Frequency of HPV detection in chagasic megaesophagus associated or not with esophageal squamous cell carcinoma

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Research Article

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Abstract

Background: Chagasic megaesophagus (clinical manifestation of chagasic disease) has been reported as an etiological factor for squamous cell carcinoma of the esophagus, as well as the presence of human papillomavirus (HPV). Objective: We accessed the prevalence of HPV DNA in a series of squamous cell carcinomas of the esophagus associated or not with the chagasic megaesophagus, and within samples of chagasic megaesophagus without cancer. Data obtained was further correlated to the pathological clinical data of affected individuals.

Methods: Retrospective study that used a total 92 samples tissue/biopsy specimens of formalin fixed and paraffin embedded tissues were retrospectively collected from the southeast region of Brazil from patients treated in three hospitals: Barretos Cancer Hospital, Barretos, São Paulo; Federal University of Triângulo Mineiro (UFTM), Uberaba, Minas Gerais; and São Paulo State University (UNESP), Botucatu, São Paulo. Cases were divided in three groups: i) 24 patients with chagasic megaesophagus associated with esophageal ESCC (CM/ESCC); ii) 37 patients with esophageal ESCC without chagasic megaesophagus (ESCC); iii) 31 patients with chagasic megaesophagus without esophageal ESCC (CM).

Results: We detected a higher prevalence of high-risk HPVs in patients from both CM (12/31, 38.8%) and CM/ESCC groups (8/24, 33.3%), as compared to individuals of the ESCC group (6/37, 16.3%), although data was not statistically significant. We further observed that HPV-16 was more prevalent in patients of the ESCC (4/9, 44.5%) and CM/ESCC groups (2/8, 25.0%). In addition, some of these samples presented infection by multiple HPV types. High-risk HPVs detected were HPV-31, 45, 51, 53, 56, 66, and 73, of which the majority was identified in patients from the CM group. Furthermore, low-risk HPV-11 and HPV70 were identified in individuals from both ESCC and CM groups.

Conclusion: This is the first report regarding the presence of HPV DNA in megaesophagus associated with esophageal squamous cell carcinoma. In the present study, HPV infection appears to be directly related to the development of esophageal squamous cell carcinoma in patients with chagasic megaesophagus. Further studies are warrantee to confirm and better understand the role of oncogenic HPV persistent infection in these patients.

Introduction

Esophageal cancer is ranked as the 8th most frequent type of cancer and the 6th most lethal cancer worldwide [1-3]. Most esophageal cancers occur in developing countries, such as in Brazil, and, according to the Brazilian National Cancer Institute (INCA), they are considered the 10th most common type of cancer (6th in men and 15th in women) with 10,000 new cases estimated for 2020[3].

Among the histological subtypes of esophageal cancer, squamous cell carcinoma (ESCC) is the most frequent, accounting for 90% of the cases [4]; this is a very aggressive malignant neoplasm with high incidence and high mortality rates even in the scenario of recent advances in treatment and diagnosis [1, 2]. The main risk factors for ESCC development are the consumption of alcohol and tobacco (particularly in combination), food and hot drinks, as well as high consumption of red meat [5-7]. Moreover, chronic diseases, such as chagasic megaesophagus, and infectious agents, such as human Papillomavirus (HPV), have been suggested to influence esophageal carcinogenesis[8-10].

Chagasic megaesophagus occurs due to Chagas' disease (American Trypanosomiasis), caused by the parasitic infection of the protozoan *Trypanosoma cruzi* [11]. It is an endemic disease in South and Central America; Brazil is one of the main endemic countries where about 4 million people now are infected with the parasite [12, 13]. However, due to the high number of Latin American immigrants, non-endemic regions, such as the United States, Canada, Europe, Australia, and Japan, may present cases of the disease, and thus this may prove to be an important public health problem worldwide [13-16]. Chagas' disease is divided into two forms: acute (initial) and chronic (late) [11]. During the acute phase, which lasts from 4 to 8 weeks, a high number of parasites circulate in the blood, but in most cases, the symptoms are absent or mild and unspecific, thus making early diagnosis difficult[11]. The chronic form occurs in 30% of the individuals infected with the parasites, and clinical manifestations include the dilation of some organs such as the colon (megacolon), esophagus (megaesophagus), and heart (cardiomegaly) [11].

