Original Article
Quantitative methylation analysis to detect cervical (pre)-cancerous lesions in high-risk HPV-positive women

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Abstract: Objective: High-risk of human papillomavirus (hr-HPV) testing have high sensitivity for cervical (pre)-cancerous lesions. However, its specificity compared with cytology is low. Therefore, a triage strategy for hrHPV positive women is needed in cervical cancer screening. Here, we evaluated the clinical performance of 5 methylation markers in cervical (pre)-cancerous lesions and tested whether methylation analysis for these genes could serve as triage markers. Methods: The hospital-based and case-control study was conducted in 259 hrHPV-positive cervical exfoliated subjects. With histopathological classification of normal uterine cervix (n=45), cervical intraepithelial neoplasia grade 1 (CIN1, n=50), CIN2 (n=46), CIN3 (n=43), and cervical cancer (n=75). Methylation levels of the genes JAM3, SLIT2, TERT, SOX1, and C13ORF18 were examined by using pyrosequencing technology. Results: DNA methylation analysis of five genes showed in the hrHPV-positive cervical exfoliated cell sensitivities 50-93% and specificities 79-90% for CIN3+. For CIN2+, the sensitivities were 34-89% and specificities of 90-98%. We select four with better effect out of five genes to joint each other that showed in the hrHPV-positive cervical exfoliated cell sensitivities for CIN3+ between 66-87% and specificities between 74-91%. For CIN2+, sensitivities between 60-84% and specificities between 64-90%. Conclusion: The current results indicated that quantitative pyrosequencing-based testing for DNA methylation of four genes (JAM3, SOX1, SLIT2 and C13ORF18), especially the JAM3 and SOX1, may serve as molecular triage strategy for hrHPV-positive women. However, further prospective population-based studies using standardized DNA methylation testing will be need to validated.

Keywords: Cervical cancer screening, cervical intraepithelial neoplasia, DNA methylation, HrHPV, pyrosequencing

Introduction

Cervical cancer, a preventable disease, is one of the most common malignant tumors and the main causes of death among women worldwide. According to Global Burden of Disease Cancer Collaboration statistics on cervical cancer, with approximately 485,000 new cases and 236,000 deaths in the year 2013 [1]. Therefore, it is critical important to establish a feasible and effective screening strategy to reduce the cervical cancer burden. As well known, cytology-based pap smear is the most widely used screening strategy that reduced incidence rate and mortality of cervical cancer for decades [2, 3]. However, its sensitivity for detection of CIN2 and high-grade lesions is low [4, 5]. As all known, high-risk human papilloma-virus (hrHPV) is the main cause of cervical cancer [6]. Persistent hrHPV infection drives development of high-grade cervical intraepithelial neoplasia (CIN2/CIN3) which may, if left untreated, progress to invasive cancer [7]. hrHPV testing is more sensitive in detecting high-grade cervical lesions, providing a superior protection against cervical (pre)-carcinoma than cytology [8-10]. Therefore, it become an attractive primary cervical cancer screening tool [11]. A drawback of hrHPV testing, however, is the modest specificity which does not distinguish cancer-relevant lesions from transient hrHPV infections (≤CIN1) [12]. Insufficient specificity of the hrHPV DNA testing leads to high false-positive rate and colposcopy referral rate, needless worry and extra medical burden [12-14]. Therefore, an effective triage strategy to
Quantitative methylation for cervical (pre)-cancerous lesions detection

Numerous studies have demonstrated that promoter hypermethylation of tumor suppressor genes is an early event in the development of cervical cancer and could serve as molecular marker for the early diagnosis [15-18]. DNA methylation analysis of tumor-suppressor genes has shown to be an effective molecular triage tool in hrHPV-positive women [19-22]. There other several existing tests to triage hrHPV-positive screening, including Pap cytology, genotyping for HPV16, HPV18 and other types, MicroRNA, immunostaining for p16 and ki-67 or combination between them [23-26]. At present, cytology-based testing is more commonly used triage tool of hrHPV-positive women [23]. Study has showed that DNA methylation analysis as triage was more sensitive for CIN3 lesions and cancer than cytology testing [20]. Therefore, DNA methylation analysis marker can serve as an alternative or supplementary tool to detect advanced lesions and cervical cancer missed by cytology in hrHPV-positive women [22, 27]. In this present study, we explored the clinical performance of 5 genes methylation status by pyrosequencing assay and to evaluated its potential value as biomarker for hrHPV-positive women in cervical cancer screening.

