HPV infection and clinical profiles in laryngeal diseases.
A preliminary study

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PURPOSE: *The study analysed the presence of HPV in samples tissue from laryngeal chronic hyperplastic inflammation, with and without pre-neoplastic potential, and from squamous cell carcinoma of the larynx. The aim of this analysis was to evaluate the presence/absence of different types of HPV and their relationship to the clinical profile of the patients studied (habit of smoking and drinking).*
METHODS: *Sixty cases were randomly selected from patients undergoing surgical treatment of the larynx for inflammatory/neoplastic lesions and of neck nodes. Patients underwent standard clinical workup, comprising medical history and physical examination, panendoscopy, whole-body CT scan (in cancer patients), diagnostic or therapeutic microlaryngoscopy with laryngeal biopsy, and HPV evaluation.*
RESULTS: *The HPV analysis showed an increased risk for heavy smokers of HPV positivity, as well as precancer lesions and cancer. Type 6 and 16 seem to be prevalent in all types of laryngeal mucosa disease, but pre-neoplastic conditions versus cancer seem to show a wider variety of HPV infections while cancer patients are invariably affected by types 6 and 66. Heavy smoking is related to HPV infection likewise alcohol in association with smoking. Advanced T is more associated with HPV positivity.*
CONCLUSIONS: *These data impose a closer follow-up of smokers and pre-neoplastic cases and the utility of the broad-spectrum polymerase chain reaction assay in laryngeal dysplastic and cancer lesions. This study may allow to develop biomarkers for early detection or recurrence surveillance, to identify therapeutic targets, and to begin individualization of treatment based on the biology of these tumours.*

KEY WORDS: HPV infection, Larynx, Laryngeal chronic hyperplastic inflammation, Squamous cell carcinoma

Introduction

Human PapillomaVirus (HPV) infection can play a major role in the development of benign and malignant upper respiratory tract tumours in humans [1]. Recent evidence suggests, infact, that papillomavirus infection may be crucial in the pathogenesis of head and neck neoplasia. Epidemiologic studies have demonstrated HPV as an etiological agent in laryngeal carcinogenesis [2]. Jacob et al. investigated the frequency of HPV infection in various neoplastic and non-neoplastic laryngeal tissues, its association with the expression of the proliferating cell nuclear antigen (PCNA) and the tumour suppressor protein p53. They concluded that changes in p53 and PCNA expression may be associated with HPV infection and could play a role in laryngeal carcinogenesis [3]. Moreover, it appears that a variety of different HPV types can be found in benign and malignant lesions of the upper aerodigestive tract. Fischer et Al. found sixteen positive cases of HPV (by polymerase chain reaction and/or nested polymerase chain reaction) out of a total of 27 laryngeal papillomas biopsies [4]. This indicates

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the utility of using a broadspectrum assay for screening HPV. The purpose of the present study was to analyse the presence of HPV in tissue samples from laryngeal chronic hyperplastic inflammation, with and without preneoplastic potential, and from malignant epithelial tumours (squamous cell carcinoma) of the larynx in order to verify the presence/absence of different types of HPV and their relationship to the clinical profile of the patients studied (habit of smoking and drinking).

Materials and Methods

Sixty cases were randomly selected from patients undergoing surgical treatment of the larynx for inflammatory/neoplastic lesions and of neck nodes (in tumour patients). The study was undertaken at the Department of Otolaryngology, University of Rome "Tor Vergata". There is no ethical question involved in this study as it is non-experimental and only anonymous data have been used. Nonetheless, proper consent has been obtained from the patients with signed permission allowing us to include their records, in accordance with the Helsinki Declaration. Patients underwent standard clinical workup, comprising medical history and physical examination, panendoscopy, whole-body CT scan (in cancer patients), diagnostic or therapeutic microlaryngoscopy with laryngeal biopsy, and HPV evaluation. The following details were recorded: sex, age, smoker/non-smoker, alcohol intake, degree of mucosal dysplasia, tumour size, node size, distant metastasis, stage (based on TNM, according to UICC staging system). Patients were divided into three groups according to their laryngeal pathology report obtained from surgical specimens: group 1 included 10 patients with laryngeal polyp/cyst; group 2 included 24 patients with chronic dyplastic laryngitis; and group 3 included 26 patients with laryngeal cancer (SCCA). Pathological reports assessed the presence/absence, and degree of, mucosal dysplasia (by LIN classification) or the presence/absence, and degree of invasive squamous cell carcinoma (SCCA) in the laryngeal mucosa and/or cervical lymph nodes, according to the TNM staging system (UICC). HPV presence in specimens from laryngeal biopsies was assessed through the following methods:

