

## RESEARCH COMMUNICATION

# Evaluation of Human Papilloma Virus Infection in Patients with Esophageal Squamous Cell Carcinoma from the Caspian Sea Area, North of Iran

Yousef Yahyapour<sup>1,3</sup>, Mahmoud Shamsi-Shahrabadi<sup>1</sup>, Mahmoud Mahmoudi<sup>2</sup>, Sepideh Siadati<sup>3</sup>, Shefaei Shahryar Shahryar<sup>3</sup>, Javad Shokri-Shirvani<sup>4</sup>, Hamid Mollaei<sup>1,5</sup>, Seyed Hamid Reza Monavari<sup>1\*</sup>

### Abstract

**Introduction:** HPV has been found repeatedly in esophageal squamous cell carcinoma (ESCC) tissues. However, reported detection rates of HPV DNA in these tumors have varied markedly. Differences in detection methods, sample types, and geographic regions of sample origin have been suggested as potential causes of variation. We have reported that infection of HPV DNA in ESCC tumors depends on anatomical sites of esophagus of the patients from Mazandaran, north of Iran. **Materials and Methods:** HPV DNA was examined in 46 upper, 69 middle and 62 lower third anatomical sites of esophageal squamous cell carcinoma specimens collected from Mazandaran province in north Iran, near the Caspian Littoral as a region with high incidence of ESCC. HPV L1 DNA was detected using Qualitative Real time PCR and MY09/MY11 primers. **Results:** 28.3% of upper, 29% of middle and 25.8% of lower third of ESCC samples were positive for HPV DNA. 13.6% for males and 14.1% for females were HPV positive in all samples. **Conclusions:** HPV infection is about one third of ESCC in this area. Findings in this study increase the possibility that HPV is involved in esophageal carcinogenesis. Further investigation with a larger sample size is necessary.

**Keywords:** HPV - esophageal squamous cell carcinoma - site of esophagus - Iran

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### Introduction

Human papilloma viruses (HPVs) have been concerned as a causal agent in a variety of human squamous cell carcinomas, including those of the cervix, anogenital region, skin, upper respiratory tract, and digestive track (Bosch et al., 2002; Gillison & Shah, 2003).

Mazandaran Province, located in the south-littoral of the Caspian Sea in northern Iran (Figure 1), has some of the highest rates of Esophageal Cancer (EC) in the world (Blot & McLaughlin, 1999). Nevertheless, the real causes and the mechanism of ESCC have not been elucidated yet.

About 4000 esophageal squamous cell carcinomas (ESCCs) are diagnosed annually in the Iran. A high incidence Asian "esophageal cancer belt" stretching from Northern Iran through the central Asian republics to North-central China, has been identified in the world (Mahboubi & Kmet, 1973). The reasons for these major regional variations in the incidence of this disease are poorly understood. In western countries, where the risk of ESCC is generally low, consumption of tobacco and

alcohol could explain more than 90% of the cases of ESCC (Brown et al., 2001). However, in countries with the highest rates of ESCC, such as Iran, China and India, only small proportion of ESCC could be attributed to smoking or alcohol consumption (Cook-Mozaffari et al., 1979; Mir et al., 2005; Tran et al., 2005).

Microbial agents, especially HPV, may be one of the factors that explain part of this high incidence of ESCC. The role of HPV in ESCC has been studied in many high-risk and low-risk areas of the world (Lavergne & De Villiers, 1999; Syrjanen, 2002). Most studies from high-risk areas, such as China and South Africa, have suggested a role of HPV in ESCC, while most studies from low-risk areas have failed to find any association (Lu et al., 1991; Syrjanen, 2002). The human Papillomavirus (HPV) has been thought of as a possible etiological factor of EC since 1982 and there is a large amount of variation in the observations of such studies which have been reviewed by Syrjanen (Syrjanen, 2002). A general review by Syrjanen published in 2002 reported that HPV is positive in 22.9% of 1485 ESCC cases tested by in situ

