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Article

Detection of High-Risk Human Papillomavirus (HPV) and Their Genotypes in Lung Cancer: Insights from the Mediterranean Region of Turkey

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Abstract: Lung cancer is the leading cause of cancer death worldwide therefore studies about the etiological factors and carcinogenesis are important. The aim of the study is to detect the presence and types of Human papilloma virus (HPV) in lung cancer and non-tumoral lung and Epidermal Growth Factor Receptor (EGFR) mutation prevalence in lung cancer with Polymerase Chain Reaction (PCR) method. Fifty patients who were histopathologically diagnosed lung cancer and 7 patients with non-neoplastic lung disease were enrolled in the study. HPV positivity was detected in 75.4% of the patients. The rate of high-risk HPV positivity was 100% in adenocarcinomas, 64.5% in squamous cell carcinomas, and 57% in non-neoplastic lung. HPV 51 was the most frequent type followed by HPV 16 (43.9%). There was no statistically significant relationship between HPV infection and age, gender and smoking history and EGFR mutation. The prevalence of HPV positivity is variable in lung cancer in different studies and this may be due to many factors including HPV detection methods, number of patients, geographic regions. New studies about the role of HPV infection need to be performed in order to obtain information about the use of prophylactic and therapeutic agents in lung cancer.

Keywords: lung cancer; human papilloma virus (HPV); polymerase chain reaction (PCR); viral oncology

1. Introduction

Lung cancer is the leading cause of cancer death in both men and women worldwide. Smoking ranks first among the etiological factors that cause the development of lung cancer [1–3]. The cases of lung cancer detected in non-smokers are more often seen in females and Asian countries. Furthermore these cases have different molecular characteristics compared to the cases of lung cancer in smokers. Genetic susceptibility, radiation, environmental pollution, occupational exposure and infectious agents, especially those of viral origin can be counted among other factors that play a role in lung carcinogenesis, apart from smoking [4–6].

It is known that about 10-15% of cancers seen in humans all over the world are caused by Epstein–Barr virus, hepatitis B or C virus, human T-lymphotropic virus-1, HPV and Merkel cell polyomavirus. The viruses can promote cancer as carcinogens or promoters. Among these viruses, the ones with a potential role in the development of lung cancer include HPV, Merkel cell polyomavirus and Epstein–Barr virus [7]. The cancers most associated with HPV infection are known as cervical cancer, anogenital cancer and head and neck (oropharyngeal) cancer [8].

HPV is a non-enveloped, small, double stranded circular DNA virus and has “low-risk” or “high-risk” types which were defined according to their relation with cancer development. HPV is known to dysregulate the cell cycle at transition from G1 to S phase and promote DNA synthesis for viral replication. The expression of the most important viral oncoproteins E6 and E7 is considered as a first step in carcinogenesis since these oncoproteins inactivate the two important tumor suppressor genes p53 and Retinoblastoma protein (Rb), respectively. [9]. E5 is the other known oncoprotein that

has supportive procarcinogenic roles in high risk HPV-related tumours by downregulating the major histocompatibility complex (MHC) Class I [9–11].

The link between HPV and bronchial lesions was first established in the 1970's by Rubel and Reynold's finding that there is cytological and histological similarity between condyloma acuminatum and squamous cell papilloma. They detected koilocytes that are characteristic of HPV infections in the sputum samples of patients with benign bronchial lesions [12]. They also observed condylomatous histological changes in bronchial epithelium and bronchial squamous cell carcinoma similar to the changes seen in the genital tract in 1979 and 1980's [4,13,14].

After these detections, the relation between HPV infection and lung cancer were researched by several studies until today. These studies revealed a great difference of HPV infection rate in lung cancer (0-78.3%) in different regions of the world. It is suggested that this wide range may be due to the difference in sensitivity and specificity of the methods used for HPV genotyping, the number of types of HPV analysed, the diagnostic criteria, the number and characteristics of patients and ethnicity.[7,8].

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that affects some signalling pathways in cell proliferation. Mutation in EGFR may cause uncontrolled growth and proliferation of cells in especially non-small cell lung cancer. EGFR mutations are detected more frequently in women, non-smokers, adenocarcinoma and Asian population [15–18].

In this study we aimed to search the status of HPV infection and its relation with EGFR mutation and clinicopathological findings in patients with lung cancer .

