Determination of human papillomavirus 16 physical status through E1/E6 and E2/E6 ratio analysis

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Human papillomavirus (HPV) 16 genome integration into the host chromosome is a crucial event during the life cycle of the virus and a major step towards carcinogenesis. The integration of HPV16 DNA promotes a constitutive high expression level of E6 and E7 oncoproteins, resulting in the extensive proliferation of the infected epithelial cells. In the present report the physical status of the HPV16 genome was studied, through determination of E1/E6 and E2/E6 DNA copy number ratios in 61 cervical samples of low- and high-grade malignancy and 8 cervical cancer samples, all of them associated with HPV16 infection. The selection of E1, E2 and E6 amplification target regions was performed according to the most prevalent deleted/disrupted sites of E1 and E2 genes. For this target selection we also considered the most conserved regions of E1, E2 and E6 genes among the same HPV16 isolates that were recently reported by our group. The analysis of HPV16 DNA form revealed a significant association among the mixed DNA forms in low-grade and high-grade malignancies, ($\chi^2$, P<0.01). The comparative analysis of E1/E6 and E2/E6 in the same cervical samples provides an accurate picture of HPV16 DNA form and may reveal whether different HPV16 DNA integrants coexist in the same cervical sample or not. This study proposes that E1/E6 and E2/E6 ratios determine with accuracy the HPV16 DNA integration pattern and may predict multiple integration events in the examined sample, thus providing significant information about the progression of cervical dysplasia.

INTRODUCTION

Cervical cancer is the third most common type of cancer among women worldwide, with a high mortality rate. The worldwide incidence of cervical carcinoma is more than 530,000 cases per year, whereas mortality reaches 275,000 deaths annually, of which approximately 85% occur in developing countries (Jemal et al., 2011; Forman et al., 2012). The aetiological agents for the development of high-grade precancerous cervical lesions and invasive cervical cancer are the oncogenic human papillomavirus (HPV) types present as persistent infections (Muñoz et al., 2003). To date, more than 150 different HPV types have been characterized and about 40 of them are related with anogenital tract malignancy, grouped as high-risk (HR) or low-risk (LR) genotypes (zur Hausen, 1996; Bernard et al., 2006, 2010). The oncogenic human papillomavirus types present as persistent infections (Muñoz et al., 2003). Persistent infection with HR HPV types is associated with an increasing risk of integration of the viral circular genome (episome) into the host chromosomes, leading to cancer development. The circular HPV genome is then linearized, but the long control region, and the E6 and E7 oncogenes, are always retained intact (Wentzensen et al., 2004; Xu et al., 2013; Akagi et al., 2014). Viral integration appears to coincide with the development of high-grade cervical intraepithelial neoplasia (CIN II, III) as a consequence of overexpression of the E6 and E7 oncogenes (Doorbar et al., 2012). In particular, the integration of the HPV16 genome usually disrupts the E2 and/or E1 regions found downstream of the early genes E6 and E7, thus resulting in functional inactivation of the E1 and E2 proteins (Chen et al., 1994; Arias-Pulido et al., 2006; Cricca et al., 2009). Disruption events within the E1 and/or E2 genes lead to interference of viral DNA replication control. Moreover, disruption of the E2 gene results in the
elimination of the E2 viral repressor function which leads to deregulation of the E6/E7 promoter activity (Kalantari et al., 1998; Arias-Pulido et al., 2006; Cricca et al., 2009; Hamid et al., 2009; Kadaja et al., 2009; Thiry, 2009). In addition, integration of the HPV genome leads to the generation of viral–cellular fusion transcripts with increased stability which results in constitutive, high-level expression of the viral E6 and E7 oncoproteins (Jean & Lambert, 1995). As a consequence, the integrated viral DNA stimulates cell cycle entry and provides a selective growth advantage to the infected cells. The extensive proliferation of infected epithelia is a key event for the accumulation of genetic errors and genome destabilization that finally results in cancer development (Moody & Laimins, 2010; Peter et al., 2010).

