Original Article
Correlation between high-risk human papillomavirus (HR-HPV) infection, DNA methyltransferase 1 (DNMT1) expression, and death-associated protein kinase (DAPK) methylation status in cervical lesions

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Abstract: The aim of this study was to investigate the correlation between high-risk human papillomavirus (HR-HPV) infection, expression of DNA methyltransferase 1 (DNMT1) protein, and methylation of death-associated protein kinase (DAPK) promoter in the development of cervical lesions. We included 175 patients at various stages of cervical lesions in our study. HR-HPV infection was investigated using surface Plasmon resonance method, immunocytochemistry assay was used to detect the expression of DNMT1 and the methylation status of DAPK was determined using methylation-specific polymerase chain reaction (PCR). The positive expression rates of DNMT1 protein in normal, cervical intraepithelial neoplasia (CIN)1, CIN2, CIN3, and squamous cell carcinoma (SCC) group were 5.71%, 57.14%, 62.86%, and 76.67%, respectively. The positive expression rates of DAPK methylation in normal, CIN1, CIN2, CIN3, and SCC group were 2.86%, 11.43%, 14.29%, 45.71%, and 60.00%, respectively. In terms of association with the lesion progression, DNMT1 protein expression and DAPK methylation demonstrated an increasing trend (P<0.05). During the development of cervical lesions, high-risk HPV, DNMT1 protein expression, and DAPK gene methylation were positively correlated with each other (P<0.05). DAPK methylation may be completed under the action of DNMT1, which might play a synergistic role with HR-HPV persistent infection in cervical cancerization.

Keywords: Cervical cancer, cervical exfoliated cells, DNA methyltransferase 1, death-associated protein kinase, methylation, human papillomavirus

Introduction
Squamous cell carcinoma of the uterine cervix remains a leading cause of morbidity and mortality worldwide, with more than 85% of the cases occurring in developing countries [1]. Infection with high risk human papillomavirus (HR-HPV) is necessary for the development of cervical cancerization. HPV infection is common, especially in young women, and frequently shows a transient course [2]. Following the HR-HPV infection, cervical cancer develops through pre-cancerous lesions (cervical intraepithelial neoplasia graded 1 to 3 (CIN1-3)) and finally, progresses to invasive cervical cancer. The molecular mechanisms involved in the progression of HPV infections to cervical cancer are poorly understood.

Epigenetic changes, including histone modifications, nucleosome occupancy and positioning, protein and non-coding RNA interactions, as well as direct DNA modifications, are important mechanisms for the development of tumors [3]. In cervical lesions, DNA methylation has gained the most attention. DNA methylation is catalyzed by DNA methyltransferases (DNMTs), which catalyze the covalent addition of methyl groups to cytosines in the CpG dinucleotide. The DNMT1, known as the “maintenance methyltransferase” is mostly used by cells to maintain a stable DNA methylation status through cell division. Aberrant expression of DNMT1 can lead to abnormal DNA methylation, especially hypermethylation of tumor suppressor genes leading to the silencing of tumor suppressor genes, contributing to uncontrolled cell growth.
Abnormal methylation of promoters of tumor suppressor genes is common in cervical cancer, and the analysis of DNA methylation as a prognostic biomarker is under intense investigation [5]. Death-associated protein kinase (DAPK) is a protein kinase associated with apoptosis, forward-induced apoptosis. Recent studies have shown that methylation of DAPK is a potential biomarker for the identification of premalignant lesions that have a high risk to evolve into invasive cervical cancer [6]. However, the direct relationship between methylation of DAPK and DNMT1 in cervical cancer has not been described before.

To evaluate the potential role of HR-HPV, DNMT1 and methylation of DAPK in the progression of CIN to cervical cancer, our study tested the expression of DNMT1 in a range of cervical exfoliated cells obtained from normal, CIN1-3 stage and diagnosed cervical cancer patients. Our findings shed light on the probable mechanism of DAPK methylation induced by abnormal expression of DNMT1, which may provide a new insight to block CIN progress to cervical cancer.

