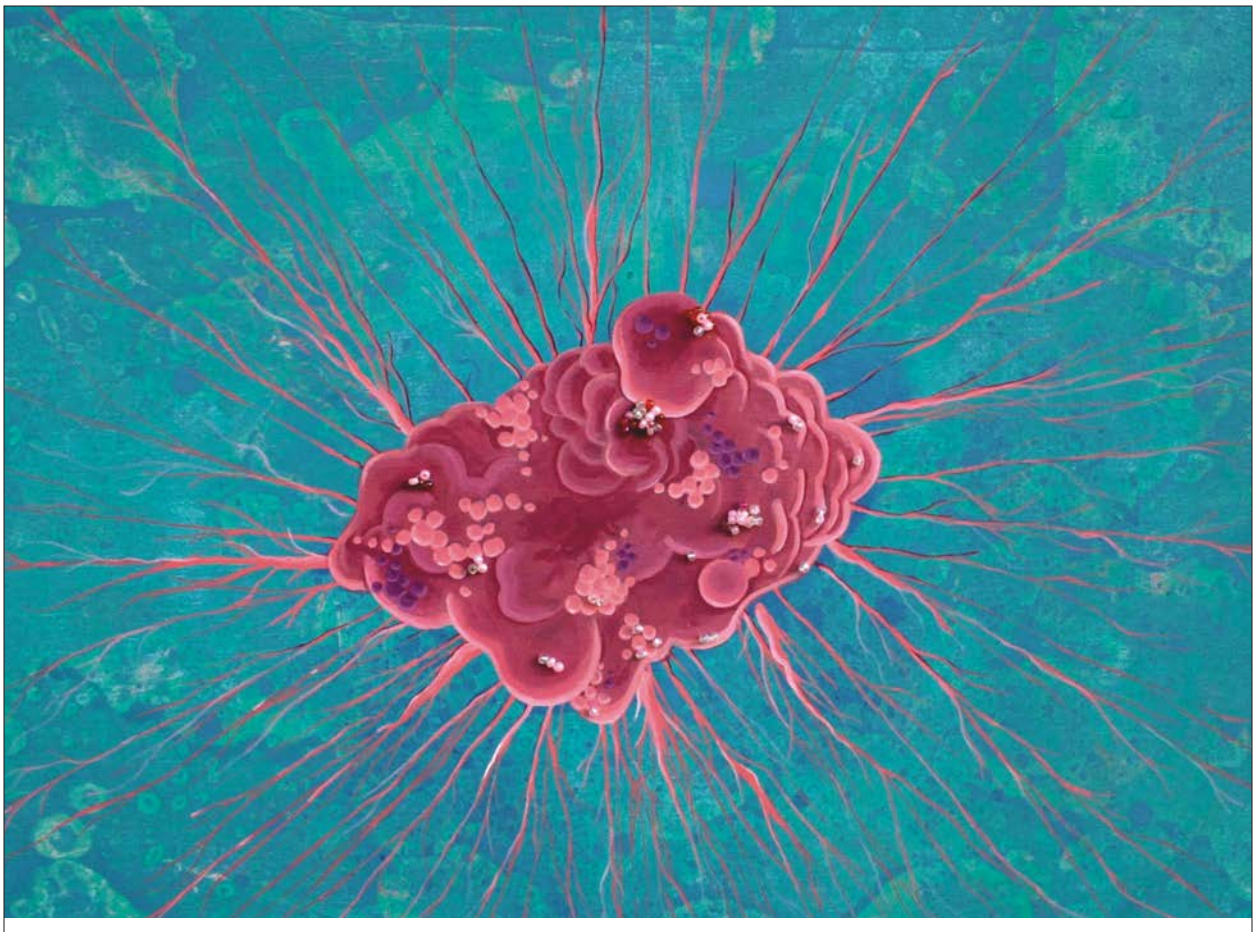

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Detection of HPV in Cancerous and Non-cancerous Esophageal Tissues from Turkmen-Sahra, Iran

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Abstract: The aim of this study were to assess the presence of HPV esophageal infection among Iranian Turkmen who live in an area located in the cancer belt in Asia. The specimens derived from 120 patients previously diagnosed for Squamous Cell Carcinoma (SCC) non-cancerous tissue derived from esophagus. All specimens were examined for the presence of HPV DNA. PCR was utilized to amplify a 150 bp segment of HPV L1 gene using the consensus primers. The amplified region was subsequently sequenced to identify the HPV genotypes. The HPV DNA was detected in 49.4% of patients with SCC, and 58% of non-cancerous tissue of esophagus. The positive samples included HPV-16 (46.6%), HPV-6 (24.6%), HPV-66 (8.2%), HPV-52 (4.1%), HPV-18 (2.7%); 14% of cases were positive for more than one type of HPV. The results confirm the presence of HPV in both esophageal cancerous and non-cancerous tissues.These results imply two different interpretations: 1) Due to non-significant difference between the rate of HPV positive in cancerous and non-cancerous tissues, HPV has no important role in esophageal cancers, which is less probable. 2). Because of a highly incidence of esophageal cancer in Turkmen-Sahra region, the HPV is a possible etiologic agent in esophageal carcinogenesis, most probably acting synergistically with physical, chemical, and/or nutritional factors that have previously been found to be related to this malignancy in Turkmen-Sahra.