In the present report we have focused on the determination of the physical status of the HPV16 genome among low- and high-grade malignancies and cervical cancer cases. Towards this end, we took into consideration previous reports that the most frequently disrupted region of the HPV16 genome during integration is the 3′ end of the viral DNA, between the E1 and E2 genes. The ratio values of E1/E6 and E2/E6 DNA copy numbers for each individual cervical sample were determined in order to discern the precise physical state of the HPV16 genome and the HPV16 integration pattern in host chromosomes. The overall goal was achieved through real-time PCR (RT-PCR) assays, using specific primer pairs hybridizing to E1 and E2 target regions, and with an E6-specific primer pair hybridizing to the E6 ORF. The E1 and E2 target regions were previously determined to be the most frequently disrupted sites during HPV16 DNA integration, whereas the E6 region is always retained intact (Łukaszuk et al., 2003; Andersson et al., 2005; Cricca et al., 2007).

**METHODS**

**Cervical sample collection and DNA isolation.** A total of 69 samples from Greek women positive for HPV16 DNA (53 from ThinPrep and 14 from cervical biopsies of paraffin-embedded tissue) were diagnosed as showing CINs of stages I, II and III and cervical cancer. Of these 69 samples, 27 were diagnosed as high-grade CIN (CIN II, III), 34 as low-grade CIN (CIN I) and 8 as cervical cancer cases.

Genomic DNA from ThinPrep samples was extracted using the chaotropically guanidine thiocyanate (Casas et al., 1995), while DNA from formalin-fixed, paraffin-embedded cervical tissues was extracted using the established proteinase K method (Tsakogiannis et al., 2012). A 250 bp GAPDH fragment was amplified by PCR as a quality control in order to assay for the integrity of the extracted DNA and for inhibition of the PCR process (Li et al., 2008). The GAPDH plasmid was constructed through PCR amplification of a 250 bp fragment. The PCR amplification was carried out using the primer set: HPV-16 41 5′-AGGGACCCAGAAATTTACC-3′/HPV-16 3912 5′-GCACACAAAGGAAAAACAAA-3′. The primers were designed with the Primer3 program (http://bioinfo.ut.ee/primer3-0.4.0/). The numbering of nucleotide positions was based upon the sequence of the prototype HPV16 strain (GenBank accession no. NC001526).

PCR was performed in a final volume of 50 μl. The PCR mixture contained 50 pmol each primer, 10 μl Taq buffer (GeneON), 2 mM MgCl₂, 1.2 mM dNTPs and 2.5 U thermostable DNA polymerase (Maximo Taq DNA Polymerase; GeneON). The cycling conditions were as follows: 40 cycles of 30 s at 95 °C, 40 s at 55 °C and 4 min at 72 °C. The first cycle was proceeded by a 5 min denaturation step at 95 °C and the last cycle was followed by a 10 min elongation step at 72 °C.

The amplicon 3791 bp in size was subjected to cloning using the Stratagene Clone PCR cloning kit (Stratagene, Agilent Technologies). The recombinant plasmid DNA was purified using the Nucleosipin plasmid kit (Macherey-Nagel) and the plasmids were subjected to sequencing at Macrogen, Korea, in order to confirm the integrity of the HPV16 sequence of pE6-E2.

**pGAPDH.** The GAPDH plasmid was constructed through PCR amplification of a 250 bp fragment. The PCR amplification was carried out using the primer set GAPDH F 5′-ACGGATTTTGGCTGCATTGGG-3′/GAPDH R 5′-TGATTGTTGAGGATCTCCTG-3′ as reported elsewhere (Li et al., 2008). The GAPDH plasmid construction was similar to that described for the construction of pE6-E2.

**pE6.** The E6 plasmid was constructed through PCR amplification of a 354 bp fragment located in the E6 ORF in order to perform the reconstitution experiments. In particular, the E6 fragment was amplified using the primer set HPV-16 5′-GCACAGATCCTGCAAACACC-3′/HPV-16 353 5′-GGTCCACCAGACCCCTTATTAT-3′. The primers were designed with the Primer3 program and the numbering of nucleotide positions was based upon the sequence of the prototype HPV16 strain (GenBank accession no. NC001526).

