Chapter 6

Physical Status of Multiple Human Papillomavirus Genotypes in Flow-Sorted Cervical Cancer Cells
Abstract

Multiple HPV infections have been detected in cervical cancer. To investigate the significance of multiple HPV infections we studied their prevalence in cervical cancer samples from a low-risk (Dutch) and a high-risk (Surinamese) population and the correlation of HPV infection with tumour cell aneuploidy. SPF10 LiPA was used for HPV detection in 96 Dutch and 95 Surinamese formalin-fixed cervical carcinoma samples. Samples with combined HPV 16/18 infections were sorted by flow cytometry and fluorescence in situ hybridisation was performed on the diploid and aneuploid subpopulations to detect HPV 16 and 18 genotypes simultaneously. Multiple HPV infections were present in 11/80 (13.8%) Dutch and 17/77 (22.1%) Surinamese carcinomas. Three cases had an HPV 16 and HPV 18 co-infection: in two cases, integrated HPV copies of HPV 16 or 18 were detected in the aneuploid fraction and in one case both HPV 16 and 18 were present solely as episomes. Based on our findings multiple HPV infections are present in cervical cancer samples from both high- and low-risk populations. Furthermore, multiple HPV types can be present in an episomal state in both diploid and aneuploid tumour cells, but integrated HPV genomes are detectable only in the aneuploid tumour cell subpopulations.
Introduction

Human papillomavirus (HPV) infection is a prerequisite for the development of cervical cancer (reviewed in\textsuperscript{1}). Although HPV infections are common, the majority are transient and are cleared by the immune system\textsuperscript{2}. When high-risk (HR-)HPV is persistently present, low grade cervical intraepithelial lesions (CIN) eventually progress to invasive cervical carcinoma\textsuperscript{3}. HPV infects the basal epithelial cells and is frequently found in an episomal state in low- and high-grade CIN. It is generally thought that viral integration into the human genome occurs during cervical carcinogenesis\textsuperscript{4,5}. Upon integration, the viral E2 repressor is disrupted, leading to continued expression of the E6 and E7 oncoproteins. They inactivate the p53 and pRb tumour suppressor proteins, leading to uncontrolled cell proliferation and ultimately to cancer\textsuperscript{5}. Viral integration frequency was shown to increase with disease severity\textsuperscript{4,6,7}. The two most common HR-HPV types found in cervical carcinoma are HPV 16 and HPV 18. While both HPV 16 episomes and HPV 16 integrated copies are able to transform normal keratinocytes, HPV 18 has been reported to be present mainly in the integrated form\textsuperscript{6,8}.

Fluorescence in situ hybridisation (FISH) has recently been used to investigate the physical state of HPV (episomal or integrated) in cells from cervical (pre)invasive lesions\textsuperscript{9,10}. Several studies suggest that a diffuse nuclear signal is indicative of the presence of episomal HPV, while a punctate signal in the nucleus is characteristic of integrated HPV\textsuperscript{11-13}. It was shown that the diffuse signal can be excluded by harsh pre-treatment protocols, allowing the clear detection of integrated HPV copies\textsuperscript{10}.

In addition to the physical state of HPV, abnormal cellular DNA content or numerical chromosome aberrations were suggested to be associated with the progression of CIN to cervical carcinoma\textsuperscript{14,15}. Despite studies into whether HPV integration or DNA instability and aneuploidisation is the first step in malignant transformation, the sequence of events is still under debate\textsuperscript{16,17}. Data on malignant transformation and progression as a result of multiple HPV infections are even more limited. Such multiple HPV infections are frequent in premalignant stages and have recently been detected in invasive cervical cancer\textsuperscript{18-20}.

