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Detection of human papillomaviruses in squamous cell carcinomas of the lung

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Abstract The aim of this study was to evaluate the possible association of human papillomaviruses (HPV) with the development of squamous cell lung carcinomas (SqCLCs). Tissue material from 52 cases of SqCLCs were studied, and the data were evaluated according to the degree of differentiation, HPV presence and type. Analysis was performed by polymerase chain reaction (PCR) method using consensus primers, and the results were confirmed by subsequent Southern blot hybridization. Overall, the results showed 69% positivity (n=32). Forty-one cases were examined for the presence of specific HPV types (6/11 and 16/18) by hybridization of the PCR products with ^{32}P-labelled probes. HPV 6/11 types were detected in 6 of the 29 positive cases (20.6%). HPV 16/18 types were the most prevalent types, and were detected in 11/29 cases (37.9%: 4/10 of well-differentiated cases, 6/25 of moderately and 1/6 of poorly differentiated carcinomas). Our results confirm the possibility that HPV might play a role in the development of SqCLCs and suggest a possible relation of high-risk HPV16/18 types to tumour differentiation.

Key words Human papillomaviruses · Squamous cell lung carcinoma · Polymerase chain reaction · Southern blot hybridization

Introduction

Pulmonary carcinomas are common cause of death from neoplasms, particularly among men, with rates rising steadily in both men and women. Lung cancer of the squamous type constitutes approximately 35% of the major subtypes of lung cancer in men [5]. Smoking is considered the most important aetiological factor, although not the only determining criterion for the prevalence of the disease, other factors also having possible aetiological significance. Carcinogenesis is a multi-step process, which may involve interactions between oncogenes, tumour suppresor genes (TSG) and oncogenic viruses, including some types of human papillomaviruses (HPV) [37].

HPV are small DNA viruses that infect squamous epithelia. More than 77 different HPV genotypes have been identified, most with specific site (cutaneous/mucosal) or lesion preferences [8]. They have been shown to be involved in both malignant and benign tumours. HPV DNA is detected in approximately 10% of all human neoplasms; such viruses may, then, be related to the aetiology of a number of tumours [20]. There is strong support for the role of specific HPV types in the pathogenesis of squamous cell carcinoma of the anogenital tract, and more than 90% of cervical carcinomas contain HPV DNA, usually of the high-oncogenic-risk HPV types 16 and 18 and less frequently of intermediate-risk HPV types 31, 33 and 35 [15, 38]. DNAs from high-risk HPV types are frequently integrated into genomic DNA, and E6/E7 oncoproteins accelerate the cell cycle by inactivation of tumour suppressor proteins [39].

HPV infection has been reported in dysplasias and carcinomas of several organs, including the skin, penis, vulva, oropharynx, and larynx [1, 11, 18]. Despite these well-recognized associations, very few data are available concerning the possible involvement of HPV in lung neoplasms. The role of HPV infection in the pathogenesis of the aerodigestive tract carcinomas is still controversial, but bronchial mucosa is probably liable to HPV infection [16, 24]. The HPV prevalence rates in lung carcinomas vary among studies, ranging from zero to 79%. Initially Syrjanen began to investigate the HPV-induced alterations in bronchial mucosa and Bejui-Thivolet et al. studied the presence of HPV in 33 cases of squamous

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cell carcinomas of the lung (SqCLCs). They found 3 positive for HPV 18, 1 for HPV 16, 1 for HPV 11 and 1 for type 6 by in situ hybridization (ISH) [3, 28, 29]. Yousen et al., using a similar method but with a larger battery of biotinylated probes, found 31% HPV positivity in primary lung neoplasms [36]. In contrast, Szabo et al. and Shamanin et al. found no HPV infection in lung cancer biopsies analysed by means of the polymerase chain reaction (PCR) [23, 30]. Recent studies using the PCR method showed variable positivity rates for HPV infection in lung carcinomas. This variability may be attributed to the sensitivity and specificity of the method used or to the geographical distribution of the disease [10, 17, 26].

