Analysis of p53 status in tonsillar carcinomas associated with human papillomavirus

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Tonsillar squamous cell carcinomas (a total of 14) were examined both for the presence of human papillomavirus (HPV) DNA and for p53 alterations. General primer-mediated HPV polymerase chain reaction (GP-PCR) revealed the presence of HPV DNA in 12/14 cases. Subsequent typing by HPV type-specific PCR and sequence or hybridization analysis of GP-PCR products revealed DNA from HPV 16 in seven cases, from HPV 33 in two cases, and from HPV 7, HPV 16/33 and HPV 33/59 each in a single case. p53 immunohistochemistry performed on nine HPV containing tonsillar carcinomas using polyclonal serum CM-1 showed elevated p53 levels in four cases. These included 3/5 HPV 16 containing carcinomas and the HPV 33/59 containing carcinoma. Analysis of p53 mutations using denaturing gradient gel electrophoresis (DGGE) of GC-clamped PCR products of exons 5 to 8 showed p53 gene alterations in 3/13 cases, including 2/11 HPV positive cases and 1/2 HPV negative cases. The alterations included a silent point mutation within exon 8 of an HPV 16 containing carcinoma, a 1 bp deletion within exon 8 of an HPV 33 containing carcinoma, and a missense mutation within exon 7 of one of the HPV negative carcinomas. There was evident discrepancy between p53 immunohistochemistry and gene analysis. Four HPV containing cases showing elevated p53 levels did not reveal the presence of exon 5 to 8 alterations affecting the amino acid code, suggesting the presence of mutations occurring in other exons or non-mutational p53 stabilization. The data indicate that HPV and elevated p53 can coexist in tonsillar carcinomas and that despite the low frequency of p53 mutations the presence of HPV is not exclusively related to the absence of mutated p53.

To date, it is widely accepted that certain high risk human papillomavirus (HPV) types play an aetiopathological role in squamous cell carcinoma of the uterine cervix (zur Hausen, 1991). In contrast, a postulated involvement of HPV in the development of squamous cell carcinomas originating from the mucosa at extragenital sites still remains at a speculative stage. This is due to the low associations demonstrated for the majority of these tumours thus far. Recently, however, a high prevalence rate of mainly HPV types 16 and 33 has been demonstrated in tonsillar carcinomas (Snijders et al., 1992a). Together with the finding that the E6/E7 region, encoding the viral oncoproteins, is consistently expressed into mRNA and that E6/E7 region transcripts are uniformly present exclusively in the neoplastic cells, this points to a possible viral aetiology for these carcinomas (Snijders et al., 1992a, b, c). In contrast, HPV can only be detected in a minority of carcinomas of the oral cavity, larynx and lung (for review see Snijders et al., 1994). An attractive hypothesis is that still unidentified HPV types, undetectable by the regular HPV detection methods, are preferentially associated with squamous cell carcinomas of these sites. However, in order to justify the search for novel HPV types, a possible HPV involvement should fit into the model of multistep carcinogenesis at these sites.

One of the most intriguing findings concerning the mechanism of HPV induced carcinogenesis is the ability of high risk HPVs to inactivate the cellular tumour suppressor gene products p53 and pRB by means of interactions with the viral oncoproteins E6 and E7, respectively (Dyson et al., 1989; Münger et al., 1989; Werness et al., 1990; Scheffner et al., 1990). Alterations of the p53 gene are the most common genetic change in a variety of human cancers (Caron de Fromentel & Soussi, 1992); in particular, the relationship between HPV and p53 has been extensively analysed during the last few years. The wild-type p53 gene encodes a nuclear phosphoprotein having a very short half-life; consequently this protein does not accumulate in levels high enough to be detected immunohistochemically. In contrast, most mutated p53 proteins exhibit an enhanced
### Table 1. Analysis of HPV status and p53 status in tonsillar squamous cell carcinomas

