

Advances in Brief

Association between Cyclin D1 (*CCND1*) Gene Amplification and Human Papillomavirus Infection in Human Laryngeal Squamous Cell Carcinoma¹

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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 HPV⁴ 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

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⁴ The abbreviations used are: HPV, human papillomavirus; SCC, squamous cell carcinoma.

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 Medical 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 anti-mouse 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

Table 1 *CCND1* gene amplification and HPV DNA detection according to the clinicopathological parameters of 75 primary laryngeal cancer patients

	No. of cases	No. of cases with amplified <i>CCND1</i>	No. of HPV DNA-positive cases
Sex			
Males	70	14	21
Females	5	1	1
Age (yr)			
≤ 60	31	7	7
> 60	44	8	15
Tumor site			
Transglottic	44	9	12
Supraglottic	26	6	10
Glottic	5	0	0
T classification			
T ₁	12	1	3
T ₂	25	3	5
T ₃	21	2	5
T ₄	17	9	9
Lymph-node status			
N0	50	8	14
N+	25	7	8
Grading			
G1	12	1	4
G2	39	5	6
G3	24	9	12
Stage			
I	8	0	1
II	23	5	5
III	18	0	3
IV	26	10	13

to prevent specimen contamination and PCR carryover were rigorously observed at every step in this analysis (14).

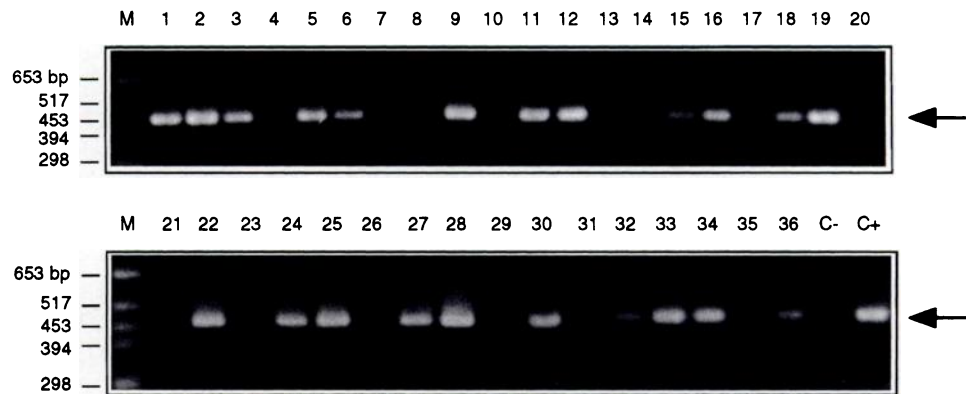
***CCND1* Gene Analysis.** *CCND1* amplification was evaluated as reported previously (9). Briefly, Southern blot analysis was performed after digestion with the restriction enzyme *EcoRI*, electrophoresis onto 0.7% agarose gels, and transfer to nylon membranes (Hybond N+; Amersham). The filters were then hybridized to a 1.4-kb cDNA insert from plasmid pPL-8 (15) and radiolabeled with [α - ^{32}P]dCTP. Each blot included placental and normal laryngeal mucosa DNA samples as negative controls. Band intensities were determined by laser scanning densitometry (UltraScan XL; Pharmacia LKB), and tumor signals were normalized against those obtained from the negative controls. Samples were scored as positive when >2 *CCND1* copies were detected. To rule out polysomy of the whole chromosome 11, densitometric analysis was subsequently performed on the same filters rehybridized to a probe for the *ETS1* locus, which maps to 11q23 (16), and to the pEJ probe for the *HRAS1* locus, which maps to 11p15.5 (17).

Statistical Analysis. The χ^2 test was used to evaluate the association between HPV DNA detection and *CCND1* gene amplification in laryngeal SCC samples.

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

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.



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 β -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*.

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).

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

Table 2 *CCND1* amplification and HPV DNA detection in 75 primary laryngeal cancer patients

	No. of <i>CCND1</i> amplification-positive cases	No. of <i>CCND1</i> amplification-negative cases	P^a
No. of HPV DNA-positive cases	12	10	<0.001
No. of HPV DNA-negative cases	3	50	
Total	15	60	

$$^a \chi^2 = 20.3.$$

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 evaluated on the same large series of laryngeal SCC specimens analyzed for the presence of HPV DNA. These analyses were conducted separately in two different laboratories on the same blinded sample set (see "Patients, Materials, and Methods"). A statistically significant correlation was observed between HPV DNA positivity and *CCND1* gene amplification. To our knowledge, this is the first description of a significant association between HPV infection and *CCND1* gene amplification in laryngeal carcinoma. In a recent report, no association between HPV DNA and *CCND1* amplification was

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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