Original Article

Limited Role of Promoter Methylation of *MGMT* and *C13ORF18* in Triage of Low-Grade Squamous Intraepithelial Lesion

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Abstract

Background: Promoter methylation of *MGMT* and *C13ORF18* has been confirmed as a potential biomarker for early diagnosis of cervical cancer. The aim of this study was to evaluate the performance of *MGMT* and *C13ORF18* promoter methylation for triage of cytology screening samples and explore the potential mechanism.

Methods: Methylation-sensitive high-resolution melting was used to detect promoter methylation of *MGMT* and *C13ORF18* in 124 cervical samples. High-risk human papillomavirus (HR-HPV) was detected by the Digene Hybrid Capture 2®. Gene methylation frequencies in relation to cervical intraepithelial neoplasia (CIN) were analyzed. Frequencies were compared by Chi-square tests. The expression of gene biomarkers and methylation regulators was analyzed by immunohistochemical staining, real-time fluorescence quantitative polymerase chain reaction, and Western blot.

Results: For triage of low-grade squamous intraepithelial lesion (LSIL), gene methylation increased specificity from 4.0% of HR-HPV detection to 30.8% of *MGMT* ($\chi^2 = 9.873$, $P = 0.002$) and to 50.0% of *C13ORF18* ($\chi^2 = 21.814$, $P = 0.001$). For triage of atypical squamous cells of undetermined significance, HR-HPV detection had higher positive predictive value of 54.8%. Either *MGMT* or *C13ORF18* methylation combined with HR-HPV increased the negative predictive value to 100.0% ($\chi^2 = 9.757$, $P = 0.002$). There was no relationship between *MGMT* and *C13ORF18* expression and DNA methylation ($\chi^2 = 0.776$, $P = 0.379$ and $\chi^2 = 1.411$, $P = 0.235$, respectively). MBD2 protein level in cervical cancer was relatively lower than normal cervical tissue ($t = 4.11$, $P = 0.006$).

Conclusions: HR-HPV detection is the cornerstone for triage setting of CIN. Promoter methylation of *MGMT* and *C13ORF18* plays a limited role in triage of LSIL. Promoter methylation of both genes may not be the causes of gene silence.

Key words: Cervical Cancer; Cervical Intraepithelial Neoplasia; DNA Methylation

Introduction

Cervical cancer ranked the 9th of all cancer death causes in female population in China.[1] Chinese cervical cancer screening strategy reduced the incidence of invasive cervical cancer and dramatically rose up the incidence of cervical intraepithelial neoplasia (CIN).[2] The sensitivity of atypical squamous cells of undetermined significance (ASCUS) for the detection of high-grade CIN is unsatisfactory, and high-risk human papillomavirus (HR-HPV) infection is always transient.[3] Early detection of high-grade CIN not only reduces the financial burdens of repeated cervical screening and HR-HPV test but also relieves the mental stress of suspected patients of CIN.

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Different tumors have characteristic signatures of methylated genes which can be used as biomarkers for early detection or monitoring of the progression of carcinogenesis.[4] MGMT is a DNA methyltransferase which inhibits cell proliferation and survival.[5] MGMT gene silencing might be one of the mechanisms of tumorigenesis.[6] Our previous

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population-based study on methylation of *MGMT* involving 667 cases showed that *MGMT* methylation may serve as a marker for early diagnosis of high-grade neoplasia in cervical cytology screening.[7,8] Promoter methylation of *C13ORF18* in cervical scrapings was strongly associated with high-grade lesions.[9] A study on methylation status of nine gene promoters revealed that methylation biomarkers distinguishing cervical precursor lesions from normal cervix were primarily *C13ORF18*.[10] This study was designed to verify the efficacy of *MGMT* and *C13ORF18* methylation and HR-HPV for the triage of cytology screening samples.

Methods

Ethical approval

This study was approved by the Research Ethics Committee of International Peace Maternity and Child Health Hospital of the China Welfare Institute. Informed written consent was obtained from all patients.

