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Human Papillomavirus Genotyping in Colombian Patients with Esophageal Squamous Cell Carcinoma and Gastric Carcinoma

Genotipificación del virus del papiloma humano en pacientes colombianos con carcinoma de células escamosas de esófago y carcinoma gástrico

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ABSTRACT

Introduction: Human papillomavirus (HPV) is one of the most important oncogenic viruses detected in different types of cancer, which includes esophageal squamous cell carcinoma (ESCC) and gastric cancer (GC). However, Colombia does not have data on the association of HPV with GC.

Objective: The aim of this study was to determine the presence HPV genotypes in patients with ESCC and GC, diagnosed between 2007 and 2014 at the Hospital Central de la Policía Nacional of Bogotá-Colombia (HOCEN).

Methods: A total of 33 paraffin embedded tissue samples were examine by polymerase chain reaction for the L1 gene of HPV. The extracted DNA was amplified using the universal primers MY09 and MY11. Then, the positive samples had high risk HPV genotyping performed on them. The samples were analyzed by Roche Cobas 4800 system.

Results: Five (15%) samples identified as positive to HPV DNA [four men (80%) and one woman (20%)], one (20%) was positive for HPV-16. The other four, whose genotypes had not detected by COBAS 4800, samples could correspond to low-risk HPV, or match to some of the high-risk genotypes that had not included in the detection panel of this test. Also, four (80%) HPV DNA positives had identified as adenocarcinoma intestinal type, and one (20%) as ESCC.

Conclusion: Our study proves the existence of HPV and p16 in Colombian patients with ESCC and GC, that could be a risk factor for the development of these pathologies in this population.

Keywords: Human papillomavirus, carcinoma, esophageal squamous cell carcinoma, polymerase chain reaction.

RESUMEN

Introducción: El virus del papiloma humano (VPH) es un virus oncogénico detectado en diferentes tipos de cáncer, como el carcinoma de células escamosas de esófago (ESCC) y el cáncer gástrico (CG). Sin embargo, Colombia no cuenta con datos sobre la asociación de VPH con CG.

Objetivo: Determinar la presencia de genotipos de VPH en pacientes con ESCC y CG que fueron diagnosticadas entre 2007 y 2014 en el Hospital Central de la Policía Nacional de Colombia.

Métodos: Se examinaron 33 muestras de tejido parafinado mediante la reacción de cadena de la polimerasa para el gen L1 del VPH. El ADN extraído se amplificó utilizando los cebadores universales MY09 y MY11. Luego se seleccionaron las muestras positivas y se les realizó genotipado para VPH de alto riesgo. Las muestras fueron analizadas por el sistema Roche Cobas 4800.

Resultados: En cinco muestras (15 %) se identificó el ADN del VPH [cuatro hombres (80 %) y una mujer (20 %)], una (20 %) fue positiva para VPH-16. Las otras cuatro podrían corresponder a VPH de bajo riesgo, cuyos genotipos no son detectados por COBAS 4800, o corresponden a un genotipo de alto riesgo que no fue incluido en esta prueba. Además, cuatro muestras (80 %) positivas para ADN de VPH correspondían a CG y una (20 %) a ESCC. Conclusión: Nuestro estudio demuestra la expresión de VPH y p16 en pacientes colombianos con ESCC y CG, que podría ser un factor de riesgo para el desarrollo de estas patologías en esta población.

Palabras clave: Papillomavirus humano, carcinoma, carcinoma de células escamosas de esófago, reacción en cadena de la polimerasa.

INTRODUCTION

Gastric and esophageal cancers are some of the most common malignant diseases worldwide. Esophageal cancer is the seventh in terms of the incidence (572,000 new cases), and sixth in mortality rate (509,000 cases); whereas GC is the fifth most frequently diagnosed cancer (1,000,000 new cases in 2018), and the third leading cause of death worldwide (783,000 deaths) (1).

