

Detection of Human Papillomavirus DNA by PCR in Esophageal Squamous Cell Carcinoma from Turkmen Sahra, North-East of Iran

Abdolvahab Moradi^{*1}, Ethel-Michele de Villiers², Talat Mokhtari-Azad³, Mahmoud Mahmoudi³, Bahman Hazrati⁴, Ezzat-Elah Ghaemi⁵ and Rakhshandeh Nategh³

¹Zahedan University of Medical Sciences, Zahedan, Iran; ²German Cancer Research Center, Heidelberg, Germany; ³Tehran University of Medical Sciences, Tehran, Iran; ⁴Gorgan Laboratory of Pathology, Gorgan, Iran; ⁵Gorgan University of Medical Sciences, Gorgan, Iran

ABSTRACT

Human papillomavirus (HPV) DNA has been identified in esophageal carcinomas. However, the incidence of HPV varies significantly in different geographical locations. In this study, neoplasms from Turkmen Sahra, a region in Golestan province in northeast part of Iran, with a high incidence of squamous cell carcinoma were analyzed for the presence of HPV DNA. Turkmen Sahra is located in the cancer belt in Asia. Eighty-five squamous cell carcinomas were examined for the presence of HPV DNA. PCR was utilized to amplify a 124-bp segment of the HPV L1 gene using the consensus primers. The amplified region was subsequently sequenced to identify the HPV. The results indicated that the rates of HPV detection in squamous cell carcinoma specimen for men and women were 52.8% and 43.7% respectively. The positive cases included HPV-16 (54.7%), HPV-18 (4.8%), HPV-6 (14.3%), HPV-66 (7.1%), HPV-52 (4.8%) and 14.3% of cases were positive for more than one type of HPV. Human papillomavirus type 16 that can be potentially oncogenic was prevalent in 54.8% of the cases of esophageal squamous cell carcinoma. Our results confirm the previously reported HPV involvement in the esophageal squamous cell carcinoma, and support the possible role of HPV as an etiological agent in esophageal carcinogenesis. *Iran. Biomed. J. 6 (1): 19-23, 2002*

Keywords: Esophageal carcinoma, HPV, Turkmen Sahra, Iran

INTRODUCTION

Esophageal carcinoma has a striking geographical distribution. The incidence is remarkably high in certain regions of Iran, China and South Africa. The highest incidence of this highly malignant tumor was encountered in the northeast of Iran, particularly in the Turkmen Sahra. (Fig. 1) The annual incidence of esophageal cancer in the Turkmen Sahra is estimated to be 8.8 in 100,000 persons for all ages, and 37 in 100,000 persons in people aged over 35 years old. Based on the data collected in 1999, the highest incidence of squamous cell carcinoma is observed in women aged 75-80 years old with an annual incidence of 192 in 100,000 persons, and in men aged 70-74 years old with an annual incidence of 145 in 100,000 persons (unpublished data).

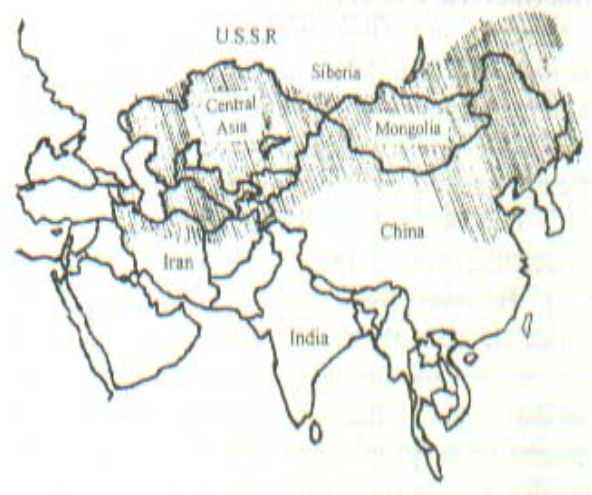


Fig. 1. Iran and its location in the esophageal cancer belt in Asia. (Ref. 3)

*Corresponding Author; Tel. (98-541) 241 8962, Fax: (98-541) 244 2481

Although a great deal of information has been obtained on esophageal cancer, the causative factor of this disease remains to be established [1-5]. Cigarette smoking and excessive alcohol intake may be the 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 [6-8]. According to the previous studies in Iran, specific nutritional deficiencies such as vitamin A, B, C and certain minerals, nitrosamines formed in moldy foodstuffs may be important [9-11]. Furthermore, opium tar has been blamed as possible risk factor [1, 7]. However, as stated earlier, it seems extremely unlikely that these factors alone could offer satisfactory explanation for the high mortality and morbidity of this malignancy in the Turkmen Sahra. It is more likely that these factors may render the esophageal mucous membrane, more susceptible to injury by carcinogens such as 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, [12] upper respiratory, and digestive tracts [13-20]. Eighty-five types of HPV have been described in full and 120 types have been partially characterized [21]. HPV types 6, 11, 16, 18, and 31 represent the most common types found in the epithelium of squamous cell hyperplasia, dysplasia, and carcinomas [14]. Based on their association with neoplasm's in the anogenital tract, HPV types can be categorized as either high-risk types (HPV-16, 18 and 31) or low-risk types (HPV-6, 11).