The aim of this study was to evaluate the possible association of HPV with the development of SqCLCs and to determine whether HPV infection was a common event in the pathogenesis of SqCLC. This study represents the first report of HPV detection and typing in SqCLCs biopsies in patients in Greece. For this purpose we examined 52 cases of SqCLCs by the PCR method using consensus primers and followed this by analysis of the PCR products with Southern blot hybridization using general and type-specific probes for low- (HPV 6/11) and high-risk (HPV 16/18) HPV types. The results of the study support a possible association of HPV with SqCLCs.

Materials and methods

Tissue material from 52 cases of squamous cell lung carcinomas diagnosed in the Pathology Department of Athens Hospital for Thoracic Diseases was analysed for the presence of HPV DNA. These specimens were mostly surgical specimens (48 cases), while 4 were bronchial biopsies obtained under bronchoscopic guidance. The cases were selected on the basis of histological evaluation according to the WHO classification. After careful examination the specimens were found to contain histopathological features suggestive for HPV association such as alterations reminiscent of condylomata (evident focally in histologically differentiated cases) and individually or dispersed koilocytic tumour cells (Fig. 1). Specimens from nonneoplastic peripheral sites and pulmonary lymph nodes without metastasis were used as negative controls. In 26 of the total number of cases the DNA was extracted from paraffin-embedded tumour specimens, while for the rest of the cases DNA was obtained from frozen tumour specimens.

To extract DNA from frozen specimens, small pieces taken from the tumour specimens were minced well and incubated overnight at 55°C in 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 0.25% SDS and 200 μg/ml proteinase K. The DNA was extracted with phenol/chloroform, precipitated and resuspended in dH_2O [14, 15].

Sections (5–10 μm) from paraffin-embedded biopsies were used for DNA extraction according to a modification of the method published previously [34]. Specifically, the sections were incubated twice with xylene to dissolve paraffin, and once with ethanol. The remaining pellet was resuspended in 300 μl of digestion buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% Tween 20 and 200 μg/ml proteinase K. The digested products were extracted with phenol/chloroform, precipitated with ethanol, and resuspended in dH_2O.

PCR reaction mixtures were prepared according to the protocol of Manos et al. [32]. A 2-μl DNA sample was amplified in 100 μl reaction mixture containing 1,5 mM $MgCl_2$, 10 mM Tris, pH 8.0, 200 μM of each dNTP, 50 pmol of each consensus primer (MY09/MY11) and 2.5 U of Taq polymerase. The PCR mixture was overlaid with paraffin oil and subjected to 35 cycles of amplification, consisting in denaturation at 95°C (50 s), annealing at 55°C (50 s) and extension at 72°C (1 min). Amplification products were analysed by agarose gel electrophoresis, followed by ethidium bromide staining and visualization under UV light.

DNAs from recombinant plasmids containing HPVs (6, 11, 16, 18) or extracted DNA from condylomata containing HPV 6/11, as determined with ISH analysis, were used as positive controls in the amplification reaction. Samples containing distilled water or DNA from pulmonary alveolar tissues and lymph nodes were used as negative controls. Different steps of PCR analysis, such as sample preparation and amplification reactions, were performed separately to avoid problems of contamination. To exclude false-negative results all specimens were tested for amplification with GH2O and PCO4 primers of the β-globin gene [2].

