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• *BRIEF REPORTS* •

Human papillomavirus in squamous cell carcinoma of esophagus in a high-risk population

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Abstract

AIM: To investigate the relation of human papillomavirus (HPV) and esophageal squamous cell carcinoma (ESCC) in Iranian patients as compared to normal controls.

METHODS: Using MY09/MY11 consensus primers, we compared the prevalence of a HPV *L1* gene in tumor tissues from 38 ESCC cases and biopsied tissues from 38 endoscopically normal Iranian individuals. We also compared the presence of HPV16 and HPV18 in the same samples using type-specific E6/E7 primers.

RESULTS: Fourteen (36.8%) of the 38 ESCC samples but only 5 (13.2%) of the 38 control samples were positive for the HPV *L1* gene ($P = 0.02$). Five (13.2%) of the ESCC samples but none of the control samples were positive for the HPV16 *E6/E7* gene ($P = 0.05$). Three (7.9%) of the ESCC samples and 5 (13.2%) of the control samples were positive for the HPV18 *E6/E7* gene ($P = 0.71$).

CONCLUSION: Our data are consistent with HPV DNA studies conducted in other high-risk areas for ESCC. HPV should be considered as a potential factor contributing to the high incidence of ESCC in Iran and other high-incidence areas of the world.

Key words: Papillomavirus; Squamous cell carcinoma of esophagus; Population

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INTRODUCTION

The role of human papillomavirus (HPV) in the etiology of esophageal squamous cell carcinoma (ESCC) has been debated in the past 20 years. Oncogenic types of HPV, most notably HPV 16 and HPV 18, are recognized as the most significant risk factors of cervical cancer[1]. A role of HPV in the etiology of cancers of vulva, anus, penis, and oropharyngeal cavity has also been established[2]. However, the role of HPV in the causation of ESCC remains controversial. Syrjanen first suggested a role of HPV in the etiology of ESCC in 1982, based on the observation of characteristic histological findings suggesting the presence of HPV in benign esophageal epithelia and malignant esophageal tumors[3]. Since then several studies have used a variety of techniques, including detection of HPV DNA in esophageal tumor tissues and serological methods, to examine the association between exposure to HPV and risk of ESCC[4]. The results of these studies are not consistent. Case series using polymerase chain reaction have found evidence of HPV in tumor tissues varying from 0 to 67%[4].

It has been suggested that the high variation in HPV DNA results may partly be explained by geographic variation. Most studies, that did not detect HPV DNA in esophageal tumors, were conducted in low-risk areas of USA or Europe. However most studies in high-risk areas for ESCC (such as China, South Africa, and Japan) found that HPV had significantly higher percentages in esophageal tumors[4].

Iran is a very high-risk area for esophageal cancer[5-8]. In some parts of northeastern Iran, the incidence rate of ESCC is reportedly over 100/100 000 person/year[5].

In order to investigate the prevalence of HPV infection in ESCC in Iran, a country with high rates of ESCC, we evaluated the tumor tissues from patients with ESCC and normal esophageal tissues from age-matched controls for the presence of HPV DNA.

MATERIALS AND METHODS

Formalin-fixed paraffin-embedded tissue samples were collected from the patients undergoing surgery for ESCC in two hospitals in Tehran (Shariati, Mehr) from 1996 to 2001. One control per case was selected from consecutive patients referred to a private gastroenterology clinic in Tehran for symptoms of dyspepsia. Only subjects who had normal endoscopy (non-ulcer dyspepsia) and matched on age (±5 years) with one of the case subjects were eligible to be controls. In the controls, two biopsies were taken from the middle third of the esophagus, about 30 cm from the incisor teeth.

The presence of the representative tumor in selected paraffin blocks was confirmed by at least two pathologists before the blocks were further processed for HPV DNA.

One block from each tumor and one block containing both biopsies from each control patient were evaluated for the presence of HPV *L1* gene using MY09/MY11 consensus (general) primers. MY09/MY11 primers are complementary to 450-bp-conserved sequences in the *L1* gene of HPV, and are able to amplify the *L1* gene from a broad range of HPV types. In samples where the *L1* gene could be amplified, further examination was performed to explore the presence of HPV16- and HPV18-specific *E6/E7* genes.