-DNA extraction and B-globin amplification: 5 to 10-um thick serial paraffin sections of tissue were cut and accurately collected in 1.5 ml sterile microtubes. Specimens were dewaxed with two xylene washes, followed by rehydrated with a decreasing concentration of ethanol. The DNA extraction was carried out using the commercial Qiamp DNA Mini Kit (Quiagen, Hilden, Germany), according to manufacturer instructions. Recovered DNA was quantified using a spectrophotometer (Ultrospec 3000, Pharmacia). To ensure adequate DNA quality, polymerase chain reaction (PCR) of the B-globin gene was performed in a separate reaction using GH20 and PC04 primers [6]. Following ethidium bromide staining, PCR products (10 ul) were analysed by electrophoresis in 2.5 agarose (Invitrogen, Carlsbad, CA, USA) gel and viewed under UV light. All PCR examinations were carried out using the appropriate precautions, with appropriate positive and negative controls, to avoid cross contamination.

-Detection and Typing of Human Papillomavirus: Broad-spectrum HPV DNA amplification was performed using the short PCR fragment (SPF10) primer set (Innogenetics, Belgium), containing a mixture of 10 sequences, and amplifying a 65bp ri fragment from the L1 region of the HPV genome [8]. Initial denaturation was 95°C (9min), followed by 40 cycles of denaturation at 94°C (30sec), annealing at 52°C (45sec) and extension at 72°C (45sec), with an additional 7min. PCR amplicons were analyzed using the Inno-line probe assay (LiPA; Innogenetics), by means of reverse hybridization [7]. The assay simultaneously recognizes twenty-four individual HPV genotypes (6, 11, 16, 18, 31, 33, 35,39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, 74) and genotype "x".

Statistical Evaluation

A personal computer equipped with the statistical package SPSS for Windows (Chicago, Illinois, release 12.0) was used to carry out the statistical evaluation. Normality

Table I - *Group 1: laryngeal polyp/cyst (10 patients.)*

Patient	Age	Sex	Smoke	Alcohol	Laryngeal pathology	HPV type
1	41	M	3	1	saccular cyst	6
2	58	M	3	1	Polyp	0
3	27	M	2	1	Polyp	0
4	24	F	3	1	Polyp	0
5	47	M	2	0	Polyp	0
6	68	M	0	0	Polyp	0
7	23	M	2	0	Polyp	0
8	45	F	1	1	Polyp	0
9	56	M	4	1	Polyp	0
10	80	M	1	1	Squamous papilloma	0

SEX = M: male; F: female;
AGE = years;
SMOKE = scoring 0: non-smokers; 1: ex-smokers; 2: 1-15 cig. /day; 3: 15-30 cig./day; 4: > 30 cigs. /day;
ALCOHOL = scoring 0: non-drinkers; 1: < 0.5 litre/day; 2: > 0.5 litre/day
LIN = 1: low dysplasia; 2: mild dysplasia; 3: severe dysplasia;
T = tumour size (according to UICC staging system);
N = node size (according to UICC staging system);
M = distant metastasis (according to UICC staging system);
STAGE = based on TNM (according to UICC staging system);
HPV = Human papillomavirus type. Scoring 0: absence of HPV infection.

Table II - *Group 2: chronic dysplastic laryngitis (24 patients.)*

Patient	Age	Sex	Smoke	Alcohol	LIN	HPV type
1	66	M	3	1	2	16
2	73	M	4	0	3	6
3	65	M	4	1	3	6 – 66
4	66	M	3	1	2	6
5	47	M	2	1	3	6 – 66
6	66	M	4	1	1	0
7	43	M	4	1	3	3
8	71	F	3	0	2	6 – 66
9	68	M	3	0	1	6
10	84	M	1	1	3	6 – 51
11	84	M	2	1	2	0
12	77	M	3	2	2	6
13	56	M	3	1	3	0
14	61	M	4	2	1	6 – 16
15	39	M	2	1	1	0
16	63	M	3	0	3	X
17	88	M	1	1	2	0
18	83	M	1	1	2	0
19	63	M	1	2	2	0
20	78	M	3	1	2	16
21	62	M	3	1	1	0
22	42	M	2	1	1	0
23	51	M	1	0	1	0
24	64	M	2	1	2	0

for each age group was tested using a non-parametric test – Kolmogorov-Smirnov – which confirmed the normality of the variable. Comparison among the three groups was also evaluated by parametric tests, namely Anova and Student's Test. A non-parametric test – the chi squared test – was used to study the association between other variables. In many cases dichotomous variables were codified, for which the Fisher's exact test was applied, in addition to using the chi squared test. For the present study, a probability value of less than 0.05 was considered statistically significant.

