Different DNA methylation pattern of HPV16, HPV18 and HPV51 genomes in asymptomatic HPV infection as compared to cervical neoplasia

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Introduction

Human papillomaviruses (HPVs) are common human pathogens that infect cutaneous or mucosal epithelia in which they may cause benign and cancer diseases. The development of cervical neoplasia is linked to the infection with at least one of 13 high-risk HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) (Cogliano et al., 2005). Epidemiologic and molecular studies revealed that persistent infections with high-risk types of HPV are essential in the pathogenesis of cervical carcinoma but additional genetic and epigenetic alterations are required for triggering the carcinogenesis (Lazo, 1999).

Epigenetic regulation of gene expression is mediated by highly specific DNA methylation and histone modification patterns. DNA methylation is one of the most studied epigenetic modifications, which occurs at the cytosine bases of CpG sequences. During this process, cytosine bases are converted to 5-methylcytosine by DNA methyltransferase. Methylated cytosines have an important impact on gene expression by either physical blocking of binding sites of transcriptional proteins involved in gene expression processes or by changing chromatin structures responsible for inhibiting transcription. Previous studies have revealed an important role of DNA methylation in the transcriptional regulation of specific genes and causal relationship between certain defects in DNA modification mechanisms and various diseases including cancer (Kulis and Esteller, 2010).

HPV long control region (LCR) contains viral promoter sequences of early genes including viral oncogenes E6 and E7, viral transcriptional enhancer and viral origin of DNA replication (Burd, 2003). DNA methylation of HPV LCR is one of possible mechanisms by which transcription of E6 and E7 genes are regulated. DNA methylation studies indicated that the degree of CpG methylation at HPV16 LCR sequences increased with the severity of the disease (Ding et al., 2009; Hong et al., 2008; Kalantari et al., 2004). HPV16 DNA sequences are found in carcinoma cells most often integrated into the host genome and in some cases integration of multiple HPV copies can occur as tandem repeats that represent efficient DNA methylation targets (Daniel et al., 1995). Such methylation of HPV LCR DNA sequences may serve as a host defense mechanism for silencing the transcription and replication of viral genomes. However, a certain degree of LCR DNA methylation was also detected in asymptomatic patients (Ding et al., 2009; Hong et al., 2008; Kalantari et al., 2004). In contrast to HPV16, a few previous studies have shown
that LCR DNA sequences of HPV18 were unmethylated in DNA samples extracted from specimens with different degrees of cervical pathologies (Turan et al., 2006; Badal et al., 2004).

The 3′ parts of HPV L1 genes located upstream of LCR have been also investigated as targets for DNA methylation. Investigation of DNA samples purified from cervical cancer specimens has shown that L1 genes of HPV16 and HPV18 are methylated and the degree of DNA methylation of target sequences increases correspondingly to the severity of the disease (Kalantari et al., 2004; Turan et al., 2006; Badal et al., 2004; Bryant et al., 2014). DNA methylation pattern of L1 gene sequences of various HPV types provides a basis for speculations that methylation of HPV L1 genes might be a result of HPV integration into the host DNA (Kalantari et al., 2004, 2008, 2010; Bryant et al., 2014; Van Tine et al., 2004).

DNA methylation frequencies of other high-risk HPV types such as HPV31, HPV33, HPV45, HPV52 and HPV58 have been investigated as targets for DNA methylation. Investigation of HPV16, HPV18 and HPV51 isolated from clinical specimens with different grades of cervical pathology and to evaluate possible correlations between HPV DNA methylation levels and the severity of cervical neoplasia. For the first time, methylation pattern of HPV51 DNA isolated from clinical specimens has been analyzed.

