Analysis of human papillomavirus type 16 E6 variants in relation to p53 codon 72 polymorphism genotypes in cervical carcinogenesis

Mark van Duin,1 Peter J. F. Snijders,1 Mireille T. M. Vossen,1 Erik Klaassen,1 Feja Voorhorst,1 René H. M. Verheijen,2 Theo J. Helmerhorst,3 Chris J. L. M. Meijer1 and Jan M. M. Walboomers1

1, 2Department of Pathology1 and Department of Obstetrics and Gynecology2, University Hospital Vrije Universiteit, PO Box 7057, 1007 MB Amsterdam, The Netherlands
3 Department of Obstetrics and Gynecology, University Hospital Rotterdam, Rotterdam, The Netherlands

This study aimed to assess the role of specific human papillomavirus type 16 (HPV-16) variants, in combination with p53 codon 72 polymorphism genotypes, in cervical carcinogenesis. An initial sequence analysis of HPV-16 long control, E6 and E7 regions of 53 well-defined cervical samples containing HPV-16 revealed that a T to G transition at nucleotide position 350 within the E6 open reading frame was the most common variation, the frequency of which seemed to decrease with increasing severity of the lesion. Therefore, a total of 246 cervical samples of residents of The Netherlands was specifically analysed for HPV-16 350G/T variants and/or p53 codon 72 genotypes. These comprised HPV-negative normal cervical scrapes (n = 40), normal cervical scrapes containing HPV-16 (n = 46), scrapes containing HPV-16 from women with abnormal cervical cytology participating in a non-intervention follow-up study without (n = 38) and with (n = 51) a histologically proven cervical intraepithelial neoplasia (CIN) III lesion at the end of the study, and cervical squamous cell carcinomas (n = 71). Neither specific HPV-16 350G/T variants nor specific p53 genotypes were associated with a higher risk of developing CIN III or cervical cancer. However, HPV-16 350T variants were significantly over-represented in p53 Arg homozygous women with cervical cancer. This suggests that, in p53 Arg/Arg women, infection with HPV-16 350T variants confers a higher risk of cervical cancer.

Introduction

Infection with so-called high-risk human papillomavirus (HPV) types, predominantly HPV-16 and HPV-18, has been recognized as the main cause of cervical cancer and its precursor lesions (cervical intraepithelial neoplasia; CIN) (IARC, 1995; Walboomers et al., 1999). However, only a subset of women infected with high-risk HPV, with or without CIN disease, will eventually develop cervical cancer. Therefore, additional, as yet unknown factors are likely to contribute to neoplastic transformation of HPV-infected cervical epithelial cells.

One of these may involve specific viral variants displaying a relatively higher risk of malignant transformation (Conrad Stöppler et al., 1996). HPV intratype variants are defined as having more than 98% nucleotide sequence identity determined over the E6, E7 and L1 open reading frames (ORFs) with the reference sequence (Van Ranst et al., 1993; Myers et al., 1995). Different studies have revealed that for HPV-16 variants both numerical and specific structural nucleotide alterations relative to the HPV-16 reference sequence may affect biological behaviour (Xi et al., 1997; Zehbe et al., 1998a, b; Londesborough et al., 1996; Sang Song et al., 1997). For example, Xi et al. (1997) have shown that HPV-16 variants with a high proportion of nucleotide alterations within the long control region (LCR) and E6 ORF, referred to as non-prototype-like, confer a higher risk of developing high-grade CIN compared to so-called prototype-like variants with no or only a few nucleotide alterations within these regions. Other
studies have described HPV-16 variants showing a subtle one nucleotide alteration at position 350 (a T to G substitution) within the E6 ORF to be associated with virus persistence and progression of premalignant cervical disease (Londesborough et al., 1996; Zehbe et al., 1998a). However, a recent study suggested that the latter phenomenon may be population-dependent (Zehbe et al., 1998a). In a Swedish population, the HPV-16 350G variants represented a high-risk factor for cervical cancer, whereas these variants displayed all the features of being a low-risk factor in an Italian population. In contrast, in a recent study performed on a German cohort neither HPV-16 variant was associated with a higher risk of developing cervical cancer (Nindl et al., 1999). Thus, the oncogenicity of HPV-16 350G/T variants may be determined by genetic differences between populations (Zehbe et al., 1998a). This may involve polymorphisms within cellular targets that interact with the HPV-16 E6 gene product. One of these is the p53 gene product; binding of this product to E6 is considered to be an important event in HPV-mediated carcinogenesis (Werness et al., 1990; Scheffner et al., 1990). The frequency of two p53 alleles, which differ at polymorphic codon position 72, encoding either arginine or proline, also seems to vary amongst populations (Beckman et al., 1994).

