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Detection of Human Papillomavirus in Osophageal Squamous Cell Carcinoma samples in Mazandaran Province, Iran

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ABSTRACT

Human papillomavirus (HPV) has been associated with several disorders of the genital tract, skin and esophagus squamous cell carcinoma. The aims of the present study were to evaluate the prevalence of HPV infection in esophageal squamous cell carcinoma in Mazandaran province, northern Iran, to identify the prevalence of HPVs. We examined 170 formalin-fixed paraffin-embedded samples from esophageal squamous cell carcinoma patients. All subjects live in Mazandaran province, a region with high incidence rate of esophageal cancer and have become known as the "Asian Esophageal Cancer Belt". Samples were tested for HPV-DNA by MY09/11 and Gp5+/6+ general primers using nested PCR. Of the 170 ESCC samples, 86 (50.6%) were male and 84 (49.4%) were female. The mean age of the subjects was 66.5±11.1 and ranged from 35 to 91 years. Totally, HPV-DNA was detected in 62 (36.5%) of the esophageal squamous cell carcinoma samples by HPV L1 consensus primers. Considering the location of esophagus specimens, of 62 positive samples, 16 (25.8%) samples were in the upper third, 28 (45.2%) in the middle third, and 18 (29.0%) in the lower third. The current study showed a relatively substantial prevalence of HPV infection in esophageal squamous cell carcinoma samples in Mazandaran province.

1. Introduction

Cancers are among the most common causes of death throughout the world. It is estimated that the overall incidence of various types of cancers will increase by 45% in developed countries by 2030. Recent reports have indicated that cancers are the second most common cause of non-accidental death in Iran, following cardiovascular death (Parkin, 2005; Naghavi, 2000; Kamangar, 2006). An estimated 51,000 cases of new cancers are diagnosed each year in Iran. About 38% of these cancers arise from the gastrointestinal tract with 6,500 of them resting in the esophagus (Sadjadi, 2005). Both histological subtypes of esophageal malignancies, squamous cell carcinoma and adenocarcinoma are highly lethal with a current five year survival of less than 10% (Samadi, 2007). Of the approximately 35,000 yearly cancer deaths in Iran, about 5,800 are due to esophageal cancers (Sadjadi, 2005).

Epidemiological studies have identified the several high incidence areas for esophageal

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cancer, including China, Singapore, Iran, Russia, Puerto Rico, Chile, Brazil, Switzerland, France and South Africa (Li, 1980; Li, 1982), but the causes for striking geographical variations in the incidence of human esophageal cancer remain obscure. In India, esophageal cancer is most common, but unevenly distributed with many regions showing a high prevalence, and, recently, an upward trend in the frequency of its occurrence has been observed (Siddiqi, 1989).

Several associated risk factors, such as dietary, cultural habits, environmental and genetic factors, nutritional deficiencies, excessive use of tobacco, alcohol consumption and infection with certain DNA tumor viruses including Human Papillomavirus (HPV) and Epstein - Barr virus (EBV) are attributed to this disease (Kodsi, 1976; Jarrett, 1987; Goff, 1988; Syrjanen, 2002).

Viral infections, in particular HPV infections, have been reported in esophageal squamous cell carcinoma (ESCC) and various rates of HPV DNA detection have been found, ranging from 0 to 80%, depending on the geographical area, ethnic group and the methods used for viral detection (Syrjanen, 2002).

A case series, from Egypt (Bahnassy, 2005), Colombia, Chile (Castillo, 2006), Brazil (Souto, 2006), Germany (Pantelis, 2007), the Republic of Korea (Koh, 2008), the Islamic Republic of Iran (Eslami, 2007), and China (Shuyama, 2007; Lu, 2008) have been reported HPV in ESCC. HPV 16 was the most common type in all studies, followed by HPV 18. Interestingly, a recent paper has confirmed that HPV DNA detection rates were 65% in samples from Gansu, a high risk area of ESCC and only 6% in samples from Shandong, a low-risk area of ESCC in China (Shuyama, 2007). A monograph of the International Agency for Research on Cancer showed that sufficient evidence was available for HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66 to be considered as class I carcinogens for humans (Cogliano, 2005). The epithelium of the esophagus is similar to the oral cavity, and papillomas have been described at this anatomical site although they are rare, and HPV is not consistently detected therein. The main object of the present study was to assess the prevalence of HPV infection in ESCC tumor samples from Mazandaran, near the Caspian Sea as an area known as the “Asian Esophageal Cancer Belt”.

