High-Risk Human Papillomavirus Affects Prognosis in Patients With Surgically Treated Oropharyngeal Squamous Cell Carcinoma

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ABSTRACT

Purpose
Human papillomavirus (HPV) DNA tumors actively integrating the E6 and E7 oncogenes have a distinct biologic behavior resulting in a more favorable prognosis. To which extent the viral integration by itself, and/or the associated wild-type (wt) TP53 status, and/or a functional p16 contribute to prognosis is unclear.

Patients and Methods
To clarify how the presence of high-risk (HR) -HPV, TP53, and p16\(^{\text{WT}}\) status interact with clinical outcome, we considered a retrospective series of 90 consecutive oropharyngeal cancer patients treated primarily with surgery.

Results
Seventeen (19%) patients showed integrated HPV 16 DNA (HPV positive), wt TP53 in all but two patients, normal p16\(^{\text{WT}}\) in 15 assessable patients, and p16 expression in all 17 patients. Thirty-five patients (39%), two of whom were HPV positive, harbored TP53 mutations. p16\(^{\text{WT}}\) deletion and p16 null immunophenotype occurred in 28 and 58 patients, respectively, and was similarly distributed in both patients with mutated TP53 (48% and 82%, respectively) and in patients with wt TP53 (46% and 77%, respectively). Statistical analysis showed that HPV-positive status significantly affects all investigated end points: overall survival (P = .0018), incidence of tumor relapse (P = .0371), and second tumor (P = .0152), whereas TP53 and p16\(^{\text{WT}}\) status and p16 expression were not prognostic by themselves.

Conclusion
Our molecular and clinical results are in agreement with previous findings but provide additional information into the biologic mechanisms involved in HR-HPV oropharyngeal cancer in comparison to HPV-negative tumors. According to the reduced risk of relapse and second tumors associated with HR-HPV positivity of oropharyngeal cancer, the therapeutic strategy and follow-up procedures should be reviewed.

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INTRODUCTION

Recent studies showed an etiologic role of infection with high-risk human papillomavirus (HR-HPV) in a subset of oropharyngeal squamous cell carcinomas (SCCs) and a distinct biologic behavior of tumors integrating E6 and E7 oncogenes, resulting in a more favorable prognosis. In some studies, HPV-positive patients were more prevalent in younger patients, and they were associated with lower exposure to alcohol or tobacco and advanced disease. These factors are implicated potentially in prognosis, regardless of HPV positivity. However, not all studies confirmed these associations, due to an insufficient sample size to adjust for these and other important prognostic factors (such as sex and treatment), so that some reports failed to detect a survival advantage of HPV-positive patients bearing tumors.

Oropharyngeal cancer is commonly treated with surgery and/or radiotherapy and, in advanced disease, with concomitant chemoradiotherapy. In retrospective studies detecting the favorable prognostic effect of HPV status, the correlation between HPV status and the type of treatment was not specifically addressed.

At the molecular level, in head and neck cancer, the presence of E6 oncoprotein, which mediates p53...
degradation, generally is coupled with wild-type (wt) TP53, although it was suggested that p53 may partly retain its functional role, despite coexpression of viral E6 oncoprotein. Moreover, the presence of E7 oncoprotein, which binds and functionally inactivates retinoblastoma protein, parallels an upregulation of p16 expression suggestive of a functional p16 protein. To which extent the viral retinoblastoma protein, parallels an upregulation of p16 expression, should be investigated. Integration by itself, and/or the specific associated p16INK4a status contribute to prognosis, and whether this is dependent on the delivery, is unclear.

This study investigated the prognostic effect of HR-HPV, TP53, and p16INK4a status and expression in oropharyngeal SCCs, based on a series of 90 consecutive patients treated at our institution with surgery, followed by radiotherapy in patients with high-risk disease.