This p53 codon 72 polymorphism has recently been linked to the risk of cervical cancer. It has been suggested that women homozygous for the arginine residue at this position (p53 Arg/Arg) are at higher risk of developing HPV-associated cervical carcinoma (Storey et al., 1998; Zehbe et al., 1999). However, other studies have not confirmed this finding (Rosenthal et al., 1998; Helland et al., 1998; Josefsson et al., 1998; Hildesheim et al., 1998; Lanham et al., 1998; Minaguchi et al., 1998; Klaes et al., 1999; Giannoudis et al., 1999). Also, in a Dutch population, no evidence could be obtained for p53 Arg/Arg homozygotes to be at increased risk of developing cervical carcinoma (Hayes et al., 1998). Still, combined analysis of HPV variants and p53 codon 72 polymorphism may explain, at least in part, this discrepancy in results.

A recently evaluated, prospective non-intervention follow-up study of women with abnormal cervical cytology offered a unique possibility to study both parameters. In this study, only women with cytomorphologically abnormal scrapes were included (Remmink et al., 1995; Nobbenhuis et al., 1999). Cytological and colposcopical examination as well as HPV testing were carried out every 3–4 months. Furthermore, to prevent interference with the natural history of CIN disease, a biopsy was taken only at the end of the study. According to the HPV testing performed and the histological diagnosis given at the end of the study, the women were categorized both according to HPV infection status (i.e. virus persistence or clearance) and end-point histology (i.e. CIN III or less than CIN III) (Remmink et al., 1995; Nobbenhuis et al., 1999).

Using this cohort, as well as a series of cytomorphologically normal cervical scrapes and cervical carcinomas collected in The Netherlands, we aimed to assess the role of HPV-16 variants in cervical carcinogenesis in this study. In addition, we considered the role of the p53 codon 72 polymorphism alone and in combination with HPV-16 variant status. Independently from each other, HPV-16 350G/T variants and p53 codon 72 genotypes were not associated with an increased susceptibility to CIN III and cervical cancer. However, a significant over-representation of HPV-16 350T variants was evident in p53 Arg/Arg women with cervical cancer. This suggests a possible differentially oncogenic effect of HPV-16 350T variants which is influenced by the p53 genotype.

Methods

Patient groups. For this study, cervical samples from residents in the north and south-western regions of The Netherlands were used. These comprised the following groups of samples which had been subjected to HPV detection and genotyping according to the general primer GP5+/GP6+ PCR-based method, as described previously (Jacobs et al., 1995).

1. Randomly chosen HPV-negative (n = 40) and HPV-16-positive (n = 46), cytomorphologically normal scrapes from a cross-sectional study of women participating in a population-based cervical cancer screening programme.

2. All available cervical scrapes containing HPV-16 (n = 89) taken from a larger prospective non-intervention follow-up study of women with CIN (n = 353) (Remmink et al., 1995; Nobbenhuis et al., 1999). Patients entered the study with abnormal cytology (mild to severe dyskaryosis). Every 3–4 months, HPV typing and cytological analysis were performed on cervical scrapes. Furthermore, colposcopical evaluation was performed without histological intervention to describe the predicted grade of dysplasia (CIN I–III) and to determine the extent of the lesions. At the end of follow-up, biopsies were taken for histological diagnosis (normal epithelium; CIN I, mild dysplasia; CIN II, moderate dysplasia; CIN III, severe dysplasia or carcinoma in situ). Details of the cohort study are described elsewhere (Remmink et al., 1995; Nobbenhuis et al., 1999). Of the scrapes analysed, 38 were from women with a histological diagnosis of less than CIN III at the end of the study, whereas 51 women showed a histologically proven CIN III at the end of follow-up. Women were also categorized on the basis of HPV infection status as follows: those with HPV-16 persistence, i.e. HPV-16 in the scrape used for analysis and the last scrape before biopsy (n = 61); and those with HPV-16 clearance, i.e. HPV-16-positive at the time of analysis and negative in the last scrape before biopsy (n = 28).