<sup>1</sup>Department of Virology and Antimicrobial Resistant Research Center Faculty of Medicine, <sup>2</sup>Department of Epidemiology and Biostatistics, School of Health, <sup>3</sup>Cellular and Molecular Research Center, Tehran University of Medical Sciences, Tehran, <sup>4</sup>Department of Pathology, <sup>5</sup>Department of Internal Medicine, Babol University of Medical Sciences, Babol, Iran \*For correspondence: hrmonavari@yahoo.com



**Figure 1. Geographic Location of Mazandaran Province in the Caspian Littoral of Iran**

hybridization (ISH) and in 15.2% of 2020 ESCC cases tested by PCR (Syrjanen, 2002). HPV has been found in variable proportions of ESCC, depending on the method and study areas (Syrjanen, 2002) (Table1).

HPV DNA has been detected in 30% of human head and neck cancers (Gillison et al., 2000; Hansson et al., 2005), and because the esophagus can be infected with these viruses in the same way as the oral cavity, tonsils, and pharynx, it is speculated that HPV infection might explain at least some esophageal cancers in humans.

HPV DNA detection rates in ESCC samples appear to be different from area to area. An explanation, offered by Syrjanen in his review, is the hypothesis that the contribution of oncogenic HPVs to ESCC risk is higher in the areas with high ESCC risks (Syrjanen, 1982). Esophageal squamous epithelium is one of the susceptible sites to HPV. So far, there is no report on the correlation of HPV infection and high incidence of esophageal carcinomas in Mazandaran province, north of Iran, and its epidemiology has not been fully studied.

In the present study, we report the infection of HPV in 177 ESCC tumors depend on anatomical sites of esophagus of the patients from Mazandaran, Iran. Indeed, recent studies conducted in areas with a high ESCC risk showed moderately high detection rates of high-risk HPV DNA in cancer specimens.

## Materials and Methods

### *Study population and samples*

Biopsy and surgery fresh samples from patients with esophageal carcinoma, referred to the Department of Pathology, University Hospital, Babol, and Amol Central Pathobiology Lab., Mazandaran province, Iran, during 2004–2011 were identified. A total of 303 specimens found, but 177 formalin-fixed paraffin-embedded tumor tissues of patients collected and entered to our study, and others patients were lost due to inadequate, absence of histological material or undefined the anatomical sites of esophagus. No patient had received chemo- or radiotherapy before endoscopies and surgery. In all cases, the histopathological type of tumor was squamous cell carcinoma. The present study is based on a retrospective examination of esophageal carcinoma diagnostic biopsy and surgery samples from clinical cases, all original hematoxylin and eosin (H&E) slides and/or H&E recuts from tissue blocks were reviewed.

**Table 1. Prevalence of HPV in ESCC: Literature Review**

Country	No. of Cases	HPV Ve+ (%)	HPV Detection Method	Reference
Iran	85	49.4	PCR	34
Iran	140	23.6	PCR	35
Sweden	100	16	Real-time PCR	37
Australia	222	3.6	PCR	38
Colombia & Chile	73	28.8	PCR	39
Iran	38	36.8	PCR	41
China	435	46.9	PCR	42
South Africa	50	46	PCR	43
China	70	33.33	Real-time PCR	44
China	700	16.9	In-situ Hybridization	45
Turkey	30	33.3	PCR	46
China	20	30	In-situ Hybridization	47
Latin American	60	25	INNO-LiPA	48
China	67	21	INNO-LiPA	49
Presence study	177	27.7	Real-time PCR	-

This project was approved by the human research ethics committee of the Babol University of Medical Sciences and participating Centers, and written informed consent was obtained from all participants. The following parameters were studied: age, gender and type of sample (biopsy or surgery). Anatomical localization of the tumor was grouped into an upper part (15–24 cm), a middle part (25–34 cm) and a lower part of the esophagus (35–46 cm).

### *Deparaffination procedure*

Paraffinated blocks from the 177 tumor samples were cut in 5- $\mu$ m sections and 8 sections/patient were collected in the same microcentrifuge tube. Samples were de-waxed in 500  $\mu$ l xylene, All microcentrifuge tube located for 10 min in a 60 °C heated block and centrifuged at 8,000 rpm, supernatant was removed. This step was then repeated 3 times. Add 500  $\mu$ l absolute ethanol, centrifuge at 10,000 rpm for 1 min, the samples were then dried in a 60 °C heated block with open lids for 10-20 min for remove residual ethanol.