Clinical samples

Clinical samples of residual liquid-based cytology (LCT, BD SurePath™, USA) specimens, which stored at ambient temperature within 1.0–1.5 years from sample collection, were selected from cytopathology archives of the International Peace Maternity and Child Health Hospital of the China Welfare Institute. Cytology diagnosis was based on the Bethesda System. Slides with an atypical squamous cells were reviewed and diagnosed by two cytopathologists independently. The Digene Hybrid Capture 2® (Qiagen, Germany) was utilized to detect 13 types of HR-HPV infection according to the manufacturers' instructions. Patients of ASCUS with HR-HPV infection, low-grade and high-grade squamous intraepithelial lesion were underwent colposcopy.

DNA extraction and sodium bisulfite modification

Genomic DNA was extracted from 1.5 ml to 5.0 ml of residual cytology collections by the DNA Tissue Extraction Kit (SK1261, Sangon Biotec, China) following the manufacturer's protocol. Genomic DNA was isolated with phenol/chloroform extraction method. Chemical modification of CpG islands was performed through sodium bisulfite treatment. The modified DNA was used immediately for analysis.

Amplification of the methylated CpG island and methylation-sensitive high-resolution melting analysis

Real-time polymerase chain reaction (PCR) followed by methylation-sensitive high-resolution melting (MS-HRM) for the detection of methylation was carried out in a LightCycler 480 (Roche, Switzerland). The primer sets for all MS-HRM assays were designed according to the principles recently set out to compensate for PCR bias. The primers were designed to amplify both methylated and unmethylated template. The sequences of specific primers are shown in Table 1. PCR was carried out in a 25-μl reaction volume with 1× PCR buffer, 1 unit of Taq DNA polymerase, 1× Evagreen dye, 250 μmol/L of dNTPs, and 250 nmol/L of each primer. The reaction cycle started with one cycle of 95°C for 5 min, followed by 30 cycles at 94°C for 25 s, 56°C for 25 s, 72°C for 30 s, followed by a final extension step at 72°C for 25 s.

MS-HRM analyses were performed following the manufacturer's instructions. Fluorescence was detected and recorded 40 times/s. For each assay, a standard dilution series of 0.1%, 10.0%, 25.0%, 50.0%, 75.0%, and 100.0% with different ratios of methylated and unmethylated template (EpiTect Control DNA methlyated and unmethlyated, Qiagen, Germany) was run to assess the quantitative properties and sensitivity of the assay. The level of gene methylation was recorded semiquantitatively as 1.0%, 10.0%, 25.0%, 50.0%, 75.0%, and 100.0%, respectively. High methylation level was defined as more than 25.0% methylation.

The protein levels of MGMT, C13ORF18, Dnmt1, Dnmt3B, and MBD2 were analyzed by immunohistochemical examination. Levels of mRNA and protein of these genes were detected using qPCR and Western blot.

Statistical analysis

Statistical analysis was performed using the SAS System for Windows (version 6.12, SAS Institute Inc., Cary, North Carolina, USA). Frequencies of gene methylation were compared by Chi-square tests. Kruskal-Wallis nonparametric statistics had been exploited for abnormal distribution of data. A $P < 0.05$ was considered statistically significant.

Results

A total of 124 tissue specimens were included in this study. The average age was 40.5 ± 9.0 years (range 20.0–65.0 years). The diagnosis of cytology and histology is shown in Table 2. The sensitivity of the MS-HRM assay was 1.0% methylated DNA in a background of unmethylated DNA.

Promoter methylation frequencies of *MGMT* and *C13ORF18* differ in high-grade cervical intraepithelial neoplasia

The average methylation level of *MGMT* was 1.0%. Samples of high methylation level account for 4.8% of *MGMT* and

Table 1: Primer sequences of *MGMT* and *C13ORF18*

Gene	Primers	Product size (bp)
MGMT-forward	5'-GCGTTTCGGATATGTTGGGATAGT-3'	173
MGMT-reverse	5'-CCTACAAAACCACTCGAAACTACCA-3'	
C13ORF18-forward	5'-GTAGTTTTTTAGAAGTTTTTTAGGGAAGT-3'	142
C13ORF18-reverse	5'-CATAAATACGTAATACTAAACCCGAAC-3'	

58.7% of *C13ORF18*. Methylation level of *MGMT* decreased with cervical neoplasia grades ($Z = 1.987$, $P = 0.044$), and methylation level of *C13ORF18* increased with cervical neoplasia grades ($Z = 1.800$, $P = 0.048$). Proportions of DNA methylation level are shown in Figure 1. Compared with normal tissues, the methylation frequencies of *C13ORF18* and *MGMT* of CIN1, and CIN2$^+$ were significantly different [$\chi^2 = 8.433$, $P = 0.002$, and $\chi^2 = 16.208$, $P = 0.001$, respectively; Table 3].