Chagasic megaesophagus affects about 3% of individuals chronically infected with Chagas' disease [11]. It occurs when the amastigote forms of the protozoa destroy the myenteric plexus, which is part of the enteric nervous system composed of a chain of neurons responsible for contractions of the gastrointestinal tract. This destruction occurs when a flagellar antigen of the parasite (similar to a protein released by these neurons) leads to cross-immunoreactivity, attracting immune cells into the ganglia, causing inflammation and leading to the deposition of dense connective tissue in interstitial fibrosis between muscle fibers [17, 18]. Consequently, uncoordinated contractions of peristalsis occur, reducing the esophageal body and altering the functioning of the lower esophageal sphincter. These changes lead to the buildup of food, resulting in the development of megaesophagus[17]. Importantly, about 2-10% of individuals with chagasic megaesophagus will develop esophageal squamous cell carcinoma [19, 20]; however, due to the scarcity of studies regarding the carcinogenic environment, the neoplastic mechanism leading to ESCC remains uncertain[21]. Nevertheless, it is believed that inflammation and chronic esophagitis may play an important role in the development of epithelial dysplasia and, subsequently in cancer [22, 23].

HPV (Human Papillomavirus) belongs to the Papillomaviridae family and has a tropism towards lining tissues such as the parenchyma of the epidermis and mucous membranes [24, 25]. Over 200 types of HPV (http://pave.niaid.nih.gov/) have been described and clustered within five genera:

alpha-, *beta*-, *gamma*-, *mu*- and *nu*-HPV. High-risk alpha(α)-HPVs are associated with the development of several tumors, including the uterine cervix and the head and neck [26]. Its oncogenic potential occurs due to the expression of viral oncoproteins that interact and block the activities of host cell cycle regulatory proteins [24, 25]. Briefly, the E6 viral oncoprotein interacts with the p53 tumor suppressor protein leading to its degradation and consequently the inhibition of DNA repair and apoptosis; additionally, the E7 oncoprotein interacts with the retinoblastoma protein (pRB) leading to the release of the E2F transcription factor, leading to a deregulated cell proliferation and consequently cancer [24, 25, 27].

In the early 1980s, esophageal tissues (benign and malignant) with cytopathological alterations characteristic to those found in HPV-infected uterine cervix tumors were first observed [9], suggesting that HPV could be involved in esophageal carcinogenesis, since the oral mucosa extends to the squamous epithelium of the esophagus being exposed to viruses such as HPV through oral transmission [10]. This relationship is still a matter of debate since HPV DNA prevalence in ESCC varies substantially within studies, ranging from 0 to 100% [28-30]. Moreover, in the megaesophagus context, solely a single study from Crema and colleagues identified a high frequency of HPV (63.3%) in individuals with chagasic megaesophagus when compared to healthy individuals without megaesophagus (13.8%)[31]. Nevertheless, to the best of our knowledge, currently there are no studies that have analyzed the presence of HPV within individuals with chagasic megaesophagus associated with esophageal cancer.

In summary, esophageal squamous cell carcinoma has as etiologic factors, the chagasic megaesophagus and possibly HPV infection. Facing the evidence of the presence of HPV in individuals with chagasic megaesophagus (whose carcinogenic mechanism is still uncertain), we hypothesized if the presence of HPV in the chagasic megaesophagus may increase the chance of these individuals to develop esophageal cancer.

Materials And Methods

Study population

The casuistic of this retrospective study was composed of patients examined between 1990 and 2016 in the Upper Digestive Tract Department of three hospitals in Southeastern Brazil: Barretos Cancer Hospital (BCH), Barretos, São Paulo; Federal University of Triângulo Mineiro (UFTM), Uberaba, Minas Gerais; and São Paulo State University (UNESP), Botucatu, São Paulo.

Inclusion criteria

We included only patients diagnosed with an image or histopathological proven chagasic megaesophagus with or without a positive serologic test for Chagas' disease; we also included patients with ESCC without chagasic megaesophagus serologic negative for Chagas' disease whose exams (image and histopathology) confirmed the malignant disease. Clinical-pathological variables of patients, as well as the molecular status of *TP53* and *PI3KCA* hotspot mutations and MSI phenotype, were previously reported from these individuals [21, 32, 33]. All clinical and pathological information was obtained through medical record´s review.

Samples and definition of study groups

We included 92 formalin-fixed paraffin-embedded (FFPE) tissue specimens from 92 patients divided into three groups: i) 24 patients with chagasic megaesophagus associated with ESCC (CM/ESCC); ii) 37 patients with ESCC without chagasic megaesophagus (ESCC); iii) 31 patients with chagasic megaesophagus without ESCC (CM).

DNA isolation

A slide containing a 4μm paraffin section of each sample was stained with hematoxylin-eosin (HE, Merck KGaA, GE) and evaluated by a specialist pathologist to delineate necrosis free areas. DNA was isolated from FFPE tissues representative of the tumor lesions in ESCC and CM/ESCC groups and esophageal tissues in the CM group, as previously described [34]. Sections were deparaffinized by heating at 80°C for 20 min, followed by sequential washing in xylol (5 min) and decreasing concentrations of ethanol (1 min - 100%, 70%, and 50%) and water free of nucleases (1 min). DNA extraction was performed using the commercial kit QIAamp DNA Micro Kit (Qiagen, USA) following the recommended protocol.