### *Tissue digestion*

According to samples (biopsy or surgery), 200–400  $\mu$ l of Tissue Lysis Buffer was added to each tube [4 M Urea, 200 mM Tris, 20 mM NaCl, 200 mM EDTA; PH=7.4 (25 °C)]. To all tubes added 20-40  $\mu$ l proteinase K, Samples were gently vortexed and located for 10 min in a 60 °C heated block, and all samples were subsequently incubated at 37 °C overnight.

### *DNA Extraction*

The next day, 200  $\mu$ l of Binding Buffer [6 M Guanidine-HCl, 10mM Urea, 10mM Tris-HCl, 20% Tritonx-100 (v/v); PH=4.4 (25 °C)] was added to each tube with gently vortex . DNA was isolated using a High Pure Template PCR kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Extracted DNA pellets were resuspended in 100  $\mu$ l of prewarmed Elution buffer and stored at -20 °C until use for Real Time PCR.

### *Quality Control*

The quality and concentration of DNA was measured

**Table 2. Sequence of Primers Used**

Set	Primers	Sequences
1	MY09	5'-CGT CCM AAR GGA WAC TGA TC-3'
2	MY11	5'-GCM CAG GGW CAT AAY AAT GG-3'
3	$\beta$ -Globulin (F)	5'-TGG GTT TCT GAT AGG CAC TGA CT-3'
4	$\beta$ -Globulin (R)	5'-AACAGC ATCAGG AGT GGACAG AT-3'

either on an ethidium bromide-stained 1% agarose gel or by standard spectrophotometric methods (Data Not Shown). Also, presence of DNA was confirmed by PCR with human  $\beta$ -globin primer as an internal control (Table. 2). Hela cell line was used as a positive control for HPV infection. Fragments of human  $\beta$ -globin gene, which served as the internal controls, were successfully amplified from 129 of the 177 cancer specimens. Therefore, in 46 samples were not present of DNA after extraction, thus DNA extractions of these specimens were done again. Also, Distilled water was used as a negative control. This control was necessary to determine if any of the reagents was contaminated with HPV DNA.

#### Real-time PCR

After DNA extraction, Real-time PCR was performed with the Corbett Rotor-Gene 6000 (Corbett Research, Australia) instrument for Real Time PCR test and three step with melt curve method. Approximately, 100-200 ng cellular DNA was utilized for Qualitative Real Time PCR. We used SYBR-Green PCR master mix (Maxima<sup>®</sup> SYBR Green qPCR Master Mix (2X), Applied Fermentas, EU). In brief, qualitative PCR was performed by 6  $\mu$ l of purified DNA from each sample was added to the 6  $\mu$ l PCR master mix with 300 pmol of each primer. The amplification conditions were 2 min at 50 °C, a three-step cycle of 95 °C for 10 min (for initial denaturation), 95 °C for 15s, 55 °C for 30 s and 72 °C for 3 s (5 repeats). For cycling 2: 95 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s for a total of 40 cycles ,then ,melting temperature was done from 70 °C to 99 °C and data stored for analysis melting curve. To examine for the presence of any HPV DNA in the tissue, MY09/MY11 primer pairs as a general primers were used to amplify the L1 gene (Table 2). For confirmed result we use gel agarose electrophoresis 1% that stained ethidium bromide.

#### Statistical analysis

X<sup>2</sup> test or Fisher's exact test was conducted using SPSS version 14 for the association between the presence of HPV genome and anatomical sites of esophagus, gender and age group (values P=0.05 were considered statistically significant).