2. Materials and Methods

2.1. Patient Selection and Data Collection

This study included 50 patients who had a histopathologic diagnosis of lung carcinoma in lobectomy specimens and 7 patients who had surgery for a non-tumoral lung pathology (bullous disease, infection) at a tertiary level hospital between January 1st 2013 and January 1st 2019. None of the patients received HPV vaccination.

The study was performed in accordance with the ethical standards of Declaration of Helsinki, 2013. The study was approved by the Ethics Committee of Health Sciences University, Antalya Education and Research Hospital. (Date and register number: 2019-242, 19/9)

Hematoxylin and eosin sections of the cases were obtained from the archive and examined histologically. The demographic and clinical characteristics of the patients including age, gender, smoking history, pathological data were obtained by searching the hospital database, patient records, and pathology reports.

2.2. DNA Extraction

Eight tissue sections of 10 µm thickness obtained from formalin-fixed paraffin-embedded blocks were soaked in xylene and vortexed. DNA was extracted and deparaffinized from sections using QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and precautions such as extensive cleaning of the work area, work with a small number of samples at a time, separating tumoral and non-tumoral samples and using appropriate protection equipment were taken to avoid contamination.

2.3. HPV DNA Detection and Genotyping

All HPV positive samples had underwent genotyping of Human Papillomavirus (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) using the "HPV Genotypes 14 Real-TM Quant kit (Nuclear Laser Medicine, Milan, Italy)" which is based on two major processes: isolation of DNA from specimens and multiplex Real Time amplification of 4 PCR tubes for each sample, each tube amplifying "16-18-31-IC", "39-45-59-IC", "33-35-56-68" and "51-52-58-66". HPV Genotypes 14 RealTM Quant kit contains the internal control (human beta-globin gene), which allows to control the presence of cellular material in the sample in order to avoid false-negative results.

HPV DNA amplification was carried out in the real-time PCR cycler (Rotor-Gene™ 3000/6000/Q (Qiagen, Hilden, Germany) and for quantitative analysis Microsoft® Excel HPV Genotype 14 Real-TM.xls was used according to enclosed instructions.

2.4. EGFR Mutation Detection

The extracted DNA samples were assessed using real-time PCR (Rotor-Gene™ 3000/6000/Q, Qiagen, Hilden, Germany) with the Easy EGFR Real Time PCR kit (Diatech Pharmacogenetics, Jesi, Italy) following the manufacturer's protocol. Each DNA sample was analyzed for mutations on exon 18 (G719X), exon 19 (ex19del), exon 20 (T790M, S768I, ex20ins), and exon 21 (L858R, L861Q). A positive control was included with the kit, and distilled water was used as the negative control.

2.5. Statistical Analysis

Statistical analyses were carried out using IBM SPSS Statistics for Windows, Version 23.0 (IBM Corp., Armonk, NY). The descriptive findings were presented with mean±standard deviation (SD) for the continuous data, and with frequency and percentage for the categorical data. The normality assumptions were controlled by the Shapiro-Wilk test. Categorical data were analyzed by Pearson chi-square test and Fisher's Exact test. Student's t-test was used for analysis of normally distributed numerical data. Two-sided p values <0.05 were considered statistically significant.

3. Results

3.1. Clinicopathological Characteristics of Patients

Clinicopathological characteristics are presented in Table 1, including age, gender, smoking history and histopathological diagnosis. The study included 57 patients with median age of 63.2 SD12.8 (range, 27-83 years). Among patients, 84.2% were men and 59.6% had smoking history. The most common two histological tumor types were as follows; squamous cell carcinoma (54.5%) and adenocarcinoma (22.8%).

Table 1. Clinicopathological characteristics of patients, Total n = 57.

Characteristics	n	%
Age (median 63.2)		
Gender		
Female	9	15.8
Male	48	84.2
Smoking history	34	59.6
Histopathological diagnosis		
Adenocarcinoma	13	22.8
Squamous cell carcinoma	31	54.4

Small cell carcinoma	4	7.0
Large cell carcinoma	1	1.8
Large cell neuroendocrine carcinoma	1	1.8
Non-neoplastic lung	7	12.3

3.2. HPV Infection and the Prevalence of HPV Subtypes

HPV positivity was detected in 75.4% of the patients regardless of the group of patients. HPV positivity rate was found as 57.1 % (4/7) and 78 % (39/50) of the non-neoplastic and neoplastic samples, respectively. In terms of the histologic types of tumours; 13/13 (100%) cases of adenocarcinomas, 20/31 (64.5%) cases of squamous cell carcinomas were positive for high-risk HPV DNA. Table 2 shows the prevalence of HPV subtypes in HPV positive cases. HPV 51 was the most frequent, representing 49.1% of the positive samples followed by HPV 16 (43.9%), HPV 31 (15.8%) and HPV 18 (12.3%).