Members of the high-risk group promote carcinogenesis and their DNA usually integrates into the host genome, whereas, the low-risk HPV types which are primarily found in benign tumors and their DNA remain extrachromosomal [19]. HPV 6, 11, 16, 18, and 31 have been described in association with esophageal squamous cell lesions [22]. However, the incidence of HPV positively varies significantly depending on the histology of the lesion and the geographical location of the patient. Studies that suggest a role for HPV in the genesis of esophageal carcinoma almost invariably involve a population which is at a high risk for development of esophageal carcinoma [13, 15].

This study was designed to determine the incidence of various HPV types in the specimen obtained from esophageal squamous cell carcinoma cases 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 85 human squamous cell carcinomas 53 men and 32 women, aged between 42 to 85 years were removed by biopsy between 1997 to 1998 from Turkmen Sahra and were studied in the Institute of Public Health and Research at the Tehran University of Medical Sciences. The patients were classified histologically and clinically based on the guideline for clinical and pathological studies on carcinoma of the esophageal diseases. All specimens were fixed in 10% buffered formalin, processed routinely, and embedded in paraffin. For each case, all available hematoxylin and eosin stained sections were reviewed, and a representative block was selected for further studies.

Sample preparation. Genomic DNA was extracted by the standard proteinase K/ phenol method [23]. After ethanol precipitation, the DNA was resuspended in TE buffer (10 mM Tris-HCl 1 mM EDTA) and quantified by measuring absorbance at 260 nm. DNA concentration of more than 10 ng/ μ l was 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 [24].

Polymerase chain reaction (PCR). A slight modification of the PCR method described by Saiki *et al.* [24] were used. PCR was performed in a total volume of 50 μ l containing 100 ng of DNA extracted from paraffin-embedded tissue, 50 mM-KCl, 10 mM Tris-HCl pH 8.3, 200 μ M of each dNTP, 2 to 4 mM MgCl₂, 1 U Taq polymerase and 50 pmol of each primer. The primers used in this study were GP5+/GP6+ pairs with the following sequences [de Roba Husman *et al.*: 1995 modified in German Cancer Research Center]:

GP5 + 5'TTGGATCCTTTGTTACTGTGGTAGA
TACTAC

GP6 + 5'TTGGATCCGAA AAA TAA ACT GTA
AATCATATTC

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 minutes 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 µl of each PCR mixture was finally analyzed by 1.5% agarose gel electrophoresis [24] (Fig. 2).

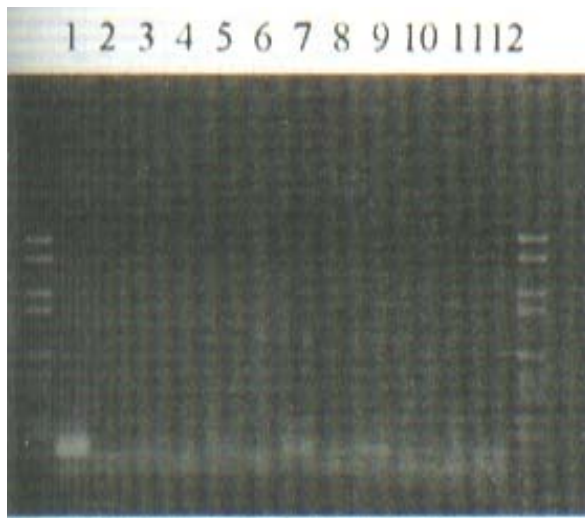


Fig. 2. PCR product of HPV DNA from esophageal carcinoma. Lane 1, positive control; Lane 2, negative control; Lanes 3, 5, 7, 8, 9, 10, 12, positive patient samples; Lane 4, 6, 11, negative patient samples.

Sequence analysis of PCR products. PCR amplified products (124 bp) were purified with commercial PCR product purification Kit (Boehringer Mannheim, Germany) according to the procedure described by the manufacturer. Approximately 25 ng of each purified amplified DNA product was subjected to nucleotide sequence analysis using 20 pmoles of M13 forward (-20) and reverse primers with the following sequences. M13 Forward (-20) priming site 5'GTAAAACGACGGCCAG, M13 Reverse Priming site 5'CAGGAAACAGCTATGAC

The sequencing was performed using commercial sequencing kite (Perkin Elmer, USA). Subsequently, X-ray film autoradiography was then performed to reveal the nucleotide of different HPV DNA.