Methods

Subjects

Cervical exfoliated cells were obtained from 175 women with a median age of 32 years (range 30-50) through colposcopic examinations between January and December of the year 2013. According to the results of cervical biopsy pathologic diagnosis was categorized into four study groups, each with 35 patients namely CIN1-3 and squamous cell carcinoma (SCC); the control group of 35 cases was negative for intraepithelial lesion or malignancy. According to the 2009 International Federation of gynecology and Obstetrics (FIGO) stage standard: 21 patients with cervical squamous cell carcinoma were categorized as Ia stage, 6 patients as Ib stage, and 8 patients as Iia stage. According to the pathological classification: 10 patients demonstrated high differentiation, 13 patients demonstrated middle differentiation, and 2 patients exhibited low differentiation. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Shanxi Medical University Second Hospital. Written informed consent was obtained from all participants.

All participants were Han Chinese living in Shanxi for more than five years and were confirmed by two independent pathological examinations. No patient had received radiotherapy or chemotherapy before. After obtaining written informed consent from all participants, cervical exfoliated cells were stored in the Thinprep cytologic test (TCT) cell preservation solution at room temperature, until further analysis.

Surface Plasmon resonance (SPR)

Genomic DNA was extracted using Genomic DNA Fast Extract kit (Bodhisattva Ka Beijing Medical Technology Co. Ltd., Beijing City, China) form the remaining TCT cell preservation solution, and measured at 260 nm, 280 nm absorbance (OD value) by a UV spectrophotometer. The OD260/OD280 (R) reflects the concentration levels of DNA i.e. if R<1.8, the solution contains organic contamination; 1.8<R<2.0, the DNA quality is better; R>2.2, DNA has hydrolyzed to single nucleotides in the solution. In this study, the extracted DNA was detected by UV spectrophotometer, R-values between 1.8 to 2.0 indicated that the DNA had a high-level purity and little protein contamination. Qualified DNA solution was detected for HPV-DNA using HPV genotyping kit (Beijing gold Bodhisattva Jia Medical Technology Co., Beijing City, China); the test results were interpreted using the biosensor chip reading device.

Immunocytochemistry

The TCT remaining cell preservation solution was used to make a thin smear using Thinprep2000 automated cell preparation instrument. Microscope identification was done to select the satisfactory smears with as recommended by the TBS diagnostic system (revised in 2001). This system was modified by using the Streptavidin/Peroxidase series kit (Zymed Laboratories, South San Francisco, CA, USA); diaminobenzidine chromogenic, instead of primary antibody in PBS buffer was used as a negative control. The positive expression of DNMT1 protein was in the nucleus, and a small part of the cell cytoplasm. The DNMT1 protein can be dyed tan or yellow granular. As a secondary scoring method for the criteria of immunohistochemistry results, we randomly selected five regions with high magnification on each cell slide and observed for the number of positive cells. The criteria for evaluation of positive
cells was as follows: zero point for positive cells ≤5%; one point for 6% to 25% positive cells; two points for 26% to 50% positive cells; three points for 51% to 75% positive cells and four points for positive cells >75%. The staining intensity grading scores ranged as follows: zero points for colorless; one point for pale yellow; two points for yellow or dark yellow and three points for brown or tan. Positive results were interpreted if the product of two points on a slide was ≥1.

Methylation specific PCR (MSP)

The qualified DNA was used to make the bisulfite modification using methylation specific PCR kit (Beijing days grace Gene Technology Co., Beijing City, China). The primer was amplified by ready-to-use PCR Kit 2.0 (days grace gene Technology Co., Beijing City, China), and the PCR products was analyzed by electrophoresis through SDS-PAGE gel preparation kit (Beijing Soledad Technology Co., Beijing City, China). Methylation criteria was classified as: permethylation, if the methylated primers amplified target fragment and unmethylated primers did not amplify the objective fragment; partially methylated (hemimethylation), if the target bands of methylated primers and unmethylated primers were amplified and non-methylation, if the unmethylated primers amplified the target band and methylated primers did not amplify.

Statistical analysis

Statistical analyses were conducted using the SPSS 13.0 software package (SPSS Inc., IL, USA). The Chi-square test and trend Chi-square test were used to describe a correlation between HR-HPV, DNMT1, DAPK methylation and clinical features. Pearson correlation analysis was used to describe a correlation between HR-HPV, DNMT1 and DAPK methylation. P< 0.05 was used as the criterion for statistical significance.