DNA integrity was evaluated by Polymerase Chain Reaction (PCR) of a region of the human β-globin gene (110pb) using primers Forward - PCO3 (5'-ACACAACTGTGTTCACTAGC-3') and Reverse - PCO4 (5'-CAACTTCATCCACGTTCACC-3') and a protocol previously established in our group [34]. Briefly, PCR was performed under the following conditions: 20mM Tris-HCl (Invitrogen, USA), 50mM KCl (Invitrogen, USA), 4mM $MgCl_2$ (Invitrogen, USA), 200nM dNTP mix (Invitrogen, USA), 200nM primers, 1,25U de Platinum ®Taq DNA Polymerase (Invitrogen, USA), and DNase RNase free water (Gibco/BRL, Life Technologies, USA) to a final volume of 25μL and 5μL of DNA at 50 ng/μl. PCR products were subjected to 1.5% agarose gel electrophoresis with Gel Red (Biotium, CA) to evaluate the amplification of the gene of interest.

HPV detection and genotyping

Detection of HPV DNA was assessed in all samples by a genotyping assay combining multiplex PCR (TS-MPG) and bead-based Luminex technology (Luminex Corp., USA), as previously described [35]. This technique is able to simultaneously identify 20 types of α-HPV: 3 low risk: HPV-6,

11, 70; 6 probably high risk: HPV- 26, 53, 66, 68, 73, 82; and 11 high risk: HPV-16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59 [36,37]. As a positive control for the quality of the template DNA, primers for the β-globin gene were included in these reactions. PCRs were performed with 10µl of template DNA in a 96-well format in 25-µl/well final reaction volume. HPV multiplex PCR was performed with Qiagen Multiplex PCR Kit (Qiagen, Germany), following the manufacturer protocol. Each reaction consisted of 45 cycles: 94°C for 30 seconds, 63°C for 3 minutes, and 72°C for 90 seconds. The first cycle was preceded by incubation at 95°C for 15 minutes, and the last cycle was extended for 10 minutes at 72°C. Hybridization reactions were performed according to Schmitt *et al* (2006) [38]. For each HPV type-specific probe, the mean fluorescence intensity (MFI) values obtained when no PCR product was added to the hybridization mixture were considered as background. The cutoffs were calculated by adding 5 MFI to 1.1 times the value of the median background. MFI values > 20 were considered positive.

Statistical analysis

Characterization of the study population was analyzed through frequency tables for qualitative variables, and measures of central tendency and dispersion (mean, standard deviation, minimum, and maximum) for the quantitative variables, comparing the different groups. To assess the association of HPV DNA detection, clinical-pathological characteristics, and the mutation status of *TP53* and *PIK3CA* between study groups, we used Chi-square or Fisher's exact tests. We performed an overall survival analysis using the Kaplan-Meier limit estimator and we applied the Log-rank test to compare overall survival curves in the groups with cancer.

The level of significance adopted was 5% ($p \leq 0.05$). Statistical analyses were performed using the SPSS software v.21.0 (SPSS, Chicago, IL).

Results

Characterization of the study population

Clinical-pathological and molecular features of the patients studied are described in Table 1 [21]. As previously reported by our group [32], most of the patients were males and under 60 years old. In relation to the principal risk factors for the development of esophageal cancer, patients from groups CM/ESCC and ESCC were statistically associated with higher consumption of tobacco and alcohol (87.5% and 86.5%; 66.7% and 81.0%, respectively). In addition, most patients had advanced stage esophageal cancer and chagasic megaesophagus (Table 1).

Detection of HPV DNA

Using a very sensitive HPV detection and typing protocol based on multiplex PCR (Luminex), we observed a high prevalence of high-risk HPVs DNA in patients of both CM (12/31, 38.8%) and CM/ESCC groups (8/24, 33.3%), when compared to patients of the ESCC group (6/37, 16.3%), although data was not statistically significant (Table 2).

We analyzed the frequency of individual HPV types within each group (Table 3). We observed that HPV-16 was more frequently detected in patients from both ESCC (4/9, 44.5%) and CM/ESCC groups (2/8, 25.0%). In addition, for some samples, HPV co-infection by more than one viral type was observed. We additionally detected several high-risk HPVs (HPV-31, 45, 51, 53, 56, 66, and 73), of which the majority was identified in patients from the CM group. Finally, low-risk HPV-11 and HPV-70 were identified in individuals from both ESCC and CM groups (Table 3).

We next thought to analyze the association between HPV prevalence and clinical-pathological features of patients independent or not of the study group. The data obtained are depicted in tables 4 and 5. Nevertheless, no significant associations were observed for any of the variables studied (Table 4 and 5).