To analyze the effectiveness of gene methylation for triage of high-grade cervical intraepithelial neoplasia, individuals were recategorized into normal tissue, CIN1, and CIN2$^+$ (CIN2-3) subgroups. *MGMT* methylation frequency decreased significantly in CIN2$^+$ subgroup of ASCUS ($P = 0.010$), and *C13ORF18* methylation frequency increased significantly in CIN2$^+$ subgroup of low-grade squamous intraepithelial lesion (LSIL) ($\chi^2 = 3.11$, $P = 0.003$). Methylation frequencies in ASCUS and LSIL are shown in Table 4.

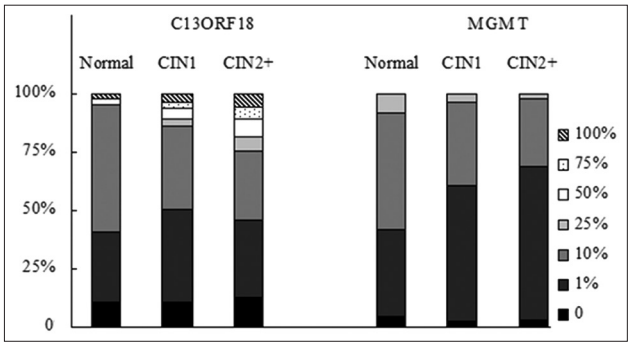

Figure 1: Proportions of DNA methylation level. CIN: Cervical intraepithelial neoplasia.

Table 2: Cytological and histological results

Items	Normal	CIN1	CIN2	CIN3	CC	Total
ASCUS, *n*	28	8	9	11	2	58
LSIL, *n*	11	15	7	8	0	41
HSIL, *n*	6	1	2	13	3	25
Total, *N*	45	24	18	32	5	124

ASCUS: Atypical squamous cells of undetermined significance; LSIL: Low-grade squamous intraepithelial lesion; HSIL: High-grade squamous intraepithelial lesions; CIN: Cervical intraepithelial neoplasia; CC: Cervical carcinoma.

Performance of gene methylation combined with high-risk human papillomavirus detection for triage of high-grade cervical intraepithelial neoplasia

The performance of *MGMT* and *C13ORF18* methylation combined with HR-HPV detection for triage of ASCUS and LSIL is shown in Table 5. For diagnosis of CIN2$^+$ in ASCUS, HR-HPV detection has the highest positive predictive value (PPV) of 54.8%. *MGMT* methylation combined with HR-HPV detection showed higher sensitivity than HR-HPV detection (88.2% vs. 82.4%, $\chi^2 = 0.620$, $P = 0.431$). Both *MGMT* and *C13ORF18* methylation combined with HR-HPV detection increased the negative predictive value (NPV) to 100.0%, which were better than single HR-HPV detection (84.2%, $\chi^2 = 9.757$, $P = 0.002$). *MGMT* and *C13ORF18* methylation combined with HR-HPV detection also increased the specificity than single HR-HPV detection (3.6% vs. 3.0%, $\chi^2 = 0.209$, $P = 0.648$).

For diagnosis of CIN2$^+$ in LSIL, compared with HR-HPV detection, *C13ORF18* methylation increased both specificity (50.0% vs. 4.0%, $\chi^2 = 21.814$, $P = 0.001$) and NPV (59.1% vs. 50.0%, $\chi^2 = 0.443$, $P = 0.506$). The sensitivity of *C13ORF18* methylation combined with HR-HPV detection reached 100.0%, which is 93.0% for single HR-HPV detection ($\chi^2 = 3.114$, $P = 0.078$). *MGMT* methylation increased specificity (30.8% vs. 4.0%, $\chi^2 = 9.873$, $P = 0.002$) and showed higher PPV than single HR-HPV detection (41.9% and 36.8%, $\chi^2 = 0.205$, $P = 0.651$).

For triage of CIN2$^+$ among all cervical samples, HR-HPV detection showed better NPV, PPV, and specificity (80.8%, 49.5%, and 33.3%, respectively). *MGMT* methylation increased the sensitivity compared with single HR-HPV detection (96.6% vs. 91.5%, $\chi^2 = 3.477$, $P = 0.062$).

