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Detection of Human Papillomavirus in Squamous Cell Carcinoma of the Lung by Semi-nested PCR

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ABSTRACT

Background: *Lung cancer is the second common malignancy in human. Human papillomavirus (HPV) has a well established association with squamous cell carcinoma of anogenital region. This study was performed in Massih Daneshvari hospital, between 1999 and 2003 to evaluate association of human papillomavirus type 16 and 18 in squamous cell carcinoma of the lung among Iranian patients.*

Materials and Methods: *Paraffin embedded block of pathology archive of Massih Daneshvari hospital with diagnosis of SCC were selected for determination of HPV DNA by semi-nested PCR. For each specimen, all hematoxyline-eosin stained slides were reviewed by two pathologists; if the initial slide was inappropriate, a new slide was prepared. All inadequate specimens were excluded from the study.*

Results: *18 out of 45 paraffin- embedded specimens with diagnosis of squamous cell carcinoma were selected. Six specimens were positive for HPV type 16, and 2 were positive for HPV type 18. None of the specimens was shown to have concurrent positivity of HPV types 16 and 18.*

Conclusion: *The present study showed that high risk HPV was associated with squamous cell carcinoma of the lung and more prevalent type was HPV 16. We suggest further investigations to evaluate this relationship.* ***(Tanaffos 2004; 3(11): 15-21)***

Key words*: Lung, HPV (Human Papillomavirus), PCR, SCC (Squamous Cell Carcinoma)*

INTRODUCTION

Lung cancer is currently the most frequent diagnosed major cancer and the most common cause of cancer mortality in the world. This cancer similar to cancer at other organ arise by a stepwise accumulation of genetic abnormalities, but the major environmental insult that inflict genetic damage is known. The evidence provides a positive relationship between tobacco smoking and lung cancer. About 87% of lung carcinoma occur in active smokers or in those who have quitted smoking recently (1, 2). However, data have shown that all smokers have not been afflicted by lung cancer during their lives. Furthermore, this malignancy may patient without

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smoking history. Thus, some other factors may have an effective role, including environmental and occupational factors, previous benign pulmonary diseases and genetic susceptibility (1,2,3). Human papillomavirus (HPV) has an important role in malignancy of squamous cells in various organs including ano-genital region and gastrointestinal tract (4, 5, 6). Among 85 known of HPV, types 6 and 11 are most common in hyperplasia and types 16 and 18 are frequently seen in dysplasia, and malignancy of squamous cell. HPV types 16 and 18 are categorized as high risk and HPV types 6 and 11 as low risk types (1, 6). It seems that the risk of lung cancer is significantly increased among women with ano-genital malignancies (4). Recent studies have demonstrated that individuals who are involved with preparation of meat products (which HPV infection is more common in them i.e., butcher's wart) are more susceptible to lung cancer (7). As a whole, epidemiologic data indicate the possible role of HPV in lung cancer (2, 8). The first study in this regard was conducted by Stremlau et al. in 1985 (9). Later, numerous conflicting results were reported across the world (10-14).

The present study was performed to access the rate of association of HPV types 16 and 18 in Iranian patients with squamous cell carcinoma of the lung.

MATERIALS AND METHODS

Between 1999 to 2003, paraffin-embedded blocks with diagnosis of squamous cell carcinoma were retrieved from pathology files of Massih Daneshvari hospital.

All specimens were fixed in 10% neutralized buffered formalin and embedded in paraffin. For each specimen, all hematoxyline-eosin stained slides were reviewed by two pathologists; if the initial slide was inappropriate, a new slide was prepared. All inadequate specimens were excluded from the study.

PCR Method

DNA detection and typing were done in the molecular laboratory which used PCR amplification methods that targeted small fragments of HPV genome in the region of E6 gene.

