Human papillomavirus (HPV) type 16 and 33 E6/E7 region transcripts in tonsillar carcinomas can originate from integrated and episomal HPV DNA


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This study was undertaken to determine whether human papillomavirus (HPV) E6/E7 gene transcription in tonsillar carcinomas is correlated with viral DNA integration. Therefore, tonsillar carcinomas containing HPV-16 (n = 2) and HPV-33 (n = 2) DNA were analysed for the viral physical state and transcription of the E6/E7 region. Southern blot analysis, DNA polymerase chain reaction (PCR) and, eventually, two-dimensional gel electrophoresis revealed indications for the presence of only episomal DNA in the HPV-16-containing biopsies and only integrated DNA in one HPV-33-containing biopsy. The second HPV-33-containing carcinoma, from which one biopsy and two resected tumour specimens were analysed, showed a rather complex physical state profile. The biopsy of this tumour contained only episomal DNA, one resected tumour part contained only integrated DNA and the remaining tumour part contained both integrated and episomal HPV-33 DNA. Independent of the viral physical state, all biopsies and resected tumour parts tested showed the presence of E6/E7 transcripts as determined by RNA PCR. The results indicate that E6/E7 transcripts in tonsillar carcinomas can originate from integrated as well as episomal HPV DNA.

Introduction

A distinct subset of human papillomavirus (HPV) types, predominantly HPV-16 and HPV-18, have been implicated in human anogenital cancer and are considered high-risk HPV types (zur Hausen & Schneider, 1987). Moreover, we have recently found a strong association between HPV-16 and HPV-33 genotypes, predominantly, and tonsillar carcinomas (Snijders et al., 1992a).

Based on accumulating experimental data the E6 and E7 open reading frames (ORFs) are supposed to be crucial for transforming properties of high-risk HPVs (Barbosa & Schlegel, 1989; Hawley-Nelson et al., 1989; Münger et al., 1989). Of these ORFs, the E7 ORF encodes a major transforming gene (Storey et al., 1988; Phelps et al., 1988; Halbert et al., 1991). Interference with E6/E7 expression has been shown to affect the tumorigenicity of cervical carcinoma cells and transformation of rodent cells (von Knebel Doeberitz et al., 1988; Crook et al., 1989). Consequently, it has been suggested that HPV E6/E7 functions are essential both for initiation and maintenance of the malignant phenotype (zur Hausen, 1991). Indeed, the E6/E7 region has been found to be consistently transcribed in cervical carcinomas and cell lines derived from cervical carcinomas (Baker et al., 1987; Schwarz et al., 1985; Smotkin & Wettstein, 1986; van den Brule et al., 1991), and also tonsillar carcinomas previously tested have been shown to contain E6/E7 region mRNA (Snijders et al., 1992a).

In the proposed model of anogenital carcinogenesis by HPV, it has been suggested that viral DNA integration plays an essential role, most likely by triggering deregulated expression of E6/E7 genes (zur Hausen, 1991). Integration of viral DNA has often been observed in HPV-16- and HPV-18-containing cervical carcinomas (Dürst et al., 1985; Cullen et al., 1991) and cell lines derived from cervical carcinomas (Schwarz et al., 1985; Awady et al., 1987; Yee et al., 1985; Shirasawa et al., 1987). In contrast, premalignant and benign cervical lesions predominantly contain episomal HPV DNA (Dürst et al., 1985; Lehn et al., 1988; Cullen et al., 1991). HPV DNA integration regularly occurs within the viral E1/E2 region (Schwarz et al., 1985; Baker et al., 1987; Choo et al., 1987; Shirasawa et al., 1989), thereby disrupting E2 expression. Since the HPV E2 ORF encodes transcriptional modulator proteins (Phelps & Howley, 1987; Cripe et al., 1987; Bernard et al., 1989; Romanczuk et al., 1990), it has been suggested that integration-mediated disruption of E2 expression would trigger uncontrolled expression of the transforming

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genes E6 and E7. Another event often resulting from HPV-16 or HPV-18 DNA integration is E6/E7 expression via virus–cell fusion transcripts (Inagaki et al., 1988; Schneider-Gädicke & Schwarz, 1986; Rohlfs et al., 1991; Smits et al., 1991).

