

Detection of HPV Infection in Non-cancer Oesophageal Lesions and Normal Tissue by Nested Polymerase Chain Reaction

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ABSTRACT

Objective: Human papilloma virus (HPV) can be the aetiologic factor in the benign or malignant oesophageal epithelium. Human papilloma virus is suspected of causing extragenital cancers, including: cancers of the oral cavity, larynx, oesophagus and the lungs. More than 40 genotypes of HPV that infect the anogenital area are associated with a large spectrum of diseases from benign proliferation to invasive cancers. The aims of our study were to evaluate the prevalence of HPV-infection in non-cancer oesophageal lesions in Mazandran, North of Iran, and identify the prevalence of HPV in benign lesions.

Methods: A total of 104 non-cancer oesophageal samples were collected in paraffinated blocks of tissue archived in pathology. After deparaffinization and DNA extraction, nested polymerase chain reaction (nPCR) method was performed by HPV L1 primer pairs (MY09/MY11 and Gp5+/GP6+).

Results: Of 104 cases, 35 (33.7%) were HPV DNA positive. By histopathology, 61.5%, 31.8%, 30.4% and 30% of dysplasia, oesophagitis, normal tissue and ulcerative lesions were positive for HPV DNA, respectively. Also, the lower-third (distal) of the oesophagus had less infection of HPV DNA (18.8%) than the upper and middle-third (about 46%). The highest prevalence of HPV DNA was found in cases > 75 years old.

Conclusion: To our knowledge, considering the highest rate of HPV-infection in people more than 75 years, we recommend the diagnostic procedures at a lower age. Also, the proximal part of the oesophagus is more infected with HPV than the distal part.

Keywords: Human papilloma virus, HPV, nested PCR, non-cancer, oesophagus

Detection of HPV Infection in Non-cancer Oesophageal Lesions and Normal Tissue by Nested Reacción en Cadena de la Polimerasa

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RESUMEN

Objetivo: El virus del papiloma humano (VPH) puede ser el factor etiológico en el epitelio esofágico benigno o maligno. Se sospecha que este virus es la causa de los cánceres extragenitales, incluyendo los cánceres de la cavidad oral, la laringe, el esófago y los pulmones. Los más de 40 genotipos de VPH que infectan el área anogenital se asocian con un amplio espectro de enfermedades, desde la proliferación benigna hasta los cánceres invasivos. Los objetivos de nuestro estudio fueron evaluar la prevalencia de la infección por VPH en lesiones esofágicas no cancerosas en Mazandran, en el norte de Irán, e identificar la prevalencia de VPH en las lesiones benignas.

Métodos: Un total de 104 muestras de cáncer esofágicos se recogieron en bloques parafinados para archivar tejidos en patología. Después de la desparafinación y la extracción de DNA, se realizó el método de la reacción en cadena de la polimerasa anidada (RCPa) fue realizada por pares de iniciadores (primers) VPH L1 (MY09/MY11 and Gp5+/GP6+).

Resultados: De 104 casos, 35 (33.7%) fueron positivos al ADN de VPH. Por histopatología, 61.5%, 31.8%, 30.4% y 30% de la displasia, esofagitis, tejido normal y lesiones ulcerosas fueron positivas al ADN de VPH, respectivamente. También, el tercio inferior (distal) del esófago tenía menos infección de ADN

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VPH (18.8%) que el tercio superior y el tercio medio (alrededor de 46%). La mayor prevalencia de ADN VPH se encontró en casos > 75 años de edad.

Conclusión: Para nuestro conocimiento, considerando la tasa más alta de infección por VPH en personas de más de 75 años, recomendamos los procedimientos de diagnóstico a una edad menor. Además, la parte proximal del esófago es más infectada con VPH que la parte distal.

Palabras claves: Virus del papiloma humano, VPH, RCP anidada, no canceroso, esófago

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INTRODUCTION

Viral infections are known to contribute to the development of several human cancers (1). In 1982, Syrjanen was the first person that reported HPV as the aetiologic agent factor for oesophageal squamous cell carcinoma (ESCC). He stated the possibility of HPV being benign or malignant oesophageal epithelium according to histological findings (2).

Human papillomavirus (HPV), a double-stranded DNA virus, is recognized as an aetiologic agent of cervical cancer (3). In addition, HPV is suspected of causing extragenital cancers, including; cancers of the oral cavity, larynx, oesophagus, and lung (4). More than 40 genotypes of HPV that infect the anogenital area are associated with a large spectrum of diseases from benign proliferative lesions to invasive cancers.

