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Study on the relationship between methylation status of HPV 16 E2 binding sites and cervical lesions

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ABSTRACT

Background: The aim of this study was to investigate the methylation status of E2BSs in the HPV 16 long control region (LCR) in clinical cervical samples.

Methods: Methylation status of the four E2BSs in 43 clinical cervical samples with HPV 16 infection was quantitatively detected using pyrosequencing. Meanwhile, Quantivirus® HPV E6/E7 RNA 3.0 assay (bDNA) was used to detect E6/E7 mRNA levels in the corresponding specimens.

Results: Our results showed that methylation status of E2BS1, 2 and 4 sites in high-grade squamous intraepithelial lesions (HSIL) and cervical cancer were significantly higher than that of asymptomatic HPV 16 infection and low-grade squamous intraepithelial lesions (LSIL) (all $P < .05$). Furthermore, methylation status of HPV 16 E2BS1 and 2 was positively correlated with E6/E7 mRNA levels ($r_s = 0.529$ and 0.512 respectively, $P < .01$). Receiver operating characteristic curve analysis was used to compare the diagnostic performance of E2BSs methylation. When the Youden index was the maximum value, the methylation level of E2BS1 and E2BS2 all demonstrated optimum diagnostic sensitivity of 77.8%, and specificity of 80% and 92%, respectively.

Conclusions: The methylation status of E2BS1 and 2 may have utility as diagnostic markers for the severity of cervical lesions in the future.

1. Introduction

Cervical cancer is one of the most common gynecological malignancies in the world and is the third leading cause of cancer death among females in developing countries [1]. Persistent infection with high-risk human papillomaviruses (HR-HPVs) is the definitive etiological agent of cervical cancer. Among the HR-HPVs, HPV 16 is one of the most prevalent types observed in cervical carcinoma [2,3].

It is generally accepted that overexpression of the oncogenic proteins E6 and E7 is necessary for the progression of cervical intraepithelial neoplasia. The primary transforming properties of E6 and E7 oncoproteins seem to be their ability to inactivate the tumor suppressor gene products p53 and pRb, respectively [4,5]. Related studies reported binding of the E2 protein to E2 binding sites (E2BSs) at the long control region (LCR) of the HPV 16 genome as transcription factors that may exert either activation or inhibition of E6 and E7 oncoproteins [6]. When E2 protein concentrations are low, it is expected that E2 will preferentially bind to E2BS4 (promoter-distal), leading to enhanced production of early viral proteins, including E2 itself and the oncoproteins E6 and E7. As E2 concentrations increase, more E2 protein will bind to promoter-proximal E2BSs relative to E2BS4, resulting in transcriptional repression through displacement of specificity protein 1 (Sp1) and TATA binding protein (TBP) from their binding sites [7–9] (note that the numbering of E2BSs in the current study may differ from

Abbreviations: HPV, human papillomavirus; E2BSs, E2 binding sites.; HR-HPV, high-risk human papillomavirus.; LCR, long control region.; Sp1, specificity pretein 1.; TBP, TATA binding pretein.; LBC, liquid-based cytology.; TBS, the Bethesda reporting system.; LSIL, low-grade squamous intraepithelial lesions.; HSIL, high-grade squamous intraepithelial lesions.; HSIL+, high-grade squamous intraepithelial lesions and squamous cell carcinoma.; SCC, squamous cell carcinoma.; ROC, receiver operating characteristic curve.; bDNA, Quantivirus® HPV E6/E7 RNA 3.0 assay.

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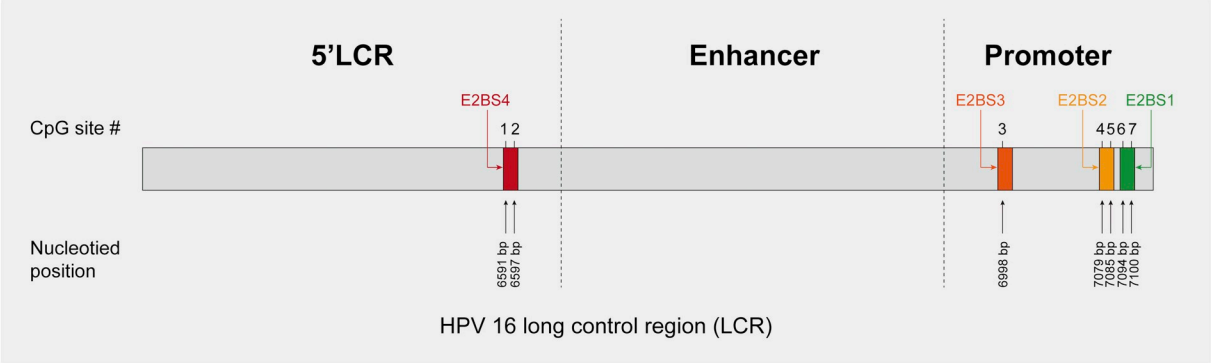

Fig. 1. Four E2BSs in the different areas of HPV16 LCR respectively, which contains seven CpG sites.

that of the references cited). Therefore, the HPV E2 protein plays a considerable role in the regulation of E6/E7 transcription, which is considered essential for the progression of cervical carcinogenesis.