**PATIENTS AND METHODS**

Between 1990 and 1999, 100 consecutive patients with oropharyngeal SCC were treated surgically at the National Cancer Institute of Milan (Milan, Italy). Ninety patients were fully assessable in terms of availability of pathologic specimen and follow-up information. Fifty-eight patients (64%) received postoperative radiotherapy according to commonly accepted postoperative pathologic risk features. Radiation doses ranged from 45 to 66 Gy. Two HPV-negative patients did not complete irradiation. During a median follow-up of 5.8 years (range, 12 to 129 months), patients were assessed for the occurrence of SCC relapse, second tumor (ST), and eventually death. ST was defined clinically as a tumor occurring more than 2 cm away from and/or more than 3 years after the treatment of the index tumor. Reliable information about smoking and alcohol habits was not available consistently.

Analyses of pathology specimen were performed on formalin-fixed, paraffin-embedded tissue samples, HR-HPV presence was investigated through real-time polymerase chain reaction (PCR), TP53 mutations status through double-gradient–denaturing gradient gel electrophoresis (dg-DGGE) analysis followed by automated sequencing, p16INK4a homozygous deletion through comparative duplex PCR, and by p16 immunohistochemical analysis.

**High-Risk HPV DNA Detection and Assessment of HPV Physical Status**

DNA was extracted from microdissected serial sections of formalin-fixed, paraffin-embedded tissues, as described previously. The samples were analyzed for detection of HR-HPV DNA type 16 and 18 by real-time quantitative PCR using TaqMan-based assay (Applied Biosystems, Foster City, CA), as described previously. The real-time PCR was performed with ABI Prism 5700 Sequence Detection System (Applied Biosystems). Each reaction contained 1X TaqMan Universal Master Mix (Applied Biosystems), 50 ng of DNA, 0.9 μmol/L each of forward and reverse primers, 0.2 μmol/L fluorogenic TaqMan probe, in a total volume of 25 μL. The probe specific to HPV 16, primers, and PCR conditions used to amplify HPV 18, we used the specific probe FAM-5'-CCGGCTTTTGGCTTTTCGGCCTACTATT-3'-TAMRA (6-carboxyfluorescin [FAM]; 6-carboxy-tetramethylrhodamine [TAMRA]). *Homo sapiens* RNaseP, detected by means of a commercially available kit (kit No. N4316831; Applied Biosystems), was used as an endogenous control and thus amplified in parallel with the samples. All of the experiments were performed in triplicate.

DNA extracted from CaSkI and HeLa cell lines was used as positive control for HPV 16 and HPV 18, respectively.

To assess the physical status of HPV, each HPV DNA-positive sample was quantified absolutely for E2 and E6 by means of a real-time PCR TaqMan assay, as described previously. Given that the integration of viral DNA into the host cell usually disrupts the viral E2 open reading frame, whereas the E6 generally remains intact, equivalent copy numbers of E2 and E6 should be detected by real-time PCR when only the episomal form is present. In contrast, an E2/E6 of more or less than 1 indicates the presence of both integrated and episomal forms, and the absence of E2 amplification indicates the presence of only the integrated form. Plasmid HPV 16 DNAs from known commercially available standards (cloning) were analyzed in parallel with the samples. Standard curves were created automatically by plotting the threshold cycle values against the logarithm of the copy numbers of plasmid DNA standards (serially diluted 10-fold from 10⁷ to 10⁷ copies of HPV DNA). The copy number in the unknown samples was evaluated by means of integrated software using regression analysis. All of the experiments were performed in triplicate.

**Detection of TP53 Gene Mutations**

Samples were screened by dg-DGGE analysis, performed as described previously. Briefly, after electrophoresis, gels were stained with ethidium bromide. Samples with mutations were identified by the presence of abnormal migration pattern, compared with a control carrying a wt TP53 (SiHa cell line) and with samples carrying well-known mutations. The samples showing an abnormal dg-DGGE pattern were amplified further and then subjected to automated DNA sequencing (ABIprism 377; Applied Biosystems; Table 1), as described previously.