3. Frozen (n = 25) and formalin-fixed, paraffin-embedded (n = 40) cervical squamous cell carcinoma specimens containing HPV-16 were obtained from the Amsterdam and Zeeland areas, respectively, after surgical resection. For the PCR-based HPV-16 variant and p53 polymorphism analyses, crude extracts of the cervical scrapes were used, as described previously (de Roda Husman et al., 1995). The first HPV-16-positive scrape was taken from women of the follow-up study. For the carcinoma specimens, consecutive sections were cut, the first and last of which were stained with haematoxylin–eosin for histomorphological examination (Snijders et al., 1998). Series of in-between sections were subjected to proteinase K digestion overnight at 37 °C in a Tris-buffered solution containing 100 µg/ml proteinase K and 0.045% Tween 20. After heat inactivation of the enzyme, 10 µl aliquots of the crude extracts were subsequently used for PCR analysis.
Table 1. HPV-16 350 polymorphism (a) in relation to cytology/end-point histology and (b) in relation to HPV-16 infection status

<table>
<thead>
<tr>
<th>(a) Cytology/histology</th>
<th>HPV-16 350 polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>HPV-16-positive normal cytology</td>
<td>46</td>
</tr>
<tr>
<td>Abnormal cytology</td>
<td></td>
</tr>
<tr>
<td>Less than CIN III</td>
<td>38</td>
</tr>
<tr>
<td>CIN III</td>
<td>51</td>
</tr>
<tr>
<td>Cervical carcinoma</td>
<td>71</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(b) HPV-16 infection status</th>
<th>HPV-16 350 polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>Abnormal cytology</td>
<td></td>
</tr>
<tr>
<td>Persistence</td>
<td>61</td>
</tr>
</tbody>
</table>

* Odds ratios (OR) calculated for each of the categories relative to HPV-16-positive normal cytology (a) and relative to clearance (b) are given with confidence intervals (C.I.) in parentheses.

The cell lines SiHa, CaSki and A431 were obtained from the ATCC. The HPV-16 reference clone was kindly provided by H. zur Hausen and L. Gissmann (Heidelberg, Germany).

**HPV-16 sequence analysis of the 3' part of the LCR, E6 and E7.** Initial HPV-16 sequence analysis was performed on 43 randomly chosen HPV-16-positive cervical scrapes from the follow-up cohort, consisting of women with end-point histology of normal epithelium (n = 11), CIN I (n = 7) and CIN III (n = 25), respectively. In addition, 10 randomly chosen HPV-16-positive cervical carcinomas were analysed. HPV-16 variant analysis was performed by sequencing of PCR products encompassing the 3’ part of the LCR and the E6 and E7 ORFs, respectively. The HPV-16 LCR, E6 and E7 regions were amplified in separate reactions. HPV-16 LCR, E6 and E7 type-specific primers were selected on the basis of sequence information from the compendium of Myers et al. (1995) (LCR1, location nt 7437, 5’ CCATTTTGATGCTTTACACTTGGGTCTGCCTTGT 3’; LCR2, location nt 119, 5’ AAGTGGTGAACTTTCTCAGTTGT 3’; E6-1, location nt 53, 5’ GAAACGGGTTAGTATAAAACGAC 3’; E6-2, location nt 533, 5’ AGCTGGGTTCCTCTACGTTGTCT 3’; E7-1, location nt 478, 5’ CCTATCATATTAAAGGAGTTGTTGAGA 3’; E7-2, location nt 923, 5’ TTTTTCACATACAGCCTTCTACT 3’). Primers and methods for sequencing E6 and E7 ORFs have recently been described (Bontkes et al., 1998). The primer combinations LCR1/2, E6-1/2 and E7-1/2 generate PCR products of 617, 503 and 468 nt, respectively. The PCR products generated with the LCR1/2 primer combination have an overlap of 94 nt with the products generated with the E6-1/2 primer combination. An overlap of 77 nt exists between the PCR products generated with the primer combinations E6-1/2 and E7-1/2. For PCR, one of the primers was biotinylated to allow for subsequent preparation of single-stranded DNAs using Dynabeads, according to recommendations of the manufacturer (Dynal). Subsequent sequence analysis was performed on the plus-strand by solid-phase sequencing, whereas the minus-strand was analysed using the supernatant (Hultman et al., 1991). Single-stranded templates were used as input for the cycle sequencing reaction using either the dideoxy termination method or the dye primer method (Thermo Sequenase Dye Terminator Cycle sequencing pre-mix kit V2.0 and Dynemic direct cycle sequencing kit; Amersham Life Science) according to recommendations of the manufacturer. Cycle sequence products were analysed on an automated sequencer (ABI 373; Perkin Elmer). For all cases, PCR and subsequent sequence reactions were performed in duplicate to ensure reliability. All duplicate reactions showed complete concordance. HPV-16 variants were denoted by nucleotide position and substituted nucleotides, as described by Yamada et al. (1997). An additional final letter denotes the nucleotide at position 350 in the E6 ORF. For instance, E-G131G encodes a European variant with a G at both nt positions 131 and 350.

