Human Papillomavirus Type 16 and TP53 Mutation in Oral Cancer: Matched Analysis of the IARC Multicenter Study

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Abstract

TP53 mutations were analyzed in 35 human papillomavirus (HPV) type 16 DNA-positive cancers of the oral cavity and oropharynx and in 35 HPV DNA-negative cancers matched by subsite, country, sex, age, and tobacco and alcohol consumption. Wild-type TP53 was found more frequently in cancer specimens that contained HPV16 DNA than in those that did not. All 14 HPV16 DNA-positive cancers in HPV16 E6 antibody-positive patients contained wild-type TP53, compared with 50% of corresponding HPV DNA-negative cancers (matched odds ratio, 1.4; 95% confidence interval, 1.4–5.4). TP53 inactivation is a major mechanism of HPV-related carcinogenesis in the oral cavity and oropharynx. The role of HPV in cancers also containing TP53 mutations remains to be clarified.

Introduction

Already known to be a necessary cause of cervical carcinoma, a growing amount of epidemiological and molecular evidence suggests that high-risk human papillomaviruses (HPVs), most notably HPV16, are also causally associated with a subset of oral cancers. Based on evidence from large case series and case-control studies, such a subset appears to be predominantly those cancers arising in the oropharynx (OP), notably in the tonsils (1–4), or alternatively as those having “basaloid” histological features (3). In the large multicenter case-control study of oral cancer coordinated by the IARC, HPV DNA (mainly of HPV16 type) was identified in 3.9% of oral cavity (OC) and 18.3% of OP cancer biopsies (2). Previous studies have reported HPV DNA in up to 55% of OP cancers (reviewed in Refs. 5–7), but the reported prevalence has been shown to vary greatly, depending on the population, type of specimen, and detection method.

It is clear that the same high-risk HPV types, most notably HPV16 and HPV18, are predominantly found in both cervical (8) and oral cancers (2, 3, 7, 9). These HPV types are able to transform both cervical and upper aerodigestive tract epithelia via similar mechanisms (10), notably E6 (early protein 6)-induced inactivation of p53, the protein encoded by the tumor suppressor gene TP53 (11). Hence, HPV E6 expression and TP53 mutation can be seen as alternative mechanisms leading to a phenotype of p53 pathway inactivation.

In cervical carcinomas, where HPV is found in over 90% of cancer specimens (8), TP53 is very rarely mutated (11). In contrast, TP53 mutations are found in approximately half of oral cancers (11), for which tobacco and alcohol are strong etiologic agents, with an attributable fraction in most world areas close to 90% (12). In some parts of Asia, paan chewing is also associated with a considerable fraction of oral cancers (13). These risk factors entail exposure to various carcinogens, some of which have mutagenic activity that results in specific mutation patterns in the TP53 (11).

In the present study, we sought to clarify the biological role of HPV in cancers of the OC and OP by comparing the frequency and type of TP53 mutations in patients with cancers positive for HPV16 DNA and in a sample of HPV-negative cancers, taking into account cancer subsite and major risk factors for OC and OP. Furthermore, hypothesizing that the expression of E6 protein is, in addition to the presence of HPV DNA, an important prerequisite for HPV-dependent inactivation of p53, we went on to compare the prevalence of TP53 mutations according to the presence of HPV E6 serum antibodies in these patients.

Materials and Methods

Case Identification. A case-control study on cancer of the OC and OP was carried out by the IARC between 1996 and 2000 in Italy, Spain, Northern Ireland, Poland, India, Cuba, Canada, Australia, and the Sudan (2). Incident cases of cancer of the OC and OP were contacted at referral centers before any cancer treatment. The lip (excluding external lip), tongue (including the base of the tongue), gum, floor of mouth, palate, and other parts of the mouth were grouped as OC, whereas the OP and tonsils were grouped as OP. When tonsil or OP were reported in combination with other subsites, cancer was classified as OP. Second primaries were not eligible, and only squamous cell carcinomas were included. Frozen cancer biopsies were obtained, and aliquots of plasma were prepared from 10 ml of heparinized blood. Biopsies and plasma were kept frozen at –70°C or –40°C until shipment on dry ice to collaborating laboratories. A standardized risk factor interview was administered (2). Obtention of informed consent, collection and storage of biological samples, and molecular analyses were performed according to protocols approved by local as well as IARC Ethical Review Boards.

