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Review

Aberrant DNA methylation in cervical carcinogenesis

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Abstract

Persistent infection with high-risk types of human papillomavirus (HPV) is known to cause cervical cancer; however, additional genetic and epigenetic alterations are required for progression from precancerous disease to invasive cancer. DNA methylation is an early and frequent molecular alteration in cervical carcinogenesis. In this review, we summarize DNA methylation within the HPV genome and human genome and identify its clinical implications. Methylation of the HPV long control region (LCR) and *L1* gene is common during cervical carcinogenesis and increases with the severity of the cervical neoplasm. The *L1* gene of HPV16 and HPV18 is consistently hypermethylated in invasive cervical cancers and can potentially be used as a clinical marker of cancer progression. Moreover, promoters of tumor suppressor genes (TSGs) involved in many cellular pathways are methylated in cervical precursors and invasive cancers. Some are associated with squamous cell carcinomas, and others are associated with adenocarcinomas. Identification of methylated TSGs in Pap smear could be an adjuvant test in cervical cancer screening for triage of women with high-risk HPV, atypical squamous cells of undetermined significance, or low grade squamous intraepithelial lesion (LSIL). However, consistent panels must be validated for this approach to be translated to the clinic. Furthermore, reversion of methylated TSGs using demethylating drugs may be an alternative anticancer treatment, but demethylating drugs without toxic carcinogenic and mutagenic properties must be identified and validated.

Key words Cervical cancer, cervical intraepithelial neoplasm, DNA methylation, human papillomavirus, tumor suppressor genes

Cervical cancer remains a common cancer in women worldwide. According to GLOBOCAN statistics on cervical cancer[1], there were approximately 529 000 new cases and 275 000 deaths in 2008, of which more than 85% occurred in developing countries. In China, there are an estimated 135 000 new cases and 80 000 deaths attributed to the disease annually. Moreover, the incidence of cervical cancer in young Chinese women (≤30 years old) is increasing by 2%–3% each year.

Epidemiologic and molecular studies support that persistent infections with high-risk types of human papillomavirus (HPV) are essential but not exclusive prerequisites for cervical carcinogenesis[2]. Additional genetic and epigenetic alterations are required for progression from precancerous disease to invasive cancer. Among the earliest and most common molecular changes in the multistep carcinogenesis process is DNA methylation[3]. DNA methylation, an important epigenetic mechanism for gene silencing, tends to accumulate with disease severity[4,5] and has been demonstrated in cervical cancer and its precursors[5,6].

In this review, I summarize DNA methylation within the human genome and HPV genome and discuss the clinical implications of this epigenetic change in cervical cancer screening, diagnosis, prognosis, and treatment.

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Human Papillomavirus Genome

High-risk HPVs (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) are causative agents of cervical

cancer and its precursors. The HPV genome is a double-stranded circular DNA that is divided into three regions: the long control region (LCR), early region (E1, E2, E4, E5, E6, and E7), and late region (L1 and L2). The LCR regulates DNA replication, and the early region is involved in viral replication and oncogenesis[7]. The *L1* and *L2* genes produce viral capsule products. The *E6* and *E7* gene products are the key HPV proteins that initiate tumors. In addition to deregulating the host cell growth cycle by binding and inactivating P53 and pRB, respectively, the two proteins interrupt multiple cellular pathways by interacting with various host cell targets, including those that regulate genomic instability (ATM, ATR and γ-tubulin), apoptosis (Caspase 8, BAX, BAK, IRF3, P53, P600, and P48), cell proliferation (E6AP, HDAC, P107, P130, and Cyclins), and immortalization (TERT, MYC, and NFX123)[8]. The *E2* gene product is a DNA-binding protein that blocks transcription of the *E6* and *E7* genes. During cervical carcinogenesis, there are two types of HPV DNA within the nucleus of the host cell: episomal and integrated. Integration of HPV DNA disrupts or deletes the *E2* gene, which results in loss of its expression. Thus, the normal function of *E2* is lost, leading to increased expression of *E6* and *E7* [8]. The function of E5 protein is less clear. Recent studies support that it enhances the functions of E6 and E7 through the activation of signaling proteins, B-cell receptor-associated protein 31 (BAP31), the vacuolar H^+-ATPase and others[8]. Consequently, impairment of those cellular processes, including apoptosis, DNA repair, and cell cycle progression, leads to stimulation of cell proliferation, accumulation of DNA damage, and eventually malignant transformation.