Although the most frequently and common causes of these cancers are smoking and the presence of Helicobacter Pylori and Epstein Barr virus (EBV) infections (2,3), HPV, particularly genotype 16, is considered as a potential factor that contributes to its high incidence, but this relation is controversial (4). Several meta-analyzes and reviews have established the association of the virus with ESCC, in a range from 11.7% to 38.9%, and with esophagus adenocarcinoma (EAC) in 3.86%; likewise, in 33-45% of colorectal cancers (CRC), and in 80-90% of anal cancers (5); with genotypes 16, 18, and 33 being the most frequently found in tumors of the digestive tract (6). Studies in Colombia and Mexico have exposed the relationship between this virus with cancers adjacent to the airways, finding an incidence of 35.6% in oropharynx and 23.5% in oral cavity (7).

In the same way, HPV can play an important role in GC tumorigenesis. Researches from Spain and Italy demonstrated the presence of genotypes 6 and 16 in ESCC and gastric adenocarcinoma (AG) in 54% and 27.8%, respectively (8). In the United States, Iyer A. et al. described the presence of HPV in 31% of the population with AG (9). In 2010, a study carried out in China showed the presence of HPV genotype 16 in the ESCC and GC in 47% and 29%, respectively (10), and, in 2016, a meta-analysis in 1,917 cases in the same country found the presence of the virus in precursor lesions (adenomas and dysplasia), with genotype 16 as the most frequently associated, and genotype 18 in second place (6). Also in 2016, a study made in Iran on paraffin blocks from 100 patients diagnosed with GC demonstrated the presence of HPV's genotypes 16, 18, and 45 in 3% of AG and 2% of the gastroesophageal junction, without establishing its definitive association (11), results that lead to new studies that clarify the role of HPV in the carcinogenesis of these tumors. However, Colombia does not have information on the association of HPV with GC and ESCC, thus, the current study was designed to determine the distribution of HPV genotypes in Colombian patients with these pathologies, using the polymerase chain reaction (PCR).

MATERIALS AND METHODS

Retrospective Cross-sectional Study

Gastric cancer sample collection: Prior to collecting the samples, a meeting to address HOCEN patients, diagnosed with GC or ESCC during from 2007 to 2014, and their family took place. Patients alive who agreed to take part by signing the informed consent and deceased patients whose relatives signed this document were included in this study. Patients were located via telephone and home visit. From the 200 formerly recognized patients, consent was obtained from 33 individuals.

A questionnaire was used to obtain information about sociodemographic data, risk factors (smoking, alcohol consumption and family history of cancer). Likewise, the histopathological diagnosis was reviewed and confirmed with rereading.

Preparation of samples: Sections (thickness 10 μm) of the paraffin embedded tissue corresponding to the biopsies of these patients were collected in sterile microtubes. To deparaffinize the samples, the paraffin-embedded tissues were saturated in xylol (30 min), then they were incubated in ethanol series (1 min each) of 100% ethanol (dehydration) to 40% ethanol, then they were for 10 sec in double-distilled water for rehydration.

DNA Extraction

DNA extraction was performed using High Pure PCR Template Preparation kit (Norgen Biotek Corp) according to the manufacturer's instructions. After DNA elution, the material was stored at -20°C until PCR.

HPV Detection and Genotyping

DNA samples were subjected to analysis by PCR for MY09/MY11 (450 bp fragment), both targets in L1 open reading frame of HPV. The cycle conditions 94°C for 1 minute followed by 40 cycles of 92°C for 1 minute, 56°C for 3 minutes, and 72°C for 1 minute, with final extension step performed at 72°C for 5 minutes. The HPV positive control was obtained from Secretaría de Salud de Bogotá, and double distilled water was used as negative control in PCR. The Cobas 4800 system (Roche Diagnostics) was used to determine the HPV genotype in positive samples. A total of 14

highrisk HPV genotypes (specifically 16 and 18, and, generically, 31, 33, 35,39, 45, 51, 52, 56, 58, 59, 66, 68) were examined (12).

