Recurrent Integration of Human Papillomaviruses 16, 45, and 67 Near Translocation Breakpoints in New Cervical Cancer Cell Lines

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ABSTRACT

Progressive chromosomal changes and integration of human papillomavirus (HPV) sequences mark the development of invasive cervical cancer. Chromosomal localization of HPV integration is essential to the study of genomic regions involved in HPV-induced pathogenesis. Yet, the available information about HPV integration loci is still limited, especially with respect to different HPV types. We have established cell lines from five cervical cancers with HPV-16, HPV-45, and HPV-67. We have determined HPV integration sites and karyotype abnormalities by using the multicolor combined binary ratio-fluorescence in situ hybridization method (Tanke et al.) with 24 chromosome-specific paints in combination with full-length HPV DNA probes.

All cell lines were cytogenetically abnormal, and exhibited numerical and structural chromosomal deviations. HPV sequences were integrated at various (segments of) chromosomes. Duplicate integration sites were seen in all multiploid cell lines, suggesting that viral integration had preceded chromosomal endoreduplication. HPV-16 was found near the t(3p14.1–14.3;14) breakpoint in cervical squamous cell carcinoma (CSCC)-7 and mainly in episomal form in CSSCC-1. HPV-45 was integrated near 3q26–29 in cervical (aden or adenosquamous) carcinoma (CC)-8 and near 1q21–23 as well as near the t(1q21;2q13) breakpoint in CC-10A and CC-10B variant lines. HPV-67 was localized near the breakpoint of t(3p23–26;13q22–31) in CC-11. Southern blot analysis showed that, except for CSSCC-1, the physical state of HPV in the cell lines was the same as in the original tumor lesions.

This set of six cervical cancer cell lines included three lines with HPV-45, a major non-Western high-risk HPV type, the first reported HPV-67-positive cell line, and two cell lines with integrated and episomal HPV-16 DNA, respectively. The novel combined binary ratio-fluorescence in situ hybridization technique enabled us to simultaneously map chromosomal rearrangements and HPV integration sites, thereby revealing recurrent integration near translocation junctions for all of these HPV types in the cell lines from three of the five primary tumors. The detection of multiple HPV integration sites at rearranged chromosomes at such high frequency in cervical cancer-derived cells may reflect events that are relevant to the development of cervical cancer.

INTRODUCTION

Cervical cancer is a common malignancy that affects women worldwide, especially in developing countries. It is strongly associated with the “high risk” HPV's, which are detected in practically all invasive cervical cancers (1). Besides the predominant HPV types, HPV-16 and HPV-18, other oncogenic HPV types, such as HPV-31, HPV-33, HPV-35, and HPV-45 have been detected in cervical malignancies. The prevalence of certain HPV types appears to correlate with geographical variation; e.g., HPV-45 may represent a major oncogenic HPV type in Western Africa (2) and Surinam (3).

Infection with HPV is an early event in the multistep cervical pathogenic process. The oncogenic potential of HPV has been attributed mainly to the continued expression of the early gene products, E6 and E7 (4). These interfere with normal cell cycle regulation through inactivation of the tumor suppressor proteins p53 and pRB, respectively (5). Although oncogenic HPV types are required for initial transformation, HPV infection and viral oncoprotein expression alone are not sufficient for the completion of the malignant conversion process. This notion correlates with the facts that (a) a long latency period precedes tumor occurrence in vivo and that (b) the incidence of tumors is less frequent than the number of HPV infections (6). In vitro models established by transfection of primary human keratinocytes with HPV-16, HPV-18, or HPV-33 have shown that cells could be immortalized by action of the E6 and E7 genes (7). The full malignant phenotype, however, was only reached after extensive prolonged culture in vitro or by cotransfection with the ras oncogene (8, 9).

Cytogenetic studies or loss of heterozygosity analyses in primary cervical cancers and pre-invasive lesions indicate that secondary events involving different chromosomes, e.g., chromosomes 1, 3, 4, 5, 6, 11, 15, 17, and 18 are progressively implicated in vivo (10–12). Yet the exact nature of cervical cancer-related genes has still to be identified.

The HPV DNA genome persists as an extrachromosomal episome in premalignant cervical intraepithelial neoplastic lesions, whereas in invasive cancers and tumor cell lines, multiple copies of the viral DNA are integrated in the host genome. In contrast with most cervical tumors, which seem to contain exclusively integrated HPV sequences, a proportion of HPV-16-positive tumors contains episomal HPV DNA either alone or in coexistence with integrated HPV sequences (13, 14).

Thus, although differences between the different HPV types may exist, integration of HPV DNA in the host genome represents an important event in cervical carcinogenesis. Besides increasing the transcript stability and protein expression of the viral E6 and E7 genes (15), integration may cause changes in the transcription and expression of cellular genes targeted by the integration event. The integration of HPV sequences in the host genome might occur randomly or with a preference in or near fragile sites or oncogenes (16). Various HPV integration sites have been identified in HPV-16- or HPV-18-containing tumor-derived cell lines (16–18), in a limited number of primary lesions (19, 20), or in HPV-immortalized primary keratinocytes (21–23). Thus far, these integration sites do not point to preferential chromosome- or HPV type-specific integration. Still, HPV may integrate at presently unidentified oncogenic loci involved in cervical carcinogenesis.

