Advances in Brief

Association between Cyclin D1 (CCND1) Gene Amplification and Human Papillomavirus Infection in Human Laryngeal Squamous Cell Carcinoma

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Abstract
Head and neck squamous cell carcinomas (SCCs) seem to follow a multistep process of carcinogenesis in which chemical and/or viral agents are associated with specific genetic alterations. The prevalence of human papillomavirus (HPV) infection and the amplification of the cyclin D1 (CCND1) gene were evaluated in a series of 75 laryngeal SCCs by PCR with HPV consensus primers and Southern blot analysis with a CCND1-specific probe, respectively. HPV DNA was detected in 22 of 75 (29.3%) tumors, and it belonged almost exclusively to the highly oncogenic HPV-16, HPV-18, and HPV-33. CCND1 gene amplification was found in 15 of 75 (20%) tumors, and it was associated with HPV infection in a statistically significant manner ($\chi^2 = 20.3; P < 0.001$). Because the viral oncoproteins E6 and E7 from high-risk HPV types are known to promote genomic rearrangements, these findings suggest that amplification of the CCND1 gene in laryngeal SCCs may occur as a consequence of the genomic instability associated with HPV infection. In turn, amplified CCND1, either alone or in conjunction with a direct action of the viral oncoproteins E6 and E7, could lead to a perturbation of the cell cycle. This model could explain the involvement of high-risk HPV types in laryngeal carcinogenesis.

Introduction
Clinical, epidemiological, and molecular studies support the oncogenic potential of a specific subset of HPV types in the development of genital and respiratory tract malignancies (1, 2). These HPV types (HPV-16, HPV-18, HPV-31, HPV-33, and HPV-39) are referred to as high-risk types because of their association with cervical carcinoma and SCC of the upper respiratory tract, including the larynx. Approximately 90% of cervical carcinomas and 20% of laryngeal SCCs are associated with HPV infection (3-5). The induction of genomic instability and the proliferative stimuli associated with chronic infection may explain the contribution of these viruses to cancer development in vivo, although additional alterations of host cell genes by chemical factors, such as tobacco and alcohol, are equally important (6).

The major determinants of the oncogenic potential of high-risk HPV types are the viral oncoproteins E6 and E7. These proteins can interfere with cell cycle control mechanisms by specifically inactivating the products of the p53 and Rb tumor suppressor genes. Both p53 and Rb proteins negatively regulate the cell cycle, inhibit the G1-S-phase transition, and contribute to genomic stability (7).

The D-type cyclins are a family of cell cycle proteins involved in the Rb pathway and p53-dependent checkpoint that are associated with cancer development (8). In particular, cyclin D1 is known to regulate cell cycle progression at the G1-S-phase checkpoint, and its overexpression in human cells is expected to drive the cells through the G1-S-phase transition, thus contributing to oncogenesis. The cyclin D1 (CCND1) gene is frequently amplified, rearranged, and overexpressed in a wide variety of human cancers, including head and neck carcinoma. In laryngeal SCC, CCND1 amplification is associated with poor prognosis (9, 10).

Because CCND1 alterations play an important role in the natural history of laryngeal SCC, and HPV infection is expected to promote cell cycle deregulation and genomic instability, in this study we determined the prevalence of both high-risk HPV infections and CCND1 gene amplification in a series of laryngeal SCCs.

A statistically significant association between HPV DNA detection and CCND1 gene amplification was observed. To our...
knowledge, this is the first report describing such an association in vivo.

**Patients, Materials, and Methods**

**Patients and Tumor Specimens.** Laryngeal carcinoma specimens were collected during the period 1989–1995 from consecutive patients undergoing surgery at the A. Gemelli Catholic University Hospital, a tertiary-care hospital in Rome, Italy. We evaluated a total of 75 primary tumor specimens from 75 patients (median age, 64 years; range, 40–85 years), 70 males and 5 females. Tumors were defined according to location as supraglottic, glottic, or transglottic (when extension of the tumor did not allow the identification of the original site), and they were staged according to the International Union Against Cancer TNM classification (11). All tumors were SCC, and they were graded as well differentiated (G1), moderately differentiated (G2), or poorly differentiated (G3). Clinicopathological characteristics of the patients are summarized in Table 1. Tissue samples were snap-frozen in liquid nitrogen and stored at −80°C until HPV DNA detection and **CCND1** gene analysis. All samples were blinded and coded by clinicians in the Division of Otorhinolaryngology. Coded samples were independently analyzed for HPV infection at the Institute of Microbiology and analyzed for **CCND1** amplification at the Institute of Molecular Genetics. The code was released upon completion of the molecular analyses.

**DNA Extraction.** Genomic DNA was extracted from tissue specimens by the standard proteinase K/phenol method, as described previously (3, 9). The DNA concentration was determined with a spectrophotometer, and the quality of DNA samples was assessed by agarose gel electrophoresis.

