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Genotyping of Human Papillomavirus and TP53 Mutaions at Exons 5 to 7 in Lung Cancer Patients from Iran

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ABSTRACT

Introduction: There is a powerful relationship between high-risk human papillomaviruses and lung cancer. In fact, inactivation of p53 is the most common genetic abnormality in lung cancer. Indeed, the frequency of HPV types and TP53 mutations in squamous cell carcinoma of lung, among patients from the northwest of Iran has been evaluated in this article. ***Methodes:*** Fifty Paraffin embedded blocks of lung SCC were selected for detection of HPV DNA by Nested PCR, and then DNA was sequenced for HPV typing. Equal numbers of positive and negative samples for the HPV DNA were examined for the presence of mutations in exons 5-7 of the TP53 gene by PCR and direct sequencing. ***Results:*** Overtly 9 (18%) of 50 samples presented the HPV DNA: eight were HPV-18 and one was HPV-6. TP53 mutations were found in 5 samples (27.7%). Of these, 4 cases showed mutations in exon 5 and one case contained a mutation in exon 7.The most frequent mutation in exon 5 was the C to G transversion (c.409C>G), and also the T to A tansversion (c.770T>A) in exon 7. ***Conclusion:*** This study showed that HPV-18 is more likely to conscequence in the development of lung cancer among some communities. Genetic alterations, alongside with environmental factors, all play a significant role in the pathogenesis of lung cancer.

Introduction

Lung cancer is the leading cause of death by cancer in both genders worldwide, causing over 28% of deaths from cancers.[1,2] Clinically, lung cancer can be divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Approximately 80% of primary lung cancers are NSCLC which include squamous cell carcinoma (SCC) and adenocarcinoma (AC).[3] Due to the interaction between environmental and genetic factors, the pathogenesis of lung cancer is very complicated. Environmental tobacco smoking, cooking oil vapors, asbestos and radon, indoor smoky coal burning, and infection with tuberculosis and human papillomavirus are those mentionable risk factors throughout different populations.[3,4] In 2002 and 2003, two separate studies of population-based cancer registries were carried out in Iran which suggest that the incidence rate of lung cancer is very low.[5]

It is worth mentioning that one of the small undeveloped and dsDNA viruses is human papillomavirus (HPV) that is epitheliotropic and related to both benign and malignant tumors such as papillomas and carcinomas.[6] E5, E6, and E7 genes are considered to be correlated with a host cell environment, which is proper for viral DNA synthesis that can cause host cellular DNA replication and suppress apoptosis. Therefore, the high-risk HPV E6 and E7 viral oncogenes encode viral proteins that are permanently produced in HPV-positive human cancers.[7] The role of HPV in the development of cervical carcinoma is well proven. It has been shown that about 15–20% of all human cancers can be associated with oncogenic HPVs.[8] It should be stated that there are more than 200 known types of HPV.[9] Epidemiological and molecular biological studies determined 15 genital HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) as high-risk HPVs (hrHPVs). They have the capability to cause invasive cancer.[10] Currently, a review by Syrjanen on HPV DNA detected that among 21.7% of lung cancers, HPV16 was the most common type reported.[11] High risk HPV types for lung cancer are 16, 18, 31, 33 and low risk types are 6, 11.[12] Recently, two studies on HPV infection and

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lung cancer were conducted in China.[13,14] They indicated that infection with HPV-16 and HPV-18 was related to lung cancer induction in the patients. Preliminary studies on the oncogenicity of HPV DNA in the lung focused on alterations of the normal cell cycle by p53 and RB inactivation due to viral infection.[12]

TP53 is a tumor suppressor gene that is located on chromosome 17p13 and plays a fundamental role in cell cycle, DNA repair, apoptosis, and in carcinogenesis.[15] In the majority of malignancies, including lung cancer, mutation or deletion, there are alterations that inactivate p53.[16-18] It has been reported that TP53 mutations occur in about 70% of SCLCs and more than 50% of NSCLCs.[18] The frequency of TP53 mutations in squamous cell carcinomas is higher than that of other NSCLC tumor types, however, among diverse populations, the frequency of TP53 mutations in lung cancers significantly vary from 20% to 60%.[17] A meta-analysis study of the experiments that examined the whole TP53 gene, indicated that more than 86% of mutations happened in exons 5-8.[19] P53 inactivation is observed in early stage of NSCLC. It has been recommended that this might provide a valuable molecular marker for early diagnosis or prognosis of this disease.[20]

The aim of this research was therefore to study the prevalence of high-risk HPV types and investigate the TP53 mutations in 50 blocks of paraffin-embedded tissue of SCC of lung by means of PCR and sequencing and to elucidate whether the TP53 mutations are associated with the HPV infection in SCC development in northwest of Iran.