2. Materials and Methods

2.1. Clinical samples

In this cross-sectional study, a total of 170 ESCC specimens were obtained from the archives of two referral pathology centers in Mazandaran province (Pathology Department of Shahid Beheshti Hospital, affiliated to Babol University of Medical Sciences and Amol Central Pathobiology Laboratory). Study group included 86 (50.6%) males and 84 (49.4%) females with age range of 38 to 91 year old, an average age of 65.7 years.

None of the patients had radical therapy or chemotherapy prior to endoscopy and surgery. The paraffin-embedded, formaldehyde fixed samples were cut into 4-8 sections of 5 µm for DNA extraction (depends on type of sample as biopsy or surgery). 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). In this study, ESCC samples were stratified by anatomical sites, 37 (21.8%) samples were located in upper third of esophagus, 66 (38.8%) were in middle third, and 67 (39.4%) the lower third. This study was approved by the Ethical Committee of Golestan University of Medical Sciences, and for all subjects, written informed consent was obtained.

2.2. Deparaffination and Tissue digestion of specimens

Paraffinated blocks from the all tumor samples were cut in 5-µm sections and 4-8 sections/patient were collected in the same microcentrifuge tube. Samples were dewaxed in 500 µl xylene for 3 times. All microcentrifuge tubes were located for 10 min in a 60°C heated block and centrifuged at 8,000 rpm, supernatants were removed. Tissue was dehydrated with absolute ethanol and digestion was done according to our previous procedure as already explained (Yahyapour, 2013).

2.3. DNA Extraction

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 µl of prewarmed elution buffer and stored at -20°C until use for Nested PCR.

2.4. *Quality Control*

The quality and concentration of DNA was measured on an ethidium bromide-stained 1% agarose gel. Also, the presence of DNA was confirmed by PCR with human β-globin primer as an internal control (Forward: 5'-TGG GTT TCT GAT AGG CAC TGA CT-3'; Reverse: 5'-AAC AGC ATC AGG AGT GGA CAG AT-3'). HeLa cell line was used as a positive control for HPV infection. Distilled water was used as a negative control.

2.5. Nested PCR by the MY and the GP primers

To examine for HPV DNA prevalence in the tissue, MY09: 5'- CGT CCM ARR GGA WAC TGA TC-3'; MY11: 5'- GCM CAG GGW CAT AAY AAT GG-3'primers and GP5+:5'- GAA AAA TAA ACT GTA AAT CAT-3'; GP6+: 5'-TTT GTT ACT GTG GTA GAT ACT- 3' primers were used to amplify the L1 gene.

Nested PCR with the MY09/MY11 (450bp) as an external and GP5+/GP6+ (150 bp) used as internal primers, such as consensus primers. 5 µl of purified DNA from each sample was added to the 20 µl PCR master mix. Each PCR master mix contained 16 mM ddH2O, 2.5mM buffer, 0.5 mM Mgcl2, 0.3 mM dNTP, 0.25 mM of each MY09 and MY11 primers, 0.2 mM Taq DNA polymerase (5 U/µL). To confirmed result we used 1.5% gel agarose electrophoresis stained by ethidium bromide.

2.6. Statistical analysis

X^2 test or Fisher's exact test was conducted using SPSS version 18 for the association between the presence of HPV genome and anatomical sites of esophagus, gender and age group (P values $\leq$ 0.05 were considered statistically significant).