The detected mutations were confirmed at least twice by independent amplifications and sequence reactions. Moreover, in all samples showing the wt TP53 gene from exons 5 to 8, but showing a p53 immunoreactivity ≥ 50% of the nuclei, molecular analysis was extended to exons 4, 9, and 10.

The primers used to amplify DNA from exons 4 to 10 are listed in Table 2. For exon 4, we performed two internal PCRs on the same external PCR.

**Homozygous Deletions of the p16INK4a Gene**

To investigate homozygous deletion (HD) of p16INK4a exons 1a and 2, a comparative duplex PCR was performed in which each exon was

<table>
<thead>
<tr>
<th>TP53 Exon</th>
<th>Sense</th>
<th>Antisense</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>CAAGGTTGGTGCTGGCAGCTT</td>
<td>AGAGGAAATCAAAAAGGTCCA</td>
<td>GAGGACCCTGCTCTCGACT</td>
<td>AAGGACAGAAGATTACGG</td>
</tr>
<tr>
<td>5</td>
<td>TGTTTCCTGTGCGCTGTC</td>
<td>CAGAGGGAGGCTGGGAGG</td>
<td>TCTTTATCTTCATCTCTCTTC</td>
<td>CAGGCCCTGCGCTTCCAG</td>
</tr>
<tr>
<td>6</td>
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<td>CTTCCATGATTTCTTCTTCTTG</td>
<td>TTAACCCCTTCTCTCAGAAG</td>
</tr>
<tr>
<td>7</td>
<td>CGTCATACAGGTGCCACCAAA</td>
<td>GTTACCAGCAGGAGGAC</td>
<td>AGGGCTACGCTGCCCTTG</td>
<td>TTTGAGGATGAGGAGAAGCT</td>
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<td>8</td>
<td>GTGGGAGGAGTGGAGGAGCTT</td>
<td>TCTGCTGTAGAATGAAACTT</td>
<td>TGTCTACTTCAGCTG</td>
<td>AATGAGAATGAGGAGG</td>
</tr>
<tr>
<td>9</td>
<td>GTGGGAGGAGTGGAGGAGCTT</td>
<td>TCTGCTGTAGAATGAAACTT</td>
<td>AGGAGCAGGAAAGAAGAAG</td>
<td>ACTTATGAAAGGTAGCTG</td>
</tr>
<tr>
<td>10</td>
<td>CAATGTGAATCCATGACCT</td>
<td>GAGATGAAGAATGAACTTAT</td>
<td>TTTGAGGATGAGGAGAAGCT</td>
<td>CTTTGAACGATAGGAGGCA</td>
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</tbody>
</table>

Abbreviation: PCR, polymerase chain reaction.
complemented with a fragment of the human β-globin gene. The primers used for p16INK4a amplification for exon 1α were forward: 5'-GCC CAA CGC ACC GAA TAG T-3'; reverse: 5'-TAC CTG ATT CCA ATT CCC CTG C-3'. The primers used for p16INK4a amplification for exon 2 were forward: 5’-CTT CCT GGA CAC GCT GGT-3’; reverse: 5’-GCA GGT ACC GTG CGA CAT-3’. The occurrence of HD was evaluated on the basis of previously described criteria.23 The K562 cell line, which has an HD at the INK4A locus, was used as a negative control.

### Immunohistochemistry

Immunohistochemistry was performed on a 2-μm slice of formalin-fixed and paraffin-embedded tumoral sections by the peroxidase-streptavidin method (1:300 in phosphate-buffered saline (DAKO, Copenhagen, Denmark) in accordance with the manufacturer’s instructions. All stains were developed in accordance with the manufacturer’s instructions. All stains were developed in accordance with the manufacturer’s instructions.