Results

Study design

DNA bisulfite modification and sequencing of target amplicons were used to determine the CpG methylation status of HPV16, HPV18 and HPV51 genomes at 3′ part of L1 gene and LCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position in HPV genome (nt)</th>
<th>Sequence, 5′–3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>16msp3F</td>
<td>7049–7078</td>
<td>AAGTCAGCTTAGGCTAAATTTAAAATTTTTT</td>
</tr>
<tr>
<td>16msp3R</td>
<td>7590–7560</td>
<td>GACAAAATAATCAATAAAAACAAACAAAAAA</td>
</tr>
<tr>
<td>16msp4F</td>
<td>7469–7493</td>
<td>CAGATTTTTTTGATATAATTAG</td>
</tr>
<tr>
<td>16msp4R</td>
<td>7732–7703</td>
<td>TAAATTTAAAAATCAATAAAAATAT</td>
</tr>
<tr>
<td>16msp5F</td>
<td>7748–7777</td>
<td>TAAATTTAAAAATCAATAAAAATAT</td>
</tr>
<tr>
<td>16msp5R</td>
<td>7158–7186</td>
<td>ATCCTAAAAATCATACCTTCTTCTTTT</td>
</tr>
<tr>
<td>18msp6F</td>
<td>6847–6871</td>
<td>TATTTGTTTAGGGATGATAT</td>
</tr>
<tr>
<td>18msp6R</td>
<td>7186–7161</td>
<td>TATTTGTTTAGGGATGATAT</td>
</tr>
<tr>
<td>18msp10F</td>
<td>7282–7293</td>
<td>TATAATTGTTTTGTGTTTTT</td>
</tr>
<tr>
<td>18msp10R</td>
<td>7747–7721</td>
<td>TATAATTGTTTTGTGTTTTT</td>
</tr>
<tr>
<td>18msp8F</td>
<td>7573–7781</td>
<td>TATTTGTTTAGGGATGATAT</td>
</tr>
<tr>
<td>18msp8R</td>
<td>7773–7815</td>
<td>TATTTGTTTAGGGATGATAT</td>
</tr>
<tr>
<td>18msp11F</td>
<td>7715–7815</td>
<td>TATTTGTTTAGGGATGATAT</td>
</tr>
<tr>
<td>18msp11R</td>
<td>7815–7815</td>
<td>TATTTGTTTAGGGATGATAT</td>
</tr>
</tbody>
</table>

PCR primers used for amplification of bisulfite-modified HPV16, HPV18 and HPV51 DNA sequences.

DNA methylation status of 17 CpG dinucleotides of the selected HPV16 DNA sequences was investigated in 157 DNA samples isolated from HPV16-infected cervical specimens. Two of these 17 CpG dinucleotides were located in 3′ part of the L1 gene (positions from 7136 nt to 7145 nt of HPV genome), 4 CpG dinucleotides were located in LCR 5′ segment sequences (positions from 7270 nt to 7461 nt), 6 CpG dinucleotides were located in DNA promoter region encoding the transcriptional enhancer (positions from 7535 nt to 7862 nt) and 5 pairs of CpG were located in E6 promoter regions (positions from 31 nt up to 58 nt) (Table 2). Investigations of DNA methylation frequency at selected CpG dinucleotides revealed high variability in cytosine methylation among selected CpG sites and among different amplicons. Investigation of HPV16 DNA methylation pattern revealed a strong correlation between the increased CpG methylation of selected DNA sequences and the severity of cervical neoplasia. In general, the methylation of CpG sequences was more prevalent in L1 gene and promoter region sequences as compared to LCR 5′ and enhancer regions of HPV16 DNA. The degree of DNA methylation of at least one CpG dinucleotide of HPV16 L1 gene ranged from 0% in a subgroup of CIN I up to 35.5% in a subgroup of cervical cancer. In a similar way, the degree of CpG methylation within the sequences of HPV16 DNA promoter region ranged from 0% in CIN I up to 32.3% in cervical cancer. Methylation frequency of CpG dinucleotides in HPV16 DNA sequences of LCR 5′ and enhancer regions ranged from 0% in CIN I up to 25.8% in cervical cancer. In addition, we detected 6.9% methylated CpG dinucleotides within the selected HPV16 DNA sequences in case of asymptomatic HPV16 infections in comparison to 22.6% methylated CpG within the same HPV16 DNA sequences in cervical cancer. Statistically significant difference after Bonferroni correction was detected between HPV16 DNA methylation frequency within the selected sequences of L1 gene in asymptomatic HPV16 infection as compared to cervical cancer (P = 0.0027, Table 3).