HPV Analysis. The procedures used for HPV DNA extraction from cancer biopsies, detection, and typing have been described previously (2). In brief, the general primer-mediated GP5+/GP6+ PCR enzyme immunoassay was used to detect and type 20 different HPV types (14). PCR products were additionally characterized by agarose gel electrophoresis followed by low-stringency Southern blot hybridization. The few cancers containing HPV types other than HPV16 were not included in our study.

The procedure used for HPV16 E6 serum antibody detection has been described previously in full (2. 15). In brief, an ELISA based on glutathione S-transferase capture with bacterially expressed full-length HPV16 E6 fused to glutathione S-transferase as antigens was used (15).

Matching and Statistical Analysis. Biopsy material for TP53 mutational analysis was available for 17 of 25 HPV16 DNA-positive cancers of the OC and for 18 of 28 HPV16 DNA-positive cancers of the OP, 16 of which...
involved the tonsils (2). Of these HPV16 DNA-positive cancers, 32 were positive for HPV16 only, and 3 were positive for both HPV16 and HPV18 (although HPV16 was the predominant type by type-specific PCR in all three cancers). Each HPV16 DNA-positive cancer was individually matched to a HPV16 DNA-negative cancer from the same study (2). Matching criteria included cancer subsite (OC or OP), gender, country, age group (<40 years, 40–49 years, 50–59 years, 60–69 years, and ≥70 years), tobacco consumption [no (N), ≤19 cigarettes/day (L), >19 cigarettes/day (H)], and alcohol consumption [no (N), ≤20 g/day (L), >20 g/day (H)]. For OP cancers, it was not always possible to identify a HPV16 DNA-negative cancer with an identical mutational status and in such circumstances, it was necessary to relax one or more of the matching criteria (Table 1).

For the matched analysis of TP53 mutational status according to presence of HPV16 DNA, odds ratios (ORs) are calculated by using Mantel-Haenszel method using only pairs discordant for TP53 status (16). Exact 95% confidence intervals (CIs) are given throughout. All P values refer to two-sided χ² tests.

**TP53 Mutation Analysis.** Exons 5–9 of TP53 were analyzed for the presence of mutations in DNA extracted from cancer biopsies using denaturing high-performance liquid chromatography, and the mutation was further determined by automated sequencing of an independent PCR product. The primers used for Touchdown PCR were as follows: exon 5–6, 5'-TGTTCACTGTGCCTGACT-3' (forward), 5'-TTAACCCCTCTCCACAGAGA-3' (reverse), length of PCR product = 467 bp; exon 7, 5'-CTTGCCACAGTCTCCCTCCAA-3' (forward), 5'-AGGGGTCAAGCGCGCAAGCAGA-3' (reverse), length of PCR product = 237 bp; and exon 8–9, 5'-TTGGGATAGTGAGACGACGCT-3' (forward), 5'-AGTGTTAGACCTGGAACATTT-3' (reverse), length of PCR product = 445 bp.

After denaturation at 94°C for 2 min, PCR consisted of 20 cycles of 30 s at 94°C, 45 s at 63°C with a decrement of 0.5°C each 3 cycles, and 1 min at 72°C; followed by 30 cycles of 30 s at 94°C, 45 s at 60°C, and 1 min at 72°C; and subsequently followed by a final extension step of 10 min at 72°C. Purified PCR products were sequenced using an ABI-Prism automated sequencer. Two HPV16 DNA-positive OP cancers and one HPV DNA-negative OC cancer with silent TP53 mutations resulting in no amino acid change were classified as having wild-type p53 protein.

**Results.**

Information on the principal matching variables for each of the 35 pairs, as well as the presence, position, and type of TP53 mutations and the presence of E6 serum antibodies, is summarized in Table 1. Only one OC pair was constituted by nonsmoking current chewers. Seventeen pairs involved OC, and 18 pairs involved OP, and 27 were constituted by men. Both individuals smoked heavily in 8 pairs and
drank alcohol in 25 pairs. E6 serum antibodies were detected in 14 (40.0%) HPV16 DNA-positive cancers and in 1 (2.8%) HPV DNA-negative cancer ($\chi^2 = 14.34; P < 0.01$). Among HPV16 DNA-positive cases, never smokers were more likely to be positive for HPV16 E6 antibodies (71.4%) than ever smokers (32.1%; $\chi^2 = 3.60; P = 0.06$). The type of TP53 mutation, when present, did not differ substantially between HPV16 DNA-positive and HPV DNA-negative cancers (data not shown).

