Detection of human papilloma virus (HPV) and K-*ras* mutations in human lung carcinomas

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Abstract. The purpose of our study was to assess the prevalence and prognostic significance of HPV infection as well as K-*ras* codon 12 point mutations in lung cancer. Patients diagnosed with lung carcinoma between 1988 and 1992 (N=99) were selected. HPV detection and typing was performed by PCR from paraffin-embedded tissues, while mutations in codon 12 of K-*ras* gene were detected using the restriction fragment length polymorphism (RFLP) analysis. The prevalence of HPV infection was 15%, while K-*ras* codon 12 point mutations were found in 18% of the specimens examined. In 50% of the HPV-positive cases, K-*ras* gene mutation co-existed. HPV 18 was the most frequent type. No correlation was found between K-*ras* mutation and HPV infection with sex, age and clinical outcome of the patient, or the histological type and the differentiation grade of the tumor. An association was found between K-*ras* codon 12 point mutations and the stage of the tumor, occurring more frequently at stage III (p=0.037). Infection with potentially oncogenic HPV types could co-operate with K-*ras* gene activation in the progression of the disease, since K-*ras* activation by point mutations seems to be a late event in lung carcinogenesis.

Introduction

Carcinoma of the lung has become increasingly frequent during the past 50 years. It is now the leading cause of cancer mortality in the Western world. This is due not only to increased recognition through better radiographic, bronchoscopic and cytologic techniques but also to an actual rise in incidence. Carcinoma of the lung is associated with various environmental factors, most importantly smoking and asbestos exposure (1-3). However, many heavy smokers remain free of this disease or other smoking-related cancers. It has been suggested that genetic factors may also contribute to the development of lung carcinoma. So far, no classical linkage analysis that correlates incidence of the disease with the inheritance of genetic markers, has been reported for lung cancer (4).

During recent years, a great deal of data has been accumulated on the role of HPV in the development of carcinoma of different anatomical sites of the body. Molecular biology techniques have disclosed that there are at least 73 different types and several subtypes of HPV (5,6). Most viral types are observed in anogenital tract and skin lesions (7). HPV has also been detected in carcinomas of the oral and nasal cavity (8), the male urethra (9), the urinary bladder (10-12), the esophagus and the respiratory tract.

With regard to the upper respiratory tract, many types of HPV (6, 11, 16, 30) have been detected in laryngeal carcinomas (13,14). Clear etiological evidence indicated that in laryngeal papillomas infection with HPV types 16 and 18 was associated with progression to malignant lesions, whereas HPV types 6b and 11 were usually associated with benign lesions (15).

In the lower respiratory tract, the causative role of HPV was suggested by morphological data already in 1979: cytological and histological similarities with lesions from uterine cervix condylomas were described in about 30% of bronchial epithelium next to invasive bronchial squamous cell carcinoma (16) or well differentiated squamous cell carcinoma (17,18). HPV sequences have also been demonstrated in primary lung squamous cell carcinomas (19-21). Patients with squamous cell papillomas exhibiting HPV 16 or 18 positivity are at high risk for the development of squamous cell carcinoma. Virus typing seems to be a better prognostic indicator than grading of dysplasia or age relationship, while virus typing by the PCR is more sensitive compared with *in situ* hybridization (ISH) (22).

Moreover, molecular tumor markers may offer clinically useful tools for diagnostic and prognostic purposes in lung cancer (23). *Ras* genes are often found activated in a variety of tumor types, although the incidence varies greatly. K-*ras* gene is by far the most frequently involved in lung tumors (24-27). Point mutations of the K-*ras* gene were observed primarily in patients with a habit of smoking. Patients with K-*ras* positive tumors have a significantly poorer prognosis than patients with K-*ras* negative tumors (28). There is evidence that K-*ras* gene may serve as a genetic marker not only in the early detection of lung cancer (29) but also in the differential diagnosis of recurrence or metastasis versus second primaries of the lung (30). In addition strong overexpression of K-*ras* gene has been reported in a high incidence of non-small cell lung carcinomas (31).

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In the current study, HPV detection and typing as well as detection of K-*ras* gene mutations was performed in 99 cases of lung carcinoma from paraffin-embedded tissues employing the PCR technique. 15% of the specimens were found positive for HPV, while 18% carried mutation in the codon 12 of the K-*ras* gene. It was also examined whether such alterations correlate with clinicopathological parameters or clinical outcome.

Materials and methods

Patients and specimens. The primary neoplasm specimens were excised either by surgical lobectomy or pneumonectomy and fixed in neutral formalin, at Sismanoglion General Hospital, Athens, between 1988-1992. Hematoxylin-eosin stained sections from all paraffin-embedded tissues examined, were reviewed to reconfirm the tumor type, differentiation grade while representative blocks (one per case) were selected for further analysis.