DNA methylation frequency of the individual CpG sites within the selected sequences of DNA ranged from 0% in asymptomatic HPV16 infection up to 11.8% in CIN I/II. Even more differences in HPV16 DNA methylation pattern were detected in amplicons generated from DNA samples isolated from specimens with CIN III/CIS and cervical cancer specimens: the degree of HPV16 DNA methylation ranged from 0% up to 35.5%, respectively. The highest frequency of HPV16 DNA methylation (35.5%) was detected at positions of 7136 nt and 7145 nt of DNA sequences generated from cervical cancer specimens. Statistically significant DNA
methylation differences after Bonferroni correction were determined at positions of 7136 nt and 7145 nt between DNA samples isolated from cervical specimens with asymptomatic HPV16 infection as compared to cervical cancer specimens ($P=0.0027$ and $P=0.0003$, respectively). The degree of HPV16 DNA methylation was low at CpG dinucleotides located at four positions: 7270 nt, 7316 nt, 7428 nt, and 7455 nt between DNA samples isolated from cervical specimens with asymptomatic HPV16 infection. In summary no statistically significant differences

CIN I, cervical intraepithelial neoplasia grade 1; CIN II, cervical intraepithelial neoplasia grade 2; CIN III/CIS, cervical intraepithelial neoplasia grade 3 or carcinoma in situ; CC, cervical cancer; Normal, asymptomatic HPV infection.

* $P=0.0027$

### Table 3
HPV16 (A), HPV18 (B) and HPV51 (C) DNA methylation frequency within the L1 3' and LCR 5' regions.

<table>
<thead>
<tr>
<th>Region</th>
<th>HPV16 CpG, nt</th>
<th>HPV18 CpG, nt</th>
<th>HPV51 CpG, nt</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1 3'</td>
<td>7136, 7145</td>
<td>6916, 7011, 7038, 7041, 7062, 7068, 7090, 7110, 7116, 7122</td>
<td>6916, 6939, 6946, 6970, 7074, 7095, 7021</td>
</tr>
<tr>
<td>LCR 5'</td>
<td>7270, 7428, 7455, 7461</td>
<td>7316</td>
<td>7228, 7245, 7277, 7283</td>
</tr>
<tr>
<td>Enhancer</td>
<td>7535, 7554, 7677, 7763, 7863, 7872</td>
<td>7460, 7468, 7511, 7573, 7575, 7586, 7597, 7631</td>
<td>7393, 7395, 7404, 7498, 7502, 7508</td>
</tr>
<tr>
<td>Promoter</td>
<td>31, 37, 43, 52, 58</td>
<td>7824, 44, 50, 54, 60, 66</td>
<td>7762, 32, 48, 54</td>
</tr>
</tbody>
</table>

**Methylation of HPV18 DNA target sequences**

The frequency of HPV18 DNA methylation was evaluated at 25 CpG dinucleotides along the selected sequences obtained from bisulfite-modified and amplified DNA isolated from HPV18-positive cervical specimens ($n=21$). Ten of the selected 25 CpG sites were located in 3' part of the L1 gene (positions from 6916 nt to 7122 nt), 1 CpG site was located in LCR 5' part of the L1 gene (position 7316 nt), and 8 CpG dinucleotides were distributed along the sequence recognized by the transcriptional enhancer (positions from 7460 nt to 7631 nt), and 6 CpG sites were located in E6 promoter sequence (positions from 7824 nt to 66 nt) (Table 2).