To determine persistence of specific HPV-16 variants, the last scrape taken during follow-up was also analysed from eight randomly chosen women with persistent HPV-16 infection and CIN III end-point histology. The sequences of the first and last scrapes were identical for all women.

**HPV-16 350G/T analysis.** HPV-16 E6 nt 350 variant-specific PCR was carried out by performing two independent PCR reactions, one specific for 350G and one specific for 350T. To ensure specificity of the PCR, oligonucleotides were designed with a mismatch next to the specific nt. The primers used were: 350-1, specific for a G at position 350 (nt 324–350, 5’ GTAGTGATAGCATTATGTTTATAG 3’); 350-2, specific for a T at position 350 (nt 324–350, 5’ GTAGTGATAGCATTATGTTTATAG 3’); and a backward primer 350-3 (nt 436–417, 5’ GACACGGTGCTTTGACAG 3’). The primers span a region of 113 nt.

PCR was initiated by a heating step at 94 °C for 4 min for DNA denaturation. Subsequently, 35 cycles of amplification were performed as
Table 2. p53 codon 72 polymorphism in women with (a) normal cytology and (b) in relation to cytology/histology

<table>
<thead>
<tr>
<th>(a) Cytology/histology</th>
<th>p53 codon 72 polymorphism</th>
<th>n</th>
<th>Arg/Arg</th>
<th>Arg/Pro</th>
<th>Pro/Pro</th>
<th>OR (C.I.)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV-negative normal cytology</td>
<td></td>
<td>40</td>
<td>18 (45%)</td>
<td>21 (52%)</td>
<td>1 (3%)</td>
<td>1 (reference)</td>
</tr>
<tr>
<td>HPV-16-positive normal cytology</td>
<td></td>
<td>46</td>
<td>31 (67%)</td>
<td>13 (28%)</td>
<td>2 (4%)</td>
<td>2·5 (1·1–6·1)†</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(b) Cytology/histology</th>
<th>p53 codon 72 polymorphism</th>
<th>n</th>
<th>Arg/Arg</th>
<th>Arg/Pro</th>
<th>Pro/Pro</th>
<th>OR (C.I.)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal cytology</td>
<td></td>
<td>86</td>
<td>49 (57%)</td>
<td>34 (40%)</td>
<td>3 (3%)</td>
<td>1 (reference)</td>
</tr>
<tr>
<td>Abnormal cytology</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less than CIN III</td>
<td></td>
<td>38</td>
<td>15 (40%)</td>
<td>18 (47%)</td>
<td>5 (13%)</td>
<td>0·5 (0·2–1·1)#</td>
</tr>
<tr>
<td>CIN III</td>
<td></td>
<td>51</td>
<td>23 (45%)</td>
<td>28 (55%)</td>
<td>0 (0%)</td>
<td>0·6 (0·3–1·2)</td>
</tr>
<tr>
<td>Cervical carcinoma</td>
<td></td>
<td>71</td>
<td>44 (62%)</td>
<td>24 (34%)</td>
<td>3 (4%)</td>
<td>1·2 (0·6–2·3)</td>
</tr>
</tbody>
</table>

* Odds ratios (OR) for each of the categories relative to HPV-negative, normal cytology (a) and relative to normal cytology (b) are given with 95% confidence intervals (C.I.) in parentheses (Arg/Arg women were contrasted to women with both Arg/Pro and Pro/Pro).
+ P = 0·05.
# P = 0·08.

Table 3. HPV-16 350 variants and p53 codon 72 polymorphism in relation to cytology/histology in (a) Arg/Arg women and (b) Arg/Pro and Pro/Pro women

