State of the art paper

Influence of standardization of human papillomavirus diagnosis in head and neck cancer treatment

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Abstract

The presence of human papillomavirus (HPV) in patients with head and neck squamous cell carcinoma (HNSCC) can guide medical management. The aim of this study was to highlight the importance of HPV diagnosis, verifying which diagnostic techniques are most used in identifying HPV and the differences between these techniques, in the research aimed at establishing a consensus on the gold standard method. We verify that HPV infection is associated with the development of HNSCC. The techniques most commonly used for diagnosis of HPV are immunohistochemistry (IHC), polymerase chain reaction (PCR), reverse transcription polymerase chain reaction (RT-PCR) and in situ hybridization (ISH). Our study concludes that detection of E6/E7 DNA by PCR is the most accepted method of diagnosis. The standardization of an accurate HPV diagnostic method can reduce morbidity and mortality in HNSCC, especially in emerging countries, where few screenings are performed, in addition to improving the social and economic impact of the disease.

Key words: p16, treatment, head and neck cancer, human papillomavirus, diagnosis.

Introduction

Head and neck squamous cell carcinomas (HNSCCs) are a diverse group of tumors classified into anatomical subsets including cancers of the oral cavity, pharynx (oropharynx, hypopharynx, and nasopharynx), larynx, paranasal sinuses, nasal cavity, and salivary glands [1]. Taking all these subsets into account, HNSCC represents the ninth-most common type of cancer worldwide, according to the World Health Organization, with approximately 700 thousand new diagnoses annually [2].

HNSCC is common in Brazil, especially for people aged more than 40 years. The high rates of HNSCC-associated morbidity and mortality in this age group, despite easy and early diagnosis, have emerged as a public health problem [3]. This form of cancer is often associated with tobacco and alcohol consumption, poor oral hygiene, and human papillomavirus (HPV) infection, which, along with alcohol and tobacco use, causes synergism in the carcinogenic action [4].

Cancers from different anatomical sites are known to possess unique epidemiology, anatomy, clinical behavior, and association with HPV in-

fection [5]. The HPV has an affinity for squamous epithelial cells, and these carcinomas, squamous cell carcinoma (SCC) being the most common histological type, represent more than 90% of HNSCC cases [6]. This could be explained by the transformation characteristics of the oral cavity, predominantly in the tonsillar tissue, the appreciable histological similarity of the latter to cervical mucosa, and its ability to immortalize oral keratinocytes and trigger the transformation of epithelial cells [7–10]. Drastic changes in sexual behavior during the 20th century probably led to increased exposure to oral HPV infection and a subsequent increase in the incidence of HPV-positive HNSCC. These factors led to the emergence of HPV as one of the most common cancer-causing pathogens worldwide, which spreads predominantly by sexual contact [11].

Since HPV classification is highly associated with the pathogenicity of HNSCC, it has substantial clinical significance in HNSCC diagnosis, prognosis, and treatment [12]. Therefore, the aim of this study was to highlight the importance of diagnosing HPV in patients with head and neck cancer, in addition to verifying which diagnostic techniques are most used in identifying HPV in HNSCC and the differences between these techniques, in the search for a consensus about the gold standard method and the difference in the progression, prognosis and treatment of this disease according to the diagnosis of HPV.

Material and methods

A systematic search was performed using the MEDLINE (PubMed) and SciELO databases. The keywords "HPV", "Head and neck cancer", "p16", "E6", and "Treatment" were included for the research and articles published in English between January 2015 and January 2021 were included. Studies defining important concepts and addressing topics such as head and neck cancer associated with the human papillomavirus virus, molecular method of virus detection in head and neck carcinoma and treatment were included in this review.

The articles that were included in this review presented the following themes: molecular methods for detecting human papillomavirus in head and neck carcinoma; head and neck cancer associated with human papillomavirus; and treatment. The articles that were excluded presented: treatments in a laboratory study; detection method in a laboratory epidemiological study; another cancer; another type of virus; and xenograft. Based on these criteria, a total of 71 articles were selected for further analysis. From the 71 papers, a total of 58 representative studies were selected as being eligible for the present review.