Results

High-risk HPV infection

175 cases of cervical lesions in cervical exfoliated cells in each phase of high-risk HPV infection were analyzed for HPV-DNA. The high-risk type HPV positive rate was 81.14%, the differences among these groups was statistically significant ($\chi^2$=35.224, P<0.001). In terms of the lesion progression, HPV infection rate is also showed an increasing trend ($\chi^2$ trend =30.658, P<0.05).

DNMT1 expression

The expression of DNMT1 protein in this experiment was 53.71%. In various types of cervical lesions the positive expression rates of DNMT1 protein have significant difference ($\chi^2$=36.339, P<0.05). The DNMT1 protein expression rate showed increasing trend with the extent of the lesion progression, ($\chi^2$ trend =26.993, P<0.05) (Figure 1).

DAPK methylation

The methylation rate of the promoter region of DAPK gene in CpG island was 26.86%, which
HPV, DNMT1, DAPK methylation in cervical lesions

Table 1. The consequences of high-risk HPV infection, DNMT1 expression and DAPK methylation in cervical exfoliated cells

<table>
<thead>
<tr>
<th></th>
<th>Total number</th>
<th>HR-HPV-positive</th>
<th>P-value</th>
<th>DNMT1-positive</th>
<th>P-value</th>
<th>DAPK-positive</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>35</td>
<td>16 (45.71)</td>
<td>&lt;0.05</td>
<td>2 (5.71)</td>
<td>&lt;0.05</td>
<td>1 (2.86)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CIN1</td>
<td>35</td>
<td>26 (74.28)</td>
<td></td>
<td>20 (57.14)</td>
<td></td>
<td>4 (11.43)</td>
<td></td>
</tr>
<tr>
<td>CIN2</td>
<td>35</td>
<td>32 (91.43)</td>
<td></td>
<td>22 (62.86)</td>
<td></td>
<td>5 (14.29)</td>
<td></td>
</tr>
<tr>
<td>CIN3</td>
<td>35</td>
<td>33 (94.29)</td>
<td></td>
<td>23 (65.71)</td>
<td></td>
<td>16 (45.71)</td>
<td></td>
</tr>
<tr>
<td>SCC</td>
<td>35</td>
<td>35 (100.00)</td>
<td></td>
<td>27 (77.14)</td>
<td></td>
<td>21 (60.00)</td>
<td></td>
</tr>
</tbody>
</table>

correlated with the extent of progression of the lesions. The DAPK gene methylation rates were 3.33%, 10.00%, 13.33%, 46.67% and 60.00%. There was significant difference among the groups ($\chi^2=38.523$, $P<0.05$), and showed an increasing trend ($\chi^2$ trend =34.278, $P<0.05$) (Figure 2; Table 1).

Correlation analysis

Through Pearson correlation analysis, HR-HPV infection was related to DNMT1 protein expression and DAPK gene methylation in the progression of cervical lesions ($r=0.340$, 0.291, $P<0.01$), the expression of DNMT1 protein and the methylation of DAPK gene was also associated ($r=0.291$, $P<0.001$).

Discussion

Cervical cancer is the most common gynecological cancer, 85% of all the new cases per year present in the developing countries. Cervical cancer incidence and mortality in China is accounted for one third of all the cases. Numerous studies show almost all of CIN and cervical cancer patients (more than 96%) with high-risk HPV infection [6]. For cervical cancer, cervical lesions secondary to HPV infection is the main factor. Among other factors, sexually active individuals, smoking, early start of sex life, sexually transmitted diseases and other related factors synergies progressive disease process. The development stages for cervical cancer include CIN1, CIN2, CIN3 and carcinoma in situ, invasive cervical cancer stages. The CIN has two different endings, only some of the lesions in the high-risk HPV long-term and persistent infection develop advanced lesions and invasive cancer. However, the molecular mechanisms involved in the progression of low-grade CIN to cervical cancer are yet poorly understood. Hence exploring the synergistic factors of HR-HPV is vital for blocking the progression of CIN to cervical cancer.

