Abstract In this retrospective study, we investigated the HPV DNA occurrence in 21 laryngeal and 26 primary lung squamous cell carcinomas. Nonisotopic in situ hybridization (NISH) technique was performed with commercially available digoxigenin-labelled DNA probes for HPV screening. Subtyping for HPV subtypes 6/11, 16/18 and 31/33 was also performed. We observed HPV DNA signals in 10 (47.6%) cases of laryngeal SCC and in only 3 (11.5%) cases of lung SCC. Typing showed signals of HPV 6/11, 16/18 and 31/33 infection in 80%, 40%, 30% of the laryngeal carcinomas, respectively. In the lung, we demonstrated type 16/18 positivity in two and type 6/11 in one of the HPV-positive cases. We found a statistically significant correlation between HPV infection and tumour recurrence \( (p<0.035) \) in laryngeal carcinomas, but not between HPV presence and tumour stage or grade in neither larynx nor lung.

Key words HPV DNA • In situ hybridization • Larinx • Lung • Intraepithelial neoplasia • Squamous cell carcinomas

Introduction

Human papillomavirus (HPV) DNA has been implicated in the pathogenesis of carcinomas of many different sites, especially the cervicovaginal area, vulva, oropharynx and upper respiratory tract. The simultaneous effects of various carcinogens on the respiratory tract epithelium are thought to generate the precursor and malignant lesions through a process termed “field cancerization” [1]. The carcinogenic agents that exert this field effect are tobacco, its derivatives, and a large number of pollutants and viruses, especially HPV.

HPVs are non-enveloped, double-stranded DNA viruses which belong to papovaviridae. Molecular cloning of viral nucleic acids, polymerase chain reaction and sequencing have demonstrated more than 70 types of HPV. Certain HPV types, particularly HPV 16 and 18 and to a lesser extent 31/33, have been shown to be associated with cervical intraepithelial neoplasia and invasive cancer. There are some studies supporting the existence of HPV 11 in histologically normal larynges, chronic laryngitis, vocal nodules, solitary papillomas, juvenile laryngeal papillomatosis and adult respiratory papillomatosis of the larynx [2-5]. HPV occurrence has been demonstrated in 11%-60% of squamous cell carcinomas (SCC) of the head and neck [6-12]. The presence of HPV DNA in SCC of the lung is more controversial, having been found in 0%-79% of sample [13-16].

The aim of this study was to examine the occurrence and the subtypes of HPV DNA in primary larynx and lung SCCs. We also studied the role of HPV DNA in the carcinogenesis of lung and larynx SCCs.

Patients and methods

We studied 21 patients with laryngeal carcinoma and 26 with lung carcinoma, all diagnosed as having primary SCC. All patients were smokers. The patients’ median age was 67 years.
(range, 56-73 years). The slides of the cases were reviewed and tumours were classified according to the WHO classification of upper respiratory tract tumours. Staging was performed according to the American Joint Committee on Cancer classification, 1999 (Philadelphia, 1992, J.B. Lippincott). They were graded into well, moderately and poorly differentiated on the basis of degree of differentiation, cellular pleomorphism, and mitotic activity. Paraffin sections (5 µm) were cut from the representative blocks for in situ hybridization.

Nonisotopic in situ hybridization

Nonisotopic in situ hybridization (NISH) technique was performed with commercially available digoxigenin-labelled DNA probes for HPV screening (Kreatech hkd38000). Subtyping for HPV subtypes 6/11, 16/18 and 31/33 was also performed (Biogenex hk863-2k and super sensitive ISH detection system Biogenex 230-ss). Signal screening was performed using 5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium (BCIP/NBT). Anal condyloma was used as a positive control.

Briefly, the sections were deparaffinized in xylene and then rehydrated in gradient ethanol washes. After H2O2 blocking, nucleic acid unmasking was assessed by incubating in proteinase K (100 µl/ml), followed by phosphate-buffered saline (PBS) washing for 5 min. The sections were dried for 5 min at room temperature. Then, the slides were incubated with prehybridization solution for 1 h at 37°C. After washing with absolute alcohol for 3 min, slides were dried at room temperature for 5 min. Hybridization was performed at 95°C for 10 min and slides were left at 37°C overnight in a hot air humidified oven. The slides were washed with two changes of double-strength (2×) saline-sodium citrate (SSC) at room temperature (5 min per change) followed by hybridization wash. They were further washed with two changes of 2× SSC at room temperature and one of 1× SSC (5 min per change). Detection was performed briefly by protein blocking solution at room temperature for 20 min, followed by three changes of PBS (5 min per change). Link 1, link 2 and label were applied for 20 min, each separated by three changes of PBS (5 min per change). Substrate working solution was applied at room temperature for 20 min, and the slides were counterstained with Mayer’s hematoxylin and mounted in glycerol jelly. Alu probe (genomic DNA) was used as a positive control. Additional negative controls were performed on adjacent sections by omitting the specific HPV DNA step of the protocol.