In this study, we investigated the prevalence of multiple HPV infections in cervical cancer for a low-risk (Dutch) and a high-risk (Surinamese) population. The cases carrying a double HPV 16 and HPV 18 infection were analysed by performing FISH on flow-sorted pure tumour cell subpopulations to determine the integration status of the multiple HPV types in relation to tumour cell aneuploidy.
Material and Methods

Patient Samples
A total of 189 patients with invasive cervical carcinoma, FIGO stage IB or IIA, were included in this study. Patients were living in the Netherlands (n = 98), a low incidence area for cervical cancer, or Suriname (n = 99), a high incidence area for cervical cancer. Patients were diagnosed with cervical carcinoma between 1989 and 1995. All Dutch patients were treated in the Leiden University Medical Centre (LUMC), Leiden, the Netherlands and the tumour tissue was stored in the Pathology Department’s archive. Of the Surinamese patients, 45 were treated in the LUMC and their tumour tissue was also kept in the LUMC laboratory. The other 49 patients were treated in Suriname and tissue samples were stored in the laboratory of the Pathology Department, Academic Hospital, Paramaribo, Suriname.

HPV Detection and Genotyping by SPF\textsubscript{10} LiPA
DNA was isolated from formalin-fixed, paraffin-embedded biopsy samples as previously described\textsuperscript{21}. Care was taken to prevent cross-contamination during preparation of the sections from the paraffin blocks. Beta-globin PCR was performed using primers RS40 and RS42\textsuperscript{21} to determine whether the isolated DNA was suitable for amplification. The DNA was subjected to a short PCR fragment assay using the SPF\textsubscript{10} primer set that amplifies a 65 base pair fragment in the L1 according to the manufacturer’s instructions (Innogenetics, Gent, Belgium). Each experiment was performed with separate positive and several negative controls. The presence of HPV was established using a microtitre plate-based hybridisation assay, and SPF\textsubscript{10}-PCR products from HPV-DNA positive cases were directly genotyped by a reverse hybridisation line probe assay (LiPA (Innogenetics)). In this assay, 25 individual HPV genotypes (HPV 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, and 74) can be identified simultaneously.

On the HPV 16- and 18-positive specimens HPV-type 16 and 18 specific PCRs were performed as described previously\textsuperscript{22}.

Flow Cytometry and Sorting
Flow cytometry sorting of formalin-fixed, paraffin-embedded samples positive for multiple HPV types was performed as described previously\textsuperscript{23}. Briefly, paraffin-embedded 60 μm sections were treated with a combined mechanical/enzymatic method to obtain single cells. Cells were then stained with a mix of monoclonal antibodies directed against keratin- and vimentin-containing clones MNF116 (anti-keratin; DAKO, Glostrup, Denmark), AE1/AE3 (anti-keratin; Chemicon International Inc., Temecula, CA, USA), and V9-2b (anti-vimentin; Department of Pathology, LUMC). A standard FACSCalibur (BD Biosciences,
San Jose, CA, USA) was used for flow cytometric analysis. Subsequently, flow sorting was performed using a FACSVantage flow-sorter (BD Biosciences). Tumour cells were sorted based on keratin and vimentin expression, combined with a gate on DNA content. Diploid and aneuploid tumour fractions and normal cell fractions were collected for DNA isolation; in addition, cell fractions were directly sorted separately onto glass slides for FISH processing.

**Interphase FISH Analysis**

FISH was modified to detect multiple HPV genotypes simultaneously. Interphase FISH analysis was performed on flow cytometry-sorted cell fractions using an adapted protocol for FISH on formalin-fixed, paraffin-embedded tissue. Approximately 400 cells were sorted by flow cytometry directly onto glass slides that had been cleaned by rinsing in 96% ethanol. After spotting, the slides were dried overnight at room temperature to ensure cell adhesion. If needed, the slides were incubated in a 0.1M solution of Na$_2$B$_4$O$_7$ to permit swelling of the nuclei. Afterwards, the slides were rinsed in phosphate buffered saline and sterile water. The HPV 16 probe was labelled with digoxigenin and the HPV 18 probe was labelled with biotin (both purchased from PanPath, Science Park Amsterdam, Amsterdam, the Netherlands). Hybridisation and immunodetection were performed as described previously for nuclei isolated from paraffin-embedded material.