This study was undertaken to determine whether transcription of E6/E7 sequences in tonsillar carcinomas is also correlated with HPV DNA integration. The results indicate that E6/E7 region transcripts in HPV-containing tonsillar carcinomas can originate both from integrated and episomal viral DNA templates.

**Methods**

**Clinical specimens and viral DNA clones.** Biopsy specimens of four tonsillar carcinomas containing HPV-16 (n = 2) or HPV-33 (n = 2) were snap-frozen and stored in liquid nitrogen until use. From one carcinoma the biopsy was taken 1 month prior to surgical treatment and two matched specimens taken from separated parts of the resected tumour were snap-frozen and also available for HPV analysis.

DNA of HPV-16 cloned into the BamHI site of pBR322 and of HPV-33 cloned into the BglII site of pBR322 was purified from vector sequences by agarose gel electrophoresis and subsequently used as probe.

**Isolation of DNA and cytoplasmic RNA.** Snap-frozen samples were serially sectioned (10 to 15 sections) on a cryostat. The first and last sections (5 μm) were used for haematoxylin and eosin staining to assess the percentage of carcinoma cells present in these samples. The intermediate sections were used for DNA and eventually RNA isolation. DNA was extracted as described by Walboomers et al. (1988). Cytoplasmic RNA was isolated according to Gough (1988). Briefly tissue sections were suspended in 200 μl cold lysis buffer containing 10 mM-Tris–HCl pH 7-5, 0.15 M-NaCl, 1-5 mM-MgCl2, 0-65%NP40 and vortexed vigorously. Nuclei and remaining cell debris were removed by centrifugation at 800 g for 5 min. The supernatant (cytoplasmic lysate) was transferred to a new tube containing 200 μl 7 M-urea, 1%SDS, 0.35 M-NaCl, 10 mM-EDTA, 10 mM-Tris–HCl pH 7.5, extracted with phenol–chloroform and precipitated with 96% ethanol. The integrity of RNA samples was determined by agarose gel electrophoresis.

**DNA polymerase chain reaction (PCR).** PCR was performed with combinations of HPV-16 or HPV-33-specific primers located within different parts of the HPV genome. Combinations of the following primers, for which sequences are numbered according to the published different parts of the HPV genome. Combinations of the following combinations of HPV-16- or HPV-33-specific primers located within the HPV-16 or HPV-33 DNA as probes.

**RNA PCR.** This was performed as described previously (van den Brule et al., 1991). One strand of cDNA was synthesized in a final reaction volume of 50 μl containing 200 ng cytoplasmic RNA, 25 pmol antisense primer, 50 pmol–Tris–HCl pH 8.3, 60 mM-KCl, 3 mM-MgCl2, 1 mM-DTT, 1 mM each dNTP, 40 units RNasin (Promega) and 7 units avian myeloblastosis virus reverse transcriptase (RT; Promega). The mixture was incubated at 42°C for 45 min and subsequently at 95°C for 5 min to inactivate the RT. In addition reactions were performed without RT to allow distinction between final RNA PCR products and products derived from eventually copurified DNA. Five μl of the cDNA mixture was used for PCR in a volume of 50 μl containing a final concentration of 50 mM-KCl, 10 mM-Tris–HCl pH 8.3, 200 μM each dNTP, 1.5 mM-MgCl2, 50 pmol of both sense and antisense primer and 1 unit thermostable DNA polymerase (AmpliTaq; Cetus). Forty cycles of amplification were performed as previously described for type-specific PCR (van den Brule et al., 1990). HPV-specific primers were used as shown above.

