, E4, E5, E6, E7, L1 and L2[9]. E1 and E2 are highly conserved and involved in viral DNA replication[13-15]. L1 and L2, which both have a high degree of sequence variation, encode for viral packaging proteins[16]. E4 releases the viral particle from the epithelial cells[17]. E6 and E7 are viral oncogenes that are involved in the integration of the HPV genome into the host genome[18]. There are more than 130 genotypes of HPV, which are categorized based on sequence variation in their L1 region[19]. Of the 130 genotypes, at least 40 genotypes infect the genital areas of humans *via* sexual transmission. HPV can also be classified into cutaneous or mucosal types[12]. The mucosal type can be subdivided into high-, intermediate-, or low-risk types[20].

HPV AND CERVICAL CANCER

The most important risk factor for cervical cancer is HPV infection, which has been found in 90.7% of cervical cancer patients worldwide[21]. HPV infection is a sexually transmitted disease. It has been estimated that more than 80% of sexually active women become infected with HPV, while more than 50% of young women become infected after they first have sexual intercourse[22]. The oncogenic potential of HPV depends on the genotype. HPV 16 and 18 are the most common types associated with invasive cervical cancer[23]. Other HPV genotypes have been found to be related to cancer, but their oncogenic risk differs among the various populations, geographic regions, and age groups.

At the country level, collecting baseline data on the local burden of specific HPV genotypes related to cervical cancer is important. This information can impact the local HPV vaccination policies. A meta-analysis revealed that HPV 16, 18, 31, 33, 45, 52, and 58 are responsible for more than 90% of cervical cancers worldwide[20]. These genotypes represent the baseline genotypes to include in a vaccine targeting the genotypes circulating in the population[4]. The current HPV vaccines were developed to prevent HPV infection, and thus prevent cervical carcinoma. HPV vaccines have been implemented in routine vaccination programs in several developed and developing countries worldwide[24]. To

date, there have been three HPV vaccines in clinical use: Bivalent, quadrivalent, and nanovalent vaccines[25].

Other independent risk factors such as immunosuppression, individual lifestyle, and smoking have been found to be associated with the development of HPV-related cervical cancer[21,26]. Most HPV infection is transient, and clearance of the virus can occur spontaneously over a 3-year period[27]. However, in some cases, persistent infection can result in cervical cancer development. The transition from dysplasia to invasive carcinoma may take several years to decades to develop. HPV initially infects the basal layers of the epithelium through micro-wounds. The virus begins to replicate, and when infected daughter cells migrate to the upper layers of the epithelium, the viral late genes are activated, and viral DNA is packaged into capsids. Progeny virions are released to re-initiate infection, which can result in persistent and/or asymptomatic infection[28]. The integration of HPV into the host genome can lead to carcinogenic transformation. Certain regions of the human genome are favored for viral DNA insertion such as fragile sites, rupture points, translocation points, and transcriptionally active regions[29]. Moreover, the virus can induce epigenetic modification of viral and cellular genes, which affect their expression, leading to malignant cell transformation[30,31].

HOST GENETIC FACTORS AND CERVICAL CANCER

Diverse immunogenetic associations with HPV infection, persistence, and transformation have been extensively investigated. Recent studies have looked at multiple genes in various populations with different environment interactions[32]. HPV infection alone might not be sufficient for the development of cervical carcinoma, and certain antigen-processing machinery (APM) and single-nucleotide polymorphisms (SNPs) may lead to a smaller immunogenic peptide repertoire for presentation to local immune cells. This can result in further attenuation of cytokine and receptor expression, which leads to an ineffective overall immune response and progression to carcinoma[33]. The Genome-Wide Association Study (GWAS) for polymorphisms of host immune response genes showed that variation in several genes contributes to different risks of cervical cancer. The integrative approach, which is also known as systems biology, could help explain the complexity of host–virus interactions and provide a better understanding that may eventually lead to personalized prevention, diagnosis, and treatment[34-36].

The detection of methylated genes in cervical specmens is a feasible technique and represents a potential source of biomarkers that are of relevance to carcinogenesis. In particular, there are methylation markers that, among HPV-infected women, indicate the presence of CIN Ⅱ+ and risk of cancer[37].

