Detection of High-Risk Cervical Intraepithelial Neoplasia and Cervical Cancer by Amplification of Transcripts Derived from Integrated Papillomavirus Oncogenes

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ABSTRACT

Cervical cancer emerges from cervical intraepithelial neoplasia (CIN) induced by high-risk HPV (HR-HPV) infections. However, the vast majority of CIN lesions regress spontaneously, and only a few lesions persist or progress to invasive carcinoma. On the basis of morphological criteria, it is not possible to differentiate high-grade lesions that will regress or persist from those that inevitably will progress to invasive cancers. In most cervical carcinomas, human papillomavirus (HPV) genomes are integrated into host cell chromosomes and transcribed into mRNAs encompassing viral and cellular sequences. In contrast, in early preneoplastic lesions, HPV genomes persist as episomes, and derived transcripts contain exclusively viral sequences. Thus, detection of HPV transcripts derived from integrated HPV genomes may specifically indicate both CIN lesions at high risk for progression as well as invasive cervical cancers. Here, we established a protocol for the amplification of papillomavirus oncogene transcripts (APOT) from cervical specimens that allows us to distinguish episomal- from integrate-derived HPV mRNAs. Cervical swab and biopsy samples from 549 patients attending outpatient clinics for cervical dysplasia were screened for the presence of HPV DNA, and 155 samples that were positive for either HPV type 16 (n = 143) or 18 (n = 12) were subjected to the APOT assay. In samples derived from normal cervical epithelia (n = 19) or low-grade cervical lesions (CIN I, n = 10), no integrate-derived HPV transcripts were found. In contrast, in 1 (5%) of 22 samples derived from CIN II lesions, in 10 (16%) of 64 samples from patients with CIN III lesions, and in 35 (88%) of 40 samples from patients with cervical cancer, integrate-derived HPV transcripts were detected. Thus, detection of integrate-derived HPV transcripts in cervical swabs or biopsy specimens by the APOT assay points to advanced dysplasia or invasive cervical cancer.

INTRODUCTION

Cancer of the uterine cervix emerges from a defined series of preneoplastic lesions with increasing cellular dysplasia, referred to as CIN grade I, II, or III. CIN lesions are induced by persistent HPVs (1–3). Therefore, the detection of HR-HPV infection has been proposed as a useful additional surrogate marker to diagnose cervical dysplasia (4, 5). However, infections induced by HR-HPV types are found in about 10% of healthy normal women without clinical evidence of cervical lesions (6). Most HR-HPV infections usually last only for several months (7). Accordingly, the vast majority of low-grade CIN lesions regress spontaneously, and only very few persist or progress to high-grade cervical dysplasia (CIN II/III). Furthermore, it was estimated that only about 10–20% of these high-grade CIN lesions finally progress to invasive cervical cancer (8). Despite the low risk for progression, currently all of the high-grade CIN lesions are removed by surgical resection (conization) to securely avoid the development of invasive carcinomas because there is no criterion that allows us to differentiate regressing or persisting lesions from those that will progress. Thus, the lack of a progression marker results in a significant number of overtreated women, whose lesions would have spontaneously cured.

In low-grade CIN lesions, HPV genomes persist as episomal molecules, and HPV-encoded mRNAs expressed in these lesions exclusively encompass viral sequences (9, 10). In cervical cancer cells, however, viral genomes are frequently integrated into chromosomes of the host cells (10–14). Integration of HPV genomes in most cases results in the generation of mRNAs comprising the viral oncogenes E6 and E7 as well as cotranscribed cellular sequences (Fig. 1; Refs. 9, 12, 15–17). Moreover, because of cis-regulatory effects on the transcriptional regulation of integrated viral genomes and an increased stability of the transcripts, the integration of viral genomes contributes to deregulated high-level expression of viral oncogenes E6 and E7 (18, 19). Finally, it was clearly demonstrated that the expression of these oncogenes is required to induce and maintain the neoplastic phenotype of cervical cancer cells (20, 21). Taken together, these data suggest that cervical epithelia expressing chromosomally integrated HPV oncogenes have a strong selective growth advantage and are more susceptible for clonal outgrowth as cancer cells. This in turn implies that the detection of HR-HPV oncogene transcripts derived from integrated viral genomes points to preneoplastic lesions with a high risk for malignant progression.

