



## Investigating the role of human papillomavirus in lung cancer



E. Argyri<sup>a</sup>, E. Tsimplaki<sup>a</sup>, C. Marketos<sup>b</sup>, G. Politis<sup>b</sup>, E. Panotopoulou<sup>a,\*</sup>

<sup>a</sup> Virology Department, Saint Savvas General Anticancer Hospital, Athens, Greece

<sup>b</sup> Pulmonary Department, Saint Savvas General Anticancer Hospital, Athens, Greece

### ARTICLE INFO

#### Keywords:

Lung cancer  
HPV  
Genotyping  
mRNA E6/E7

### 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.

<http://dx.doi.org/10.1016/j.pvr.2016.12.002>

Received 9 September 2016; Received in revised form 11 November 2016; Accepted 6 December 2016

Available online 07 December 2016

2405-8521/© 2016 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## 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 quit 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<sup>®</sup> 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<sup>®</sup> 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<sup>®</sup> 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<sup>®</sup> 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<sup>™</sup> (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<sup>®</sup> EasyQ<sup>®</sup> 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  $2.3 \times 10^2$ – $3 \times 10^4$  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<sub>2</sub>). 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 <sup>a</sup>	31	1 (3.2, 0–9.8)	30 (96.8)	
SCC <sup>b</sup>	20	0 (0.0)	20 (100)	
NSCLC <sup>c</sup> [not defined]	4	0 (0.0)	4 (100)	
SCLC <sup>d</sup>	12	1 (8.3, 0–26.7)	11 (91.7)	

<sup>a</sup> Adenocarcinoma,

<sup>b</sup> Squamous Cell Carcinoma,

<sup>c</sup> Non Small Cell Lung Carcinoma,

<sup>d</sup> Small Cell Lung Carcinoma.

**Table 2**  
Patients' characteristics and E6/E7 mRNA status of HPV- positive lung cancers.

Gender	Age (years)	Tobacco	DNA test	mRNA test	Histology
Male	81	Yes	HPV 16	Negative	SCLC
Female	65	Yes	HPV 53	Negative	AdenoCa

tobacco and type of cancer).

#### 4. Discussion

The role of HPV has been firmly confirmed in cervical cancer and in a subset of oropharyngeal cancers, but such link has not yet been identified for lung cancer. This is due to the great variation in HPV prevalence presented by investigators worldwide, especially across different geographic regions. The goal of the present study was to detect HPV DNA, as well as hrHPV E6/E7 mRNA, in Greek patients with lung cancer.

The findings in our study indicate a very low frequency of HPV prevalence according to the HPV DNA test (2/67, 3%). It is important to note that lung samples were always analysed separately from other tissue samples to avoid DNA contamination and only advanced molecular techniques were used. Other studies conducted in Greece showed discrepant results regarding HPV infection in lung cancers, with the HPV DNA prevalence rate ranging from 0–61% [15–18].

A recent meta-analysis of 27 studies on lung cancers showed an HPV prevalence of 16.9% in Europe, 12.5% in North America and 18.5% in Australia, while in Taiwan and China the rate was very high (37.7%) [19]. Notably, the statistical analysis performed showed significant heterogeneity between studies concerning the same geographic region and between the geographical regions as well.

In our study, the types detected were HPV 16 and HPV 53. According to a recent international meta-analysis, HPV 16 was the most frequent type found in HPV positive lung carcinomas, followed by HPV 18, (3.8% and 2.8% respectively), while other high-risk HPV types were also detected [20]. Concerning HPV 53, which is a possibly carcinogenic type (class 2B) [21], has also been detected in studies from Europe [22] and North America [23,24].

Relatively few studies have demonstrated active transcription of HPV by detecting the mRNA of the E6/E7 oncogenes or the expression of E6/E7 [25–28]. As observed in cervical cancer and oropharyngeal carcinomas the presence of HPV DNA in the tumour does not infer its causal relation to oncogenesis. The transformation potential of HPV relies on the expression of E6/E7 HPV oncoproteins and this has been established in many studies regarding the cervix and the oropharynx.

In our study, no E6/E7 mRNA of hrHPVs 16,18,31,33 and 45 was detected, possibly indicating inactive infections. Furthermore, it is important to note that regarding the HPV 53 case no E6/E7 mRNA was found. This is either due to the method used, which does not detect E6/E7 mRNA from HPV 53, or due to the fact that HPV 53 was transcriptionally inactive.