In each experiment, negative control slides spotted with sorted vimentin-positive nuclei not infected with HPV were stained simultaneously. Positive control slides of paraffin-embedded and sorted CasKi (HPV 16-positive), SiHa (HPV 16-positive), and HeLa (HPV 18-positive) cells were also included in all experiments. Centromere 1 (pUC1.77) and centromere 6 (p308) probes were used to ensure sufficient quality of the flow-sorted paraffin samples.

**Results**

**HPV Detection and Genotyping**

Beta-globin PCR was used as a control to ensure the quality of DNA in all samples. DNA in 2/98 (2.0%) of the Dutch samples and 4/99 (4.0%) of the Surinamese samples was found to be unsuitable for PCR and therefore excluded. Using SP10-LiPa PCR, similar HPV-positivity was found in both the low- and high-risk populations (**TABLE 1**). Among the Dutch HPV-positive samples, 11 different HPV types were detected, all of which were HR-HPV types. The Surinamese HPV-positive samples contained 17 different HPV types, of which two were low-risk HPV types. These low-risk HPV types were found in combination with (at least) one HR-HPV type.
Multiple HPV infections were more prevalent in the Surinamese cervical carcinoma samples, but this difference was not significant (OR 1.61, 95% CI 0.71 – 3.65) (TABLES 1 and 2). All but 2 samples were infected with at least 2 HR-HPV types. Occurrence and combinations of multiple infections are presented in TABLE 2. In 2 cases HPV 16 and HPV 18 double infection was detected and in 1 additional case next to HPV 16 and HPV 18 a co-infection with HPV 52 was present.

### TABLE 1
**HPV Prevalence in Dutch and Surinamese Cervical Carcinoma Samples**

<table>
<thead>
<tr>
<th>HPV</th>
<th>Surinamese n (%)</th>
<th>Dutch n (%)</th>
<th>Total n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>77 (81.1)</td>
<td>80 (83.3)</td>
<td>157 (82.2)</td>
</tr>
<tr>
<td>Single</td>
<td>60 (77.9)</td>
<td>69 (86.2)</td>
<td>129 (85.3)</td>
</tr>
<tr>
<td>Multiple</td>
<td>17 (22.1)</td>
<td>11 (13.8)</td>
<td>28 (14.7)</td>
</tr>
</tbody>
</table>

### TABLE 2
**HPV type combinations detected in Dutch and Surinamese Cervical Carcinoma Samples**

<table>
<thead>
<tr>
<th>HPV type</th>
<th>Surinamese n (%)</th>
<th>Dutch n (%)</th>
<th>Total n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16+18</td>
<td>1 (5.9)*</td>
<td>1 (9.1)*</td>
<td>2 (7.1)</td>
</tr>
<tr>
<td>16+33</td>
<td>1 (5.9)</td>
<td>-</td>
<td>1 (3.6)</td>
</tr>
<tr>
<td>16+52</td>
<td>2 (11.8)</td>
<td>-</td>
<td>2 (7.1)</td>
</tr>
<tr>
<td>18+31</td>
<td>-</td>
<td>2 (18.2)</td>
<td>2 (7.1)</td>
</tr>
<tr>
<td>18+33</td>
<td>-</td>
<td>2 (18.2)</td>
<td>2 (7.1)</td>
</tr>
<tr>
<td>18+52</td>
<td>1 (5.9)</td>
<td>2 (18.2)</td>
<td>3 (10.7)</td>
</tr>
<tr>
<td>16+18+52</td>
<td>1 (5.9)*</td>
<td>-</td>
<td>1 (3.6)</td>
</tr>
<tr>
<td>16+33+52</td>
<td>1 (5.9)</td>
<td>-</td>
<td>1 (3.6)</td>
</tr>
<tr>
<td>18+33+52</td>
<td>2 (11.8)</td>
<td>-</td>
<td>2 (7.1)</td>
</tr>
<tr>
<td>Other</td>
<td>8 (47.1)</td>
<td>4 (36.4)</td>
<td>12 (42.9)</td>
</tr>
<tr>
<td>Total</td>
<td>17 (100)</td>
<td>11 (100)</td>
<td>28 (100)</td>
</tr>
</tbody>
</table>

* cases used in further analyses

Multiple HPV infections were more prevalent in the Surinamese cervical carcinoma samples, but this difference was not significant (OR 1.61, 95% CI 0.71 – 3.65) (TABLES 1 and 2). All but 2 samples were infected with at least 2 HR-HPV types. Occurrence and combinations of multiple infections are presented in TABLE 2. In 2 cases HPV 16 and HPV 18 double infection was detected and in 1 additional case next to HPV 16 and HPV 18 a co-infection with HPV 52 was present.