DNA extraction

Serial tissue sections (3-5 sections, each 10-20-μm thick) were cut from each paraffin block using disposable microtome blades. After rehydration, DNA was extracted using a lysis buffer containing 10 mmol/L Tris–HCl (pH 8), 100 mmol/L NaCl, 1% sodium docecyl sulfate, 200 μg/mL proteinase K, and 0.01% EDTA at 56 ℃ for 4 h and then incubated overnight at 37 ℃ in a lytic solution. After proteinase K digestion of the tissue, proteinase K was inactivated by incubation at 95 ℃ for 8-10 min. After vortex with phenol and 150 μL chloroform-isoamylalcohol and spun for 2 min at high speed, the upper phase was transferred to a new tube.

Sample suitability

Suitability of samples for PCR amplification was ascertained by testing for the beta-globin gene. Successful amplification of the beta-globin gene fragments indicated that the DNA sample was adequate for PCR analysis and that no PCR inhibitors were present.

Primers

To examine for the presence of any HPV DNA in the tissue, MY09/MY11 primer pairs were used to amplify the *L1* gene. To look for HPV types 16 and 18, the type-specific primer pairs for the *E6/E7* gene were used (Table 1). Distilled water was used as a negative control. This control was necessary to determine if any of the reagents was contaminated with HPV DNA.

Table 1 Primer sequences used for the amplification of HPV *L1*, HPV16 *E6/E7*, and HPV18 *E6/E7* genes

Target	Primer sequence	Approximate size (bp)
HPV *L1* gene (MY09)	5′ CGTCC{C/A}A{G/A}{G/A}GGA{T /A}ACTGATC 3′	450
HPV *L1* gene (MY11)	5′ GC{C/A}CAGGG {T/A} CAT AA {T/C}AATGG 3′	450
HPV16 *E6/E7* gene (sense)	5′ GAACAGCAATACAACAAACCCG 3′	240
HPV16 *E6/E7* gene (antisense)	5′ CCATGCATGATTACAGCTGG 3′	240
HPV18 *E6/E7* gene (sense)	5′ TGCCAGAAACCGTTGAATCC 3′	250
HPV18 *E6/E7* gene (antisense)	5′ CAATGTCTTGCAATGTTGCC 3′	250

Amplification

Master mixtures contained PCR buffer, 10 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 0.01% gelatin, 0.2 mmol/L of each dNTP (dATP, dCTP, dGTP and dTTP), 0.5 mmol/L of each primer and 2.5 units of Taq polymerase (Amp Taq). The PCR mixture was subjected to 30 cycles of amplification (using Genius thermal cycler) each consisting of an initial denaturing step at 94 ℃ for 1 min, annealing at 60 ℃ for 30 s and extension at 72 ℃ for 1 min.

The PCR products were then detected by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. Results were saved by a documentation system along with a transilluminator.

Statistical methods

We used the χ^2 test or the Fisher exact test, wherever appropriate, to compare the proportions of cases and controls that were positive for HPV *L1* gene and HPV16 and HPV18 (type-specific *E6/E7* genes).

The study protocol was approved by the Ethics Committee of the Digestive Disease Research Center, Tehran University of Medical Sciences, and informed consent was obtained from all controls before endoscopy.

RESULTS

Tissue samples were available from 40 cases of ESCC operated between 1996 and 2001. After DNA extraction, two samples were found unsuitable for PCR and excluded. The other 38 samples (22 males and 16 females) were included in the study as cases. Thirty-eight control subjects (16 males and 22 females), age-matched to cases, were selected from patients who were endoscopied for dyspepsia and had normal endoscopies. Mean ± SD age was 54.2±13 years in cases (range 25-75 years), and 51.6±11.3 years in controls (range 22-78 years).