Regarding the route of transmission, it has been speculated that the virus reaches the lungs via the bloodstream [29], whereas it is commonly known that HPV does not cause a generalized viremia. Additionally, our patients did not report any previous HPV associated disease. It has also been suggested that infected particles or cell complexes are carried through the air stream to the lungs [30]. It has been observed that oral sexual practices increase the risk of HPV infection by 4.6 fold [31], possibly contributing to the spread of the virus to the lungs.

#### 5. Conclusion

Considering our results, they indicate the absence of an etiological link between HPV and lung cancer, which is in line with what is seen in

many Western populations. However more patients need to be recruited in order to reach safe conclusions.

#### Conflict of interests

None.

#### Sources of support

This project was supported by St. Savvas, Regional Anticancer Oncology Hospital of Athens, Athens, Greece.

#### References

- [1] World Health Organization. International agency for research on cancer. GLOBOCAN 2012. Cancer fact sheets. Available at: ([http://globocan.iarc.fr/Pages/fact\\_sheets\\_cancer.aspx](http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx))
- [2] D.M. Parkin, F. Bray, J. Ferlay, P. Pisani, Global cancer statistics, 2002, *CA Cancer J. Clin.* 55 (2005) 74–108.
- [3] J.E. Tyczynski, F. Bray, D.M. Parkin, Lung cancer in Europe in 2000: Epidemiology, prevention, and early detection, *Lancet Oncol.* 4 (2003) 45–55.
- [4] J. Subramanian, R. Govindan, Lung cancer in never smokers: a review, *J. Clin. Oncol.* 25 (2007) 561–570.
- [5] K.J. Syrjänen, Condylomatous changes in neoplastic bronchial epithelium. Report of a case, *Respiration* 38 (1979) 299–304.
- [6] S.M. Schwartz, J.R. Daling, D.R. Doody, G.C. Wipf, J.J. Carter, M.M. Madeleine, E.J. Mao, E.D. Fitzgibbons, S. Huang, A.M. Beckmann, J.K. McDougall, D.A. Galloway, Oral cancer risk in relation to sexual history and evidence of human papillomavirus infection, *J. Natl. Cancer Inst.* 90 (1998) 1626–1636.
- [7] E.M. Smith, L.M. Rubenstein, T.H. Haugen, E. Hamsikova, L.P. Turek, Tobacco and alcohol use increases the risk of both HPV-associated and HPV-independent head and neck cancers, *Cancer Causes Control.* 21 (2010) 1369–1378.
- [8] N. Peña, D. Carrillo, J.P. Muñoz, J. Chnaiderman, U. Urzúa, O. León, M.L. Tornesello, A.H. Corvalán, R. Soto-Rifo, F. Aguayo, Tobacco smoke activates human papillomavirus 16 p97 promoter and cooperates with high-risk E6/E7 for oxidative DNA damage in lung cells, *PLoS One* 10 (2015) e0123029.
- [9] K.J. Syrjänen, HPV infections and lung cancer, *J. Clin. Pathol.* 55 (2002) 885–891.
- [10] C. Ziegert, N. Wentzensen, S. Vinokurova, F. Kisseljov, J. Einkenel, M. Hoeckel, M. von Knebel Doeberitz, A comprehensive analysis of HPV integration loci in anogenital lesions combining transcript and genome-based amplification techniques, *Oncogene* 22 (2003) 3977–3984.
- [11] N. Wentzensen, S. Vinokurova, M. von Knebel Doeberitz, Systematic review of genomic integration sites of human papillomavirus genomes in epithelial dysplasia and invasive cancer of the female lower genital tract, *Cancer Res.* 64 (2004) 3878–3884.
- [12] S. Cheng, D.C. Schmidt-Grimminger, T. Murant, T.R. Broker, L.T. Chow, Differentiation-dependent up-regulation of the human papillomavirus E7 gene reactivates cellular DNA replication in suprabasal differentiated keratinocytes, *Genes Dev.* 9 (1995) 2335–2349.
- [13] N. Dyson, P.M. Howley, K. Munger, E. Harlow, The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product, *Science* 243 (1989) 934–937.
- [14] C.A. Moody, L.A. Laimins, Human papillomavirus oncoproteins: pathways to transformation, *Nat. Rev. Cancer* 10 (2010) 550–560.
- [15] A. Noutsou, M. Koffa, M. Ergazaki, N. Sifakakos, D. Spandidos, Detection of human papilloma virus (HPV) and K-ras mutations in human lung carcinomas, *Int. J. Oncol.* 8 (1996) 1089–1093.
- [16] K. Papadopoulou, V. Labropoulou, P. Davaris, P. Mavromara, H. Tsimara-Papastamatiou, Detection of human papillomaviruses in squamous cell carcinomas of the lung, *Virchows Arch.* 433 (1998) 49–54.
- [17] V.G. Gorgoulis, P. Zacharatos, A. Kotsinas, A. Kyroudi, A.N. Rassidakis, J.A. Ikononopoulos, C. Barbatis, C.S. Herrington, C. Kittas, Human papilloma virus (HPV) is possibly involved in laryngeal but not in lung carcinogenesis, *Hum. Pathol.* 30 (1999) 274–283.
- [18] E.D. Papadakis, N. Soultziz, D.A. Spandidos, Association of p53 codon 72 polymorphism with advanced lung cancer: the Arg allele is preferentially retained in tumours arising in Arg/Pro germline heterozygotes, *Br. J. Cancer* 87 (2002) 1013–1018.
- [19] K. Syrjänen, Detection of human papillomavirus in lung cancer: systematic review and meta-analysis, *Anticancer Res.* 32 (2012) 3235–3250.
- [20] M. Srinivasan, E. Taioli, C.C. Ragin, Human papillomavirus type 16 and 18 in primary lung cancers—a meta-analysis, *Carcinogenesis* 30 (2009) 1722–1728.
- [21] V. Bouvard, R. Baan, K. Straif, Y. Grosse, B. Secretan, F. El, Ghissassi, L. Benbrahim-Tallaa, N. Guha, C. Freeman, L. Galichet, V. Coglian, WHO international agency for research on cancer monograph working group, a review of human carcinogens—Part B: biological agents, *Lancet Oncol.* 10 (2009) 321–322.
- [22] L. Giuliani, T. Jaxmar, C. Casadio, M. Gariglio, A. Manna, D. D'Antonio, K. Syrjanen, C. Favalli, M. Ciotti, Detection of oncogenic viruses SV40, BKV, JCV, HCMV, HPV and p53 codon 72 polymorphism in lung carcinoma, *Lung Cancer* 57 (2007) 273–281.
- [23] R.N. Pillai, C. Ragin, G. Sica, M. Behera, Z. Chen, S. Kim, W. Mayfield, R. Hermann, N.