Results

Mean age of the whole cohort was 58 years, with an age range between 23 and 93 years (mean age for females was lower, though is of no statistical relevance). HPV positive patients were statistically older than those with negative results (t test, p= 0,013) (mann-whitney, p=0,037) (Fig. 1). Group 1 included 10 patients, 8 males (80%) and 2 (20%) females, with an average age of 46.9 years old; 10% (n=1) of patients were non-smokers, 20% (n=2) ex smokers, 30% (n=3) light smokers (1-15 cig. /day), 30% (n=3) heavy smokers (15-30 cig. /day), 10% (n=1) very heavy smokers (> 30 cig. /day). Regarding the consumption of alcohol, 30% (n=3) of patients were no drinkers, 70% (n=7) light drinkers (< 0.5 litre/day) and no patient was heavy drinker (> 0.5 litre/day). Taking into consideration their laryngeal pathology, 10 % (n=1) patients presented saccular cyst, 80% (n=8) developed polyps, 10% (n=1) with squamous papilloma. In group 2, in a whole sample of 24 patients, 23 were males (95.84%) and 1 female (4.16%), with an average age of 65 years old; no patient was non-smoker; 20.83 % of patients (n=5) were ex-smokers; 20.83% (n=5) were light smokers; 37.5 % (n=9) patients were heavy smokers and 20.83% (n=5) were very heavy smokers. Of 24 patients, 20.83% (n=5) were no drinkers, 66.66% of patients (n=16) were light drinkers and 12.5 % (n=3) were heavy drinkers. The 29.16% (n=7) resulted to be LIN1 (low dysplasia), 41.66 % (n=10) presented L2 (mild dysplasia) and the remaining 29.16% (n=7) developed L3 (high dysplasia). Group 3 was composed of 23 (88.46%) males and 3 females (11.53%), with an average age of 62.23 years old. No patient was no-smokers; 15.38% (n=4) of patients were ex smokers 15.38% (n=4) were light smokers; 50% (n=13) were heavy smokers and and 19,23% (n= 5) were very heavy smokers. 30.76 % (n=8) of patients were no drinkers, 46.15 % (n=12) were light drinkers and 23.07% (n=6) were heavy drinkers. According to UICC staging system, 46.15% (n=12) presented first stage, 23.07% (n=6) presented second stage, 7.69% (n=2) a third stage and 23.07% (n=6) fourth stage (Tab. 1-2-3). No correlation was found between age or sex and smoking or drinking habits. Prevalence of HPV positive cases seems to be significantly higher among heavy smokers (> 15 cigarettes/day) as compared to light (0-15 cigarettes/day) or non-smokers (p=0,044). Similar findings emerged for heavy drinkers (>0,5 litres day) vs. light drinkers (<0,5 litre/day) or nondrinkers (p=0,020) (Tables IV and V). According to pathology reports, HPV positive results revealed a statistical association with both pre-cancer and cancer lesions (p=0,026): matching pathology versus HPV by crosstabulation 17/26 laryngeal cancer (65.38%) were HPV positive and 13/24 (54.16%) precancer lesions were HPV positive. 1/10 laryngeal polyp/cyst were HPV positive (10%). HPV positive versus mucosal dysplasia grade (LIN) did not show any statistical correlation. On the contrary, matching tumour size (T) versus HPV by crosstabulation, 13/13 tumours T3-T4 were correlated with HPV positivity (p=0.038).

No correlation was found between nodal status (N) or tumour stage (S). Distant metastases (M) were never present. In benign laryngeal chronic inflammation cases, an HPV prevalence rate of 54.16% (n=13) was revealed. In accordance with available literature [1,] the presence of HPV rose to 26% (n=13) in precancer and cancer lesions (HPV type 6). Analysis of HPV types revealed that HPV 6 was present in 14 cases (23.33 %); HPV 6-66 in 4 cases (6.66%); HPV X in 4 cases (6.66%); HPV 16 in 6 cases (10 %); HPV 3 in 1 case (3,4%); HPV 6-16 in 3 cases (5%), HPV 6-51 in 1 case (1.66%), HPV 3 in 1 case (3.4%), HPV 11 in 1 case (3.4%).