High expression levels of certain oncoproteins in cervical cells have been found to be associated with cervical carcinoma. One study found a strong correlation between centromere protein H (CENP-H) expression and cervical carcinoma in a Chinese population[38]. Another study found that expression of the B-cell-specific Moloney leukemia virus insert site 1 (Bmi-1), P16, and CD44v6 (a CD44 variant) were significantly higher in cervical carcinoma tissues compared with precancerous lesions and normal tissues[39]. In addition, abnormalities in the phosphatidylinositide 3-kinase (PI3K) pathway induced by mutations in PI3K catalytic subunit α (PIK3CA) were associated with shorter survival in cervical cancer patients[40]. Recently, deep sequencing of somatic mutations has identified several novel mutations in carcinoma cells, including E322K in the mitogen-activated protein kinase 1 (*MAPK1*) gene, inactivating mutations in the major histocompatibility complex, class I, B (HLA-B) gene, and mutations in F-box and WD repeat domain containing 7 (*FBXW7*), tumor protein p53 (*TP53*), and Erb-B2 receptor tyrosine kinase 2 (*ERBB2*)[41].

EPIGENETIC MECHANISMS AND RISK OF CANCER DEVELOPMENT

Recent studies also investigated epigenetic mechanisms related to HPV infection, including methylation of the host and viral genes, and chromatin modification in host cells[42]. Epigenetic mechanisms affect gene regulation without changing the genetic sequences, and these mechanisms have been increasingly found to be associated with cancer development[43]. The main epigenetic mechanism is methylation patterning, which occurs to various extents in different DNA and proteins. DNA methylation is a mechanism of gene regulation that typically occurs in CpG dinucleotide contexts, resulting in genomic instability. Methylation of heterochromatin and promoter regions is associated with decreased gene transcription. Several studies have found that DNA methylation frequently occurs in cervical cells but rarely in normal cells, suggesting that their methylation is highly related to the severity of cervical neoplasia[44]. Several markers have been evaluated extensively in studies involving women with precancerous and cancerous cervical lesions[44-46]. Epigenetic methylation in the promoter region of several tumor suppressor genes (TSGs) has been detected in precancerous cervical cells[47,48]. Genes that were found to be significantly associated with promoter methylation were RASSF1A and MGMT (involved in DNA repair), CDKN2A (involved in cell cycle control), PYCARD (involved in apoptosis), and APC and SFRP1 (involved in Wnt signaling)[49].

One striking conclusion of previous studies was that methylation frequencies for the same gene vary widely between studies. It was difficult to identify highly consistent results for most genes even when restricting analyses to studies of similar size or those that used common specimen sources or similar assays.

This suggests that the frequency of certain methylation markers may also vary for reasons related to differences in populations, specific features of assay protocols, chance, or other unidentified factors. The most important prerequisite for a potential biomarker is that it must be reliable in its measurement. There is a possibility that the wide range of frequencies reported for some genes (in contrast to the more consistent measurement of a few other genes in similar studies) could be related to unreliable assays for these specific genes rather than biological variation. Another prerequisite for a good biomarker is that it has high sensitivity and high specificity for disease detection, resulting in a high positive predictive value. Several studies have proposed the use of methylated gene panels in order to obtain optimal assessment performance for cervical cancer screening[47,50].

Retinoic acid (RA) is an essential regulator of normal epithelial cell differentiation. The effect of RA is mediated by two types of nuclear receptors, the retinoic-acid receptor (RAR) family and retinoid-X receptor (RXR) family. Both of these receptor families have three members (alpha, beta, and gamma), which are encoded by distinct genes in vertebrates. The retinoic acid receptor beta (*RARβ*) gene encodes a nuclear receptor that binds RA and mediates cellular signaling. It is important during differentiation of stratified squamous epithelium, including cervical epithelium. It is considered to be a potential TSG. The *RARβ* gene is usually expressed in normal epithelial tissue. The direct roles of the RARβ protein include regulating gene expression and differentiation, immune modulation, and inducing apoptosis. Previous studies revealed that the *RARβ* gene is downregulated in high-grade lesions[51]. *RARβ* gene silencing was observed in carcinoma cells[52]. Recent research suggested that the RARβ protein can suppress cervical carcinogenesis and may play a role in the early development of cancer[51]. CpG methylation of the 5' region of the *RARβ* gene contributes to gene silencing, and this methylation is associated with increased grades of SIL and invasive cervical cancer. Many studies have revealed that methylation of CpG islands in the promoter region of the *RARβ* gene induces repression of *RARβ* expression in several epithelial carcinomas, including cervical cancer[53-55].