Finally, in order to better understand the role of HPV in these patients, we analyzed if there was any correlation between the presence of high-risk HPV DNA and the mutation status of *TP53*, which was reported previously in our studies [32]. Despite the lack of significant associations, we observed that patients who tested positive for HPV were more prone to lack mutation within the *TP53* gene (18/25, 72.0%) (Table 4). However, when disease groups were analyzed independently, only HPV positive samples from individuals from the CM group did not present mutations within *TP53* (12/12, 100.0%), as opposed to patients of the CM/ESCC and ESCC groups, among which HPV frequencies were similar in patients with or without *TP53* mutation (Table 5).

Overall survival analysis

Finally, we compared the Kaplan-Meier survival curves and the Log Rank test among all groups. As expected, we observed that individuals in the group with only megaesophagus (CM) had a significant better survival when compared with the other groups with cancer (Figure 1). Regarding the HPV status, HPV positive cases exhibited a better outcome; however, it was not statistically significant (Figure 2). We performed the Cox regression test to assess the survival ratio between the group ESCC/CM and the positive HPV (Table 6). We observed that the hazard ratio is significantly associated with the ESCC/MEC group.

Discussion

There are several well-known risk factors for the development of esophageal squamous cell carcinoma, but two remain controversial: chagasic megaesophagus caused by neglected Chagas disease, endemic in Latin and South America regions and considered a significant public health problem[16, 17, 39]; and persistent HPV infection, which has been suggested since the early 1980s as a potential risk factor [9, 10]. However, no studies have until now investigated the possible association between HPV detected in patients with chagasic megaesophagus associated with esophageal cancer. In this sense, the present study assessed, for the first time. the presence of HPV in patients with chagasic megaesophagus associated with esophageal squamous cell carcinoma (CM/ESCC) and compared with patients with esophageal squamous cell carcinoma without chagasic megaesophagus (ESCC) and chagasic megaesophagus without esophageal squamous cell carcinoma (CM).

The results obtained in this study showed a higher prevalence of high-risk HPVs in the context of the chagasic megaesophagus without (12/31, 38.8%) or associated with ESCC (8/24, 33.3%). These findings are partially in concordance with the previous Brazilian study that reported an HPV frequency of 63.3% in individuals with chagasic megaesophagus without esophageal cancer [31]. Moreover, the high frequency of HPV is similar to that reported in esophageal tumors in regions in China considered to be at high risk (~32-63,6%) [40]. In addition, we observed low HPV prevalence in patients with esophageal cancer of the ESCC group (6/37, 16.3%). These results agree with those reported by Brazilian studies in which frequencies reached 15% [41-44], and with a recent study from our group that identified a frequency of 13.8% using the same methodology, in patients with esophageal squamous cell carcinoma [34, 45].

The HPV type more commonly detected within samples analyzed in the current study was HPV-16, present within the three groups, but more frequently detected in the ESCC group (4/9, 44.5%) followed by CM/ESCC (2/8, 25.0%) and CM groups (2/14, 14.3%). Notably, persistent HPV infection, mainly HPV-16, is associated with carcinogenesis in several anatomical sites such as cervical cancer worldwide [46-48]. In addition, we identified a low frequency of several other high-risk HPV types, including HPV-31, 39, 45, 51, 52, 53, 56, 59, 66, 68, and 73 (Table 3). Some of the HPV types detected in our analysis coincide with that reported in our previous study, also using the Luminex technique [34]. These types identified with low frequency are little studied in the literature [49].

Importantly, the oncogenic potential of high-risk HPV is well-established and related to the expression of the E6 and E7 oncoproteins that interact and cause degradation of p53[24, 25]. Therefore, in an attempt to better understand the role of HPV in the groups, we evaluated the presence of HPV and the mutation status of the *TP53* gene previously reported in a study of our group[31]. We observed that most HPV positive patients did not have *TP53* mutations. What caught our attention was the fact that the HPV positive CM group of patients was 100% *TP53* wild-type, while in the CM/ESCC and ESCC groups HPV frequencies were similar in the mutated or wild-type patients for the *TP53* gene. According to the literature, it is expected that HPV associated cancers do not present *TP53* mutations, being mutually exclusive events. [50, 51]. Some studies report that in cervical tumors, HPV infection and *TP53* mutations are mutually exclusive, evidencing that such infection leads to the development of this tumor type, but in head and neck tumors both HPV infection and *TP53* mutations can occur simultaneously. In these cases, the study shows that HPV did not play a causal role in tumor development, but it was only casually present[52-57].

We also assessed the relationship between survival between study groups and HPV status. We observed that HPV status does not influence patient's survival, although HPV positive individuals have a longer survival in relation to HPV negative individuals. These results are in line with previous reports on esophageal cancer, that shown that HPV infection is not associated with improved survival, suggesting that the presence of HPV may not be useful for a possible prognostic assessment related to fact or that contribute to cancer of the esophagus[58, 59].