<table>
<thead>
<tr>
<th>(a) Cytology/histology</th>
<th>Arg/Arg</th>
<th>n</th>
<th>350T</th>
<th>350G</th>
<th>OR (C.I.)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal cytology</td>
<td></td>
<td>31</td>
<td>12 (39%)</td>
<td>19 (61%)</td>
<td>1 (reference)</td>
</tr>
<tr>
<td>Abnormal cytology</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less than CIN III</td>
<td></td>
<td>15</td>
<td>6 (40%)</td>
<td>9 (60%)</td>
<td>1·1 (0·3–3·7)</td>
</tr>
<tr>
<td>CIN III</td>
<td></td>
<td>23</td>
<td>12 (52%)</td>
<td>11 (48%)</td>
<td>1·7 (0·6–5·1)</td>
</tr>
<tr>
<td>Cervical carcinoma</td>
<td></td>
<td>44</td>
<td>28 (64%)</td>
<td>16 (36%)</td>
<td>2·8 (1·1–7·2)†</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(b) Cytology/histology</th>
<th>Pro/Pro and Pro/Arg</th>
<th>n</th>
<th>350T</th>
<th>350G</th>
<th>OR (C.I.)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal cytology</td>
<td></td>
<td>15</td>
<td>8 (53%)</td>
<td>7 (47%)</td>
<td>1 (reference)</td>
</tr>
<tr>
<td>Abnormal cytology</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less than CIN III</td>
<td></td>
<td>23</td>
<td>12 (52%)</td>
<td>11 (48%)</td>
<td>1·0 (0·3–3·5)</td>
</tr>
<tr>
<td>CIN III</td>
<td></td>
<td>28</td>
<td>16 (57%)</td>
<td>12 (43%)</td>
<td>1·2 (0·3–4·1)</td>
</tr>
<tr>
<td>Cervical carcinoma</td>
<td></td>
<td>27</td>
<td>12 (44%)</td>
<td>15 (56%)</td>
<td>0·7 (0·2–2·5)</td>
</tr>
</tbody>
</table>

* Odds ratios (OR) for each of the categories relative to normal cytology are given with confidence intervals (C.I.) in parentheses.
† P = 0·04.
results: 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C. The final elongation step was prolonged for another 4 min at 72 °C. Amplions were analysed by gel electrophoresis (1.5% agarose gel) followed by Southern blot analysis. Hybridization was performed overnight using an [γ-32P]ATP 3’-end labelled oligonucleotide probe 350.4 (nt 366–415, 5’ CCGTTGTGTATTGCATATTACCGAGGAATTAAT 3’) at 55 °C (Snijders et al., 1998). Membranes were subsequently washed three times with 3 x SSC/0.5% SDS (1 x SSC: 0.15 M sodium chloride, 0.015 M sodium citrate). Autoradiography was performed for 4–48 h at −80 °C using intensifying screens.

Of seven randomly chosen women with persistent HPV-16 infection and end-point histologies of either normal epithelium (n = 4), CIN I (n = 2) or CIN III (n = 1), HPV-16 350/G/T PCR analysis was also carried out on the last scrape. The PCR data of the first and last scrapes of each case were identical.

p53 codon 72 polymorphism analysis. Allele-specific PCR for p53 codon 72 polymorphism was carried out by performing two PCR reactions, one specific for C (encoding Pro) and one specific for G (encoding Arg) at nt 734. Essentially the same protocol was used as for the HPV-16 350C/T variants, i.e. backward primers were designed with a mismatch next to the specific nucleotide at the 3’ primer end to ensure specificity. The primers used were a universal forward primer p53-3 (nt 617–635, 5’ TCCCCCTTGGCCTGCCCAA 3’) and specific backward primers for the Pro residue (p53-1: nt 751–734, 5’ CTGTGTCAGGCAGGGCACCCTC 3’) and for the Arg residue (p53-2: nt 751–734, 5’ CTGTGTCAGGGCACCACGT 3’). The primers span a region of 117 nt.

PCR conditions and subsequent evaluations were performed as described for the HPV-16 350G/T-specific PCR assays, except that the annealing temperature was 64 °C. Hybridization was performed with oligonucleotide probe p53.4 (nt 654–683, 5’ CTGTCCCCGGACGATATTGAAACATGTC 3’).

Statistical analysis. To determine the magnitude of differences with respect to the frequencies of the reference sequence, HPV-16 350T (Table 1) and p53 Arg/Arg (Table 2) and HPV-16 350T frequency in relation to the p53 genotype (Table 3), odds ratios (OR) were calculated and Woolf’s confidence intervals (CI) were given. Contrasts between frequencies were tested statistically by the Fisher exact test where appropriate. P values of less than 0.05 were considered significant. Logistic regression analysis was performed using SPSS 8.0 software to establish the differences in results between the cervical carcinoma group taken from the Amsterdam region and the Zeeland region. This analysis indicated that the results from these regions are not significantly different (P = 0.7).

Results

Sequence variation within the LCR, E6 and E7

HPV-16 variant analysis was initiated by sequence analysis of the 3’ part of the LCR and the E6 and E7 ORFs. This was performed on 43 cervical scrapes containing HPV-16 randomly taken from the follow-up study as well as 10 cervical carcinomas containing HPV-16. Of the women from the follow-up study, 25 developed or maintained a CIN III lesion (i.e. CIN III end-point histology), whereas 18 had end-point histology consistent with normal cervical mucosa (n = 11) or CIN I (n = 7). The sequence variations relative to the HPV-16 reference sequence are shown in Fig. 1. Overall, one or more base substitutions relative to the reference sequence were found in the LCR in 83% of cases, involving mainly a G to A substitution at position 7521. Variations within E6 were found in 58% of cases, involving mostly a T to G substitution at position 350. E7 appeared to be the most conserved region in which the most prevalent alteration, a T to G substitution at position 795, was found in 15% of cases. In contrast to E6, the majority of alterations found within E7 were silent mutations.