Materials and methods

Patients

We conducted a hospital-based and case-control study in Tianjin Central Hospital of Gynecology Obstetrics. The cervical exfoliated cell specimens collected from 259 patients. All the included 259 patients have received HCII hrHPV DNA test and had positive results. A cervical brush was used to collected exfoliated cell and stored at 4°C until DNA extraction for the quantitative methylation analysis of JAM3, SLIT2, TERT, SOX1 and C13ORF18, before patients first referred for a colposcopy examination and cervical biopsy. With histopathological classification of normal uterine cervix (n=45), cervical intraepithelial neoplasia grade 1 (CIN1, n=50), CIN2 (n=46), CIN3 (n=43), and cervical cancer (n=75). The exclusion criteria included pregnancy, a history of cervical lesions and surgery, presence of other cancers, or therapy with radiation and chemotherapy. Tianjin Central Hospital of Gynecology Obstetrics approved this study, and all subjects signed a written informed consent.

Table 1. Primer sequences for methylation markers

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Sequence primer</th>
<th>Tm (°C)</th>
<th>Amplification size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAM3</td>
<td>5'-GGTAGTAGGTTTGGTAGTTGTT-3'</td>
<td>5'-biotin-CCCAACCCACACTCC-3'</td>
<td>5'-GTTGTAAGGTGTTTTAGTATTGT-3'</td>
<td>59</td>
<td>206</td>
</tr>
<tr>
<td>SLIT2</td>
<td>5'-GAGGAGGGAGTGTTAGTTAGATAT-3'</td>
<td>5'-biotin-CCCAACCCACACTCC-3'</td>
<td>5'-GAGGAGGGAGTGTTAGTTAGATAT-3'</td>
<td>56</td>
<td>152</td>
</tr>
<tr>
<td>TERT</td>
<td>5'-TGGAGGTTGGTTGGTTGGTT-3'</td>
<td>5'-biotin-ACCTCCCACTCCACACT-3'</td>
<td>5'-TGGAGGTTGGTTGGTTGGTT-3'</td>
<td>60</td>
<td>112</td>
</tr>
<tr>
<td>SOX1</td>
<td>5'-GGTGGTTGTTGTTGTTGTTGTT-3'</td>
<td>5'-biotin-ACCTCCCACTCCACACT-3'</td>
<td>5'-GGTGGTTGTTGTTGTTGTTGTT-3'</td>
<td>58</td>
<td>87</td>
</tr>
<tr>
<td>C13ORF18</td>
<td>5'-AGGAGGTTGTTGTTGTTGTT-3'</td>
<td>5'-biotin-ACCTCCCACTCCACACT-3'</td>
<td>5'-AGGAGGTTGTTGTTGTTGTT-3'</td>
<td>56</td>
<td>163</td>
</tr>
</tbody>
</table>

JAM3: junctional adhesion molecule 3; SLIT2: neuronal guidance factor 2; TERT: telomerase reverse transcriptase; SOX1: sex-determining region Y, box1; C13ORF18: chromosome13 open reading frame 18.

Table 2. The values of methylation in the cervical specimens in the 5 genes

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>JAM3 (%)</th>
<th>SLIT2 (%)</th>
<th>TERT (%)</th>
<th>SOX1 (%)</th>
<th>C13ORF18 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>45</td>
<td>4.42±1.55</td>
<td>5.74±1.19</td>
<td>6.46±1.65</td>
<td>5.78±1.16</td>
<td>1.13±0.60</td>
</tr>
<tr>
<td>CIN1</td>
<td>50</td>
<td>4.31±1.82</td>
<td>6.96±2.98</td>
<td>6.25±1.55</td>
<td>5.28±1.37</td>
<td>1.35±0.68</td>
</tr>
<tr>
<td>CIN2</td>
<td>46</td>
<td>9.58±3.47</td>
<td>8.94±3.26</td>
<td>5.05±2.72</td>
<td>8.02±3.96</td>
<td>2.53±1.74</td>
</tr>
<tr>
<td>CIN3</td>
<td>43</td>
<td>13.77±4.87</td>
<td>11.39±5.98</td>
<td>7.63±2.42</td>
<td>16.38±8.08</td>
<td>4.27±3.12</td>
</tr>
<tr>
<td>Cancer</td>
<td>75</td>
<td>25.88±13.92</td>
<td>24.38±15.75</td>
<td>12.73±9.45</td>
<td>33.01±17.37</td>
<td>8.92±7.94</td>
</tr>
</tbody>
</table>

SD indicates standard deviation; JAM3: junctional adhesion molecule 3; SLIT2: neuronal guidance factor 2; TERT: telomerase reverse transcriptase; SOX1: sex-determining region Y, box1; C13ORF18: chromosome13 open reading frame 18. CIN: cervical intraepithelial neoplasia. *P<0.001 (difference between CIN3 and other groups); jP<0.001 (difference between cancer and other groups).
Quantitative methylation for cervical (pre)-cancerous lesions detection

HR-HPV infection was detected using HCII kits (Digene) in this study according to the manufacturer’s protocol. 13 high-risk HPV subtypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) are able to be detected. Samples with a ratio of RLU (relative light units) higher than 1.0 were recorded as positive.