DNA Methylation of the HPV Genome

The carcinogenic role of HPV lies mainly with the HPV E6 and E7 oncoproteins, whose expression is controlled by the promoter *p97* and its enhancer and silencer sequences in the HPV LCR[7]. DNA methylation of the HPV16 LCR is one mechanism by which E6 and E7 are down-regulated and might be a host defense mechanism[9,10]. The LCR is also the location of E2 binding sites and contains CpG sites for potential methylation, resulting in inhibition of E2 function. Ding *et al.*[9] found that CpG hypermethylation of the HPV16 LCR increased with the severity of the cervical neoplasm [low grade squamous intraepithelial lesion (LSIL): 5.9%; high grade squamous intraepithelial lesion (HSIL): 33.3%; squamous cell carcinoma (SCC): 53.3%]. Hong *et al.*[10] found that HPV16 LCR methylation in the promoter and enhancer core was more common in invasive cervical carcinomas and cervical intraepithelial neoplasia (CIN) III than in CIN I–II (84.6% and 46.2% vs. 29.4%, respectively). However, it was also present in 71.4% of asymptomatic infection cases. Thus, the clinical implication of LCR methylation needs further investigation. Studies of invasive cervical cancers consistently show that the *L1* gene of HPV16 and HPV18 is hypermethylated[11-13], and hypermethylation increases with the severity of the disease. Moreover, Turan *et al.*[12] found that *L1* hypermethylation was more common than *LCR* hypermethylation. *L1* gene hypermethylation might be a consequence of HPV genome integration into cellular DNA and could potentially be used as a clinical marker of cancer progression[11].

CpG Methylation in Tumor Suppressor Gene (TSG) Promoters in Cervical Cancer

CpG methylation of TSG promoters, an important epigenetic mechanism for gene silencing, is an early and frequent alteration in carcinogenesis [14] and provides a growth advantage to tumor cells [4]. CpG island hypermethylation is widespread in the human genome, but only a subset of affected loci play critical roles in tumorigenesis. Indeed, CpG island hypermethylation is not randomly distributed in carcinogenesis; it is gene- and cancer type-specific [15-18]. Thus, specific methylation patterns among tumor types may provide a useful signature for tumor diagnosis and prognosis[17].

CpG island hypermethylation has been reported in cervical carcinomas[5,19] and occurs independently of HPV infection status. The affected genes are involved in many cellular pathways[5]. Some are associated with squamous cell carcinomas, whereas others are associated with adenocarcinomas[20-22]. Here, we review genes commonly methylated in cervical cancers.

TSGs associated with the cell cycle

The p16 protein is a cyclin-dependent kinase inhibitor and loss of its expression is common in most malignancies. However, p16 overexpression increases with the severity of cervical dysplasia [23], and this is due to HPV-mediated oncogenesis. In cervical carcinogenesis, the HPV E7 protein binds to cellular pRB, disrupting its functions and altering cell cycle regulatory pathways. Reduced levels of free Rb lead to overexpression of p16. *p16* hypermethylation is common in cervical neoplastic progression. Huang *et al.* [24] reported that it was present in 17.6% of CIN I, 42.1% of CIN II, 55.0% of CIN III, and 65.0% of invasive cancers. However, the loss of *p16* due to epigenetic silencing is overcome by the HPV viral mechanism. Indeed, *p16* hypermethylation was found to be independent of the

presence of HPV DNA and might be associated with smoking in cervical carcinogenesis[25]. Furthermore, it may be a useful biomarker with which to predict progression in the follow-up of low-grade dysplasia and recurrence of high-grade intraepithelial neoplasias after conization[26].

The *CCNA1* gene encodes Cyclin A1, which binds to both the CDK2 and CDC2 kinases and regulates the cell cycle. Cyclin A1 was also found to bind to the Rb family of proteins, the transcription factor E2F-1, and the p21 family of proteins[27]. Cyclin A1 methylation, which leads to down-regulation of its expression, is common in cervical cancer. Kitkumthorn *et al*.[28] observed *CCNA1* promoter hypermethylation in 36.6%, 60%, and 93.3% of HSIL, microinvasive cancers, and invasive cancers, respectively. In other studies, *CCNA1* was rarely methylated in normal cervices and LSILs[29]. Therefore, *CCNA1* methylation may be a good marker to distinguish normal/LSIL from HSIL/cancer lesions.