For Southern blot analysis, 10 μl of the PCR products and 5 μl of amplified cloned HPV 6, 11, 16, and 18 were separated in 1.5% agarose by electrophoresis, denatured in 0.5 M NaOH, 1.5 M

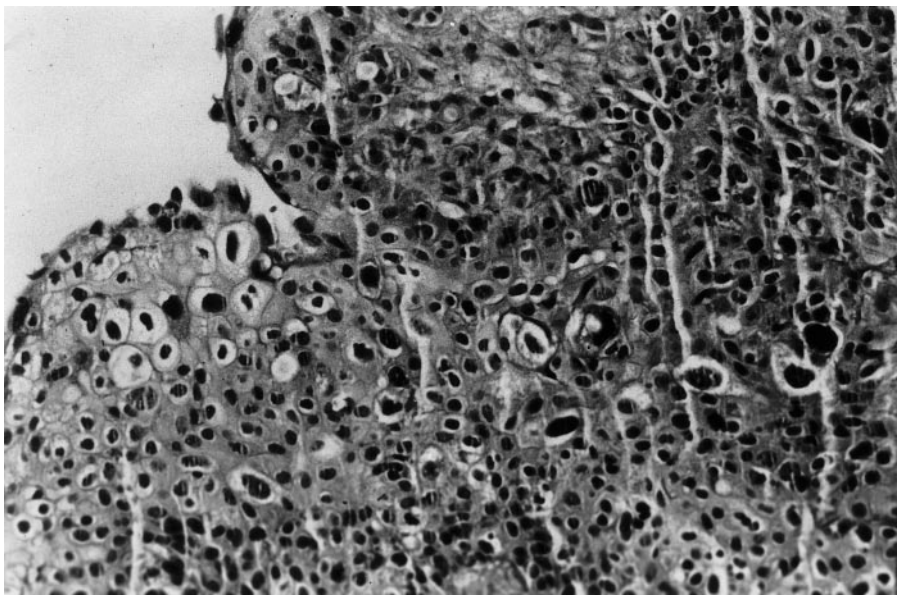

Fig. 1 Squamous cell carcinoma with koilocytic features. Haematoxylin-eosin stain, ×400

NaCl, neutralized in 1.5 M NaCl, 0.5 M Tris, pH 7.2, 0.001 M EDTA and transferred to a nylon membrane filter (Hybond N, Amersham) overnight in 2×SSC. Filters were incubated for 2 h at 55°C in prehybridization solution containing 6×SSC, 5×Denhardt's, 0.1% SDS and 100 μg/ml salmon sperm DNA. Hybridization was carried out in the same buffer for 4 h at 55°C, with the addition of labelled probe mix. Oligonucleotide probe mix (GP1 and GP2) and type-specific probes (MY12, MY13, MY14, WD74) for the PCR products of HPV 6, 11, 16, and 18 respectively, were end-labelled with [γ-^{32}P] ATP using T4 polynucleotide kinase (USB). Membranes were washed twice for 15 min in 2×SSC, 0.1% SDS at 50°C for hybridization with the general probes and at 56°C or 58°C for MY12/MY13- and for MY14/WD74-specific probes respectively. Autoradiography was performed for 3 h or 24 h on Kodak XR-Omat film, using an intensifying screen at –70°C. It should be noted that hybridization with the specific probes was carried out using the same membrane. The membrane was first hybridized with HPV 6/11 probes and then rehybridized with HPV 16/18 probes.

The data were evaluated according to the following variables: degree of differentiation, HPV presence and HPV 6/11 and HPV 16/18 types. As 49 of the 52 cases referred to male patients, no evaluation was needed regarding the sex of the patients.

Results

Tissue material from 52 cases of SqCLC of various degrees of differentiation was analysed by PCR analysis combined with Southern blot hybridization for the presence of HPV DNA sequences. Table 1 shows the degree of differentiation of the study cases and the results based on the hybridization with general and type-specific probes of the PCR products amplified with consensus primers. The specimens were classified as follows: those that gave no HPV amplification products detectable by gel electrophoresis or by hybridization (HPV negative), those whose PCR products were detectable with the general probe but not with any of the type-specific probes (presumably reflecting HPV types other than HPV6/11/16/18), and those that yielded PCR products detectable with both the general probe mix and with the type-specific probes.

