Human Papillomavirus and Anal Cancer: Prevalence, Genotype Distribution, and Prognosis Aspects from Midwestern Region of Brazil

Research Article

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Background. Approximately 90% of all anal cancers are associated with human papillomavirus (HPV), especially high-risk genotypes such as HPVs 16 and 18. Objective. To investigate the clinical and prognostic aspects of anal cancers associated with the presence, as well as the genotypic distribution of human papillomavirus (HPV). Methods. A retrospective study carried out over a 10-year period, using clinical and molecular data, with PCR analysis and reverse hybridization (INNO-LIPA kit), in anal cancers. The data analysis was done using descriptive univariate statistics, and the survival curves were made using the Kaplan–Meier and log-rank methods. Results. Of the 81 formalin-fixed and paraffin-embedded specimens, HPV prevalence was 69% and was significantly higher in squamous cell carcinomas (SCC) than in other anal tumors \((p = 0.0001)\). Female patients had a higher prevalence of HPV \((p = 0.01)\). Multiple infections were detected in 14.3% of cases. The most prevalent genotypes were HPVs 16, 33, and 18. The overall survival at 60 months was 44.3%, and the prognostic factors included gender \((p = 0.008)\) with greater survival for men (52.9%) in comparison to women (29.6%), histological type \((p = 0.01)\), SCC (54.4%), adenocarcinomas (37.5%), other carcinomas (14.2%), and the presence of distant metastasis \((p = 0.01)\). Survival was not influenced by the presence of HPV \((p = 0.54)\). Conclusions. The association of HPV to anal cancer was found in this study, especially in SCC. However, the presence of HPV did not influence the prognosis of patients with anal cancer.

1. Introduction

Approximately 5% of all cancers worldwide are associated with human papillomavirus (HPV), and the proportion of anal cancer attributed to HPV is 90%, with genotypes 16 and 18 found in more than 70% of these cancers [1–5]. Anal cancer is a rare tumor that corresponds to approximately 2% of cancers that affect the gastrointestinal system [6]. About
The goal of this study was to evaluate the prognostic, sociodemographic, and clinical aspects of individuals with anal cancer associated with the detection and genotypic distribution of HPV, as well as its influence on the clinical prognosis of anal carcinomas [14–18].

The role of HPV in the prognosis of anal carcinomas is poorly understood, as well as its influence on the clinical aspects of this tumor [14–16]. Although patients diagnosed with primary cancers associated with HPV respond well to treatment, there is a risk for a second exposure or relapse of HPV infection [14, 18]. Classical aspects of staging, such as tumor size, metastatic lymph node involvement, and distant metastasis, remain the main factors that influence the prognosis of anal carcinomas [6, 19, 20].

The most common histological type of anal cancer is squamous cell carcinoma (SCC), followed by adenocarcinoma [2, 6, 13]. HPV can be considered an essential factor for the development of SCC, as well as its precursor lesions such as anal intraepithelial neoplasia (AIN) [14, 15]. Several factors contribute to HPV-induced anal carcinogenicity, such as early onset of sexual activity, sexual practices involving anal intercourse, prior exposure to high-risk HPV genotypes, history of anogenital injury, and other cancers associated with HPV. The major risk groups include men who have sex with men, transsexual women, and individuals carrying human immunodeficiency virus (HIV) [14–18].

2. Methodology

2.1. Type of Study and Sample Selection. This is a retrospective study that investigated HPV prevalence and genotypic distribution in a group of anal cancer patients assisted in a cancer reference center from Goiânia, a mediumsized city in the Midwest region of Brazil, during a period of 10 years.

A sample size calculation was not performed since we aimed to include in the study all the cases that were diagnosed as anal cancer in the Pathology Department of the center. Our reference sample came from the registry of the Pathology Laboratory, so initially, a list of 140 patients diagnosed with anal cancers from 2000 to 2010 was consulted. After pathological/clinical review, 85 cases of anal cancers were considered eligible. A description of the inclusion and exclusion criteria of the cases was presented in Figure 1.

The selected cases were those that presented histopathological diagnoses of anal cancer confirmed by two pathologists, those with clinical data available in the medical records, and those with paraffin blocks available and sufficient for molecular analyses. Cases that were not confirmed as primary anal cancer were excluded. Since we aimed to evaluate five-year overall survival, the retrospective study that was initiated in 2016 considered patients that were diagnosed until 2010.