HPV: general characteristics and oncogenicity

HPV belongs to the *Papillomaviridae* family, formed by a group of small non-enveloped tumor viruses that infect their respective hosts in a species-specific manner. The virus is approximately 55 nm in diameter and its viral genome can be functionally divided into 3 parts: the initial region (*E*) responsible for coding proteins necessary for viral replication and transcription, the late region (*L*) involved in coding the structural capsid proteins (*L1* and *L2*), and a segment of non-coding region called the long control region which contains cis-regulatory elements necessary for the regulation of viral DNA transcription and replication [13].

HPV represents one of the most common sexually transmitted viruses, where the transmission occurs through direct contact with the skin or mucous membranes, and infection is mediated by micro-injuries during intercourse or at other anatomical sites with accessible basal epithelium. The latter provides a permissive environment for initial replication of the viral genome, where new viruses can be synthesized if the target cell is mitotically active [14, 15]. HPV DNA can be produced inside human cells in three ways: via integration into the cellular genome; free in the cell nucleus, that is, episomal; and a combination of both [7]. The integrated viral transcripts are more stable, and are known to have a stronger association with increased proliferative capacity of the affected cells [1]. The virus must reach the basal layer corresponding to the site of trauma in the tissue, to ensure contact of the capsid proteins (L1) with the cell surface [8]. Viral integration leads to loss of expression of the *E1* and *E2* genes, constituting a critical stage for HPV-associated carcinogenesis, since the loss of *E2* leads to the expression of the main viral oncoproteins, E6 and E7 [8, 14]. The E6 and E7 proteins are the major factors contributing to the transformed phenotype and are responsible for the malignancy process, synergistically immortalizing primary cells and overriding the mitotic checkpoint and p53-mediated cell cycle arrest in response to DNA damage [8, 14].

The synthesis of oncoproteins represents the most well-characterized aspect of oncogenic contribution of HPV [1].

The viral life cycle of HPV is intimately linked to the differentiation stage of the infected epithelial cell [13]. In the case of HPV-infected cells, a positive feedback mechanism is believed to be deployed in the infected cells in an attempt to interrupt cell cycle progression, which would lead to increased p16 expression. The p16 protein has been shown to be an important marker of HPV activity, since it is a tumor suppressor protein overexpressed in HPV-associated oncogenesis [16–18].

HPV leads to the formation of certain neoplasms, including cervical, oropharyngeal, anal, vaginal, vulvar, and penile cancer [13, 14, 18, 19]. Cancers of the mouth and oropharynx show an upward trend in incidence [8]. In sinonasal tumorigenesis, HPV infection causes 21% of carcinomas [20, 21]. The former is considered to be the second most common anatomical site associated with carcinoma due to high-risk HPV infection, being transcriptionally active in more than a fifth of these cases [20]. However, the frequency of detection of the virus in the larynx varies between 8 and 60%, raising doubts regarding the non-viral origins of carcinoma of this subsite [22]. Unfortunately, HPV infections in the head and neck have not been studied to the same extent as infections of the genital tract [23].

Most infections of these cancers caused by the HPV are cleared from the body in 1 to 2 years, resulting in an asymptomatic transient oral infection. However, in a small percentage of cases the infection remains in a latent stage for years or decades, preventing immune release for reasons that remain unknown. Therefore, depending on the HPV genotype, a persistent oral infection could culminate in benign or malignant diseases [10, 24].

The α-papilloma viruses 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66 were classified by the World Health Organization (WHO) as carcinogenic in humans [25]. In contrast, low-risk, or non-oncogenic infections of types 6, 11, 40, 42, 43, 44, and 54 have been shown to be associated with the development of genital warts [26]. High-risk HPV-16 was shown to be the predominant type [27]. An example of geographical variation can be observed in Ecuador, where the incidence pattern is different, with 33 and 67 being the most common viral subtypes [28].