DNA extraction and bisulfite modification

Genomic DNA was extracted from the cervical exfoliated cell using the TIANamp Micro DNA Kit (TIANGEN BIOTECH Co.Ltd, Beijing, China) following the manufacturer’s recommendations. The DNA concentration and purity was measured using NanoDrop 2000 spectrophotometer (Thermo Fisher ScientificInc, Valencia, CA). Ratio of $A_{260}/A_{280}>1.8$ was the qualified samples required. Sodium bisulphate treated of Genomic DNA samples was prepared using the EZ DNA Methylation-Gold kit™D5006 (ZYMO RESEARCH, USA) according to the manufacturer’s instructions. Bisulfite-converted DNA was stored at -20°C until further use.

Pyrosequencing

Quantitative DNA methylation analysis of the 5 genes were conducted by pyrosequencing. Genes fragments were amplified from bisulphite-converted DNA by PCR that needed primers were in Table 2. The PCR was performed in a 50 μl reaction volume, which contained 3 μl of the bisulfite-modified DNA template, 25 μl Thermo Scientific Dream Taq Green PCR Master
Quantitative methylation for cervical (pre)-cancerous lesions detection

Mix (Thermo Scientific, Lithuania, EU), 10 uM each of gene-specific forward and reverse primer, nuclease-free water. Thermocycling was conducted using the following conditions: 95°C for 10 minutes, followed by 40 cycles at 95°C for 30 seconds, 56-60°C for 30 seconds, 72°C for 35 seconds and final extension at 72°C for 7 minutes. Following amplification, 45 μl of PCR product was mixed with 3 μl streptavidin-conjugated sepharose beads (GE healthcare, Sweden) in 47 μl binding buffer, then shock blending for 15 minutes. The beads were released by a vacuum prep workstation, 1.2 μl sequencing primer (Table 2) added in 38.8 μl annealing buffer, and heated to 80°C for 3 minutes on the ThermoPlate, then cooling to room temperature. Methylation levels of each CpG loci of the 5 genes were quantitatively measured by a PyroMark Q-CpG 1.0.9 software.

Statistical analysis

Statistical analysis and Graphs drawn were performed by SPSS Version 20.0 (IBM, NY) and GraphPad Prism 6 statistical software. Measurement datas were expressed as mean ± standard deviation. Differences in methylation levels between different groups were analyzed by the One-way ANOVA. Receiver operating characteristic (ROC) curves (for CIN2+, CIN3+ and cervical cancer) were made and the area under the curve (AUC) were determined for the detection of the cervical (pre)-cancerous lesions. Specificity and sensitivity were calculated with CIN2+, CIN3+ and cervical cancer as cutoff. P values were lower than 0.05 were considered statistically significant.

Results

Methylation levels of five genes in cervical specimens of hrHPV-positive women

A total of 259 women were enrolled in this study, including normal uterine cervix subjects (n=45), cervical intraepithelial neoplasia grade 1 patients (CIN1, n=50), CIN2 (n=46), CIN3 (n=43), and cervical cancer patients (n=75). They were all tested HPV positive, and histological diagnoses were proved by two pathologists. The characteristics of enrolled patients and genes methylation levels are shown in Table 1. The mean age of cervical cancer had significant higher than anyother groups (P<0.001). Quantitative measurement of DNA methylation for each gene in line with disease severity is shown in Table 2 and Figure 1. The mean methylation levels for all genes, except for TERT, was lower in normal and CIN1 groups than other groups, which also displayed a tendency closely related to the progression of cervical carcinogenesis (Figure 1).
Quantitative methylation for cervical (pre)-cancerous lesions detection

Table 4. Combined testing of DNA methylation for the detection of CIN2+ and CIN3+ in cervical specimens