Most people will never know that they are infected by HPV, because they have no symptom and their immune system inactivates the virus. Sometimes HPV-infections are not cleared. This can lead to cell changes that over many years may develop into cancer (5). The association of HPV-infection and oesophageal cancer (EC) has been reported in the last 30 years, especially in geographic areas with a high incidence of EC. However, unlike cervical carcinoma, with its almost conclusive association with HPV, the causal role of HPV-infection in EC remains controversial. This lack of association is due in part to the wide variation in reported infection rates among different studies (from 0% to more than 82%) and few studies have related HPV status to genetic changes (6, 7).

The last several studies from different geographical areas of the world including; China by Wang (8), Guo (9), Qi (10), Zhang (7), Zhou (11), Shen (12); from India: Mohiuddin (13), Katiyar (14); from Italy: Tornesello (15); Australia: Antonsson (16); Brazil: Moreira Antunes (17), Souto Damin (18); Lublin, Dabrowski (19) and many studies from Iran, by Emadian (20), Tahmasebi Fard (21), Farhadi (22) and Mostafalou (23) reported many findings of infection to HPV in cancerous specimens and non-cancerous or normal lesions used as control cases. Most of them indicated infection of HPV DNA in some of the non-cancerous or normal tissue samples.

Therefore, we conducted a study on oesophageal specimens to see the presence of the HPV in normal and non-cancerous tissue in the North of Iran, a high-risk area for oesophageal cancer.

SUBJECTS AND METHODS

Study population

The study included, formalin-fixed and paraffin embedded tissue samples of 107 non-cancer oesophageal cases. Specimens were obtained from patients of Shahid-Beheshti Hospital in Babol city and a Reference Laboratory in Amol city. Demographic and medical information including age, area and pathological diagnosis collected from patients medical records. All samples were biopsies. Finally, three patients were excluded, due to lack of data and 104 subjects were enrolled.

DNA extraction

A total of 5–10 slides about 5 µm thick were deparaffinized in xylene and absolute ethanol. Then DNA was extracted using High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany). β-Globin was used as a control to DNA viability with PCO3/PCO4 primers.

Nested polymerase chain reaction

Human papilloma virus DNA was amplified through MY09/MY11 and GP5+/GP6+ primers by Nested PCR (Table 1).

Table 1: The sequences of MY09/MY11 and GP5+/GP6+ primers

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	GP5+	5'-TTT GTT ACT GTG GTA GAT ACT AC-3'
4	GP6+	5'-GAA AAA TAA ACT GTA AAT CAT ATT C-3'

MY09/MY11 which amplifies the L1 gene of HPV was capable of amplifying a wide spectrum of HPV types to produce a PCR product of 450 bp. The PCR reaction for MY09/MY11 primers was performed using the following steps; four minutes at 94 °C; then 35 cycles at 94 °C for 45 seconds, 52 °C for one minute and 72 °C for 45 seconds. Finally reactions were carried out to a final extension for 10 minutes at 72 °C. Also, nested PCR was performed by amplified GP5+/GP6+ primers. The GP5+/GP6+ primers set that detects a wide range of HPV types produces a PCR product of approximately, 150 bp. The second round of PCR reactions were as follows; four minutes at 94 °C; then 35 cycles at 94 °C for 45 seconds, 55 °C for one minute and 72 °C for 45 seconds.

Finally, reactions were carried out to a final extension of 10 minutes at 72 °C. Each batch of samples included negative controls without a DNA template; and positive controls containing HPV-18 was extracted from HeLa cell line. Polymerase chain reaction products were separated by electrophoresis through 1.5% agarose gel and then HPV-positive samples were detected. All statistical analysis was performed with SPSS 18 software. The significance level was set at $p < 0.05$ by χ^2 test.

RESULTS

A total of 104 samples were enrolled in this study with an age-range of 17–91 (mean \pm SD 62.5 \pm 14.49) years. The non-cancer samples included 20 patients with ulcer, 44 with oesophagitis, 13 with dysplasia, four with Barrett's oesophagus and 23 with normal tissues of the oesophagus. From 104 cases, 35 (33.7%) were HPV DNA positive by nested PCR (Table 2). According to Table 2, the percentage of dysplastic changes in tissue contaminated with HPV was 61.5% (8/13) versus 30.4% (7/23) of normal oesophageal tissue. Of the 104 samples, 41% (16/39) of HPV-infected samples were rural, but 29.2% (19/65) were urban. There is no significant relationship between HPV DNA positive and anatomical sites of the oesophagus by demographic variables ($p > 0.05$).