## Results

Of 177 confirmed ESCC enrolled to our study, which is divided in 26%, 39% and 35% of upper, middle and lower third of ESCC patients, respectively. 58.8% of the patients in upper, 56.5% in middle and 50% in lower part of the esophagus were male. The age ranges in patients were between 35 and 91 years old. 44.1% of all patients were between 65 and 75 years old. Also, the table has shown

**Table 3. Absolute and Relative Frequency of HPV-DNA in Different Anatomical Sites of Esophageal SCC by Age Group**

Third of Esophagus	Age group (year)				P-Value
	<55 No. %	55-64 No. %	65-74 No. %	$\geq$ 75 No. %	
Upper: Ve+	1 17	4 40	5 25	3 30	$\chi^2=1.20$ df=3 q=0.754
Ve-	5 83	6 60	15 75	7 70	
Total	6 100	10 100	20 100	10 100	
Middle: Ve+	7 41	1 11	5 20	7 39	$\chi^2=4.463$ df=3 q=0.216
Ve-	10 59	8 89	20 80	11 61	
Total	17 100	9 100	25 100	18 100	
Lower: Ve+	5 42	4 44	2 8	5 29	$\chi^2=7.15$ df=3 q=0.067
Ve-	7 58	5 56	22 92	12 71	
Total	12 100	9 100	24 100	17 100	

many demographic data of patient by different parts of anatomical sites of ESCC patient. 42.4% of patients were living in urban and 57.6% were live in rural areas.

#### Detection of HPV in ESCC patients

L1 consensus primer was used to detect all HPV infection in a total of 120 esophageal tumor biopsies and 57 esophageal tumor surgeries obtained from Mazandaran province, geographic regions of Iran (Table 3). Also, there is a significant correlation between biopsy resections versus surgical in middle and lower anatomical sites of esophagus (P= 0.048; P=0.052) (Table 3).

The distribution of the HPV positivity in cases with esophageal cancer, based on gender of the patients, were 25.9%, 23.1% and 25.8% for males in upper, middle and lower part of ESCC patients. Also, the rates of HPV-DNA in upper, middle and lower part of ESCC in female were 31.6%, 36.7% and 25.8%, respectively. The table that shown data of HPV infection in different third part of esophagus by age group in less than 55, 55 to 64, 65 to 74 and +75 years old. HPV-DNA presence in ESCC was not significantly associated with gender and age. But, there is significant difference between lower part of ESCC and age group by HPV-DNA positive (q=0.067). As the shown in table 6; 20%, 30.6% and 29.2% of patients in upper, middle and lower part of ESCC infected with HPV-DNA were living in urban.

#### Effect tumor sites on HPV prevalence

Tumor site data was available for squamous cell carcinomas from the Mazandaran regions, and evaluation of the association between anatomic location and HPV prevalence was carried out in these cases. L1 was detected in 13 (28.3%) of carcinomas located in the upper third of esophagus, 20 (29%) in the middle third and 16 (25.8%) in the lower third. No statistically significant relationship between HPV infection and tumor site was found in this study (q= 0.91).

## Discussion

In Western countries, where the risk of ESCC is generally low, consumption of tobacco and alcohol could explain more than 90% of the cases of ESCC (Li & Cheng, 1984; Lu et al., 1991). However, in countries with the

highest rates of ESCC, such as Iran and China, other risk factors such as microbial agents especially HPV may be some of the factors that explain part of this high incidence of ESCC (Lu et al., 1991; Rosai, 2004).

HPVs are oncogenic viruses and show oncogenic activity through spoiling mucosal immune resistance and destroying tumor suppresser genes (Chang et al., 1990; Chen et al., 1997).

The role of HPV in ESCC has been studied in many high-risk and low-risk areas of the world (Gravitt et al., 1998; Chan et al., 2001; Syrjanen, 2002; Katiyar et al., 2005; Murtaza et al., 2006). Most studies from high-risk areas, such as China and South Africa, have suggested a role of HPV in ESCC, while most studies from low-risk areas have failed to find any association (Kok et al., 1997; Lavergne & De Villiers, 1999; Mir et al., 2007).

There is increasing evidence suggesting that HPV infection is an important etiological factor in esophageal cancers (Chang et al., 1990; Shen et al., 2002; Ting-Ting et al., 2005). An association of HPV with esophageal carcinoma has been previously reported in many countries. The incidence of infection differs markedly depending on the different geographical location of the population under study and within different studies (Chang et al., 1990; Williamson et al., 1991; Cooper et al., 1995).