Key words: HPV, esophageal tissue, Turkmen-Sahra, Iran

Introduction

Esophageal carcinoma has a striking geographical distribution. The incidence is remarkably high in certain region of Iran, in China and South Africa. The highest incidence of this highly malignant tumor is encountered in the northeast of Iran, particularly in the Turkmen Sahra (Kemt *et al*., 1980; Oneill *et al*., 1980; Kmet and Mahboubi, 1972).

The causative factor of this disease remains to be established (Kemt *et al*., 1980; Oneill *et al*., 1980; Kmet; Mahboubi, 1972; Munoz *et al*., 1982; Muhboubi *et al*., 1973). Cigarette smoking and excess alcohol intake may be risk factors in some areas (e.g. Western countries and South Africa), especially when the two factors are combined. Nevertheless, these factors do not appear to be a problem in Iran (Editorial, 1978; IARC, 1977; Crespi, 1979). Previous studies in Iran show, specific nutritional deficiencies such as vitamin A, B, C and certain minerals, as well as nitrosamines formed in moldy food stuffs my be important (Hormozidari *et al*., 1975; Cook-Mozaffari *et al*., 1979;

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Ghadirian, 1987). Furthermore, opium tar has been blamed as possible risk factor (Kemt *et al*., 1980; IARC, 1977). It is more likely that these factors may render the esophageal mucus membrane, more susceptible to injury by carcinogens including mycotoxins, nitrosyl compound, and possibly viruses.

HPV plays an important role in the development of squamous cell carcinomas in various body sites, including the anogenital (Hausen and Villiers, 1994), upper respiratory and digestive tracts (Chen *et al*., 1994; Chang *et al*., 1990; Chang *et al*., 1993; Kulski *et al*., 1986; Loke *et al*., 1990; Benzmouzig *et al*., 1992; Toh, 1992; Togawa *et al*., 1994). Eighty-five types of HPV have been described in full and 120 types have been partially characterized (Hausen, 1999). HPV types 6, 11, 16, 18 and 31 represent the most common types found in the epithelium of squamous cell hyperplasia, dysplasia, and carcinomas (Chang *et al*., 1990). Based on their association with neoplasm's in the anogeital tract, HPV types can be categorized as high-risk types (HPV-16, 18 and 31) and low risk types (HPV-6, 11).

Members of the high-risk group promote carcinogensis and their DNA usually integrates in to the host genome, whereas the low risk HPV types which are primarily found in benign tumors and their DNA remain extra chromosomal (Toh *et al*., 1992). HPV-6, 11, 16, 18, and 31 have been described in association with esophageal squamous cell lesions (Maniatis *et al*., 1989). However, the incidence of HPV positively varies significantly depending and the geographic location of the patient. Studies that suggest a role for the HPV play in the genesis of esophageal carcinoma almost invariably involve a population, which is at a high risk for development of esophageal carcinoma (Chen *et al*., 1994; Chang *et al*., 1993). Our aim was to determine the incidence of various HPV types in the specimen obtained from esophagus (cancerous and non-cancerous tissues) based on the DNA sequence of L1 gene, which is the most widely gene used in PCR assay and can be detected a segment of the gene encoding the L1 major capsid protein, that is common to all HPV types.

Materials and Methods

A total of 116-esophageal samples, 74 men and 42 women, aged between 42 to 85 years were removed by biopsy from patient in Turkmen Sahra and were studied in the Institute of Public Health and Research at the Tehran University of Medical Sciences. All specimens received were fixed in 10% buffered formalin, processed routinely, and embedded in paraffin. For each case, all available hematoxylin and eosin stained sections were reviewed. Eightyfive samples were diagnosed cancerous (squamous cell carcinoma), and thirty-one non-cancerous tissues (esophagitis, and moco-epithelial cell) of esophagus.

Sample preparation

Genomic DNA was extracted by the standard proteinase K/phenol method (Saiki *et al*., 1985). After ethanol precipitation, the DNA was resuspended in TE buffer (10 mM Tris-HCL 1mM EDTA) and quantified by measuring absorbance at 260 nm. DNA concentration of more than 10 ng μL^{-1} were judged as suitable for further studies. DNA samples were then checked by agarose gel electrophoresis to verify that degradation had not occurred during the extraction. Samples containing 100 ng of purified DNA were used for PCR amplification according to the following procedure (Saiki *et al*., 1988).