Statistical analysis: For the statistical analysis, absolute and relative frequencies were used for the description of the nominal variables, and for their distribution average and standard deviation. The statistical program SPSS version 17 was used.

Ethical criteria: This work has the approval from ethics committee of Hospital Central de la Policia (oficios S-2017-049605 y S-2017-431448). Also, it complied with the ethical principles and moral value judgments of medical research on human beings presented in the Declaration of Helsinki of the World Medical Association; 64th General Assembly, Fortaleza, Brazil, October 2013.

RESULTS

The characteristics of the patients who were included in the study are presented in table 1.

Table 1. Demographic, clinical and histopathological characteristics of the study group

Feature	Value		P value
Gender	Female	6	<0,0001
	Male	27	
Age	Mean	66 yr	Does not apply
	Range	45-84 yr	
Sample type	Biopsies	26	<0,0001
	Surgical specimen	7	
Anatomic location	Esophagus	5	<0,0001
	Stomach	28	
Histopathological type	Adenocarcinoma intestinal type	18	<0,0001
	Gastric diffuse carcinoma	10	
	Esophagus Adenocarcinoma	4	
	Esophagus squamous cellular	1	
Smoking	Smoker	13	0,55
	Nonsmoker	10	

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Alcohol usage	Alcohol use	18	0,32
	Non-alcohol use	15	
Family history of gastric cancer	Yes	14	0,33
	No	19	

Source: applied questionnaire y HOCEN Pathology Department.

The positive rate of HPV by PCR in the specimens was 5% (4 men (12.1%) and 1 woman (3 %)), without finding significant difference between genders for HPV infection (P=0,6). The mean age for these patients was 61,8 years with a range of 51-75 years. The pathological diagnosis showed 4 (80%) gastric adenocarcinomas, one of them located in gastro esophageal union. The rest of them were squamous cell carcinoma in the esophagus. The H. Pylori infection and gastritis were identified in 50% (2) of gastric adenocarcinomas. Cobas-4800 genotyping showed positivity for HPV-16 in one sample (20%) and the others (80%) were negative for HPV-AR detected in this test. However, they could correspond to high or low risk genotypes that are not included on Cobas 4800 (table 2).

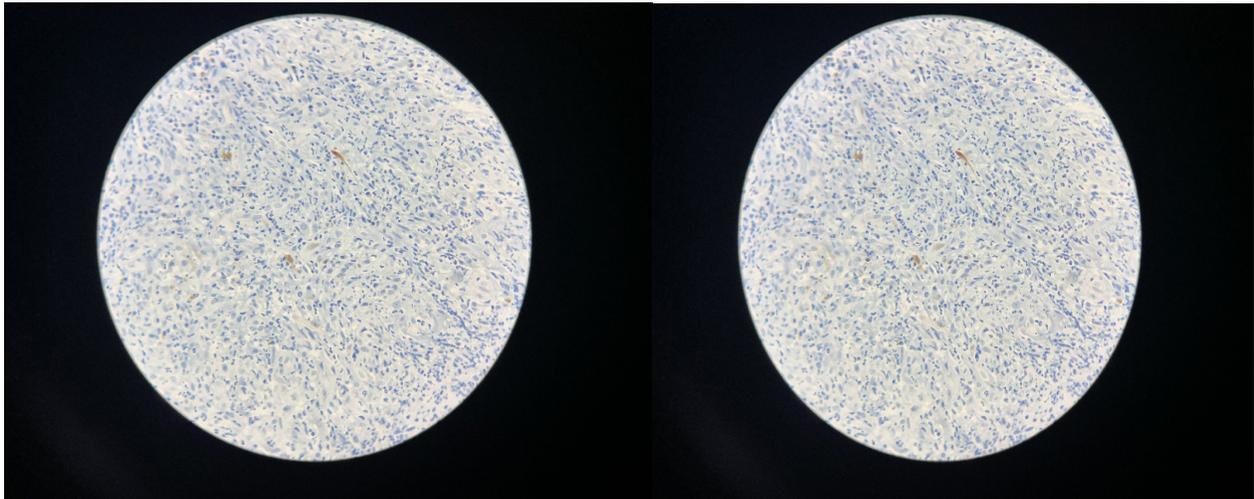
Table 2. HPV positive samples description