To test this hypothesis, we developed a RT-PCR assay—amplification of papillomavirus oncogene transcripts, or APOT—that allows the discrimination of HPV mRNAs derived from integrated and episomal viral genomes. Using the APOT assay, we defined the prevalence of integrate-derived HR-HPV (HPV16/18) transcripts in cervical swab or biopsy samples that originated from patients with dysplastic cervical lesions of different progression stages.

MATERIALS AND METHODS

Cervical Carcinoma Cell Lines and Clinical Samples. Cell lines derived from HPV16- (SiHa, CaSiK) and HPV18-associated cervical cancers (HeLa, C-4 I, C-4 II, SW756) were cultured under standard conditions in DMEM supplemented with 10% fetal bovine serum. Clinical samples were collected from patients attending the outpatient clinic for cervical dysplasia at the Departments of Obstetrics and Gynecology of the Universities of Jena and Heidelberg (Jena and Heidelberg, Germany) with informed consent. From a total of 549 patients, punch biopsy samples (n = 83), cervical swabs (n = 361), or paired samples (n = 105) were obtained. Punch biopsy samples were divided into two parts; one was kept for histopathological analysis, and the other one was transferred into cryotubes, immediately shock-frozen in liquid nitrogen, and stored until further use at −70°C. From each patient, two independent cervical swab samples were collected using cytobrushes. One of the swab specimens was subjected to routine cytological analysis (Pap smear) and reviewed by an experienced pathologist. The second brush was transferred...
Fig. 1. Structure of HPV16/18 genomes, in either their episomal (left) or their integrated form (right), and transcripts derived thereof. Locations of primers used for RT [(dT)17-p3], for PCR APOT (p1, p2, and p3), and for hybridization analysis (h1, specific for HPV E7; h2, specific for HPV E4) are indicated. Integrate-derived transcripts differ either in lacking (type A) or in containing E4 sequences (type B).

Fig. 2. APOT from cervical carcinoma cell lines. Both HPV16-positive cell lines [SiHa (Lane 1) and CaSkí (Lane 2)] and HPV18-positive cell lines [HeLa, C-4 I, C-4 II, and SW56 (Lanes 3–6)] were subjected to the APOT assay specific for HPV16 (left) or HPV18 (right). One-tenth of the RT-PCR products were separated on 1.2% agarose gels (top), transferred to nylon membranes, and subsequently hybridized with either an E7-specific (middle) or an E4-specific (bottom) oligonucleotide. No amplification products were generated from the HPV18-positive cell lines when applying the HPV16-specific APOT assay nor from the HPV16-positive cell lines when applying the HPV18-specific APOT assay.
Table 1  HPV detection in cervical swab and biopsy samples

<table>
<thead>
<tr>
<th>Histology</th>
<th>HPV status</th>
<th>n</th>
<th>Negative n (%)</th>
<th>Low risk n (%)</th>
<th>High risk n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td>203</td>
<td>148 (72.9)</td>
<td>18 (8.9)</td>
<td>37 (18.2)</td>
</tr>
<tr>
<td>CIN I</td>
<td></td>
<td>90</td>
<td>51 (56.7)</td>
<td>19 (21.1)</td>
<td>20 (22.2)</td>
</tr>
<tr>
<td>CIN II</td>
<td></td>
<td>81</td>
<td>30 (37.0)</td>
<td>7 (8.6)</td>
<td>44 (54.4)</td>
</tr>
<tr>
<td>CIN III</td>
<td></td>
<td>122</td>
<td>23 (18.9)</td>
<td>7 (5.7)</td>
<td>52 (42.4)</td>
</tr>
<tr>
<td>CxCa(^a)</td>
<td></td>
<td>53</td>
<td>2 (3.8)</td>
<td>2 (3.8)</td>
<td>49 (92.4)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>549</td>
<td>254</td>
<td>53</td>
<td>242</td>
</tr>
</tbody>
</table>

\(^a\) HPV types 6b, 11, 13, 41, 42, 44, 53, 54, 58, 59, 61, 62, 66, 69, MM 4, MM 7, MM 9, LVX 160, and CP 8304.