The genotyping of HPV-DNA has revealed more than 120 kinds of this pathogen. According to the size of its virulence, they are divided into two kinds namely high-risk and low-risk. Low-risk type mainly causes exogenous wart lesions,
the flat warts class lesions and CIN1 at genital tract, peri anal skin, and the lower part of the vagina. These generally do not progress to cancer. The high-risk types can lead to CIN2, CIN3 lesions and cervical cancer [7]. In this study, plasma resonance technology could detect 16 HR-HPV type (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 81), the positive rates of infection were 45.71% in the normal control group, 74.28% in the CIN1 group, 91.43% in the CIN2 group, 94.29% in the CIN3 group, and 100.0% in the SCC group. The HR-HPV infection positive rate gradually increased with the progress of cervical lesions, and they are most similar to the results reported in the literature [6]. Nevertheless, the ratio of cervical lesions developing into cancer is much lower than HPV infection rate in the population, and the majority of HPV-positive patients will be negative in about one year, meaning that in addition to HPV infection, there are other synergistic mechanisms that play an important role in the occurrence and progression of cervical lesions.

Current epigenetics research has mainly concentrated on the aberrant methylation of the tumor suppressor gene promoter CpG island. As the DNMT causes abnormally high methylation of the CpG island at the 5' end of tumor suppressor gene, it causes a change in the spatial structure of the chromatin leading to structural alterations and silencing of the expression of tumor suppressor genes resulting in tumorigenesis [8]. Epigenetic changes are the key events in the early tumor formation [9]. As the core content of epigenetics, DNA methylation affects the gene expression pattern and plays an important role in tumor development, closely associated with silencing of tumor suppressor gene transcription [10]. Especially in recent years, several methylation inhibitor compounds have been approved by the FDA for the treatment of certain cancers and show promise as effective anticancer drugs in the future [11]. Currently, the change in DNA methylation patterns has become the focus of attention in the process of cancer development. DNA methylation is a type of DNA modification generally associated with transcriptional silencing, including the global DNA hypomethylation in non-promoter regions and the hypermethylation of CpG islands in the promoter regions. Where the former cause the activation of proto-oncogene and the latter cause the silence of tumor-suppressor gene transcription [12]. The core of the present study focused on the promoter regions of CpG island methylation leading to tumor suppressor gene transcription silencing. Accumulating evidence suggests that alterations of DNA methylation are involved even in the early and precancerous stages [13].

Apoptosis is genetically controlled programmed cell deaths. Under normal circumstances, the proliferation and apoptosis of cells are in dynamic equilibrium. Once the balance is disturbed, the cells show infinite proliferation state, which may lead to cancer. Apoptosis related protein kinase, DAPK is a calcium/calmodulin-dependent serine/threonine kinase and has an important and regulatory role in many signaling pathways [14]. The DAPK located in the chromosome 9q34.1 is a calcium/calmodulin dependent kinase. As a kind of apoptosis positive phase adjustment factor, DAPK participates in multiple apoptosis pathways including the p19ARF/p53, Fas, TNF-α and γ-INF induced pathways [15], and maintained the steady state of dynamic balance between the proliferation and apoptosis of tissue cells [16]. Promoter methylation of the tumor suppressor gene, DAPK, has been reported for several cancers including cervical cancer [17]. However, a link with HR-HPV persistence has not been reported previously but there is a plausible mechanism for a causal relationship. Our study found that the methylation positive rate of DAPK gene promoter in cervical cells was 2.86% in the normal control group; 11.43% in the CIN1 group; 14.29% in CIN2 group; 45.71% in CIN3 group and 60.00% in the SCC group. With the development of cervical lesions, the DAPK methylation rate has shown an increasing trend. Additionally, DAPK gene methylation and HR-HPV infection were positively correlated in the process of cervical cancerization (r=0.291, P<0.01), suggesting that hypermethylation of DAPK gene promoter region may cause synergistic effect with HR-HPV persistent infection in the process of cervical cancerization.

Accumulating studies have shown that the promoter methylation of the tumor suppressor gene is closely related with the activity of DNMTs [18]. The DNMT1 is the major enzyme responsible for maintenance of the DNA methylation pattern and often referred to as mainte-
HPV, DNMT1, DAPK methylation in cervical lesions

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Disclosure of conflict of interest

None.

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