It is known that infection by the protozoan *Trypanosoma cruzi* in the esophageal mucosa leads to several physiological changes that result in the development of the chagasic megaesophagus. The altered microenvironment of chagasic megaesophagus has been shown to be a favorable environment for HPV. In the present study, HPV was associated with esophageal squamous cell carcinoma in patients with chagasic megaesophagus. Further studies are needed to confirm and better understand the potential role of oncogenic HPV persistent infection in patients with chagasic megaesophagus.

Abbreviations

ESCC – esophageal squamous cell carcinoma

HPV - human Papillomavirus

CM – chagasic megaesophagus

PCR - Polymerase Chain Reaction

Declarations

Authors' contributions

FFM participated in the conception of the study, data collection, analysis and interpretation of the results, and draft of the manuscript. ACC, CFL, ATTO, CSN, SRMS, EC, SJA, MAMR, MACAH, LS, EMN and LLV participated in the acquisition and quality assessment of the data collection and results interpretation. ALF and DPG participated in the, data collection, analysis and interpretation and draft of the manuscript. RMR participated in the design, supervision, data interpretation, drafting and final revision of the manuscript. All authors gave final approval of the manuscript.

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Tables

Table 1 - Clinical-pathological and molecular features of the three study groups.

		Groups (n= 92)			
		CM/ESCC (n= 24)	ESCC (n= 37)	CM (n= 31)	
Variable	**Category**	**n (%)**	**n (%)**	**n (%)**	**p-value**
Gender	Female	5 (20.8)	6 (16.2)	4 (12.9)	0.676*
	Male	19 (79.2)	31 (83.8)	27 (87.1)	
Age	≤ 60 years	11 (45.8)	21 (56.8)	23 (74.2)	0.096*
	≥ 60 years	13 (54.2)	16 (43.2)	8 (25.8)	
Tobacco consumption	No	3 (12.5)	5 (13.5)	17 (54.8)	**<0.001***
	Yes	21 (87.5)	32 (86.5)	14 (45.2)	
Alcohol consumption	No	8 (33.3)	7 (18.9)	22 (73.3)	**<0.001***
	Yes	16 (66.7)	30 (81.1)	8 (26.7)	
	Missing	0	0	1	
Tumor differentiation	Well differentiated	5 (23.8)	7 (20.0)	NA	0.921**
	Moderate differentiated	14 (66.7)	23 (65.7)	NA	
	Poorly differentiated	2 (9.5)	5 (14.3)	NA	
	Missing	3	2	-	
TNM Staging	I/II	4 (22.2)	16 (44.4)	NA	0.142*
	III/IV	14 (77.8)	20 (55.6)	NA	
	Missing	0	1	-	
Megaesophagus grades	GI/GII	10 (41.7)	NA	4 (12.9)	**0.027***
	GIII/GIV	14 (58.3)	NA	27 (87.1)	

*Chi-square association test; **Fisher's exact test.

CM/ESCC – Chagasic megaesophagus associated with esophageal squamous cell carcinoma; ESCC – Esophageal squamous cell carcinoma; CM - Chagasic megaesophagus without esophageal squamous cell carcinoma. n – Casuistry; NA – Not applicable.

Table 2 – Analysis of high-risk HPV DNA prevalence among the three study groups.

		Groups (n= 92)			
		CM/ESCC (n= 24)	ESCC (n= 37)	CM (n= 31)	
Variable	**Category**	**n (%)**	**n (%)**	**n (%)**	**p-value**
High-risk HPV[(a)]	Negative	16 (66.7)	31 (83.8)	19 (61.3)	0.107*
	Positive	8 (33.3)	6 (16.2)	12 (38.7)	

*Chi-square association test.

CM/ESCC – Chagasic megaesophagus associated with esophageal squamous cell carcinoma; ESCC – Esophageal squamous cell carcinoma; CM - Chagasic megaesophagus without esophageal squamous cell carcinoma; n – Casuistry; [(a)] The types of high-risk HPV analyzed were: HPV-16, 31, 45, 51, 53, 56, 66 and 73.

Table 3 – Frequency of all types of HPV detected by Luminex in three study groups.

