Human Papillomavirus Infection and Epidermal Growth Factor Receptor Expression in Primary Laryngeal Squamous Cell Carcinoma

Giovanni Almadori, Gabriella Cadoni, Paola Cattani, Jacopo Galli, Francesco Bussu, Gabriella Ferrandina, Giovanni Scambia, Giovanni Fadda, Maurizio Maurizi
Institutes of Otolaryngology [G. A., G. C., J. G., F. B., M. M.], Microbiology [P. C., G. Fa.], and Gynecology [G. Fe., G. S.], Catholic University of the Sacred Heart, Rome 00168, Italy

ABSTRACT

Purpose: This study was designed to add new data about laryngeal carcinogenesis, a multistep process in which chemical and/or viral agents induce and promote successive alterations in growth factor-linked signal transmission pathways, genetic instability, and mutations in key genes involved in cell growth control. Epidemiological evidence suggests that human papillomavirus (HPV) infection may be associated with the development of laryngeal cancer.

Experimental design: In this report, we have analyzed the prevalence of HPV infection and epidermal growth factor receptor (EGFR) expression in a series of 42 laryngeal squamous cell carcinomas by PCR with HPV consensus primers and by a radioligand receptor assay, respectively.

Results: HPV DNA was detected in 15 of 42 (35.7%) tumors, and it belonged almost exclusively to the highly oncogenic HPV-16, HPV-18, and HPV-33 genotypes. At analysis by Mann-Whitney nonparametric statistical test, EGFR level was found to be significantly higher in HPV-infected than in HPV-negative cases (T = 440; P = 0.002). EGFR overexpression (EGFR-positive status > 6 fmol/mg protein, the arbitrary cutoff value chosen) was found in 20 of 42 (47.6%) tumors, and it was associated with HPV infection in a statistically significant extent (χ² = 4.686; P = 0.03).

Conclusions: Viral oncoproteins have been shown to induce a perturbation of the cell response to signals for growth and differentiation; these findings confirm that enhanced EGFR expression and activation in laryngeal squamous cell carcinoma may occur also as a consequence of HPV infection and support the hypothesis of an involvement of HPV infection in laryngeal carcinogenesis.

INTRODUCTION

LSCC is one of the most common head and neck cancers and accounts for almost 2% of all new cancers in the United States (1). Although early-stage laryngeal cancer is often curable with surgery or radiotherapy, the majority of patients present with advanced disease, and, despite therapeutic advances, the outcome has not improved dramatically in the last two decades. To further improve survival and cure, it is important to better understand the carcinogenic mechanisms, thereby allowing the identification of high-risk patients for prevention, early diagnosis, and better therapeutic options.

According to the modern theory of carcinogenesis, a complex and multistep process is likely in the development of LSCC. There is evidence that altered dominant and recessive oncogenes, genetic instability, and growth factor-linked signal transmission pathways are probably involved in such a process (2–4).

Although it is known that cigarette smoking and chronic alcohol consumption are the best established risk factors in laryngeal cancer development, a number of reports recently have also suggested a role for HPV infection in pathogenesis of laryngeal and other head and neck cancers (5, 6). The transforming activity of high-risk HPV seems to depend primarily on deregulated expression of E6 and E7 HPV oncoproteins. The E5 HPV oncoprotein also seems to play a role in early growth stimulation of HPV-infected cells by an interaction with some cellular proteins, and in particular with EGFR, and to alter the cell response to signals for growth and differentiation (7).

The EGFR, a potent stimulator of the mitogen-activated protein kinase signaling pathway, seems to play a role in the development of laryngeal cancer (8). Higher EGFR expression in primary LSCC than in normal laryngeal mucosa has been reported (9). Moreover, EGFR overexpression was shown to be a predictor of tumor aggressiveness and invasiveness in LSCC (10–12). Recently, experimental studies have demonstrated that HPV infection elevates EGFR levels by posttranslational mechanisms in keratinocytes and laryngeal papilloma cells (13).

Although HPV DNA infection and EGFR expression have been largely studied in LSCC patients, no reports have investigated both in the same patient population.

The aim of this paper was to study the association between EGFR expression and HPV-DNA infection (genotypes HPV-6, HPV-11, HPV-16, HPV-18, HPV-31, and HPV-33), in tumor specimens from primary LSCC patients.