An extremely high frequency of HPV18 DNA methylation was detected in L1 3' region sequences of the subgroups diagnosed with CIN III/CIS and cervical cancer. The degree of HPV18 DNA methylation at positions from 6916 nt to 7122 nt (10 CpG dinucleotides in a part of L1 3' region) reached 100% in DNA samples obtained from subgroups of cervical cancer and CIN III/CIS. Only one HPV18 DNA sample in the subgroups of asymptomatic HPV18 infections and CIN I was methylated within the 3' part of L1 gene. In contrast to this unique HPV18 DNA sample, no one HPV18 DNA sample from a subgroup of CIN II was methylated along the 3' part of L1 gene. Only one HPV18 DNA sample isolated from the subgroup of CIN III/CIS and only 2 HPV18 DNA samples purified from cervical cancer specimens were found to be methylated along the LCR 5' region sequences at 7316 nt. HPV18 DNA enhancer and promoter region sequences were methylated at positions from 7460 nt to 66 nt only in one DNA sample representing the subgroup of cervical cancer. Similarly to HPV16, unmethylated cytosines were identified at positions 7824 nt and 50 nt in all analyzed HPV18 DNA samples. In contrast to clinical specimens, the selected DNA sequences of HPV18-transformed HeLa cells used as a positive control were methylated at all CpG dinucleotides along the selected L1 gene and LCR 5' part sequences. No methylated cytosines were detected within other selected parts of DNA isolated from cervical specimens with HPV18 infection. In summary no statistically significant differences of HPV18 DNA methylation frequency at all selected CpG positions (from 6916 nt to 66 nt) were determined when HPV18 DNA
samples isolated from specimens with different grades of cervical pathology were compared (Fig. 2; Supplementary Table 4).

DNA methylation of HPV51 DNA target sequences

HPV51 DNA methylation pattern at 27 selected CpG sites were investigated using 24 DNA samples purified from cervical specimens infected with HPV51. Eight of these 27 CpG sites were located within the 3’ part of L1 gene sequence from 6916 nt to 7021 nt, 4 CpG dinucleotides were selected from LCR S’ segment sequence from 7228 nt to 7283 nt, 11 CpG sites were selected within the sequence recognized by the transcriptional enhancer (from 7393 nt to 7594 nt) and 4 CpG sites were selected from the sequence from 7228 nt to 7283 nt, 11 CpG sites were selected within the sequence recognized by the transcriptional enhancer (from 7393 nt to 7594 nt) and 4 CpG sites were selected from the sequence of E6 promoter (from 7762 nt to 54 nt) (Table 2).

Unexpected pattern of HPV51 DNA methylation was detected in almost all DNA samples purified from 24 HPV51-positive cervical specimens: DNA sequences were unmethylated along all amplicons selected for detailed analysis. The exception was detected only in one HPV51 DNA sample representing the subgroup of cervical cancer: in this specimen all cytosines were methylated within the 3’ part of L1 gene sequences from 6916 nt to 7021 nt. Data on HPV51 DNA methylation frequency along the selected target sequences are presented in Table 3.

Discussion

HPV infection is the most common viral infection of the reproductive tract that may cause cervical cancer and precancerous pathology (Longworth and Laimins, 2004; Hoory et al., 2008). According to the World Health Organization, cervical cancer is the second most common cancer in women worldwide. Every year, 530,000 new cases of cervical cancer are reported and more than 270,000 women die from cervical cancer (World Health Organisation, 2014). Therefore it is important to investigate the mechanisms of carcinogenic progression induced by HPV16, HPV18 and other high-risk HPV types.

In the current study, DNA methylation status of HPV16, HPV18 and HPV51 genomes representing the selected sequences encompassing CpG dinucleotides within the 3’ part of L1 gene and LCR region sequences were investigated in clinical specimens with confirmed HPV infection and different grades of cervical pathology. To our knowledge, this is the first study to investigate the DNA methylation pattern of HPV51. The obtained data revealed that DNA methylation of 3’ part of HPV L1 gene is much more prevalent than that of viral LCR. Moreover, HPV DNA methylation frequency is much higher in DNA samples isolated from carcinoma specimens than in those isolated from cervical specimens with asymptomatic HPV infection. The highest degree of HPV16 DNA methylation (35.5%) was determined along the 3’ part of the L1 gene in case of cervical cancer. The lowest HPV DNA methylation frequency was detected in the selected DNA sequences of the subgroups of CIN I (0%) and asymptomatic HPV16 infection (3.4%).

