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ORIGINAL ARTICLE

Case Control Study

Detection of human papillomavirus DNA in esophageal carcinoma in Greece

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Abstract

AIM: To detect human papillomavirus (HPV) in the esophageal mucosa and the possible relationship with esophageal cancer in Greece.

METHODS: Forty-nine patients underwent esophagogastroduodenoscopy (EGD) and esophageal biopsy at a university hospital that acts as a referral center for Northern Greece. Nineteen of these patients (14 male and 5 female) had esophageal squamous cell carcinoma (ESCC) and 30 (15 male and 15 female) did not have any reported esophageal malignancy. Histopathological assessment was followed by polymerase chain reaction analysis of all the samples. Patient demographic data (age, sex, and place of birth) and information regarding smoking habits, alcohol consumption or sexual habits were collected. A method of statistical interference, verification of hypotheses based on homogeneity and independent χ^2 test, was used.

RESULTS: From the 49 patients that underwent EGD and biopsy, 19 had ESCC and 30 had normal esophageal mucosa, with a mean age of 65.2 years. Regarding the prevalence of oncogenic risk factors for esophageal carcinoma, an interesting conclusion was that 78% of the patients used tobacco and almost one-third had multiple sexual partners, whereas only 20% of the patients consumed alcohol, which was not statistically significant, when compared to the control group. In the ESCC group, the only two positive samples were among the male patients (2/14 male patients with ESCC, 14.5%). No HPV was identified in the control group. The predominant HPV types identified were 11 and 31, which have a low malignancy potential. The presence of HPV DNA in the ESCC group was not statistically significant, 95% confidence interval (χ^2 = 3.292, P = 0.07).

CONCLUSION: This is the first relevant study in Greece, and despite the lack of statistical significance, the issue of HPV infection and ESCC does merit further investigation.

Key words: Esophageal cancer; Esophageal squamous cell carcinoma; Human papilloma virus; Polymerase chain reaction

Core tip: A similarity between the esophagus and cervix is the presence of squamous cells at the gastroesophageal junction. This has led to the hypothesis that at the lower esophagus there may be a transformation zone where insults, such as human papillomavirus (HPV) infection, can have a carcinogenic effect (similar to HPV in cervical cancer). The relationship between esophageal carcinoma and HPV was investigated by polymerase chain reaction analysis of esophageal biopsies in a case-control study. Although there was a non-significant correlation between esophageal squamous cell carcinoma and HPV, the issue merits further attention.

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INTRODUCTION

Esophageal cancer is the eighth most common cancer worldwide, with an estimated 500000 deaths yearly[1]. Esophageal squamous cell carcinoma (ESCC) is the most common histological type, although in the past three decades there has been an increase in the incidence of adenocarcinoma[2]. Additionally, there are many histological similarities between the esophagus and the cervix, especially at the gastroesophageal junction, with the mucosa in both organs populated by squamous cells. As a result, it is possible to identify at the lower esophagus a zone of transformation, similar to that in the cervix. This has led some to suggest that similar pathological events can occur in the esophagus and the cervix, with human papillomavirus (HPV) infection being a likely explanation[3]. The etiology of HPV infection is thought to be multifactorial, including tobacco and alcohol use, leading to a persistent mucosal injury due to bacteria or chemicals, and raising the possibility of HPV acting as a carcinogen[4]. Although many studies have examined the potential causal role of HPV regarding its effect on the squamous cell epithelium and the malignant transformation of the esophageal mucosa, the evidence is not conclusive[3,5]. This is an active debate, because there have been meta-analyses showing that HPV may play a role in carcinogenesis in some regions, especially in ESCC[6,7]. The high geographical variation in ESCC incidence observed worldwide may be a reflection of the exposure to specific environmental, dietary and cultural factors, which may not have been fully investigated[8-11]. In China, India, Central Asia and South Africa, ESCC predominates, with the highest incidence and mortality reported in the Kazakh populations, especially in Xinjiang, China[12,13]. Even in low-risk areas, such as Europe and North America, the incidence of esophageal cancer is increasing[14]. Similar to the case of ESCC, data regarding the relation of esophageal adenocarcinoma (EA) and HPV remain limited[15]. These findings have led the International Agency on Research in Cancer not to make any conclusive statements about the relationship between ESCC and EA and HPV, in contrast to other types of cancer, such as oropharyngeal cancer.