The reference HPV-16 sequence was found in 11% of cases with end-point histology of less than CIN III, 8% of cases with CIN III and 20% of cervical carcinomas, but these differences were not significant (P > 0.1). Variants with a high proportion of nucleotide alterations which could be considered non-prototype-like, were found in 6% of cases with less than CIN III, in 16% of CIN III cases and in 20% of cervical carcinomas. These differences were also not significant (P > 0.1).

Detailed analysis of specific sequence alterations revealed that the T to G substitution at nt 350 within E6 seemed to be the most common variation that showed a different distribution amongst the three categories of cases. Variants with a G at this position (350G) were found in 56% of cases less than CIN III, 32% of CIN III cases and 30% of cervical carcinomas. Although these differences were not statistically significant (P > 0.1), the possible relationship of these E6 variants with the p53 polymorphism prompted us to extend our study group for analysis of the HPV-16 350G/T variation only.

HPV-16 350G/T analysis

To study specifically the HPV-16 350G/T polymorphism, the series of samples was extended to a total of 206 samples containing HPV-16, which were grouped into four categories: (1) scrapes containing HPV-16 with normal cytology (n = 46); (2) scrapes containing HPV-16 of cases less than CIN III from the follow-up study (n = 38); (3) scrapes containing HPV-16 of CIN III cases from the follow-up study (n = 51); and (4) cervical carcinomas containing HPV-16 (n = 71). For HPV-16 350G/T polymorphism analysis, two independent PCR reactions, each specific for the detection of one of the two possible variants, were performed. The method was validated on samples in which the HPV-16 E6 ORF was sequenced, including the cervical cancer cell line SiHa (350G) and the plasmid clone containing the reference HPV-16 sequence (pHPV-16; 350T). Representative results are shown in Fig. 2(a).

In Table 1(n), the results of HPV-16 350G/T analysis are shown in relation to cytology and (end-point) histology. The frequency of HPV-16 350T variants in cytomorphologically normal cervical scrapes was 43%. Within the follow-up cohort, HPV-16 350T variants were found in 47 and 55% of less than CIN III and CIN III cases, respectively. HPV-16 350T variants were furthermore found in 56% of cases with cervical carcinomas. When women with cervical cancer were contrasted to those with normal cytology, the OR for HPV-16 350T compared to HPV-16 350G was 1.7 (CI = 0.8–3.5).
Fig. 1. Sequence alterations relative to the HPV-16 reference sequence (bold) for cytomorphologically abnormal scrapes from the follow-up cohort with end-point histology of less than CIN III ($n_{fl} 18$), CIN III ($n_{fl} 25$) and cervical carcinomas are shown. Novel variants based on sequence variants listed in Myers et al. (1995) and Yamada et al. (1997) are underlined. Dashes indicate nucleotides identical to the reference sequence; 0, in sample E-C169G-del indicates a deletion. Capital letters point to alterations that result in an amino acid change, whereas lower case letters point to silent mutations.

After categorization of women from the cohort study according to HPV-16 infection status, 350T variants were found in 61% of cases with HPV-16 clearance and 47% of cases with persistent HPV-16 infection (Table 1; $OR_{fl} 0–6; C.I. 0–2–1–5$). None of the aforementioned differences was statistically significant.

### p53 codon 72 polymorphism analysis

For the p53 codon 72 polymorphism analysis, allele-specific PCR was developed similar to the HPV-16 350G/T polymorphism analysis. The method consisted of two independent PCR reactions, one specific for the Arg allele and one specific for the Pro allele. The test was validated on the cell lines CaSki (Arg/Pro) and A431 (Pro/Pro). Representative results are shown in Fig. 2(b). A significant over-representation of p53 Arg/Arg carriers was found in the group of women with HPV-16-positive normal cervical scrapes compared to those with HPV-negative normal scrapes (Table 2a; $OR = 2.5; C.I. = 1.1–6–1; P = 0.05$). This suggests either that p53 Arg/Arg
carriers are more susceptible to cervical HPV-16 infection, or that women containing at least one p53 Pro allele are less prone to HPV-16 infection. For further analysis of cervical carcinoma patients and women of the follow-up cohort, the use of the group of cytologically normal HPV-negative individuals as a control population may bias results, as possible differences may reflect different susceptibilities to HPV-16 infection. The control population, therefore, consists of a combination of both HPV-negative and HPV-16-positive women with normal cytology. The frequency of p53 Arg/Arg in cervical carcinomas was similar to the p53 Arg/Arg frequency in this control group (OR = 1.2; C.I. = 0.2–2.3). Furthermore, a significant difference in p53 Arg/Arg frequency was not found in any of the studied categories either in comparison to this mixed control population or in comparison to the HPV-negative women with normal cytology (Table 2a; data not shown). Moreover, within the follow-up cohort, frequencies of p53 Arg/Arg were similar in the group of women with less than CIN III compared to the group of women who maintained or developed a CIN III lesion.