	HPV positives (n= 31)		
	CM/ESCC (n= 8)	ESCC (n= 9)	CM (n= 14)
Variable/Category	**n (%)**	**n (%)**	**n (%)**
Low-risk HPV			
HPV-11	0 (0.0%)	2 (22.2%)	1 (7.1%)
HPV-70	0 (0.0%)	1 (11.1%)	1 (7.1%)
High-risk HPV			
HPV-16	2 (25.0%)	4 (44.5%)	2 (14.3%)
HPV-31	0 (0.0%)	0 (0.0%)	1 (7.1%)
HPV-45	0 (0.0%)	0 (0.0%)	1 (7.1%)
HPV-51	0 (0.0%)	0 (0.0%)	1 (7.1%)
HPV-53	1 (12.5%)	0 (0.0%)	0 (0.0%)
HPV-56	0 (0.0%)	0 (0.0%)	2 (14.3%)
HPV-66	0 (0.0%)	1 (11.1%)	0 (0.0%)
HPV-73	1 (12.5%)	0 (0.0%)	1 (7.1%)
Coinfection	4 (50.0%)*	1 (11.1%)**	4 (28.6%)***

*HPV-16, 58; HPV-16, 68; HPV-39, 59; HPV- 52, 73.

**HPV-6, 31, 56, 73.

***HPV-16, 45; HPV-16, 51, 59; HPV- 52, 73; HPV- 6, 51, 66.

CM/ESCC – Chagasic megaesophagus associated with esophageal squamous cell carcinoma; ESCC – Esophageal squamous cell carcinoma; CM - Chagasic megaesophagus without esophageal squamous cell carcinoma. n – Casuistry.

Table 4 – Overall analysis of the frequency of high-risk HPV in patients related to patients' clinical-pathological features.

		High-risk HPV		
		Negative (n= 66)	Positive (n= 26)	
Variable	**Category**	**n (%)**	**n (%)**	**p-value**
Gender	Female	14 (21.2)	1 (3.8)	0.059**
	Male	52 (78.8)	25 (96.2)	
Age	≤ 60 years	39 (59.1)	16 (61.5)	1.000*
	≥ 60 years	27 (40.9)	10 (38.5)	
Tobacco consumption	No	20 (30.3)	5 (19.2)	0.314*
	Yes	46 (69.7)	21 (80.8)	
Alcohol consumption	No	25 (38.5)	12 (46.2)	0.637*
	Yes	40 (61.5)	14 (53.8)	
	Missing	1	0	
Tumor differentiation	Well differentiated	9 (20.5)	3 (25.0)	1.000**
	Moderate differentiated	29 (65.9)	8 (66.7)	
	Poorly differentiated	6 (13.6)	1 (8.3)	
	Missing	22	14	
TNM Staging	I/II	17 (38.6)	3 (30.0)	0.728**
	III/IV	27 (61.4)	7 (70.0)	
	Missing	22	16	
Megaesophagus grades	GI/GII	6 (17.1)	8 (40.0)	0.106*
	GIII/GIV	29 (82.9)	12 (60.0)	
	Missing	31	6	
TP53 gene [31]	WT	42 (66.7)	18 (72.0)	0.800*
	MUT	21 (33.3)	7 (28.0)	
	Missing	3	1	

*Chi-square association test; **Fisher's exact test.

CM/ESCC – Chagasic megaesophagus associated with esophageal squamous cell carcinoma; ESCC – Esophageal squamous cell carcinoma; CM - Chagasic megaesophagus without esophageal squamous cell carcinoma. n – Casuistry.

Table 5 – Analysis of the frequency of high-risk HPVs in the three study groups related to their clinical-pathological features.

		High-risk HPV								
		CM/ESCC group (n= 24)			ESCC group (n= 37)			CM group (n= 31)		
		Negative (n=16)	Positive (n=8)		Negative (n=31)	Positive (n=6)		Negative (n=19)	Positive (n=12)	
Variable	**Category**	**n (%)**	**n (%)**	**p-value**	**n (%)**	**n (%)**	**p-value**	**n (%)**	**n (%)**	**p-value**
Gender	Female	4 (25.0)	1 (12.5)	0.631**	6 (21.9)	0 (0.0)	0.330**	4 (21.1)	0 (0.0)	0.139**
	Male	12 (75.0)	7 (87.5)		25 (78.1)	6 (100.0)		15 (78.9)	12 (100.0)	
Age	≤ 60 years	7 (43.8)	4 (50.0)	1.000**	18 (56.3)	4 (66.7)	1.000**	15 (78.9)	8 (66.7)	0.676**
	≥ 60 years	9 (56.2)	4 (50.0)		14 (43.8)	2 (33.3)		4 (21.1)	4 (33.3)	
Tobacco consumption	No	3 (18.8)	0 (0.0)	0.526**	5 (15.6)	0 (0.0)	0.570**	12 (63.2)	5 (41.7)	0.288*
	Yes	13 (81.2)	8 (100.0)		27 (84.4)	6 (100.0)		7 (36.8)	7 (58.3)	
Alcohol consumption	No	5 (31.2)	3 (37.5)	1.000**	6 (18.8)	1 (16.7)	1.000**	14 (73.7)	8 (66.7)	0.678**
	Yes	11 (68.8)	5 (62.5)		26 (81.3)	5 (83.3)		4 (21.0)	4 (33.3)	
Tumor differentiation	Well differentiated	4 (26.7)	1 (16.7)	1.000**	5 (17.2)	2 (33.3)	0.664**	NA	NA	NA
	Moderate differentiated	9 (60.0)	5 (83.3)		20 (69.0)	3 (50.0)		NA	NA	
	Poorly differentiated	2 (13.3)	0 (0.0)		4 (13.8)	1 (16.7)		NA	NA	
	Missing	1	2		2	0		-	-	
TNM Staging	I/II	4 (28.6)	0 (0.0)	0.524**	14 (45.2)	3 (50.0)	1.000**	NA	NA	NA
	III/IV	10 (71.4)	4 (100.0)		17 (54.8)	3 (50.0)		NA	NA	
	Missing	2	4		0	0		-	-	
Megaesophagus grades	GI/GII	5 (31.2)	5 (62.5)	0.204**	NA	NA	NA	1 (5.3)	3 (25.0)	0.272**
	GIII/GIV	11 (68.8)	3 (37.5)		NA	NA		18 (94.7)	9 (75.0)	
TP53 gene (31)	WT	7 (50.0)	3 (42.9)	1.000**	18 (58.1)	3 (50.0)	1.000**	17 (94.4)	12 (100.0)	1.000**
	MUT	7 (50.0)	4 (57.1)		13 (41.9)	3 (50.0)		1 (5.6)	0 (0.0)	