This study was approved by the Research Ethics Committee of the Association to Combat Cancer in Goiás (CEP/ACCG) under CEP: 272,288.

2.2. Preparation of Samples. After selection of the paraffin blocks containing the tumor specimens, each block was serially sectioned with the use of a microtome and the sections packed in properly identified 2 ml sterile microtubes. The microtome knives were changed between samples, and the equipment was cleaned with ethanol for each new block. From each block, slides containing tumor fragments were prepared and stained using hematoxylin and eosin and reviewed by a pathologist. The diagnosis of anal carcinoma was confirmed for each case, based on the classification criteria for tumors from the World Health Organization [21].

2.3. DNA Extraction. Viral DNA extraction was performed using the phenol-chloroform-isooamyl alcohol method; the paraffin removal was done with the organic solvent xylol and cell digestion performed with proteinase-K. The final DNA precipitation was done with isopropanol and DNA purification with 70% ethanol. As dewaxing of the sample can lead to tissue loss and consequent degradation of the DNA present in the sample [22], the amount of DNA extracted and its purity were evaluated by spectrophotometry (Thermo Scientific NanoDrop Products). The samples were submitted to polymerase chain reaction (PCR) to amplify the endogenous control, a fragment of the human gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Samples negative for endogenous control were re-extracted.

2.4. Detection of HPV Genotype. Our study employed the INNO-LiPA HPV Genotyping Extra test (Innogenetics NV, Ghent, Belgium) to detect and genotype HPV DNA, following the manufacturer’s instructions. This assay can identify 28 different HPV genotypes, including all known HR-HPV genotypes and probable HR-HPV genotypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82), as well as several LR-HPV genotypes (6, 11, 40, 43, 44, 54, and 70) and a number of additional types (69, 71, and 74), based on nested PCR amplification of a fragment (65 base pairs) of the L1 region of the HPV genome. Amplified
products were denatured under alkaline conditions and immediately incubated with the test strips in hybridization buffer. The results were visually interpreted by two independent investigators by comparing them with a template provided with the assay. The kit allows simultaneous detection of multiple genotypes in a single sample. Several publications have already proved the performance of the assay in cervical scrapes and in formalin-fixed and paraffin-embedded (FFPE) tissue.

The study employed the INNO-LiPA HPV Genotyping Extra test (Innogenetics NV, Ghent, Belgium) to detect and genotype HPV DNA. This assay is a reverse line hybridization assay validated by several previous studies. The physical state of HPV was not investigated.

In genotyping, only those samples that presented a single genotype of HPV were considered as single infections, and the samples that had more than one HPV genotype were considered multiple infections. In cases of multiple HPV infections with at least one high-risk genotype, the result was considered to be high-risk HPV. In cases that contained only low-risk genotypes, the sample was considered low-risk HPV.

The entire laboratory procedure, from sample handling to HPV detection and genotyping, followed the international standards for HPV testing by the World Health Organization [23].

2.5. Statistical Analysis. Sociodemographic and clinical and pathological data were collected on appropriate forms and transferred to spreadsheets, Microsoft Excel, version 2013. The database was digitized by two independent researchers and compared for data verification and database cleanup. The data were transferred to GraphPad Prism version 4.0 and analyzed using descriptive statistics, in order to generate prevalence estimates with respective confidence intervals.

For the age group, the mean and standard deviation were calculated. In order to evaluate the possible associations between the analyzed variables, a univariate analysis was performed considering the level of significance \( p < 0.05 \) and chi-square test (\( \chi^2 \)). In order to evaluate the associations between the results obtained for HPV detection and the other variables, odds ratios (OR) were calculated with a 95% confidence interval (CI) and significance level of 5%.

The Kaplan–Meier method was used to calculate survival, and the log-rank test was used to compare survival curves against prognostic factors for anal cancer. Death was considered independent of its cause.

The cases included in the study did not present HIV infection status registered in the patient files, and therefore, these data were not used as a prognostic factor.
3. Results

3.1. Sample Characteristics. Sampling included 81 cases of anal cancer. The characteristics of patients with anal cancer are presented in Table 1.

The majority of the patients were female (63%). Age ranged from 36 to 92 years, and the overall mean age at diagnosis was 61.57 years (±12.73); mean age for women was 62.47 years (±13.01) and for men 60.03 years (±12.30).