Similar pattern of HPV16 DNA methylation was detected within the promoter region sequences. The degree of DNA methylation ranged from 0% in HPV16 DNA samples representing CIN I to 32.3% in HPV16 DNA samples representing cervical cancer. Statistically significant differences in HPV16 DNA methylation of L1 region sequences were detected between the subgroups of asymptomatic HPV16 infection and cervical cancer (P=0.0027). Lower HPV16 DNA methylation frequency (ranging from 0% to 25.7%) were determined within other LCR regions (Fig. 1, Supplementary Table 4). Previous studies on HPV16 DNA methylation reported similar positive relationship between the degree of methylation in the L1 gene and the severity of cervical pathology (Kalantari et al., 2009; Fernandez et al., 2009; Brandsma et al., 2009; Sun et al., 2011; Mirabello et al., 2012). However, previous data on the association between HPV DNA methylation in the LCR region and the grade of cervical pathology are rather controversial. Several studies have revealed a decreased DNA methylation frequency in viral LCR region sequences generated from DNA samples isolated from HPV-infected specimens with cervical pathology (Ding et al., 2009; Badal et al., 2003; Xi et al., 2011; Mazummer et al., 2011; Snellenberg et al., 2012; Vinokurova and von Knebel, 2011; Bhattacharjee and Sengupta, 2006). Despite these controversial results on the pattern of HPV DNA methylation in cervical pathology, several findings are common: in most previous studies the level of DNA methylation at 7862 nt (CpG) in the enhancer region was found to be very low. In line with these data, in the current study all analyzed HPV DNA samples were found to be unmethylated at position 7862 nt. The indicated CpG position (7862 nt) overlaps with E2 binding site. It is well-documented that E2 gene repress the transcription of oncogenes E6 and E7. Therefore, the methylation of E2 binding site might lead to the loss of E2 repression and sustained E6/E7 expression (Ding et al., 2009; Kalantari et al., 2004; Vinokurova and von Knebel, 2011; Bhattacharjee and Sengupta, 2006). In line with many previous studies, the current research has demonstrated that HPV16 DNA methylation within the viral promoter region is increased in case of cervical cancer as compared to other types of cervical pathology or asymptomatic HPV infection. (Ding et al., 2009; Hong et al., 2008; Badal et al., 2003; Xi et al., 2011; Snellenberg et al., 2012;
of HPV51 genome was analyzed in DNA samples purified from cervical cancer. Noteworthy, we detected methylated cytosines in HPV51-positive specimens included only 1 case of cervical cancer. In contrast, DNA methylation levels at the selected sequences of HPV18 LCR DNA sequences (Turan et al., 2006; Badal et al., 2004; Fernandez et al., 2009).

The current study also revealed the highest methylation frequency of HPV18 DNA along the 3' part of L1 gene sequences. DNA methylation frequency reached 100% in the selected sequences of HPV DNA isolated from specimens with CIN III/CIS and cervical cancer. In contrast, DNA methylation levels at the selected sequences of HPV18 LCR were low among all tested cervical HPV DNA samples (Fig. 2; Supplementary Table 4). The obtained data on the pattern of HPV18 DNA methylation are in a full concordance to previous reports, demonstrating an intense (often exceeding 80%) methylation at CpG positions within the 3' part of L1 gene sequences in DNA samples isolated from cervical cancer specimens, and very low methylation (often none), in the HPV18 LCR DNA sequences (Turan et al., 2006; Badal et al., 2004; Fernandez et al., 2009).