In this context, we have mapped HPV DNA integration sites in six recently established cervical cancer cell lines containing HPV-16, HPV-45, and HPV-67. In addition, we have characterized the numerical and structural chromosome abnormalities in the cell lines and compared them with the original tumors with respect to the physical

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4 The abbreviations used are: HPV, human papillomavirus; FISH, fluorescence in situ hybridization; mAb, monoclonal antibody; DEAC, diethylnitrosamumin; DAPI, 4,6-diamino-2-phenylindole; DI, DNA index; CSSCC, cervical squamous cell carcinoma; CC, cervical (adeno or adenosquamous) carcinoma.
status of HPV and the overall DNA content. The multicolor COBRA- 
FISH technique (24), which was extended with HPV hybridization9,
enabled us to map HPV s at unique chromosomal integration loca-
tions, including chromosomal translocation junctions. These sites may
reflect events important in HPV-related carcinogenesis.

**MATERIALS AND METHODS**

**Clinical Background.** Initially, tumor specimens from 74 FIGO stage
IIA/IB cervical cancer patients who had undergone a Wertheim’s hysterectomy
at Leiden University Medical Center were brought into cell culture. This group
distributed of 19 patients (26%) of multiple ethnicities, such as Creoles, Java-
inese, and Hindustani, who were all from the former Dutch colony Surinam
(South America) and were referred for treatment in The Netherlands. The other
55 (74%) were Caucasian. The mean patient age was 45.5 years in a range of
23–76 years. All tumors were examined histologically on paraffin-embedded
sections and pathologically diagnosed as having cervical squamous cell car-
cinoma (55/74), adenocarcinoma (10/74), or adenosquamous carcinoma (9/74).

**Patient clinical characteristics, histopathology of the original tumor, and HPV types in primary lesions and derivative cell lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Patient age</th>
<th>Federation Internationale de Gynécologie et Obstétrique (FIGO) stage</th>
<th>Histopathological diagnosis</th>
<th>LNM*</th>
<th>Survival</th>
<th>Primary tumor</th>
<th>cell line*</th>
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<tr>
<td>CSCC-1†#</td>
<td>34</td>
<td>IIA</td>
<td>NKLSCC§</td>
<td>+</td>
<td>Disease-free</td>
<td>16</td>
<td>16</td>
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<tr>
<td>CSCC-2†#</td>
<td>33</td>
<td>IB/IIA</td>
<td>NKLSCC</td>
<td>–</td>
<td>Disease-free</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>CC-1#</td>
<td>30</td>
<td>ASC</td>
<td>GCC#</td>
<td>+</td>
<td>Disease-free</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>CC-10/A/B#</td>
<td>45</td>
<td>IB</td>
<td>+</td>
<td>4 mo</td>
<td>58 + 67</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* LNM, status of lymph node metastasis, either positive (+) or negative (–).
† HPV typing results as determined by GP-PCR with consensus primer sets GP5/GP6+, or MY09/11 followed by sequence analysis. Additional HPV-16-specific PCR confirmed HPV-16 where listed and was negative in CC-8, CC-10, and CC-11 primary tumor and lines.
* Typing was done on both early (5/18) and late passages (>20).
• Caucasian patient.
# NKLSCC, nonkeratinizing large cell squamous cell carcinoma.
† Surinamese patient.
§ ASC, adenosquamous carcinoma.
* GCC, glassy cell carcinoma. It is classified within the group of mixed (adenosquamous) carcinomas (25).

**HPV Detection and Typing.** General primer-mediated PCR and subse-
quent sequencing in combination with type-specific PCR or oligonucleotide
probe-hybridization was used for the detection and typing of HPV DNA. The
β-globin gene was used as an internal control for PCR amplification (28). An
initial general primer-mediated PCR using the HPV consensus primer set
(CPI/CPII) to amplify a 188-bp fragment in the highly conserved E1 ORF
region was performed as described previously (29). Inconclusive or negative
samples were subsequently tested with additional HPV consensus primer sets,
i.e., GP5+/6+ (30) or MY09/11 (29). To determine the HPV subtype,
PCR products were subjected to direct sequence analysis. In short, after
purification of the PCR products using the “EasyPrep” kit (Amersham Phar-
macia Biotech, Uppsala, Sweden), the products were sequenced directly with
the cycle-sequencing kit (Perkin-Elmer, Norwalk, Connecticut) using the PCR
primers end-labeled with 32P-ATP. For nucleotide sequence analysis and compara-
tions, the programs Seqed, Fasta, and MAP of the Wisconsin Genetics
Computer Group (version 8.1) sequence analysis software package were used.