RESULTS

A total of 85 specimens from 85 patients with squamous cell carcinoma of the esophagus were available for examination. The neoplasm was histologically graded as, well differentiated (56.3%), moderately differentiated (28.6%) and poorly differentiated (25.1%). DNA was amplifiable in every specimen as determined by amplification of the beta-globin gene. Forty-two specimens were PCR positive (49.4%) using primers of the HPV L1 gene. The distribution of the HPV positive cases with esophageal cancer based on sex of the patients were 52.8% for male and 43.7% for female (Table 1).

Table 1. Frequency of HPV types in esophageal squamous cell carcinoma from north-east of Iran (Turkmen Sahara).

HPV Types	Frequency	Percent
HPV-6	6	14.3
HPV-16	23	54.7
HPV-18	2	4.8
HPV-66	3	7.1
HPV-52	2	4.8
HPV-6,16	4	9.5
HPV-16,18	2	4.8
Total	42	100.0

DISCUSSION

Esophageal carcinoma has a distinct geographical distribution with a high prevalence in certain regions of Iran, China, Africa and France [19]. The basis for the variation in geographical distribution of the disease stems in part from environmental factors such as the mineral content of the soil, dietary practices, occupational factors, and personal habits [25]. Viral infections may also play a role in the genesis of esophageal carcinoma in some populations [26]. In 1982, Syrjanen published his observation on the presence of histological changes, consistent with those of condyloma in esophageal squamous cell cancer [14]. 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 [13-15], France [18], Italy [20], Japan [27], South Africa [28, 29], Hong Kong [19], Slovenia [30], Portugal [31], and the United State [20]. However, the incidence of infection differs based on the

geographical location of the patient population under study. HPV detection rate varies from none up to 70%, depending on the regions, under study [32]. The highest incidence of HPV isolation has been reported from high-risk regions of China and South Africa [22]. Turkmen Sahra located in the cancer belt in Asia is also a high-risk region for esophageal carcinoma.

In our study, HPV-16, which has a potential for oncogenesis, was the most prevalent among the esophageal cancer cases examined together with HPV types 52 and 66. These types have not been previously recovered from esophageal tumors.

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. 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.

ACKNOWLEDGMENTS

This work would not have been possible without the sustained support, interest and encouragement of Prof. H. Zur Hausen, from the German Cancer Research Center, Heidelberg, Germany.

REFERENCES

1. Kmet, J., McLaren, D.S. and Siassi, F. (1980) Epidemiology of esophageal cancer with special reference to nutritional studies among the Turkmen of Iran. In: *Advances in modern human nutrition*. Pathotox, New York, pp. 343-365.
2. O'Neill, C.H., Hodges, G.M. and Riddle, P.N. (1980) A fine fibrous silica contaminant of flour in the high oesophageal cancer area of north-east Iran. *Int. J. Cancer* 26: 617-628.
3. Kmet, J. and Mahboubi, E. (1972) Esophageal cancer in the Caspian littoral of Iran: Initial Studies. *Science* 17: 846-853.
4. Munoz, N., Grassi, A., Qiong, S., Crespi, M., Wang G.Q. and Li, Z.C. (1982) Precursor lesions of esophageal cancer in high- risk populations in Iran and China. *Lancet Apr.* 17: 876-879.
5. Mahboubi, E., Kemet, J., Cook, P.J., Day, N.E., Ghadirian, P. and Salmasizadeh, S. (1973) Oesophageal cancer studies in the Caspian littoral of Iran: the Caspian cancer registry. *Br. J. cancer* 28: 197-214.
6. Editorial (1978) Oesophageal Cancer in the Caspian littoral. *Lancet (I)* 641-642.
7. Joint Iran/IARC Study Group (1977) Esophageal cancer studies in the Caspian littoral of Iran: results of population studies-a prodrome. *J. Nat. Cancer Inst.* 59: 1127-1138.
8. Crespi, M., Munoz, N., Grassi, A., Aramesh, B., Amiri, G., Mojtabai, A. and Casale, V. (1979) Esophageal lesions in Northern Iran: A pre-malignant condition? *Lancet Aug. 4:* 217-220.
9. Hormozdiari, H., Day, N.E., Aramesh, B. and Mahboubi, E. (1975) Dietary factors and esophageal cancer in the Caspian littoral of Iran. *Cancer Research* 35: 3493-3498.
10. Cook-Mozaffari, P.J., Azordegan, F., Day, N.E., Ressicaud, A., Sabai, C. and Aramesh, B. (1979) Esophageal cancer studies in the Caspian littoral of Iran: Results of a case-control study. *B. J. Cancer* 39: 293-309.
11. Ghadirian, P. (1987) Thermal irritation and esophageal cancer in North of Iran. *Cancer* 60: 1909-1914.
12. Zur Hausen, H. and de Villiers, E.M. (1994) Human papillomaviruses. *Ann. Rev. Microbiol.* 48: 427-447.
13. Chen, B., Yin, H. and Dhurandhar, N. (1994) Detection of human papillomavirus DNA in esophageal squamous cell carcinomas by the polymerase chain reaction using general consensus primers. *Hum. Pathol.* 25: 920-923.
14. Chang, F., Syrjanen, S., Shen, Q., Hongxiu, J.I. and Syrjanen, K. (1990) Human papillomavirus (HPV) DNA in esophageal precancer lesions and squamous cell carcinomas from China. *Int. J. Cancer* 45:21-25.
15. Chang, F., Syrjanen, S., Shen, Q., Wang, L. and Syrjanen, K. (1993) Screening for human papillomavirus infections in esophageal squamous cell carcinoma by *in situ* hybridization. *Cancer* 72: 2525-2530.
16. Kulski, J., Demeter, T., Sterret, G.F. and Shilkin, K.B. (1986) Human papillomavirus DNA in esophageal carcinoma. *Lancet* 2: 683-684.
17. Loke, S.L., Ma, L., Wong, M., Srivastava, G., Lo, I. and Bird, C.C. (1990) Human papillomavirus in esophageal squamous cell carcinoma. *J. Clin. Pathol.* 43: 909-1012.
18. Benamouzig, R., Pigot, F., Quiroga, G., Validire, P., Chaussade, S., Catalan, F. and Couturier, D. (1992) Human papillomavirus infection in esophageal squamous cell carcinoma in western countries. *Int. J. Cancer* 50: 549-552.
19. Toh, Y., Kuwano, H., Tanaka, S., Baba, K., Matsuda, H., Sugimachi, K. and Mori, R. (1992) Detection of human papillomavirus DNA in