For the first time, DNA methylation pattern of the selected parts of HPV51 genome was analyzed in DNA samples purified from cervical specimens with a various grade of cervical pathology. This collection of HPV51-positive specimens included only 1 case of cervical cancer. Noteworthy, we detected methylated cytosines within the 3' part of L1 gene sequences (from 6916 nt to 7021 nt) only in this single HPV51 DNA sample representing a case of cervical cancer (Table 3). The obtained data are in line with data on HPV16 and HPV18 DNA methylation at the same locus. All other analyzed HPV51 DNA sequences were found to be unmethylated in all tested samples representing the subgroups of asymptomatic HPV51 infection, CIN I/II and CIN III/CIS. Methylation pattern of other high-risk HPV types, such as HPV31, HPV33, HPV45, HPV52 and HPV58 have been analyzed in recent studies. Obtained data demonstrated that HPV DNA methylation frequency of the L1 gene is much more prevalent than that of the LCR sequences and correlates with the severity of cervical pathology (Murakami et al., 2013; Vasiljevic et al., 2014; Wentzensen et al., 2012). However, the biological basis for the association between HPV L1 gene methylation and cervical cancer progression is still unclear. A few studies showed that the L1 gene methylation is associated with HPV integration into cellular DNA (Kalantari et al., 2004, 2008; Bryant et al., 2014; Van Tine et al., 2004). Although DNA methylation represents gene repression mechanism, the product of L1 gene is HPV capsid protein not involved into carcinogenesis processes. HPV DNA methylation events can be interpreted as a genomic epigenetic alterations in high-risk HPV genomes may serve as a potential biomarker for HPV-induced carcinogenesis.

Materials and methods

Materials

Maxima™ Hot Start PCR buffer, Hot Start Taq DNA polymerase, 2 mM dNTP, 25 mM MgCl2, agarose, BSA, O’GeneRuler™ 50 bp DNA Ladder, “GeneJet™ Genomic DNA Purification Kit”, “GeneJET Gel Extraction and DNA Cleanup Micro Kit” were purchased from Thermo Fisher Scientific Baltics UAB (Vilnius, Lithuania). “DNA Methylation–Gold Kit” was obtained from Zymo Research (Irvine, CA, USA). All oligonucleotides were obtained from Metabion (Steinkirchen, Germany). Cervical cell lines used in the study: CaSki (60–660 copies of HPV16 per genome) and HeLa (10–50 copies of HPV18 per genome) were obtained from American Type Culture Collection (Manassas, VA).

Clinical specimens and study groups

In total, 824 women (aged from 18 to 80 years) with known histological and/or cytological diagnosis were included into the study. Cervical samples were collected from the cervix using cervical brush during our previous study on the prevalence of oncopgenic HPV types among Lithuanian women with cervical pathology (Simanaviciene et al., 2015). The study was approved by Vilnius Regional Committee of Biomedical Research (Lithuania, permission no. 158200–6–062–16, 2009–06–03). In total, 202 clinical specimens with known histological diagnosis were included into the study: 157 HPV16-positive clinical specimens, 21 HPV18-positive clinical specimens and 24 HPV51-positive clinical specimens. HPV16-positive clinical specimens (n=157) consisted of 10 cases of grade 1 cervical intraepithelial neoplasia (CIN I), 17 cases of grade 2 cervical intraepithelial neoplasia or carcinoma in situ (CIN II/CIS), 31 cases of cervical cancer and 29 cases of asymptomatic HPV16 infection. HPV18– positive clinical specimens (n=21) included 3 cases of CIN I, 2 cases of CIN II, 2 cases of CIN III/CIS, 6 cases of cervical cancer and 8 cases of asymptomatic HPV18 infection. HPV51–positive specimens (n=24) consisted of 6 cases of CIN I, 4 cases of CIN II, 5 cases of CIN III/CIS, 1 case of cervical cancer and 8 cases of asymptomatic HPV51 infection.