Table 2. HPV subtypes.

HPV	number	%
HPV positive cases	43	75.4
Type 16	25	43.9
Type 18	7	12.3
Type 31	9	15.8
Type 39	1	1.8
Type 45	2	3.5
Type 51	28	49.1
Type 52	1	1.8
Type 56	1	1.8
Type 58	1	1.8
Type 59	2	3.5

Type 66

1

1.8

More than one subtype of HPV was detected in 2/4 (50%) of the non-neoplastic and in 23/39 of the neoplastic samples and the most frequently detected multiple HPV subtypes were HPV 16 and 51 in ½ (50 %) of the multiple infected non-neoplastic and 11/23 (52 .6 %) of the multiple infected neoplastic samples. Table 3 shows the frequency of Multiple HPV genotypes.

Table 3. The Frequency of Multiple HPV Infections in Neoplastic and Non-Neoplastic Lung Tissue.

HPV types	Number of cases
HPV16-HPV51	12
HPV16-HPV18	1
HPV16-HPV31	2
HPV18-HPV31	1
HPV18-HPV51	1
HPV51-HPV56	1
HPV51-HPV59	1
HPV16-HPV18-HPV31	2
HPV16-HPV18-HPV51	1
HPV16-HPV45-HPV51	1
HPV16-HPV18-HPV39-HPV45-HPV59-HPV51	1
HPV51-HPV52-HPV58-HPV66	1

3.3. The Relationship between HPV Genotypes and Clinicopathological Parameters

Table 4 shows the clinicopathologic characteristics of patients according to HPV status. The frequency in adenocarcinoma was higher in HPV positive cases. There was no relationship between HPV infection and age (p=0.38), gender (p=0.42) and smoking history (p=0.68).

Table 4. The The relationship between HPV infection and clinicopathological parameters.

	HPV negative	HPV positive	
	(Total n = 14)	(Total n = 43)	
	n (%)	n (%)	p
Median age	60.6±15.4	64.1±12.0	0.381
Gender			
Men	13 (92.9)	35 (81.4)	0.427
Woman	1 (7.1)	8 (18.6)	
Smoking history	9 (64.3)	25 (58.1)	0.684
Histopathological diagnosis			
Adenocarcinoma	0 (0.0)	13 (30.2)	0.014
Squamous cell carcinoma	11 (78.6)	20 (46.5)	
Non-neoplastic lung	3 (21.4)	4 (9.3)	
Other tumors	0 (0.0)	6 (14.0)	

Of the 50 patients with lung cancer 36 (72%) patients were smokers and 25 of these smokers were found HPV positive. Consequently 50% of the patients with lung cancer were both smokers and HPV-infected. The higher rates of HPV 51 and 56 positivity had been detected in women but there was no statistically significant difference between gender and HPV types. Table 5 shows the HPV subtype distribution in tumors and non-neoplastic lung tissue.

Table 5. Histological diagnosis and HPV subtypes.

HPV subtypes	Squamous cell Non-neoplastic				p
	Adenocarcinoma	carcinoma	lung tissue	Other tumors	
	(Total n = 13)	(Total n = 31)	(Total n = 7)	(Total n = 6)	
	n (%)	n (%)	n (%)	n (%)	
Type 16	11 (84.6)	11(35.5)	2(28.6)	1(16.7)	0.006
Type 18	0 (0.0)	6 (19.4)	0 (0.0)	1 (16.7)	0.221
Type 31	1(7.7)	8 (25.8)	0 (0.0)	0 (0.0)	0.220
Type 39	0 (0.0)	1 (3.2)	0 (0.0)	0 (0.0)	0.999
Type 45	0 (0.0)	2 (6.5)	0 (0.0)	0 (0.0)	0.999
Type 51	11 (84.6)	8 (25.8)	3 (42.9)	6 (100.0)	<0.001
Type 52	0 (0.0)	1 (3.2)	0 (0.0)	0 (0.0)	0.999
Type 56	0 (0.0)	0 (0.0)	1(14.3)	0 (0.0)	0.226
Type 58	0 (0.0)	1 (3.2)	0 (0.0)	0 (0.0)	0.999
Type 59	0 (0.0)	2 (6.5)	0 (0.0)	0 (0.0)	0.999
Type 66	0 (0.0)	1 (3.2)	0 (0.0)	0 (0.0)	0.999

HPV 16 positivity was more common in adenocarcinomas (p=0.006). Furthermore, HPV 51 was more frequent in adenocarcinomas and other histological types of carcinomas including large and small cell carcinoma and large cell neuroendocrine carcinoma (p<0.001) and was the only subtype that had been detected in histological types of carcinomas other than adenocarcinoma and squamous cell carcinoma. There was no significant difference between smoking history and HPV subtypes.