Polymerase Chain Reaction (PCR)

A slight modification of the PCR method described by Saiki *et al*. (1988) was used. PCR was performed in a total volume of 50 µL containing 100 ng DNA of extracted from paraffin-embedded tissue, 50 mM KCL, 10 mM Tris-HCL pH 8.3, 200 µM of each dNTP, $MgCl_2$ at between 2 and 4 mM, 1 U Taq polymerase and 50 pmol each primer. The primers used in this study were GP5+/GP6+ pairs with the following sequences (de Roda Husman *et al*. (1995) modified in German Cancer Research Center):

GP5+5'TTGGATCCTTTGTTAACTGTGGTAGATACTAC
GP6+5'TTGGATCCGAAAAATAAACTGTAAATCATATTC

The mixture was denatured at 94°C for 5 min, followed by 40 cycles of amplification using a PCR processor Bio-Med (Perkin Elmer Cetus USA). Each cycle consisted of 94°C for 1.5 min 40°C for 2 min and 72°C for 1.5 min. The final elongation step was prolonged for 4 min to ensure complete extension of the amplified product. Samples processing prior to and after the amplification reactions were performed in strictly separated rooms to avoid contamination by PCR products. Samples containing distilled water were used as negative controls. Ten microliter of each the PCR mixture was finally analyzed by 1.5% agarose gel electrophoresis (Saiki *et al*., 1995).

Sequence analysis of PCR products

PCR amplified products (124 bp) were purified with commercial PCR product purification Kit (Boeringer Mannheim Germany). Approximately, 25 ng of the purified amplified DNA product was subjected to nucleotide sequence analysis with 20 pmoles of M13 forward (-20) and reverse primers with the following sequences. M13 forward (-20) priming sit 5'GTAAAACGACGGCCAG, M13 reveres priming sit 5' CAGGAAACAGCTATGAC. The sequencing was performed using commercial sequencing Kit (Perkin Elmer USA). Subsequently, X-ray film autoradiography was then performed to reveal the nucleotide of different HPV DNA.

Results and Discussion

DNA of 85 patients with squamous cell carcinoma and 31 non-cancerous of esophagus was amplifiable in every sample as determined by amplification of β-globin gene. Forty-two samples of squamous cell carcinoma and eighteen of non-cancerous tissue were PCR positive using primers of the HPV L1 gene. The sex distribution of the patient with esophageal cancer HPV was positive 52.8% male and 43.7% female, in non-cancerous 66.6 and 33.3%, respectively (Table 1).

Table1: Frequency of HPV types in esophageal squamous cell carcinoma and non-cancerous tissue of esophagus

	Squamous cell carcinoma		Non-cancerous tissue of esophagus	
HPV types	Frequency	%	Frequency	%
HPV-6	6	14.3	8	44.4
HPV-16	23	54.7	7	38.8
HPV-18	2	4.8	-	-
HPV-66	3	7.1	1	5.5
HPV-52	2	4.8	2	11.1
HPV-6, 18	4	9.2	-	-
HPV-16, 18	2	4.8	-	-
Total	42	100	18	100

Esophageal carcinoma has a distinct geographic distribution with a high prevalence in certain regions of Iran, china, Africa and France (Toh *et al.*, 1992). This variation of the diseases stem in part from environmental factors such as the mineral content of the soil, dietary practices, occupational factors, and personal habits (Whelan *et al.*, 1990). Viral infections may also play a role in the genesis of esophageal carcinoma in some population (Chang *et al.*, 1992). In 1982, Syrjanen published his observation on the present of histologic changes consistent with those on condyloma in esophageal squamous cell cancer (Chang *et al.*, 1990). This observation showed etiological role of HPV infection in the development of esophageal carcinoma. An association between HPV and esophageal carcinoma has been previously reported in China (Chen 1994; Chang *et al.*, 1990, 1993), France (Benamouzig *et al.*, 1992), Italy (Togawa *et al.*, 1994), Japan (Mori *et al.*, 1989), South Africa (Williamson *et al.*, 1991; Copper *et al.*, 1995), Hong Kong (Toh *et al.*, 1992), Slovenia (Polijak *et al.*, 1998), Portugal (Fidalgo *et al.*, 1995), and the United State (Togawa *et al.*, 1994). However the incidence of infection differs based on the geographical location of the patient population under study. HPV detection rates vary from 0-70%, depending on the regions under study (Benamouzing *et al.*, 1995). The highest of HPV isolation has been reported from high-risk regions of china and South Africa (Maniatis *et al.*, 1989).

In present study, HPV-16, which has a potential for oncogensis, was the most prevalent among the esophageal cancer cases examined together with HPV types 52 and 66. The data indicate that the infection with oncogenic DNA viruses such as HPV, may be a factor in development of cancer by itself or in synergism with other factors including environmental carcinogens, foods, health habits, and hereditary factors. But we also found various type of HPV in 58% non-cancerous tissues of esophagus.These results imply two different interpretations: 1) Due to non-significant difference between the rate of HPV positive in cancerous and non-cancerous tissues, may be HPV has no important role in esophageal cancers, which is less probable. 2) Because of a highly incidence of esophageal cancer in Turkmen-Sahra region, the HPV is a possible etiologic agent in esophageal carcinogenesis, most probably acting synergistically with physical, chemical and/or nutritional factors that have previously been found to be related to this malignancy in Turkmen-Sahra.

We don't have any evidence to determine whether infection with HPV preceded or followed the development of squamous cell carcinoma. Further studies are required to pinpoint the cases of esophageal cancer in this area.

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