TABLE III - *Group 3: laryngeal cancer (SCCA) (26 patients.)*

Patient	Age	Sex	Smoke	Alcohol	T	N	M	Stage	HPV
1	71	M	3	1	2	0	0	1	0
2	73	M	4	0	3	0	0	2	6
3	77	M	4	0	3	0	0	2	6
4	39	F	4	0	2	0	0	1	0
5	63	M	3	0	4	0	0	3	X
6	76	M	3	1	1	0	0	1	6
7	65	M	4	2	3	0	0	2	6 – 66
8	51	F	3	1	3	0	0	2	6
9	93	M	1	1	2	0	0	1	X
10	50	M	3	2	1	0	0	1	6
11	58	M	3	2	3	2	0	4	6
12	55	M	3	2	4	2	0	4	6
13	55	F	2	1	1	0	0	1	X
14	72	M	3	1	1	2	0	4	0
15	38	M	3	1	1	0	0	1	0
16	54	M	2	2	4	1	0	4	6
17	61	M	4	1	3	0	0	2	16
18	58	M	3	1	4	1	0	4	16
19	74	M	1	2	1	0	0	1	0
20	84	M	2	1	1	0	0	1	0
21	55	M	1	0	1	0	0	1	0
22	65	M	1	0	3	0	0	2	16
23	51	M	3	1	4	0	0	4	16
24	61	M	2	1	2	0	0	1	0
25	65	M	3	0	3	0	0	2	6
26	54	M	3	0	2	0	0	1	11

TABLE IV - *Smoke vs. HPV. Crosstabulation*

Smokers	HPV negative	HPV positive	Total
No/light smokers(scoring 0,1,2)	18	6	24
Heavy smokers(scoring 3,4)	10	26*	36
Total	27	32	60

*p= 0.044

Legend: No/light smokers = scoring 0 (non-smokers), 1 (ex-smokers), 2 (0-15 cigarettes/day);
Heavy smokers = scoring 3 (>15 cigarettes/day), 4 (> 30 cig./day).

TABLE V - *Alcohol vs. HPV. Crosstabulation*

Alcohol	HPV negative	HPV positive	Total
No/light drinkers(scoring 0,1)	27	24	51
Heavy drinkers(scoring 2)	2	7*	9
Total	29	31	60

* p= 0.020

Legend: No/light drinkers = scoring 0 (0 litres/day), 1 (< 0.5 litres/day)
Heavy drinkers = scoring 2 (>0.5 litres/day)

Discussion

HPV infection has become one of the most common sexually transmitted diseases in adults [5]. The oncogenic potential of many HPV types can derive from the persistence of viruses in the upper respiratory mucosa since childhood, which may lead to neoplasia later in adulthood [6]. The virus infects primarily epithelial cells, where it can persist actively (even subclinically) or exist as a long-term latent infection that can become active, with resultant accumulation of host chromosomal mutations [7-8]. The most frequent presence of HPV in dysplastic and neoplastic lesions of the mouth (75%) and of the larynx (66%) is reported in the literature [9]. The results of previous studies analysing the prevalence of human papillomaviruses (HPVs) in squamous cell carcinomas of the head and neck region often vary depending on the different molecular biological methods applied [10,11]. Data on human papillomavirus (HPV) and its involvement in oral, oropharyngeal, sinonasal and laryngeal carcinomas have been reviewed by Syrjanesn in 2005 [14]. The most recent meta-analyses of epidemiological works, in addition to multicentre case-control studies, have confirmed HPV as an independent risk factor for oral cancer [12,13]. The data available in the literature are difficult to compare due to the great variability of techniques used for the determination and typing of the virus, and for the different anatomical site of the lesions. HPV is chang-