3.4. Presence of EGFR Mutation and its Relation with HPV

EGFR mutation was detected in 3/50 (% 6) of lung cancer cases, and all cases with EGFR mutation revealed exon 19 deletion. All of the EGFR-mutated patients had a histopathological diagnosis of adenocarcinoma and revealed multiple infection of HPV types 16 and 51 and two of them were smokers. There was no significant relationship between HPV positivity and the presence of EGFR mutation in lung cancer cases (p>0.999).

4. Discussion

Since lung cancer is the leading cause of cancer death worldwide, studies about the etiological factors and carcinogenesis are important especially for guiding prophylactic therapy with vaccines and also targeted-therapy. After Rous's experiments suggesting that the virus is a possible transmissible agent the searching field for virus-associated cancer started to expand everyday [12,19]. Although supporting information about the relation between HPV infection and lung carcinogenesis is increasing everyday, the subject is still controversial and because of this, studies about this issue is ongoing.

The rate of HPV positivity in lung cancer reported in several studies is between 0-100%. This wide range depends on several factors such as geographic regions, smoking history and other patient characteristics and the method used for detection of HPV [1,6,7,20–23]. Some of the studies compared the presence of HPV in lung cancer versus non-neoplastic lung. In a study of MM Tsayganov et al searching the presence of HPV in non-small cell lung cancer patients analyzing normal and tumor tissues as well as blood from each patient; HPV was identified in 35 patients (12.7%) regardless of the group of patients and the type of material. [24]. The most recent meta-analysis study reported the prevalence difference of HPV as 22% in lung cancer compared to control cases [20]. According to another international pooled analysis HPV positivity in lung cancer tissues was found nearly 4-fold of normal lung tissue [25]. In different meta-analysis studies of Xiong et al. and Kan Zhai et al. the rate of positivity of HPV was found higher in cancer than non-neoplastic controls [8,26].

In our study HPV positivity was detected in 75.4% of all the patients. The positivity rate among cases with lung cancer and cases with non-neoplastic lung was found as 78% and 57%, respectively. The high rate detected in our study in both cancer and non-neoplastic tissue may be due to geographic characteristics, the number and the vaccination status of the patients.

Some of the studies report that there is a close relationship between HPV and lung cancer [3,6,7,20,22,26], while some of them report very low prevalence of HPV in lung cancer [1,2,21,23,27]. HPV seems to be more associated with lung cancer in certain geographical regions of the world [6]. Syrjanen et al. reported that the HPV infection rate in lung cancer is the highest in China as 37.7%, 23.9% in South America, 18.5% in Australia, 17.2% in other Asian countries, 16.9% in Europe and 12.5% in North America [28]. In many studies it is well established that the prevalence of HPV in lung cancer is higher in Asian countries than other continents [8,29–33]. Furthermore, the HPV prevalence rate ranging from 0-61% in different studies performed in Greece indicates that the results even in the same country may be discrepant [7,21]. The relationship between presence of HPV and some clinicopathologic characteristics of patients was searched in some studies. HPV prevalence was found higher in men between ages of 56-81 who were smokers or ex-smokers [3]. In a study of Zafer et al. which reported the prevalence of HPV among lung cancer cases as 2%, all of the patients were smokers [16]. (24) In our study there was no relationship between HPV infection and age ($p=0.38$), gender ($p=0.42$) and smoking history ($p=0.68$) similar to the results of some other studies [1,21].