The risk of cervical cancer due to *RARβ* methylation remains inconsistent across different studies[51,52,56]. Therefore, we reviewed previously published articles and summarized the relationship between *RARβ* promoter methylation and cervical cancer (Table 1).

Among the 14 articles reviewed, the majority of them (11/14) demonstrated that the frequency of *RARβ* promoter methylation was significantly correlated with severity of cervical epithelium abnormalities. Three studies did not concur with this finding. The first study was conducted in 2003 with a small sample size and no cancer patients were involved[37]. The other two studies were conducted in 2010 and 2015. Both studies found that normal tissue also had *RARβ* promoter methylation, which made it a poor predictor of progression to severe disease[62,64]. However, one of the two studies also investigated the level of methylation using quantitative methylation-specific PCR and found that although normal cells were methylated, the level of methylation increased in LSIL, HSIL, and invasive cancer tissue[62].

In addition, both Narayan *et al*[56] and Choi *et al*[60] found that *RARβ* promoter methylation was associated with cervical cancer prognosis. Narayan *et al*[56] found that 80% of the patients with *RARβ* methylation either died of cancer or only partly responded to treatment, while Choi *et al*[60] found that absence or reduction of RARβ protein expression was associated with a higher level of SCC antigen (P = 0.04) and more frequent lymph node metastasis (P = 0.023).

A study of the frequency of *RARβ* promoter methylation in urine and cervical samples from Senegalese women and cervical epithelial cell abnormalities found that methylation was significantly greater in abnormal specimens (and the results from the urine samples correlated with the results from the cervical swab samples)[58,65]. Another study by Zhang *et al*[52] compared the frequency of methylation with *RARβ* mRNA expression. The authors found that in normal cervical cells, the *RARβ* gene was highly expressed. In contrast, among 17 samples from patients with invasive cervical carcinoma, *RARβ2* expression was completely repressed in 13 samples, highly repressed in 2 samples, and moderately downregulated in 2 samples. Among the 13 samples with completely repressed *RARβ2* expression, the *RARβ* promoter region was methylated in 9 samples and unmethylated in 4 samples. The authors then further investigated the silencing mechanism and discovered that apart from methylation, repressive histone modifications also played a role in gene silencing, which could contribute to the development of cervical carcinoma.

Four studies performed a quantitative assessment of methylation. The first study was conducted in 2006 by Wisman *et al*[59], who found that the *RARβ2* promoter was more methylated in cervical cancer than in control tissue. Four years later, Kim *et al*[61] found that the *RARβ* methylation level in normal tissue was 1.59% ± 3.51% whereas, in HSIL and SCC, it was 21.93% ± 20.10% and 19.06% ± 19.39%, respectively. The third study, by Yang *et al*[62], also highlighted that although the percentage of methylated samples was very high in normal tissue, the level of methylation correlated with disease severity. The last study was conducted by Sun *et al*[51] in 2015. They found that among 250 cervical samples from healthy individuals and patients with various stages of cervical epithelium abnormalities, the percentage of methylation in patients showed that 68.8% had no *RARβ* promoter methylation, 26.4% had 0%-5% methylation, and 4.8% had 5%-25% methylation. No samples had methylation levels above 25%.