**FISH Probes.** Probes for all chromosomes were obtained from Cytocell,
United Kingdom. Dr. Nigel Carter (Sanger Institute, Cambridge, United King-
dom) kindly provided additional probes for chromosomes 1–8, 13–20, 22, and
X. The probe with the best performance was chosen for each chromosome.
All DNA probes were amplified by degenerate oligonucleotide-primed-PCR (32)
to generate a set of 24 human chromosome painting probes for enzymatic
labeling. Full-length DNA probes for HPV-16, HPV-18, and HPV-45 were
kindly provided by Dr. H. Zur Hausen (Heidelberg, Germany), and full-length
DNA probes for HPV-58 and HPV-67 were provided by Dr. T. Matsukura
(Tokyo, Japan).

**Multicolor FISH Staining (COBRA).** To visualize each separate chro-
omosome as well as the respective HPV genome, we applied the COBRA-
FISH technique as described in detail elsewhere (24). This method allows
for staining of all 24 human chromosomes in distinguishable colors using
four primary fluorophores versus using five as it was reported thus far for
other multicolor FISH techniques. Thus, the fifth color (in this case, Cy7)
is available for the identification of the integrated HPV. In short, each of
the 24 human chromosomes were fluorescently labeled by incorporating
labeled dUTP-5S. This was done by using either PCR or nick translation and
by using the following various combinations of four fluorophores/haptens-
dUTP according to the COBRA protocol (24): Fluorescein (Boehringer
Mannheim, Germany), Lissamine (NEN Life Science Products, Boston,
MA), Cy5 (Amersham), and Digoxigenin (Roche, Basel, Switzerland).
The HPV probes were labeled with Biotin-dUTP (Sigma) during nick trans-
lation. Metaphase chromosomes were prepared according to standard proce-
dures, and they were pretreated with RNase A and pepsin according to
Wiegant et al. (33). After denaturing, 5 ng of the biotin-labeled HPV probe
and a total of 4 μg of a cocktail with each chromosome represented were
hybridized for 120 h at 37°C in a humidified chamber. After washing, a
Table 2  Chromosomal location of HPV and karyotype description in cervical cancer cell lines

<table>
<thead>
<tr>
<th>Cell line passage</th>
<th>HPV Probe</th>
<th>HPV status</th>
<th>Karyotype abnormalities</th>
<th>Modal range</th>
<th>Figure</th>
</tr>
</thead>
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<tr>
<td>SCC-1 p26/ p38</td>
<td>HPV-16</td>
<td>Episomal</td>
<td>At normal 14q (&gt;4) in one complex metaphase</td>
<td>&lt;3n&gt;XXXXX, +1X&gt;2, +2, +3&gt;1, +3&gt;2, der(3) del(3p), der(4) t(4;17)X2; +5, der(5) del(3q), der(7) del(7q), +9&gt;3, +10, +11&gt;2, der(11) t(3;11), +12, der(12) t(12;15); +13, +14, +15, +16&lt;2, +17&gt;2, t(17;20), +18, +20&gt;2X2, +21, +22, der(2)X2</td>
<td>68-79</td>
</tr>
<tr>
<td>SCC-7 p49</td>
<td>HPV-16</td>
<td>No</td>
<td>Near breakpoint of der(14): t(3;14) (p14.1–14.31)X2</td>
<td>&lt;4&gt;3X, der(2) t(2;6), –3&gt;4, der(3) t(3;7)X2, dic(3;7), der(3) t(3;5)X2, –4&gt;2X2, +5, der(5) t(5;17), der(5) ins(5;6), –6&gt;2, der(6) t(3;6)X2, –7&gt;2, der(7) t(2;3;7)X2, der(7) t(7;7), –8, –9, –11&gt;2, der(11) t(11;X)X2, –13&gt;2, der(13) t(X;13)X2, –14&gt;4, der(14) t(13;4)X2, dic(14;14)X2, –15&gt;2, –17&gt;2, der(17) t(17;17), –18&gt;2, der(20)X4, –22</td>
<td>76-91</td>
</tr>
<tr>
<td>CC-8 p21</td>
<td>HPV-45</td>
<td>No</td>
<td>At normal 3: (3q26–29)X2</td>
<td>&lt;4&gt;n&gt; –X&gt;4, der(X) t(X;1), t(X;2), der(X) t(X;20), –2&gt;3X, der(3) t(3;4)X2, der(3) t(3;11), der(3) t(3;17), –4&gt;3, der(4) iso4q, +5&gt;2, der(6)X2, der(6) t(6;14), 9&gt;2, der(9)X2, der(9) t(9;5), –10, –12, –13&gt;3, der(14), –15&gt;4, der(15) t(13;15)X2, –16, –17, der(17), –19, +20, der(20) t(7;20), –21X2, der(21) t(12;1)X2, der(21) t(11;21), –22X2, der(22) t(7;15;16), der(22) t(11;22;4;17), der(22) t(5;22;4;17) [cp 13]</td>
<td>83-93</td>
</tr>
<tr>
<td>CC-10A p25</td>
<td>HPV-45</td>
<td>No</td>
<td>At normal 1: (1q21–23)X3, near breakpoint of der(22): t(11;22) (q21;1q13)X2</td>
<td>&lt;5&gt;n&gt; –XXXXX, +1, der(1) t(1;22), der(2)X2, der(2) t(2;3), der(3), der(6), +7, –8, der(8) t(8;20)X4, +9&gt;2, der(11) (9;11), –13, –14&gt;2, –15&gt;4, dic(15;15)X2, –18, –20, –21, –22X2, der(22) t(11;22)X3 [cp 17]</td>
<td>112-119</td>
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<td>CC-10B p25</td>
<td>HPV-45</td>
<td>No</td>
<td>At normal 1: (1q21–23)X3, near breakpoint of der(22): t(11;22) (q21;1q13)X2</td>
<td>&lt;3&gt;n&gt; –XXX, +X, der(1) t(1;9), +5, +4, der(7) t(7;8), der(8) t(8;20)X2, +9, der(9) t(9;20), –15, dic(15;15), +19, +20, der(22) t(11;22)X2 [cp 13]</td>
<td>65-75</td>
</tr>
<tr>
<td>CC-11 p28</td>
<td>HPV-67</td>
<td>No</td>
<td>Near breakpoint of der(3): t(3;13) (p23–26;q22–31)</td>
<td>&lt;2&gt;n&gt;XX, –1, der(1) t(2;12;13), der(1) t(1;13), der(4) t(4;19), der(5), –7, der(7) t(7;15), –10, der(10) t(10;22), –13, der(3) t(3;13), –15, +20, –22</td>
<td>46-51</td>
</tr>
</tbody>
</table>