This strain generally affected younger age groups, with men predisposed to infection in the absence of traditional risk factors and revealing better prognosis and response to treatment [27, 29, 30], with a reduction of up to 72% in the risk of death [31].

HPV infection and development of HNSCC

HNSCC exhibits enormous heterogeneity since it is associated with one of the highest mutation rates and a high degree of genomic instability. Although it is a form of cancer with high immunological infiltration, some HNSCC subsets are characterized by an immunosuppressive environment, with altered cytokine levels, defects in antigen presentation, dysfunction of T cells, low levels of $CD4^+$ and $CD8^+$ T cells, and increased T-regulatory cells [1, 32].

In HPV-positive cases, in addition to the integration of the virus containing the E6 and E7 oncoproteins, HPV-positive HNSCCs are characterized by shortened or deleted *RAF3*, a gene encoding a protein involved in regulating the immune response; amplification of the *E2F1* gene responsible for encoding one of the transcription factors of the E2F family associated with cell cycle regulation; mutations in *PIK3CA*; and increase in levels of miR-106b and miR-9, the latter being 16 times higher than in normal tissues [29, 33]. These tumors also differ significantly with respect to the methylation pattern of the CpG1 island. The absence of p53 is a hallmark of HPV-positive HNSCCs, since p53 inactivation results mainly from somatic mutations caused by alcohol and tobacco consumption, leading to the expression of inactive p53 protein [34]. Interestingly, HPV-positive oropharyngeal squamous cell carcinoma (OPSCC) appears to be linked to the change in epidermal growth factor (EGF) copy number; therefore, the tumor with no gain in copy number has a favorable prognosis, regardless of the status of HPV [35]. The association with epithelial-mesenchymal transition (EMT) is more complex, pointing to an unfavorable marker of carcinogenic evolution, although the former remains a reliable approach in the prognosis and management of cancer [36].

So, HPV-positive OPSCC pathogenesis is characterized by the presence of at least one copy of the viral genome per tumor cell, interaction of viral oncoproteins with cell cycle regulators and apoptosis inducers, active transcription of viral oncogenes E6 and E7, a genetic pattern without mutations in the *TP53* gene, elevated *p16 INKa*, decreased pRb, low levels of cyclin D1 expression, and less occurrence or complete loss of heterozygosity. The consequences and markers corresponding to these defining characteristics are described in the table below [37] (Table I).

HPV-negative and HPV-positive HNSCCs are molecularly distinct at the genomic, transcriptomic, and methylomic levels [1, 33]. In HPV-negative HNSCCs, 11q13 and 11q22 are coamplified, an event that probably promotes the expression of *BIRC2* and *FADD* genes, which together work to inhibit cell death and, when related to tobacco consumption, induce mutations in *TP53*, inactivation of *CDKN2A*, and frequent changes in gene copy numbers [33]. A critical change for this type of tumor with a poor prognosis, which induces alterations in copy numbers or overexpression, is related to the EGFR. The EGFR is the most overexpressed receptor in HNSCC and is associated with tumor growth, invasion, and metastasis [35]. In addition, these tumors are characterized by the expression of genes that control cell motility, angiogenesis, and the EMT, all resulting in aggressive clinical behavior [20].

Table I. Pathogenesis of HPV-positive OPSCC

Necessary resources		Consequences		Markers
≥ 1 viral genome in each tumor cell	→	Presence of HPV DNA	→	HPV-DNA detection by polymerase chain reaction (PCR) or in situ hybridization (ISH)
Active transcription of E6 and E7 oncogenes	→	Presence of E6 mRNA and E7 mRNA	→	Detection of HPV *E6* and *E7* mRNA by PCR
Interaction of viral oncoproteins with regulatory proteins of the cell cycle	→	Degradation of the *pRb* oncosuppressor	→	Low levels of *pRb* expression by immunohistochemistry (IHC)
		Degradation of the *p53* oncosuppressor	→	Low levels of *p53* expression by IHC with wild-type TP53
		Positive regulation of the *CDNK2a* gene	→	High levels of *p16 INK4a* expression by IHC
		Negative regulation of the *CCND1* gene	→	Low levels of cyclin D1 expression by IHC