Missing	2	1		0	0	1	0

*Chi-square association test; **Fisher's exact test.

CM/ESCC – Chagasic megaesophagus associated with esophageal squamous cell carcinoma; ESCC – Esophageal squamous cell carcinoma; CM - Chagasic megaesophagus without esophageal squamous cell carcinoma. n – Casuistry; NA – not applicable.

Table 6- Multivariable survival analysis (Cox regression model) (n= 62)

	Overall survival		
Variables	**HR**	**95% confidence interval**	***P***
Grupo (CCE/CM)	1.952	1.003 to 3.797	0.049
HPV (positive)	1.130	0.575 to 2.224	0.723

CM/ESCC – Chagasic megaesophagus associated with esophageal squamous cell carcinoma

Figures

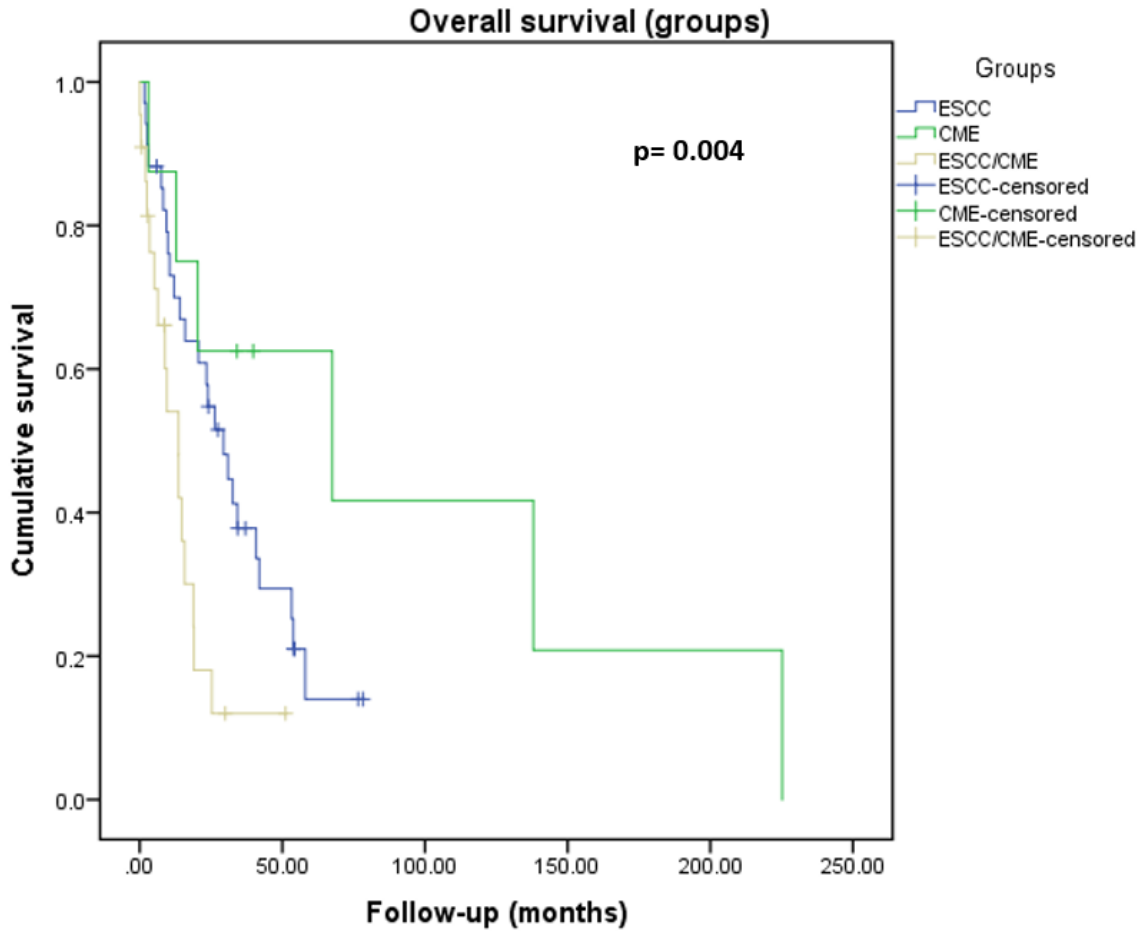

Figure 1

Kaplan-Meier curve for assessing the estimated overall survival probability of follow-up time among patients from diagnosis to death per group. ESCC: group with squamous cell carcinoma of the esophagus; MEC/ESCC: chagasic megaesophagus group associated with cancer.

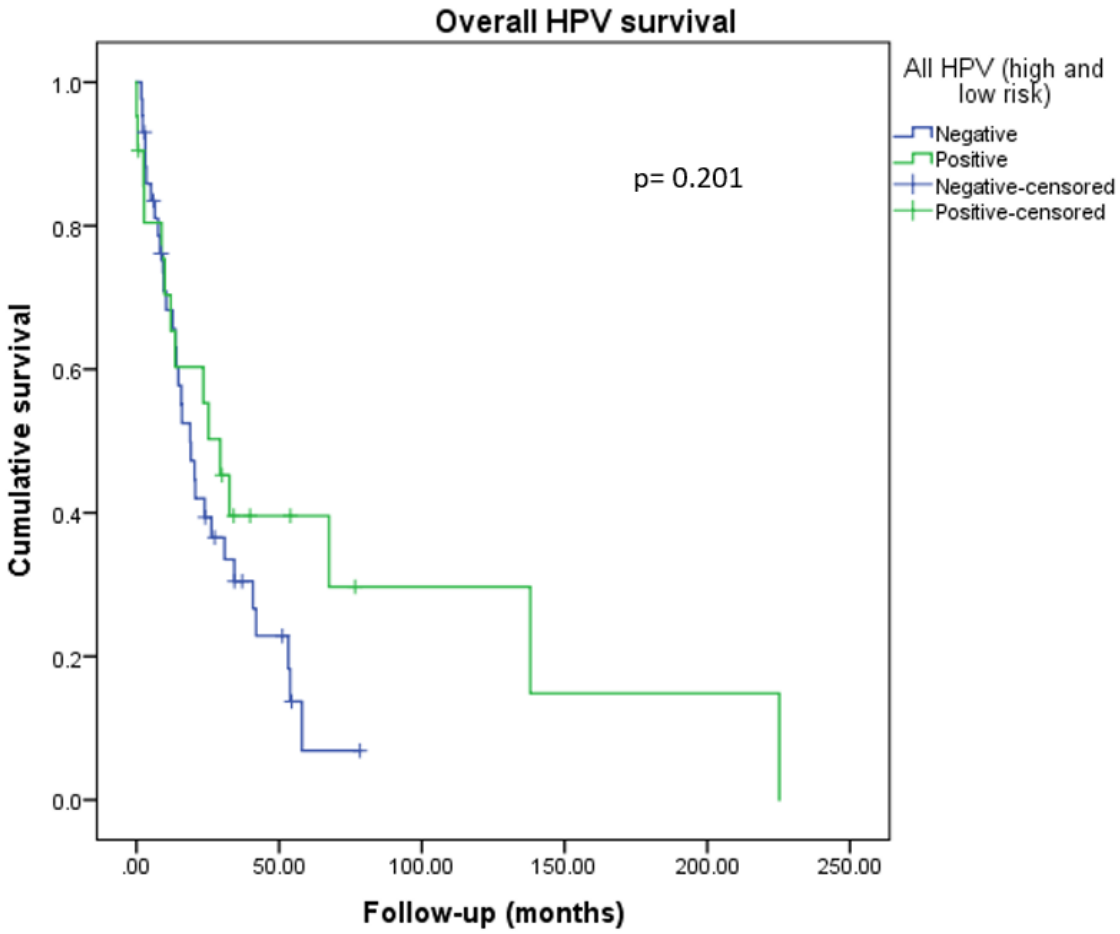

Figure 2

Kaplan-Meier curve for assessing the estimated overall HPV survival probability of follow-up time among patients (all groups) from diagnosis to death.