* This value is the modal number as calculated from DI (Table 3) × 46 chromosomes.

mAb against digoxin followed by a sheep anti-mouse mAb conjugated to DEAC (Molecular Probes, Eugene, Oregon) was added to detect the Digoxigenin-labeled probes. The Biotin-labeled HPV probes were detected using streptavidin-Cy3 conjugates (Amersham) followed by incubations with a biotinylated mAb against streptavidin (Life Technologies, Inc.) and streptavidin-Cy3 (Amersham). Chromosomes were counterstained with DAPI solution. The slides were embedded in Vectashield (Vector) prior to microscopic evaluation. Digital fluorescence imaging and analysis were done as described (24, 34). Multicolor FISH karyotyping of the cell lines was simultaneously done by the same procedure (24).

**Analysis of the Physical State of HPV DNA/Southern Blot Analysis.** The restriction enzymes used were EcoRI (Roche), HindIII (Roche), and PstI (Amersham). With each restriction enzyme, 10 μg of genomic DNA were digested, electrophoresed on 1% agarose gel, and transferred to nylon filters (Hybond N+, Amersham) by Southern blotting. Full-length HPV DNA probes were radiolabeled with 20 μCi of [α-32P]dCTP using a random-primed labeling kit (Amersham Pharmacia Biotech). Southern blots were prehybridized at 65°C in hybridization mix (5× Denhardt’s, 6× SSC, 0.5% SDS) and after 2 h, they were hybridized with the HPV probes overnight at 67°C. Blots were washed for 30 min at 65°C in 2× SSC/0.1% SDS, for 30 min in 1× SSC/0.1% SDS, and finally for 15 min in 0.5× SSC/0.1% SDS. Membranes were exposed to Kodak X-Omat AR films with intensifying screens at −70°C.

**Ploidy Analysis.** For paraffin-embedded primary tumor material, the pepsin digestion method of Hedley et al. (35) was used for nuclear isolation from 40-μm sections. Nuclei from the cell lines were isolated using the detergent trypsin method of Vindelov et al. (36). Propidium iodide was used as a DNA stain for both methods. DNA content was measured on a FACSscan flow cytometer (Becton Dickinson, Mountainview, CA) and expressed as a DI (37).

**RESULTS**

**Establishment of Six Novel Human Cell Lines From Five Cervical Carcinomas.** In our attempts to establish long-term cell cultures from cervical carcinoma specimens obtained from 74 patients, we established six continuous cell lines from five primary tumours. The reasons for the lack of success in establishing cell lines from the other 69 primary cultures were (a) the lack of adequate numbers of tumor cells, (b) overgrowth by fibroblasts, (c) senescence of short-term tumor cell cultures after 3–6 passages, or (d) microbial contamination. Long-term cultures, i.e., cultures that could be maintained beyond at least 20 passages, were established from five primary cultures. Histopathology, patient characteristics, and lymph node status of these cases are listed in Table 1.