However, inactive HPV tumors do not show gene expression, and therefore are somewhat similar to HPV-negative ones, although some differences have been detected [20]. Some cytomorphological changes supporting the presence of neoplasm associated with HPV infection include increased nucleus-cytoplasm ratio, syncytial cytoplasm without intercellular bridges, minimal keratinization, and basaloid appearance. The detection of these changes could help to initiate molecular studies on the presence of HPV with greater precision [8]. Macroscopically, lymph nodes exhibit extensive necrosis and cystic changes, since HPV-positive OPSCC are more prone to cervical lymph node metastasis compared to the HPV-negative ones [35, 36].

HPV and HNSCC: association with tobacco and alcohol consumption

According to Simonato *et al.*, 2016 [38], HPV infection alone is not sufficient for the induction of carcinogenesis, and its oncogenic potential associated with risk factors such as infection with other oncogenic viruses, exposure to sunlight, tobacco consumption, and alcohol dependence must be considered [38, 39]. The consumption of tobacco and alcohol represents a distinct biological and clinical entity that can induce HNSCC through trauma inflicted on extensive areas, which results in field cancerization and subsequent formation of an altered epithelial field, which, in turn, increases the possibility of formation of a new carcinoma [6, 40]. Smoking is known to suppress immune function and facilitate persistent infection, with the magnitude of risk being proportional to the intensity of exposure [40, 41]. A recent analysis indicated that the *CDKN2A* gene, responsible for coding the p16 protein, is often mutated or deleted in smoking-related HNSCCs; as a result, p16 overexpression is prevented, even if HPV is transcriptionally active [42]. HPV-positive HNSCC smoking groups exhibit an increased risk of recurrence of distant metastases, as well as reduced survival, compared to the non-smoking group. For alcoholics, the greatest risk is the development of cancer in the oral cavity and pharynx, which is 5.13 times more likely compared to the non-alcoholic group. Therefore, there is an additive effect of smoking and drinking habits and HPV infection with worse prognosis in HNSCC patients, possibly resulting from DNA breaks induced by these habits, thereby increasing the carcinogenic potential of HPV [43].

Diagnosis of HNSCC and its relationship with HPV infection

Immunohistochemistry (IHC)

Head and neck pathology experts from the College of American Pathologists as well as other professionals met to develop recommendations for all newly diagnosed patients with OPSCC in primary tumors or cervical metastases, although these were not commonly recommended for other HNSCC. The recommendation outlines the use of the p16 immunohistochemistry technique with a 70% true positive rate for nuclear and cytoplasmic staining; thus pathologists should report tumors as HPV-positive or p16-positive [44]. Based on p16 expression and the presence or absence of viral DNA, a model consisting of 3 classes was developed to stratify HNSCC patients. These included class I tumors, those with low levels of p16 and absence of HPV DNA; class II, those with low levels of p16 and presence of viral DNA; and class III, for those showing high p16 expression and viral DNA. Class III tumors show better prognosis compared to other classes [35]. In HNSCC cases, the high expression level of p16 is used as a marker of HPV in-

fection, although it may be derived from other carcinogenic processes, in addition to those induced by the virus, unlike cervical cancer, where the rate of infection is higher, but it is used as a marker of carcinogenic progression [45] (Figure 1).

The detection of p16 by IHC has been widely used due to its reproducibility, ease of handling, availability, and suitability for small samples. However, in the case of HPV, it is a substitute marker, which simply detects distinct oncogenic pathway(s) based on the upregulation of p16 expression [44, 46, 47]. The high expression of p16 in OPSCC demonstrates high sensitivity and a correlation between the infection and resultant transcriptional activity [48]. However, p16 upregulation is not a reliable marker for HPV in anatomical subsites other than the oropharynx, since the true positive rate for p16 staining for these subsites falls below 50%, reflecting the rarity of tumors associated with viruses outside the oropharynx [1, 47]. Still, there is a percentage of OPSCC cancers that test positive for p16 by IHC, but do not harbor active HPV, suggesting that other non-oncogenetic branches of the HPV pathway could lead to increased p16 expression, impairing the specificity of the test [48].

