PREVALENCE AND GENOTYPES OF HUMAN PAPILLOMA VIRUS IN LARYNGEAL CANCER IN A SMALL COHORT OF PATIENTS FROM SOUTHERN ITALY

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ABSTRACT

Objective*: An important and consistent association between HPV and oropharynx tumors has emerged across different countries and ethnic groups, whereas the worldwide HPV prevalence of other head and neck sites is still unclear. The aim of this study was to evaluate the prevalence and genotype of human HPV in laryngeal SCC treated by surgery in a Southern Italian population.*

Methods*: Previously untreated patients affected by laryngeal carcinoma who underwent surgical treatment were considered for this retrospective study. Formalin fixed paraffin-embedded tumor cell blocks were taken either from primary resections or biopsies of the histological confirmed laryngeal squamous cell carcinoma. Tissue specimens were processed for immunohistochemical p16 protein (surrogate HPV marker) detection and Human papillomavirus DNA detection by PCR.*

Results*: Nuclear expression of p16 in tumor cells was detected in ten of the 40 (25%) laryngeal carcinomas. Genotypic identification was performed on 22 out of the total of 40 starting patients. HPV DNA sequences were detected in 6 (27.27%) of the 22 patients examined. High-risk viral genotypes 16, 39, 52 and 67 were identified in 4 patients, one of which showed coinfection with 16-52 genotypes. Low-risk HPV 61 and 62 were detected in 2 cases.*

Conclusions*: This study, concerning a small southern Italian patients series, confirms that HPV could be involved in the development of a subset of laryngeal tumors.*

Keywords: *Laryngeal cancer, p16 overexpression, HPV DNA, HPV prevalence.*

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Introduction

Laryngeal carcinoma is the most frequent tumor of the upper aero-digestive tract (28%), representing about 2.5% of all malignant tumors in man and 0.5% in women. In Italy its incidence is estimated in 3 cases/100,000/year[(1)]. The aspects that link laryngeal carcinoma to the remaining part of head and neck tumors are some risk factors and histological aspects, since more than 95% of laryngeal tumors are squamous cell carcinomas (scc). The pathogenesis of LSCC has always been associated with classic risk factors such as the patient's lifestyle (smoking and alcohol abuse)[(2)].

In particular, smoking and alcohol cause about a third of head-neck tumors[(3)] and have a strong synergistic effect when present simultaneously, multiplying by 80 times the risk of developing a carcinoma of the oral cavity and 12 times a carcinoma of the larynx. However, evidence has accumulated over the years of the possible oncogenic role of human papilloma virus (HPV) in head and neck cancers[(4)].

The prevalence of HPV in head and neck cancers appears to be higher in studies performed in North America compared to those performed in Europe and Asia[5-8]. An important and consistent association between HPV and oropharynx tumors has emerged across different countries and ethnic groups. HPV involvement in these head and neck cancers has been associated with a better prognosis and linked to a better response to chemo-radiotherapy treatment[8]. HPV-positive oropharynx tumors are therefore a subset of head and neck tumors, also referred to as HPV-related tumors, whereas the worldwide HPV prevalence of other head and neck sites is still unclear. Recently, an epidemiological study showed an increase in the incidence of potential HPV-related carcinomas, especially in females[9].

In this study, there is no clear role for HPV infection in laryngeal carcinomas in the Italian population. Furthermore , these data on the prevalence of HPV in head and neck cancers mainly concern the population of central-northern Italy[9-11]. The aim of this study was to evaluate the prevalence and genotype of human HPV in laryngeal SCC treated by surgery in a Southern Italian population.

Materials and methods

Patients

Previously untreated patients affected by laryngeal carcinoma who underwent surgical treatment between January 2011 and December 2013 were considered for this retrospective study. Patients with metastatic disease or with synchronous tumors at the time of diagnosis were excluded. For each patient, clinical-anamnestic data, including age, sex, primary tumor site, histopathology, tumor (T) lymph node (N) metastases (M) classification were collected in a database. The stage was determined in accordance with the 7th edition of the TNM classification established by the American Joint Committee for Cancer. Furthermore, data about smoking and alcohol habits were included. To collect data on smoking habits, non-smokers were defined as patients who never smoked or had stopped. Smokers were defined as patients who had smoked regularly before or were still active smokers. Patients were asked how many cigarettes they smoked and the pack years were then calculated. Patients who declared to drink alcohol regularly more than 2 drinks per day were defined as alcohol drinkers. Non-alcohol drinkers were defined as patients who never drank alcohol or only on vary rare occasions and less than 2 drinks per day. Formalin fixed paraffin-embedded (FFPE) tumor cell blocks were taken either from primary resections or biopsies of the histological confirmed laryngeal squamous cell carcinoma.