- F.Saba, A.A.Gal, F.R.Khuri, S.S.Ramalingam, T.K.Owonikoko, Human Papillomavirus (HPV)- Associated Early Stage Non- Small Cell Lung Cancer (NSCLC), ASCO Meeting Abstracts, Chicago IL, 31, Abstract no. 7560
- [24] R.Mehra, B.Egleston, D.Yang, W.Scott, H.Borghaei, C.Ragin, A pilot study of the association and prevalence of the human papillomavirus (HPV) in non- small cell lung cancer (NSCLC), AACR Meeting Abstracts, Washington, DC., Abstractno. 4785
- [25] M. Ciotti, L. Giuliani, V. Ambrogi, C. Ronci, A. Benedetto, T.C. Mineo, K. Syrjänen, C. Favalli, Detection and expression of human papillomavirus oncogenes in non-small cell lung cancer, *Oncol. Rep.* 16 (2006) 183–189.
- [26] I. Kinoshita, H. Dosaka-Akita, M. Shindoh, M. Fujino, K. Akie, M. Kato, K. Fujinaga, Y. Kawakami, Human papillomavirus type 18 DNA and E6-E7 mRNA are detected in squamous cell carcinoma and adenocarcinoma of the lung, *Br. J. Cancer* 71 (1995) 344–349.
- [27] D. Krikelis, G. Tzimagiorgis, E. Georgiou, C. Destouni, T. Agorastos, C. Haitoglou, S. Kouidou, Frequent presence of incomplete HPV16 E7 ORFs in lung carcinomas: memories of viral infection, *J. Clin. Virol.* 49 (2010) 169–174.
- [28] L. Giuliani, C. Favalli, K. Syrjänen, M. Ciotti, Human papillomavirus infections in lung cancer. detection of E6 and E7 transcripts and review of the literature, *Anticancer Res.* 27 (2007) 2697–2704.
- [29] H.L. Chiou, M.F. Wu, Y.C. Liaw, Y.W. Cheng, R.H. Wong, C.Y. Chen, H. Lee, The presence of human papillomavirus type 16/18 DNA in blood circulation may act as a risk marker of lung cancer in Taiwan, *Cancer* 97 (2003) 1558–1563.
- [30] F. Klein, W.F. Amin Kotb, I. Petersen, Incidence of human papilloma virus in lung cancer, *Lung Cancer* 65 (2009) 13–18.
- [31] A.C. de Freitas, A.P. Gurgel, E.G. de Lima, B. de França São Marcos, C.M. do Amaral, Human papillomavirus and lung carcinogenesis: an overview, *J. Cancer Res. Clin. Oncol.* 142 (2016) 2415–2427.