In the present study, using qualitative Real-time PCR with MY09/MY11 primers, we detected HPV DNA in 27.7% of samples different from of HPV DNA was positive in 49.4% of samples from north-east of Iran (Turkmen Sahra) in the east of Mazandaran (Golestan Province) (Moradi et al., 2002), where ESCC incidence is much higher than in Mazandaran. While, in another study in Tehran (South of Mazandaran), HPV DNA was positive in 23.6% and 36.8 % of samples in tumoral region of patients (Farhadi et al., 2005; Eslami et al., 2007).

According to our results, imply that HPV is not a predominant risk factor for ESCC in Iran because 49 (27.7%) of all 177 samples of ESCC were positive for the common indicator of HPV (L1 gene). But, this is the first study in Iran that reports HPV infection in different anatomical sites of ESCC patient.

HPV prevalence carries a close correlation to the incidence of SCC, being low (0-3%) and high (up to 80%) in the respective geographic regions (Syrjanen, 2006). In China the frequency of HPV in ESCC were reported between 6.7% and 83.3% in different parts of this country (Chang et al., 1990; Ting-Ting et al., 2005). However, the frequency of HPV infections varies significantly in different geographic locations in China. In a cross-sectional study conducted in a high-risk region (Linxian) of China, HPV positivity was found in 16% of patients with severe dysplasia but never in patients with ESCC (Gao et al., 2006).

Our study and Previous studies have shown HPV infection in different area of Iran, were nearly close together and different from to others reports of many countries. In our study prevalence of HPV L1 DNA were detected in 28.3%, 29% and 25.8% of upper, middle and lower part of esophagus in patients with ESCC. In China study reported, 7.5% in upper, 63.4% in middle and 29% in lower part of ESCC patients (Wang et al., 2010). But, In

Sweden study, HPV L1 DNA were reported in the rates of 27.3%, 18.2% and 6.4% of upper, middle and lower part of esophagus in patients with ESCC (Dreilich et al., 2006).

There was high incidence of HPV infection in the esophageal epithelium in eastern Guangdong, southern China, where esophageal carcinoma is prevalent. The results indicate that the high incidence of esophageal carcinoma in this area is associated with HPV infection (Shen et al., 2002).

In many Western countries the results show that, in contrast to geographic regions where ESCC is prevalent, HPV infection occurs infrequently in association with ESCC (Turner et al., 1997).

HPV L1 DNA was detected in 24.5% of male and 31.3% in female of patients that inrolled to our study. Wang and colleagues in China reported HPV L1 DNA in 26.6% of male and 73.3% of female (Wang et al., 2010); while Dreilich and colleagues in Sweden ESCC patients detected HPV infection in 14.1% and 20.7% in male and female (Dreilich et al., 2006). This rate in Colombia and Chile reported by Castillo and colleague in male and female ESCC patients 23.1% and 35.3%, respectively (Castillo et al., 2006). The male: female ratio in HPV positive ESCC decreased in comparison with HPV negative cases (0.96:1 vs 1.2:1). This ratio is nearly similar to that reported from Colombia and Chile (0.75:1 vs 1.1:1) (Castillo et al., 2006). However, the gender-ratio between HPV positive and HPV negative cases was not statistically significant.

34.9% of HPV L1 DNA positive in ESCC patients in this study were less than 65 years and 23.7% of ESCC patients were more than 65 years old. This rate in our study was similar to almost others studies that HPV infection in younger were more than to older patients (Castillo et al., 2006; Dreilich et al., 2006; Wang et al., 2010).

Our results are consistent with HPV DNA studies conducted in other high-risk areas for ESCC which showed evidence of HPV in tumor tissues from 20% to 50% of ESCC cases and provided more evidence to support a causal association of HPV infection with esophageal squamous cell carcinoma. Also, our finding suggests possibility of a strong geographical difference in the proportion of HPV-associated ESCCs. Note, however, that our ESCC cases were convenient samples and, therefore, may not represent the ESCC cases in the study areas.

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