Table 3: Methylation frequencies in relation to cervical intraepithelial neoplasia, *n* (%)

Items	*MGMT*					*C13ORF18*				
	Normal (*n* = 45)	CIN1 (*n* = 24)	CIN2$^+$ (*n* = 55)	χ^2	*P*	Normal (*n* = 45)	CIN1 (*n* = 24)	CIN2$^+$ (*n* = 55)	χ^2	*P*
10.0% methylation	25 (46.7)	6 (25.0)	19 (34.5)	16.208	0.001	25 (55.6)	8 (33.3)	33 (60.0)	4.927	0.085
25.0% methylation	4 (8.9)	0 (0.0)	2 (3.6)	–	0.342*	2 (50.0)	2 (8.3)	13 (23.6)	8.433	0.002

–: Not applicable. *Fisher's exact test. CIN: Cervical intraepithelial neoplasia; CIN2$^+$: CIN2–3.

Table 4: DNA methylation for triage of high-grade cervical neoplasia in ASCUS and LSIL, *n* (%)

Items	*MGMT* methylation				*C13ORF18* methylation			
	Normal/CIN1 (*n* = 36)	CIN2$^+$ (*n* = 22)	χ^2	*P*	Normal/CIN1 (*n* = 36)	CIN2$^+$ (*n* = 22)	χ^2	*P*
ASCUS*	35 (97.2)	16 (72.7)	–	0.010‡	34 (94.4)	19 (86.4)	–	0.357‡
LSIL†	7 (26.9)	2 (13.3)	1.117	0.311	15 (57.7)	15 (100.0)	3.11	0.003

–: Not applicable. *Cut-off value is 1.0% methylation; †Cut-off value is 10.0% methylation; ‡Fisher's exact test. ASCUS: Atypical squamous cells of undetermined significance; LSIL: Low-grade squamous intraepithelial lesion; CIN: Cervical intraepithelial neoplasia; CIN2$^+$: CIN2–3.

Table 5: Performance of gene methylation combined with HR-HPV detection for triage of CIN2+

Items	Cut-off value (%)	ASCUS				LSIL			
		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
HR-HPV	1.0	82.4	3.0	54.8	84.2	93.0	4.0	36.8	50.0
MGMT methylation	1.0	72.7	3.6	31.4	5.9	86.7	30.8*	41.9	38.5
C13ORF18 methylation	10.0	45.5	3.1	37.3	5.9	40.0	50.0†	27.3	59.1
MGMT + HR-HPV	1.0/1.0	88.2	3.6	46.9	100.0‡	91.7	0.0	32.4	0.0
C13ORF18 + HR-HPV	10.0/1.0	76.5	3.6	41.9	100.0‡	100.0	0.0	37.5	0.0

*Compared with high-risk HPV detection, $P = 0.002$; †Compared with high-risk HPV detection, $P = 0.001$; ‡Compared with high-risk HPV detection, $P = 0.002$. CIN: Cervical intraepithelial neoplasia; CIN2+: CIN2–3; ASCUS: Atypical squamous cells of undetermined significance; LSIL: Low-grade squamous intraepithelial lesion; PPV: Positive predictive value; NPV: Negative predictive value; HR-HPV: High-risk human papillomavirus.

Regulatory mechanisms of promoter methylation

Immunohistochemical staining of MGMT, C13orf18, and mentylation regulators (Dnmt1, Dnmt3b, and MBD2) is shown in Figure 2. The expression of mRNA and protein level is shown in Figure 3.

Promoter methylation in relation to gene expression

There was no relationship between MGMT and C13orf18 immunohistochemical staining and DNA methylation in all cervical samples ($\chi^2 = 0.776$, $P = 0.379$ and $\chi^2 = 1.411$, $P = 0.235$). C13ORF18 protein expression in cervical cancer, especially in epithelial cells of basal layer, was significantly stronger than that in normal cervical tissue ($P = 0.023$). MGMT protein expression both in cervical cancer and normal cervical tissue showed no significantly difference [$P = 0.367$; Figure 2]. The expression of mRNA and protein level of MGMT and C13ORF18 between cervical cancer and normal cervical tissue has no significant difference [$P > 0.05$; Figure 3].