In addition, two studies performed immunohistochemistry staining of the RARβ protein in cervical cells. Narayan *et al*[56] found that in the LSIL group, 11% had

Table 1 The summary of the articles that investigated the methylation of *RARβ* gene in tumor tissue from women diagnosed with squamous intraepithelial lesion and cervical cancer

Ref.	Year of publication	Nationality of participants	Sample size	Source of samples	Lab technique	*RARβ* methylation results
Virmani *et al*[57]	2001	American	Normal/LSIL = 37	Normal/LSIL/HSIL from liquid-based cytology specimen	MSP	*RARβ* methylation positive in
			HSIL = 17 ICC = 19	ICC from biopsy tissue		Normal/LSIL = 11% HSIL = 29% ICC = 26%
Narayan *et al*[56]	2003	Colombians	Normal = 8	Normal = cells from cervical swab LSIL/HSIL = formalin-fixed and paraffin-embedded cervical tissues	MSP	*RARβ* methylation positive in
		German	LSIL = 9			Normal = 0% SCC/AC = 29.3%
		American	HSIL = 30	SCC/AC = tumor biopsies	Immuno-histochemistry of *RARβ* protein	Immunohistochemistry
			SCC = 77 AC = 5			LSIL; 11% showed low expression HSIL; 60% showed complete lack of expression
Gustafson *et al*[37]	2004	American	Normal = 11	Liquid-based cytology specimen	Nested MSP	*RARβ* methylation positive in
			LSIL = 17 HSIL = 11			Normal = 0% LSIL = 0% HSIL = 9.1%
Feng *et al*[58]	2005	Senegalese	Normal/ASCUS = 142 CIN Ⅰ = 39 CIN Ⅱ = 23 CIN Ⅲ = 23 ICC = 92	Exfoliated cervical cells and tissue biopsy	MSP	*RARβ* methylation positive in Normal/ASCUS = 3.2% CIN Ⅰ = 0% CIN Ⅱ = 0% CIN Ⅲ = 15.8% ICC = 38.2%
Wisman *et al*[59]	2006	Dutch	Normal = 19 SCC = 20 AC = 8	Cervical scraping	QMSP	The percentage of *RARβ* methylation level above control ratio were detected in Normal = 0% SCC = 15% AC = 25%
Choi *et al*[60]	2007	Korean	Normal = 37	Normal cells were from hysterectomy due to myoma	MSP	*RARβ* methylation positive in
			SCC = 37	Cancer cells were from tissue after surgery	Immuno-histochemistry of *RARβ* protein	Normal = 0% SCC = 41% Immunostaining normal = strong staining SCC = 43% absent staining
Zhang *et al*[52]	2007	Japanese and Chinese	Normal = 6 ICC = 17	Cervical tissue by biopsy or surgery	Real-time PCR for *RARβ* mRNA	*RARβ* expression level among normal cells: All were highly expressed RARb2 expression level among cancer cells:
					Semi-nested MSP	13/17: Completely repressed 2/17: Highly repressed 2/17: Moderately down-regulated Among 13 samples with completely repressed mRNA expression 9 promoter methylated, 4 unmethylated
Flatley *et al*[2]	2009	English	Normal = 58 CIN Ⅰ = 68 CIN Ⅱ = 56 CIN Ⅲ = 76 ICC = 50	Exfoliated cervical cells and cervical biopsy	Nested MSP	*RARβ* methylation positive in Normal = 6.5% CIN Ⅰ = 42.6% CIN Ⅱ = 6.3% CIN III = 0% ICC = 15.9%
Kim *et al*[54]	2010	Korean	Normal = 41 LSIL = 32 HSIL = 67 SCC = 69	Liquid based cytology specimen	Multiplex nested MSP	*RARβ* methylation positive in Normal = 4.9% LSIL = 15.6% HSIL = 46.3% SCC = 53.6%
Kim *et al*[61]	2010	Korean	Normal = 28	Liquid based cytology specimen	Multiplex QMSP	*RARβ* methylation level