**HPV-16 350G/T variants in relation to p53 codon 72 genotypes**

In Table 3, the frequency of HPV-16 350G/T variants is shown in relation to p53 codon 72 genotyping. In normal cytology, HPV-16 350T variants were detected in 39% of p53 Arg/Arg women (Table 3a). However, in the p53 Arg/Arg carriers who developed cervical carcinoma, HPV-16 350T variants were significantly over-represented and found in 64% of cases (OR = 2.8; C.I. = 1.1–7.2; P = 0.04). Comparison with the groups from the follow-up cohort revealed that the presence of HPV-16 350T in p53 Arg/Arg women was associated neither with a low-grade cervical lesion nor with the development or maintenance of CIN III lesions (Table 3a). In contrast, no significant differences in HPV-16 350G/T frequencies were evident for the different categories of p53 Arg/Pro and p53 Pro/Pro women (Table 3b).

After categorization of women of the follow-up study according to HPV status, no major difference was found between the distribution of HPV-16 350T variants in p53 Arg/Arg subjects with virus clearance versus those with persistence (data not shown).

**Discussion**

In this study, both HPV-16 intratype variations and p53 codon 72 polymorphism were investigated in a follow-up study of women with abnormal cervical cytology. In addition, cervical scrapes representing normal cytology and cervical carcinomas were analysed. Sequence analysis revealed that the number of nucleotide alterations relative to the HPV-16 reference sequence was highest in the LCR, followed by E6 and E7, respectively. This finding is in agreement with other studies (Yamada et al., 1997; Xi et al., 1997).

Both the HPV-16 reference sequence and non-prototype-like variants tended to be over-represented in cervical carcinomas compared to samples from the follow-up cohort and cytomorphologically normal cervical scrapes. However, these differences were not significant. Our data on the reference sequence seem to contradict data of Zehbe et al. (1998b), who found that in the Swedish population cervical carcinomas were almost exclusively associated with non-reference HPV-16 variants, based on the E6 and E7 ORFs. On the other hand, our results were in agreement with a larger worldwide study on HPV-16 E6 variants, which revealed that the HPV-16 reference sequence was present in 34% of cervical carcinomas containing HPV-16 from Germany, Poland and Spain (Yamada et al., 1997). Similarly, in a German population, the HPV-16 reference sequence was detected in 32% of HPV-16-positive cancer cases (Nindl et al., 1999).

Non-prototype-like variants were generally detected at a low frequency in all categories. This finding is in agreement with other studies (Yamada et al., 1997; Xi et al., 1997). Although the non-prototype-like variants may confer a higher risk of developing cervical carcinoma, as found by Xi et al. (1997), the sample size is too small to draw conclusions about this. In contrast to other studies (Londesborough et al., 1996; Zehbe et al., 1998b), we ultimately did not find an association of HPV-16 350G/T variants with either virus persistence or development of cervical cancer, as reported by others. However, the observed differences may reflect a population-dependent oncogenicity of HPV-16 350G/T variants (Zehbe et al., 1998b). In conclusion, we did not find any significant association between specific HPV-16 variants and the risk of developing CIN III or cervical cancer. Similar results were obtained after analysis of a smaller group of samples from the follow-up cohort for HPV-16 E6 and E7 sequence variations (Bontkes et al., 1998).