Specifically, HPV DNA was detected in 36 out of 52 cases (69%). Twenty-one cases (40%) showed the presence of HPV DNA sequences by PCR after ethidium bromide stained agarose gel electrophoresis of PCR products (data not shown). However, 15 other specimens showed positivity only when Southern blot hybridization was performed using the general probe mix GP1 and GP2. HPV DNA sequences were detected in 6 out of 8 poorly differentiated, in 22 out of 32 moderately differentiated and in 8 out of 12 well-differentiated SqCLCs. There was an equal distribution of HPV sequences in samples taken from frozen (n=19) and those taken from paraffin-embedded tissue (n=17).

From the total number of cases, 41 specimens were examined for the presence of specific HPV types (6/11 and 16/18) by hybridization of the PCR products with ^{32}P-labelled probes. HPV 16/18 was detected in 4 out of 10 well-differentiated cases (40%), and in 6 of 25 moderately (24%) and in 1 of 6 poorly differentiated cases

Table 1 Differentiation and results based on hybridization with general and type-specific probes of PCR products amplified with consensus primers. (*ND* not done, 1–26 frozen samples, 27–52 paraffin samples)

Case no.	Degree of differentiation	HPV status	Papillomavirus type		Case no.	Degree of differentiation	HPV status	Papillomavirus type	
			6/11,	16/18				6/11	16/18
1	Poor	+	–	+	27	Well	+	–	+
2	Well	+	+[a]	+[a]	28	Moderate	+	–	–
3	Moderate	+	+[a]	+[a]	29	Moderate	+	+	–
4	Poor	+	–	–	30	Moderate	–	–	–
5	Poor	+	+	–	31	Moderate	+	+	–
6	Moderate	+	–	–	32	Moderate	+	–	+
7	Moderate	–	–	–	33	Poor	+	–	–
8	Moderate	+	–	–	34	Moderate	+	–	+
9	Moderate	–	–	–	35	Moderate	–	–	–
10	Well	+	–	+	36	Moderate	–	–	–
11	Well	–	–	–	37	Moderate	+	–	+
12	Moderate	+	–	–	38	Moderate	–	–	–
13	Moderate	+	–	–	39	Well	–	–	–
14	Moderate	+	–	–	40	Moderate	+	–	+
15	Well	+	–	–	41	Moderate	+	+	–
16	Poor	–	–	–	42	Moderate	+	ND	ND
17	Moderate	–	–	–	43	Moderate	+	ND	ND
18	Poor	+	–	–	44	Moderate	–	ND	ND
19	Well	+	–	–	45	Poor	+	ND	ND
20	Well	+	–	+	46	Well	–	ND	ND
21	Moderate	+	–	–	47	Well	+	ND	ND
22	Well	+	–	–	48	Moderate	–	ND	ND
23	Moderate	+	–	–	49	Poor	–	ND	ND
24	Moderate	+	–	+	50	Moderate	+	ND	ND
25	Moderate	–	–	–	51	Moderate	+	ND	ND
26	Well	–	–	–	52	Moderate	+	ND	ND