To determine 3′ cDNA ends the method described by Frohman et al. (1988) was applied using a 35 base (dT)7-adaptor primer (′-GACTCGAGGATCCTGCAGCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT′) for first-strand cDNA synthesis. In the subsequent PCR with single-sided specificity this primer was replaced by a 19 base adaptor primer lacking the (dT) stretch and combined with an HPV-specific primer. PCR products were analysed on 1.5% agarose gels. Southern blot analysis was carried out as described above using cloned HPV-16 or HPV-33 DNA as probes.

**Results**

**Comparative analysis of HPV-33 physical state and E6/E7 transcription in a biopsy specimen and matched resected tumour specimens from the same tonsillar carcinoma**

From one HPV-33-containing tonsillar carcinoma a biopsy specimen (designated T6) and two resected tumour specimens (T6.1 and T6.2) were available for combined HPV DNA and RNA analysis. Histological examination revealed that all three specimens were morphologically identical, consistent with poorly differentiated squamous cell carcinoma. Furthermore all these samples consisted of approximately 50% carcinoma cells. Results of HPV DNA and RNA studies are summarized in Table 1. Standard Southern blot analysis of biopsy sample T6 using the single-cut enzyme BglII
Table 1. DNA and RNA analysis on tonsillar carcinoma specimens

<table>
<thead>
<tr>
<th>Specimen</th>
<th>HPV type</th>
<th>Southern blot*</th>
<th>2D analysis</th>
<th>DNA PCR†</th>
<th>RNA PCR‡</th>
<th>Physical state</th>
</tr>
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<tr>
<td>T6§</td>
<td>33</td>
<td>Prototype</td>
<td>Episomal</td>
<td>Positive</td>
<td>E6*I</td>
<td>Episomal</td>
</tr>
<tr>
<td>T6.1§</td>
<td>33</td>
<td>Prototype</td>
<td>Episomal and integrated</td>
<td>Positive</td>
<td>E6*I</td>
<td>Episomal</td>
</tr>
<tr>
<td>T6.2§</td>
<td>33</td>
<td>Aberrant</td>
<td>ND</td>
<td>Disruption E2 ORF</td>
<td>E6*I</td>
<td>Integrated¶</td>
</tr>
<tr>
<td>T4</td>
<td>33</td>
<td>Aberrant</td>
<td>ND</td>
<td>Disruption E1 ORF</td>
<td>Positive</td>
<td>Episomal</td>
</tr>
<tr>
<td>T2</td>
<td>16</td>
<td>Prototype</td>
<td>Episomal</td>
<td>Positive</td>
<td>E6*I and unspliced</td>
<td>Episomal</td>
</tr>
<tr>
<td>T7</td>
<td>16</td>
<td>Prototype</td>
<td>Episomal</td>
<td>Positive</td>
<td>ND</td>
<td>Episomal</td>
</tr>
</tbody>
</table>

* Prototype restriction fragments are according to the published sequence data for HPV-16 (Seedorf et al., 1985) and HPV-33 (Cole & Streeck, 1986). † Positivity indicates successful reaction with all primer combinations used. ‡ Concerns RNA PCR detection of transcripts with E7-coding potential. § Samples T6, T6.1 and T6.2 were obtained from the same carcinoma. ¶ Not done.