PCR was performed in a final volume of 50 μl. The PCR mixture contained 50 pmol each primer, 10 μl Taq buffer (GeneON), 1.5 mM MgCl₂, 1 mM dNTPs and 2.5 U thermostable DNA polymerase (Maximo Taq DNA Polymerase; GeneON). The cycling conditions were as follows: 40 cycles of 40 s at 95 °C, 30 s at 60 °C and 4 min at 72 °C. The first cycle was proceeded by a 2 min denaturation step at 95 °C and the last cycle was followed by a 5 min elongation step at 72 °C. The amplicon 354 bp in size was subjected to cloning, as described above.

**Quantitative RT-PCR assays.** A total of 69 cervical samples were investigated in order to determine the integrated, mixed or episomal physical status of the HPV16 genome. RT PCR assays were performed using the Mx3000P instrument (Stratagene). To determine the physical state of the viral DNA, the E1, E2 and E6 genes were quantified and both E1/E6 and E2/E6 ratios were calculated for each individual cervical sample. The principle of this approach is based on the assumption that E1, E2 and E6 genes are present in equal amounts in episomal HPV16 genome, while integration most often induces the deletion of the E2 and/or E1 target regions but not of E6.
Extensive mapping analysis of the E2 gene in the 69 cervical samples was conducted using 3 overlapping primer sets (a1–a2, b1–b2, c1–c2) through PCR assay, in order to locate sites of disruption in the E2 gene, as described elsewhere (Li et al., 2008). However, in order to map the sites of disruptions within the E1 gene, the complete E1 gene was amplified using the primer sets that were designed in a previous study in separate reactions (Arias-Pulido et al., 2006). In the present study, the most commonly disrupted region of the E2 ORF was that encoding the hinge region of the E2 protein (from 3172 to 3649), while for the E1 gene the most prevalent region of disruption was located within the 5′ end of the E1 gene (from 1059 to 1323) (data not shown). The selection of primer pairs was conducted according to the following two criteria: the primer pairs (i) must target the most frequently disrupted regions of E1 and E2 genes, and (ii) must hybridize to conserved regions of E1, E2 and E6 genes. As a consequence, the amplification reaction for the E2 target was carried out using the primer pair E2F12–E2R12, which targets the hinge region between nucleotides 3243 and 3539. For the E1 target, the primer pair E1F2–E1R2 was used, which targets the region between nucleotides 1059 and 1323. Moreover, the amplification reaction for the E6 target was performed using the primer pair HPV16 1–HPV16 353.

The construction of standard curves for E1, E2, E6 and GAPDH genes was carried out for each individual gene target separately. Each RT-PCR assay was performed in a final volume of 25 μl. The RT-PCR mixture contained 10 pmol each primer set, 2 μl KAPA SYBR FAST qPCR master mix (KAPA SYBR FAST qPCR kit; Kapa Biosystems) and ROX reference dye. The cycling conditions for E1, E2, E6 and GAPDH genes were as follows: 40 cycles of 3 s at 95 °C and 30 s at 60 °C. The first cycle was preceded by a 1 min denaturation step at 95 °C. Data acquisition at 510 nm was performed at 72 °C (Table 1).

One standard curve for GAPDH was constructed through amplification of a 10-fold dilution series of 10^6 to 1 copy of pGAPDH. In addition, three distinct standard curves were constructed for E1, E2 and E6 targets using only the pE6-E2 plasmid construct, which contains the viral fragment from E6 to E2 ORFs through primer sets E1F2–E1R2, E2F12–E2R12 and HPV16 1–HPV16 353, respectively. The three standard curves were constructed through amplification of a 10-fold dilution series ranging between 10^6 to 1 copy of pE6-E2 per reaction. All standard curves were performed in triplicate in three independent RT-PCR assays, in order to minimize unreliability of the quantification of results. All cervical samples were examined in triplicate. The copy number of E1, E2 and E6 genes was determined according to the number of cells (RT-PCR targeting the GAPDH gene) and results were expressed as E1, E2, E6 copy number per 500 cells.