The relationship of TP53 mutations with markers of HPV infection is shown in Table 2. HPV16 DNA-positive cancers were more likely to contain wild-type TP53 than corresponding HPV DNA-negative cancers (matched OR, 2.75; 95% CI, 0.8–11.8), but this association was not statistically significant. The matched OR was 2.0 (95% CI, 0.3–22.1) for OC cancers and 3.5 (95% CI, 0.7–34.5) for OP cancers. Adjusting matched ORs, where possible, for residual differences in smoking and alcohol exposure using conditional logistic regression methods did not materially alter these results (data not shown).

When cancers were stratified by the presence of E6 antibodies in the HPV16 DNA-positive cases (Table 2), all 14 cancers positive for both HPV16 DNA and HPV E6 contained wild-type TP53 (matched OR, $\infty$; 95% CI, 1.4–$\infty$). Despite the higher prevalence of E6 antibodies in OP cancers than in OC cancers, the strong relationship between the presence of HPV16 E6 antibodies and TP53 wild-type status was evident for both OC (matched OR, $\infty$; 95% CI, 0.02–$\infty$) and OP cancers (matched OR, $\infty$; 95% CI, 1.2–$\infty$). In contrast, in patients without E6 antibodies, there was no relationship between the presence of HPV16 DNA and TP53 wild-type status (matched OR, 1.0; 95% CI, 0.2–5.4; Table 2). This held true for either OC (matched OR, 1.5; 95% CI, 0.2–18.0) or OP cancers (matched OR, 0.5; 95% CI, 0.01–9.6).

The findings concerning TP53 mutations by the presence of HPV16 DNA and E6 antibodies are shown in Table 3, stratified by smoking. The only pair of OC patients who were nonsmoking current chewers was combined with current smokers. The association between HPV16 DNA and wild-type TP53 status seemed to be stronger among the few individuals who had never smoked or chewed (matched OR, $\infty$; 95% CI, 0.9–$\infty$) than among those who reported such high-risk behavior (matched OR, 1.5; 95% CI, 0.4–7.2).

### Table 2 TP53 mutations and matched odds ratios (ORs) for 35 pairs of human papillomavirus (HPV) type 16 DNA-positive and HPV DNA-negative oral cancers, by subsite and TP53 wild-type status

<table>
<thead>
<tr>
<th>HPV DNA (–)</th>
<th>HPV DNA (+)</th>
<th>HPV DNA (+), E6 antibody (+)</th>
<th>HPV DNA (+), E6 antibody (–)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TP53 wild-type</td>
<td>TP53 mutant</td>
<td>Matched OR (95% CI)</td>
</tr>
<tr>
<td>All cancers</td>
<td>TP53 wild-type</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>TP53 mutant</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>Mouth</td>
<td>TP53 wild-type</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>TP53 mutant</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Oropharynx</td>
<td>TP53 wild-type</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>TP53 mutant</td>
<td>7</td>
<td>2</td>
</tr>
</tbody>
</table>

* CI, confidence interval.

### Table 3 TP53 mutations and matched odds ratios (ORs) for 35 pairs of human papillomavirus (HPV) type 16 DNA-positive and HPV DNA-negative oral cancers, by tobacco exposure and HPV16 E6 serum antibodies

<table>
<thead>
<tr>
<th>HPV DNA (–)</th>
<th>HPV DNA (+)</th>
<th>HPV DNA (+), E6 antibody (+)</th>
<th>HPV DNA (+), E6 antibody (–)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Never smokers/chewers</td>
<td>TP53 wild-type</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>TP53 mutant</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Ever smokers/chewers</td>
<td>TP53 wild-type</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>TP53 mutant</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

Discussion

Our study provides further evidence of an inverse association between the presence of TP53 mutations and HPV16 infection in tissue specimens from cancers of the OC and OP. Its novelty, however, in comparison with previous reports (3, 14, 17–19), lies in the ability to rule out, by means of a careful matched design, the influence of possible confounders on the observed correlation, most notably anatomical subsite and smoking.