Gender	Age	Anatomic location	Histopathological diagnosis	Gastritis	H. Pylori	Alcohol usage	Smoking	Virus genotypes
M	75	Stomach	Gastric-type extremely well-differentiated adenocarcinoma	No	(-)	Yes	No	Others
M	67	Stomach	Gastric intestinal type well differentiated adenocarcinoma	Yes	(+/+++)	Yes	No	Others
M	54	Stomach	Gastric intestinal type poor differentiated adenocarcinoma	No	(-)	No	Yes	VPH-AR 16
F	51	Gastro esophageal union	Adenocarcinoma of GE junction	Yes	(+/+++)	Yes	Yes	Others
M	62	Esophagus	Squamous cell carcinoma	No	Not reported	No	No	Others

Note. M: Male; F: Female.

Source: HOCEN Pathology Department.

The patient HPV 16 positive medical history did not present previous cervical pathology. Her immunohistochemical staining p16^{INK4a} protein showed strong focal expression, associated most likely viral integration (figure).



Source: HOCEN Pathology Department.

Figure. p16^{INK4a} expression

DISCUSSION

It is estimated that 4,8% (610,000) of cancer diagnoses in the world involve HPV, especially cervix, vulva, vagina, anus, and penis cancers (13,14). It has been observed that by increasing the sensitivity of diagnostic techniques it is possible to identify the presence of this viral agent in other regions of the body such as the stomach, lung, and upper respiratory tract (5,7,15). These locations suggest the possibility that it behaves not just as an etiological agent but as a commensalism / opportunistic infection (11).

Looking through the databases, this work represents one of the first studies in Colombia related with the presence of HPV in GC and ESCC. In which it was found the virus in 5 of 33 (15%) biopsies of patients from Hospital Central de la Policia with an average age of 61,8 +/- 15 years.

Roesch-Dietlen et al. (16) reported similar data with 53 patients in Mexico City, where the presence of HPV was detected in 11,32% of the cases with a diagnosis of cancer in the digestive tract,

specifically squamous cell carcinoma and adenocarcinoma. Studies with a larger sample size, such as one published by Zeng et al. (17), shows the presence of HPV in 35% of 1,363 patients with GC originating from 5 countries in Asia, Europe, and South America, in which 83% they corresponded to regions of China. As it was reported by Erol et al. (18), in Turkish patients with gastrointestinal tract tumors, the presence of HPV was detected in 44,7% of the samples corresponding to GC. Likewise, they are similar to those of a meta-analysis carried out in the Iranian population (19), where a prevalence of 16,4% was obtained in patients with GC include in 17 studies, and it is higher than that found by Candido et al. (20) in Brazilian patients with GC, who showed a prevalence of 10% in the analyzed cases.

The variation in the percentages of presence of the associated virus responds to sociodemographic and geographical differences and the determinants in public health typical of the regions.

In relation to gender, for this study, HPV infection is adjusted to the global statistics of GC incidence, which regardless of the etiology, reflect a higher incidence of the disease in the male gender (21,22).

The presence of HPV in the analyzed samples was 80% (4) for men and 20% (1) for women. This finding is related to other studies that investigated the presence of high-risk HPV in paraffin tissue samples from patients with GC in countries such as China (100%), positive (10) for the male gender; Iran (11), 70% of men and 30% of women; and Brazil (20), with a frequency of 11.1% for men and 7.7% for women with this neoplasia.