**In Vitro Characteristics of the Cervical Cancer Cell Lines.** All cell lines have now undergone >100 population doublings over at least 20–30 passages in keratinocyte serum-free medium. Phase-contrast microscopy revealed flat adherent monolayers with epithelial cell morphology; cells ranged in size and shape and were arranged in a pavement-like architecture (not shown). One of the cell lines, CC-10, was split into two subpopulations (A and B) on morphological grounds at an early passage. At later passages beyond p30, the resulting variant cell lines, CC-10A and 10B, no longer showed obvious morphological differences but appeared distinct by other parameters (Tables 2 and 3).

**Ploidy Analysis.** To determine whether the cell lines had changed their overall chromosome content during culturing, we studied DNA ploidy in the primary tumor and in early and late passage cell lines. The DIs of the cell lines versus the paraffin-embedded tumor material are listed in Table 4. Allowing for technical variability due to the different methods used to obtain and analyze the DNA from the different sources, the DI of CC-11 could represent the stemline DI detected in the primary tumor. In the other cell lines, the DNA content was different from the corresponding primary tumors. This was probably a result of endoreduplication and subsequent DNA loss during

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**HPV INTEGRATION IN CERVICAL CARCINOMA CELL LINES**

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HPV INTEGRATION IN CERVICAL CARCINOMA CELL LINES

Table 3 Chromosomes involved in genomic rearrangements and HPV integration

| Chr | CS-CC-1 | CS-CC-7 | CC-8 | CC-10A | CC-10B | CC-11 | Total
<table>
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<td>1</td>
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<td>21</td>
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<td>22</td>
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<td>T</td>
<td>T/ntHPV45 (q)</td>
<td>4</td>
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<td></td>
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<tr>
<td>x</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>T/ntHPV45 (q)</td>
<td>4</td>
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<tr>
<td>Total</td>
<td>10</td>
<td>13</td>
<td>18</td>
<td>10</td>
<td>7</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

* a, chromosome.
| b Total number of cases per chromosome.
| c Only in 1/38 studied metaphases.
| d Total number of chromosomes involved in changes per cell line.

culturing. In most cases, this was evident already at early passages (Table 4).

Karyotypes. Description of the COBRA-FISH karyotypes in each cell line was done according to the standard nomenclature (Table 2). Modal chromosome numbers varied from the hyperdiploid range (CC-11) to the triploid (CSCC-1, CC-10B), tetraploid (CSCC-7, CC-8), and pentaploid range (CC-10A), and they were in a similar range as those calculated from the measured DIs (Table 3). In addition to gross numerical changes, structural chromosomal abnormalities were abundant in all cell lines. Table 3 lists the chromosomes according to their involvement in structural changes as studied by multicolor FISH. Most chromosomes showed structural rearrangements in three or more of the six cell lines. Chromosomes 3, 7, 15, and 20 were most frequently affected, but no single chromosome was structurally aberrant in all cell lines. Chromosomes 10, 12, 16, 19, 21, and X were only occasionally affected, and chromosome 18 appeared unchanged in all cell lines.

HPV Typing of Primary Tumors and Cell Lines. HPV was detected in 69 (93%) of the 74 primary tumors. The overall distribution of HPV sequences was 55.4% HPV-16 (n = 41), 20% HPV-18 (n = 15), 6.8% HPV-45 (n = 5), 4% HPV-33 (n = 3), and one each of HPV-31, HPV-35, HPV-52, HPV-56, and HPV-58 + HPV-67 (7%). Five (7%) cases remained HPV negative. In CSCC-1 and CSCC-7, HPV-16 was detected, and in CC-8 and both CC-10 variants, HPV-45 was found. Identical typing results were found in the corresponding primary tumors (Table 1). In CC-11, HPV-67 was detected by using all of the CPI/II, GP5+/6+, or My09/My11 primer sets. In the primary tumor, however, HPV-58 was detected with the CPI/II primer set, whereas HPV-67 was found with the GP5+/6+ and the My09/11 sets. In consecutive culture passages (p3-p27), CC-11 was consistently positive for only HPV-67 with all three primer sets. Additional HPV-58-specific hybridization performed on the primary tumor and early and late passages of CC-11 were positive on the primary tumor only. Apparently, the primary tumor contained both HPV-58 and HPV-67, and the cell line CC-11 was cultured from the HPV-67-containing tumor cells.

Chromosomal HPV-integration Sites. To analyze the localization of HPV integration, we applied the modified multicolor COBRA-

Table 4 Cell line growth rates and ploidy in cervical tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Doubling time (h)</th>
<th>DNA index</th>
<th>Passages studied and number of measurements (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS-CC-1</td>
<td>~74 ± 5</td>
<td>1.0</td>
<td>p21–26 (5)</td>
</tr>
<tr>
<td>CS-CC-7</td>
<td>~50 ± 4</td>
<td>1.10</td>
<td>p17–20 (6)</td>
</tr>
<tr>
<td>CC-8</td>
<td>~55 ± 7</td>
<td>1.10</td>
<td>p15–19 (3)</td>
</tr>
<tr>
<td>CC-10A</td>
<td>~58 ± 1</td>
<td>1.10</td>
<td>p10–16 (4)</td>
</tr>
<tr>
<td>CC-10B</td>
<td>~31 ± 2</td>
<td>1.10</td>
<td>p20–30 (4)</td>
</tr>
<tr>
<td>CC-11</td>
<td>~62 ± 10</td>
<td>1.23 1.35</td>
<td>p37–43 (10)</td>
</tr>
</tbody>
</table>

a DIs were determined on primary tumors from paraffin-embedded tissue sections as described in “Materials and Methods.”

b DIs were determined on cell line suspensions as described in “Materials and Methods.”