Various studies have shown that epigenetic modification of the HPV genome, especially DNA methylation, could serve as an alternative mechanism for the viral life cycle and cancer progression [10,11]. DNA methylation primarily occurs on cytosine bases of CpG dinucleotides, leading to gene silencing in response to chromatin structure remodeling [12]. Our previous studies have shown that the increase in HPV 16 L1 gene methylation is associated with cervical dysplasia, consistent with other findings [13,14]. However, it is highly controversial in describing the association between the methylation status of HPV 16 LCR and the grade of cervical lesions. The methylation status of four E2BSs may alter binding affinity to the E2 protein, which affects transcriptional regulation of E2 on E6 and E7 oncoproteins. Therefore, to better understand HPV-induced carcinogenic effects, it is imperative to carry out further studies on HPV DNA methylation.

In this study, the methylation status of four E2BSs in HPV 16 LCR in clinical samples was evaluated by pyrosequencing technology. Pyrosequencing provides quantitative assessments of CpG methylation, without cloning, for large-scale molecular epidemiology studies. The aim of the present study is to investigate methylation patterns of E2BSs contained in the HPV 16 LCR. At the same time, the effect of HPV 16 E2BS methylation on the expression of E6/E7 mRNA was also observed. There is a possible correlation between HPV DNA methylation and the severity of cervical neoplasia, suggesting that HPV DNA methylation may be a biomarker for cervical cancer diagnosis.

2. Materials and methods

2.1. Patients

The present prospective study enrolled consecutive patients attending routine outpatient liquid-based cytology (LBC) screening using the ThinPrep system (Cytyc Corporation, MA, USA) at the Third Affiliated Hospital of Zhengzhou University, Zhengzhou, China, between September 1, 2016, and September 1, 2017. Cytology were classified using the 2014 Bethesda Reporting System (TBS) by three experienced cytopathologists. The patients with abnormal cytological diagnosis were further performed by colposcopy biopsy. Histology diagnosis using the current World Health Organization (WHO) classification. Detection of HPV genotyping was performed by HybriBio HPV GenoArray test (HybriBio Ltd., Hong Kong SAR, China) using the residual LBC, as previously described [15] , and only HPV 16 single infection were included in the study. Additionally, follow-up the histological diagnosis, the histological diagnoses were consistent with the cytological diagnoses. Finally, a total of 43 clinical specimens (median age 37 years, range 25–65 years) were included in this study, including 10 cases of asymptomatic HPV 16 infection (Normal), 15 cases of low-grade squamous intraepithelial lesions (LSIL), and 18 cases of high-grade squamous intraepithelial lesions and squamous cell carcinoma (SCC) (HSIL+). This study was approved by the institutional Ethics Committee of Bioscience, Zhengzhou University. Informed consent was obtained from all participants prior to inclusion.

2.2. DNA extraction and bisulfite modification

DNA was isolated and purified from all 43 LBC samples using the General AllGen Kit (CWBIO, Beijing, China) and according to the manufacturer's instructions. DNA concentrations were quantified on a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Bisulfite conversion of DNA (500 ng) was performed using the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany), and the bisulfite-modified DNA was purified on columns provided with the kit. During modification, unmethylated cytosines were converted to uracils, whereas methylated cytosines remained unmodified.

2.3. Amplification and pyrosequencing

Quantitative methylation by pyrosequencing was focused on the target sequence from nucleotide (nt) 6293 to nt 7124, encompassing 8 CpG sites of HPV 16 LCR (NCBI accession no. NC_001526.4). Within the LCR, two sites (nucleotide positions 6591 and 6597) are located in E2BS4, one site (position 6998) is located in E2BS3, two sites (nucleotide positions 7079 and 7085) are located in E2BS2 and two sites (nucleotide positions 7094 and 7100) are located in E2BS1 (Fig. 1). Because optimal read length of pyrosequencing per primer is limited to < 60 nucleotides, three pairs of amplification and sequencing primers were designed for the current study using commercial assay design software (PyrosequencingTM Assay Design Software, Qiagen, Germany) (Table 1). One of the primers in each amplification reaction was labeled with biotin at its 5′ end, and the choice of primer was based on the direction of pyrosequencing.