and the multi-cut enzyme *Pst*I yielded the prototype HPV-33 fragments, an 8 kb *Bgl*II fragment and *Pst*I fragments of 4-6, 2-0 and 1-2 kb (Fig 1a). Two-dimensional analysis with *Bam*HI, a no-cut enzyme for HPV-33, revealed the presence of circular HPV DNA in addition to linear fragments of approximately 8 and 16 kb in size (Fig. 2a). The linear fragments also appeared after 2D analysis of undigested DNA (not shown) indicating that they represent linearized monomeric and dimeric episomal DNA rather than integrated HPV DNA. In contrast to the biopsy, resected tumour specimen T6.1 yielded an off-sized 15 kb *Bgl*II fragment and an off-sized 6 kb *Pst*I fragment in addition to the prototype HPV-33 fragments (Fig. 1b). Two-dimensional analysis of sample T6.1 showed the presence of linear HPV-33 DNA of 22 to 23 kb in addition to the spots of circular DNA (Fig. 2b). This finding suggests the presence of both integrated and episomal HPV-33 DNA in this sample, the integrated DNA most likely giving rise to the off-sized restriction fragments. Apart from these, the specimens T6 and T6.1 also showed differences in HPV-33 copy number: sample T6 contained approximately 20 copies per carcinoma cell, and sample T6.1 contained 0-5 and 1 genome equivalent per carcinoma cell of integrated and episomal HPV-33 DNA, respectively. The second resected tumour specimen T6.2 showed a restriction enzyme pattern consistent with the presence of integrated HPV-33 DNA exclusively and the estimated copy number was 1 genome equivalent per carcinoma cell (Fig. 1c). Analysis with *Bgl*II revealed the off-sized 15 kb fragment only, whereas the prototype 8 kb fragment was missing. Moreover, the prototype 4-6 kb *Pst*I fragment was replaced by the off-sized 6 kb fragment. The missing 4-6 kb *Pst*I fragment contains sequences from the upper regulatory region to the E2/E4 ORFs. Although the lack of sufficient DNA did not allow ultimate 2D analysis of this sample, these findings, together with *Bam*HI analysis which revealed a single 22 kb fragment (Fig. 1c), strongly support the assumption that this specimen exclusively contained integrated HPV-33 DNA. This
was substantiated by DNA PCR analysis with different primer pairs encompassing the whole genomic region (Table 1). Although samples T6 and T6.1 reacted with all primer combinations employed, sample T6.2 failed to react with primers spanning the E2/E4 region and a disruption was mapped between nt 2870 and 3589.

To study E6/E7 region transcription, RNA PCR was applied to these samples. The location of PCR primers utilized in relation to the HPV-33 early region genome structure is depicted in Fig. 3. Primer combination E6/E7, spanning a region of 513 bp at the DNA level, revealed an amplified 236 bp cDNA fragment for all specimens from this carcinoma (Fig. 4a). This cDNA fragment represents putative HPV-33 E7 mRNAs containing an intron within the E6 ORF (E6*I mRNAs), of which two species previously have been mapped (Fig. 3, species a and b; Snijders et al., 1992b). From biopsy sample T6 the full-length 513 bp fragment was also amplified (Fig. 4a, lane T6). However this fragment also was generated in a separate reaction without RT added (not shown) suggesting that it represents copurified DNA rather than unspliced E6/E7 mRNA. With the E6-E2/E4 primer pair, only samples T6 and T6.1 revealed amplified cDNA fragments of 714, 438 and 328 bp (Fig. 4b), representing cDNA species a (E6*I), c (E6*II) and d (E6*III), respectively (Fig. 3). The nature of the E7 mRNA from sample T6.2 was determined by 3' cDNA end mapping via RNA PCR with single-sided specificity. For this sample the E6-oligo(dT) primer combination revealed a single hybridizing cDNA fragment, approximately 500 bp in size, whereas sample T6.1 in addition revealed fragments of higher Mr (Fig. 4c). The 500 bp fragment represents cDNA species b (Fig. 3) that utilizes a second early region poly(A) signal at nt 862 and has a coding potential limited to E6*I and E7 proteins (Snijders et al., 1992b).