Table 2  HPV16 and 18 oncogene transcripts in cervical swab and biopsy samples

<table>
<thead>
<tr>
<th>Histology</th>
<th>Only episomal n (%)</th>
<th>Integrated n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>19 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>CIN I</td>
<td>10 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>CIN II</td>
<td>22</td>
<td>1 (4.5)</td>
</tr>
<tr>
<td>CIN III</td>
<td>64</td>
<td>10 (15.6)</td>
</tr>
<tr>
<td>CxCa(^b)</td>
<td>40</td>
<td>35 (87.5)</td>
</tr>
<tr>
<td>Total</td>
<td>155</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Episomal and integrated HPV transcripts were coexpressed in 1 CIN II lesion, in 7 of 10 CIN III lesions, and in 9 of 35 carcinoma samples.

\(^b\) CxCa, cervical carcinoma.

**RESULTS**

**Differentiation between Episome- and Integrate-derived HR-HPV Transcripts by APOT Analysis.** The APOT assay is based on the structural differences among the 3′-ends of viral oncogene transcripts. Integration of HR-HPV genomes in cervical carcinoma cells usually results both in disruption of the E1 or E2 open reading frames (25) and in disruption or deletion of the viral early-region polyadenylation signal from the viral oncogene-encoding sequences (Fig. 1). Consequently, transcripts derived from the integrated E6 and E7 oncogenes commonly encompass viral sequences at their 5′-ends and flanking cellular sequences at their 3′-ends. These transcripts usually use the splice donor site downstream from the E1 translational start codon (Fig. 1, transcripts of type A; Refs. 13, 15). In a few cervical carcinoma cell lines, integrated HPV genomes are disrupted within the E4 gene, which results in the generation of viral-cellular fusion transcripts that encompass E6-E7-E1 sequences at their 5′-ends followed by E4 sequences and cellular sequences at their 3′-ends (Fig. 1, transcripts of type B; Refs. 11). In contrast, in lesions retaining only episomal HPV genomes, the most prevalent HPV E6-E7 encompassing transcript is spliced at the E1-splice donor signal to the E4-splice acceptor site and terminated at the viral polyadenylation site (Fig. 1, episome-derived transcript; Ref. 26).

These structural differences of integrated or episomal viral oncogene transcripts are detectable by the APOT assay. Using an oligo(dT)\(_{17}\)-primer coupled to a linker sequence ((dT)\(_{17}\)-p3), the RT of the complete mRNAs is initiated by binding to their poly(A) tail. Subsequently, both episome- as well as integrate-derived HPV oncogene transcripts are amplified by nested PCR reactions using E7-specific forward primers in combination with primers p3 and (dT)\(_{17}\)-p3, respectively (Fig. 1). Integrate-derived transcripts can now be differentiated from the abundant episome-derived transcript (1050 bp) because of their deviating size. Furthermore, the obtained PCR fragments can be verified by Southern blot hybridization analysis using HPV E7- and E4-specific oligonucleotides (Fig. 1, h1 and h2).

**Evaluation of the APOT Assay Using Cervical Carcinoma Cell Lines.** We first examined the applicability of the APOT assay using two HPV16-positive (SiHa, CaSk) and four HPV18-positive cervical carcinoma cell lines (HeLa, C-4 I, C-4 II, and SW756; Fig. 2). From SiHa cells, three amplification products were obtained (Fig. 2, Lane I), which all hybridized to the HPV16 E7-specific oligonucleotide but not to the HPV16 E4-specific oligonucleotide (integrate-derived transcripts of type A, Fig. 1). Two PCR fragments were generated from CaSk cells (Fig. 2, Lane 2). The fragment at 650 bp hybridized both to the HPV16 E7 and E4 oligonucleotides, although its size was different from the expected size of episome-derived transcripts of about 1050 bp (Fig. 1). Thus, it was presumably derived from integrated transcript type B (Fig. 1), which was subsequently confirmed by direct DNA sequence analysis. In contrast, the PCR fragment of 250-bp length hybridized with the E7- but not with the E4-specific probe. Sequence analysis of this fragment revealed that it was generated by misannealing of the (dT)\(_{17}\)-p3 primer during RT to an