**Cut-off value.** An experimental cut-off value was established in order to determine the physical state of the HPV16 DNA (episomal, integrated, mixed integrated and episomal forms). The ratio cut-off was obtained by preparing DNA plasmid solutions containing a standard quantity of 100 copies of the pE6-E2 plasmid and different copy numbers of pE6 ranging from 0 to 1 × 10^6 copies (Table 2). The plasmid solution that contained only the pE6-E2 was considered to be the episomal DNA, while the solution containing only the pE6 plasmid was considered to be the integrated DNA. However E1, E2 and E6 copy numbers were determined in all plasmid solutions in triplicate in three independent RT-PCR assays and the E1/E6 and E2/E6 ratios were calculated for each DNA solution (Table 2).

**Statistical analysis.** The association between the physical status of the HPV16 genome and the grade of cervical malignancy was determined through χ²-test. The statistical analysis was conducted with GraphPad Prism version 4.00 software for Windows. P values were considered significant if they were less than 0.05.

**RESULTS**

In the present study a total of 69 cervical samples that were positive for HPV16 DNA were investigated in order to determine the physical state of the HPV16 genome through E2/E6 and E1/E6 ratio analysis. An extensive mapping analysis of E1 and E2 genes was carried out in all cervical samples through PCR assay, using 12 overlapping primer sets (Arias-Pulido et al., 2006; Li et al., 2008). According to our findings, the most frequently disrupted site was located in the E2 hinge region (from nucleotide 3172 to 3649), while the most prevalent site of disruption within the E1 gene was located between nucleotides 1059 and 1323. Moreover, nucleotide analysis of the E1 and E2 genes in the

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**Table 1.** Primer pairs and assay conditions used to amplify the target regions for E1, E2, E6 and GAPDH genes with quantitative RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→3′)</th>
<th>Position</th>
<th>Product size (bp)</th>
<th>Assay conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1 target</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1F2</td>
<td>GACAGCACATGCGTTGTTTAC</td>
<td>1059</td>
<td>265</td>
<td>95 °C 1 min; 40 cycles of 95 °C 3 s, 60 °C 30 s; 55–95 °C denaturation</td>
<td>Arias Pulido et al. (2006)</td>
</tr>
<tr>
<td>E1R2</td>
<td>AGTCTCAGTGGGCACCTTC</td>
<td>1323</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2 target</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2F12</td>
<td>GAATACGAAACATATTGTGTACG</td>
<td>3243</td>
<td>297</td>
<td></td>
<td>Arias Pulido et al. (2006)</td>
</tr>
<tr>
<td>E2R12</td>
<td>CTGAGTCCTCTGTGCAACCAGTTT</td>
<td>3539</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E6 target</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV16 1</td>
<td>GCACAGAGCTGCAAACACT</td>
<td>150</td>
<td>353</td>
<td></td>
<td>Present study</td>
</tr>
<tr>
<td>HPV16 353</td>
<td>GGTCCACCGACCCCTATATT</td>
<td>502</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH target</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH F</td>
<td>ACGGATTGTGCTGATTGGG</td>
<td>107</td>
<td>250</td>
<td></td>
<td>Li et al. (2008)</td>
</tr>
<tr>
<td>GAPDH R</td>
<td>TGATTCTGAGGGGATCCTGC</td>
<td>356</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
same cervical samples, which were derived from Greek women, revealed that the region between nucleotides 3172 and 3649 (E2 gene) and the region between nucleotides 1059 and 1323 (E1 gene) are conserved among distinct HPV16 isolates that are circulating in the Greek population (Tsakogiannis et al., 2012, 2013, 2014). Taking these data into consideration, we targeted the E2 domain between nucleotides 3243 and 3539, and the E1 domain between nucleotides 1059 and 1323, through RT-PCR, in order to assign the copy numbers of the E2 and E1 genes, respectively.

Reconstitution experiments were performed using plasmid solutions containing from 0 to $10^5$ copies of the pE6 plasmid (integrated form of the HPV16 DNA) combined with 100 copies of the pE6-E2 plasmid (episomal form of the HPV16 genome) in order to determine the cut-off value and establish the physical state of the HPV16 genome. The E1, E2 and E6 gene copy numbers were determined through quantitative RT-PCR assay, using the standard curves that were constructed for this purpose. According to our findings, the ratio values close to zero (E2/E6 ratio value < 0.001, E1/E6 ratio value < 0.003) were observed when the pE6 plasmid was in 10-fold excess of the pE2-E6 plasmid. The E2/E6 ratio value of 0.01 and the E1/E6 ratio value of 0.03 were observed in plasmid solutions that contained 100 copies from each. However, when the pE6-E2 plasmid was in 10-fold excess in the plasmid solution the E2/E6 value was 0.82, and the E1/E6 value was 0.7. Finally, the E2/E6 ratio value of 0.96 and the E1/E6 ratio value of 0.98 were encountered in plasmid solutions that contained only the pE6-E2 plasmid (episomal DNA) (Table 2).