**Integrated HPV in Flow-Sorted Aneuploid Cancer Cells**

The 3 cervical tumours that were positive for both HPV 16 and HPV 18 (TABLE 2) according to LiPA genotyping were flow-sorted based on keratin expression and DNA content. Diploid and aneuploid tumour cell fractions were typed separately by HPV-specific PCR. The results are shown in TABLE 3.

In case 1, both HPV 16 and 18 were detected by LiPA in DNA extracted from the unsorted sample. Only HPV 16 was found after sorting in the diploid and aneuploid cell fractions, indicating that HPV 18 was not involved in malignant transformation in this
tumour. FISH was positive for HPV 16 exclusively in the aneuploid tumour cell fraction (FIGURE 1A and 1B). The punctate signal pattern observed is indicative of integrated HPV 16 DNA (FIGURE 1B).

Case 2 also consisted of diploid and aneuploid tumour cell fractions. While in the diploid fraction both HPV 16 and HPV 18 were detected by HPV-specific PCR typing, the aneuploid fraction was exclusively HPV 18-positive. FISH showed integration of HPV 18 in the aneuploid fraction, and no integration of either HPV 16 or HPV 18 DNA in the diploid fraction (FIGURE 1D and 1E).

Case 3 was found to contain only an aneuploid tumour cell population. Both HPV 16 and HPV 18 were detected by HPV-specific PCR in the sorted tumour cells. However, FISH did not show integration of either of the two HPV types (FIGURE 1G).

Simultaneous FISH control experiments were performed using centromere 1 and centromere 6 probes to ensure sufficient quality of the sorted nuclei and were always positive for both diploid and aneuploid sorted tumour cell fractions (FIGURE 1C, 1F and 1H). In addition, positive control cervical carcinoma cell lines containing integrated HPV 16 and HPV 18 copies were always positive (FIGURE 2).

**Discussion**

HPV is the causative agent of cervical carcinoma and approximately 80% of cervical cancer deaths occur in developing countries. The incidence is highest in Latin America, the Caribbean, Sub-Saharan Africa, and South Asia and considerably lower in North America and Western Europe. It is known that the prevalence of HPV infection is tightly linked to cervical cancer incidence, and we have previously shown that Suriname is a high-risk
The prevalence of multiple HPV infections in our study group was 13.8% in Dutch and 22.1% in Surinamese cervical cancer samples, which confirms recent studies showing rates between 9% and 32%, depending on ethnicity\textsuperscript{18-20}. Multiple infections were often seen in high-risk population studies, but high- and low-risk populations have not been compared. In our study, no significant difference was observed between the low-risk (Dutch) and high-risk (Surinamese) cases, although the odds of

**FIGURE 1**
Interphase FISH on flow-sorted cervical carcinoma cells of the HPV 16/18 positive cases. Case 1 (A): the diploid cells are negative for HPV; (B) The aneuploid tumour cells show punctate signals for HPV 16 (green); (C) Control centromere 1 (red) and centromere 6 (green) signals. Case 2 (D): the diploid tumour cell fraction is negative for HPV; (E) The aneuploid tumour cells show punctate signals for HPV 18 (red); (F) Control centromere 1 (red) and centromere 6 (green) signals. Case 3 (G): the aneuploid tumour cell fraction is negative for HPV; (H) Control centromere 1 (red) and centromere 6 (green) signals.
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having multiple HPV infections was higher for the high-risk population (OR 1.61, 95% CI 0.71 – 3.65).