Eighty-seven of the 99 patients were smokers, 3 were non-smokers and for the remaining 9 no reliable data on smoking habits were available. Most of the smokers were heavy smokers (>60 py). The age range was 44-77 years, and the mean age 62.8 years.

DNA extraction. Five or six 10 μm thick sections from formalin-fixed, paraffin-embedded tissues were lysed in 400 μl digestion buffer, containing 100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% SDS pH 8.0, 0.1 mg/ml proteinase K. Samples were incubated for 24 h at 37°C. Fresh proteinase K was added and the incubation was continued for another 24 h. The samples were then extracted once with phenol/chloroform and once with chloroform. DNA was precipitated with the addition of 20 μl 5 M NaCl and 1 ml ethanol, recovered by centrifugation for 15 min at 4°C, washed once with cold 70% ethanol and resuspended in 30 μl double distilled water.

Oligonucleotide primers and PCR amplification. For the detection and type distinction of the HPV, multiplex PCR was employed using four pairs of primers stimultaneously (for HPV types 11, 16, 18 and 33), providing different lengths of amplified DNA for each virus type (32), while the results were confirmed using the general primers GP5 and GP6 (33) followed by RsaI digestion giving a different pattern for each type (34). The oligonucleotides used for K-*ras* have been previously described (35). One μl of the extracted DNA of each sample was amplified in a reaction solution of 50 μl containing 20 mM $(NH_4)_2SO_4$, 2.0 mM $MgCl_2$, 75 mM Tris-HCl pH 9.0, 0.01% (w/v) Tween, 200 μM of each dNTP, 0.5 μM of each primer and 1.25 U *Taq* polymerase (Advanced Biotechnologies). The mixture was heated for 1 min at 95°C, and then subjected to 35 cycles of amplification under the following conditions.

HPV amplification: Using the specific set of primers each cycle consisted of 50 sec at 94°C, 40 sec at 56°C and 45 sec at 72°C, increasing the elongation time 1 sec per cycle. To establish type specificity of primer-directed amplification, each set of primers was tested with template plasmid DNA of the five HPV types 6b, 11, 16, 18 and 33.

Using the general primers GP5 and GP6 each cycle consisted of 50 sec at 94°C, 50 sec at 52°C and 40 sec at 72°C, increasing the elongation time 1 sec per cycle.

K-*ras* amplification: Each cycle consisted of a denaturation step at 94°C for 50 sec, an annealing step at 58°C for 45 sec and an elongation step at 72°C for 50 sec, increasing the elongation time 1 sec per cycle.

RFLP analysis. HPV-general primers: Confirmation of the typing was achieved by digestion of the amplification products with 30 U of RsaI giving a different pattern for each type. Digestion products were electrophoresed through a 10% polyacrylamide gel. As control the amplified product of plasmid DNA of the HPV types 6b, 11, 16, 18 and 33 were used.

K-*ras:* 10-20 μl were digested for 3 h with 30 U of BstNI. Digestion products were electrophoresed through an 8% polyacrylamide gel. Gels were stained with ethidium bromide and photographed on a UV light transilluminator. Enzymes were supplied by New England Biolabs and the conditions followed for digestions were those recommended by the supplier. Incubation temperatures were 37°C for RsaI and 60°C for BstNI.

Statistical analysis. The presence of HPV and mutations at codon 12 of K-*ras* gene were analysed for significant correlation with histological type, grade, TNM stage and age at day of operation, by Fisher's exact test. Survival curves were drawn up using the Kaplan-Meier method. Differences between survival times were analysed by the log rank method.

Results

In the current study we examined 99 specimens from patients with lung carcinomas. Tumors were classified according to their histological type: 41 adenocarcinomas, 41 squamous cell, 10 undifferentiated large cell, 5 small cell and 2 adenosquamous carcinomas. According to the degree of histological differentiation: 7 well, 42 moderate and 33 poorly differentiated carcinomas. Tumors were also staged according to the TNM system as: 46 in stage I, 37 in stage II and 16 in stage III. Follow-up was available for 42 patients. 32 have died of the disease while 10 patients are still alive (after 1-63 months following surgery). Although no statistically significant correlations were found, the survival was shorter in patients with *ras* mutations (average survival 665.1 days), HPV infection (a.s. 718.2 days) and HPV infection simultaneously with *ras* mutations (a.s. 773.1 days), compared to those with no such alterations (a.s. 929.7, 956.7 and 871.2 days respectively).

The type of HPV as well as the presence of point mutations in codon 12 of the K-*ras* gene were examined. The results of the PCR analysis are summarized in Table I.