DNA Extraction

DNA was extracted from four to five 10 μm tissue sections of paraffin blocks by standard phenol-chloroform procedure after treatment with proteinase K. To avoid carry-over of samples the microtome blade was carefully cleaned with xylene between sectioning of blocks and scalpel was changed for each case. Paraffin was removed with two rounds of warm xylene extraction (60°C) followed by two 90% ethanol washes. After high-speed centrifugation of the tissues, samples were incubated in digestion buffer (10 mM Tris-HCl, pH 8.5, 25 mM EDTA, 0.5% SDS and 0.2 mg/ml Proteinase K) for overnight at 55^0C with shaking. Proteinase K was inactivated at 95°C for 10 min, and An equal volume of phenol: chloroform (1:1) added, mixed gently and centrifuged. The aqueous phase was mixed with an equal volume of chloroform. DNA was precipitated from aqueous phase by adding an equal volume of isopropanol in the presence of 30 μl of 3 M Na-acetate (pH5.2). The precipitated DNA was washed twice with 70% ethanol to remove salt. The pellets were vacuum-dried for 15 min, dissolved in deionized distilled water and used directly for PCR. To further purification of DNA, a purification kit (Amplisens, Ltd., Moscow, Russia) with a sorbent was used in accord with recommendation.

Integrity of purified DNA and the absence of inhibitors of Taq DNA polymerase was assessed with the human HFE gene specific primers. Cases with a negative amplification of the HFE gene were excluded from the analysis. Semi-nested PCR with HPV type specific primers was used for the HPV detection and typing studies. In brief, for HPV16 and 18 viral genotyping, PCR products were successively

analysed by using two pairs of type-specific primers to unique sequences of the E6 regions of HPV-16 (16f , 5'-tcaaaagccactgtgtcctg-3'; 16r, 5'-cgtgttcttgatgatctgca-3') and HPV-18 (18f, 5'-gacacattggaaaaactaac-3'; 18r 5'-tagtgcccagctatgttgtg-3') as described previously (2, 3, 4). To increase the sensitivity of the assay an additional "inner" primer was introduced to both HPV16 (5'-acaagacatacatcgaccgg-3') and HPV18 (5'-ctaactaacactgggttatac-3'). Standard precautions were taken to keep the risk of false-positive results in the PCR reactions at a minimum. Plasmids containing HPV-16 and -18 DNA were used for titration and evaluation of the sensitivity of the detection and typing method.

Table 1. The PCR results of HFE gene and HPV types 16, 18 gene.

No. of specimen	type	HFE	HPV-16	HPV 18
1	Biopsy	+	-	-
2	CT-guided Biopsy	+	+	-
3	Biopsy	+	-	-
4	Lobectomy	+	-	+
5	Lobectomy	+	-	-
6	Biopsy	+	-	-
7	Lobectomy	+	-	+
8	Lobectomy	+	-	-
9	Pneumonectomy	+	-	-
10	Biopsy	+	+	-
11	Biopsy	+	-	-
12	Pneumonectomy	+	+	-
13	Biopsy	+	-	-
14	Biopsy	+	+	-
15	Biopsy	+	-	-
16	Biopsy	+	+	-
17	Biopsy	+	+	-
18	Lobectomy	+	-	-
Total	-	18	6	2

PCR was performed in a final reaction volume of 25 ul containing 5 ul of the isolated DNA, 16.6 mM $(NH_4)_2SO_4$ 66.7 mM Tris-HCl (pH8.8), 2.0 mM $MgCl_2$, 200 uM each deoxynucleoside triphosphate, 15 pmol of each of the forward and reverse primers, and 1 U of Taq DNA polymerase (Roche). For seminested PCR, 1 ul of PCR product from the above amplification was added to PCR mix containing "inner" combination of primers. Conditions and thermocycling parameters were identical both for the first and the second amplification: after heating at 94°C for 3 min the mixture was subjected to 30 cycles of PCR amplification (denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min). The last cycle was followed by an additional 5 min of extension at 72°C. The amplified fragments were resolved by electrophoresis on the 2.5% agarose gel in 80 volts for 90 min and stained with ethidium bromide and visualized on a UV transilluminator (Uvitec, UK).

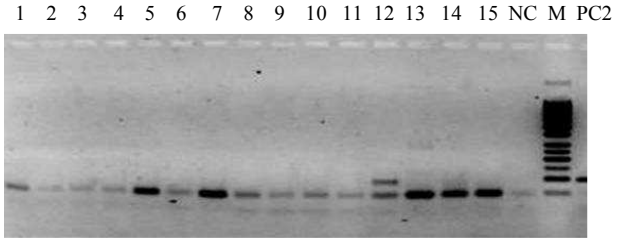

Figure 1. The electrophoresis results for HPV -18 in paraffin- embedded specimens by semi- nested PCR. PC2 is positive control. M is marker and NC is negative control. No 12 is positive case.