Statistical analysis

Statistical analysis was based on two-sided Fisher’s exact test. The computations were performed using the GraphPad InStat version 2.04 (GraphPad Software, San Diego, California, USA) The statistical difference was considered significant if the p value was less than 0.05.

Results

We observed HPV DNA signals in 10 (47.6%) cases of laryngeal SCC and in only 3 (11.5%) cases of lung SCC (Fig. 1). We did not observe HPV DNA in 11 (52.4%) cases of larynx SCC (Table 1). Typing showed signals of HPV 6/11, 16/18 and 31/33 infection in 80%, 40%, 30% of the laryngeal carcinomas, respectively (Table 2). There was coinfection with 6/11 and 16/18 in 2 cases; 6/11, 16/18 and 31/33 in one case and 6/11 and 31/33 in another case (Table 2). Our results demonstrated statistically a significant correlation between HPV infection and tumour recurrence (p<0.035), but not between HPV presence and tumour stage or grade. In the lung, we demonstrated type 16/18 positivity in 2 and type 6/11 in 1 of the HPV-positive cases. There was no correlation between HPV positivity and clinical behaviour (tumour stage or recurrence).

Discussion

HPVs induce unrestricted cellular proliferation by integrating into the host genome, leading to uncontrolled expression of the viral genes E6 and E7, and amplification of c-myc and c-ras-1 oncogenes. It has been postulated that HPV acts as a promoter in cancer with other carcinogenic agents including other viruses (e.g. herpes simplex), chemical carcinogens (e.g. tobacco smoke, nitrosamines) or immunodeficiencies [17]. Park et al. [18] showed that sequential exposure of oral keratinocytes to HPV-16 DNA and benzo(a)pyrene converts normal cells to tumorigenic cells in vitro [18]. This finding enforced the results of in vivo studies.

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HPV coinfection and its subtypes are predictors of an aggressive clinical course in laryngeal SCCs [10, 12, 13]. In our series, there was co-infection in 4 cases of laryngeal SCCs. Two of these cases, which were stage III and I, had co-infection with 6/11 and 16/18 subtypes. One was stage II with 6/11, 16/18 and 31/33 positivity. The last case was stage III infected with 6/11 and 31/33 subtypes. There was recurrence only in the last of these 4 cases. This recurrence may be related to tumour stage rather than to HPV coinfection.

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In our study, 80% of HPV-positive laryngeal SCCs showed HPV 6/11 presence, and all of the coinfected cases demonstrated HPV 6/11 positivity. However, the importance of HPV 6/11 presence in laryngeal carcinomas is not well
known. HPV 11 has been found not only in histologically normal or inflamed larynges but also in benign tumours of the larynx [2, 4, 5]. Certain HPV types, namely 16, 18, 31, 33 and 35, occur more frequently in carcinomas [9, 12, 13]. We also demonstrated that 60% of HPV-positive cases had these high-risk types of HPVs. Although we did not reveal a correlation with either the tumour stage or grade, there was a statistically significant correlation of HPV DNA occurrence the tumour recurrence ($p<0.35$).

Iwamasa et al. [19] demonstrated that virus-infected pulmonary SCCs were histologically well differentiated with a better prognosis compared to noninfected SCC cases. We did not reveal any significant correlation between HPV DNA positivity and prognosis in lung SCCs. These discrepancies may be attributable to either the sensitivity of various methods or to epidemiological differences.

In conclusion, our study suggests that HPV may be an important factor in the carcinogenesis and prognosis of SCC of the larynx, but not of the lung.

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**Table 1** HPV-negative laryngeal SCC cases. There were no recurrences in this group

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**Table 2** HPV-positive laryngeal SCC cases. There were no cases of lymph node metastasis in this group

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**Riassunto** In questo studio retrospettivo abbiamo studiato la presenza di HPV DNA in carcinomi squamosi primitivi di laringe e polmone. È stata condotta una ibridazione in situ non isotopica (NISH) con sonde commerciali DNA HPV legate con digossigenina. È stata inoltre condotta una sottotipizzazione per HPV 6/11, 16/18, 31/33. Abbiamo osservato segnale HPV DNA in 10 casi di carcinoma del laringe (47.6%) e in 3 casi di carcinoma squamoso del polmone (11.5%). Nel carcinoma del laringe la sottotipizzazione ha mostrato segnale nell’80% di HPV 6/11, 40% di 16/18 e 30% di 31/33. Nel polmone abbiamo dimostrato positività 16/18 in due casi e 6/11 in un caso. Abbiamo osservato una correlazione statisticamente significativa fra l’infezione da HPV e la recidiva tumorale (p<0.0351) nei carcinomi del laringe, ma non correlazioni con lo stadio o il grado dei vari tumori.

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**References**