Regulator of gene methylation

The average expression levels of MBD2 protein were decreased significantly in cervical cancer compared with normal cervical tissue ($t = 4.11$, $P = 0.006$). The average expression levels of MBD2 mRNA and immunohistochemical staining showed no significant difference ($P = 0.157$ and $P = 0.277$, respectively). The average expression levels of DNMT3b mRNA, protein, and immunohistochemical staining showed no significant difference in cancer and normal tissue ($P = 0.295$, $P = 0.151$, and $P = 0.337$, respectively).

Discussion

MS-HRM was a semiquantitative method and used for sensitive and high-throughput assessment of DNA methylation.[11,12] The efficacy of MS-HRM was to detect 1.0% of methylated DNA in residual cervical cytology specimens within 1 year after sample collection. The high sensitivity of this method makes it convenient for clinical usage of residual specimens after cytology diagnosis. The semiquantitative characteristics provide detection and comparison of different methylation levels. Furthermore, it is preferable for short fragments of DNA derived from formalin-fixed paraffin-embedded biopsies.

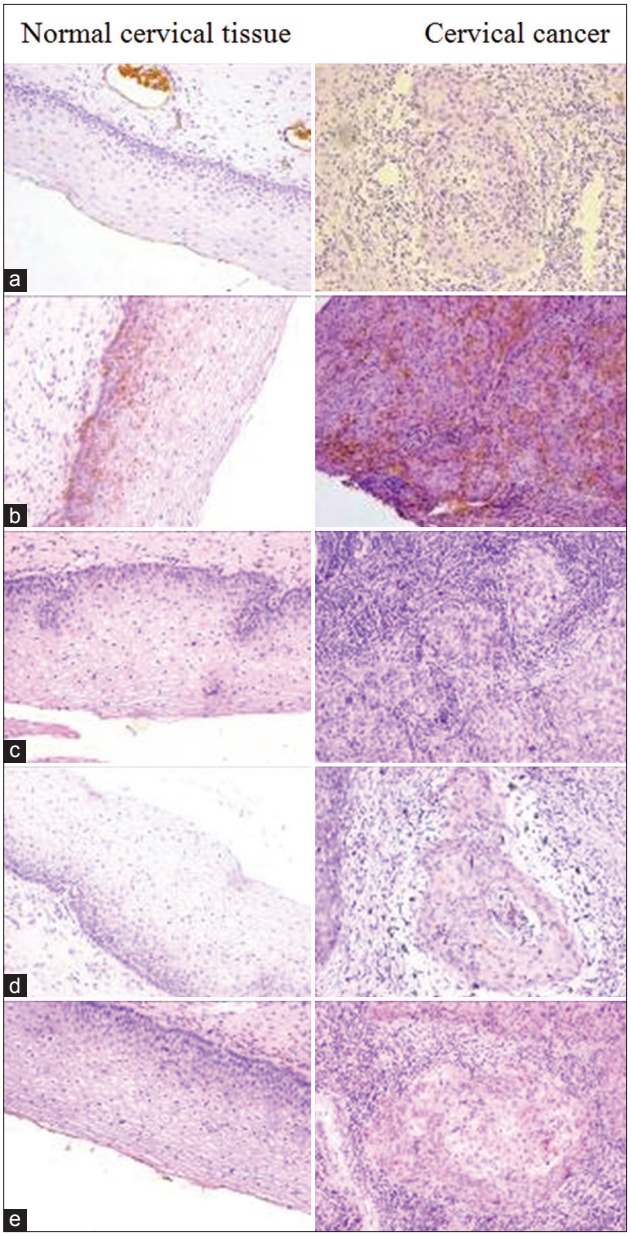

Figure 2: Immunohistochemical staining of (a) MGMT, (b) C13orf18, (c) Dnmt1, (d) Dnmt3b, and (e) MBD2 methylation regulators in normal tissue and cervical cancer tissue (original magnification ×200).