**HPV DNA Detection.** Genomic DNAs (0.5 μg) were analyzed with an assay that allows the detection of a broad spectrum of HPV genotypes by consensus primer-mediated PCR. MY11 and MY09 primers, which were selected from a highly conserved region (L1) within the HPV genome, were used as described by Resnick et al. (12). An aliquot of amplified DNA was analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining to visualize fragments of the expected size (450 bp). The amplification products were then subjected to a DNA enzyme immunoassay routinely used for clinical diagnostics (Gen-Eti-K-DEIA-HPV: Sorin, Saluggia, Italy) for HPV typing (genotypes HPV-6, HPV-11, HPV-16, HPV-18, and HPV-31), according to the manufacturer's protocols. This assay is based on the hybridization of denatured PCR-amplified DNA to a specific HPV oligonucleotide probe immobilized on microtiter plates through a streptavidin-biotin bond. The resulting hybrid is revealed by incubation with a monoclonal antibody directed against double-stranded DNA. After incubation with a horseradish peroxidase-labeled antimouse IgG antibody, a colorimetric assay identifies wells in which hybridization occurred. Type-specific PCR was used to detect HPV-33 DNA as described by Van den Brule et al. (13). To assess the integrity of the genomic DNA of HPV-negative samples, a region of the human β-globin gene was amplified as described previously (3). Negative controls included reaction mixtures lacking any DNA template and reaction mixtures containing human DNA without HPV target sequences. Procedures to prevent specimen contamination and PCR carryover were rigorously observed at every step in this analysis (14).

**CCND1 Gene amplification and HPV DNA detection according to the clinicopathological parameters of 75 primary laryngeal cancer patients**

<table>
<thead>
<tr>
<th>Sex</th>
<th>No. of cases with amplified CCND1</th>
<th>No. of HPV DNA-positive cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>70</td>
<td>14</td>
</tr>
<tr>
<td>Females</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤60</td>
<td>31</td>
<td>7</td>
</tr>
<tr>
<td>&gt;60</td>
<td>44</td>
<td>8</td>
</tr>
<tr>
<td>Tumor site</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supraglottic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glottic</td>
<td>26</td>
<td>6</td>
</tr>
<tr>
<td>Lymph-node status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>50</td>
<td>8</td>
</tr>
<tr>
<td>N+</td>
<td>25</td>
<td>7</td>
</tr>
<tr>
<td>Grading</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>G2</td>
<td>39</td>
<td>5</td>
</tr>
<tr>
<td>G3</td>
<td>24</td>
<td>9</td>
</tr>
<tr>
<td>Stage</td>
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<td></td>
</tr>
<tr>
<td>I</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>23</td>
<td>5</td>
</tr>
<tr>
<td>III</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>IV</td>
<td>26</td>
<td>10</td>
</tr>
</tbody>
</table>

**Results**

HPV DNA was found in 22 of 75 (29.3%) laryngeal SCC specimens examined (Fig. 1). In the HPV-positive specimens, the high-risk HPV types 16 and 18 were predominantly represented. HPV-16 DNA was detected in nine cases (12%), and
amplification

Discussion

Carcinogenesis of the respiratory and gastrointestinal tracts is thought to be a complex, multistep process (18). Tumors of the aerodigestive epithelium are largely caused by environmental factors, and they occur almost exclusively in individuals with prolonged exposure to tobacco and alcohol (19, 20). Although it is known that these factors are the main causal agents, a number of reports have suggested a role for HPV infection in the pathogenesis of aerodigestive tract tumors. Thus, laryngeal papillomas may contain HPV DNA (usually HPV-6 or HPV-11; Ref. 21), and a clear association between HPV and verrucous carcinomas of the larynx has been reported (22). More recently, high-risk HPV infection has been detected in laryngeal SCC (3–5, 23). In the present study, 22 of 75 (29.3%) laryngeal SCC samples were found to contain HPV DNA. Our results were obtained on fresh frozen specimens, which represent the most advantageous source for detection of viral DNA compared to paraffin-embedded tissues (24). In accordance with previous studies, high-risk HPV types 16 and 18 were predominantly present, suggesting that HPV could be involved in laryngeal carcinogenesis (1, 6, 7, 20).

Deregulation of cell cycle control mechanisms is involved in the etiology and natural history of laryngeal cancer, as suggested by the alterations of p53, Rb, and cyclins that are frequently observed in head and neck SCC (9, 10, 25–27). CCND1 gene amplification was found in 15 of 75 (20%) cases of laryngeal SCC, with the gene copy number ranging from 3 to 18, as determined by densitometric analysis, compared to normal tissue and normalized to the control probes for ETS1 and HRAS1.