PCR was performed in a 50 μL reaction mixture containing 5 × PCR buffer, 1 μL of 10 mM dNTP, 0.2 μL of 5 U Taq polymerase, 50 pmol of each primer and 2 μL of bisulfite-modified DNA. PCR reaction was carried out using the following conditions: preheating at 95 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min, and 1 cycle of 72 °C for 7 min. Amplified products were verified in 1.8% agarose gel electrophoresis.

Pyrosequencing of the PCR amplified products was performed using the PyroMark Q96 ID instrument (Qiagen) as directed by the manufacturer. Assay setup and sequence run, as well as results analysis, were performed by the Pyro Q-CpG TM software.

2.4. HPV E6/E7 mRNA testing

The Quantivirus® HPV E6/E7 RNA 3.0 assay (bDNA) (DiaCarta, CA, USA) is a sandwich nucleic acid hybridization procedure for direct quantitation of HPV E6/E7 mRNA in residual LBC samples without RNA

Table 1
Primer sequences for methylation study of HPV 16 by pyrosequencing.

Amplicon (bp) amplification primers (5′ → 3′) sequencing primers[a]	CpG position (nt)
A (185) FW (bio): ATTGTGTTGTGGTTATTTATTGTA S1: **ACATTTTATACCAAAAAACATAC**	6597, 6591
RV: ACAAATTTAAACCATAATTACTAACATAA *AACCRAATTCRATTAAAACTACAAAATAACC*	
B (204) FW: TGTTTATTTGTAAAATTGTATATGGGTGTG S2: **ATGGGTGTGTGTAAAT**	6998
RV (bio): CCTATAAATCCTAAAACATTACAATTCTCT *YGTTTTGGGTTATATATTTATAAGTA*	
C (204) FW (bio): TGTTTATTTGTAAAATTGTATATGGGTGTG S3: **ACATAAAATATCTACTTTTATACT**	7100, 7094, 7085, 7079, 7073
RV: CCTATAAATCCTAAAACATTACAATTCTCT *AACCRATTTCRATTCAACCRATTTCRA TTACRCCCTTAATTTTATACATAA*	

[a] Sequencing primers are indicated in bold. Sequences to analyze are italicized with the CpG sites underlined.

purification and amplification. Particular housekeeping genes were detected in parallel with HPV mRNA to calculate the effective concentration of cells from each clinical sample at the same time. The specific testing methods for the detection of HPV mRNA were described in our previous report [16].

2.5. Statistical analysis

SPSS 21.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. Spearman rank correlation was used to evaluate the association among HPV 16 E2BSs methylation, E6/E7 mRNA levels and severity of cervical lesions. Kruskal-Wallis and Mann-Whitney tests were used for comparison of HPV 16 E2BSs methylation and E6/E7 mRNA levels among the different grade cervical lesions. Diagnostic accuracy of E2BSs methylation was assessed using the ROC curve. P-values < .05 were considered significant.

3. Results

3.1. Analysis of methylation status of eight CpGs in HPV 16 LCR

The methylation level of each of the eight CpG dinucleotides of HPV 16 LCR, in 43 DNA samples isolated from HPV16 infected cervical LBC specimens, was detected using pyrosequencing (Fig. 2). Progressive methylation of HPV 16 LCR was noted with increasing severity of cervical lesions, and the methylation status of 6597, 7079, 7085, 7094 and 7100 CpG sites differed significantly among the cervical lesion groups (all $P < .05$; Fig. 3). In general, methylation levels of all five CpGs among HSIL+ specimens were significantly higher than that in asymptomatic HPV 16 infection samples (all $P < .05$).

3.2. Analysis of the relationship between E2BSs methylation levels and cervical lesions

From the investigation of the HPV 16 E2BSs methylation patterns, except for E2BS3, there was a better correlation between the progressive methylation and the severity of cervical lesions. The methylation levels of E2BS1, 2 and 4 showed an increasing trend from asymptomatic HPV 16 infection to LSIL and HSIL+ ($r_s = 0.644$, 0.714 and 0.511, respectively, all $P < .01$). The methylation level of E2BS2 was most highly correlated with the degree of cervical lesions ($r_s = 0.714$, $P < .01$). However, the correlation between the methylation level of E2BS3 and cervical lesions was not significant ($r_s = 0.250$, $P = .11$). In addition, as shown in Table 2, the degree of E2BS1, 2 and 4 methylation showed significant variation among the different groups of clinical cervical specimens (all $P < .05$). Overall, the HSIL+ group was significantly more methylated than the other two groups (all $P < .05$).