3. Results

Of the 170 ESCC samples, 86 (50.6%) were male and 84 (49.4%) were female. The youngest ESCC patient was 35 years and the oldest ESCC patient was 91 years. The mean age of the subjects was 66.5±11.1. No significant difference was observed between distribution of patients gender in our study group (86 male vs 84 female) (P=0.98). There was no statistically significant difference between male and female subjects regarding mean age (p=0.098). In terms of urban/rural residence, 100 (58.8%) samples were from rural areas, and 70 (41.2%) of samples were from the urban areas (Table 1).

Of the 170 ESCC samples, the HPV DNA was detected in 62 (36.5%) samples by nested PCR with L1 consensus primers. Five samples of HPV DNA were positive by MY09/11 primers and 62 samples for GP5+/6+ primers. There were no significant differences among the HPV detection rates in the samples from the three anatomical sites of ESCC samples (P=0.49). No statistically significant association was found between urban/rural residence status and ESCC (P=0.34).

4. Discussion

Esophageal cancer is one of the most common cancers in the digestive system and the fifth most common cancer in the developing world (Katiyar, 2005). Several studies have noted that in addition to genetic factors, environmental factors and biological factors also play an important role in the development of esophageal cancer. Many microbial agents including papillomavirus in esophageal cancer has been confirmed (Lu, 2008). The presence of HPV in esophageal cancer study showed in 1982 by Syrjanen. A large study by Syrjanen in 2002 showed that HPV in 29.22% of 1485 cases of esophageal cancer using the technique of in situ hybridization. 15.2% of 2020 cases of esophageal cancer using PCR analysis reviewed by Syrjanen in areas where there was high risk of esophageal cancer and also with more papillomavirus infection (Katiyar, 2005).

Several studies showed (Table 2), the presence of HPV in esophageal cancer tissues from 0 to 82% of the notes (Shen, 2002). Most studies have revealed areas with a low risk of esophageal cancer, the association between HPV and esophageal cancer was not observed. Iran has been considered a very high risk area for osophageal cancer.

The studies of Andres Castillo presented 73 cases of osophageal cancer in which 21 samples

(29%) were positive for HPV. In our study, the prevalence of HPV in esophageal cancer tissue was 36.7%, which corresponded high-risk areas (Castillo, 2006). Therefore in our study, the role of HPV can be an etiological factor in the development of esophageal cancer confirmed. Andrzej Dabrowski showed 56 cases of esophageal cancer; 28 (50%) cases were positive for HPV (Dabrowski, 2012).

Torneselloa's study by using Nested-PCR techniques, showed that 12 (21.1%) of 57 cases of esophageal cancer were positive for HPV genome. The average age was 61.3 including 44 (77.2%) male and 13 (22.8%) were female (Tornesello, 2009). Yahyapour et al. reported 27.7% HPVDNA in ESCC samples using SYBR Green Real-PCR in the north of Iran (Yahyapour, 2013).

Table 1. Demographic description of OSCC patients in Mazandaran, near the Caspian Sea, Iran and detection of HPVDNA by nested PCR.

Characteristics	Anatomical Sites						Total		P value
	Upper third		Middle third		Lower third				
	No.	%	No.	%	No.	%	No.	%	
Sex									
Male	21	24.4	35	40.7	30	34.9	86	100	0.46
Female	16	19	31	36.9	37	44	84	100	
Resident									
Urban	12	17.1	34	48.6	24	34.3	70	100	0.09
Rural	25	25	32	32	43	43	100	100	
Age group									
<45	-	-	3	50	3	50	6	100	0.75
45-60	12	25.5	20	42.6	15	31.9	47	100	
61-75	18	22.2	28	34.6	35	43.2	81	100	
>75	7	19.4	15	41.7	14	38.9	36	100	
Histological Diagnosis									
SCC [a]	28	20.9	51	38.1	55	41	134	100	0.49
In Situ	3	42.9	1	14.3	3	42.9	7	100	
Dif [b]. poorly	1	25	1	25	2	50	4	100	
Dif. Well	5	20	13	52	7	28	25	100	
HPV DNA									0.10
Positive	16	25.8	28	45.2	18	29	62	100	
Negative	21	19.4	38	35.2	49	45.4	108	100	

[a] Squamous Cell Carcinoma
[b] Differentiated

Table 2. Prevalence of HPV DNA in case series of esophageal carcinoma (>50 cases).