Lymph node metastases were reported in 25.9% of patients, and inguinal lymph nodes were the most compromised (data not shown). Distant metastases were described in 8.6% of the group, with the liver and lung being the most affected organs. At the end of 60 months following diagnosis, 55.6% of the patients had reported deaths.

Most of the samples (52%) were diagnosed as anal squamous cell carcinoma, followed by adenocarcinomas (39.5%). The majority of SCC and adenocarcinomas were in T1-T2 stages (p < 0.01). The other types of anal cancers were in advanced stages, but without lymph node spread. Both SCCs and adenocarcinomas presented cases with distant metastasis.

3.2. Prevalence of HPV DNA. The prevalence of HPV DNA for the evaluated group was 69.1%. Only 25 patients were negative for HPV DNA. Table 2 presents the prevalence of HPV and its association with the social, demographic, clinical, and pathological characteristics investigated. HPV was more prevalent in women than in men (OR 3.18 95% CI 1.19–8.48). The mean age of the group at diagnosis was similar in HPV-negative (63 years ±11.6) and HPV-positive patients (61 years ±13.2). HPV was significantly associated with anal SCC (OR 9.51 95% CI 2.96–30.50) (Table 2).

3.3. HPV Genotype Distribution. Of all patients with anal cancer positive for HPV DNA, 85.7% had a single HPV-type infection, while 14.3% had multiple HPV types. The genotypic distribution of HPV and the presence of single and multiple infections of all genotyped samples are described and presented in Figure 2. The most prevalent genotypes in squamous cell carcinomas and anal adenocarcinomas were HPVs 16, 18, and 33. In the other types, the most common were HPVs 16 and 33 (Table 3).

3.4. Survival. Overall survival at 60 months for patients with anal cancers was 44.3% (Figure 3). The mean follow-up was 31 months (±59.4) with a minimum of 1 month and a maximum of 191 months. The prognostic factors were being female (Figure 4(a)), squamous cell carcinoma (Figure 4(b)), and the presence of distant metastasis (Figure 4(c)). Survival was not influenced by the presence of HPV (Figure 4(d)), lymphatic dissemination (p = 0.84), or tumor size (p = 0.08); however, all individuals with T3 or larger tumors were deceased after 38 months.

4. Discussion

HPV was present in 69% of anal cancer samples, and HPV 16 was the most prevalent genotype (78.5%), followed by HPV
Although the prevalence of HPV in the anal cancers evaluated was lower than the overall percentage reported in other studies [2, 8, 14, 15, 20–25], it is worth noting that this study included a relatively large number of anal adenocarcinomas (39.5%) and that there are few studies investigating the association of HPV with this
### Table 3: Distribution of HPV genotypes according to histological type of anal carcinoma.

<table>
<thead>
<tr>
<th>HPV</th>
<th>SCC (37/42)</th>
<th>Adenocarcinoma (14/32)</th>
<th>Other carcinomas (5/7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>HPV 16 only</td>
<td>25</td>
<td>67.6</td>
<td>9</td>
</tr>
<tr>
<td>HPV 16 and others</td>
<td>4</td>
<td>10.8</td>
<td>1</td>
</tr>
<tr>
<td>HPV 18 only</td>
<td>2</td>
<td>5.4</td>
<td>1</td>
</tr>
<tr>
<td>HPV 18 and others</td>
<td>0</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>HPV 16 and HPV 18</td>
<td>1</td>
<td>2.7</td>
<td>1</td>
</tr>
<tr>
<td>Others single HPV</td>
<td>5</td>
<td>13.5</td>
<td>2</td>
</tr>
<tr>
<td>HPV negative</td>
<td>5</td>
<td>11.9</td>
<td>18</td>
</tr>
</tbody>
</table>

Other HPV: HPV 6, 11, 33, 35, 39, 52, 56, 58, and 74. Other carcinomas: basaloid carcinoma, neuroendocrine, and cloacogenic.

![Figure 3: Five-year overall survival for patients with anal cancer (Kaplan–Meier method).](image)

![Figure 4: Continued.](image)
histologic type of anal cancer [23–26]. SCC is the histological type most associated with the presence of HPV. In our study, the prevalence of the virus in this histological type was 88%, and HPV 16 was present in 82% of the genotyped samples. The high prevalence of HPV in anal SCC has also been observed in 15 other studies, in which the overall prevalence of HPV ranged from 60.6% to 100% [1, 2, 8, 14–16, 20–28]. These large number of SCC cases positive for HPV suggest that virus infection is a necessary cause for this type of anal cancer, as well as cervical cancer, mainly because the transition zone of the anal canal is very similar to the cervical squamocolumnar junction. Because HPV is a virus that is highly tropic in regions covered by squamous epithelium or high proliferative cell activity, these sites become more vulnerable to viral infection [6, 29].