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**Table 3  Accordion of APOT results of paired cytobrush and biopsy samples**

<table>
<thead>
<tr>
<th>Cytobrush</th>
<th>Biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Episomal</td>
<td>Episomal</td>
</tr>
<tr>
<td>Integrated</td>
<td>Integrated</td>
</tr>
<tr>
<td>Episomal</td>
<td>Integrated</td>
</tr>
<tr>
<td>Integrated</td>
<td>Episomal</td>
</tr>
<tr>
<td>Total</td>
<td></td>
</tr>
</tbody>
</table>

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**Fig. 3.** APOT from HPV16-positive clinical samples of normal cervical epithelia, preneoplastic lesions (CIN I, II, and III), and primary cervical carcinomas (CxCa). RT-PCR products were separated on 1.2% agarose gels (A), transferred to nylon membranes, and subsequently hybridized with either an E7- (B) or an E4- (C) specific oligonucleotide. All of the amplifiers that are visible in the agarose gel hybridized with the E7-specific oligonucleotide, thus confirming the specificity of the assay. The PCR products of about 1050-bp length hybridized with both the E7- and E4-specific probes and represent the abundant episomal transcript. Amplifiers with a different size and not hybridizing with the E4-specific oligonucleotide display integrate-derived transcripts of type A (Lanes 11–15). Both one CIN III lesion and one carcinoma sample show episme-as well as integrate-type transcripts (Lanes 12 and 13), whereas two cervical carcinomas show more than a single integrate-type transcript (Lanes 13 and 14). The 250-bp APOT product obtained from various samples, including normal cervical epithelia, is derived from misannealing of the (dT)\(_{17}\)-p3 primer to an adenosine-rich sequence stretch within the HPV16 E1 gene during RT.

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Reference:

Statistical Analysis. Differences in the frequency of the occurrence of integrate-derived transcripts among the subgroups of patients with normal histology, increasing grades of the cervical dysplasia, or invasive cervical cancer was determined by the Exact Cochran-Armitage Trend test.
adenosine-rich nucleotide sequence stretch within the HPV16 E1 gene (nucleotides 941–951). Similarly, by using the HPV18-specific APOT assay, a 200-bp fragment was amplified from HeLa cells (Fig. 2, Lanes 3), which results from misannealing of the (dT)_{17}-p3 primer to HPV18 E1 sequences (nucleotides 985–997). All but one of the amplimers of the HPV18-positive cell lines hybridized only with the E7-specific oligonucleotide, representing integrate-type A transcripts. However, a single fragment, with a length of about 1000 bp amplified from SW756 cells, encompasses both E7 and E4 sequences as demonstrated by both hybridization (Fig. 2, Lanes 6) and sequence analysis (data not shown). The structure of this transcript corresponds to the episome-derived transcript (Fig. 1) and is presumably derived from integrated head-to-tail tandem repeats of the HPV 18 genome in SW 756 cells (27).

C-4 I and C-4 II cells were established from a cervical carcinoma biopsy sample as two independent lines (28). APOT amplimers generated from these two cell lines showed exactly the same pattern (Fig. 2, Lanes 4 and 5), thus, underlining their common clonal origin. The APOT assay, therefore, reveals a molecular fingerprint for clonal HPV-transformed cells.