The situation is similar in Greece, because there is not sufficient data to determine the possibility of an etiological role for HPV in esophageal carcinogenesis. As a result, we have decided to investigate the detection of HPV in esophageal mucosa, in patients with and without esophageal malignancy, in order to determine the possible existence of a relationship between esophageal cancer and HPV in our geographical region.

MATERIALS AND METHODS

Patients and tissues

Between 2010 and 2012, upper gastrointestinal endoscopy was performed in 49 patients: 19 with esophageal cancer and 30 patients with no reported esophageal malignancy, and biopsies were taken in all of them. For the control group, biopsies were taken from healthy tissue in the middle of the esophagus, from patients undergoing upper gastrointestinal endoscopy for reasons other than esophageal malignancy and with no microscopic or gross esophageal pathology. In the protocol used, the esophagogastroduodenoscopies and the esophageal biopsies were performed by specialized gastroenterologists, who had performed at least 500 esophageal biopsies each. Areas that were considered suitable for biopsy were those that had erythema or a nodular appearance or friable tissue or ulcerations, scars or neoepithelialization. The endoscope provided a high-definition picture and could also be used for therapeutic purposes, if the need arose. Special biopsy forceps (called jumboforceps) were used for the esophageal biopsies, which can take a significant amount of tissue both in terms of volume, as well as depth from areas with noted pathology. From each region, four random samples surrounding the lesion were taken in a radius of 1-2 cm. The study was conducted at the University Hospital (Aristotle University of Thessaloniki) and the patients came from Northern Greece, because the hospital is a referral center for the whole of Northern Greece, and thus a significant part of the population. Patient demographic data (age, sex and place of birth) and information

Table 1 Clinical and demographic characteristics and risk factors of the patients *n* (%)

	ESCC	Normal
Age (yr)		
mean ± SD	68.1 ± 8.7	64.2 ± 11.2
Sex		
Male	14 (73)	15 (50)
Female	5 (26)	15 (50)
Smoking		
Current	15 (78)	13 (43)
Never smoked	5 (26)	17 (56)
Alcohol use		
Current	4 (20)	7 (23)
Never used alcohol	15 (78)	23 (77)
Sexual partners		
Multiple	6 (31)	9 (30)
Single	13 (68)	21 (70)

ESCC: Esophageal squamous cell carcinoma.

regarding smoking habits, alcohol consumption or sexual habits were collected. Histopathological assessment of all samples was carried out by the Department of Histopathology.

DNA extraction and polymerase chain reaction

After the biopsies were taken, the DNA was extracted using the Qiagen QIAamp DNA Mini Kit (Qiagen, Netherlands) according to manufacturer's instructions. DNA was extracted using careful measures to avoid contamination of the samples and false-positive results. DNA quantification and purity were determined by optical density in a spectrophotometer. We amplified the human conserved genes GAPGDH, G3PDH and β-globin, whose presence indicated that the sample was appropriate for DNA analysis. The sequences of the *HPV* L1 gene were amplified by nested-PCR using two general primer sets: MY09/MY11 (MY09: 5'-GTCCMARRGGAWACTGATC-3', MY11:5' GCMCAGGGWCATAAYAATGG-3') in the amplification step to produce a 450-bp fragment. Final volume of PCR was 50 μL, including 5.0 μL of the genome from the extracted DNA sample, 5.0 μL 10× PCR buffer, 5.0 μL dNTP (2.5 mmol/L), 1.2 μL $MgCl_2$ (50 mmol/L), 0.5 μL Taq DNA polymerase and 0.2 μL (500 pmol/μL) MY09 and MY11 primers. The PCR mixture was subjected to 40 amplification cycles, each consisting of an initial denaturation step at 94 ℃ for 30 s, anneal at 56 ℃ for 1 min and extension at 72 ℃ for 1 min. The PCR products were separated by electrophoresis on 2% agarose gel and visualized by staining with ethidium bromide.

Statistical analysis

From the statistical analysis of the results obtained from HPV (+) and HPV (-) patients, correlation tables containing structure indices were compiled. A method of statistical interference, verification of hypotheses based on homogeneity and independent χ^2 test, was used. The statistical analyses were performed using SPSS.