To further analyze the relationship between HPV DNA methylation and cervical lesions, the differences in methylation levels at the four E2BSs within the same cervical lesions were studied. Among the group of HSIL+ samples, significant differences were found in the methylation levels of all four E2BSs ($\chi^2 = 18.627$, $P < .05$). The methylation degree of E2BS3 was significantly lower than that of E2BS1, E2BS2 and E2BS4 (all $P < .05$). However, E2BS1 showed higher methylation compared to E2BS4 ($Z = -2.499$, $P = .01$). In LSIL, a significant difference among four E2BSs was also observed ($\chi^2 = 11.296$, $P = .01$), with the level of E2BS3 methylation being the lowest in this group as well. However, no significant difference was observed among the four E2BSs in asymptomatic HPV 16 infection, although E2BS4 tended to be more methylated than E2BS1, 2 and 3 ($\chi^2 = 5.971$, $P = .11$).

3.3. Analysis of the relationship between E2BS methylation status and the expression level of E6/E7 mRNA

In this study, the Quantivirus® HPV E6/E7 RNA 3.0 assay (bDNA) was performed with total RNA from all 43 samples to detect HPV E6/E7 mRNA expression level, and the results were compared with E2BS methylation status. The E6/E7 mRNA levels were significantly increased, corresponding with the severity of cervical neoplasia ($r_s = 0.662$, $P < .05$). Moreover, the expression levels of HPV 16 E6/E7 mRNA varied significantly among the different groups of clinical cervical specimens ($\chi^2 = 19.970$, $P < .05$). Of note, the E6/E7 mRNA levels increased with the progression of HPV 16 E2BS1 and 2 methylation ($r_s = 0.512$ and 0.529, respectively, $P < .05$). However, the correlation between E6/E7 mRNA and the methylation levels of E2BS3 and 4 were not significant ($r_s = 0.195$ and 0.287, respectively, $P > .05$).

3.4. ROC curve

Receiver operating characteristic curve (ROC)analysis was used to compare the diagnostic performance of the E2BSs methylation. We analyzed the clinically relevant portion of the area under the curve (AUC), which was greater for the methylation of E2BS1 and 2, compared to that of E2BS3 and 4 (Table 3). The methylation level of E2BS1 and E2BS2 exhibited higher diagnostic value for predicting HSIL+ (AUC = 0.836 and 0.901, respectively). When the Youden index was the maximum value, the methylation level of E2BS1 and E2BS2 both demonstrated optimum diagnostic sensitivity of 77.8%, and specificity of 80% and 92%, respectively(Fig. 4).

4. Discussion

Persistent infection with HR-HPVs and corresponding increased expression of E6 and E7 oncogenes are essential in the pathogenesis of cervical carcinoma. However, epigenetic alterations, especially DNA methylation, are required for triggering carcinogenesis. DNA methylation primarily occurs on cytosines located on CpG dinucleotides and directly leads to gene silencing in response to chromatin structure remodeling [10]. Each E2BS of the HPV 16 LCR contains one or two CpG dinucleotides in a conserved palindromic sequence, which is a potential target for DNA methylation in the host cell. Previous studies have shown that methylation of E2BSs may alter its binding affinity for E2 proteins, thereby enhancing E6 and E7 transcription by activating the p97 promoter [17,18].