[a] Mixed 6/11 and 16/18

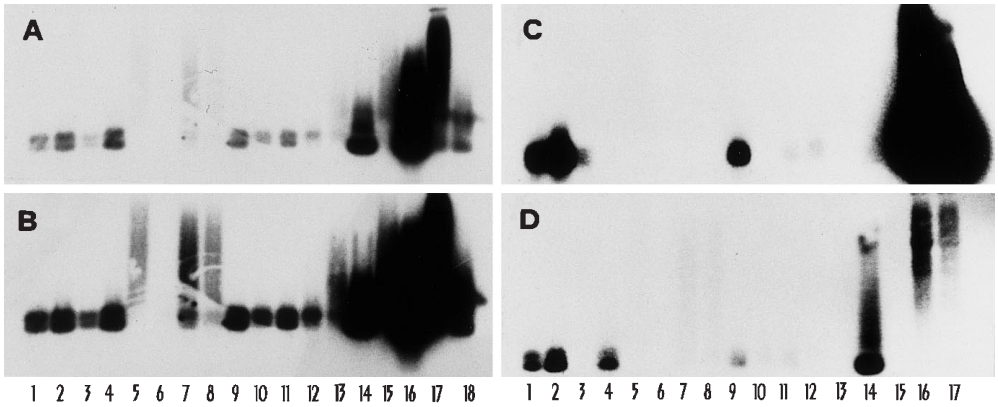

Fig. 2A, B Southern blot hybridization of PCR amplified DNAs from squamous cell lung carcinoma specimens, using GP1/GP2 probes after (**A**) 3 h and (**B**) 24 h exposure. Type-specific hybridization (**C**) with HPV 16/18 and (**D**) with HPV 6/11 probe mixture after 24 h exposure. *Lanes 1–13* specimens from patients, *lane 14* HPV6/11-positive sample processed in the same way as the lung specimens and used as positive control, *lane 15* negative PCR control, *lanes 16, 17* HPV 16 and HPV 18 plasmid DNA, respectively. *Lane 18* HPV 6/11-positive sample (**A**, **B**). Hybridization with type-specific probes was performed using the same membrane, i.e. hybridized with HPV 6/11 probe mixture (**D**) followed by probe removal (70% formamide) and subsequently hybridized using HPV 16/18 probe mixture (**C**)

Table 2 HPV typing in 29 HPV-positive squamous cell lung carcinomas (*ND* type not determined)

HPV type	6/11	16/18	Mixed[a]	ND
Positivity	6/29 (20.6%)	11/29 (37.9%)	2/29 (7%)	14/29 (48%)

[a]Two specimens contained both type 6/11 and type 16/18

(16.6%). HPV 6/11 was detected in 1 of the well-differentiated cases (10%), in 4 of the moderately differentiated cases (16%), and in 1 of the poorly differentiated (16.6%) carcinomas.

Figure 2 shows representative hybridization results of the PCR-amplified HPV DNA sequences with the general probes after 3 h (Fig. 2A) and 24 h (Fig. 2B) exposure as well as with type-specific probes for HPV types 16/18 and 6/11 after 24 h exposure (Fig. 2C, D). Most of the positive cases showed a strong hybridization signal with the general probes only (Fig. 2B, lanes 3, 7 and 11), while negative specimens and negative PCR controls showed no hybridization signals (Fig. 2A, lanes 5, 6 and 15). Cases in lanes 10–12 (Fig. 2C, D) gave low-intensity bands in the hybridization with type-specific probes (all lanes have equal amounts of DNA) and represent positive specimens, most probably with HPV 6/11- and HPV 16/18-related types. Two specimens produced hybridization signals for HPV 6/11- and 16/18-specific probes (lanes 1 and 2) and were therefore scored positive for both sets of probes.

The prevalence of HPV 6/11 and HPV 16/18 is shown in Table 2. HPV 6/11 was detected in 6 cases (20.6%) and HPV 16/18 in 11 of 29 positive cases (37.9%). Two cases, 1 well and 1 moderately differentiated, were positive for both HPV 6/11 and HPV16/18 probes.

Discussion

It has been a decade since several investigators reported the presence of HPV DNA in carcinomas of the upper digestive and respiratory tracts [7, 25]. Using Southern blot and/or ISH techniques, HPV DNA was initially detected in carcinoma samples of the oral cavity, larynx and lung [13, 19, 27]. These initial observations were followed by a number of publications confirming the presence of HPV in such tumours and demonstrating the transforming potential of the high-risk HPV types on cultured epithelial cells derived from these organs [22, 33].

In the present study a close association between HPV infection and squamous carcinomas of the lung was demonstrated. HPV DNA sequences were detected by PCR analysis using consensus primers, and the resulting products were analysed by Southern blot hybridization with general and type-specific radioactive probes. These techniques are presently considered to be the most sensitive methods of HPV detection and typing.