- esophageal carcinoma in Japan by polymerase chain reaction. *Cancer* 70 (3): 2234-2238.
20. Togawa, K., Jaskiewicz, K., Takahashi, H., Meltzer, S.J. and Sugimachi, K. (1994) Human papillomavirus DNA sequences in esophageal squamous cell carcinoma. *Gastroenterology* 107: 128-136.
 21. Zur Hausen, H. (1999) Papillomaviruses in human cancer. *Pro. Assoc. Am. Physicians (United States). Nov. Dec. 111 (6): 581-587.*
 22. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1989) *Molecular cloning: A laboratory manual.* Cold Harbor Laboratory Press, New York.
 23. Saiki, R.K., Scharf, S. and Faloona, F. (1985) Enzymatic amplification of the beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230: 1350-1354.
 24. Saiki, R.K., Gelfand, D.H. and Stoffel, S. (1988) Primer directed enzymatic amplification of DNA with a Taq DNA polymerase. *Science* 239: 487-491.
 25. Whelan, S.L., Parkin, D.M. and Masuyer, E. (1990) *Pattern of cancer in five continents.* IARC Scientific Publications. No.102, Lyon: International Agency for Research on Cancer, pp.14-104.
 26. Chang, F., Syrjanen, S., Shen, Q., Wang, L. and Syrjanen, K. (1992) Infections agents in the etiology of esophageal cancer. *Gastroenterology* 103: 1336-1348.
 27. Mori, M., Shimono, R., Inoue, T., Kuwano, H., Sugimachi, K. and Imachi, K. (1989) Papillomavirus and esophageal cancer in the Japanese and Chinese. *Am. J. Gastroenterol.* 84: 1126-1127.
 28. Williamson, A.L., Jaskiesicz, K. and Gunning, A. (1991) The detection of human papillomavirus in esophageal lesions. *Anticancer Res.* 11(1): 236-265.
 29. Cooper, K., Taylor, L. and Govind, S. (1995) Human papillomavirus DNA in esophageal carcinomas in Africa. *J. Pathol.* 175 (3): 273-277.
 30. Polijak, M., Cerar, A. and Seme, K. (1998) Human papillomavirus infection in esophageal carcinoma a study of 121 lesions using multiple broad-spectrum PCR and literature review. *Human Pathology* 29 (3): 266-271.
 31. Fidalgo, P., Cravo, M. and Chaves, P. (1995) High prevalence of human papillomavirus in squamous cell carcinoma and matched normal esophageal mucosa. *Cancer* 76: 1522-1528.
 32. Benamouzing, R., Jullian, E., Chang, F., Robaskiewicz, M., Flejou, J.F., Raoul, U.L., Coste, T., Couturier, D., Pompidou, A. and Rautkureau, J. (1995) Absence of human papillomavirus DNA detected by polymerase chain reaction in French patients with esophageal carcinoma. *Gastroenterology* 109 (6): 1876-1881.