			LSIL = 26			Normal = 1.59+3.51% LSIL = 3.67+9.09%
			HSIL = 45 SCC = 63			HSIL = 21.93+20.10% SCC = 19.06+19.39%
Yang *et al*[62]	2010	Dutch	Normal = 20	Biopsy tissue	QMSP	*RARβ* methylation positive (from tissue) in
			LSIL = 20			Normal = 85% LSIL = 65%
			HSIL = 20	Cervical scraping only available in subset of samples		HSIL = 75% SCC = 85% AC = 85%
			SCC = 40			*RARβ* methylation positive (from scraping) in
			AC = 20			Normal = 44% LSIL = 37.5% HSIL = 55.6% SCC = 83.8% AC = 100% The median methylation level increased significantly with the severity of lesion ($P < 0.05$)
Pathak *et al*[63]	2012	Indian	Normal = 35	Normal cells from hysterectomy SIL from excision ICC from tissue biopsy	MSP	*RARβ* methylation positive in
			SIL = 27			Normal = 11.4% SIL = 55.5% ICC = 57.8%
			ICC = 38			
Milutin Gašperov *et al*[64]	2015	Croatian	Normal = 40	Cervical scraping	MSP	*RARβ* methylation positive in
			CIN Ⅰ = 40			Normal = 62.5%
			CIN Ⅱ = 40			CIN Ⅰ = 35%
			CIN Ⅲ = 42			CIN Ⅱ = 61.5%
			SCC = 8 AC = 3			CIN Ⅲ = 61.9% SCC/AC = 90%
Sun *et al*[51]	2015	Chinese	Normal = 48	Liquid based cytology specimen	Methylation specific high resolution melting analysis (Quantitative)	*RARβ* methylation positive in
			CIN Ⅰ = 54			Normal = 31.3% CIN Ⅰ = 35.2%
			CIN Ⅱ = 47			CIN Ⅱ and Ⅲ = 28.2% SCC = 33.3%
			CIN Ⅲ = 56			*RARβ* methylation level: none = 68.8%
			SCC = 45			0-5% methylation = 26.4% 5-25% = 4.8%

CIN: Cervical intraepithelial neoplasia; SIL: Squamous intraepithelial lesion; LSIL: Low-grade squamous intraepithelial lesion; HSIL: High-grade squamous intraepithelial lesion; SCC: Squamous cell carcinoma of the cervix; AC: Adenocarcinoma of cervix; ICC: Invasive cervical cancer; MSP: Methylation-Specific Polymerase Chain Reaction; QMSP: Quantitative methylation-specific polymerase chain reaction; ASCUS: Atypical squamous cells of undetermined.

low RARβ expression whereas, in the HSIL group, 60% had a complete lack of RARβ expression. This finding suggested that the downregulation of the *RARβ* gene occurs early in the development of cervical carcinoma[56]. The second study was carried out by Choi *et al*[60], who discovered that all normal tissues highly expressed the RARβ protein, whereas no staining was detected in 43% of the SCC tissues.

Almost of cancer cell lines and primary cancer tissues examined, the *RARβ2* was repressed. The repression was frequently associated with promoter methylation, which causes lack of gene expression. These results strongly support the hypothesis that promoter methylation is the epigenetic cause of *RARβ2* repression in cervical cancers harboring methylated *RARβ2* promoters. A DNA demethylating reagent can reactivate gene expression by inducing drastic demethylation of the promoter in repressed cells carrying a methylated promoter[44]. This consistency between promoter demethylation and *RARβ2* derepression strongly suggests that the primary cause of *RARβ2* repression is indeed promoter methylation.

Several hypotheses have been proposed regarding the mechanisms of DNA methylation that lead to silencing of genes. In some cancer cells and tissues examined, *RARβ2* was repressed without promoter methylation. These facts indicate that although DNA methylation is the major epigenetic mechanism for gene silencing, there are other epigenetic silencing pathways independent of DNA methylation. *RARβ2* is frequently silenced in cervical cancers by one of two epigenetic mechanisms. One is DNA methylation, a well-known epigenetic mechanism leading to transcriptional silencing of genes, while the other involves the formation of repressive histone modifications near the promoter, by unknown mechanisms independent of DNA methylation. At present, the initial causes of these epigenetic changes during carcinogenesis are unclear. *RARβ2* silenced by promoter methylation can be reactivated by promoter hypomethylation. This result indicates the importance of examining promoter methylation if epigenetic modulation drugs are to be used for chemotherapy in patients with cervical cancers.

In conclusion, DNA methylation of TSGs likely contributes to the development of cancer. Although DNA

methylation of only one gene may not represent the complete process of epigenetic silencing, it has been shown to be significantly correlated with cervical cancer. Analyzing combinations of aberrant hyper- or hypo-methylation of multiple genes may increase the sensitivity of prognoses. Thus, this approach may serve as a better tool for predicting disease progression. Risk factors should also be further characterized to better understand the pathogenesis of cervical carcinoma.

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