<table>
<thead>
<tr>
<th>Genes</th>
<th>CIN2+ (AUC (95% CI), P)</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>CIN3+ (AUC (95% CI), P)</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAM3/SLIT2</td>
<td>0.925 (90%, 95%) &lt;0.001</td>
<td>76%</td>
<td>92%</td>
<td>0.910 (88%, 93%) &lt;0.001</td>
<td>74%</td>
<td>91%</td>
</tr>
<tr>
<td>JAM3/C13ORF18</td>
<td>0.841 (81%, 87%) &lt;0.001</td>
<td>78%</td>
<td>70%</td>
<td>0.839 (81%, 87%) &lt;0.001</td>
<td>75%</td>
<td>75%</td>
</tr>
<tr>
<td>JAM3/SOX1</td>
<td>0.940 (92%, 96%) &lt;0.001</td>
<td>84%</td>
<td>91%</td>
<td>0.957 (94%, 97%) &lt;0.001</td>
<td>87%</td>
<td>90%</td>
</tr>
<tr>
<td>SLIT2/C13ORF18</td>
<td>0.776 (74%, 82%) &lt;0.001</td>
<td>71%</td>
<td>64%</td>
<td>0.790 (75%, 83%) &lt;0.001</td>
<td>66%</td>
<td>77%</td>
</tr>
<tr>
<td>SLIT2/SOX1</td>
<td>0.891 (86%, 92%) &lt;0.001</td>
<td>77%</td>
<td>90%</td>
<td>0.927 (91%, 95%) &lt;0.001</td>
<td>76%</td>
<td>91%</td>
</tr>
<tr>
<td>C13ORF18/SOX1</td>
<td>0.793 (76%, 83%) &lt;0.001</td>
<td>60%</td>
<td>82%</td>
<td>0.830 (80%, 87%) &lt;0.001</td>
<td>67%</td>
<td>90%</td>
</tr>
</tbody>
</table>

JAM3: junctional adhesion molecule 3; SLIT2: neuronal guidance factor 2; TERT: telomerase reverse transcriptase; SOX1: sex-determining region Y, box1; C13ORF18: chromosome 13 open reading frame 18. CIN: cervical intraepithelial neoplasia. AUC: area under the ROC curve.

Figure 3. ROC curve of DNA methylation analysis of combined testing for detection of CIN2+ (A) and CIN3+ (B) in hrHPV-positive women.

Performance of DNA methylation analysis of five genes for detection of CIN2+ and CIN3+ in cervical specimens

Sensitivity and specificity for detection CIN2+ and CIN3+ in cervical specimens for the triage of hrHPV-positive women were shown in Table 3 and ROC curve were computed in Figure 2 for each marker. At the optimal cut-off values, the gene JAM3 showed the highest sensitivity (89%) and specificity (91%) for the detection of CIN2+, while for SLIT2, SOX1 and C13ORF18, the sensitivity for CIN2+ was between 61 and 77%. However, these genes showed high specificity between 90 and 99%. The area under the ROC curves (AUC) were 0.967 (95% CI, 95-99%) for JAM3, 0.872 (95% CI, 83-92%) for SLIT2, 0.901 (95% CI, 86-94%) for SOX1, and 0.907 (95% CI, 87-94%) for C13ORF18. For one gene (TERT) an AUC of 0.575 (95% CI, 51-64%), sensitivity and specificity were 61% and 45%, respectively. For CIN3+, SOX1 showed the highest sensitivity (93%) and specificity (90%) among the five genes, with accuracy of 0.968 (95% CI, 95-99%). While for JAM3, SLIT2, C13ORF18, the sensitivity for CIN3+ between 70% and 86%, the specificity between 79% and 90%, nd accuracy ranged from 0.866 to 0.946 (95% CI, 82-97%). For TERT, accuracy, sensitivity and specificity were 0.754 (95% CI, 69-81%), 86% and 40%, respectively. Therefore TERT was excluded from further analysis, although it had high sensitivity for CIN3+, with low specificity for detection CIN2+ and CIN3+.
Performance of DNA methylation analysis of combined testing for detection CIN2+ and CIN3+ in cervical specimens

The clinical performance combined testing was calculated in Table 4 and ROC curve were computed as shown in Figure 3 for detection of CIN2+ and CIN3+. The combination of genes with the highest combined sensitivity (84%) and specificity (91%) for CIN2+ were JAM3/SOX1, and also for CIN3+, with sensitivity and specificity of 87% and 90%, respectively. The area under the ROC curve (AUC) were 0.940 (95% CI, 92-96%) for CIN2+, 0.957 (95% CI, 94-97%) for CIN3+. Table 4 shows that for other combinations, sensitivities for detecting CIN2+ lesions are between 60% and 78%, with a combined specificity between 64% and 92%. For the detection of CIN3+, the sensitivity decreased (66-76%), whereas the specificity slightly increased (75-91%). The area under the ROC curve (AUC) ranged from 0.790 (95% CI, 75-83%) to 0.927 (95% CI, 91-95%) for CIN2+, 0.839 (95% CI, 81-87%) to 0.927 (95% CI, 91-95%) for CIN3+.