ing many aspects of head and neck cancer diagnosis, treatment and prognosis [15,16]. Compared to the past, in which the habit of smoking and alcohol were recognized as the only risk factors, the cases of laryngeal lesions in non-smokers and non-drinkers subjects increased; therefore, the attention has grown to the search for additional risk factors, among which the HPV16 infection [17]. These patients respond better to treatments, such as chemoradiation, tend to remain in remission, and are often cured of the disease [18-19]. Positive HPV patients show better survival compared to the negative HPV group: the integrity of the apoptotic response due to the presence of p53 "wild type" genes, would allow a better response to radio- and chemotherapy [22]. Given these findings, there is a clear need to assess HPV in all head and neck cancer patients to determine its relevance, and to use HPV status as a stratification criterion in designing the next generation of clinical trials. In our study, the analysis of HPV presence in laryngeal chronic hyperplastic inflammation with and without pre-neoplastic potential and in squamous cell carcinoma of the larynx has shown that heavy smoking increases probability of HPV positivity, as well as precancer lesions and cancer. Type 6 and 16 seem to be prevalent in all types of laryngeal mucosa disease, but pre-neoplastic conditions versus cancer seem to show a wider variety of HPV infections while cancer patients are invariably affected by types 6 and 66. In the population of benign/dysplastic lesions of the larynx, it was found that when laryngeal dysplasia is present, the HPV rate rises sharply, as well as in SCCA. It was also evident that heavy smoking is related to HPV infection likewise alcohol in association with smoking is related. In addition, in cancer lesions, advanced T is associated with HPV positivity. These results point out the importance of a closer follow-up of smokers and pre-neoplastic cases exhibiting these subtypes of HPV and the utility of the broad-spectrum polymerase chain reaction assay for human papillomaviruses in laryngeal dysplastic and cancer lesions. A meta-analysis published in 2001 by Miller et al. of 4680 samples between normal mucosa, leukoplasic lesions, dysplastic, and neoplastic head-neck documents a progressive increase in the prevalence of HPV infection with the increase of the atypia [23]. Our observations are superimposable with regard to the progressive increase in infection, even if the greater sensitivity of the technique used in our study has led to numerically higher prevalence values; therefore, the possible responsibility of HPV in the neoplastic transformation of laryngeal mucosal cells seems to be confirmed.

Conclusions

Laryngeal papilloma and respiratory papillomatosis are well-known HPV-induced tumours, though the role of HPV in laryngeal carcinomatosis remains controversial [20-21]. The HPV 6 is present in both the preneoplastic lesions and in the cancerous ones and compared to the 16 and 18 genotypes, could be evaluated in a larger scale. Heavy smoking increases the probability of HPV positivity, frequent in precancer and cancer lesions. The higher prevalence of other risk factors (smoking, alcohol) in subjects with benign or leukoplasic lesions compared to those with dysplasia, may suggest that HPV infection is responsible for the more advanced stages of neoplastic transformation in a partially altered mucosa from other oncogenic agents. In the staging of the malignancy of laryngeal neoplasies, the analysis of the presence of HPV is now essential and in HPV positive preneoplastic patients is mandatory a close follow-up. Understanding of the differences between HPV^{+} and HPV^{-}LSCC tumors may allow to develop biomarkers for early detection or recurrence surveillance, to identify therapeutic targets, and to begin individualization of treatment based on the biology of these tumors. Evaluation of patients with positive HPV lesions and comparison with similar negative HPV patients will allow to better define in the future whether HPV-induced neoplasms actually constitute a more favorable prognosis subtype in order to develop a specific therapeutic protocol.

Riassunto

Il seguente studio si è proposto di analizzare in campioni estratti da tessuto laringeo affetto da infiammazione cronica iperplastica, con e senza potenziale pre-neoplastico, e in campioni di carcinoma a cellule squamose laringeo, la presenza/assenza dei diversi genotipi di HPV e la loro relazione con il profilo clinico dei pazienti studiati (abitudine tabagica e abuso di alcol). Sessanta casi sono stati selezionati in modo randomizzato da pazienti sottoposti a trattamento chirurgico della laringe per patologie infiammatorie / neoplastiche e dei linfonodi del collo. I pazienti sono stati sottoposti a un esame clinico standard comprendente anamnesi ed esame fisico, endoscopia, TC total-body (in pazienti oncologici), microlaringoscopia diagnostica o terapeutica con biopsia laringea e valutazione dell'HPV. L'analisi HPV ha mostrato un aumento del rischio di positività all'HPV per i fumatori definiti "pesanti", nonché di lesioni precancerose e carcinoma. I tipi 6 e 16 sembrano prevalere in tutti i tipi di malattia della mucosa laringea, ma le condizioni pre-neoplastiche rispetto al carcioma sembrano mostrare una più ampia varietà di infezioni da HPV mentre i pazienti oncologici sono invariabilmente affetti dai tipi 6 e 66. Allo stesso modo del fumo "pesante", l'abuso di alcol in associazione con il fumo sono correlato ad un aumento del rischio di infezione da HPV. Carcinomi con T di alto grado sono maggiormente associati a positività di HPV. Questi dati impongono, pertanto, un follow-up più stretto dei fumatori e dei casi pre-neoplastici e l'utilità del test di reazione a catena del-

la polimerasi ad ampio spettro nelle lesioni displastiche e tumorali laringee. Questo studio potrebbe consentire di sviluppare biomarcatori per la rilevazione precoce o la sorveglianza di recidiva, per identificare bersagli terapeutici e per iniziare l'individualizzazione del trattamento basato sulla biologia di questi tumori.

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