HPV was detected in about 40% of SqCLCs by PCR analysis alone, and in 69% by Southern blot hybridization of the PCR products. This suggests that a limited amount of HPV DNA could be detected by a more sensitive method than the PCR alone. In this study the HPV prevalence rate in lung carcinomas is higher than that previously reported [4, 12, 31, 36]. This may reflect a higher sensitivity of the PCR method, combined with the ability of consensus PCR primers to detect a broader spectrum of HPVs. Nevertheless, our results are compatible with those of Hirayasu et al., who found 34 cases (79.1%) positive for HPV DNA in patients from Okinawa, using PCR [10]. Moreover, Soini et al. found HPV DNA in 13 out of 43 cases of lung carcinomas by ISH and reported in the same study an additional 13 positive cases detected by nested PCR [26]. In addition antibodies to E6 and E7 proteins of HPV 16 were found in a higher proportion of lung cancer patients (42%) than of controls (13%) [25]. In contrast, two recent studies based on PCR analysis us-

ing a different set of primers and probes for HPV detection found no HPV-positive cases [23, 30].

The fact that none of the specimens from nonneoplastic peripheral sites or pulmonary lymph nodes without metastasis, which were used as negative controls, showed hybridization with any of the probes excludes the possibility of false-positive PCR results caused by possible infection of neighbouring non-neoplastic cells or by cross-contamination between specimens.

In the present study, classic morphological alterations characteristic of HPV infection, such as a papillomatous growth pattern similar to that described by Bejui-Thivolet et al. (koilocytosis in focal areas or in individual cells), were found in the tumours examined (Fig. 1) [3, 4]. Moreover, earlier ISH studies have shown a focal HPV presence in neoplastic cells of the lung [12]. Such a mosaic pattern may be attributed to a nonclonal development of HPV in the tumour, and this could explain the low copy number found in these tumours. The association of the virulence of the HPV with the tumour development is not well characterized, since no clear transmission pattern of the infection from cell to cell has been documented histologically. Yeudall et al. suggested a hit-and-run mechanism following their examination of oral carcinoma-derived cell lines, which revealed low copy number HPV16 DNA (fewer than 1 copy per cell) at early passage while at later passages no viral DNA was detectable [35]. According to the authors, these data suggest that only a subpopulation of cells initially harboured HPV 16 DNA and that culturing of those cells resulted in a loss of viral nucleic acids or caused a possible negative selection. If such a hit-and-run mechanism does occur in vivo it will have far-reaching consequences, since the implication is that HPV-negative carcinomas may also develop from HPV-containing lesions. In fact, HPV nucleic acids have been detected in squamous metaplasia of the bronchus and hyperplastic and dysplastic epithelium of the lung [4, 29, 36].

Our results are also in agreement with those of other studies referring to the prevalence of high-risk HPV types 16/18, which were found in 37.9% of cases in this study. Similarly, HPV 6/11 types were detected in 6 of the 29 positive cases (20.6%), while 2 of the cases were positive for both set of probes (7%). Although infections with types 6/11 rarely occur in invasive tumours and are considered a remote risk in tumours of the anogenital tract, the presence of HPV 6 or 11 sequences has been reported especially in nongenital malignant lesions [37]. Extensive analysis of these sequences showed duplication of transcriptional regulatory elements or mutations [6]. This probably means that such molecular changes may be necessary to confer malignant potential. Similarly, duplications in HPV 6 and HPV 11 genomes have also been reported in the lung [9, 21].

It should be noted that in our study, some HPV infection was shown in poorly differentiated SqCLCs. However high-risk HPV types 16/18 were detected mostly in well-differentiated squamous cell carcinomas, a finding compatible with that reported by Hirayasu et al., who found an association between detection of HPV 16 and 6 and the histological differentiation of carcinomas [10]. Although the history of the gradual identification of HPV infection seems to associate such infection with more highly differentiated lesions, the evaluation of larger numbers of tumours of varying degrees of differentiation, combined with the use of a broader panel of HPV types, is needed to confirm this hypothesis.

We conclude that among other factors contributing to the development and progression of squamous lung cancer, HPV infection might have a role.

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