Discussion

More and more studies have demonstrated that DNA methylation could be a potential biomarker for detection of cervical cancer. Currently, numerous western countries (USA, Netherlands, Australia, Italy, Spain, etc.) are switching from previously programs cytological-based screening to HPV testing in women with the age of 30-35 years for early detection of cervical cancer. High-risk HPV assay has a high sensitivity for detection of CIN3+, so that it can early find high-grade lesions and prevent cervical cancer [9]. However, the specificity of hrHPV testing is relatively low compared with cytology, it may lead to high false-positive rates, which will increase referrals for colposcopy, overtreatment, and higher costs. Therefore, with a convincing and feasible triage tool for hrHPV-positive women is urgently needed. Recently, studies have proved that DNA methylation analysis as a biomarker for hrHPV-positive women is valuable and it can alternative the role of cytology-based test [19-21]. Cytology is an objective, morphological test that is largely variable between countries and even within a country [10, 23].

Most researches detected DNA methylation in cervical cancer have depend on the methods of methylation-specific PCR (MSP) or quantitative methylation-specific PCR (QMSP). However, these methods have been reported to overrate aberrant methylation prevalence [28]. Quantitative pyrosequencing technique have been proved that could be a preferred tool for detection DNA methylation and feasible to use in clinical setting [29]. In the present study, we used the method of quantitative pyrosequencing to detected the clinical performance of five methylation markers in cervical exfoliated cell of hrHPV-positive subjects. Here, we found the JAM3 gene alone had the highest performance for the detection of CIN2+, with sensitivity and specificity of 89% and 91%, respectively. SOX1 and C13ORF18 gene alone have the highest specificity of 98% and 99% for CIN2+, but the sensitivity, is relatively low (77% and 73%). For CIN3+, SOX1 gene single had the highest performance, with the sensitivity of 93% and specificity of 90%. JAM3 had the same specificity with SOX1, but the sensitivity is 83%. C13ORF18 and SLIT2 also have better performance in detection of CIN3+. TERT had high sensitivity for CIN3+, but the specificity is very low for CIN2+ and CIN3+. To find the methylation panel with the highest combined sensitivity and specificity, different combinations of four genes (JAM3, SLIT2, SOX1, and C13ORF18) were analyzed. We found that the combination of genes with the highest combined sensitivity and specificity for CIN2+ and CIN3+ was JAM3/SOX1, with sensitivity and specificity of 84% and 91% for CIN2+, 87% and 90% for CIN3+. The panel of JAM3/SLIT2 and SOX1/SLIT2 also have higher sensitivity and specificity for detection of CIN2+ and CIN3+.

The JAM3 (Junction adhesion molecule 3) is the third member of adhesion molecules family, played an important role in maintain cell polarity of epithelial and endothelial cells, belonging to the immunoglobulin superfamily [30, 31]. One research had suggested that JAM3 methylation marker may be used as a triage marker for hrHPV-positive patients [32]. Methylation analysis of JAM3, C13ORF18, and TERT for CIN2+ and CIN3+ in scrapings of hrHPV-positive women also had been reported. For CIN2+, the sensitivity and specificity of 68% and 94% for JAM3, 43% and 94% for C13ORF18, 81% and 47% for TERT. For CIN3+, the sensitivity and specificity were 80% and 76% for JAM3, 54% and 88% for C13ORF18, 90% and 43% for TERT [30]. In another study, a four genes (JAM3,
The present studies have some limitations: 1) Almost all of cervical cancer are squamous cell carcinoma, we have collected some exfoliated cells of adenocarcinoma but not many. 2) The cut-off value for each gene was based on a hospital-based, case-control study from a research background, which may not be suitable for clinical setting from large population-based screening test. 3) Moreover, all patients enrolled in this study were Chinese. Methylation analysis of the markers on cervical exfoliated cells was conducted on smaller quantity patients. 4) The collection of cervical exfoliated cells taken immediately before colposcopy may be not appropriate, because of bad vision due to cervical bleeding.

In conclusion, the current results indicated that quantitative pyrosequencing-based testing for DNA methylation, especially the JAM3 and SOX1 may serve as molecular triage strategy for hrHPV-positive women in cervical cancer screening. However, further prospective population-based studies using standardized DNA methylation testing will be need for validation.

Acknowledgements

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Disclosure of conflict of interest

None.

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