P16 Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue specimens were obtained from the surgical pathology and processed at the Department G.F. Ingrassia, section of Pathology, University of Catania, Catania, Italy. From formalin-fixed and paraffin-embedded tissue specimens, multiple sections (at least five) were obtained. Sections were processed as previously described[12]. Briefly, the slides were dewaxed in xylene, hydrated using graded ethanol mixtures, were incubated for 30 min in 0.3% hydrogen peroxide (H_2O_2)/methanol to quench endogenous peroxidase activity, and then rinsed for 20 min with phosphate-buffered saline (PBS; Bio-Optica, Milan, Italy). The sections were heated (5 min × 3) in capped polypropylene slide-holders with citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0; Bio-Optica, Milan, Italy) using a microwave oven (750 W) to unmask antigenic sites. The blocking step was performed before application of the primary antibody with 5% bovine serum albumin (BSA; Sigma, Milan,Italy) in PBS for 1 h in a humid chamber. BSA was used as a blocking agent to prevent non-specific antibody binding. The sections were then incubated overnight at 4 °C with rabbit polyclonal anti-p16 antibody (CINtec® INK4a; Roche Diagnostics, Indianapolis, Indiana, USA) ready to use in PBS (Sigma, Milan, Italy). The secondary antibody, biotinylated anti-rabbit antibody, was applied for 30 min at room temperature followed by the avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for a further 30 min at room temperature. The immunoreaction was visualized by incubating the sections for 4 min in a 0.1% 3,3'-diaminobenzidine (DAB) and 0.02% H_2O_2 solution (DAB substrate kit, Vector Laboratories, CA, USA). The sections were lightly counterstained with Mayer's hematoxylin (Histolab Products AB, Göteborg, Sweden), mounted in glycerol vinyl alcohol mounting solution (GVA, Zymed Laboratories, San Francisco, CA, USA), and observed with a Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany).

Evaluation of immunohistochemistry

Immunostained slides were separately evaluated using light microscopy by two

pathologists who were blinded to patient identity, clinical status, and group identification. The p16-staining status was identified as either negative or positive. Immunohistochemistry-positive staining was defined as the presence of brown chromogen detection within the nucleus. The staining intensity of p16 was classified as negative (0), weak (1), moderate (2), or strong (3) as described previously (12). The percentage of positive cells was used to score the specimens: (1) none (score 0); (2) 1%–10% stained cells (score 1); (3) 11%–30% stained cells (score 2); (4) 31%–50% stained cells (score 3); (5) 51%–80% stained cells (score 4); and (6) >80% stained cells (score 5). The staining intensity was multiplied by the percentage of positive cells to obtain the intensity reactivity score (IRS). The patients were classified into three groups based on their IRSs: (1) IRS = 0 (no expression); (2) IRS <10 (low expression); and (3) IRS >10 (high expression).

Human papillomavirus (HPV) DNA detection by PCR

Five to seven slices (5 μm) from each Formalin-fixed, paraffin-embedded tissue sample were collected using a microtome, with precautions taken to ensure that there was no contamination between cases. Genomic DNA was extracted from tissue samples using a Neasy bold and tissue kit (Qiagen, Valencia, CA), according the manufacture's protocol. This protocol use a deparaffinization method with xylene. Eliminated ethanol, pellets were resuspended overnight at about 56 °C with 20 μl of proteinase k and 180 μl of ATL lysis buffer. Proceeding with the protocol the next morning an AL buffer was added to the solution for 10 minutes at 70 ° C.

The protocol adopted (AmpliqualityHpv-type Express v3.0), for detection and genotyping of HPV by Single-Step PCR and Reverse Line Blot, involves the amplification of the L1 region of the viral genome with biotinated primers. The kit used allows the identification of the following viral genotypes: HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68 (68a and 68b), 69, 70, 71, 72, 73, 81, 82, 83, 84, 87, 89, 90. PCR reagents for this assay are provided in a ready-to-use PCR master mix that contains all reagents needed for amplification of the HPV and TST targets. To verify the efficiency of the DNA extraction, the housekeeping gene Thiosulphate SulphurTransferase (TST) was also amplified. Samples negative for TST were considered inadequate and a new sample was requested.