Materials and methods

Materials

Ethanol, Tris, EDTA, phenol/chloroform and MgCL2 were obtained from merk Co., (Darmstadt, Germany). Primers were purchased from Takapuzist Co., (Tehran, Iran). All other chemicals (not mentioned) used in this study were from Sinagen Co., (Tehran, Iran).

Clinical specimens

All of 50 formalin-fixed paraffin-embedded (FFPE) tissue specimens of patients with SCC of lung were collected and diagnosed in the Imam Reza Hospital, Faculty of Medicine, Tabriz University of Medical Sciences, Iran, between 2006 and 2009.

Methods

FFPE sample processing

*A*ll Formalin-fixed paraffin-embedded samples were cut into 10µm slices and prepared according to the method described previously.[21]

Deparaffinization

One ml of xylene was added to the contents of the tube under a fume hood. The tubes were vortexed and the xylene was decanted and repeated two more times. To remove the remaining xylene, all samples were washed with ethanol at room temperature. The ethanol wash was then repeated one more time and the tubes were then left in a 55ºC oven for 20 min to dry the tissues.

Digestion

Two hundred µL of the lysis solution contained: Tris, EDTA , 0.5% Tween-20 and proteinase K, pH 8.5 was added to each tube (final concentration 200 µg/ml) and digested the tube for 3h at 55˚C with gentle agitation per hour. To inactivation of the proteinase K cell lysate was then heated at 95ºC for 10 min.

DNA extraction with phenol/chloroform

The same volume of Tris-saturated phenol was added and the tubes were left on a rotating wheel for 10 minutes and then centrifuged at 10500 rpm for 15 min. The above phase was transferred to a new tube and after adding the same volume of phenol/chloroform mixture, the tube contents were shaken and incubated for 10 min at room temperature and were centrifuged at 10500 rpm for 15 min. After the transfer of aqueous phase to a new tube, 2 original volume of cold and absolute ethanol was added and the tube was left overnight at -20ºC for DNA to precipitate. The tubes were then centrifuged for 30 min at 4ºC and ethanol was decanted. The DNA pellet was washed two times with 70% ethanol and dried at room temperature to remove any traces of ethanol. Then, the dried pellet was resuspended in 60 µl of TE buffer. To complete the solubilization, the tubes were left in a 40ºC water bath for 1 h. Finally, to verify the extracted DNA, 5 µl of the solution was electrophoresed on a 1% agarose gel. In all samples, the quality of DNA in each specimen for PCR amplification was determined by GAPDH gene amplification.

Identification of HPV DNA by nested PCR and direct DNA sequencing

Primer pairs that were used for Nested PCR are described in Table 1. PCR was performed in final reaction of 25 µL, containing 10 µL of template DNA, 2.5 µL 10X PCR buffer, 2 µL MgCl2 (50 Mm), 1 µL dNTPs (100µM), 3 µL of MY09 Primer (10 µM), 3µL MY11 Primer (10 µL) and 0.5 µL of taq DNA polymerase (5 U/µL). PCR condition was as follows: the initial denaturation at 94˚C for 5 min, 40 cycles of 94˚C for 1 min, 55˚C for 1 min and 72˚C for 1 min and a final extension of 5 min at 72˚C.

HPV detection by PCR was carried out in a nested PCR system, using MY09-MY11 as outer and GP5+- GP6+ as inner primers. Nested PCR was performed in final volume of 60 µL, containing 10 µL of the first reaction, 6 µL 10X PCR, 3 µL MgCl2 (50Mm), 1 µL dNTPs (100µM), 6 µL of GP5+ Primer (10 µM), 6 µL GP6+ Primer (10 µL) and 0.5 µL of taq DNA Polymerase (5 U/µL). PCR condition was as follows: preheating at 94˚C for 5 min, 2 min at 40˚C and 2 min at 72˚C was followed by 43 cycles of 94˚C for 1 min, 40˚C for 1 min and 72˚C for 4 min.