The fragile histidine triad (FHIT) protein is another protein involved in cell cycle regulation and apoptosis. Inactivation of FHIT is common in cervical cancer. Epigenetic silencing of the *FHIT* gene by promoter hypermethylation has been suggested to be more common than genetic alterations[30]. Re-expression of *FHIT* after treatment with 5-aza-CdR in cervical cancer cell lines resulted in inhibition of cell proliferation[31]. Whether it could be an effective epigenetic target for the treatment of cervical cancer warrants further study to confirm.

TSGs involved in cell adhesion

Cell adhesion molecule 1 (CADM1) is involved in epithelial cell adhesion, and loss of its expression is associated with cancer invasion and/or metastasis. Reduced *CADM1* expression associated with promoter hypermethylation has been demonstrated in high-grade CIN and SCC[32]. Overmeer *et al*.[32] reported that frequency and density of *CADM1* methylation increased with the severity of cervical dysplasia associated with high-risk HPV infection. Moreover, significantly decreased *CADM1* expression was observed in tumors with two or more methylated regions within the gene ($P < 0.001$)[32]. Their recent study further supported that *CADM1* promoter methylation may be a useful epigenetic marker for the triage of high-risk HPV-positive women at risk for high-grade dysplasia[33].

E-cadherin is a calcium-dependent transmembrane glycoprotein involved in maintaining epithelial adherent junctions. It plays an important role in cell growth and development via the control of tissue architecture and integrity. Numerous studies have demonstrated that down-regulation of E-cadherin expression in carcinomas associates with invasion and metastasis. Silencing E-cadherin by promoter hypermethylation has been found in cervical cancer cell lines and specimens[34]. E-cadherin gene hypermethylation occurs according to the severity of cervical dysplasia. Shivapurkar *et al*.[35] found this epigenetic change in 89% of invasive cervical cancers whereas only in 26% of CIN III cases and none of normal tissues. Thus, E-cadherin gene hypermethylation might be a candidate epigenetic marker for early detection of cervical cancer.

TSGs associated with apoptosis

DAPK, a pro-apoptotic serine/threonine kinase, plays a role in tumor pathogenesis and metastasis[36]. *DAPK* gene inactivation, mainly by promoter hypermethylation, has been reported in cervical cancer[37,38]. In our previous study, we demonstrated that *DAPK* hypermethylation was detectable in 60% of cervical cancers. Furthermore, this epigenetic alteration could be detected in plasma from cancer patients[39,40]. Using quantitative methylation-specific PCR, Reesink-Peters *et al*.[41] confirmed that *DAPK* methylation levels in cervical scraping samples were concordant to the levels in paired tumor tissue specimens. Using the same method, Yang *et al*.[29] reported that frequency of *DAPK* methylation increased with the severity of cervical dysplasia. Thus, evaluation of *DAPK* methylation levels in cervical scraping samples may be useful for cervical cancer detection.

TSGs involved in cell signaling pathways

The retinoic acid receptor-β2 (*RARβ2*) gene is a retinoic acid-regulated TSG and belongs to the nuclear receptor superfamily. *RARβ2* is associated with the cell growth suppression function of retinoic acid. Epigenetic silencing of *RARβ2* by promoter hypermethylation leads to retinoic acid therapy failure in breast cancers[42]. In cervical cancer cell lines SiHa and HeLa, demethylation of the *RARβ2* promoter by the natural compounds curcumin and genistein led to re-expression of the gene[43]. Thus, *RARβ2* might be an epigenetic therapeutic target of natural compounds.