Fifteen of the 99 specimens (15%) were found positive for HPV. The prevalence of HPV infection was 20% in adenocarcinomas, 9.7% in squamous cell carcinomas, 20% in undifferentiated large cell carcinomas, while HPV was also found in one of the two adenosquamous carcinomas. Results from the type distinction of HPV (Fig. 1) indicated that HPV 18 was the most frequent type (in 8% of the cases), while HPV 16 was found in 4%, HPV 11 in 3% and HPV 33 in 2% of the cases examined (Table II). Samples found positive with the multiplex PCR (amplifying a region from the E6 ORF) were also positive with the general primer PCR, indicating that the L1 region was present.

Table I. Detection of HPV and K-*ras* codon 12 point mutations in lung carcinomas by PCR.

Histological type	No. of patients	HPV positive (%)	K-*ras* mutations (%)	HPV+K-*ras* (%)
Adenocarcinoma	41	8 (20)	8 (20)	4 (9.8)
Squamous cell carcinoma	41	4 (9.8)	7 (17)	3 (7.3)
Undifferentiated large cell carcinoma	10	2 (20)	2 (20)	1 (10)
Small cell carcinoma	5	0	0	0
Adenosquamous carcinoma	2	1	1	1
Total	99	15 (15)	18 (18)	9 (9.1)

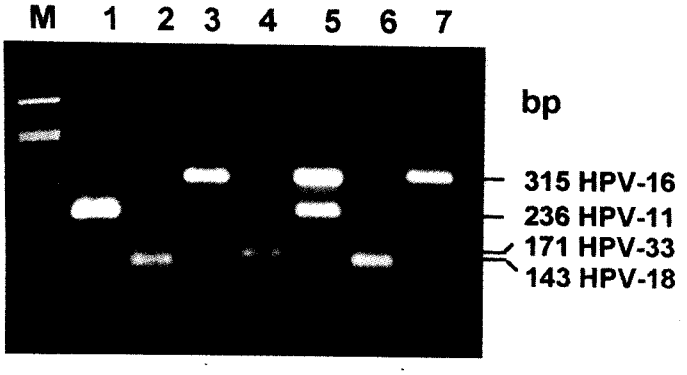

Figure 1. Type distinction of HPV employing a multiplex PCR. Products of different size (315 bp, HPV 16; 236 bp, HPV 11; 171 bp, HPV33; and 143 bp, HPV18) were analysed by agarose gel electrophoresis. Lane M, molecular weight marker pUC18/HaeIII; lane 1, sample positive for HPV 11; lanes 2,6, samples positive for HPV 18; lanes 3,7, samples positive for HPV 16; lane 4, sample positive for HPV 33; lane 5, sample positive for HPV 16-HPV 11.

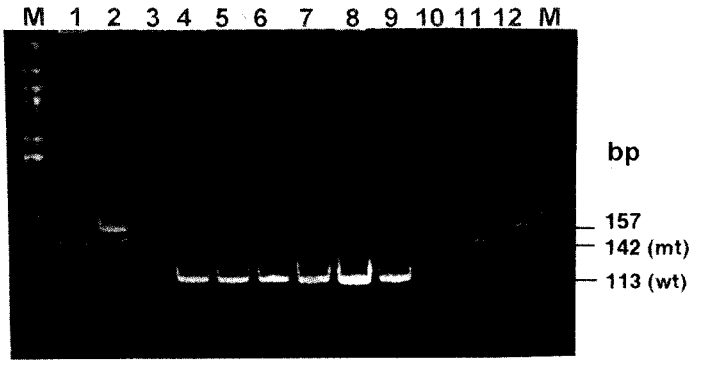

Figure 2. K-*ras* amplification products (157 bp) were digested with the restriction endonuclease BstNI and electrophoresed through an 8% polyacrylamide gel. Lanes M, molecular weight marker pUC18/HaeIII; lanes 1,11, positive control SW480 cell line (142 bp); lanes 2,12, undigested PCR product; lane 3, negative control; lanes 4,7-9, positive samples; lanes 5,6,10, negative samples (113 bp).

Table II. Incidence of HPV type in primary lung carcinomas.

Type of HPV	HPV positive carcinomas[a]
HPV 18	8
HPV 16	4
HPV 11	3
HPV 33	2

[a]in a total of 99 samples.

K-*ras* codon 12 point mutations were found in 18 of the 99 specimens (18%) examined (Fig. 2). Mutations were found in 8 (20%) of the 41 cases of adenocarcinoma, in 7 (17%) of the 41 cases of squamous cell carcinoma, in 2 (20%) of the 10 cases of undifferentiated large cell carcinoma and in 1 of the 2 cases of adenosquamous carcinoma.