Whereas TP53 mutations were found more frequently in oral cancer specimens from smokers than nonsmokers (20), the prevalence of HPV16 DNA was, in a few studies, more frequent among nonsmokers than smokers (2, 3). This is, perhaps, not surprising because at least some high-risk HPV types and chemical carcinogens can inactivate p53 suppressor function and thus can be considered alternative pathways to chromosomal instability, uncontrolled proliferation, and malignant transformation in precursors of OC and OP cancers.

Our present findings, however, suggest that, for the relationship between TP53 mutations and HPV16 infection to be mutually exclusive, it is not sufficient to find HPV DNA in cancer specimens, but some marker of E6 expression must also be identified. In other words, the ability of HPV16 DNA to predict wild-type TP53 (69%) is substantially improved (to 100%) by taking into consideration information on the presence of HPV16 E6 antibodies. Two previous studies (14, 19) have also shown that HPV-positive oral cancers expressing HPV16 E6 almost always contained wild-type TP53. Contrary to the findings of van Houten et al. (14) and Wiest et al. (19), we could not evaluate E6 expression by means of reverse transcription because the quality of cancer biopsies did not allow RNA extraction. One possible explanation is that the presence of HPV DNA in cancer biopsies as detected by PCR cannot distinguish between viral infections causally related to cancer and concomitant, harmless HPV infections (14). The proportion of cancers in which HPV DNA is detected by PCR, therefore, may overestimate the fraction of cancers of the OC and, to a lesser extent, of the OP attributable to the virus. Conversely, E6 antibodies are markers of HPV E6 oncoprotein mRNA expression that is likely to be clonally related to the tumor.
Indeed, high specificity and good sensitivity of E6 antibodies as markers of HPV-associated malignancies have been demonstrated in studies of cervical carcinoma (15, 21). E6 antibodies in our study were usually accompanied by E7 antibodies (2), and only one individual who was negative for E6 antibodies was positive for E7 antibodies.

High-risk HPV types, however, can promote carcinogenesis through mechanisms other than inactivation of p53, such as via the interaction of the viral protein E7 with pRb, the product of the retinoblastoma suppressor gene RB1 (22). Therefore, caution must be exerted in discarding a role of HPV in cancers containing TP53 mutations, even more so because the vast majority of cancers of the OC and OP, including those positive for HPV DNA, arise in smokers or chewers (2, 3). These individuals are chronically exposed to high levels of chemical mutagens, and hence TP53 mutations may act in conjunction with non-p53-mediated mechanisms of HPV carcinogenesis. Some support for this hypothesis comes from the finding that, in the IARC case-control study (2), positivity for HPV16 E6 serum antibodies also increased the risk of OC and OP cancers among smokers or chewers, although to a lesser extent than among individuals who did not report these habits.

In the present study, we have been able to evaluate a series of cancers of the OC and OP from many different countries where a uniquely broad range of information had been accurately documented, including histological type, anatomical subsite, major risk factors, and, most notably, the presence of serum E6 antibodies and HPV DNA. Unfortunately, although we drew our sample from a large study including over 1000 HPV-tested cancer cases, we eventually had to restrict the evaluation of TP53 mutations to 35 pairs of HPV16 DNA-positive and HPV16 DNA-negative cancers because of the relative rarity of HPV DNA detection and the depletion of histological material for other study purposes. It was, in particular, very difficult to evaluate the mutational pattern of individuals who did not smoke nor chew because, in the lack of such exposures, the occurrence of cancer of the OC and, even more so, of the OP is extremely rare in any population (12). As a consequence of limited study power, some moderate or even strong associations did not attain statistical significance. They did point, however, to a consistent cluster of HPV16 DNA, wild-type p53, and lack of exposure to smoking.

Notwithstanding these limitations, the demonstration of a role of HPV in oral carcinogenesis raises the hope that prophylactic and therapeutic vaccines against oncogenic HPV types (23) may be effective not only against the vast majority of cervical cancer but also in a subset of cancers of the OC and OP where the HPV E6 gene is a key player. It remains to be seen whether control of HPV infection can also reduce the far greater burden of cancers of the OC and OP where HPV and smoking coexist and TP53 mutations are often found.

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