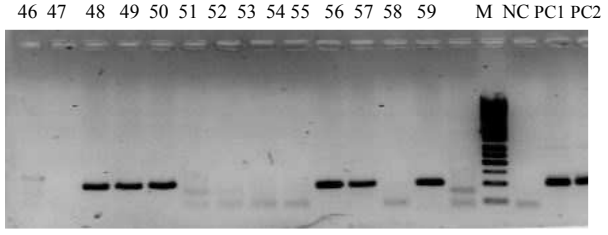

Figure 2. The electrophoresis results for HPV types 16 in paraffin-embedded specimens by semi-nested PCR. PC1 and PC2 are positive controls. M is marker. Lanes 48, 49, 50, 56, 57, 59 are positive cases.

RESULTS

Eighteen out of 45 paraffin-embedded specimens with diagnosis of pulmonary squamous cell carcinoma were selected considering adequacy of the specimens.

The selected specimens belonged to 15 men and 3 women ranging from 45-81 years old. Seven specimens were lobectomy and pneumonectomy and 11 were biopsy specimens. Integrity of purified DNA and the absence of inhibitors of tag DNA polymerase was confirmed by using HFE gene primers in all cases. Six specimens were positive for HPV-16 and 2 were positive for HPV-18 by semi nested PCR. No specimen was positive for both types of HPV.

For conformation of results all specimens were evaluated again for PCR of HFE gene and HPV types 16 and 18, those which showed significant electrophoresis band, in both runs, for HPV types 16 or 18 were considered positive and those showing electrophoresis band only in one run, were considered negative.

DISCUSSION

Lung cancer has multifactorial pathogenesis, the most common factor is smoking. However, it is obviously clear that no risk factor can cause lung cancer by it self (1, 14). Human papillomavirus (HPV) has an important role in malignancy of squamous cell of various organs (4-6). It seems that this virus involves squamo-columnar junctions like uterocervical junction and anorectal junction (14). The possibility of association of HPV with squamous cell carcinoma of the lung is enhanced with detection of this virus in malignancy of upper respiratory tract (15- 17). Smoking causes squamous metaplasia and subsequently numerous squamo-columnar junctions in upper and lower respiratory tract, and prepare an appropriate soil for HPV infection (14).

The present study evaluated the association of HPV DNA in squamous cell carcinoma of the lung. We detected HPV in 8 out of 18 specimens, using semi nested PCR; HPV 16 in six cases and HPV 18 in two cases. The results were similar to previous studies. Bejui-Thivolet et al. (11) in 1990, detected HPV genome in 5 out of 33 SCCs of lung using in situ hybridization method; of which three cases were HPV 18, one case was HPV 16 and one case was positive for both HPV 16 and 18. Li Q et al (12) in 1995, also detected HPV 16 and 18 genomes in 48% of SCC of lung by PCR method and dot-blot hybridization, which is almost compatible with our study. On the other hand, Welt A et al (14) in 1997, can not demonstrated HPV DNA in 32 SCC and 6 small cell carcinoma of the lung.

In this study, prevalence of HPV- 16 infection was more significant than that of HPV-18, which is similar to most studies carried out in Western and Asian countries (including Iran) (6, 11, 12, 13, 18, 19). Another study which has been concurrently conducted in our center, showed that in 15% of 33 laryngeal squamous cell carcinoma of Iranian patients were positive for HPV DNA types 16 and 18 which is less than our results (20). Regarding positivity of all laryngeal carcinoma for HFE gene, the method of DNA extraction could not be responsible for this difference. It should be mentioned that all stages of both studies (laryngeal and lung carcinoma studies) were carried out by same staffs and methods. Thus, one possibility is the length of lower respiratory tracts comparing to larynx which may increase the possibility of creating more squamo-columnar junctions which prepare appropriate soil for penetration of virus. One remarkable note is that HPV type 16 is prevalent type in both studies.

CONCLUSION

The present study showed that high risk HPV was associated with squamous cell carcinoma of the lung and more prevalent type was HPV 16. We suggest further studies with more specimens, using of in situ hybridization, performance of case-case studies with regard to all known risk factors, and finally case-control studies.

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