In terms of the relation between presence of HPV and histopathological subtype of lung cancer the results reported are discrepant in different studies. HPV prevalence was found higher in squamous cell carcinoma compared to adenocarcinoma with significant differences in geographic patterns [3,7,8,20]. In a study of Aguayo et al. HPV was found in 46% and 9% of cases of squamous cell carcinoma and adenocarcinoma, respectively in South America [34]. A study evaluating the HPV prevalence in patients with lung cancer in Brazil reported the HPV prevalence as 39.4% in squamous cell carcinoma, 33.3% in adenocarcinoma, 18.2% in small cell lung carcinoma and 9.1% in large cell carcinoma [3]. Compared to these studies mentioned above there are diverging results because some other studies found higher HPV prevalence in adenocarcinoma than squamous cell carcinoma [35–37]. In our study 13/13 (100%) cases of adenocarcinomas, 20/31 (64.5%) cases of squamous cell carcinomas were positive for HPV. In 6 patients with other histological types of tumor, HPV was also found positive. A study analysing the HPV infection in patients of Taiwan reported the prevalence of HPV infection higher in adenocarcinoma (55.6%) compared to squamous cell carcinoma (35.6%),

similar to our study [37]. These different results may be due to geographic region, prevalence of the histopathological subtype of cancer in the region, ethnicity and other variables [3].

The most frequently detected HPV subtypes worldwide are types 16 and 18 [3,20,26,27]. Although a number of studies found that HPV 16/18 is significantly associated with squamous cell carcinoma, some reported that there is no statistically significant relation between HPV 16 /18 and histopathological subtypes of cancer [3,26]. The other commonly detected high-risk types are HPV 31 and 33, low-risk types are HPV 6 and 11 [29]. In a study of Baba et al. which reported HPV positivity rate higher in adenocarcinoma than squamous cell carcinoma, HPV type 16 was the most frequently detected type both in adenocarcinoma and squamous cell carcinoma [36]. In our study HPV 51 was the most frequent, representing 49.1% of the positive samples followed by HPV 16 (43.9%), HPV 31 (15.8%) and HPV 18 (12.3%). HPV 16 positivity was more common in adenocarcinomas ($p=0.006$). Furthermore, HPV 51 was more frequent in adenocarcinomas and other histological types of carcinomas ($p<0.001$).

The relation between HPV infection and lung cancer in terms of pathogenesis is still not completely understood. There are some different hypotheses about the way the virus reaches the lung. One of the mostly accepted theories include the entrance of the virus from oral cavity and going to lung cell by cell. On the other hand transfer of the virus from the genital region to lung by blood is also acceptable since various types of the virus have been detected in blood cells, plasma, umbilical cord artery. Chiou et al. detected high HPV prevalence in blood of non-small cell lung cancer patients in their study [38].

It is supposed that after reaching the lung several oncogenic mechanisms play a role in transformation to the cancer cell. HPV oncogenes such as E6 and E7 may play a role in regulating the expression of many target proteins and genes that play a role in proliferation and death of the cell and angiogenesis by taking part in various signalling pathways [3,8,20]. Some of these target proteins and genes are known to be pRb, p53, bcl-2, EGFR, ROS-1 [8].

Some studies in the literature revealed that HPV infections were more commonly detected in patients with EGFR mutation. In the study of Liang H. et al, HPV infection was suggested to be associated with EGFR mutations in Asian cases with non-small cell lung cancer [22]. Li M et al reported that there was a significant association of HPV DNA with EGFR mutations in advanced lung adenocarcinoma, in their study [18]. It is supposed that the EGFR/PI3K/AKT pathway may play an important role in HPV-associated lung carcinogenesis in EGFR-mutated patients [39]. In our study, EGFR mutation was detected in 6% (3/50) of the patients with cancer and all of the EGFR-mutated patients had a histopathological diagnosis of adenocarcinoma and multiple HPV infection with HPV types 16 and 51. There was no significant relationship between HPV positivity and the presence of EGFR mutation in lung cancer cases ($p>0.999$). This result may be due to the small number of EGFR-mutated patients detected in this study.

This study had some limitations, the small number of patients being the most important limitation. However the results show the existence of HPV in the neoplastic and non-neoplastic lung tissue and therefore a link between HPV infection and non-small cell lung cancer can be considered possible.

We suggest that our study will contribute to the literature since unlike other studies about HPV genotyping in lung cancer we searched multiple HR-HPV types instead of limited HR-types such as HPV 16 and HPV 18.

5. Conclusions

In conclusion, our study demonstrated that HPV was positive in 78 % (39/50) of lung cancer cases. HPV infection may be one of the factors playing a role in development of lung cancer. However further studies are necessary to understand the importance of detection of HPV by reliable methods , in larger number of patients, in different geographical regions, comparing patients with cancer and normal population, in order to improve especially prophylactic therapy of lung cancer by vaccines or medicine

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Conflicts of Interest: The authors declare no conflicts of interest.

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