In order to genotype HPV, the positive PCR products were extracted from the agaros gel and the extracted DNA was analyzed by direct sequencing. PCR products for purification and direct sequencing were sent to

Table 1. Primers and PCR product length for Nested PCR

Product length	Primer sequences (5′ _ 3′)	Direction	Primers
450 bp	CGTCCMARRGGAWACTGATC	F	MY9
	GCMCAGGGWCTATAAYAATGG	R	MY 11
150 bp	TTTGTTACTGTGGTAGATACYAC	F	GP5+
	GAAAAATAAACTGTAAATCATATTC	R	GP6+

Table 2. Primers and PCR product length for PCR and sequencing analysis in exons 5, 6 and 7 of TP53 gene

Product length	Primer sequences 3′→ 5′	Direction	Exon
467 bp	Tgttcacttgtgccctgact	F	
	Ttaacccctcctcccagaga	R	Exons 5-6
177 bp	Cttgccacaggtctccccaa	F	
	aggggtcagcggcaagcaga	R	Exon 7

Macrogen Inc (Seoul, Korea, Macrogen). Finally for HPV genotyping, obtained sequencing data were processed by blast software (http://www.ncbi.nlm.nih.gov/blast).

Analysis of TP53 mutations by direct sequencing

Positive samples for HPV DNA and same number of negative samples were examined for the presence of mutations in exons 5–7 of the TP53 gene by PCR and direct sequencing. Primer pairs described in Table 2 were extracted from "Detection of TP53 mutations by direct sequencing".[22] The PCR reactions were performed in a total volume of 20 mL containing 100 ng of genomic DNA, 0.2 mM of each primer, 0.2 mM dNTPs, 1 unit of *Taq* polymerase, and 1×reaction buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, and 1.5 mM MgCL2). The PCR cycle conditions consisted of an initial denaturation step at 94°C for 2 min, followed by 20 cycles at 94°C for 30 sec, 45 sec at 63°C, 1 min at 72°C, then 30 cycles at 94°C for 30 sec, 45 sec at 60°C, 1 min at 72°C, and a final elongation at 72°C for 10 min. The primers and conditions for PCR reactions are shown in Table 2. PCR products for purification and direct sequencing were sent to Macrogen Inc (Seoul, South Korea).

Results

Table 3 summaries the clinical and existing demographic parameters from each 50 patients with SCC of lung, including age, gender, histological grade, as well as the presence/absence of HPVs. As shown in Table 3, there were 13 (26%) female patients and 37 (74%) male patients. The mean age of the patients was 65.42 ± 10.31.

Genotyping of human papillomavirus in lung cancer patients with SCC

Nine out of the 50 (18%) patients' samples, investigated for HPV DNA using nested PCR, were positive for HPV (Fig. 1). After direct sequencing of the selected nine samples, eight samples were recognized as HPV18, and one as HPV6; therefore, none of them were positive for HPV 16 DNA. Seven (77.8%) out of 9 FFPE samples with HPV positive were males and 2 (22.2%) were females; the mean age of them was 65.44 years. Furthermore, form pathological point of view, seven cases were well-differentiated.

Table 3. Patient characteristics and HPV DNA detection in lung squamous cell carcinomas

Variable	Total	HPV positive	HPV negative
Age	65.42 ± 10.31	65.44 ± 13.1	65.41 ± 9.79
Gender			
Male	37	7	30
Female	13	2	11
Grade of differentiation			
Well	38	7	31
Moderately	8	1	7
Poorly	4	1	3

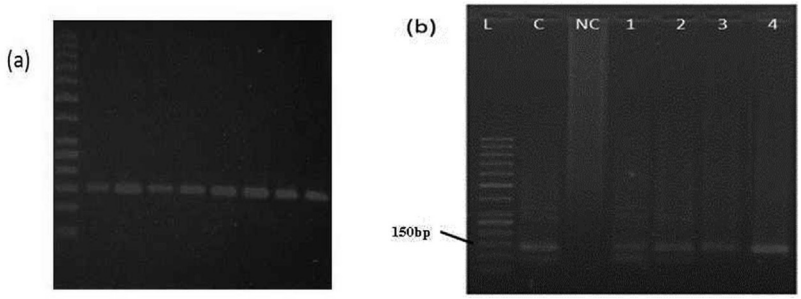

Fig. 1. (a) GAPDH gene was amplified to check the quality of the samples (b) Agarose gel electrophoresis of PCR products. (L) 50-bp ladder as DNA size marker, (C) positive control, (NC) negative control, (1, 2, 3 and 4) PCR products for HPV positive detection whit GP5+- GP6+ as inner primers

Genotyping of TP53 mutaions at exons 5 to 7 in lung cancer patients

Table 4 summarizes demographic parameters from 9 HPV positive samples and also 9 HPV negative samples that were examined for mutations in exons 5-7 by PCR and direct sequencing.