The main detection strategies for HPV in OPSCC include HPV polymerase chain reaction (PCR) for viral oncogenes E6/E7, quantitative reverse transcription PCR (qRT-PCR) for detection of HPV mRNA E6/E7 transcription, and RNA in situ hybridization (ISH) [44, 46]. The PCR-based assay is highly sensitive and could lead to false positive results, whereas the p16 IHC and ISH methods individually result in diagnostic errors in approximately one fifth of the tested cases [31].

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

RT-PCR is considered the gold standard for detecting HPV infection in HNSCCs, either by detecting DNA or mRNA [43, 45, 37], although the suitability of this method is yet to be comprehensively validated. In addition, methods can vary considerably between laboratories, irrespective of its use for detecting HPV nucleic acids, serum antibodies, oncoproteins, or cellular proteins [48]. The consensus to establish a standardized approach remains challenging due to issues of sensitivity/specificity of the methods, the cost/benefit ratio, and the fact that most of the available trials were initially designed for cervical cancer [45, 46, 48]. Detecting HPV DNA in human tissues is easier and more sensitive than other methods due to the exponential amplification in DNA sequences that the method offers [38]. It is important to note that the detection of HPV DNA is not sufficient to identify an OPSCC associated with HPV, as the presence of its DNA may reflect a transient infection or one unrelated to the oncogenic process and also does not mean that the virus is transcriptionally active, a fact that needs to be considered when categorizing tumors associated with HPV [45, 49, 50]. In contrast, since the viral proteins E6 and E7 contribute to malignant transformation of cells, HPV infection is considered oncogenic only when the viral mRNA E6/E7 is detected. Therefore, the detection of E6/E7 transcripts could be considered as the gold standard for detecting HPV infection, since the former reflects active viral transcription [50, 51].

In situ hybridization (ISH)

The ISH approach based on mRNAs is highly sensitive and shows higher specificity compared to the p16-based method for detecting HPV. This allows direct visualization of viral transcript molecules in tissue sections, confirming the presence of the transcriptionally active virus and reducing the percentage of false positive results. A comparative analysis of the sensitivity of the ISH DNA-based assay with the mRNA-based one revealed

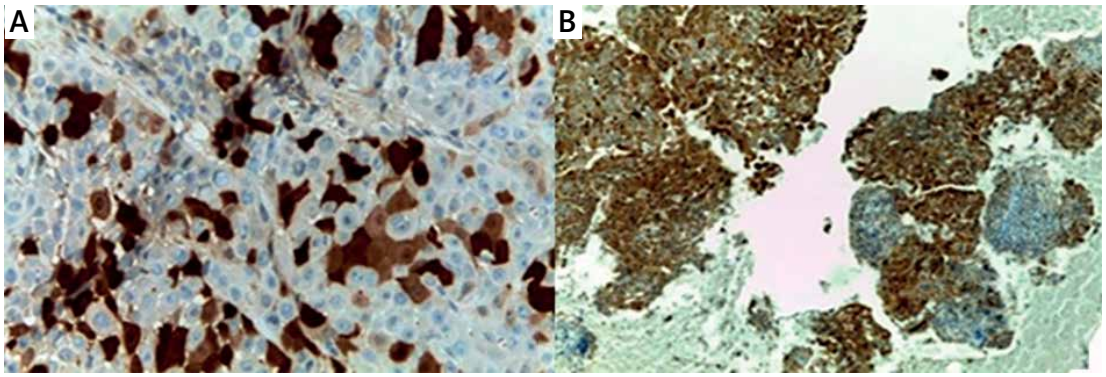

Figure 1. Immunohistochemical staining for detection of p16 protein. Brown stained cell is signal of p16 protein. **A** – Magnification 40×. **B** – Magnification 10×. p16 antibody clone [E6H4] from Ventana/Roche Company. Source: Personal archive (photos taken at the Pathological Anatomy Service of Base Hospital)