Fourteen (36.8%) out of the 38 ESCC samples but only 5 (13.2%) of the 38 control samples were positive for HPV *L1* gene ($P = 0.02$). Five (13.2%) of the ESCC samples but none of the control samples were positive for HPV16 *E6/E7* gene ($P = 0.05$). Three (7.9%) of the ESCC samples and 5 (13.2%) of the control samples were positive for HPV18 *E6/E7* gene ($P = 0.71$). No sample was positive for both HPV16 and HPV18.

DISCUSSION

ESCC has become the sixth most common cause of cancer death worldwide[9]. 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[6,10]. However, in countries with the highest rates of ESCC, such as Iran and China, only a small proportion of ESCC cases could be attributed to smoking or alcohol consumption[6,11]. So other risk factors must be responsible for the high incidence of ESCC in these areas. Microbial agents, especially HPV, may be one of the factors that explain part of this high incidence of ESCC.

The etiologic role of oncogenic HPV types has been

established in many epithelial cancers, most notably cervical cancer[1,2]. Previous studies have shown that HPV16 and HPV18 are the most important risk factors for cervical cancer[1]. The mechanisms through which HPV can induce epithelial neoplasia have been extensively studied[12-15]. Some of the proteins produced by HPV, notably E6 and E7, are oncoproteins that could immortalize various human cell types, inactivate host proteins (such as p53 or pRb), and induce mutations in the host cell DNA[14,16,17].

The role of HPV in ESCC has been studied in many high-risk and low-risk areas of the world[4,18]. 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[4,19-21]. To the best of our knowledge, this is the first study that reports the association between DNA markers of HPV and the risk of ESCC in Iran, a high-risk area for ESCC.

Our results imply that HPV is not a predominant risk factor for ESCC in Iran because only 14 (36.8%) of 38 samples of ESCC were positive for the common indicator of HPV (*L1* gene). However, this was higher than the percentage of positive samples in controls (13.2%) and the difference was statistically significant ($P = 0.02$). Higher prevalence of this HPV marker in ESCC cases than in controls may be confounded by other factors. But in the light of known mechanisms of carcinogenicity established for HPV and previous studies associating HPV with epithelial cancers, it is unlikely that the virus is a mere innocent bystander, and HPV should be considered as a potential factor contributing to high incidence of ESCC in Iran.

The prevalence of HPV16 was significantly higher in ESCC cases than that in controls ($P = 0.05$), but there was no statistically significant difference in the prevalence of HPV18 between cases and controls. This implies that only HPV16, but not HPV18, may be a risk factor for ESCC in Iran. A similar Chinese study by Zhou *et al* found a similar result. We found markers for HPV16 and HPV18 in only 8 out of 14 ESCC samples in which HPV *L1* gene was present. Therefore, it is possible that other HPV types, not tested in this study, may also be associated with the risk of ESCC in this area. Another line of evidence that argues against high exposure of the Iranian population to HPV16 and HPV18, and hence against these two types of HPV being major risk factors for ESCC in Iran, is the low prevalence of cervical cancer in Iran[7]. Low exposure to HPV16 and HPV18 in Iran is possibly related to the lifestyle and sexual behaviors in this religious society.

A potential shortcoming of this study, as well as other retrospective studies, is their limited ability to find an association between HPV and ESCC, if HPV has a "hit-and-run" mechanism for inducing ESCC, as some studies in a bovine model have suggested[22]. These studies have found that bovine papillomavirus is essential in the early stages of carcinogenesis of the bovine foregut, but is not needed for progression to the malignant state. Therefore, although we found evidence for the presence of HPV in only 38% of our case samples, it is possible that such evidence in other cases has disappeared. This hypothesis can only be tested in prospective studies with tissue or serum samples. So far, no prospective studies using tissue samples have examined this hypothesis, but two small prospective serologic studies have found a strong association between serologic HPV markers and the risk of ESCC[23,24].

In summary, our data 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. We think that HPV should be considered as a potential factor contributing to the high incidence of ESCC in Iran and other high-incidence areas of the world. Further prospective studies are needed to test the hypothesis of a "hit-and-run" phenomenon, the hypothetical mechanism suggested for the disappearance of HPV from tumors after initial DNA damage.

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