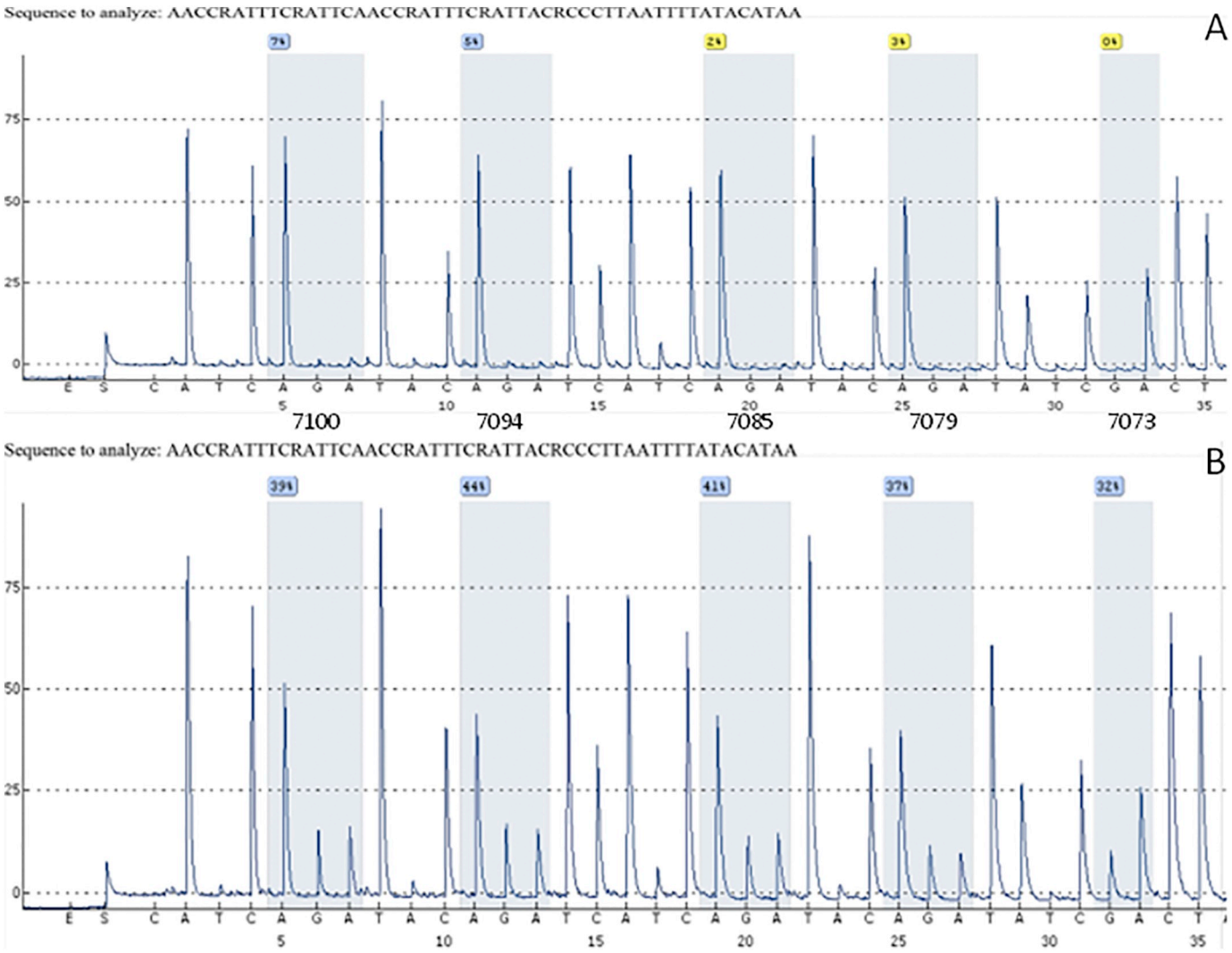

Fig. 2. Methylation levels of eight CpG dinucleotides in HPV 16 LCR were detected using pyrosequencing. CpGs in the E2BS1 and E2BS2 (nucleotide positions 7100, 7094, 7085 and 7079) were detected in amplicon C with sequencing primer S3. A. Methylation level of asymptomatic HPV 16 infection (normal). B. Methylation level of invasive squamous cell carcinoma (SCC).

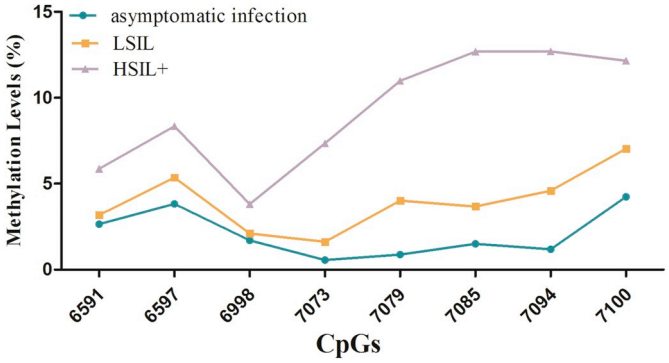

Fig. 3. Methylation levels of the eight CpG sites of HPV 16 LCR in different cervical lesions. Progressive methylation of HPV 16 LCR was noted with the severity of cervical lesions. Methylation levels (%) for each CpG sites are presented as the mean (± S.D.). LSIL, low-grade squamous intraepithelial lesion. HSIL+, including HSIL and SCC. HSIL, high-grade squamous intraepithelial lesion. SCC, invasive squamous cell carcinoma.

However, previous studies aimed at describing the methylation status of HPV 16 LCR and the therein-contained E2BSs in clinical samples are limited with controversial results. Some studies show a decrease in DNA methylation frequency in HPV LCR, which is associated with cervical cancer progression [19,20]. In contrast, this study and some early reports have suggested that HPV LCR methylation is more common in cervical cancer than in asymptomatic infections or that patients with high E2BS methylation exhibit a trend for higher risk of cancerous lesions [14,21–24]. There are two main reasons for these inconsistencies. First, DNA methylation was detected using a variety of techniques, and some assays could not quantify methylation adequately at individual CpG sites. Second, the source of the samples used for these

Table 2
The methylation levels of the four E2BSs of HPV 16 in cervical lesions.