Ethics

The study was approved by the Ethics Committee of Aristotle University and written informed consent was obtained from all the patients.

RESULTS

Patient characteristics

The 49 patients that were examined were divided into two groups: the study group, which was the patients with the esophageal carcinoma; and the control group, which was the patients without esophageal malignancy. In the control group there were 30 patients, whereas in the study group there were 19 patients. From the 19 patients with esophageal cancer, 14 were male with a mean age of 66.9 years and five were female with a mean age of 69.6 years. The control group consisted of 30 patients (15 male with a mean age of 63.2 years and 15 female with a mean age of 65.2 years).

Histology

All specimens underwent histopathological examination at the Department of Pathology of the University Hospital, which confirmed the diagnosis of ESCC in the 19 patients. The biopsies were multiple and were taken from the lesion, as well as the adjacent areas, according to the standard protocol. The histological report classified the specimens into high differentiation (6/19, 31%), mid differentiation (8/19, 42%) and low differentiation (5/19, 26%). The 30 specimens in the control group were obtained from patients with benign disease, including dyspepsia (20/30, 66%) and gastroesophageal reflux (10/30, 33%) (Table 1). The specimens were obtained from different parts of the esophagus and usually from the middle and lower third. The biopsies taken from the esophagus revealed no malignancy in any of the specimens, and there was normal tissue in any of them.

PCR analysis

The average DNA concentration in the samples was 192 ng/μL. PCR products were run in agarose electrophoresis gel and visualized with ethidium bromide by electrophoresis. Results were analyzed, confirming the presence of the product. The quantity of the samples was adequate, so as to draw conclusions. In the ESCC group, the only two samples positive for HPV were from men (2/14, 14.5%), and that reached statistical significance, when compared to the number of negative samples in the same population, that is male patients with ESCC (2/14 *vs* 0/5). No HPV was identified in the control group, including none of the high-risk types (HPV 16 and 18). The predominant HPV types identified were 11 and 31, which have a low malignancy potential. According to the χ^2 analysis

used, the correlation came close to reaching statistical significance one more positive sample was needed to meet criteria, 95% confidence interval (χ^2 = 3.292, P = 0.07).

Risk factors

We have also studied the prevalence of oncogenic risk factors for esophageal carcinoma, such as tobacco and alcohol use and a history of multiple sexual partners. One interesting conclusion is that 78% of the patients used tobacco, and almost one-third had multiple sexual partners, whereas only 20% of the patients consumed alcohol, which was not statistically significant, when compared to the control group.

DISCUSSION

HPV is a double-stranded DNA virus with tropism for the squamous epithelium, and with oral sex as a possible mode of infection in the case of the esophagus[16,17]. There are > 150 subtypes of the virus, with HPV 16 and 18 linked to the highest risk of malignancy, whereas in the case of the esophagus, many other types have been investigated such as HPV 11 and 31. On the one hand, there have been studies revealing a possible role for HPV in the early stages of carcinogenesis for ESCC, such as a review and a meta-analysis from Australia and China that demonstrated a threefold increase in the risk for esophageal cancer after HPV infection[7,18,19]. On the other hand, reports from other countries have questioned these data[14,15,20]. These findings make it difficult to draw any firm conclusions, with one of the reasons being the different methods used to identify specificity. Another possibility is that the global geographical spread of HPV may be contributing to these contradictory results, given that there are endemic regions with an increased incidence of HPV infection, as well as other regions with increased incidence of esophageal cancer[6].