Mutations were found in 5 samples after direct sequence of exons 5, 6 and 7 of TP53 (27.7%). Of these, 4 cases showed mutations in exon 5 and one case contained a mutation in exon 7. One of HPV negative samples had a mutation in exon 5. Three HPV positive cases demonstrated a mutation in exon 5 and one in exon 7. The frequent mutation in exon 5 was the C to G transversion (c.409C>G), and the T to A transversion (c.770T>A) in exon 7. Both DNA variations have been reported as missense mutations, L137V and L257Q. Fig. 2 shows a sequencing result of our samples with L137V mutation.

Discussion

Similar to other cancers, lung cancer occurs due to accumulation of genetic abnormalities; however, all environmental factors which cause genetic damage are known.[23]

HPV has a significant role in malignancy of squamous cell carcinoma of lung, however its infection alone is not sufficient for carcinogenesis; virus-infected cells must also undergo additional genetic changes.[24]

Our understanding about the significance of HPV infection in the pathogenesis of lung cancer has been established by several studies to screen patients with lung cancer.

A wide range of HPV infection (from none to almost

Table 4. Patient characteristics, HPV DNA detection and TP53 mutatin in selected patients

Predicted effect	Region	Nucleotide change	HPV(type)	Grade of differentiation	Sex	Age	Case
		-	-	Well	F	71	1
		-	-	Moderate	M	75	4
		-	+(18)	Well	M	55	6
		-	-	Well	F	69	8
L257Q	7-exon	+ (c.770T>A)	+(18)	Poor	M	60	9
		-	+(6)	Well	M	83	11
		-	-	Well	F	50	14
L137V	5-exon	+(c.409C>G)	+(18)	Well	F	71	15
		-	-	Well	M	82	18
		-	+(18)	Moderate	F	54	21
L137V	5-exon	+(c.409C>G)	+(18)	Well	M	78	22
		-	-	Well	M	63	24
		-	-	Well	M	75	26
		-	-	Well	M	55	30
		-	+(18)	Well	M	74	31
L137V	5-exon	+(c.409C>G)	+(18)	Well	M	71	33
L137V	5-exon	+(c.409C>G)	-	Well	M	65	36
		-	+(18)	Well	M	43	47