Group	Normal (n = 10)	LSIL (n = 15)	HSIL+ (n = 18)	χ^2	P
E2BS4	3.24(1.82)	4.00(2.01)	7.34(4.93)	11.414	0.003
E2BS3	1.70(2.38)	2.10(2.76)	3.82(3.62)	2.858	0.240
E2BS2	1.19(1.77)	2.96(2.45)	12.56(11.86)	21.808	0.000
E2BS1	2.72(2.27)	5.32(2.51)	12.83(11.66)	17.476	0.000

Data (%) are presented as the mean methylation percentage (± SD) of all samples included in that group.
Normal, asymptomatic HPV infection; LSIL, low-grade squamous intraepithelial lesion; HSIL+ (including HSIL and SCC), high-grade squamous intraepithelial lesion and cervical squamous carcinoma.

Table 3
AUC, sensitivity and specificity of HPV 16 E2BSs methylation for prediction of high-grade histological diagnosis.

Assay (n = 43)	AUC	95% CI		P	sensitivity	specificity
		LB	UB		(%)	(%)
E2BS4 methylation	0.798	0.665	0.930	0.001	88.9	68.0
E2BS3 methylation	0.642	0.472	0.812	0.115	50.0	80.0
E2BS2 methylation	0.901	0.801	1.000	0.000	77.8	92.0
E2BS1 methylation	0.836	0.708	0.963	0.000	77.8	80.0

AUC, the area under the ROC curve; CI, confidence interval; LB, lower bound; UB, upper bound.

experiments is different. Some studies have been performed on fresh-frozen cervical tissues, whereas others have been conducted on cervical exfoliated cells or cervical cancer cell lines. In the current study, methylation levels of four E2BSs in HPV 16 LCR in cervical LBC specimens were successfully detected using pyrosequencing. The results showed that HPV 16 E2BSs methylation appeared at a high frequency in HSIL +. In particular, E2BS2 methylation was significantly associated with the severity of cervical disease. At the same time, E6/E7 mRNA levels were found to increase with progression of HPV 16 E2BS methylation. Taken together, these data further indicate that the hypermethylation status of the HPV genome, especially in E2BSs, may potentially block the inhibitory function of E2, leading to upregulated expression of E6 and E7 oncogenes.

Expression of E6 and E7 oncogenes is regulated by the LCR region of the HPV genome, to which cellular transcription factors can bind, as well as the viral protein E2 [6,25,26]. The E2 protein is the master regulator of the viral life cycle, since it has high affinity for E2BS4, leading to transcriptional activation of E6/E7 oncoproteins through loss of inhibition on the p97 early promoter. In contrast, when E2 binds to the other three sites proximal to the TATA box, especially E2BS1 and E2BS2, transcription of E6 and E7 is repressed [7,26–30]. Thus, this study further compared the differential methylation patterns of the four E2BSs. Consistent with previous studies, in HSIL+, E2BS1 (proximal to the promoter) was significantly more methylated than E2BS4 (distal to the promoter) and thereby activated transcription. In combination with the expression levels of E6/E7 mRNA, our results showed that E2BS1 was more methylated than E2BS4 during cervical carcinogenesis, further inhibiting the ability of E2 protein to transcriptionally repress E6/E7 oncoproteins.

In this study, the methylation status of eight CpGs in HPV 16 LCR was investigated in clinical specimens with confirmed HPV infection and various grades of cervical lesions using pyrosequencing. Furthermore, bDNA was used to detect expression levels of E6/E7 mRNA in the corresponding specimens. The data showed that both HPV 16 E2BS methylation and E6/E7 mRNA levels increase with disease severity. In addition, there may be a positive correlation between E6/E7 mRNA levels and the methylation status of HPV 16 E2BS1 and E2BS2. The results of this study further support the hypothesis that the methylation status of the HPV LCR, especially in E2BSs, might contribute to the upregulated expression of E6 and E7 oncogenes [31].

The limitation of this study was that the small sample size may affect the research results. In the future, we will continue to follow-up with these patients and a larger-scale population is needed. Moreover, we would investigate HPV genome methylation in animal models, to further understand the correlation between HPV 16 LCR methylation and cervical cancer.

In conclusion, the current study presented new data on the DNA methylation status at the four E2BSs sequences of HPV 16 LCR in clinical cervical lesions. The methylation levels of HPV 16 E2BSs and the expression of E6/E7 mRNA both increased with cervical lesion severity. The results of this study further support the notion that epigenetic and genetic alterations of E2BSs in the HPV 16 LCR may