During HPV integration, HPV oncogenes E1 and/or E2 are frequently disrupted, while E6 and E7 are mostly preserved\(^4,5,26\). Some studies have used PCR techniques that compare expression of E2 and E6 to determine the integration prevalence. Other studies, however, have used other viral oncogenes for integration determination\(^27-29\). It remains difficult to accurately determine the incidence of HPV integration using PCR methods, due to the variability of disrupted and preserved viral oncogenes.

Here, we studied HPV integration using a FISH method that was modified to detect 2 different HPV types simultaneously. Previously, it was shown that harsh pre-treatment of samples washes away episomal forms of HPV DNA, leaving only the integrated HPV DNA to be detected by FISH\(^10\). This method appears very sensitive, but a detection threshold could account for missing single copy HPV infections. However the paraffin-embedded cell line SiHa (containing just 1-2 integrated copies of HPV 16\(^30\)) was positive in all our experiments, showing that the technique can detect a single copy of integrated HPV DNA.

We investigated cervical carcinomas infected with both HPV 16 and HPV 18 more thoroughly. It was previously established that HPV 16 is present exclusively in episomal form in 30-70% of cervical cancers\(^6,8,31\), while HPV 18 has been reported to be mainly integrated\(^8,32,33\), indicating that these HPV types might have different biological characteristics. Badaracco et al. observed a remarkable increase from 20 to 54% in the prevalence of exclusively episomal forms of HPV 16 when coinfection with HPV 18 existed, but the physical status of HPV 18 was not investigated\(^6\). In the cases described in the current study, integration of either one of the HPV types was seen in 2 of 3 HPV 16- and HPV 18-positive tumours. In the third HPV 16- and HPV 18-infected tumour, no integration of either type was observed.

**FIGURE 2**

Interphase FISH on flow-sorted cervical cancer cell lines. (A) SiHa: 2 copies of HPV 16 are visible in green; (B) CaSki: multiple copies of HPV 16 are visible in green; (C) HeLa: multiple copies of HPV 18 are visible in red.
In addition to the variation in integration status of the HPV 16- and HPV 18-coinfected cervical carcinomas in our study, we also observed differences in DNA ploidy of the tumour cells. All three tumours had an aneuploid fraction, in all cases positive for HPV using HPV-specific PCR. The only case in which the aneuploid tumour cells tested positive for both HPV 16 and 18 using HPV-specific PCR was the case in which the HPV genome was not integrated, as indicated by the absence of a punctate FISH signal. In the other two cases, aneuploidy was associated with integrated HPV, either type 16 or 18.

Melsheimer et al. concluded that aneuploidisation precedes HPV integration in cervical carcinogenesis, as their study found that 19/20 lesions with integrated HPV were aneuploid17. Malignant progression of cervical neoplasia is associated with the expression of the HPV oncoproteins E6 and E7, and expression of these genes does not require HPV integration34. E6 and E7 deregulate cell cycle control mechanisms, create genomic instability, and can eventually cause aneuploidy14,16. However, near-diploid cervical carcinoma tissue with integrated HPV was observed previously. In two HPV integration studies using COBRA-FISH, evidence was found supporting both integration preceding aneuploidisation and vice versa35,36. COBRA-FISH is a method that is able to investigate tumour ploidy and HPV integration sites most elegantly and accurately. In one of these studies, Koopman et al. observed one aneuploid cell line with an episomal HPV pattern, but also a diploid cell line with HPV integration35. In the other study, Brink et al. studied fresh cervical carcinoma tissue and showed HPV integration in one diploid, two near-diploid, and one aneuploid cervical tumour sample36. These divergent results suggest that HPV integration can either precede or follow aneuploidisation.

In conclusion, cervical carcinomas that are infected with multiple HPV types are present in populations from both high- and low-risk areas for development of cervical cancer. The previously established association between HPV integration and aneuploidy is supported by our study; however, the succession of events seems to be flexible. Although a small number of samples were studied, this work shows that the use of archival, flow-sorted tumour subpopulations may contribute to our understanding of the variety of possible mechanisms leading to invasive cervical cancer.
References