The current cervical screening strategies, particularly those combined with HPV test, have good NPVs. However, the

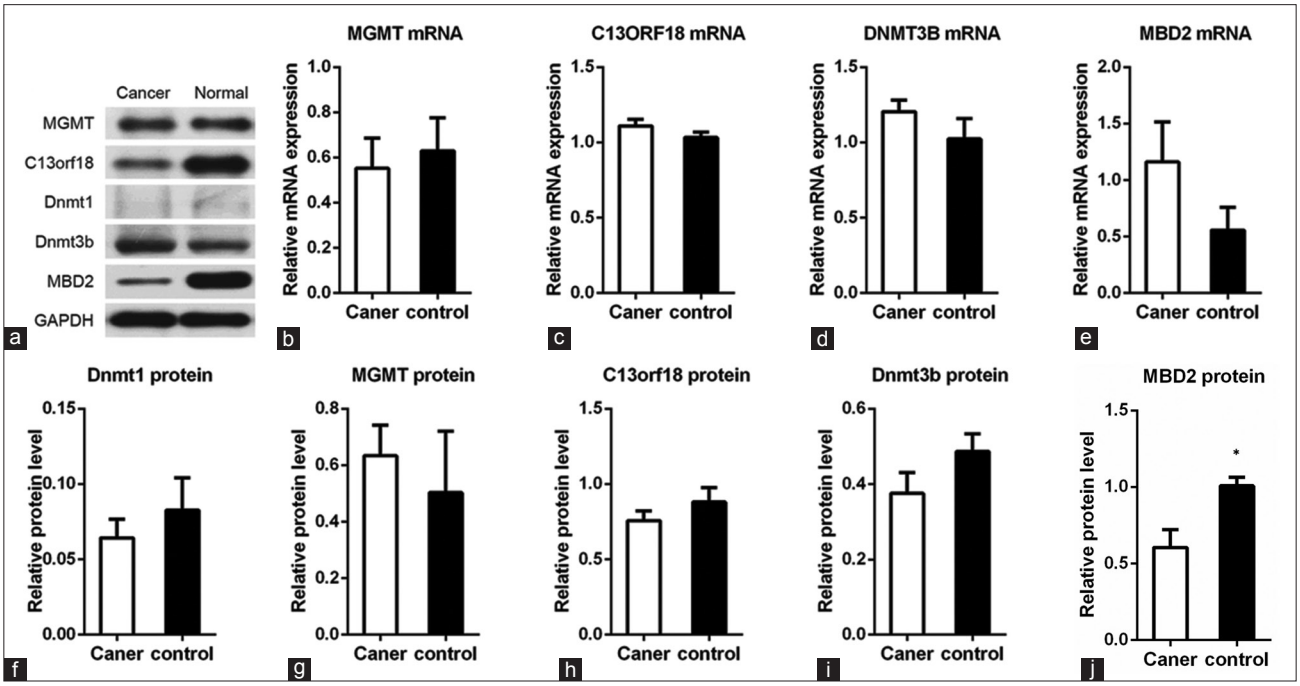

Figure 3: Expression of MGMT, C13ORF18, Dnmt1, Dnmt3b, and MBD2 in normal tissue and cervical cancer. (a, f-j) Western blot analysis were emlpoyed to analyze the expression of the MGMT, C13ORF18, DNMT1, DNMT3B and MBD2 gene in cervical cancer and normal tissue. (b-e) MGMT, C13ORF18, DNMT3B and MBD2 expression were analyzed by quantitative polymerase chain reaction. *MBD2 protein level in cervical cancer is significantly lower than normal cervical tissue ($t = 4.11$, $P = 0.006$).

PPVs are moderate since only the women with diagnostic findings of oncologic significance will develop invasive cancer if left untreated.[13] In this study, HR-HPV detection ultimately has the highest PPV for triage setting of cervical neoplasia. The methylation level and trends of variation of the two genes differed in cervical neoplasia, so the cutoff value of methylation is different in ASCUS and LSIL, and the analysis could not be performed as a whole. For triage of ASCUS, either *MGMT* or *C13ORF18* methylation combined with HR-HPV could increase the NPV of high-grade neoplasia to 100.0% ($P = 0.002$). For triage of LSIL, compared with HR-HPV detection, gene methylation is helpful for increasing the specificity of cytology screening ($P = 0.002$ for MGMT and $P = 0.001$ for C13ORF18, respectively).