### Statistical Analysis

Patients and disease characteristics were tabulated by means of frequency tables. Variable comparisons were carried out using the Wilcoxon or χ² tests. The end points of interest were overall survival, tumor relapse, and second primaries. Time to occurrence of any of these events was computed from the date of surgery to the date when the event was recorded, or censored at the latest follow-up date available in event-free patients. To investigate the pattern of occurrence of any of the aforementioned end points over time, descriptive analyses were carried out by estimating Kaplan-Meier overall survival curves and crude cumulative incidence curves of tumor relapse or second primaries,28 whereas inferential analyses relied on cumulative hazards. In particular, unadjusted P values for testing the prognostic effect of HPV/TP53 status were obtained from the log-rank test, and adjusted P values were obtained from the likelihood ratio test in a multivariable Cox regression model. The covariates entered into the latter for the purpose of adjustment (treatment for overall survival; patient age at surgery and tumor stage for tumor relapse) were chosen from a number of predictors as those that maximized the Cox model fit, as estimated by the Akaike information criterion. For second primaries, because of the limited number of events, only unadjusted P values are given. P values below the conventional 5% threshold were regarded as significant. All of the analyses were carried out using SAS software (SAS Institute Inc, Cary, NC).

### Results

**High-Risk HPV DNA Detection and Assessment of HPV Physical Status**

Out of the 90 investigated patients, 17 (19%) showed integrated HPV 16 DNA (HPV positive) and none were positive for HPV 18 DNA. We observed an E2-to-E6 transcript ratio consistent with full viral integration in two HPV-positive patients and the presence of both episomal and integrated viral forms in the remaining patients.

**TP53 Mutation**

Thirty five of 90 patients (39%) harbored TP53 mutations. We identified 24 missense mutations, resulting in an amino acid substitution, six nonsense mutations, three deletions, one intronic substitution, and one silent mutation. Two patients coexpressed HPV 16 and TP53 mutations. The remaining 55 patients (61%) carried wt TP53, of whom 15 patients were HPV positive (Table 2).

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**Table 2. Patient, Disease, and Treatment Characteristics**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total (N = 90)</th>
<th>HPV Positive or Negative/TP53 Mutation</th>
<th>HPV Negative/TP53 wt</th>
<th>HPV Positive/TP53 wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Patients</td>
<td>%</td>
<td>No. of Patients %</td>
<td>No. of Patients %</td>
<td>No. of Patients %</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>69</td>
<td>27</td>
<td>33</td>
<td>9</td>
</tr>
<tr>
<td>Female</td>
<td>21</td>
<td>8</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>58</td>
<td>26</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>Range</td>
<td>32-76</td>
<td>74</td>
<td>75</td>
<td>67</td>
</tr>
<tr>
<td>Subsite</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tonsil, tongue base</td>
<td>66</td>
<td>26</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>Other (palate, uvula, posterior wall)</td>
<td>24</td>
<td>9</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Stage, 1997</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2</td>
<td>—</td>
<td>1</td>
<td>7</td>
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<tr>
<td>II</td>
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<td>3</td>
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<td>1</td>
</tr>
<tr>
<td>III</td>
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<td>IV</td>
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<td>—</td>
</tr>
<tr>
<td>2</td>
<td>55</td>
<td>19</td>
<td>26</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>9</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surgery</td>
<td>31</td>
<td>17</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Surgery plus RT</td>
<td>59</td>
<td>18</td>
<td>30</td>
<td>11</td>
</tr>
<tr>
<td>Second tumor</td>
<td>10</td>
<td>7</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

Abbreviations: HPV, human papillomavirus; wt, wild type; RT, radiation therapy.