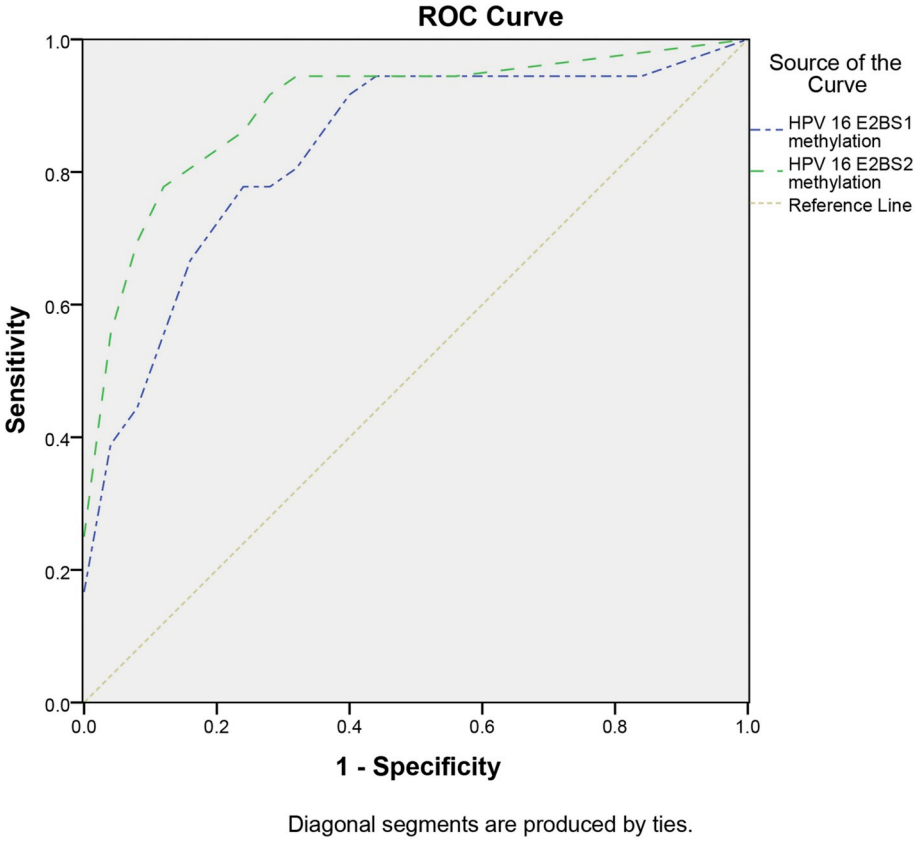

Fig. 4. ROC curves for HPV 16 E2BS1 and E2BS2 methylation level in HSIL+ with sensitivity and specificity.

potentially block the inhibitory function of E2 and lead to upregulated expression of E6 and E7 oncogenes. In addition, the methylation level of E2BS1 was significantly higher than that of E2BS4 and may facilitate the progression from asymptomatic HPV 16 infection/LSIL to HSIL+. By comparing the area under the curve as well as the sensitivity and specificity, the methylation status of E2BS1 and 2 may be used as diagnostic markers for the severity of cervical lesions.

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Declarations of interest

None.

Conflicts of interest

The authors declare that they have no competing interests.