The Ras association domain family 1 isoform A (*RASSF1A*) gene expresses a RASSF1 family protein involved in cell cycle suppression, apoptosis, and genetic instability. It plays a key role in suppressing Ras-mediated oncogenesis[44]. Promoter hypermethylation is the most common mechanism for transcriptional silencing of the *RASSF1A* gene and has been found in various epithelial tumors[45]. Notably, however, *RASSF1A* promoter hypermethylation was present in less than 10% of cervical squamous cell carcinomas and none of the HPV16/18-positive squamous cell carcinomas. In contrast, *RASSF1A* promoter methylation was identified

in 20%–45% of adenocarcinomas and adenosquamous carcinomas[46,47]. Thus, epigenetic silencing of *RASSF1A* may contribute to the development of cervical adenocarcinoma.

TSGs in the Wnt/β-catenin pathway

Adenomatous polyposis coli (APC) is a tumor suppressor protein involved in the Wnt/β-catenin pathway. Loss of *APC* expression leads to overexpression of β-catenin targets, including cyclin D, c-Myc, ephrins, and caspases[48]. Transcript silencing by promoter hypermethylation is one mechanism for reduction or loss of *APC* expression. *APC* hypermethylation has been documented in 60% of cervical adenocarcinomas, significantly higher than in squamous cell carcinomas (13%)[49]. *In vitro*, methylated *APC* was identified in the cervical adenocarcinoma cell line HeLa but not in the squamous cell carcinoma cell line SiHa. Demethylation of HeLa cells with hydralazine results in *APC* re-expression[50]. Thus, *APC* methylation is a potential epigenetic marker for cervical adenocarcinoma.

TSGs in the p53 signaling pathway

p73, a member of the *p53* TSG family, shares sequence and functional homology with *p53*. It is also involved in cell cycle regulation and apoptosis. Unlike *p53*, *p73* mutation is rare in human cancers. Promoter hypermethylation is an essential mechanism for silencing the *p73* gene. Liu *et al*. [51] found that promoter hypermenthylation was present in 38.8% of cervical cancers and associated with a decrease or loss of *p73* expression. Furthermore, this epigenetic alteration was more common in radioresistant cancers than in radiosensitive cancers (58.0% vs. 20.8%, $P < 0.001$). Thus, *p73* hypermethylation might be a therapeutic target for radiotherapy sensitization in cervical cancers.

TSGs involved in DNA repair

The O^6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair protein that protects the human genome from mutagenic and carcinogenic actions of endogenous carcinogens. Because it removes alkyl adducts from the O^6 position of guanine in DNA, MGMT also induces resistance to alkylating agents[52]. Loss of *MGMT* expression has been found in approximately 17% (5%–92%) of cervical cancers[53]. Promoter hypermethylation and histone deacetylation influence *MGMT* expression. In a recent study, the frequency of *MGMT* promoter hypermethylation increased with the severity of the cervical lesions (2.4% in normal, 3.1% in LSIL, 11.9% in HSIL, and 26.1% in SCC, $P < 0.001$)[54]. Furthermore, the specificity of *MGMT* hypermethylation in distinguishing NLM/LSIL from HSIL/SCC was 97%[54]. In gliomas, tumors with loss of *MGMT* expression because of hypermethylated promoter were more sensitive to alkylating drugs than those with *MGMT* expression[55]. Nevertheless, whether *MGMT* promoter hypermethylation can be used as a clinical predictive marker for therapeutic response in cervical cancer is not clear.

Application of Methylated TSGs as Tumor Markers in Cervical Cancer

Studies show that genes frequently hypermethylated in tumors could be used as molecular biomarkers for the detection of tumor cells in bodily fluids such as urine and plasma. Thus, these markers would supply additional targets for noninvasive early diagnosis and monitoring for cancers[18]. Because DNA methylation can be reversed by demethylating agents, genes that are always methylated in tumors could be used as therapeutic targets in cancer management.