*All HPV negative.*
HDs of the p16\textsuperscript{INK4a} Gene

p16\textsuperscript{INK4a} HD assay was performed successfully in 74 patients, of whom 15 were HPV positive and 59 were HPV negative. In HPV-positive patients, p16\textsuperscript{INK4a} gene proved normal in all assesseable patients (100%). p16\textsuperscript{INK4a} HD occurred in 28 (47%) HPV-negative assessable patients similarly distributed among the mutated TP53 and wt TP53 group: 15 (48%) of 31 and 13 (46%) of 28 patients, respectively. The remaining 31 HPV-negative assessable patients carried normal p16\textsuperscript{INK4a} gene.

p16 Immunohistochemistry

p16 immunophenotype results were positive in all 17 HPV-positive patients, whereas only 15 of 73 HPV-negative tumors were p16 positive (100% v 21%; P < 0.0001). A p16 null immunophenotype occurred in 58 (79%) HPV-negative patients, similarly distributed among the mutated TP53 and wt TP53 group: 27 (82%) of 33 and 31 (78%) of 40 patients, respectively. By crossing molecular analysis and immunohistochemistry, all 28 patients with deleted p16\textsuperscript{INK4a} gene were p16 negative, whereas the remaining 46 were p16 negative in 19 (41%) patients and positive in 27 (59%) patients.

Statistical Analysis

The limited overlap of HPV positivity and TP53 mutation (only two patients showed both) denoted the presence of a significant negative association between the two factors (Kendall's $\tau_b = -0.27$; $P = .0125$). Consequently, HPV and TP53 could not be investigated reliably as two independent factors. Rather, they were investigated after splitting the whole series into three subsets: HPV positive/TP53 wt (15 patients), HPV positive or negative/TP53 mutation (35 patients), and HPV negative/TP53 wt (40 patients). Patients, disease, and treatment characteristics according to HPV/TP53 and are listed in Table 2. The three subsets were matched for all investigated characteristics (Table 2). When HPV-positive and -negative patients were compared, median age was 58 years (range, 32 to 76 years) in HPV-negative patients and 57 years (range, 51 to 75 years) in HPV-positive patients ($P = .8285$ for the difference). The male-to-female ratio was slightly higher in HPV-negative patients (ratio, 3.9) than HPV-positive patients (ratio, 1.8; $P = .1954$). HPV positivity was not significantly associated with tumor stage ($P = .1930$), and the proportion of patients receiving postoperative radiation, indicating HR-HPV disease, was similar for HPV-positive (71%) and HPV-negative (64%) patients ($P = .6280$).

During a median follow-up of 5.8 years, 49 deaths, 43 locoregional relapses (of which seven also occurred with distant metastasis), and 10 STs were recorded. STs were diagnosed mainly in the lung (five patients), in the head and neck area (four patients), and in the urinary bladder (one patient; later, this patient developed a third primary in the lung). Overall survival, crude cumulative incidence of tumor relapse, and STs were 63.9%, 41.6%, and 2.2% at 3 years, and 50.2%, 47.6%, and 10.2% at 5 years.

By testing the prognostic role of HPV/TP53 status, using either univariable or multivariable analyses (Table 2), significant results were obtained for each investigated end point, namely overall survival ($P = .0018$ at the multivariable analysis), occurrence of tumor relapse ($P = .0371$), and ST ($P = .0152$). Figures 1 to 3 show overall survival curves and crude cumulative incidence curves of tumor relapse and STs according to HPV/TP53 status, whereas Table 3 lists corresponding probability estimates at 3 and 5 years. A more favorable outcome was generally observed in the HPV-positive subset. In contrast, for HPV-negative patients, overlapping outcomes were observed in mutated TP53 and wt TP53 subsets. The survival benefit observed in HPV-positive patients, amounting to a 61% relative mortality reduction at 5 years, occurred irrespective of tumor stage (not shown). Likewise, the reduction in crude cumulative incidence of primary tumor relapse was associated with HPV-positive status both in patients who did or did not receive radiotherapy (Fig 4). Finally, the presence of HPV in the primary tumor apparently removed the risk of developing an ST (Fig 3), and wt TP53 status seemed to be associated with a lower incidence of ST, as compared with mutated TP53 status, although this did not reach statistical significance ($P = .182$).