In our study, from 104 cases of non-malignant and normal oesophageal mucosa, the prevalence of HPV DNA was 33.7% by nested PCR. Other studies, from many countries, for the detection of HPV DNA in non-cancer tissue sample of the oesophagus have been reported from China [0–71%]: (7–12); India [7.7–50.6%] (13, 14); Brazil (0.0%). (17, 18) and Australia [0.0%] (16). Also, in studies in Iran, the rate of HPV DNA was reported as about 36.3% in 80 non-cancer oesophageal samples by SYBR Green real-time PCR (23). However, in the present study, the rate of HPV DNA was reported in 30% of ulcerative mucosal lesion of the oesophagus, 31.8% in esophagitis, 61.5% in dysplastic lesions and 30.4% was found in HPV DNA positive in normal tissue samples without any lesion.

In a study from Italy in 2009, Tornesello *et al* reported 29.6% positivity (8/27) of non-cancer oesophageal tissue samples by conventional PCR (15). Moreira Antunes, in 2013 on study of specimens of Brazilian patients, similar to our study methods and histopathological diagnosis, reported that there were no HPV-infections in oesophagitis, as a non-cancer tissue sample [0/37] (17).

In India, in 2013, Mohiuddin and his colleague, from 85 non-cancer tissue samples including dysplasia, esophagitis and normal tissue of the oesophagus, reported HPV-infection in

Table 2: Prevalence of HPV infection and demographic description in non-cancer of oesophageal lesion in Mazandaran, North of Iran

Anatomical sites Characteristics	Upper-third		Middle-third		Lower-third		Total	
	No	HPV (%)	No	HPV (%)	No	HPV (%)	No	HPV (%)
Gender								
Male	17	8 (47.1)	21	10 (47.6)	27	7 (25.9)	65	25 (38.5)
Female	7	3 (42.9)	11	5 (45.5)	21	2 (9.5)	39	10 (25.6)
Age groups								
< 45	3	2 (66.7)	5	0 (0.0)	6	0 (0.0)	14	2 (14.3)
45 to 60	5	2 (40)	13	8 (61.5)	21	4 (19.1)	39	14 (35.9)
61 to 75	11	4 (36.4)	8	3 (37.5)	11	2 (18.2)	30	9 (30)
> 75	5	3 (60)	6	4 (66.7)	10	3 (30)	21	10 (47.6)
Resident								
Urban	15	5 (26.3)	21	9 (42.9)	29	5 (17.2)	65	19 (29.2)
Rural	9	6 (66.7)	11	6 (54.5)	19	4 (21.1)	39	16 (41)
Histopathological diagnosis								
Ulcer	5	1 (20)	5	3 (60)	10	2 (20)	20	6 (30)
Oesophagitis	10	4 (40)	12	6 (50)	22	4 (18.2)	44	14 (31.8)
Barrett's	0	0 (0.0)	3	0 (0.0)	1	0 (0.0)	4	0 (0.0)
Dysplasia	6	4 (66.7)	3	2 (66.7)	4	2 (50)	13	8 (61.5)
Normal tissue	3	2 (66.7)	9	4 (44.4)	11	1 (9.1)	23	7 (30.4)

DISCUSSION

Human papilloma virus is an oncogenic virus and show oncogenic activity by destroying mucosal immune resistance and tumour suppresser genes (24). The incidence of infection differs markedly depending on the different geographical location of the population under study and within different studies (25, 26).

71.4% (5/7), 57.8% (11/19) and 45.7% (27/59), respectively, by conventional PCR (13). In our study, the highest prevalence was in dysplastic lesions (61.5%; 8/13) similar to the Mohiuddin study. However, in comparison to this study, we found 31.8% (14/44) and 30.4% (7/23) HPV-infection in oesophagitis and normal tissue samples, respectively, by nested-PCR.

In 2013, in China, Wang *et al* studied 40 patients with atypical hyperplasia (dysplasia) and without pathological

changes, they showed HPV-infection in 10% of atypical hyperplastic mucosa and 0% in normal tissue by fluorescence *in situ* hybridization [FISH] (8).

In our study, HPV prevalence in patients < 45 years of age was 14.3%, 45–60 years were 35.9%, 61–75 years were 30% and > 75 years 47.6%. Also, infection with HPV in man was detected in 38.5% versus 25.6% in women. In comparison, Qi study in China, HPV prevalence in persons ≤ 55 years was 25% and > 55 years of age was detected in 30.9%; also, they reported HPV-infection in men about 26.5% versus 41% in women (10).

According to Table 2, the highest prevalence of HPV infectious was seen in patients more than 75 years of age (47.6%). Since, HPV-infection can cause malignancy after many years, it may be that these people were infected with HPV in youth or in adolescence (5). Also, the highest prevalence of HPV-infection had been found in tissue samples with dysplastic lesions (61.5%), that could progress to malignancy. Human papilloma virus infection prevalence in the lower-third (18%) of the oesophagus is significantly less than the upper and middle-third. Also, HPV-infection in males was more than in females, and was more in urban patients than in rural patients (41% versus 29.2%).

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