<table>
<thead>
<tr>
<th>Sample*</th>
<th>HPV type</th>
<th>p53 exon altered†</th>
<th>Mutation</th>
<th>Amino acid expression</th>
<th>Elevated p53 expression</th>
<th>Elevated p53 expression and/or p53 gene alteration</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCC T3</td>
<td>HPV 7</td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SCC T1</td>
<td>HPV 16</td>
<td>-</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SCC T2</td>
<td>HPV 16</td>
<td>-</td>
<td></td>
<td></td>
<td>ND§</td>
<td>-</td>
</tr>
<tr>
<td>SCC T5</td>
<td>HPV 16</td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SCC T7</td>
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<td></td>
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<td>-</td>
</tr>
<tr>
<td>SCC P31</td>
<td>HPV 16</td>
<td>Exon 8</td>
<td>Codon 294: GAG to GAA</td>
<td>Silent</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SCC 2888</td>
<td>HPV 16</td>
<td>-</td>
<td></td>
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<td>+</td>
</tr>
<tr>
<td>SCC P44</td>
<td>HPV 16</td>
<td>-</td>
<td></td>
<td></td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>SCC T6</td>
<td>HPV 33</td>
<td>-</td>
<td></td>
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<td>-</td>
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<tr>
<td>SCC T9</td>
<td>HPV 33</td>
<td>Exon 8</td>
<td>Codon 271-272: AGGT to AGT</td>
<td>Frame-shift</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SCC T4</td>
<td>HPV 16/33</td>
<td>‡</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SCC T8</td>
<td>HPV 33/59</td>
<td>-</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SCC 379</td>
<td>HPV negative</td>
<td>Exon 7</td>
<td>Codon 234: TAC to TGC</td>
<td>Tyr to Cys</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>SCC 1467</td>
<td>HPV negative</td>
<td>-</td>
<td></td>
<td></td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>12/14 HPV positive</td>
<td>3/13</td>
<td></td>
<td></td>
<td>4/9</td>
<td>6/14</td>
</tr>
</tbody>
</table>

* SCC, squamous cell carcinoma.
† - , None of exons 5 to 8.
‡ Insufficient DNA available for p53 DGGE.
§ ND, Not done.

stability which enables immunohistochemical detection. Therefore, immunohistochemical p53 detection has been considered an appropriate means of detecting p53 mutational alterations. Initial studies on cervical carcinomas and cell lines have revealed an inverse correlation between HPV presence and p53 gene alterations. Mutated p53 appeared to occur preferentially in rare HPV negative carcinomas and hardly at all in HPV containing carcinomas (Crook et al., 1991, 1992; Scheffner et al., 1991). This has strengthened the idea that inactivation of wild-type p53 function is essential for carcinogenesis and that the presence of high risk HPV E6 proteins obviates the requirement for mutational p53 inactivation and vice versa. In contrast, p53 alterations measured either by gene analysis or by immunohistochemistry are rather common in ororespiratory cancers (Iggo et al., 1990; Field et al., 1991; Brachman et al., 1992; Maestro et al., 1992; Miller et al., 1992; Ogden et al., 1992; Brambilla et al., 1993). This might contradict the postulated HPV involvement in the majority of ororespiratory carcinomas and tumours lacking p53 alterations could, more likely, be associated with HPV.

This study was undertaken to examine the relationship between HPV and p53 status in ororespiratory squamous cell carcinoma using tonsillar carcinoma as a model. HPV containing tonsillar carcinomas, some of which were previously typed (Snijders et al., 1992a, b, c), were analysed both for elevated p53 expression by immunohistochemistry and for the presence of p53 gene alterations by denaturing gradient gel electrophoresis (DGGE; Beck et al., 1993) of GC-clamped polymerase chain reaction (PCR) products of p53 exons 5 to 8 (B. Top and others, unpublished data). In particular, these are the exons most commonly affected by mutation (Caron de Fromentel & Soussi, 1992). Using a general primer-mediated polymerase chain reaction assay (GP-PCR) which allows the detection of a broad spectrum of HPV genotypes (Snijders et al., 1990), 12/14 tonsillar squamous cell carcinomas examined showed positivity for HPV DNA. Type-specific PCR for HPV 6, 11, 16, 18, 31 and 33 (van den Brule et al., 1990) revealed that seven samples contained HPV 16 DNA, two samples contained HPV 33 DNA and one sample contained DNA of both HPV 16 and HPV 33. Moreover, sequence comparison of the GP-PCR product of one sample with all HPV sequences present in the EMBL database revealed the presence of HPV 7 DNA. Further sequence comparison of the GP-PCR product of another sample with the sequence of the GP-PCR product obtained from cloned HPV 59 (kindly provided by Dr T. Matsukura, Tokyo, Japan) generated in our laboratory revealed the presence of HPV 59 in addition to HPV 33. Data about the HPV DNA status are included in Table 1. These results revealed a strong association of mainly genital high risk HPV types (predominantly HPV 16 and HPV 33) with tonsillar carcinoma, which is in agreement with previous data (Niedobitek et al., 1990; Snijders et al., 1992a). Remarkably, one carcinoma appeared to contain DNA of HPV 7, a type that has been associated with butcher’s warts and infrequently detected in benign oral lesions (Greenspan et al., 1988; Syrjänen et al., 1989). This isolated finding confirms data of previous studies in which apparently low risk HPV types (e.g. HPV 2, 4, 6 and 11) have been found in ororespiratory
cancers (Byrne et al., 1987; de Villiers et al., 1985; Lindeberg et al., 1989) including tonsillar carcinoma (Bercovich et al., 1991).