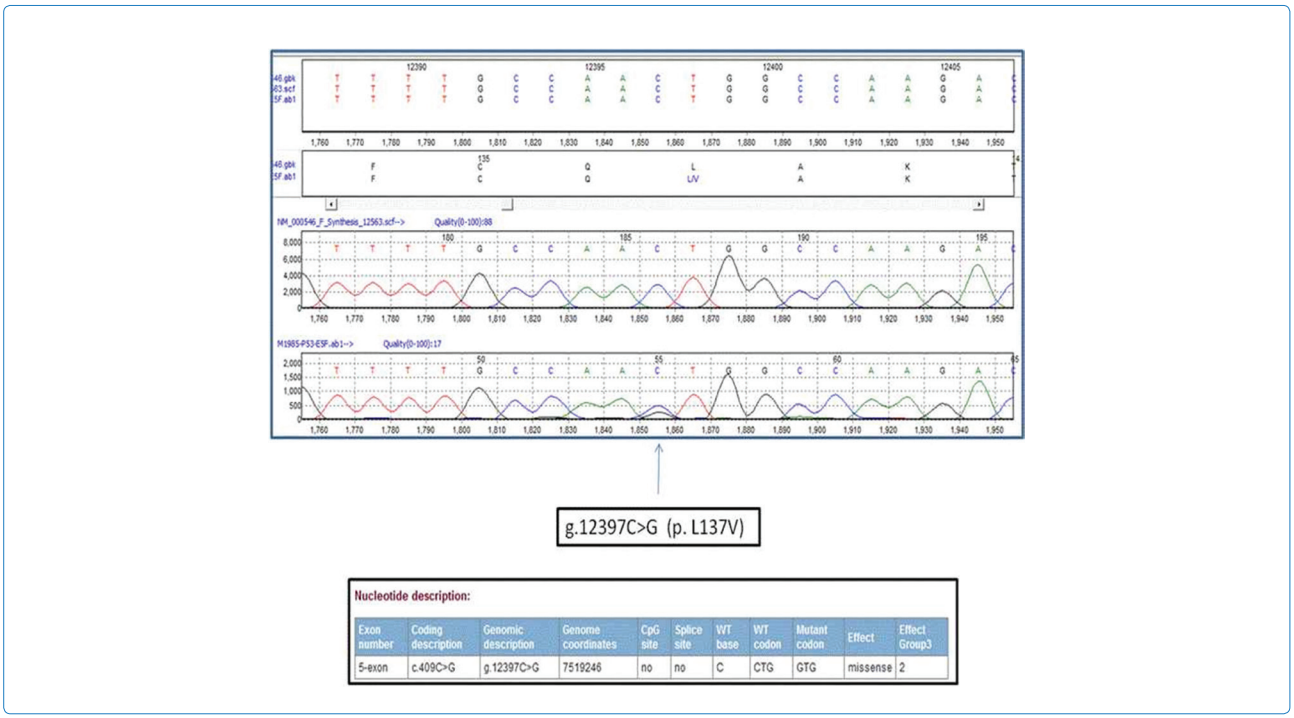

Fig. 2. Sequencing result of one sample with L137V mutation

80%) in patients with lung cancer has been observed by molecular methods.[14,24] Diversity between the positive results may be due to the different sizes, sample modes, their detection methods and geographical variations. In a review published in 2002, HPV DNA was detected in 536 out of 2468 (21.7%) patients with lung cancers.[11]

In the present study, HPV was detected by using nested-PCR. Then, PCR product was sequenced to identify the HPV type. HPV detection rate was 18% that is lower than the value of 22% in bronchial carcinomas reported by Syrjanen[11] and the value of 25.3% and 44.4% in lung SCC reported by Najdi and Shafaghi[23], respectively.

Another study, which has been recently conducted in Tabriz University of Medical Sciences, showed that in only 5.3% of saliva samples of 94 control subjects, cancer-free subjects were positive for HPV DNA.[25] In the present study, percentage of HPV infection in the SCC patients was significantly higher than that of their non-cancer control subjects. HPV detection rate was not correlated with gender, age and histological grade.

A study carried out in France reported that only 4 out of 218 samples were positive for HPV detection; one poorly differentiated SCC and three large CC.[6]

Nadji *et al* reported that the prevalence of high risk HPV types (HPV 16 and 18) was higher than that of other HPV types in lung cancer. They also suggested that especially high risk of HPV infection is strongly linked to the lung cancer development. In their study, HPV-16 was the recurrent type; however in our cases, HPV-18 was the most frequent type. Noutsou *et al* showed that the incidence of HPV in SCC was 8% and their 2 positive cases were HPV-18.[24]

Yan Yu *et al* found that the risk of lung SCC was higher among HPV-positive people compared with HPV-negative people and the risk was 3.5 times higher among people who were infected by HPV-16. The present study was in agreement with their report in that there was no correlation between HPV infection and sex, age and the differentiation grade of the tumor.[14]

In a majority of carcinomas associated with high-risk HPV, the E6 and E7 proteins act as viral oncoproteins. High-risk E6 binds to the p53 tumor suppressor protein and E7 binds to the retinoblastoma (Rb) tumor suppressor family and transform cells.[7] Five of our SCC of lung patients (27.7%) harbored a TP53 mutation in exons 5 or 7 and 4 out of 5 patients were positive for HPV type18. All of the mutations were missense.

Mohammadi *et al* reported a genetic alteration of the TP53 gene (in exon 5) in 81.1% of SCC of Lung cancer patients; however, in this study 4 (22.2%) out of 18 samples, which were examined for exon 5, had the mutations.[20] Lee *et al* reported a genetic alteration of the TP53 gene (in exons 5 and 7) in 26.15% of NSCLC in Korean patients. They found TP53 gene mutations in exon 5 among 20% of their samples.[26]

Yu Y *et al* found that p53 mutation and HPV 16/18 infection might coordinate in the development of lung SCC and their coexistence is related with poor prognosis.[27]

Conclusion

Researches similar to the present study could be useful in early diagnosis of infectious agents that have an important role in carcinogenesis. On the other hand, the prognostic value of TP53 has been demonstrated and analysis of TP53 mutations could help clinicians to choose the best therapeutic strategies.

In conclusion, HPV infection specially HPV-18 type may have an important role in pathogenesis of SSC of lung in East-Azerbayjan province of Iran.

Ethical issues

This study was approved by ethical committee of Tabriz University of Medical Sciences.

Competing interests

Authors declare no conflict of interest.

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