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Methylation of human papillomavirus-52 and -58 is a candidate biomarker in cervical neoplasia

Isao Murakami[a], Takuma Fujii[a,*,1], Katsuaki Dan[b], Miyuki Saito[a], Akiko Ohno[a], Takashi Iwata[a], Daisuke Aoki[a]

[a] Department of Obstetrics and Gynecology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
[b] Collaborative Research Resources, Core Instrumentation Facility, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

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ABSTRACT

Background: Previous studies of human papillomavirus (HPV)16/18 genome methylation have concluded that methylation status of the L1 gene might act as a biomarker for cervical intraepithelial neoplasia (CIN).
Objectives: We investigated the correlation between methylation status in the L1 gene and the long control region (LCR) of HPV52/58 and CIN.
Study design: Exfoliated cervical cells were taken from 54 HPV52-positive and 41 HPV58-positive women. The HPV genome was examined using bisulfite modification, polymerase chain reaction amplification, and sequencing.
Results: The CpGs were unmethylated or hypomethylated in the HPV52/58 LCR. In contrast, the methylation status of the HPV52 L1 gene was correlated with the severity of cervical neoplasia, with average percentages of 15%, 34%, and 52% for cervicitis/CIN1, CIN2, and CIN3, respectively ($P < 0.05$). Methylation status of the HPV52 L1 gene was also correlated with the prognosis of CIN1/2, with median percentages of 15% and 35% for regression and persistence/progression, respectively ($P < 0.05$). The methylation status of the HPV58 L1 gene was correlated with the severity of cervical neoplasia, with average percentages of 12%, 38%, and 61% for cervicitis/CIN1, CIN2, and CIN3, respectively ($P < 0.05$).
Conclusions: The increased methylation at the CpG sites in the HPV52/58 L1 gene was correlated with the severity of cervical neoplasia, similar to HPV16/18 in previous studies. These data suggest that HPV methylation status of the L1 gene is a candidate biomarker of CIN for detecting CIN2 and CIN3.

1. Background

Cervical cancer is the second most common malignant tumor in women worldwide and is associated with persistent infection with high-risk human papillomavirus (HPV) [1]. High-risk HPV types are associated with 99% of all cervical carcinomas. Among these types, HPV16 and HPV18 account for about two-thirds of these malignant lesions worldwide [1]. The available prophylactic bivalent or quadrivalent vaccine is expected to reduced cervical cancer in ~70% of women at present. However, other high-risk HPV types, such as HPV52 and HPV58, are one of the major causes of cervical intraepithelial neoplasia (CIN) and squamous cell carcinoma (SCC) in Asia, especially in Japan. Masumoto et al. performed HPV typing in 3000 Japanese women who attended hospital for cervical cancer screening, or follow-up or treatment of previous cervical neoplasia. HPV52 DNA was detected in 3.9% of patients with CIN1, 12.1% with CIN2, 22.0% with CIN3, and 7.1% with SCC [2]. Onuki et al. performed HPV typing in 2282 Japanese women who attended hospital for cervical cancer screening, treatment of cervical neoplasia, or for other reasons [3]. HPV52 DNA was detected in 9.4% of women with normal cytology, 11.4% with CIN1, 17.5% with CIN2 and CIN3, and 8.4% with SCC. HPV58 DNA was detected in 7.0% of women with normal cytology, 6.8% with CIN1, 10.7% with CIN2 and CIN3, and 3.1% with SCC. Currently, there is no prophylactic vaccine for HPV52 and HPV58. Therefore, it is important to investigate new diagnostic biomarkers for CIN caused by HPV52 and HPV58 to prevent cervical cancer.

Epigenetic mechanisms, which influence chromatin conformations that favor or repress gene expression, have been shown to play a major role in modulating HPV transcription [4,5]. DNA methylation at CpG sites is one of several mechanisms that affect

Abbreviations: CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; LCR, long control region; PCR, polymerase chain reaction.

* Corresponding author. Tel.: +81 3 3353 1211x62397; fax: +81 3 3226 1667.
E-mail addresses: fujiit44@fujita-hu.ac.jp, fujiit44@gmail.com (T. Fujii).
[1] Present address: Department of Obstetrics and Gynecology, Fujita Health University School of Medicine, 1-98 Dengakugakubo, Kutsukake-cho, Toyoake, Aichi 470-1192, Japan.

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chromatin conformation. Methylated CpG sites bind repressors that alter the conformation of nucleosomes through their interaction with histone deacetylases in a manner unfavorable to transcription. There is thus increasing interest in understanding the relationships between HPV DNA methylation and viral gene expression, viral life cycle, virus persistence and replicative behavior, and immune responses [6]. The methylation status of HPV16 and HPV18 has recently been studied by several groups investigating the HPV life cycle and potential new biomarkers [7–22]. In these previous studies, the L1 genes of HPV16 and HPV18 were shown to be hypermethylated in carcinomas, in contrast to their hypomethylated state in premalignant or HPV-positive lesions. They reported that increased methylation at CpG sites in the L1 genes of HPV16 and HPV18 is correlated with severity of cervical neoplasia. These findings suggest that the methylation status of the L1 gene may be a biomarker of clinical progression in HPV16- and HPV18-associated neoplastic lesions. So far, the methylation status of other HPV types, such as HPV52 and HPV58, has not been reported.

2. Objectives

The methylation status of HPV52 and HPV58 was examined to establish whether it was correlated with the severity and regression or progression of cervical neoplasia.