Minor subpopulations with DIs as listed were present beside prominent peaks in the sample. Multiple ploidy analyses were performed at indicated passages to obtain mean DIs ± SE. For CC-10, uncultured primary suspension was available for DNA analysis. For the remaining cell lines, only early passage samples were available.

d Susp, uncultured primary suspension.

e The DI 2.9 peak in CC-10A gradually disappeared within the range of passage 20–30, whereas it emerged at late passage in CC-10B.

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HPV integration in cervical carcinoma cell lines

FISH technique (24) in combination with HPV probe hybridization. Single cross-hybridizations using unique HPV-16, HPV-18, HPV-45, HPV-58, and HPV-67 probes on all studied cell lines completely matched the HPV-PCR data (not shown). The set-up for the COBRA analysis in combination with HPV hybridization is shown in Fig. 1 for cell line CSCC-7. Combined results for the other cell lines are depicted as metaphase spread images in Fig. 2.

HPV was integrated in four of the five cell lines, when, by origin, considering CC-10A and CC-10B as one cell line (Table 2). Both CC-10 variants contained integrated HPV-45 sequences near the breakpoint of derivative chromosome 22, t(1q;22q), and at chromosome 1q (Fig. 2, i-j). Despite differences in the modal chromosomal number and the DI (Table 4), these variant lines showed no differences in the HPV integration pattern, which probably represented that of the primary tumor. In CSCC-7, the integration of HPV-16 was detected in the derivative chromosome 14 near the (3p;14) translocation breakpoint (Fig. 1). In CC-8, the integration of HPV-45 was observed on chromosome 3q26–29 (Fig. 2h). CC-11 contained integrated HPV-67 sequences near the breakpoint region of derivative chromosome 3 t(3p;13q); Fig. 2k). This cell line was near-diploid (DI~1.1), and the integration was consistently found in one chromosome copy. In metaphase spreads of the hyperdiploid cell lines, on the other hand, the integration sites were seen in multiple chromosome copies, thereby indicating that the integration of HPV at these sites had preceded chromosomal endoreduplication (Fig. 2; Table 4). The pattern observed in the interphase nuclei of CSCC-1 was typical for the presence of episomal virus particles (Fig. 2, a and b). In 37 of the 38 metaphases studied from this cell line, only scattered signals and no integration sites were visible (Fig. 1g). In one metaphase spread however, integration was observed on four copies of chromosome 14q (Fig. 2, c-d).

**Southern Blot Analysis.** To investigate whether the physical status of HPV in the cell lines represents the HPV status of the original tumors, we performed Southern blot analysis (Fig. 3). In cases where no frozen primary tumor material was available for this method, the earliest available culture passage was used. The restriction patterns in late passages of cell lines CSCC-7, CC-8, CC-10, and CC-11 (Fig. 3, B-E) were in agreement with the presence of integrated HPV DNA as determined by HPV-FISH (Fig. 1; Fig. 2, h-k). Moreover, the patterns were identical to those observed in the primary tumor material or early passages.

In CSCC-1, however, the restriction pattern changed upon culturing from passage p6 to p20 (Fig. 3A). The digestion of genomic DNA from CSCC-1 at p20 with HindIII or EcoRI indicated the presence of episomal HPV forms, as was also found by HPV-FISH analysis at late passage. HindIII digests of p6 showed several additional bands that may represent the coexistence of integrated HPV sequences. When EcoRI was used, the restriction patterns between p6 and p20 also differed, but both patterns contained a fragment of approximately 7.3 kb, which could have resulted from the disruption of circular episomal HPV-16 DNA. The difference in restriction patterns observed between early and late passage may have resulted from the selection for episomal HPV-16 and/or may represent a change in the constitution of the extrachromosomal episomal genomes that may have occurred during culturing.

**DISCUSSION**

By using a novel combination of complete chromosome painting by COBRA (24) and HPV-FISH, we simultaneously mapped sites of HPV integration and chromosomal rearrangements in cell line metaphase spreads from five cervical cancers. Integrated HPV was found at unique chromosomal sites in each cell line, with no single preferred site for either HPV-16, HPV-45, or HPV-67. Also, among multiple cell lines with the same HPV type, integration sites were different (Table 2). Interestingly, however, HPV-16, HPV-45, and HPV-67 were integrated near translocation junctions in the cell lines from three of the five tumors (Fig. 1 and 2; Table 3). Chromosome 3 was involved in integration in three cases, two of which involved translocation (Table 3). HPV-16 was found at the junction of chromosome 3p14, with a whole chromosome 14 in CSCC-7, whereas HPV-67 was found near the junction of chromosome 3p23–26, with chromosome 13q in CC-11. HPV-45 was integrated at chromosome 3q in CC-8 and at 1q21–23 as well as at the junction of 1q21 and 22q13 in CC-10A and CC-10B. In CSCC-1, HPV-16 was present mainly in episomal form, although integration at chromosome 14q was also observed in one metaphase.