**HPV physical state and E6/E7 transcription in biopsies from different tonsillar carcinomas**

Biopsies obtained from three additional tonsillar carcinomas also were examined for E6/E7 transcription and viral physical state. This concerned one HPV-33-containing sample (sample T4) and two HPV-16-containing cases (samples T2 and T7). As shown in Table 1, RNA PCR of the HPV-33 and HPV-16 E6–E7 primer pairs revealed the presence of HPV-33 E6*I mRNA and of HPV-16 E6*I (Smotkin et al., 1989) and unspliced E6/E7 mRNA in samples T4 and T2, respectively, while degradation of RNA within sample T7 did not allow HPV RNA analysis. By standard Southern blot analysis sample T4 revealed an off-sized 11 kb BglII fragment. DNA PCR analysis of this sample with different HPV-33-specific primer combinations revealed a disruption within the E1 ORF which maps between nt 1126 and
Fig. 4. RNA PCR on samples T6, T6.1 and T6.2 from the same HPV-33-containing tonsillar carcinoma. (a) PCR with E6-E7 primer combination. Levels of full-length 513 bp fragment and spliced E6*I 236 bp fragment are indicated by arrowheads. (b) PCR with HPV-33 E6-E2/E4 primer combinations. Agarose gel pattern of PCR products and hybridization analysis with a HPV-33 probe are indicated on the left and right, respectively. Levels of hybridizing cDNA fragments corresponding with cDNA species a (714 bp), c (438 bp) and d (328 bp) (see Fig. 3) are indicated by arrowheads. (c) Agarose gel pattern (left) and hybridization analysis (right) of RNA PCR products generated by the E6-oligo(dT) adaptor–primer combination. Arrowheads point to the level of the 500 bp cDNA fragment representing cDNA species b (see Fig. 3). M, pBR322 DNA digested with Hinfl.

Discussion

A strong association between certain HPV genotypes and tonsillar carcinomas has been found recently (Snijders et al., 1992a). HPV-16- and HPV-33-containing biopsies of tonsillar carcinomas have been found to express putative E7 mRNAs that contain an intron within the E6 ORF. In addition, RNA in situ hybridization has demonstrated the presence of E7 transcripts exclusively localized within the carcinoma cells, suggesting an aetiologic role for HPV in tonsillar carcinoma. This study was undertaken to find out whether these E6/E7 mRNAs are expressed from integrated or episomal HPV DNA. The viral physical state was studied by genomic Southern blot analysis, DNA PCR and, if sufficient DNA was available, by additional 2D analysis.

The HPV-33-containing carcinoma from which a biopsy and two resected tumour parts were obtained showed a rather complex physical state profile reflected by subpopulations of tumour cells containing episomal DNA and different subpopulations containing integrated HPV-33 DNA. Biopsy specimen T6 from this tumour showed a profile consistent with the presence of episomal DNA. No evidence for the presence of clonally integrated HPV-33 was obtained. Resected tumour specimen T6.1 contained episomal as well as integrated DNA, resected tumour specimen T6.2 contained integrated HPV-33 DNA exclusively. The off-sized restriction fragments of specimens T6.1 and T6.2 were equal in size suggesting that they represent the same integrated viral DNA status. PCR data from sample T6.2 suggest that integration resulted in the interruption of the E2/E4 region. The reason for the heterogeneity observed in this carcinoma is still unknown. One explanation could be that viral DNA integration, coupled with the loss of episomes, had taken place at a late stage of tumour development in a single cell. This event may have provided this cell with greater proliferation potential than its neighbours leading to a tumour with subpopulations containing only integrated HPV-33 DNA. Alternatively, viral DNA integration might have been an early event followed by the loss of integrated DNA in some tumour cells, although this possibility seems unlikely since in biopsy specimen T6 there was absolutely no sign of viral integration.