3. Study design

3.1. Clinical specimens and HPV52/HPV58 DNA genotyping

HPV52-infected women with diagnoses of cervicitis ($n = 8$), CIN1 ($n = 9$), CIN2 ($n = 15$), and CIN3 ($n = 22$) of the uterine cervix and HPV58-infected women with diagnoses of cervicitis ($n = 10$), CIN1 ($n = 10$), CIN2 ($n = 9$), and CIN3 ($n = 12$) of the uterine cervix were included. All 95 patients were recruited at the Department of Obstetrics and Gynecology, Keio University Hospital, Tokyo, Japan, between November 2006 and March 2010. Exfoliated cervical cells were treated with proteinase K and buffer containing 150 mM NaCl, 10 mM Tris–HCl (pH 8.0), 10 mM EDTA, and 0.1% sodium dodecyl sulfate for 1 h at 55 °C, followed by overnight treatment at 37 °C. DNA was then extracted by phenol/chloroform extraction followed by ethanol precipitation. HPV52 and HPV58 DNA detection was performed using the Clinichip HPV (Sekisui Medical, Tokyo, Japan) [23].

3.2. Bisulfite modification

DNA samples extracted from exfoliated cells were modified using the DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The converted DNA was eluted with 30 μL TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA). The bisulfite-modified DNA was stored at −20 °C until use.

3.3. Amplification of bisulfite-treated DNA and DNA sequencing

Six primer pairs were designed to amplify the bisulfite-modified HPV52 containing part of the L1 gene or the long control region (LCR), and seven primer pairs were designed to amplify the bisulfite-modified HPV58 containing part of the L1 gene or LCR, using the MethPrimer Design program (http://www.urogene.org/methprimer/index1.html). The primers are listed in Table 1. Twenty-six CpG sites in the HPV52 L1 gene, 15 in the HPV52 LCR, 25 in the HPV58 L1 gene, and eight in the HPV58 LCR were included. Polymerase chain reaction (PCR) was performed using the bisulfite-modified DNA samples in a final volume of 50 μL. The PCR amplification conditions for each primer set were optimized for $MgCl_2$ concentration (2.0–4.0 mM) and annealing and elongation temperature (54 or 60 °C). Each PCR contained 1.25 U AmpliTaq Gold (Roche Applied Science, Indianapolis, IN, USA). The PCR conditions were 95 °C for 10 min, followed by five cycles of 1 min at 95 °C, 2 min at 54 or 60 °C, and 3 min at 72 °C, and 35 cycles of 1 min at 95 °C, 2 min at 60 °C, and 2 min at 72 °C. A final extension step was performed for 10 min at 72 °C. PCRs were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The presence of each PCR product was verified by electrophoresis in ethidium-bromide-stained agarose gels. PCR products were sequenced by Big Dye Terminator v3.1 Cycle Sequencing (Applied Biosystems), which shows cytosine if the original cytosine is methylated, or thymine if the original cytosine is unmethylated. To determine that CpG sites were methylated or unmethylated, the PCR products were sequenced in both directions.

3.4. Follow-up study

Follow-up data for patients with CIN1 and CIN2 who had not undergone any treatments were examined retrospectively. Follow-up visits occurred every 4–6 months and histology, cytology, and colposcopy examinations were performed at each visit. If cytology was abnormal, the patients had colposcopically directed biopsy in their following visit, even if the colposcopic impression was normal in the previous visit. Regression was defined as one negative histological result. Persistence was defined as several continually positive histological results indicating CIN1 or CIN2 for at least 1 year. Progression was defined as the appearance of histologically confirmed CIN3 during follow-up.

3.5. Statistical analysis

Differences were analyzed using two-sided Student's t tests. Differences with $P < 0.05$ were considered significant.

4. Results

4.1. HPV52 and HPV58 methylation status of the L1 gene and LCR

Five CpGs in the HPV52 L1 gene (positions 5730, 5763, 5884, 5972, and 6883), two CpGs in the HPV52 LCR (positions 7557 and 7563), three CpGs in the HPV58 L1 gene (positions 5730, 6868, and 7035), and three CpGs in the HPV58 LCR (positions 7489, 7495, and 7511) could not be amplified by PCR. Therefore, we determined the methylation frequencies at each of the 21 CpG sites in the HPV52 L1 gene, 13 in the HPV52 LCR, 22 in the HPV58 L1 gene, and five in the HPV58 LCR. The average methylation percentages at each measured CpG site in the HPV52/HPV58 L1 gene in DNA from exfoliated cervical cells from patients with cervicitis/CIN1 (A/D), CIN2 (B/E), and CIN3 (C/F) are shown in Fig. 1. The average methylation percentages at all measured CpG sites in the HPV52/HPV58 L1 gene and the LCR in DNA from exfoliated cervical cells from patients with cervicitis/CIN1, CIN2, and CIN3 are shown in Table 2. As shown in Fig. 1 and Table 2, CpGs were hypermethylated in the HPV52 and HPV58 L1 gene with increasing cervical dyskaryosis. The average percentages of all CpGs that were methylated in the HPV52 L1 gene were 15% (range 0–43%) for cervicitis/CIN1, 34% (range 0–83%) for CIN2, and 52% (range 8–85%) for CIN3 (Fig. 1 and Table 2A). The average percentages of all CpGs that were methylated in the HPV58 L1 gene were 12% (range 0–61%) for cervicitis/CIN1, 38% (range 0–100%) for CIN2, and 61% (range 11–100%) for CIN3 (Fig. 1 and Table 2B). The methylation percentages at each CpG site in the HPV52 and HPV58 L1 genes differed significantly among different grades of cervical neoplasia ($P < 0.05$). However, the average percentages of all CpGs that were methylated in the HPV52 LCR were 2.5% (range 0–16%) for cervicitis/CIN1, 1.3% (range 0–16%) for CIN2, and 3.2% (range 0–25%) for CIN3 (Table 2A). The CpG sites were unmethylated in