Taking these data into account, it was concluded that the E2/E6 ratio gave evidence for viral integration when its value was less than 0.001. However, E2/E6 ratios less than 0.96 (range 0.001 to 0.96) indicated the presence of both integrated and episomal forms of the viral DNA (mixed DNA forms), while ratio values equal to or greater than 0.96 indicated the presence of the episomal form of the HPV16 genome (Table 2). Similarly, E1/E6 ratio analysis revealed that viral integration was indicated when the E1/E6 ratio value was less than 0.003. E1/E6 ratios ranging from 0.003 to 0.98 indicated the presence of mixed DNA forms, while ratio values greater or equal to 0.98 showed the presence of the episomal form of the viral genome (Table 2).

In the present study, E1/E6 and E2/E6 ratio analysis was performed in the same cervical specimens in order to identify the HPV16 DNA form. E1/E6 and E2/E6 ratio values analysis in the 69 cervical samples showed that the pure episomal DNA form was present in 6 out of 34 (17.6 %) low-grade CINs and high-grade malignancies and cervical cancer specimens. The mixed DNA form was identified in 27 out of 34 (79.5 %) low-grade cases and in 24 out of 27 (88.8 %) high-grade CIN cases (Table 3). Moreover, the mixed DNA form was identified in two out of eight (25 %) cervical cancer specimens. In addition, the pure integrated viral form was identified in 3 out of 27 (11.1 %) high-grade cases and in 6 out of 8 (75 %) cervical cancer samples (Table 3). Finally, the pure integrated viral form was reported in the low-grade sample 747 (2.9 %) (Table 4). Taking these data into account, it was concluded that the pure episomal DNA form is exclusively detected in low-grade malignancies and cervical cancer specimens. However, a significant association among the high-grade CINs and the mixed DNA form was recorded, when compared with the episomal (0 %) and the integrated viral forms (11.1 %) ($\chi^2 = 16.333, P = 0.0001$). In addition, a strong association among the low-grade cases and the mixed DNA form was identified, when compared with the episomal (17.6 %) and the integrated viral form (2.9 %) ($\chi^2 = 11.7, P = 0.0006$).

Nevertheless, in seven low-grade malignancies an important difference among E1/E6 and E2/E6 ratio values was
recorded. In particular, in five low-grade cervical cases (PNL3, ATT8, ATT18, ATT12, ATT46) the E2/E6 ratio values analysis showed the presence of the mixed HPV16 DNA form, while the E1/E6 ratio values analysis revealed the presence of the episomal HPV16 DNA form (Table 4). In contrast, in two low-grade cervical cases (PNL2, ATT54) the E2/E6 ratio analysis showed the episomal viral DNA form, while the E1/E6 ratio analysis revealed the mixed form of viral DNA (Table 4). According to these results, it was concluded that the mixed HPV16 DNA form is encountered in these cervical cases, suggesting that the integration of HPV16 DNA may occur through disruption/deletion of E2 and E1 genes. In consequence, we propose that both the E1/E6 and E2/E6 ratio analyses should be considered in order to accurately identify the viral form, especially in low-grade malignancies, as HPV16 integration events also occur through disruption of the E1 gene.

**DISCUSSION**

HPV integration into the host chromosome is the key event for cervical cancer development and has been proposed to occur early in the development of cervical malignancy (Hudelist et al., 2004; Arias-Pulido et al., 2006; Cricca et al., 2007; Li et al., 2008; Matovina et al., 2009). In the present study, a total of 61 precancerous cervical samples and 8 cervical cancer cases associated with HPV16 infection were investigated in order to study the physical status of the HPV16 genome through quantitative RT-PCR and E2/E6 and E1/E6 ratio values analysis. However, in order to achieve more reliable results, the selection of E1, E2 and E6 targets was based on the most frequently disrupted sites of the E1 and E2 genes, and according to the most conserved E6 regions among the same HPV16 isolates, which were recently reported by our group (Tsakogiannis et al., 2012, 2013, 2014).