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1 To whom requests for reprints should be addressed, at Institute of Otolaryngology, Catholic University of the Sacred Heart, Largo A. Gemelli, 8, Rome 00168, Italy. Phone: 0039-06-3015443; E-mail: iclot@rm.unicatt.it.
2 The abbreviations used are: LSCC, laryngeal squamous cell carcinoma; HPV, human papillomavirus; EGFR, epidermal growth factor receptor.
PATIENTS, MATERIALS, AND METHODS

Patients and Tumor Specimens. Forty-two untreated consecutive primary laryngeal SCC patients were studied, and their clinicopathological features are shown in Table 1. Three patients were not smokers (2 males and 1 female), whereas 39 patients had a long-standing history (>20 years) of cigarette smoking. Of these, 35 had smoked >20 cigarettes/day (regular smokers) and 37 had consumed alcoholic beverages daily. Twenty had been heavy drinkers (>1 liter/day of wine). Tumors were classified as supraglottic, glottic, and transglottic, and they were staged according to the International Union Against Cancer Tumor-Node-Metastasis classification (14) and graded as well (G1), moderately (G2), or poorly differentiated (G3). At our institution, all primary laryngeal SCC patients received standard therapeutic management: curative surgery of the primary tumor (T) related to the lesion extension; therapeutic neck node dissection when there was lymph node involvement (N+), according to the “wait and see” policy, under strict follow-up conditions; postsurgical radiotherapy for local advanced tumors (T4) and neck lymph node metastasis with extranodal spread. All patients of this study have been treated according to this standard procedure. Twenty-four patients underwent total laryngectomy, 16 underwent supraglottic laryngectomy, and 2 cordectomy. At surgery, nine patients with clinically positive neck nodes underwent a therapeutic neck dissection. Twelve patients with advanced tumors (4 T4N0, and 8 N+: stage IV) had postsurgical radiotherapy.

Sample Preparation. Tumor fresh tissue specimens were immediately frozen on dry ice shortly after surgical removal and stored at −80°C until processed. A representative section of specimens was retained for histopathological examination. 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, Rome, Italy, and analyzed for EGFR expression at the Institute of Gynecology and Obstetrics, Rome, Italy. The code was released upon completion of the molecular analyses.

Preparation of Cytosolic and Membrane Fractions. Cytosolic and membrane fractions were prepared as described elsewhere (15, 16). Briefly, tumor specimens were finely minced and homogenized in 5 volumes of ice-cold buffer consisting of 25 mM Tris, 1.5 mM EDTA, 5 mM NaCl, 0.1% monothioglycerol, and 20% glycerol by applying several intermittent bursts of an Ultra-turrax homogenizer. The crude homogenate was centrifuged at 7000 × g for 20 min at 0°C, and the supernatant was further centrifugated at 105 × g for 75 min at 0°C to obtain a cytosolic fraction for a membrane fraction for EGFR assay.

EGFR Measurements. The membrane pellet was resuspended in 25 mM Tris, 1.5 mM EDTA, 5 mM NaCl, 20% glycerol, and 10 mM MgCl2. Aliquots of the suspension (100 μl containing 300–500 μg of protein) were incubated with 125I-EGF (2.6 μM; 800,000 Ci/mmol; NEN, DuPont, Wilmington, DE) in the presence or absence of unlabeled EGF (1 μg) for 12–16 h at room temperature in a final volume of 400 μl. Binding was blocked by the addition of 3 ml of ice-cold 25 mM Tris, 20% glycerol, 5 mM NaCl, and 0.1% BSA. After centrifugation at 2000 × g for 20 min at 0°C, the supernatant was carefully aspirated, and pellets were counted in a gamma counter. Results were expressed as fmol/mg protein. EGFR status was defined using the arbitrary cutoff value of 6.1 fmol/mg protein, corresponding to the median value of EGFR levels (EGFR expression median value = 6.1 fmol/mg protein; range = 1.2–169.9 fmol/mg protein). The cutoff value of 6.1 fmol/mg protein of EGFR levels was chosen without knowing the HPV detection results and the clinicopathological characteristics of the patients.