Additionally, it includes the dUTP/UNG system in order to prevent carry-over contamination. HPV was amplified in a PCR machine under the following conditions: 50˚C for 2 minutes, 95˚C for 10 minutes, followed by denaturation at 95˚C for 30 seconds, annealing at 50˚C for 30 seconds, and extension at 72˚C for 30 seconds for a total of 50 cycles.

At the end of the amplification cycle have been obtained two types of amplified with the dimensions of: - TST: 202 bp; - HPV: 139-145 bp. For HPV genotyping, the amplified products were hybridized on a support in which specific probes were fixed (ReverseLine Blot). After the strips have dried, each of these were evaluated using a colorimetric visualization and judged positive/negative by direct observation. All steps were performed manually in the presence of appropriate controls provided by the manufacturer.

Statistical analysis

Statistical analyses were performed with the MedCalc software (Belgium) using the chi-squared and Fisher's exact tests. Data collected are described as means, median, and standard deviations. A Pearson's chi-square or Fisher's exact test was used to identify differences in demographic and clinicopathologic data between cohorts. Correlation between p16 marker and clinical data was examined with Mann-Whitney U test. Finally, multivariate analysis was performed using multiple regression to determine independent factors.

Results

The study was carried out on 40 patients consisting of 36 men and four women; their median age was 62.5 years (range 43–85). Twenty-eight of 40 (70%) were smokers >10 sig/day and twelve were non-smokers or light mokers. Twenty-four of the 40 (60%) patients were ≤2 drinks/die drinkers, and sixteen (40%) were >2 drinks/die drinkers. Histological grade was G1 in 4 patients (10%), G2 in 26 (65%) and G3 in 10 (25%). Fourteen of the 37 (37.8%) tumors had a transglottic location, ten (27%) tumors had a supraglottic location, and thirteen (35.2%) had glottic tumors. Twenty-seven patients were classified as T1–T2 (12 T1 ,15 T2) while 13 were classifed as T3–T4 (9T3,4T4). The lymph node clinical status was classified as N0 in fifthteen patients, N1 in 3, and N2 in 22. Seven patients were submitted to Transoral Laser Microsurgery (TLM), 3 sopraglottic laryngetomy, 5 crico-hyoidoepiglottopexy and 25 total laryngectomy.

A total of 28 of the 40 patients received neck dissection, twelve received adjuvant chemotherapy and six underwent to adjuvant chemo-radiotherapy. Clinical-anamnestic patients data are reported in table 1.

Clinical-anamnestic data	N. (%)
Sex Male Female	 36 (90) 4 (10)
smoking never/no more 10-20 pack/year >20 sigarette/die	 12 (30) 14 (35) 14 (35)
Alcohol ≤ 2 drinks/ die >2 drinks/ die	 24 (60) 16 (40)
Primitive site Glottic Supraglottic Transglottic	 18 (45) 10 (25) 12 (30)
Histological grade G1 G2 G3	 4 (10) 26 (65) 10 (25)
T classification T1 T2 T3 T4	 12 (30) 15 (37,5) 9 (22,5) 4 (10)
N classification N0 N1 N2 N3	 15 (37,5) 3 (7,5) 22 (55) 0 (0)
Surgical treatment Cordectomy Partial laryngectomy Total laryngectomy	 7(17,5) 8(20) 25 (62,5)
Chemotherapy Yes No	 6 (15) 34 (85)
Radiotherapy Yes No	 18 (45) 22 (55)

Table 1: Clinical-anamnestic patient's data.

p16 expression

Nuclear expression of p16 in tumor cells was detected in ten of the 40 (25%) laryngeal carcinomas. All cases showed a high expression of p16 (IRS >10). As reported in the literature[13], we have excluded the fraction of laryngeal SCC that have low p16 expression (IRS <10). The correlation between clinical data and p16 expressions are reported in Table 2. P16 expressions correlated significantly with the patient's sex (p=0.04).