Several reports have investigated the HPV16 genome status through E2/E6 ratio analysis and demonstrated that this ratio is a significant molecular marker for evaluating the risk of progression of CIN of stage I, II and III to cervical cancer (Hudelist et al., 2004; Cricca et al., 2007; Briolat et al., 2007; Li et al., 2008; Boulet et al., 2009; Theelen et al., 2013). As a result, the HPV16 integration was determined only through disruption of the E2 gene, while the integrity of the E1 gene was not investigated. In addition, it has been demonstrated that disruption of both E1 and E2 genes is involved in high-grade CIN cases due to HPV16 DNA integration, while in low-grade cases only the E1 gene is involved (Cricca et al., 2009). Considering the prevalence of E1 gene disruption in low-grade cases, a comparative analysis along with E1/E6 and E2/E6 ratio values on the same cervical samples was performed in order to accurately determine the HPV16 DNA form and to explore for additional integration events that have been induced through disruption of the E1 gene.

Previous analyses have reported that the integration of the viral genome is exclusively identified in high-grade lesions and cervical cancer cases (Tonon et al., 2001; Hudelist et al.,

**Table 3.** HPV16 genome status according to patient cytological diagnosis

<table>
<thead>
<tr>
<th>Status</th>
<th>HPV16 DNA form</th>
<th>Low-grade CIN [n (%)]</th>
<th>High-grade CIN [n (%)]</th>
<th>Cancer [n (%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Episomal</td>
<td></td>
<td>6 (17.6)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Mixed</td>
<td></td>
<td>27 (79.5)</td>
<td>24 (88.8)</td>
<td>2 (25)</td>
</tr>
<tr>
<td>Integrated</td>
<td></td>
<td>1 (2.9)</td>
<td>3 (11.1)</td>
<td>6 (75)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>34 (100)</td>
<td>27 (100)</td>
<td>8 (100)</td>
</tr>
</tbody>
</table>