DNA Extraction and HPV DNA Detection. Genomic DNA was extracted from tissue specimens by the standard proteinase K/phenol method, as described previously (17, 18). The DNA concentration was determined with a spectrophotometer, and the quality of DNA samples was assessed by agarose gel electrophoresis. 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. (19). 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-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. Type-specific PCR was used to detect HPV-33 DNA, as described by Van den Brule et al. (20). To assess the integrity of the genomic DNA of HPV-negative samples, a region of the human β-globin gene

<table>
<thead>
<tr>
<th>Table 1</th>
<th>HPV DNA detection and EGFR levels according to the clinicopathological parameters of 42 primary LSCC patients</th>
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</thead>
<tbody>
<tr>
<td>No. of cases</td>
<td>EGFR+</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>40</td>
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<tr>
<td>Females</td>
<td>2</td>
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<tr>
<td>Age (yr)</td>
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<tr>
<td>≥60</td>
<td>22</td>
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<tr>
<td>&lt;60</td>
<td>20</td>
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<tr>
<td>Tumor site</td>
<td></td>
</tr>
<tr>
<td>Transglottic</td>
<td>23</td>
</tr>
<tr>
<td>Supraglottic</td>
<td>16</td>
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<tr>
<td>Glottic</td>
<td>3</td>
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<tr>
<td>Tumor classification</td>
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<tr>
<td>T1</td>
<td>5</td>
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<td>T2</td>
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<td>T4</td>
<td>6</td>
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<tr>
<td>Lymph-node status</td>
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<tr>
<td>N0</td>
<td>33</td>
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<tr>
<td>N+</td>
<td>9</td>
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<tr>
<td>Grading</td>
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<tr>
<td>G1</td>
<td>10</td>
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<td>G2</td>
<td>21</td>
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<td>G3</td>
<td>11</td>
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<tr>
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<td>I</td>
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<td>II</td>
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<td>III</td>
<td>7</td>
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<td>IV</td>
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was amplified as described previously (17). 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 (21).

Statistical Analysis. Values of EGFR level in HPV/H11001 and HPV- groups do not fit with a normal distribution and have different SDs (14.23 and 32.07 respectively). We thus analyzed differences between groups by non parametric Mann-Whitney test. The $\chi^2$ test was used to evaluate the association between HPV DNA detection and EGFR status in laryngeal SCC samples.

RESULTS

HPV DNA was found in 15 of 42 (35.7%) laryngeal SCC specimens examined. HPV-16 DNA was detected in six cases (14.2%), and HPV-18 DNA was detected in eight cases (16.6%); one case was found to contain both HPV-16 and HPV-18 DNA (Fig. 1; Table 2). The remaining 27 LSCC specimens, which were negative for HPV DNA amplification, yielded visible $\beta$-globin gene amplification products by PCR (data not shown); thus they were considered sufficient and appropriate for molecular analysis. EGFR levels ranged from 1.2 to 169.9 fmol/mg protein, with a median value of 6.1 fmol/mg protein. Using arbitrary cutoff values of 6.1 fmol/mg protein to assess EGFR status, 47.6% (20 of 42 cases) of tumors were considered to be EGFR-positive (with EGFR level $\leq$6.1 fmol/mg protein). The mean value for EGFR level in the whole population was 15.3, with a SD of 27. The association between clinicopathological characteristics of the patients and the HPV and EGFR status are shown in Table 1. No differences were found between HPV-positive and HPV-negative cancer patients with regard to age or alcohol consumption, but all HPV-positive patients had a long-standing history of cigarette smoking. The distribution of HPV DNA-positive cases and EGFR status according to the clinicopathological characteristics of the patients is shown in Table 1. Eleven of 15 HPV samples (73.3%) were also EGFR-positive, whereas 18 of 27 (66.6%) HPV DNA-negative specimens were associated with EGFR-negative status. The diagram in Fig. 2 shows the correlation between EGFR level and HPV status. Mean EGFR level was 19.54 fmol/mg protein in the HPV-positive group and 12.93 fmol/mg protein in HPV-negative group. The SD of EGFR level in the HPV-negative group has an higher value (SD, 32.07; range, 1.2–169.9 fmol/mg protein) than in HPV-positive group (SD, 14.23; range: 4–49.9). When compared by Mann-Whitney test, the EGFR level is significantly higher in the HPV-positive than in the HPV-negative group ($T = 440; P = 0.002$). Furthermore a statistically significant association between HPV infection and EGFR-positive status ($\chi^2 = 4.686; P = 0.03$) was detected (Table 3).