The chromosomal locations for the HPV integration described here are different from the integration sites that have been previously described in a number of cervical cancer derived cell lines, a few primary cervical cancer cell cultures, and in HPV-immortalized keratinocyte models. Although the latter are fundamentally different from tumor-derived immortal cells, they provide useful models for studying transformation in vitro. Integration of HPV-16 DNA has been found at multiple sites including chromosomes 3, 4, 7, 11, 13, 15, 17, and 20 (19, 21, 38). HPV-18 integration has been reported at 3p21, 8q21–22, and 12q14–15 (17, 39) and in the HeLa cell line at multiple sites including normal and rearranged 8q24 (18), a site also implicated in HPV-16 and HPV-18 integration in two primary cervical cancers (20). HPV-68 integration at 18q21 was recently described in a cancer cell line (40). In HPV-33-immortalized cell lines, integration was recurrently found at chromosomes 13q33–34 and 9p13 (22, 23). These previous reports and our present findings do not point toward the existence of a single preferential chromosomal integration site for HPV. However, recurrent integrations of different HPV types at chromosomal breakpoint regions in three of the five cervical cancers as described in this study provide novel perspectives on the role of HPV in cervical cancer. To our knowledge, HeLa is the only tumor-derived cell line in which HPV was detected at rearranged sites (18). Over the past few years, almost 20 new cervical cancer cell lines with HPV-16, HPV-18, HPV-31, and HPV-33 have been established, but the chromosomal localization of HPV was not assessed (41, 42). Thus, chromosomal integration sites involving multiple HPV types in cervical cancer-derived cells are narrowly explored.

By using a novel combination of HPV detection with simultaneous multicolor-FISH analysis as applied on our tumor-derived cell lines, not only the chromosomal HPV integration sites but also the related breakpoint regions and chromosomes putatively involved in HPV-related immortalization can be identified (Table 3). The cytogenetically abnormal chromosomes described in this study include those that are most frequently described in primary cervical cancers, such as chromosomes 1, 3, 5, 11, and 17 (10). Chromosomes 3, 11, and 17, as well as other chromosomes such as 18q and 6p have also been noted in loss of heterozygosity studies (11). Chromosome 3 was involved in HPV integration at three different sites: 3q (HPV-45), 3p23–26 (HPV-67), and 3p14 (HPV-16; Table 3). The 3p14 region contains the FR3A3B fragile site located within the FHIT gene locus, which is commonly altered in cervical carcinomas (43, 44). Interestingly, this region coincides with an earlier reported HPV-16 integration site in a primary cervical cancer (45). To shed light on the involvement of possible fragile sites or specific cellular sequences targeted by HPV integration in the present cell lines, further fine mapping of the chromosomal abnormalities with regard to the exact chromosomal loci is in progress. Because our (and other) established cell lines from already invasive cervical cancers constitute the starting point of our analyses, we cannot provide solid evidence concerning the sequence
Fig. 1. Set-up for the simultaneous COBRA and HPV FISH analysis. A representative COBRA-FISH karyogram of cell line CSCC-7 is shown. 

a, image (12 colors) resulting from the three primary dyes (Fluorescein, Lissamine, and Cy5) used in ratio-labeling. 

b, DEAC image (fourth dye, so-called binary-1 image) that is used to identify the odd and even numbered chromosomes that carry the same set of three primary labels (a). For instance, chromosome 1 (odd, binary 1-negative) and 4 (even, binary 1-positive, purple) have the same color probe (a) but a different binary color label (b). 

c, Cy7 image (fifth dye, so-called binary 2 image) shows the HPV-16 hybridization signals (arrows). 

d, DAPI image (used as a technical reference image, not for banding purposes). 

e, Overlay of the images in a and c reveals the chromosomal HPV integration sites. In this case, the integration of HPV-16 is near the translocation region of chromosomes 3p (red) and 14 (blue), as indicated by arrows. In all panels, the boxed chromosomes represent derivative chromosomes 14 [t(3;14)] involved in translocation and HPV integration.
Fig. 2. COBRA-HPV FISH analysis using relevant full-length HPV probes on cervical cancer cell lines. HPV-16 is present in episomal form in the interphase nuclei of CSCC-1. a. DAPI image. b. HPV signals. One analyzed metaphase was near-octaploid (c) and contained four chromosome 14q copies with HPV-16 integration signals (inset, d, arrows). e. DEAC image. f. DAPI image. g. Representative metaphase spread with scattered extra-chromosomal HPV signals (arrows). h. HPV-45 integration on two copies of chromosome 3q (arrows) in CC-8. i-j. HPV-45 integration on two copies of chromosome 1q (arrows with * and on two copies of derivative chromosomes 22 [t(1q;22q)] near the translocation junction (arrows) in CC-10A and CC-10B. k. HPV-67 integration on one copy of derivative chromosome 3 [t(3p;13q)] near the translocation junction (arrow) in CC-11. An interphase nucleus (below) shows one HPV-67 integration signal.