Country	First Authors	Year	HPV Detection and genotyping Method	N0. of Cases	Total HPV positive (%)
Australia	Bahnassy AA	2005	PCR for GP5+/GP6+	222	3.6
Colombia & Chile	Castillo A	2006	PCR for GP5+/GP6+	73	28.8
Korea	Koh JS	2008	PCR consensus primers of E6 and E7 and type-specific primers	129	0.0
Iran	Eslami Far	2007	PCR for GP5+/GP6+	140	23.6
China	Shuyama K	2007	PCR by GP5+/GP6+	59	32.2
China	Lu XM	2008	INNO-LiPA, HPV genotyping V2 test and ISH[a]	67	20.9
Iran	Yahyapour Y	2012	Real time PCR	177	27.7
India	Katiyar S	2005	PCR by MY09/MY11 and sequencing L1 consensus primer	101	26.7
Poland	Dabrowsky	2012	PCR for Gp5+/GP6+	56	50
Italy	Tornesello ML	2009	PCR for MY09/MY11	57	21.1
Sweden	Lofdahl	2012	Multiplex PCR for Gp5+/GP6+ and ISH	204	10
Iran	Moradi A	2002	PCR for GP5+/GP6+	85	49.4
Sweden	Dreilich M	2006	Real-time PCR	100	16.0
China	Wang X	2010	PCR for SPF1/GP5+/GP6+	347	54.8
South Africa	Matsha T	2002	PCR for MY09/MY11,GP5+/GP6+	50	46.0
Mexico	Herrera-Goepfert R	2009	PCR for L1C1/L1C2, MY09/MY11 and GP5+/GP6+	60	25.0
Iran	This Study		Nested PCR for Gp5+/GP6+ and MY09/MY11	170	36.5

[a]In-situ Hybridization

Tahmasebi et al. reported in Tehran the presence of HPV in 36% of esophageal cancer tissue and 13.2% of normal tissue cells of the esophagus were positive (Tahmasbi, 2004). The studies of Farhadi in 2005 indicated that 36.8% of esophageal cancers were HPV positive (Farhadi, 2006). Emadian et al. reported in Mazandaran Province the presence of HPV in 37.5% of ESCC cases and 12.5% of non-malignant specimens of the esophagus (Emadian, 2011). Oliveira in a study in Brazil found that the presence of HPV with the age and sex of the patient also location does not have a significant association (Oliveira, 2009). Lofdahl was reported in patients who were positive for HPV in esophageal cancer and its location was in the middle of the esophagus (55%) (Lofdahl , 2012), which is consistent with our study. There was no significant association between HPV infections with the anatomical location of the esophagus. In simpler terms, the presence of human papillomavirus has no role in the development of squamous cell carcinoma of the different sites of esophagus.

It seems that, several factors including nutritional and environmental factors and genetic heterogeneity of the population, different sampling methods, sample type, sensitive laboratory techniques, and diagnostic methods and the most important regions of the genome of HPV-induced cancers in helping , E6 and E7 regions of the initial binding of the transcriptional regulator tall (LCR) pointed out, and possible contamination in sensitive PCR

reaction time an important factor in obtaining different results. The etiology of cancer in relation to the area should be identified, so it can be used to design an effective screening program, the management of the target population vaccinated against the disease and the most common viral types in the population of the machine.

A number of limitations exist in the current study that should be noted. First, lack of matching non- malignant or normal esophageal samples may affect the reliability of results. Second, due to our current sample set (Formalin-fixed paraffin-embedded samples) and lack of high quality DNA, false negative results were possible.

In conclusion, the current study is a preliminary work that showed a relatively substantial prevalence of HPV infection in esophageal squamous cell carcinoma samples in Mazandaran province. Further epidemiological investigation on esophageal fresh biopsy samples and in case-control setting should be done to understand the role of HPV in ESCC.

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Refereces

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