DISCUSSION

Laryngeal cancer is largely caused by environmental factors, and, in particular, it occurs almost exclusively in individuals with prolonged exposure to tobacco and alcohol. The involvement of HPV in the development of LSCC is still unclear.

A prevalence of 4% of HPV DNA infection in normal...
laryngeal mucosa of healthy patients has been reported (22), and there are several contradictory studies concerning HPV DNA detection in laryngeal premalignant lesions (23, 24). HPV-induced tumors are primarily benign. Malignant transformation occurs occasionally and is usually associated with a loss of HPV regulation (25–28). HPV types 16 and 18 have been detected in squamous cell carcinoma of the oral cavity, tonsil, pharynx, and maxillary sinus. In particular, HPV-positive oropharyngeal cancers seem to represent a distinct molecular and pathological entity that is causally associated with HPV infection and has a markedly improved progression. The reported incidence of HPV DNA presence in LSCC ranges from 8 to 54% (29–33), and HPV-positive LSCCs have not been demonstrated to be statistically significantly different from HPV-negative LSCCs with regard to the well-established risk factors of alcohol consumption and tobacco exposure and survival (34).

To our knowledge, this is the first study analyzing the association between HPV infection and EGFR expression in laryngeal SCC.

In this study, HPV DNA was determined in 35.7% of tumor specimens with a concomitant positivity for EGFR expression in 73.3% of cases. Anyway, it cannot be excluded that among both HPV-negative and EGFR-positive specimens, some might have been infected by nonidentified HPV DNA types. In fact, HPV DNA detection by consensus primers might have failed to recognize some HPV types among the numerous different HPVs that can infect humans.

The transforming activity of high-risk HPVs, particularly well studied in HPV-16 and -18, seems to depend mainly on the expression of E6 and E7 HPV oncoproteins and on their ability to interact with some cell components (such as p53 and pRb) and alter cell cycle-control mechanisms (26, 28, 35–37). Nevertheless, the E5 HPV oncoprotein also seems to play a role in the early growth stimulation of HPV-infected cells by an interaction with some cellular proteins and by altering the cell response to signals for growth and differentiation (7). Thus, the expression of the E5-HPV oncoprotein seems to destabilize the cells at an early stage, rendering them more susceptible to the action of E6 and E7 HPV proteins (28).

In particular, there is experimental evidence that the E5 protein, an 83-amino acid membrane protein associated with Golgi apparatus and plasma membrane, can induce an enhancement of EGFR activation in a ligand-dependent manner (38, 39). Such an effect has been ascribed to an impaired down-regulation caused by an inhibition of endosomal acidification by E5 (13, 39) and concomitant recycling of receptors in the cell surface. Nevertheless in other studies, EGFR overexpression in epithelial cancer HPV-infected cells has been reported to be not a consequence of an E5-mediated posttranslational mechanism (40, 41), and it could depend on the increase of EGFR mRNA levels induced by HPV-16 E6 and E7 (42).

Our previous observation of an association between HPV infection and CCND1 gene amplification (43) and the present observation of a statistically significant association between HPV and EGFR overexpression, with a lower SD of EGFR levels in HPV-positive cancers, support the hypothesis that a well-defined homogeneous subset of LSCCs may be etiologically linked to HPV infection. In such a subset of LSCCs, HPV infection would be an early event in a multistep process of laryngeal carcinogenesis and could address tumoral progression toward a peculiar molecular pattern with clinical and maybe therapeutic implications. Additional investigations are in progress to better understand the molecular mechanism of laryngeal HPV infection and to evaluate by epidemiological and biological evidence the role of HPV in a multistep model of laryngeal carcinogenesis.

If such role will be confirmed, HPV DNA detection in laryngeal mucosa could be performed in all patients with prolonged exposure to tobacco and alcohol. The presence of high-risk HPV genotypes in the larynx of these subjects could identify patients at high risk to develop LSCC to submit to strict follow up and primary and secondary prevention (chemoprevention).

### REFERENCES


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