the higher sensitivity of the latter. Variations of this technique have also been reported. An example is the RNAscope, which is designed to detect mRNA in high-risk HPV tumor cells using a colorimetric method and complements diagnostic algorithms, accurately identifying these tumors and efficiently distinguishing between active and inactive ones [20, 48]. This test is the first diagnostic tool that has not been developed for cervical oncology and transferred to HNSCC. This approach has significant advantages, including direct visualization in formalin-fixed and paraffin-embedded tissue samples, minimal risk of contamination, and relevant clinical correlations with a single test; however, it is expensive, has limited automation capabilities, and requires a highly skilled technical workforce in the case of the manual procedure [45] (Figure 2).

Another variation of this technique was described by Augustin *et al*., 2018 [52], where the emerging RNA chromogenic in situ hybridization technique was employed as an independent prognostic marker in p16-positive OPSCC and helped guide therapy.

Our review analyzed the methods considered reliable by other researchers or those preferred by them. Only the standard tests have been described. Although the p16 IHC assay was reported in several studies, it has been omitted from our analysis since it is considered as a substitute diagnostic method (Table II) [53–57].

The general objective of HPV detection goes beyond classifying the presence or absence of the

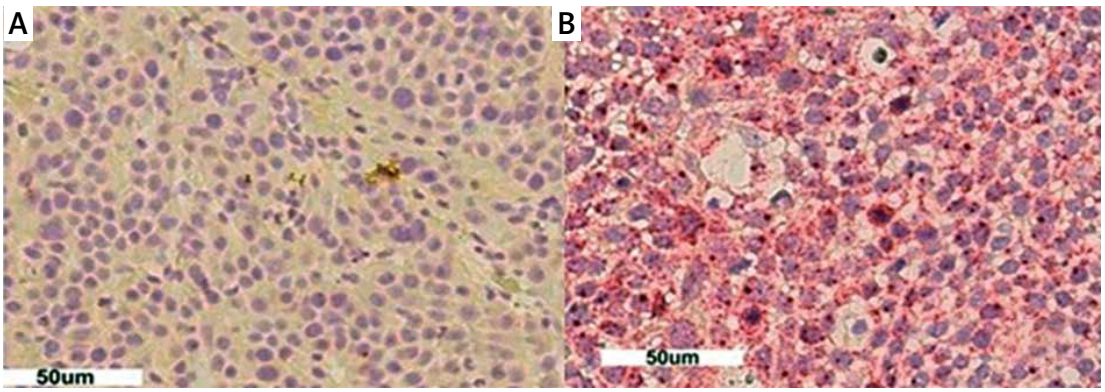

Figure 2. In situ hybridization technique (ISH) for staining E6/E7 mRNA. **A** – mRNA ISH assay negative for E6/E7 mRNA, **B** – mRNA ISH assay positive or control. Source: Ilardi G *et al*., 2018 [20]

Table II. Methods preferred by researchers to determine the gold standard method of detection