C13ORF18 is located on chromosome 13 q14.12 and represents a gene with unknown function. Sequence comparisons suggest a role as phosphatase inhibitor which fit with the function of a tumor suppressor gene inactivated in cancer by hypermethylation.[9] Promoter methylation of C13ORF18 leads to disruption of cell cycle and seems to be an early event in cervical cancerogenesis.[10] In this study, no relationship was found between MGMT and C13ORF18 expression and DNA methylation. Either promoter hypermethylation or histone deacetylation could inactivate or deregulate MGMT and C13ORF18. Promoter methylation may not be the cause of gene silence. On the contrary, there may be gene silence first, and promoter methylation acts as epigenetic marks.[14,15]

CpG island methylation is closely linked to histone modifications by interacting with Methyl-CpG-binding domain protein 2 (MBD2) and DNA methyltransferases (DNMTs). Aberrant DNMTs expression is the dominant mechanism for the genome instability which associates with a wide range of diseases including a cancer. The methylation of mammalian genomic DNA appears to be established by a complex interplay of DNA methyltransferases (DNMTs), including *de novo* (DNMT3A and DNMT3B) and maintenance (DNMT1) enzymes. DNMT1 and DNMT3B were both shown to be important for cancer cell survival and tumorigenesis.[16] Overexpression of DNMT1 and DNMT3B has been found to be associated with HPV infections and common in human tumors.[17,18] Studies showed that DNMT1 and DNMT3B were significantly increased in primary advanced cervical carcinoma compared to noncancer counterparts.[16,19] The average expression levels of DNMT3b mRNA in this study increased in cervical carcinoma, which showed no significant difference from noncancer counterparts. These samples were selected from well-differentiated squamous carcinoma, rather than primary advanced carcinoma. Immunohistochemical staining showed higher expression density in basal layer cell than in well-differentiated squamous cancer cells. MBD2 protein, with the exception of MBD3, is capable of binding specifically to methylated DNA, which is so-called DNA reader. MBD2 contains a transcription repressor domain. The knock-down of MBD2 resulted in both transcriptional activation and repression.[20] This study showed significant decreasing expression levels of MBD2 protein in cervical cancer. The unifying model of MBD2 acting as a transcriptional repressor is missing and is required further experimental data.

In conclusion, HR-HPV detection is the cornerstone for triage setting of CIN. Promoter methylation of *MGMT* and *C13ORF18* plays a limited role in triage of LSIL. Promoter methylation of both genes may not be the causes of gene silence.

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Conflicts of interest

There are no conflicts of interest.

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*MGMT*和*C13ORF18*基因甲基化对分流低级别宫颈上皮内病变起有限作用

摘要

研究背景：*MGMT*和*C13ORF18*基因甲基化是宫颈癌早期诊断潜在的分子标记。本研究目的是评价*MGMT*和*C13ORF18*基因甲基化对宫颈细胞学筛查样本的分流作用及机制探讨。

材料与方法：应用甲基化敏感性高通量溶解曲线法（MS-HRM）对124例宫颈脱落细胞学样本行*MGMT*和*C13ORF18*启动子甲基化检测，采用HC2法检测高危型HPV感染，分析基因甲基化与各级宫颈上皮内瘤变的关系。采用卡方检验进行率的比较。采用蛋白印迹法、RT-PCR及免疫组化法检测MGMT、C13ORF18以及甲基化调节基因的表达。

结果：对低级别宫颈上皮内病变（low-grade squamous intraepithelial lesion，LSIL）的分流，高危HPV检测的特异性为4.0%，*MGMT*甲基化将诊断高级别病变的特异性提高至30.8% ($\chi^2 = 9.873$, $P = 0.002$)，*C13ORF18*甲基化将特异性提高至50.0% ($\chi^2 = 21.814$, $P = 0.001$). 对不能明确意义的非典型鳞状细胞（atypical squamous cells of undetermined significance，ASCUS）的分流，高危HPV检测具有较高的阳性预测值（54.8%），*MGMT*或*C13ORF18*甲基化联合高危HPV检测均可将阴性预测值显著提高至100.0% ($\chi^2 = 9.757$, $P = 0.002$)。*MGMT*和*C13ORF18*甲基化与该基因表达无关($\chi^2 = 0.776$, $P = 0.379$；$\chi^2 = 1.411$, $P = 0.235$)。宫颈癌组织MBD2蛋白表达显著低于正常宫颈组织 ($t = 4.11$, $P = 0.006$)。

结论：高危HPV检测是宫颈病变分流的基石，*MGMT*和*C13ORF18*启动子甲基化对分流低级别宫颈上皮内病变起有限作用。*MGMT*和*C13ORF18*甲基化可能不是基因沉默的原因。