Unlike SCC, adenocarcinoma of the anus is not intrinsically related to HPV infection. In some studies, this histological type is not analyzed because it is considered an extension of rectal cancer [30]. In this study, the adenocarcinomas included (39.5%) were classified histologically and clinically as primary anal cancers, of which 43.8% were infected by HPV, mainly by high-risk genotypes, such as HPV 16.

Samples from two individuals were classified as low-risk HPV (HPV 6 and HPV 11) during genotyping. These genotypes are responsible for papillomatous lesions and are not considered carcinogenic [29, 31]. It is complicated to define the association of cancer or a precursor lesion with a specific HPV genotype, since the molecular methods used in genotyping generally do not preserve the tissue architecture [22]. Moreover, the PCR method detects the HPV genome in the tumor but does not distinguish whether or not HPV is transcriptionally active, making it impossible to conclude which genotype(s) were actually involved in the carcinogenesis [1, 4, 17].

Multiple infections were observed in 14.3% of patients, a considerably higher value than in the other studies [25, 28, 32, 33]. The genotyping used in this study is based on the technique of reverse hybridization, which in this study identified a larger number of HPV genotypes and the presence of multiple infections. This method uses SPF10 primer oligonucleotides that are able to amplify a broad spectrum of HPV genotypes by virtue of their high sensitivity [2, 13, 32].

As expected, HPV 16 was the most prevalent genotype and was present in 100% of multiple infections. Genotype 16 is considered to be high risk and tends to persist in the host for a longer time [34]. Its oncogenic potential is related to its high expression of the viral oncoproteins E6 and E7, and its ability to integrate the viral genome into host cell DNA [29, 31]. Cell targets of HPV oncoproteins are primarily pRb and p53; however, underlying mechanisms associated with other cellular proteins may occur during carcinogenesis leading to cell cycle progression, evasion of apoptosis, DNA damage, and suppression of the immune response [35].

In the analyzed anal carcinomas, HPV 33 was the second most prevalent genotype (10.7%), followed by HPV 18 (8.92%). The presence of HPVs 16 and 18 is already well established, and both are targets of HPV prophylactic vaccines [13, 36–39]. In our research, only two samples showed both genotypes. The high prevalence of HPV 33 has been found before in other studies, ranging from 2 to 11.9% [20, 24, 25, 27] and suggests that the vaccine might have greater coverage if this genotype were included in the free vaccination program in developing countries such as Brazil, similar to, for example, the introduction of the nonavalent vaccine that includes the genotypes HPVs 16, 18, 31, 33, 45, 52, 58, 6, and 11 [36].

Regarding the analysis by gender, the prevalence of HPV in anal cancers was higher in women (78.4%), as observed in other studies [1, 2, 25, 32, 37]. Persistent long-term HPV
It is unclear whether samples negative for HPV DNA are actually negative or whether these cases have actually been triggered by other carcinogens. At the molecular examination, only five of 42 SCC samples were negative for HPV. The rest of the negative anal cancers were adenocarcinomas or cloacogenic, basaloïd, and neuroendocrine carcinomas.

The lack of some relevant information in the medical records limited the ability to investigate some variables, such as sexual behavior, which is known to increase the risk of HPV infection [14, 43, 47]. Even so, our data adequately represented the reality of individuals with anal cancer in the Midwestern region of Brazil, in the service area of this referral hospital.

The association of HPV to anal cancer has been demonstrated in this study, especially in SCC. However, the presence of HPV did not influence the prognosis of patients with anal cancer. The most prevalent genotypes were HPV 16, 33, and 18. Further research on the role of HPV and its genotypes in anal carcinogenesis needs to be planned, and the prognostic aspects of anal cancer need to be better elucidated. Risk groups considered for anal cancer comprised mostly of HIV-positive individuals and men who have sex with men; however, as shown in this study, women need to be included in new public policies of this group.

Data Availability

The medical records, histopathological reports, and protocols of analysis of the sample data used to support the findings of this study are available from the corresponding author upon request or are included within the article. For any additional information, request should be given to larisse.dalla@gmail.com.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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