mRNA					
PCR			RT-PCR		ISH
E6	E6/E7	E7	E6/E7	E7	E6/E7
Boscolo-Rizzo P *et al*., 2016 [37]	Linge A *et al*., 2018 [15]	Mena M *et al*., 2018 [49] Yang D *et al*., 2016 [22]	Bussu F *et al*., 2019 [45] Delgado Ramos GM *et al*., 2018 [28] Minami K *et al*., 2017 [34] Schroeder L *et al*., 2017 [43] Contreras *et al*., 2015 [8]	Sannigrahi MK *et al*., 2017 [54] Erira A *et al*., 2016 [7]	Randén-Brady R *et al*., 2019 [48] Yakin M *et al*., 2019 [51] Ilardi G *et al*., 2018 [20] Channir HI *et al*., 2018 [53] Bhosale PG *et al*., 2016 [50]
DNA					
PCR			RT-PCR		ISH
E6/E7			E6/E7		E6/E7
Quijano Gutiérrez R *et al*., 2018 [55] Petito G *et al*., 2017 [6] Broglie MA *et al.,* 2017 [31] Bhosale PG *et al*., 2016 [50] Beck TN *et al*., 2016 [1] Simonato L *et al*., 2016 [38] Blumberg J *et al*., 2015 [56]			Miller ED *et al*., 2017 [21] Schroeder L *et al*., 2017 [43] Lefevre M *et al*., 2017 [36] Hong A *et al*., 2015 [57] Contreras W *et al.,* 2015 [7]		Augustin J *et al*., 2018 [52] Quijano Gutiérrez R *et al*., 2018 [55] Beck *et al*., 2016 [1]

virus, and encompasses discrimination between transient, transcriptionally silent, and biological infection relevant in the context of carcinogenesis. In addition to detecting hidden primary site carcinomas, a reliable method of HPV detection should include stratification of patients for scale reduction in treatment, customization of postoperative surveillance strategies, and early detection of primary SCCs and recurrences [37]. Dong *et al*., 2021 [58] also reviewed diagnostic methods, considering advanced methods of detection through antibodies and HPV protein antigens, and innovative treatments.

Regardless of the types of methods reviewed, our studies agree that a study is urgently needed to develop the best molecular typing method.

HPV infection and the response to HNSCC treatment

The specifics of treatment of HNSCC vary depending on the anatomical location and stage of the disease. In the initial stage, treatment includes surgery and subsequent radiotherapy (RT) if positive margins of the surgery are detected; the advanced stage consists of surgery followed by radiation or chemoradiotherapy. In cases of metastasis or recurrent forms of the disease that are not amenable to a local approach, chemotherapy is performed with or without a biological agent [1, 39]. The relative 5-year survival rate is 70–80% for HPV-positive HNSCC [1].

As there is no defined therapy, there is great interest in a therapy with low toxicity [21] for the treatment of HNSCC associated with HPV infection, in which there are different treatment results. However, radiotherapy is preferred in patients with HPV infection, as it results in a smaller functional impact and a better response, seeming to be related to impairments in DNA repair [15, 41]. Clinical trials have been started to verify whether patients with HPV-positive OPSCC could benefit from reducing the radiation dose to mitigate adverse effects [15].

Discussion

HPV infection is associated with the development of head and neck cancer and differs macroscopically from the molecular one, and may also have synergistic effects with the use of alcohol and tobacco. Diagnosis of HPV in patients with head and neck cancer is performed through tests that confirm the presence of the virus. The main diagnostic techniques are immunohistochemistry (IHC), polymerase chain reaction (PCR) and polymerase chain reaction reverse transcription (RT-PCR) and in situ hybridization (ISH).

The IHC technique detects the virus from the distinct oncogenic pathway based on p16 protein upregulation, but it is recommended only for primary tumors of oropharyngeal cancer; the PCR and RT-PCR techniques perform detection through exponential amplification of DNA sequences. The ISH technique is a method that allows direct visualization of viral transcript molecules in tissues, confirming the presence of the active virus. Knowing the status of HPV in HNSCC guides medical management, since HPV-positive HNSCCs present greater tumor aggressiveness and a better therapeutic response. The medical staff has not yet established an accurate technique for this type of diagnosis. Our study concludes that detection of E6/E7 DNA by PCR is the most accepted method, analyzing issues of sensitivity/specificity and cost versus benefit. The standardization of an accurate HPV diagnostic method can reduce morbidity and mortality in head and neck cancer, especially in emerging countries, where few screenings are performed, in addition to improving the social and economic impact of the disease.

Conflict of interest

The authors declare no conflict of interest.

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