DNA purification

DNA samples from cell lines HeLa (ATCC NO. CCL-2) and Caski (ATCC NO. CRL-1550) were purified using “GeneJET™ Genomic DNA Purification Kit” according to manufacturer’s instruction. Detailed description of collected cervical specimens and the procedure of DNA extraction from cervical specimens have been presented previously (Simanaviciene et al., 2015).

Conclusions

The current study presents new data on DNA methylation pattern at 3' parts of the L1 gene and the LCR sequence of HPV16, HPV18 and HPV51. For the first time, DNA methylation of HPV51 was investigated. The methylation of cytosines in CpG sites was more prevalent in the L1 gene than in the LCR sequence of all analyzed HPV DNA samples. Moreover, the frequency of HPV DNA methylation correlated with the severity of cervical neoplasia. The current study also revealed an increased DNA methylation level of HPV16 promoter region in case of cervical cancer as compared to other types of cervical pathology and asymptomatic HPV16 infection. The identified epigenetic alterations in high-risk HPV genomes may serve as a potential biomarker for HPV-induced carcinogenesis.
Bisulfite DNA modification, PCR and DNA sequencing

DNA extracted from cervical specimens was used for bisulfite modification using “DNA Methylation-Gold Kit” according to manufacturer’s instructions. DNA samples were stored at −20 °C until use. Bisulfite DNA modification may cause DNA fragmentation, therefore amplification of large genomic DNA segments is limited in most cases by specific features of procedure used for bisulfite DNA treatment. Taking it into account, target HPV DNA sequences selected for methylation analysis were split into 3–4 genomic segments. Three sets of primer pairs were used to amplify bisulfite-modified HPV16 DNA sequences containing 3’ part of L1 gene and LCR sequences (Kalantari et al., 2004). Modified DNA samples were amplified in the form of 3 separate amplicons located at the following positions of viral genome: 7049–7590 nucleotides (nt), 7465–7732 nt and 7748–115 nt. According to the same order, 3 sets of primer pairs were used to amplify HPV18 DNA sequences of 3’ part of the L1 gene and the LCR (Badal et al., 2004). Three target sequences for generation of amplicons from DNA samples of HPV18-infected specimens were located at the following positions of viral genome: 6847–7186 nt, 72829–7747 nt and 7753–186 nt. Other 4 sets of primer pairs were designed to amplify HPV51 DNA sequences containing 3’ part of the L1 gene and LCR, using the MethPrimer Design program (http://www.urogene.org/methprimer/). The beginnings and the ends of 4 amplicons of HPV51 bisulfite-modified DNA were selected at the following positions of viral genome: 6848–7064 nt, 7139–7354 nt, 7325–7639 nt and 7668–99 nt. Table 1 summarizes all sets of primers used for specific amplification of HPV16, HPV18 and HPV51 bisulfite-modified DNA samples. All PCR procedures were performed in a final volume of 25 μl with the following composition: 22 μl of Master Mix (consisted of Hot Start PCR buffer, MgCl2, Hot Start Taq DNA polymerase, dNTP, BSA, primer pair, and water) and 3 μl of bisulfite-modified DNA. PCR was run according to the following protocol: 94 °C for 5 min, 40 cycles at: 94 °C 10 s, 54 °C 30 s, 68 °C 1 min, and a final extension at 68 °C for 7 min. Obtained PCR products were analyzed in ethidium bromide-stained agarose gels and sequenced directly using reagent kit BigDye Terminator v3.1 Cycle Sequencing Kit and Applied Biosystems 3730 × 1 DNA Analyzer (Baselcare, Leiden, The Netherlands).

Statistical analysis

All HPV DNA methylation data were analyzed using Microsoft Excel and Statistica 8 programs. The frequency of methylation of CpG positions of different HPV DNA segments were compared using Fisher’s exact test. In total, 17 CpG positions of HPV16, 25 CpG positions of HPV18 and 27 CpG positions of HPV51 were analyzed in the corresponding HPV DNA sequences. A high number of statistical comparisons were performed, therefore we adjusted the level of significance for the statistical tests by using Bonferroni’s adjustment for multiple comparisons.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.06.008.

References