Clinical-anamnestic data	P16		P
	Negative	Positive	
Age ≤ 50 > 50	4 (10%) 26 (65%)	2 (5%) 8 (20%)	0,62
Sex Male Female	29 (72,5%) 1 (2,5%)	7 (17,5%) 3 (7,5%)	0,04
Smoke Never/no more 10-20 packs / day ≥ 20 packs / day	11 9 10	1 5 4	0.25
Alcohol ≤ 2 drinks/ die >2 drinks/ die	11 19	5 5	0.48
Primitive site Glottic Supraglottic Transglottic	13 (32,5%) 9 (22,5%) 8 (20%)	5 (12,5%) 1 (2,5%) 4 (10%)	0,42
Grading G1 G2 G3	3 (7,5%) 18 (45%) 9 (22,5%)	1 (2,5%) 8 (20%) 1 (2,5%)	0,43
T classification 1-2 3-4	18 (45%) 12 (30%)	9 (22,5%) 1 (2.5%)	0,12
N classification N0 N+	10 (25%) 20 (50%)	5 (12,5%) 5 (12,5%)	0,45
pT classification 1-2 3-4	16 (40%) 14 (35%)	6 (15%) 4 (10%)	1.00
pN classification N0 N+	15 (37,5%) 15 (37,5%)	7 (17,5%) 3 (7,5%)	0,46

Table 2: Correlation between p16 expression and clinic-pathological patient's data.

The subdivision of the test population into p16 positive and p16 negative, for the Mann-Whitney test, showed a statistically significant difference for clinical-anamnestic parameters such as the sex of patients ($p = 0.029$) and the value of T ($p = 0.016$).

The multivariate analysis of the 40 patients indicates that the expression of p16 doesn't correlate in a statistically significant way to the other independent variables such as the clino-anamnestic data. p16 is therefore an independent variable and isn't related to the others ($p = 0.0252$).

HPV DNA

Genotypic identification was performed on 22 out of the total of 40 starting patients.

Eighteen samples were excluded because of the presence of unsuitable or insufficient material for the execution of the work. HPV DNA sequences were detected in 6 (27.27%) of the 22 patients examined (Table 3). In 16 cases (72.73%) no viral genome was detected.

High-risk viral genotypes 16, 39, 52 and 67 were identified in 4 patients, one of which showed coinfection with 16-52 genotypes. Low-risk HPV 61 and 62 were detected in 2 cases.

The detection of HPV DNA sequences isn't statistically correlated with clinical-anamnestic parameters.

HPV genotype	Positive patients
16	1
39	1
61	1
62	1
67	1
16 – 52	1
Total	6

Table 3: HPV genotype detected in the HPV positive patients.

Correlation between p16 expression and HPV DNA

Of the 22 patients in which both p16 and the evaluation of HPV DNA were performed, the percentage of positive patients for p16 alone was 22.72% (5/22), a value that doesn't differ from what was found on the percentage of p16 positive (25%) on the total of 40 patients. The positive patients for HPV DNA alone are 6/22 (27.27%).

Both HPV and p16 are positive in 3 of 22 examined patients (13.63%) and negative in 14 (63.63%; Table 4). Mc Nemar test demonstrate that there isn't a statistically significant difference between p16 nuclear expression an HPV DNA infection (p=1.00). For this reason, p16 is confirmed as a good indirect HPV marker.

P16	HPV – DNA		P
	Negative	Positive	
Negative	14	3	0,10
Positive	2	3	

Table 4: Correlation between p16 overexpression and HPV-DNA.

Discussion

Laryngeal squamous cell carcinoma (LSCC) represents the second malignant tumor of the head and neck with 151,000 new cases per year worldwide[2-14]. Evidence suggests that alcohol and tobacco are causative agents in the development of LSCC; however, over the past few decades the overall incidence of these carcinomas in non-smokers and non-drinkers has been increasing. Studies conducted to evaluate the role of HPV as a risk factor in laryngeal tumors are still insufficient and discordant, reporting widely divergent results in terms of prevalence. In fact, according to a recent meta-analysis, the prevalence of HPV in laryngeal carcinomas varies from 4.9 to 74.6%[2]. This variability can be attributed to the geographic differences among the subjects examined in the different studies, to the inadequate separation of patients with laryngeal carcinoma from the rest of head and neck tumors and to the different sensitivity of the methods used for HPV determination. All these factors, not analyzed in a timely and statistically significant way, didn't allow to evaluate the real prevalence of HPV infection in patients with laryngeal carcinoma[15].