For p53 analyses, we used clinical specimens instead of blood samples. In theory, this approach may result in an over-estimation of homozygous p53 Arg/Arg or p53 Pro/Pro women, due to the possible occurrence of allelic loss at the p53 locus. Loss of heterozygosity at this locus has been reported in 8–15% of cervical carcinomas (Minaguchi et al., 1998; Storey et al., 1998; Mullokandov et al., 1996). However, we anticipate that in abnormal cervical scrapes, dyskaryotic cells are admixed with normal cells. Moreover, carcinoma samples contained at least 5% stroma (data not shown). Therefore, the fraction of normal cells present in the samples should ensure correct p53 genotyping. Since the methodology used for p53 genotype detection was based on two separate PCR reactions, the detection of low copy numbers of a certain p53 allele is not affected by the possible presence of high copy numbers of the other allele. This was supported by reconstruction experiments of CaSki (Arg/Pro) cells serially diluted in A431 (Pro/Pro) cells showing that the p53 Arg allele was still clearly detectable.
after a 100-fold dilution (data not shown). Therefore, we believe that, at most, an extremely small number of samples would have been misinterpreted due to a possible allelic loss at the p53 locus. In addition, the allele frequencies we found amongst the various categories of women were similar to those found by Hayes et al. (1998) who studied blood samples of Dutch women.

We found that the distribution of p53 alleles in the cytomorphicologically normal, HPV-negative population, although not significantly different from the Hardy–Weinberg equilibrium, was characterized by a relatively high frequency of heterozygotes in comparison to p53 Pro homozygotes (Beckman et al., 1994). Therefore, these frequencies may not be completely representative and may be subject to change when a larger sample size is analysed. With this restriction in mind, we found the frequency of p53 Arg/Arg to be significantly increased in women with cytomorphicologically normal scrapes containing HPV-16. Therefore, p53 Arg/Arg women may be more prone or p53 Pro/Pro or Arg/Pro women could be less prone to HPV-16 infection. Despite this finding, p53 Arg/Arg alone did not represent a significant risk factor for the development of HPV-16-associated CIN III or cervical carcinoma. The control group used for this analysis consists of both HPV-16-positive and -negative normal cervical scrapes. We believe this control group to be more representative for the general Dutch population than the HPV-negative group alone. This is supported by the fact that the allele distribution found in this mixed group is similar to the one described by Hayes et al. (1998) for a random Dutch control population. Moreover, although we specifically focused on cases containing HPV-16, the lack of correlation between the p53 codon 72 polymorphism and risk of cervical cancer is in agreement with many other studies on this subject (Helland et al., 1998; Rosenthal et al., 1998; Hildesheim et al., 1998; Jøsefsson et al., 1998; Lanham et al., 1998; Minaguchi et al., 1998; Hayes et al., 1998; Klaes et al., 1999; Giannoudis et al., 1999).

Interestingly, we found a weak, but significant over-representation of HPV-16 350T in p53 Arg/Arg women who developed cervical cancer. Therefore, a possible differentially oncogenic effect of HPV-16 350T may be influenced by the p53 genotype. If this were true, it may be due to a higher susceptibility linked to p53 Arg, with respect to HPV-16 350T-mediated carcinogenesis. It is noteworthy that a similar observation has been made in a different study performed on a population from Manchester, UK (C. Brady, M. Duggan-Keen & P. Stern, personal communication). Both studies have in common an over-representation of p53 Arg/Arg women amongst cancer patients infected with HPV-16 350T. Still, it is far from clear whether these observations reflect the property of p53 Arg to be more susceptible to degradation by HPV-16 E6 350T variants than by HPV-16 E6 350G variants. Before addressing this item in larger population-based studies, more functional studies are required regarding the susceptibility of HPV-16 350G variants to the different p53 variants. Furthermore, it should be kept in mind that the observed over-representation may reflect the action of a different, as yet unknown polymorphism that is linked to either the 350T genotype in the viral genome or the p53 genotype in the host cell genome (Yamada et al., 1997).

In contrast, HPV-16 350T over-representation was not found either in p53 Arg/Arg women who developed or maintained CIN III lesions or in p53 Arg/Arg women with virus persistence compared to those with virus clearance. Therefore, the HPV-16 350T/p53 Arg/Arg combination does not confer a higher risk of virus persistence or CIN III development but rather an increased risk of the transition of CIN III to invasive cervical cancer. The rather weak association may reflect the multistep nature of this transition process, in which many other variables are likely to influence the outcome of the disease. Moreover, it is noteworthy that for the final HPV-16 350G/T analysis, we did not consider other variations that may occur in the E6 region. Correction for E6 multivariants that potentially would display differential biological activities may have led to a stronger association. Finally, the association observed in this study warrants further investigations on HPV variables and host polymorphisms to identify populations at risk of cervical cancer.

We are indebted to Marcel Jacobs, Miranda Meijer and Henri Schrijnemakers for excellent technical assistance. We thank Renée Roosendaal for helpful discussions and Ans Remmink and Frisé de Schipper for clinical data. This work was supported by grant VU96-1151 of the Dutch Cancer Society.

References


Received 21 September 1999; Accepted 12 October 1999