Independent of the viral physical state, expression of E7-encoding HPV-33 E6*I mRNA was evident for all 5 μg DNA of the SiHa cell line (which contains one copy of integrated HPV-16 DNA per cell) mixed with 5 μg human placental DNA and cloned pH7V-16 DNA at a copy number of 50 genome equivalents per cell, allowed the detection of the integrated HPV-16 DNA (data not shown).
tumour cell populations represented by the three different specimens obtained from this carcinoma. No evidence for the presence of unspliced HPV-33 E6/E7 mRNA with E6-coding potential could be obtained in this carcinoma. This indicates that HPV-33 E6 mRNA, if present, exists at very low levels. Since HPV-33 integration within this carcinoma probably resulted in the disconnection of upstream E6/E7 sequences from the putative early region poly(A) signal which maps to nt 4176, it was suggested that the integration event would affect the transcriptional pattern. Indeed, further comparison of early region transcripts between samples T6.1 and T6.2 revealed that mRNAs encompassing most of the early region (Fig. 3, species a, c and d) were not generated from integrated HPV-33 DNA. In fact, the shorter E6*I species b, which utilizes a poly(A) signal at the 3' end of the E7 ORF, was the only mRNA originating from integrated HPV-33 DNA that could be detected. This finding is consistent with data obtained from cervical carcinomas and cell lines containing HPV-16 and HPV-18 DNA, showing that transcription of E6/E7 region sequences is preferentially selected or retained after viral DNA integration (Schneider-Gädicke et al., 1986; Inagaki et al., 1988; Shirasawa et al., 1988; Broker et al., 1989). No evidence could be obtained for the involvement of a cellular poly(A) signal in the transcription of E6/E7 region sequences. It is likely that integrated HPV-33 DNA can generate E7 transcripts independent of cellular poly(A) signals due to the presence of a second early region poly(A) motif. In contrast, HPV types 16 and 18 do not contain an equivalent second early region poly(A) signal and in a similar integrated state these types would require a cellular poly(A) signal to provide their E7 expression via polyadenylated mRNA.

It should be noted, however, that this heterogeneous distribution of integrated and episomal HPV DNA could reflect an uncommon rather than a general event. Still this finding suggests that tonsillar carcinoma cells can acquire the E6/E7 transcripts both from integrated and episomal DNA. To test this assumption, biopsies of three additional tonsillar carcinomas were analysed. Of these samples, only one HPV-33-containing biopsy (T4) showed an aberrant Southern blot pattern. In conjunction with the abnormal DNA PCR profile this is suggestive of the presence of integrated HPV DNA in this sample. However although a disruption was mapped within the E1 ORF, it still can not be excluded that this sample contained altered episomal HPV-33 forms rather than integrated HPV-33 DNA. Analysis of sample T4 by DNA PCR with single-sided specificity (Riley et al., 1990) for the detection of putative virus–cell junctions is presently in progress to find out whether the HPV DNA is integrated.

The two HPV-16-containing tonsillar carcinoma biopsies revealed hybridization and PCR results consistent with the presence of only episomal HPV-16 DNA in a clonal arrangement. In the model system of SiHa and human placental DNA half a copy per cell of integrated HPV-16 DNA could be demonstrated in the presence of excess (50 copies per cell) HPV-16 DNA; therefore a clonal distribution of single copy integrated HPV-16 DNA would have been detected in these biopsies.

The results obtained from the physical state analyses suggest that viral DNA integration is not a prerequisite for malignant growth and that viral integration may occur as a late event in the development of tonsillar carcinoma. However, an answer to the question of whether integration of HPV DNA is generally an early or a late event in the development of tonsillar carcinoma will require the analysis of more tissue samples, preferably from both malignant and premalignant lesions.

Furthermore, this study implies that transcription of HPV-16 and HPV-33 E6/E7 mRNA in tonsillar carcinomas is not necessarily dependent on viral DNA integration. Consequently, the maintenance of the malignant phenotype of tonsillar carcinomas does not appear to require integrated HPV DNA.

Recently, similar data have emerged with respect to HPV-16-containing cervical carcinomas, a proportion of which have been found to contain episomal HPV DNA only (Fuchs et al., 1989; Matsukura et al., 1989; Cullen et al., 1991). Furthermore, Nasseri et al. (1991) have found that the presence of HPV-16 E2-encoding transcripts does not affect the expression levels of the E7 protein in immortalized cervical keratinocytes suggesting that E2 interruption triggered by integration is not causally related to increased E7 expression. HPV DNA integration could nevertheless be important in carcinogenesis. However, it is likely also that viral intragenomic modifications or changes in host cell gene expression affecting viral E6/E7 expression may take place as alternative events to gain an equivalent effect from viral episomes.

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