p53 immunostaining was performed on nine formalin-fixed, paraffin-embedded tissue sections with rabbit polyclonal antibody CM-1 (1:1000; Novostra Laboratories) using the avidin–biotin–peroxidase complex method essentially as described previously (Cromme et al., 1993). CM-1 recognizes full-length protein of both wild-type and mutated p53 (Midgley et al., 1992). Elevated p53 levels, reflected by dense nuclear staining limited to neoplastic cells, were demonstrated in four of nine tonsillar carcinomas (Table 1). An example of elevated p53 expression is shown in Fig. 1. This frequency is roughly similar to values found for squamous cell carcinomas at other sites within the ororespiratory tract. In general, between 50 to 70% of head and neck carcinomas (Field et al., 1991) including oral (Ogden et al., 1992) and laryngeal carcinomas (Maestro et al., 1992; Anwar et al., 1993), as well as lung squamous cell carcinomas (Brambilla et al., 1993; Marchetti et al., 1993) have shown elevated p53 levels.

For PCR-DGGE, a two-step PCR assay was used to amplify p53 exon sequences and subsequently attach a universal 50 bp GC-clamp to the PCR products according to Top (1992). The first PCR was performed for 20 cycles using exon specific primers, of which the forward primer was provided with a 15 bp GC-rich linker sequence at the 5' end. A 1 μl volume of this reaction mixture was then subjected to a second PCR for 35 cycles in which the forward primer was replaced by the universal GC-clamp. Primer sequences used are indicated in Table 2. GC-clamped PCR products were subjected to electrophoresis on the appropriate denaturing gradient gel (Table 2) for 6 h at 100 V and 60 °C. In addition, PCR products from a sample with wild-type p53 and samples with known p53 mutations were included during the runs: cervical cancer cell line HeLa representing wild-type p53 (Scheffner et al., 1991; Crook et al., 1991), none small cell lung cancer samples representing mutations in exons 5 and 6 (B. Top and

Table 2. p53 primers and DGGE conditions

<table>
<thead>
<tr>
<th>p53 exon</th>
<th>Primers*</th>
<th>Fragment size†</th>
<th>Denaturant range</th>
</tr>
</thead>
</table>
| 5        | Forward 5' (GC)TTCCTCTTCTTCAGTACTC 3'  
Reverse 5' CTGGGCAACCAGGCGGCTGCTG 3' | 282 | 60–80% |
| 6        | Forward 5' (GC)AGACGGAGGGGCTGG 3'  
Reverse 5' GCCACTGACAACCCACCCCTA 3' | 280 | 30–55% |
| 7        | Forward 5' (GC)CCAGGGGCAGCTGGG 3'  
Reverse 5' CAAGGAGGGCTGGGGGCA 3' | 260 | 35–65% |
| 8        | Forward 5' (GC)TTGACTTTACTCCTTGG 3'  
Reverse 5' AATCTGAGGCATAACTGCAC | 290 | 45–65% |
| GC-clamp | 5' CGGCCCGCCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCC 3' | | |

* Primer sequences for exon 5 are from Beck et al. (1993). The other primer combinations were devised by B. Top and others (unpublished). (GC) is 5' CGGCCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCC GCC