A similar investigation of the joint prognostic effect of HPV and p16 expression (or p16\textsuperscript{INK4a} deletion) yielded similar results (not shown in detail): HPV status retains its favorable prognostic effect, whereas it did not influence the clinical outcome of HPV-negative patients.

<table>
<thead>
<tr>
<th>No. at risk</th>
<th>HPV+/ TP53wt</th>
<th>HPV+/ TP53mut</th>
<th>HPV-/ TP53wt</th>
<th>HPV-/ TP53mut</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV+/ TP53wt</td>
<td>15</td>
<td>13</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>HPV+/ TP53mut</td>
<td>40</td>
<td>27</td>
<td>19</td>
<td>7</td>
</tr>
<tr>
<td>HPV-/ TP53wt</td>
<td>35</td>
<td>26</td>
<td>15</td>
<td>6</td>
</tr>
</tbody>
</table>

HPV Prognostic Role in Oropharyngeal Cancer

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The presence of HR-HPV together with the analysis of TP53 and p16INK4a status and p16 immunophenotyping was assessed in a series of 90 oropharyngeal SCCs, all of which were primary tumors treated homogeneously with surgery with or without radiation. The results showed that 17 (19%) of 90 patients harbored integrated HPV 16 DNA, mostly coupled with wt TP53, normal p16INK4a gene, and a p16-positive immunophenotype. Thirty-five (39%) of 90 patients harbored TP53 mutations and not surprisingly, HR-HPV and TP53 mutation coexisted in only two patients, who retained both normal p16INK4a and expression of its encoded protein. p16INK4a homozygous deletion occurred in 28 patients and p16 null immunophenotype occurred in 58 HPV-negative patients, both similarly distributed among mutated TP53 (48% and 82%, respectively) and wt TP53 (46% and 77%, respectively) groups.

The correlation between p16 immunophenotyping and HR-HPV as well as p16INK4a molecular analyses confirmed that immunohistochemistry is a good surrogate for HR-HPV infection, with a p16 overexpression in all HPV-positive patients, and for p16INK4a inactivation, with evidence of loss of p16 nuclear staining in all p16INK4a-deleted patients. However, p16 immunostaining may be misleading because it does not entirely mirror molecular findings. Indeed, in our series, despite the significant correlation between p16 expression and presence of HR-HPV (P < .0001), 12 p16-positive patients were HR-HPV negative. Conversely, p16 null patients outnumber the molecular deleted patients, suggesting the existence of other inactivating p16INK4a mechanisms such as promoter methylation.

The outcome analysis of the different biomarker association resulted in the definition of three independent prognostic groups among which patients harboring HR-HPV showed a significant improvement in terms of survival, occurrence of relapse, and ST incidence. This apparently was independent from tumor stage as well as the delivered treatment, confirming the observation that HPV-positive tumors represent a distinct disease among oropharyngeal cancers. In contrast, TP53 mutations seem not to be significant in prognostication of oropharyngeal SCCs primarily undergoing surgery, a notion consistent with the fact that TP53 mutations act as a predictive factor under particular treatment conditions such as chemotherapy and radiotherapy, rather than as a prognostic factor.29-34 In our study, deregulation of the p16INK4a gene and its encoded protein also seem to lack prognostic significance (in the absence of HPV infection). Indeed, the role of this deregulation in head and neck tumors is not yet well defined.35,36

Few studies concluded that the reason for a better prognosis would be explained by an enhanced radiosensitivity of HPV-positive patients in comparison to HPV-negative patients.2,29,37-39 It was suggested that the interaction between E6 and p53 does not result in its full functional abrogation as compared with mutated TP53 patients.20,40 This would justify an improved radiosensitivity sustained by a functioning p53 protein20,40 to which we can add a functional p16 protein. To our knowledge, this is the first clinical series in which


39. Friesland S, Mellin H, Mu acknowledgment