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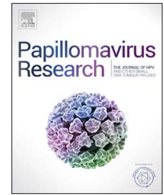

Investigating the role of human papillomavirus in lung cancer

E. Argyri[a], E. Tsimplaki[a], C. Marketos[b], G. Politis[b], E. Panotopoulou[a,*]

[a] Virology Department, Saint Savvas General Anticancer Hospital, Athens, Greece
[b] Pulmonary Department, Saint Savvas General Anticancer Hospital, Athens, Greece

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ABSTRACT

Lung cancer is the leading cancer worldwide among men and women with morbidity reaching 1.6 million. Human Papillomavirus is the causal factor of cervical cancer while its association with others is still under investigation. The purpose of our study is to examine the presence of HPV DNA as well as high-risk E6/E7 mRNA in patients with lung cancer.

Lung tissues were collected during bronchoscopy and tested for HPV DNA and E6/E7 mRNA.

67 lung tissue samples were analysed. The age range was 49–85 years old (y.o) with a mean age of 67.6 y.o. 9 patients were female and 58 were male. The study included 12 Small Cell Lung Cancers (SCLC) and 55 Non Small Cell Lung Cancer (NSCLC). HPV DNA was detected in 3.0% (2/67) of lung cancer cases, while no E6/E7 mRNA of five high-risk HPV types was found in tissue samples examined. The two positive patients had no prior history of an HPV related disease.

Using the mRNA test as a gold standard for the association of HPV with malignant transformation, the present results showed no association of HPV status with lung cancer. Further investigation of more lung cancer tissues is required to reach safe conclusions.

1. Introduction

Lung cancer is the leading cancer worldwide among men and women with 1.8 million new cases in 2012 accounting for nearly 1.6 million deaths in both sexes [1]. Tobacco smoke remains to be the major etiological factor for lung carcinogenesis, being responsible for more than 90% of cancer cases in men and 75% in women in the United States and Europe [2,3]. However, there have been recorded many cases of lung cancer that were developed among non-smokers. Several agents have been studied for causing lung cancer in never smokers including radon, asbestos, environmental tobacco smoke, air pollution, human papillomavirus, certain gene mutations, chromosomal aberrations and DNA methylation [4].

Human papillomavirus has been established as the transforming factor in cervical cancer and a proportion of vulvar, vaginal, anal, penile and oropharyngeal cancers. However, its role is still under investigation regarding oral, laryngeal and lung cancers. This was originally supported by the fact that lung tumors had morphological similarities with anogenital cancers caused by HPV. Syrjanen was the first to report a case with condylomatous changes in neoplastic bronchial epithelium [5]. Moreover, taking into account the fact that several studies have demonstrated a synergistic association of smoking and high-risk (hr) HPV types with head and neck cancers [6,7] it is possible that tobacco and HPV infection could interact for lung cancer development. It has been observed that regarding HPV 16, cigarette smoke with E6 and E7 can activate HPV 16 p97 promoter in lung epithelial cells [8]. In addition squamocolumnar junction, which can occur by cigarette smoke apart from natural causes, is the preferred entry site of HPV [9].

The molecular mechanisms of HPV transformation have been studied extensively over the last decade. HPV genome is maintained as an episome and the virus is depending on the host cell replication enzymes to complete its DNA synthesis. In high grade lesions, HPV is found integrated into the host chromosome [10,11]. Many cellular proteins, the most distinguished of which are the retinoblastoma family proteins (RB, p107, p130) are controlling the proliferation process by regulating the host's cell cycle. E7 high-risk HPV proteins bind Rb proteins with high affinity causing E2F-mediated replication [12,13]. As a result E6 HPV protein has developed the ability to target the tumor suppressor protein p53, thus preventing apoptosis of the infected cell. These two proteins have high transformation potential and act cooperatively in promoting S-phase in the infected cell and avoid apoptosis. This environment provides high malignant potential through genomic instability and interference into the cell cycle [14].

The purpose of our study is to examine the presence of HPV DNA as well as high-risk E6/E7 mRNA in patients with lung cancer.

* Corresponding author.

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2. Material and methods

2.1. 1 Sample collection and study sample

Biopsies were collected from 67 patients during bronchoscopy between May 2013 and December 2015 and sent for routine histopathological analysis. The patients were randomly selected to cover a broad range of age and all of them provided informed consent. A part of the tumorous tissue was kept in liquid storage medium (Thin-Prep PreservCyt Solution; Hologic, Inc. Ltd. West Sussex, UK) in 4 °C. Subsequently, all samples had histologically-confirmed, lung carcinomas and were analysed for the presence of HPV DNA and E6/E7 mRNA. Patients were eligible if they had not received any previous treatment. Also, they were defined as smokers if sometime in their lifetime smoked and who, at the time of study, smoked either every day or some days. Former smokers were those patients who had quitted smoking prior to their inclusion in the study. Finally, never smokers were those who had smoked less than 100 cigarettes in their lifetime and did not smoke at the time of the study.

2.2. Extraction of nucleic acids

Total nucleic acids were extracted using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA quality test was carried out using Human Globin, Beta Primer set kit (Maxim Biotech, Inc., South San Francisco, CA, USA) according to the manufacturer's instructions. To assess RNA integrity, 5 µg of RNA per sample were separated on 1% formaldehyde-agarose gel.