† Size of the PCR product after attachment of the GC-clamp.
additional DGGE analysis to confirm the abnormal clamp primer and the appropriate backward primer. A consisting of 20 cycles using a combination of the GC-normally on DGGE were excised from the DGGE gel To determine the mutations, fragments migrating ab- supernatant was subjected to a second round of PCR and eluted overnight at 4 °C. A 5 gl volume of the gene is not clonally present in all carcinoma cells. No alterations affecting exons 5 and 6 were demonstrated, in exon 8 (Table 1, samples T3, T5 and T7). Furthermore, one more samples have to be tested this may reflect a tendency of p53 mutations being less frequent in HPV containing carcinomas of the ororespiratory tract. However, a similar tendency initially described for cervical carcinomas has recently been contradicted by several groups (Choo & Chong, 1993; Fujita et al., 1992; Helland et al., 1993; Kessis et al., 1993) and the data collected suggest that clonal p53 mutations are very rare events in cervical cancer and not strictly correlated with HPV status. Remarkably, a discrepancy was observed between elevated p53 levels and p53 mutational status in the group of tonsillar carcinomas examined. Of the eight samples which were analysed for both p53 expression and p53 gene alteration, three samples did not show elevated p53 expression and mutation within exons 5 to 8 (Table 1, samples T3, T5 and T7). Furthermore, one
sample showed elevated p53 and an exon 8 silent point mutation (Table 1, sample P31), whereas the sample with the 1 bp deletion within exon 8 did not show elevated p53 levels (Table 1, sample T9). Three remaining samples showed elevated p53 expression but no sign of mutation within exons 5 to 8 (Table 1, samples T1, T8, 2888). Such findings are not uncommon and several possible explanations have been raised (Wyndford-Thomas, 1992). For the sample with the exon 8 deletion the failure of immunohistochemical p53 detection might be due to the fact that the truncated protein is either not recognized by the CM-1 antibody or is not sufficiently stabilized to allow immunohistochemical detection. However, it is more difficult to explain overexpression in the absence of mutations. Since all samples analysed consisted of at least 20% neoplastic cells (data not shown) it is unlikely that clonal p53 mutations in exons 5 to 8 were missed. The PCR-DGGE method performed in this study appeared to detect 10% of DNA with mutated p53 in a background of 90% normal DNA (B. Top and others, unpublished data). Although mutations in other exons cannot be excluded, it has been demonstrated that mutations outside the exon 5 to 8 region only occur in a minor percentage of malignancies (Caron de Fromentel & Soussi, 1992). Consequently, an accumulation of wild-type p53 should be considered. This, however, would be striking given the fact that all four samples showing elevated p53 without relevant mutation contained HPV DNA. These include 3/5 HPV 16 positive samples and the HPV 33/59 containing carcinoma (Table 1). In these cases an HPV E6-mediated enhancement of p53 degradation rather than stabilization of wild-type p53 would be expected (Scheffner et al., 1992; Lechner et al., 1992; Hubbert et al., 1992; Medcalf & Milner, 1993). However, for two reasons the present data are not sufficient to draw conclusions about this item. First, it is unknown whether in these carcinomas a full-length E6 protein is expressed. In a recent study performed to map HPV 33 transcripts in a tonsillar carcinoma, only spliced E6* transcripts of the E6/E7 region could be demonstrated whereas no transcript encoding full-length E6 has been detected (Snijders et al., 1992b). Second, it is presently not known whether the E6 proteins of HPV 33 and HPV 59 can bind p53 and target it for degradation.

Of the tonsillar carcinomas with elevated p53 levels only one sample showed focal staining whereas the other samples showed nuclear staining throughout the carcinoma (data not shown). Consequently, the discrepancy between immunohistochemistry and mutation analysis cannot always simply be explained by a low percentage of neoplastic cells containing exon 5 to 8 mutations. Unless the immunohistochemical staining patterns reflect mutations in other exons or a heterogeneous distribution of different exon 5 to 8 mutations, mechanisms of non-mutational p53 stabilization by cellular or viral factors should be considered in these cases. Recently, Demers et al. (1994) have shown higher steady-state levels of wild-type p53 protein in two HPV 16 E7 immortalized-cell lines, suggesting that HPV 16 E7 expression leads to the stabilization of wild-type p53. However, it is still not known whether an E7 mediated p53 stabilization mechanism can play a role in these tonsillar samples. In such a case, elevated non-mutated p53 in the presence of HPV would point either to a lack of functional E6 expression or to interference of the presumed E7 mediated stabilization with the degradation pathway mediated by E6.

In conclusion, this study has demonstrated that in tonsillar squamous cell carcinoma HPV genotypes and elevated p53 can coexist and that despite the low frequency of p53 mutations the presence of HPV is not exclusively related to the absence of mutated p53.

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