The simultaneous presence of HPV DNA and K-*ras* mutation was observed in almost 50% of the HPV positive cases: in 9.8% of adenocarcinomas, in 7.3% of squamous cell carcinomas, in 10% of undifferentiated large cell carcinomas and in one of the two adenosquamous carcinomas.

No correlation was found between HPV infection and K-*ras* mutation either with sex and age of the patient, or the histological type and the differentiation grade of the tumor. Survival curves determined by Kaplan-Meier method demonstrated that HPV infection and codon 12 point mutation of K-*ras* gene did not correlate with the clinical outcome of the patients. An association was found between K-*ras* codon 12 point mutations and the stage of the tumor, occuring more frequently at stage III ($p=0.037$) than stage I. These data suggest that infection with potentially oncogenic HPV types could be associated not only with anogenital tumors, but also with a subset of lung tumours, while HPV could co-operate with K-*ras* mutations in the progression of the disease.

Discussion

The potential role of HPV in the development of lung carcinomas emerged after the description of condylomatous-like lesions in the bronchial epithelium adjacent to squamous cell carcinomas. Intranuclear HPV-like particles were demonstrated by electron microscopy in squamous cell papillomas of the bronchus (36).

The presence of HPV in lung carcinomas has been reported in several studies. The rate of positivity varied between 4.2% and 31% (19-21), while in carcinomas with condylomatous changes the rate was higher (42%) (20). In contrast other investigators reported absence of HPV in lung cancer (37,38), this divergence may be due to the differences in the sensitivity and specificity of the methods applied, as well as to epidemio-

logical factors. In the current study we confirm the presence of HPV in lung carcinomas, using primers from both the E6 and L1 ORFs. The use of primers located in the E6 ORF, which remains intact in the case of high risk HPV type integration into the host genome, allows better diagnostic sensitivity. No difference was found in the detection rate of the virus using the two sets of primers from the E6 and L1 ORFs.

It is noteworthy that HPV was also detected in cases of adenocarcinoma. Recently, there has been increasing interest in the association between HPV and glandular neoplasia. In many studies HPV DNA has been found in adenocarcinomas and adenosquamous carcinomas as frequently as in squamous cell carcinomas of the cervix (39-41).

In our study we found that multiple subtypes of HPV may be related to lung cancer. However HPV 18 was the predominant type, which is in agreement with other studies reporting that high-risk HPV types were more frequently detected (19,20,22). Their identification in both squamous cell carcinomas and adenocarcinomas as well as in adenosquamous and undifferentiated large cell carcinomas suggests a key role in the process of lung carcinogenesis. Moreover, it has been suggested that infection with high risk HPVs could serve as a prognostic indicator of malignant progression in lung squamous cell papillomas (22).

Multiple molecular changes have also been documented in lung cancer. These include activation of dominant oncogenes, such as the *ras* gene family (23), *myc* and HER-2/*neu* genes, as well as loss of recessive growth regulatory genes, or onco-suppressor genes (p53, rb) (23,26).

Several studies have reported an incidence of 15-33% of activated K-*ras* gene in lung carcinomas, while more recent studies have revealed a higher incidence of more than 50% (42-44). In the current study we found K-*ras* codon 12 point mutations in 18% of the specimens examined. In cases of squamous cell carcinomas the incidence of K-*ras* mutations was higher than in other studies (24,42), propably due to the more sensitive assay used. Activation of the K-*ras* gene was examined, since point mutations of this member of the *ras* family are found more commonly in lung cancer and occur predominantly at codon 12.

It has been suggested that K-*ras* mutations arise predominantly at the non-invasive stage of lung carcinogenesis, and may be associated with the transformation of dysplastic cells to neoplastic (45). Moreover, detection of K-*ras* mutations in sputum may precede diagnosis of lung cancer (29). On the other hand, a significant number of lung adenocarcinomas harbor *ras* mutations in only a small percentage of the cancer cells, indicating that K-*ras* mutations represent a late event in lung carcinogenesis (23). This concept is consistent with the association of *ras* mutations with increased tumor growth and invasiveness, as suggested by the poorer prognosis of mutation-positive than mutation-negative cases treated by surgical resection (44,46). We found an association between K-*ras* mutations and the stage of the tumor, occuring more frequently at cases with stage III. However, no correlation was found between K-*ras* mutations and the clinical outcome of the patients examined. In 50% of the HPV-positive cases, K-*ras* mutation co-existed. This might suggest that HPV infection is not sufficient by itself for malignant transformation, but requires co-operation of an activated *ras* gene. In addition, carcinogen exposure may induce transformation in several fields of the bronchial epithelium through the induction of different genetic changes.

Our results indicate that HPV infection and K-*ras* gene activation may play a role in a subset of lung tumors. The possibility of synergic mechanisms between HPV, *ras* gene mutations and chemical or physical carcinogens such as cigarette smoking should also be considered.

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