Methylated TSGs as adjuvant biomarkers in cervical cancer screening

Cytological morphologic assessment of cervical scrapings is the standard method for screening cervical neoplasia and early-stage cervical cancer in the general population. Since the introduction of the Pap smear, the incidence and mortality of cervical cancer have declined worldwide. However, the maximum sensitivity of the conventional Pap smear and liquid-based cytology for identifying CIN II+ was 55.2% (95% confidence interval: 45.5%–64.7%) and 57.1% (95% confidence interval: 46.3%–67.2%), respectively[56]. Moreover, equivocal diagnosis in cervical cytology occurs in approximately 5% of samples, which require further testing before referral for colposcopy. High-risk HPV DNA testing has been accepted as the best triage testing. However, 40%–60% of the women with atypical squamous cells of undetermined significance are HPV-positive, among which less than 1% are confirmed to have cancer. Thus, other tests are needed to increase accuracy. Recently, Lin *et al*. [57] compared the efficacy of methylation detection for protocadherin-10 (*PCDH10*), wilms' tumor suppressor 1 (*WT1*), and paired boxed gene 1 (*PAX1*) with that of HPV DNA testing by hybrid capture 2 (HC-2) in the triage of 321 women with atypical squamous cells of undetermined significance in a nationwide cohort. They found that detection of *PCDH10* or *WT1* methylation was more accurate than HPV DNA testing for CIN III+ lesions, with a lower false-positive rate (11% vs. 33%, $P = 0.002$), a lower referral rate to colposcopy (39% vs. 47%, $P < 0.001$), and a comparable false-

negative rate (20% vs. 16%, $P = 0.62$)[57]. Chao *et al.*[58] compared the accuracy of HC-2 HPV testing and *PAX1* methylation analysis by Methylight in the identification of HSIL during the follow-up of women with atypical squamous cells. Better performance of *PAX1* methylation analysis by Methylight was observed compared with HC-2 HPV testing, with higher sensitivity (87.5% vs. 62.5%) and specificity (98% vs. 86%)[58]. In the Netherlands, high-risk HPV DNA testing has been accepted as a primary screening for cervical cancer. However, the specificity is limited for the identification of high-grade lesions. Eijsink *et al.*[59] identified a panel of methylation markers, junctional adhesion molecule 3 (*JAM3*), erythrocyte membrane protein band 4.1-like 3 (*EPB41L3*), *C13ORF18*, and telomerase reverse transcriptase (*TERT*), in the triage of high-risk HPV positive cases. They found that further detection of the four-gene methylation panel in high-risk HPV positive cases could identify more CIN III+ cases than did high-risk HPV testing in combination with conventional cytology, resulting in higher percentageof correct referral to colpscopy[59]. Similarly, Overmeer *et al.*[33] reported that additional methylation analysis for *CADM1* and T-lymphocyte maturation associated protein (*MAL*) in high-risk HPV positive patients could improve the specificity from 33% to 78%. These results suggest that analysis of DNA methylation markers can be used as a triage test in high-risk HPV-positive cases. Because methylation markers varied among studies, no consistent panels can be derived for the translational use in clinic. New candidate markers need to be identified and validated.

Methylated TSGs as therapeutic biomarkers in cervical cancer

Re-expression of silenced TSGs by demethylating agents has been widely confirmed in various cancers in both *in vitro* and *in vivo* models to inhibit cancer cell growth. Thus, reversion of methylated TSGs may be an alternative anticancer treatment. However, the carcinogenic and mutagenic properties of demethylating drugs, such as 5-azacytidine and its analog 5-aza-CdR, prevent their use in clinic. Less toxic demethylating agents are needed. Hydralazine and procainamide, antihypertensive and antiarrhytimic agents, have been confirmed as DNA methylation inhibitors. In a phase I trial, hydralazine at doses between 50 and 150 mg/day was confirmed to be well tolerated among untreated cervical cancer patients. Reactivation of TSGs occurred in 75% of the cases, with no change of the imprinted *H19* gene or global DNA methylation[60]. Recently, in a randomized phase III trial, Coronel *et al.*[61] compared chemotherapy alone with epigenetic therapy with hydralazine and the deacylation agent valproate plus cisplatin and topotecan chemotherapy in advanced cervical cancer. In that study, patients treated with combination therapy had longer median progression-free survival than did patients treated with chemotherapy alone (10 months vs. 6 months, $P = 0.038$)[61]. Nevertheless, before epigenetic therapy with demethylating agents is accepted in cancer treatment, more clinical data are needed.

Conclusions

DNA methylation in HPV and the host cellular genome commonly occurs in cervical carcinogenesis. Once identified, hypermethylated genes in cervical exfoliated cells could be used in cervical cancer early detection and in prediction of therapeutic response and prognosis. Optimal panels of methylated targets need to be validated for clinical applications.

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