Prevalence of Integrate-derived Transcripts in Dysplastic and Neoplastic Lesions of the Uterine Cervix. To determine the prevalence of integrate-derived HPV transcripts in clinical specimens from cervical lesions, we collected a total of 655 cervical swab and/or biopsy samples from 549 patients attending outpatient clinics for cervical dysplasia. All of the specimens were first analyzed for the presence of HPV infection (22). Overall, HPV DNA was found in 295 patients (53.7%), and HPV detection rates ranged from 27.1% in women with either normal Pap smears or normal histological diagnosis to 96.2% in women with invasive cervical carcinomas (Table 1). HR-HPV infections were detected in 242 samples (44.1%), of which 155 were positive for HPV16 or -18 (Tables 1 and 2). These latter samples (59 punch biopsies, 44 swabs specimens, and 42 paired samples) were subsequently subjected to the APOT assay. Representative results of the HPV16-specific APOT assay for clinical samples displaying different progression stages of cervical neoplasia are shown in Fig. 3.

In total, 29 of the 155 HPV type 16- or 18-positive samples were obtained from lesions either histologically or cytologically classified as normal epithelium or CIN I (Table 2). APOT analyses of these samples revealed exclusively episomal transcripts, whereas only 1 of 22 CIN II lesions displayed an integrate-derived transcript. Sixty-four specimens were derived from CIN III lesions, 10 (15.6%) of them showing expression of integrate-derived transcripts. The prevalence of integrate-derived transcripts is further increased significantly in invasive cervical carcinoma samples (35 of 40; 87.5%). In addition to the integrate-derived HPV transcripts, episome-derived transcripts were coexpressed in 1 of 1 CIN II lesion, in 7 of 10 CIN III lesions but in only 9 of 35 carcinoma samples. Statistical analysis of the data by the Exact Cochran-Armitage Trend test revealed a significant correlation between the detection of integrate-derived transcripts and the increasing grade of the cervical dysplasia or neoplasia \( P < 0.0001 \). These analyses suggest that the neoplastic progression of cervical lesions is associated with the selection of cell clones that preferentially express integrate-derived transcripts. The detection of integrate-type transcripts, thus, clearly points to advanced dysplasia or invasive carcinoma.

From 42 patients with HPV16 or -18 infection, paired samples of cytobrushes and biopsies were available. In all but one patient, APOT analyses showed identical results (Table 3). Thus, the validity of the APOT assay performed on noninvasively obtained samples (swabs) is high when compared with test results of biopsy material. Among these 42 patients, 1 patient had a negative Pap test, although APOT analysis from a cervical swab of this patient displayed integrate-derived transcripts. Subsequently taken biopsy samples from this patient histologically revealed an invasive cervical carcinoma and confirmed the integrate-derived APOT pattern as found for the swab sample.

Sequence Analysis of APOT-derived Viral-Cellular Fusion Transcripts. Until now, 37 integrate transcripts were further examined by cloning and sequencing. These analyses revealed that 34 (91.9%) of these fusion transcripts were spliced from the HPV16 or -18 E1 splice donor site to cellular sequences (integrate-derived transcripts of type A; Fig. 1 and Fig. 3, Lanes 11–15). Three (8.1%) integrate transcripts contained E7-E1 sequences at their 5’-ends, followed by E4 sequences and cellular sequences at their 3’-ends (integrate-derived transcripts of type B).

DISCUSSION

Integration of HR-HPV DNA into the host cell genome was hypothesized to play an important role in the pathogenesis of cervical cancer (3). Several in vitro experiments revealed that the integration of the viral genomes resulted in elevated expression levels of the E6 and E7 oncoproteins (18, 19), most probably due to increased stability of the integrate-derived viral-cellular fusion transcripts (19, 29). Because the growth rate of HPV-transformed cells is strongly dependent on the expression level of the E6 and E7 oncoproteins (20, 21), integration may confer a significant growth advantage to the affected host cells and, thus, represent an important decisive event in the progression of HR-HPV-induced cervical dysplasia. The detection of integrated HR-HPV genomes, therefore, may represent a suitable molecular marker for the identification of the small proportion of preneoplastic cervical lesions that possess a particularly high potential for progression to invasive carcinoma.