HPV INTEGRATION IN CERVICAL CARCINOMA CELL LINES

Research. on April 14, 2017. © 1999 American Association for Cancer Research.
of events, i.e., the causal relationship between HPV integration and genomic rearrangements during malignant transformation. For this purpose, HPV-immortalized keratinocytes might be suitable models for the study of sequential stages of malignancy with application of the methodology presented here.

Southern blot results indicated that the HPV integration status in all of the present cell lines, except for CSCC-1, is representative of the HPV integration status in the original primary tumors. Thus, although gross numerical changes occur in most cell lines through endoreduplication and chromosomal losses (Table 4), the physical HPV status is unchanged. This was also demonstrated by the multiplication of integration sites seen in the metaphase spreads (Fig. 2). In CSCC-1, HPV-16 was predominantly present in episomal form. The changes in the HPV-16 restriction pattern in CSCC-1 from early to late culture passages (Fig. 3A) suggest that the physical status of HPV sequences changed during culturing. From these data, it is not clear whether this is caused by a change in nature and/or relative contributions of episomal and/or integrated HPV DNA. Integrated HPV-16 was found at chromosome 14q in only one metaphase spread, which contained about four times as many chromosomes as the other metaphases analyzed (Fig. 2A).

The DNA index of CSCC-1, which was measured at the same passage numbers as was used for the preparation of chromosome spreads, was in the triploid range (Table 4). This suggests that the octaploid cells in which integration was found probably represent a negligible subpopulation. The changed restriction pattern may, however, be explained by an aberrant physical nature of the episomal HPV-16 DNA, as has been reported in a cervical carcinoma cell line harboring a 10-kb HPV-16 genome that was maintained as an extrachromosomal episome (46). The presence of extrachromosomal HPV DNA has been reported in cervical cancers and a few other cervical cancer cell lines (13, 14, 46, 47) and seems restricted to HPV-16 DNA. This is in contrast with the general idea that oncogenic HPV types integrate in cervical neoplastic lesions as they acquire an invasive character. Viral integration enhances the expression of E6/E7 mRNAs, presumably by disrupting the HPV E2 gene, which encodes a transcriptional repressor of the E6/E7-specific promoter (15). Integration events may thus be required for the continued expression of the E6/E7 oncogenes and consequent genomic instability. The presence of high copy numbers of extrachromosomal HPV DNA, however, may lead to lower but sufficient levels of E6/E7 mRNAs in comparison with cells that contain...
fewer, but integrated HPV copies (47). It will be interesting to further investigate these aspects in CSCC-1 at consecutive passages.

Well-known HPV-16- or HPV-18-containing cell lines such as HeLa, SiHa, and CasKi (48–50) have been used in many studies world-wide and have contributed a great deal to our understanding of HPV-induced pathogenesis. Because of their establishment, a number of new cell lines, including mostly HPV-16 and HPV-18-positive cell lines, have been reported. Still, the relative difficulty to propagate tumor cells in vitro (51, 52) has limited the representation of various HPV types and histological backgrounds. Although HPV-16 and HPV-18 are present in the majority of cervical cancers in the Western world, other HPV types may predominate in non-Western countries, where cervical cancer is even more abundant (2, 53). It is in this context that we point out that four of the five primary tumors from which the present cell lines were derived were from Surinamese patients, whereas only 26% of the 74 primary tumors were of Surinamese origin. Although we did not systematically assess the variables that may have contributed to the culture success of Surinamese tumors, one explanation may be the relatively large tumor size observed in these patients (3) and consequently, the increased amount of tumor material usually brought into initial culture. These patients also appear to present with non-HPV-16 or HPV-18 at a 2-fold higher frequency than the Dutch population (3). In the present study, 14% of the Dutch primary cancers were non-HPV-16/18 versus 35% among the Surinamese cancers, which may have contributed to the establishment of four non-HPV-16/18-positive cell lines.

To date, only one HPV-45-positive cell line has been described (54). CC-11 is the first known cell line with HPV-67. This HPV was only recently cloned from a vaginal intraepithelial neoplasia grade I and identified as a new HPV type phylogenetically clustered with HPV-16, HPV-31, HPV-33, HPV-35, HPV-52, and HPV-58 (55).


HPV INTEGRATION IN CERVICAL CARCINOMA CELL LINES

Recurrent Integration of Human Papillomaviruses 16, 45, and 67 Near Translocation Breakpoints in New Cervical Cancer Cell Lines


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