The distribution of HPV DNA-positive cases and CCND1 gene amplification-positive cases according to the clinicopathological characteristics of the patients is shown in Table 1. HPV-positive and CCND1 gene amplification-positive specimens were mostly high-grade and advanced-stage cases. We then examined the correlation between CCND1 amplification and HPV infection. Twelve of the 15 samples (80%) that were positive for CCND1 gene amplification were also HPV DNA positive, whereas 50 of the 53 (94.3%) HPV DNA-negative specimens were associated with the normal CCND1 gene copy number. CCND1 gene amplification was detected in only 3 of the 53 HPV DNA-negative samples. A statistically significant association between HPV DNA detection and CCND1 gene amplification ($\chi^2 = 20.3; P < 0.001$) was detected (Table 2).

Table 2

<table>
<thead>
<tr>
<th>No. of HPV DNA-positive cases</th>
<th>No. of HPV DNA-negative cases</th>
<th>$P^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>60</td>
</tr>
</tbody>
</table>

$^* \chi^2 = 20.3.$

HPV-18 DNA was detected in eight cases (10.5%); one tumor sample was found to contain both HPV-16 and HPV-18 DNA. HPV-33 DNA was amplified in a single case of laryngeal SCC. Four specimens yielded amplification products of the expected size (450 bp) using the consensus primers, but they did not hybridize to any of the type-specific internal oligonucleotide probes used in this study.

The remaining 53 laryngeal SCC specimens, which were negative for HPV DNA amplification, yielded visible $\beta$-globin gene amplification products and displayed intact CCND1 bands by Southern blot (see below); thus, they were considered sufficient and appropriate for molecular analysis.

Southern blot hybridization analysis of EcoRI-digested tumor DNAs with the CCND1 probe demonstrated the presence of the expected bands of 4.0-, 2.2-, and 2.0-kb, as shown previously (9). CCND1 gene amplification was found in 15 of 75 (20%) cases of laryngeal SCC, with the gene copy number ranging from 3 to 18, as determined by densitometric analysis, compared to normal tissue and normalized to the control probes for ETS1 and HRAS1.

Fig. 1 Examples of HPV detection in laryngeal carcinomas. Agarose gel electrophoresis of the PCR products of 22 HPV DNA-positive and 14 HPV DNA-negative laryngeal SCC specimens (Lanes 1–36). Arrows, the 450-bp HPV-specific fragment. The size of the molecular weight marker VI (Boehringer Mannheim) is indicated. C− and C+, negative and positive PCR controls.

Table 2 CCND1 amplification and HPV DNA detection in 75 primary laryngeal cancer patients
detected in a series of head and neck cancers (28). The apparent discrepancy with our results may be due to both the low number of laryngeal SCCs investigated in this report (n = 11) and to the use of archival material that may be prone to false negative results for virus detection (24).

Our data on the association between HPV infection and CCND1 amplification are consistent with a model by which expression of the high-risk HPV E6 and E7 proteins undermines cell cycle checkpoints, contributing to the accumulation of genetic alterations during tumorigenesis (29). In carcinomas, HPV DNA is usually integrated into the cell genome; as a consequence of the integration, the E6 and E7 genes are constitutively expressed (6, 7). It has been shown that the HPV E6 protein binds and promotes the degradation of p53; the inactivation of p53 by E6 enhances the frequency of genomic rearrangements and, in particular, promotes gene amplification (30). Similarly, the expression of E7, which binds and inactivates Rb, is associated with gross chromosomal rearrangements (29, 30). Thus, the inactivation of p53 and Rb by HPV oncoproteins has different and complementary effects on genomic integrity (29). The HPV-induced genomic instability would set the stage for the accumulation of mutations in tumor suppressor genes and oncogenes, including CCND1 amplification. The combined inactivation of p53 and Rb by HPV may explain why a correlation between CCND1 amplification and p53 mutation only has not been established in head and neck SCC.

Our data suggest that CCND1 amplification is a frequent consequence of the pathogenic process initiated by HPV infection in laryngeal SCC. This conclusion, however, is apparently paradoxical. The main function of the cyclin D1/cyclin-dependent kinase 4 complex is to promote the phosphorylation and consequent inactivation of the tumor suppressor protein Rb in late G1, thus allowing S-phase entry (8). If Rb is already inactivated by HPV E7, why is CCND1 amplification selected for in laryngeal SCC tumorigenesis? Additional studies are required to address this question, but the paradox could be explained by the Rb-independent effects of cyclin D1 activation. It has been shown recently that cyclin D1 blocks muscle differentiation by a mechanism at least partially independent of Rb hyperphosphorylation (31). It is possible that similar Rb-independent mechanisms, perhaps leading to the inhibition of differentiation, play a role in laryngeal SCC. This paradox is based on the assumption that HPV infection precedes CCND1 amplification; however, an alternative possibility to consider is that CCND1 amplification and the subsequent deregulation of the cell cycle promote viral entry.

In conclusion, we have shown a statistically significant association between CCND1 amplification and HPV infection. This provides further support to the involvement of high-risk HPV types in a multistep model of laryngeal carcinogenesis. In this model, CCND1 gene amplification may occur as a late event after HPV infection and the onset of genomic instability.

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References


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