In Mexico (16), they reported positivity in men on 56.60% of cases and 43.40% in women, likewise, in a meta-analysis (6) carried out with cases of GC from Japan, Brazil, Poland, India, and China, HPV was detected in 44,4% of men and 29,7% of women, that demonstrated through logistic regression studies that being male constitutes a risk factor for GC cancer associated with HPV (OR 1,698; 95% CI: 1,007-2,862). Genotyping studies showed that 20% (1) of the samples positive for HPV corresponded to HPV 16. In the remaining 80% (4), high-risk genotypes were not detected with the used methodology (COBAS 4800), which allows to deduce that they could correspond to a high-risk HPV (HPV-AR) not included in the test, or a low-risk HPV (HPV-BR), which is not detected by this technique.

Previous studies that use molecular tests demonstrated the presence of high-risk HPV genotypes in patients diagnosed with GC, both in tumor tissue and in the adjacent non-tumor mucosa. In China, the presence of HPV 16 was detected in 37,5% of the tumor samples studied and in 5% of the adjacent non-tumor mucosa (23). In the same way, in China, HPV 16 was identified in 29% (5) of GC cases through a multicenter study (10). In northern Iran, the presence of HPV 16 was found in 3 samples (60%), one (20%) for HPV 18 and one (20%) for HPV 45 (11). Additionally, in patients with GC from Europe, South America, and Asia, HPV 16 is reported in 21% of cases and HPV 18 in 7% (6). While, in the meta-analysis carried out in Iran (19), high-risk genotypes 16,18, 31, 33, 39, 45, 51, 52, 56, 58, and 59 are found.

Consequently, the HPV 16 genotype remains as the most frequent, followed by HPV 18. Alternatives for the diagnosis of HPV from paraffin material should be taken into account to improve the prognosis, for example the application of the next generation sequencing (24), that makes possible to demonstrate the molecular characteristics of tumors and thus determine gene fusion or integration.

In contrast to histopathological differentiation, the presence of HPV infection was observed in 25% of well differentiated (1) and moderately differentiated (1) adenocarcinomas and in 50% (2) of poorly differentiated ones. HPV 16 was found in one of the cases diagnosed as poorly differentiated adenocarcinomas. A finding that agrees with that shown in Iranian patients (6), where the prevalence of HPV was 30,3% (142/468) in the well-moderately differentiated and 43% in the poorly-differentiated-undifferentiated (150/349), with an OR of 1,569 (CI: 1,148, 2,143), which shows a significant association between the presence of HPV and the histological grade of the tumor, being higher in the poorly differentiated-undifferentiated, a finding that warrants investigating HPV infection in patients with a diagnosis of poorly differentiated adenocarcinomas.

In general, the characterization of the population included in the study shows evidence of risk factors such as alcohol intake, three of the five positive cases affirm the consumption of this substance, as well as the history of cancer in the family line, and smoking. The origin of the tumors in this study corresponds to cancer of the stomach and gastroesophageal junction; in general, the studies include colon and rectal cancer in the characterizations, which favors the possibility of finding a diversity of genotypes and association with a history of risky sexual behaviors (6, 16, 19).

The findings of this study are relevant if it takes into account that, in Colombia, GC occupies the second place of incidence in men and the fourth in women, as Nariño remains the department with the highest number of cases, followed by Boyacá and Cundinamarca (25). Although, the study does not characterize the expression of the E6 and E7 oncoproteins, which makes it difficult to explain the direct association of HPV with especially late tumor formation, it is important to consider the presence of the virus in the tissue as a possible risk factor and consequently perform subsequent studies that analyze its possible integration into it.

Finally, it is important to stress that, due to the type of pathology addressed, a high percentage of the patients with the possibility to be part of the study had already died, a situation that made it difficult to obtain consent from family members.

CONCLUSION

This study showed the presence of HPV and p16 expression in Colombian patients with ESCC and GC, that could constitute a risk factor for the development of these pathologies in this population, for this reason further investigations are required.

Conflict of interest and financing: This work does not present a conflict of interest and was financed by Universidad Colegio Mayor de Cundinamarca (Agreement 62 from 9th September of 2014).

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