The detection of viral integrates has previously been attempted with various, different techniques and has led to controversial results. Some investigators detected integrated HPV genomes in various preneoplastic lesions (9, 17, 30), whereas others observed HPV integration almost exclusively in invasive carcinoma samples (14, 31). This discrepancy was attributed to the different methodological approaches used for the detection of integrated HPV DNA (31). In particular, different PCR-based protocols have been described, in which the failure to amplify distinct fragments within the E1 or E2 open reading frames of the HPV genome was regarded as evidence for HPV integration (30, 32–34). However, these assays can only detect integrated viral genomes in the absence of episomal HPV genomes. Because in many primary cervical cancers integrated HPV genomes coexist with episomal genomes in the same cells, this strategy obviously results in a drastic underestimation of the prevalence of integrate-positive lesions. Carmody et al. (35) reported a different experimental approach, which is based on the selective PCR amplification of integrated HPV DNA sequences using primers specific for viral sequences and for cellular interspersed ALU repeats. This assay is specific for integrated viral genomes, however, in cells in which the integrates are located in a long distance from the next adjacent ALU repeat, this assay presumably fails because of limitations of the PCR amplification efficiency of large DNA fragments. Here, we established an alternative technique for the detection of integrated HPV genomes based on the structural difference of transcripts encoding the viral oncoproteins E6 and E7. The APOT assay allows us to distinguish between integrate- and episome-derived transcripts encompassing HR-HPV E7 sequences.

Applying the APOT assay to 155 clinical samples infected with HR-HPV types 16 and 18, we found a strong correlation between the detection of integrate-derived transcripts and the progression stage of the cervical dysplasia. The high prevalence of integrate-derived amplimers in cervical cancer specimens in this study confirms previous reports (30–32, 36) and supports the hypothesis of preferred selective outgrowth of those HPV-infected cells in preneoplastic lesions that express integrated viral oncoproteins E6 and E7. Interestingly, 10 (15.6%) of 64 CIN III lesions included in this study displayed inte-
egrate-derived transcripts. This percentage resembles the fraction of CIN III lesions expected to progress to invasive cervical cancer (8). However, because all of the CIN III lesions in our patient cohort were removed by surgical resection (conization), we cannot prove whether the dysplastic lesions expressing integrate-derived transcripts would have progressed to invasive carcinomas, whereas those exclusively displaying episome-derived transcripts may have regressed. Nonetheless, the strong discrepancy between the prevalence of integrate-derived transcripts in high-grade preneoplastic CIN III lesions and invasive carcinomas (15.6% versus 87.5%) suggests that the detection of integrate-derived transcripts represents a suitable molecular marker for preneoplastic lesions with a high risk for progression. Ongoing prospective clinical studies on large patient cohorts will help to define whether preneoplastic cervical lesions with integrated HPV genomes are indeed at a higher risk for progression to cervical cancer.

The strong accordance of APOT test results from punch biopsies and corresponding cervical swabs observed in our study demonstrates that noninvasive sampling will provide adequate material for the sensitive analysis of viral transcript patterns. Furthermore, because the assay is based on RT-PCR protocols, it has a high potential for automation and may, thus, be an effective tool for the routine testing of dysplastic lesions. Due to the nondirected integration of HPV sequences into different chromosomal loci of the host cells,6 the obtained APOT fragment patterns are specific molecular fingerprints for individual cervical cell clones (Figs. 2 and 3). Thus, the identification of specific viral-cellular fusion transscripts by the APOT assay may also provide a valuable diagnostic tool during the follow-up period of patients treated for invasive cervical cancers. The detection of a specific integrate-derived transcript pattern may mark the outgrowth of residual cancer cells and, thus, the relapse of the disease. Similarly, the detection of specific integrate-derived transcripts in surgical resection margins and adjacent lymph nodes indicates the existence of residual cancer cells, which may guide further therapeutic decision-making.

ACKNOWLEDGMENTS

We acknowledge the continuous support of Ch. Herfarth and H. zur Hausen. We are grateful to A. Benner for performing the statistical analyses and to U. Schäfer for skillful technical assistance.

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