Authors such as Smeets et al. (2007)[16] and Marur et al. (2010)[17] expressed some doubts about the use of p16 as a surrogate marker for HPV infection in head and neck carcinomas. However, in many other studies, the expression of p16 was considered to be equivalent to the genotypic evaluation of HPV in all sites of the head and neck district, even those outside the oropharynx[18], where the prevalence of proven HPV infection is significantly lower. In general, for head-neck tumors the immunohistochemical evaluation of p16 is associated with a lower specificity in the diagnosis of high-risk HPV infection than what has been observed in the cervix. P16, encoded by the CDKN2A gene (9p21.3), is a protein that inhibits tumor growth. It blocks the progression of the cell cycle in the S phase by inhibiting the cyclin-dependent kinases (CDK4 and 6), maintaining Rb in its hypo-phosphorylated state and preventing its dissociation from the transcription factor E2F. Oncogenes E6 and E7 induce the degradation of p53 and pRb tumor suppressors leading to uncontrolled cell proliferation. Since pRb normally inhibits the expression of p16, pRb-free cells over-express this protein that is commonly used as a surrogate marker for high-risk HPV infection[19]. The consequent increase of p16 protein makes it a rational objective of research, using immunohistochemistry, as a prognostic and a surrogate marker for transcriptionally active HPV infection. One of the most relevant aspects that emerged from our study is that the percentage of p16 positive patients correlates significantly with the percentage of positive HPV DNA patients.

Our data reflect what is reported in the literature by authors such as Lasse et al. (2009)[20] and Licitra et al. (2006)[21]: in fact, since no statistically significant difference was shown between the two markers, p16 is confirmed as a reliable marker of HPV infection. Comparing our data with those of Kreimer et al., (2005)[22] the prevalence that we found of HPV in laryngeal carcinomas in our series is similar to the

rest of Europe. These data are also close to that found by other authors, in particular to that found by Hernandez et al. (2014)[23], which conducted studies on the population of the United States, and Bussu et al. (2013)[24] in the valutation of the population of central Italy; while it differs from that of Baboci et al. (2016)[25] who took a Northeastern Italian population into consideration.This confirms the great variability of the prevalence of HPV in various geographical areas and different races in relation to the different life habits[26]. As suggested by Kreimer et al. (2005)[22] geographic heterogeneity must, however, be interpreted with caution because, despite the consumption of smoking varies between countries and different cultures, the predominant cause of HNSCC remains in most of the world linked to these factors and probably has not yet able to explain the differences found in the prevalence of HPV between the different regions and its role in tumorigenesis. No correlation we found between the presence of HPV and clinical-anamnestic data such as age, presence of positive lymph nodes or stage at diagnosis, as for other studies[27].

The last significant data reported in our study is related to the description of the HPV DNA serotypes found. HPV DNA sequences were detected in 6 (27.27%) of the 22 patients examined. Four of the 6 viral genotypes found are high risk: 16, 39, 52 and 67 and are identified in 3 patients. This data assumes an important meaning regarding the possible role of these serotypes on the oncogenesis of laryngeal carcinomas. Overall, HPV 16 is confirmed as one of the most widely reported viral genotypes in these tumors[23]. Furthermore, this is one of the few studies, as well as Barreco et al. (2016)[28] in which HPV 62 was detected at the laryngeal site. This species of alpha-papillomavirus is classified as having a low oncogenic risk and is generally found in skin and mucosal lesions but not in the region of the head and neck. These HPV types, retained as lacking the ability to induce neoplastic transformation, may simply be present in the tumor material but may not be causally related to tumorigenesis.

What emerged could open the door to future research concerning the real significance of high-risk and low-risk genotype and their possible role in the development of laryngeal tumors compared to classic factors such as smoking and alcohol. This our study presents some limitations represented by the limited number of patients taken into consideration. However, the strengths of the study are represented by the homogeneity of the selected patients, who were all subject submitted to surgical treatment in a limited period of time from the same team.

Conclusion

Data collected suggest that the immunohistochemical determination of p16 represents a reliable surrogate for HPV infection in laryngeal tumors. Despite the small sample of patients on which the study was conducted, the results obtained could be considered useful. Although data on HPV infection in Italy are nowadays limited and discordant, what we have found in our study about southern Italian population is similar to European studies, and confirm that HPV could be involved in the development of a subset of laryngeal tumors. Further and new investigations on a larger number of patients could contribute to confirm the importance of the studies conducted so far.

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