**Table 4.** HPV16 genome physical state of eight low-grade cases

<table>
<thead>
<tr>
<th>Sample</th>
<th>Source</th>
<th>CIN</th>
<th>E2</th>
<th>E1</th>
<th>E6</th>
<th>E2/E6 Ratio value</th>
<th>HPV16 DNA form</th>
<th>E1/E6 Ratio value</th>
<th>HPV16 DNA form</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNL2</td>
<td>ThinPrep</td>
<td>Low grade</td>
<td>800</td>
<td>721</td>
<td>820</td>
<td>0.98</td>
<td>Episomal</td>
<td>0.88</td>
<td>Mixed</td>
</tr>
<tr>
<td>PNL3</td>
<td>ThinPrep</td>
<td>Low grade</td>
<td>110</td>
<td>240</td>
<td>240</td>
<td>0.46</td>
<td>Mixed</td>
<td>1</td>
<td>Episomal</td>
</tr>
<tr>
<td>ATT8</td>
<td>ThinPrep</td>
<td>Low grade</td>
<td>60</td>
<td>260</td>
<td>265</td>
<td>0.23</td>
<td>Mixed</td>
<td>0.98</td>
<td>Episomal</td>
</tr>
<tr>
<td>ATT18</td>
<td>ThinPrep</td>
<td>Low grade</td>
<td>12</td>
<td>172</td>
<td>175</td>
<td>0.07</td>
<td>Mixed</td>
<td>0.98</td>
<td>Episomal</td>
</tr>
<tr>
<td>ATT12</td>
<td>ThinPrep</td>
<td>Low grade</td>
<td>150</td>
<td>4400</td>
<td>4500</td>
<td>0.03</td>
<td>Mixed</td>
<td>0.98</td>
<td>Episomal</td>
</tr>
<tr>
<td>ATT46</td>
<td>ThinPrep</td>
<td>Low grade</td>
<td>11</td>
<td>27</td>
<td>27</td>
<td>0.4</td>
<td>Mixed</td>
<td>1</td>
<td>Episomal</td>
</tr>
<tr>
<td>ATT54</td>
<td>ThinPrep</td>
<td>Low grade</td>
<td>208</td>
<td>163</td>
<td>214</td>
<td>0.97</td>
<td>Episomal</td>
<td>0.76</td>
<td>Mixed</td>
</tr>
<tr>
<td>747</td>
<td>ThinPrep</td>
<td>Low grade</td>
<td>12</td>
<td>7</td>
<td>86475</td>
<td>0</td>
<td>Integrated</td>
<td>0</td>
<td>Integrated</td>
</tr>
</tbody>
</table>
low-grade malignancies (Andersson et al., 2005; Kulmala et al., 2006; Cricca et al., 2007; Huang et al., 2008). Our findings demonstrate that the pure integrated viral form is preferentially identified in high-grade malignancies and cervical cancer cases, while the pure episomal DNA form is exclusively identified in low-grade lesions. In addition, it was demonstrated that both high-grade and low-grade malignancies are significantly related with the mixed DNA viral form (χ², P<0.001) (Kulmala et al., 2006). The high prevalence of HPV16 integration as the mixed DNA form in low-grade dysplasia reinforces earlier studies that proposed that HPV16 integration occurs at the early stage of the viral life cycle (Andersson et al., 2005; Kulmala et al., 2006; Cricca et al., 2007; Huang et al., 2008). In addition, in one low-grade sample the pure integrated viral form was identified, through complete disruption of E1 and E2 genes (E1/E6 and E2/E6 ratio values were close to zero) (Table 4). Considering the implication of E1 gene disruption/deletion during HPV16 integration in low-grade malignancies (Cricca et al., 2009) and the elevated prevalence of the mixed DNA form in low-grade cases, we conclude that single E2/E6 analysis might miss integration events through disruption of the E1 gene. This assumption was confirmed in two low-grade cases (PNL2, ATT54), in which E2/E6 ratio analysis showed the episomal viral DNA form, while the E1/E6 ratio analysis revealed the mixed form. Taking these data into account, we propose that both E1/E6 and E2/E6 ratio value analysis should be carried out simultaneously, in order to accurately determine the form of the HPV16 genome, and this is especially needed in low-grade malignancies.

The outcome of E1/E6 and E2/E6 ratio value analysis also demonstrates significant information about the HPV16 DNA integration pattern. In particular, it was proposed that distinct HPV16 integrants coexist in the same cervical sample, when both E1/E6 and E2/E6 ratio values predict the presence of mixed DNA forms. The HPV16 DNA episomes coexist with HPV16 DNA molecules that have been integrated into the host chromosome through disruption of the E1 gene and with HPV16 DNA molecules that have been integrated into the host chromosome through disruption of the E2 gene. These results were encountered in 58.8% of low-grade malignancies, in 88.8% of high-grade malignancies and in 25% of cervical cancer cases. The identification of distinct HPV16 integrants through E1/E6 and E2/E6 ratio value analysis constitutes serious evidence for the presence of multiple integration sites in the investigated cervical cases. Multiple integration events have been recently reported in tumour cell lines and cervical cancer cases (Yu et al., 2005; Li et al., 2013; Xu et al., 2013; Akagi et al., 2014). The identification of multiple integration events in low- and high-grade malignancies might provide valuable information about the progression of cervical malignancy to cancer disease. Although the number of samples for the low- and high-grade malignancy groups was adequate (34 and 27, respectively), the cancer group comprised only 8 samples, a fact that indicates a limitation of the present study.

In conclusion, the present study suggests a combined analysis of both E1/E6 and E2/E6 ratio values in order to accurately examine the physical state of the HPV16 genome. We propose that E1/E6 and E2/E6 ratio value analysis constitutes an important tool for investigating multiple integration events in the selected samples. Considering that the integration of the HPV16 genome into the host chromosome is a crucial step towards carcinogenesis, measuring both the E1/E6 and E2/E6 ratio values is a prospective prognostic tool to provide valuable information about HPV16 integration and the HPV16 physical state in the investigated cervical samples.

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