Our data have shown that Greece is a low-risk region for HPV-related cancer of the esophagus. The virus does not appear to be a significant etiological factor for ESCC, at least in the Greek population. With the limitation of a small sample, the incidence of HPV infection in patients with ESCC was not significantly higher, compared to the control group with the normal esophageal mucosa. Another limitation is the fact that, even though the University Hospital is a referral center for the whole of Northern Greece, and that Greece is a relatively homogeneous country, it does represent only part of the country and so a sampling issue could be raised. Additionally, HPV types that were detected in our study are known to be of low oncogenic potential. On another note, men are known to have a higher incidence of esophageal cancer compared to women, which coincides with the finding in our study of all positive samples (2/19) having been identified in male patients[3,21]. The data regarding the prevalence of risk factors, such as tobacco and alcohol use and the existence of multiple sexual partners, correlated well with the established theory of the causal relationship between tobacco use and ESCC, through the epithelial damage exposing basic cells to the virus, and allowing the integration of the virus into the DNA, which can lead to carcinogenesis. Additionally, the high number of sexual partners, further increases the possibility of oncogenesis, especially through oral sex that would affect the esophageal mucosa. Although the use of alcohol was not found to have a statistically significant association, this could be a matter of not incorporating the increased use of alcohol in more recent years.

Another factor that could explain the discrepancy seen between the various studies worldwide are the different identification techniques used. In our study the primers set that was used was MY09-MY11, which amplified a wide spectrum of HPV genotypes. Studies using this primer set have been mainly from North America, with a solid evidence basis[22-24]. The MY11-MY09 primer is capable of detecting multiple HPV types within a given sample, even in small quantities of tissue involved[25]. Furthermore, even though these specific primers have a low sensitivity for certain HPV types, such as 35, these are not known to be oncogenic, and have limited clinical significance. Finally, these specific primers are easy to replicate between different laboratories with similar results, thus increasing their credibility[26].

Despite the significant limitation of a small sample size, this is the first study of its kind in our region, and although it does not show a relation between HPV infection and ESCC, the issue does merit further investigation, given the fact that there are common contributing factors, such as tobacco use, and thus a synergistic effect cannot be excluded. The latter may take many years to appear, given the chronic nature of the mucosal injury[20]. Finally, although there may not be a relation between HPV infection and ESCC, the possibility of a relation with esophageal adenocarcinoma, should be further investigated. It is important to realize that the ideal way to reach a conclusion, or at least a more satisfactory result, on this issue, is the use of meta-analysis. However, the limited number of studies, in addition to the significant variation between studies and the factor of the geographical variability, make their use rather difficult.

COMMENTS

Background

The incidence of esophageal adenocarcinoma is rising worldwide, with esophageal squamous cell carcinoma (ESCC) being one of the more frequent types. Additionally, there are many histological similarities between the esophagus and the cervix, especially at the gastroesophageal junction, with the mucosa in both organs populated by squamous cells. As a result, it is possible to identify at the lower esophagus a zone of transformation, similar to that in the cervix. This has led some to suggest that similar pathological events can occur in the esophagus and the cervix, with human papilloma virus (HPV) infection being a likely explanation. However, the evidence from the worldwide literature has not been conclusive, with part of the reason being possible geographic variations, as well as different testing techniques for the presence of HPV.

Research frontiers

The relation between HPV infection and esophageal carcinoma has received a lot of attention, especially given the success of the HPV vaccine in the fight against cervical cancer.

Innovations and breakthroughs

The goal of this study was to investigate the relation between HPV infection and esophageal carcinoma, by analyzing esophageal biopsies for the presence of HPV from patients with esophageal carcinoma, as well as patients without any cancer who are serving as controls. The importance of this study is that it is the first of its kind for Greece and essentially for the whole region of the Balkans.

Applications

Identifying whether there is a relationship between HPV infection and esophageal carcinoma would be important, because it would provide a target for cancer prevention. Given the aggressive nature of esophageal carcinoma, it is obvious that any advantage that we can achieve is welcome.

Terminology

HPV is a DNA virus from the papilloma virus family. Although most infections by HPV are subclinical, it has been linked to cervical cancer, becoming a critical risk factor. One way to look for HPV DNA is through the use of polymerase chain reaction (PCR), which is a technique in molecular biology used to amplify a single copy or a few copies of a piece of DNA, in order to generate thousands to millions of copies, thus allowing its identification.

Peer-review

In this work, the authors investigated the detection of human papillomavirus in 19 patients with ESCC and in 30 individuals without esophageal pathology in their geographical location of Northern Greece. The aim was to investigate the possible relationship between esophageal cancer and HPV in their geographical region. The authors used PCR to detect viral genome which is the most reliable method of investigation. This is an interesting topic because of the unproven role of HPV infection and ESCC in regions with low incidence of HPV infection and ESCC such as Greece.

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