2.3. HPV detection and genotyping

The PapilloCheck® HPV genotyping assay (Greiner Bio-One GmbH, Frickenhausen, Germany) was used. This technology is based on a DNA chip for the type-specific identification of 24 types of HPV (hr: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82; probable hr: 53 and 66; and low-risk (lr): 6, 11, 40, 42, 43, 44/55, 70) with analytical sensitivity of 30–750 copies/reaction, according to manufacturer's manual. Nucleic acids were extracted from lung biopsies preserved in ThinPrep. E1-based polymerase chain reaction (PCR) was performed according the manufacturer's guidelines. For each sample, 19.8 µl PapilloCheck® MasterMix, 0.2 µl HotStarTaq DNA polymerase (5 U/µl) and 5 µl DNA from the sample were mixed. Hybridization followed by mixing 30 µl of the PapilloCheck® Hybridization buffer in a fresh reaction tube with 5 µl of the PCR product at room temperature and transferring 25 µl of the hybridization mix into each compartment of the chip. The chip was incubated for 15 min at room temperature in a humid atmosphere, then washed in three washing solutions (30 s, 1 min and 30 s, respectively), centrifuged and scanned on a CheckScanner™ (Greiner Bio-One GmbH). The kit contains a sample control system that tests for a successful DNA extraction and the template quality. Primers for a fragment of the human ADAT1 gene in the MasterMix lead to the generation of a PCR-product which subsequently gives a signal on the sample control spots.

2.4. HPV E6/E7 mRNA expression

Real-time nucleic acid sequence based amplification (NASBA) and detection assay NucliSENS® EasyQ® HPV (BioMerieux Hellas, Athens, Greece) was performed for the qualitative detection of E6/E7 mRNA of five hrHPV types (16, 18, 31, 33 and 45), with analytical sensitivity of 2.3×10^2-3×10^4 copies/ml. The NucliSENS EasyQ HPV assay was performed according to the manufacturer's instructions (BioMerieux). Firstly, three pre-mixes were made by adding reagent sphere diluent (Tris-HCl, 45% dimethyl sulfoxide (DMSO)) to reagent spheres (nucleotides, dithiothreitol and $MgCl_2$). To each premix U1A/HPV 16, HPV 33/45, or HPV 18/31 primer and molecular beacon mixes, KCl stock solution and nucleic acid sequence-based amplification (NASBA) water were added. Secondly, 10 µl of this pre-mix were distributed to each well in a reaction plate and the addition of 5 µl RNA followed. The plates were incubated for 4 min at 65 °C to destabilize secondary structures of RNA, followed by cooling to 41 °C. The reaction was started by addition of enzymes (AMV-RT, RNase H, T7 RNA polymerase, and bovine serum albumin) and measured in real time using the Lambda FL 600 fluorescence reader (Bio-Tek, Winooski, VT, USA) at 41 °C for 2 h and 30 min. To avoid false negative results due to degradation of RNA, a primer-set and probe directed against the human U1 small nuclear ribonucleoprotein (snRNP) specific A protein (U1A mRNA) was used as the performance control.

2.5. Statistical analysis

Data were analysed using SPSS 20 (IBM, Armonk, New York). Absolute and relative frequencies were used to present HPV positivity. 2×2 contingency tables and Fisher's exact test were performed to estimate p values. One way ANOVA was performed to examine differences among three or more groups with a Bonferroni post-test for pairwise comparison. P value of less than 0.05 was considered statistically significant.

3. Results

67 lung tissue samples were analysed. The patients' age range was 49–85 years old (y.o) with a mean age of 67.6 y.o. 9 patients were female and 58 were male. The study included 12 Small Cell Lung Cancers (SCLC) and 55 Non Small Cell Lung Cancer (NSCLC) (31 AdenoCa, 20 SCC and 4 not defined NSCLC). 58 patients were smokers, 8 were former smokers and a non smoker (Tables 1 and 2).

All samples were beta globin positive. In addition internal sample controls for both assays used worked properly (positive results for ADAT1 and U1A).

HPV DNA was detected in 3.0% (2/67) of lung cancer cases, while no high-risk E6/E7 mRNA was found in tissue samples examined. More specifically, two patients were found positive in the HPV DNA test harboring the high-risk types 16 and 53 each and subsequently subjected to E6/E7 mRNA test that showed negative results. The two HPV DNA positive patients regarded a male, smoker with SCLC and a female smoker with AdenoCa with no prior history of an HPV related disease. Furthermore, no association was found between the presence of HPV DNA and any patients' or cancer characteristics (gender, age,

Table 1
Association of patients' characteristics with human papilloma virus (HPV) status.

Characteristics	N	HPV DNA (+) (%, 95% CI)	HPV DNA (-) (%)	*p*-Value
Gender				0.252
Male	58	1 (1.7, 0–5.1)	57 (98.3)	
Female	9	1 (11.1, 0–36.7)	8 (88.9)	
Age				1.000
≤67 years	32	1 (3.1, 0–9.5)	17 (100)	
> 67 years	35	1 (2.9, 0–8.7)	48 (96.0)	
Tobacco				1.000
No	1	0 (0.0)	1 (100)	
Yes	66	2 (3.0, 0–7.3)	64 (97.0)	
Histology				0.603
AdenoCa[a]	31	1 (3.2,0–9.8)	30 (96.8)	
SCC[b]	20	0 (0.0)	20 (100)	
NSCLC[c] [not defined]	4	0 (0.0)	4 (100)	
SCLC[d]	12	1 (8.3,0–26.7)	11 (91.7)	

[a] Adenocarcinoma,
[b] Squamous Cell Carcinoma,
[c] Non Small Cell Lung Carcinoma,
[d] Small Cell Lung Carcinoma.

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