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References

[1] L.A. Torre, F. Bray, R.L. Siegel, et al., Global cancer statistics, 2012, CA Cancer J. Clin. 65 (2) (2015) 87–108.
[2] S.S. De, W.G. Quint, L. Alemany, et al., Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study, Lancet Oncol. 11 (11) (2010) 1013–1014.
[3] N. Muñoz, F.X. Bosch, S.S. De, et al., Epidemiologic classification of human papillomavirus types associated with cervical cancer, N. Engl. J. Med. 348 (6) (2003) 518–527.
[4] B.A. Werness, A.J. Levine, P.M. Howley, Association of human papillomavirus types 16 and 18 E6 proteins with p53, Science 248 (4951) (1990) 76–79.
[5] S.N. Boyer, D.E. Wazer, V. Band, E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway, Cancer Res. 56 (20) (1996) 4620–4624.
[6] F. Thierry, Transcriptional regulation of the papillomavirus oncogenes by cellular and viral transcription factors in cervical carcinoma, Virology 384 (2) (2009) 375–379.
[7] G. Steger, S. Corbach, Dose-dependent regulation of the early promoter of human papillomavirus type 18 by the viral E2 protein, J. Virol. 71 (1) (1997) 50–58.
[8] C. Demeret, C. Desaintes, M. Yaniv, et al., Different mechanisms contribute to the E2-mediated transcriptional repression of human papillomavirus type 18 viral oncogenes, J. Virol. 71 (12) (1997) 9343–9349.
[9] R.S. Hegde, The papillomavirus E2 proteins: structure, function, and biology, Annu. Rev. Biophys. Biomol. Struct. 31 (1) (2002) 343.
[10] M. Kulis, M. Esteller, DNA methylation and cancer, Adv. Genet. 70 (10) (2010) 27–56.
[11] P.A. Lazo, The molecular genetics of cervical carcinoma, Br. J. Cancer 80 (12) (1999) 2008–2018.
[12] S.M. Leonard, W. Wei, S.I. Collins, et al., Oncogenic human papillomavirus imposes an instructive pattern of DNA methylation changes which parallel the natural history of cervical hpv infection in young women, Carcinogenesis 33 (7) (2012) 1286–1293.
[13] C. Sun, L.L. Reimers, R.D. Burk, Methylation of HPV16 genome cpg sites is associated with cervix precancer and cancer, Gynecol. Oncol. 121 (1) (2011) 59–63.
[14] L. Mirabello, M. Schiffman, A. Ghosh, et al., Elevated methylation of HPV16 DNA is associated with the development of high grade cervical intraepithelial neoplasia, Int. J. Cancer 132 (6) (2013) 1412–1422.
[15] S. Liu, R. Leung, K. Chan, et al., Evaluation of a newly developed GenoArray human papillomavirus (HPV) genotyping assay and comparison with the Roche linear Array HPV genotyping assay, J. Clin. Microbiol. 48 (3) (2010) 758–764.
[16] Y. Shen, J. Gong, Y. He, et al., Quantivirus(R) HPV E6/E7 RNA 3.0 assay (bDNA) is as sensitive, but less specific than hybrid capture 2 test, J. Virol. Methods 187 (2) (2013) 288–293.
[17] K. Kim, P.A. Garner-Hamrick, C. Fisher, D. Lee, P.F. Lambert, Methylation patterns of papillomavirus DNA, its influence on E2 function, and implications in viral infection, J. Virol. 77 (23) (2003) 12450–12459.
[18] M. Reuschenbach, C.U. Huebbers, E.S. Prigge, et al., Methylation status of HPV16 E2-binding sites classifies subtypes of HPV-associated oropharyngeal cancers, Cancer 121 (12) (2015) 1966–1976.
[19] V. Badal, L.S. Chuang, E.H. Tan, et al., Cpg methylation of human papillomavirus type 16 DNA in cervical cancer cell lines and in clinical specimens: genomic hypomethylation correlates with carcinogenic progression, J. Virol. 77 (11) (2003) 6227–6234.
[20] I.D. Mazumder, R.K. Singh, S. Mitra, et al., Genetic and epigenetic changes of HPV16 in cervical cancer differentially regulate E6/E7 expression and associate with disease progression, Gynecol. Oncol. 123 (3) (2011) 597–604.
[21] B. Bhattacharjee, S. Sengupta, Cpg methylation of HPV 16 LCR at E2 binding site proximal to p97 is associated with cervical cancer in presence of intact E2, Virology 354 (2) (2006) 280–285.
[22] M. Kalantari, I.E. Callejamacias, D. Tewari, et al., Conserved methylation patterns of human papillomavirus type 16 DNA in asymptomatic infection and cervical neoplasia, J. Virol. 78 (23) (2004) 12762–12772.
[23] S. Snellenberg, D.M. Schütze, D. Claassenkramer, et al., Methylation status of the E2 binding sites of HPV16 in cervical lesions determined with the luminex® xmap™ system, Virology 422 (2) (2012) 357–365.
[24] S. Vinokurova, M.V.K. Doeberitz, Differential methylation of the HPV 16 upstream regulatory region during epithelial differentiation and neoplastic transformation, PLoS One 6 (9) (2011) e24451.
[25] F. Hoppe-Seyler, K. Butz, Cellular control of human papillomavirus oncogene transcription, Mol. Carcinog. 10 (3) (2010) 134–141.
[26] P. Howley, W. Phelps, Transcriptional trans-activation by the human papillomavirus type 16 E2 gene product, J Virol 61 (5) (1987) 1630–1638.
[27] N. Dostatni, P. Lambert, R. Sousa, et al., The functional bpv-1 E2 trans-activating protein can act as a repressor by preventing formation of the initiation complex, J. Genes & Development 5 (9) (1991) 1657–1671.
[28] E. Soeda, M.C. Ferran, C.C. Baker, et al., Repression of HPV16 early region transcription by the E2 protein, Virology 351 (1) (2006) 29–41.
[29] S.H. Tan, L.E. Leong, P.A. Walker, et al., The human papillomavirus type 16 E2 transcription factor binds with low cooperativity to two flanking sites and represses the E6 promoter through displacement of sp1 and tfiid, J. Virol. 68 (10) (1994) 6411–6420.
[30] S. Bellanger, C.L. Tan, Y.Z. Xue, et al., Tumor suppressor or oncogene? A critical role of the human papillomavirus (HPV) E2 protein in cervical cancer progression, Am. J. Cancer Res. 1 (3) (2011) 373–389.
[31] A. Chaiwongkot, S. Vinokurova, C. Pientong, et al., Differential methylation of E2 binding sites in episomal and integrated HPV 16 genomes in preinvasive and invasive cervical lesions, Int. J. Cancer 132 (9) (2013) 2087–2094.