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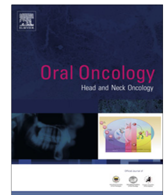

Review

Human papilloma virus testing in oropharyngeal squamous cell carcinoma: What the clinician should know

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SUMMARY

High risk Human Papilloma virus (HR-HPV) associated oropharyngeal cancers are on the increase. Although, the scientific community is aware of the importance of Human Papilloma Virus (HPV) testing, there is no consensus on the assays that are required to reliably identify HR-HPV related tumors. A wide range of methods have been developed. The most widely used techniques include viral DNA detection, with polymerase chain reaction (PCR) or In Situ Hybridization, and p16 detected by immunohistochemistry. However, these tests provide different information and have their own specific limitations. In this review, we summarize these different techniques, in light of the recent literature. p16 Overexpression, which is an indirect marker of HPV infection, is considered by many head and neck oncologists to be the most important marker for patient stratification. We describe the frequent lack of concordance of this marker with other assays and the possible reasons for this. The latest developments in HPV testing are also reported, such as the RNAscope™ HPV test, and how they fit into the existing framework of techniques. HPV testing must not be considered in isolation, as there are important interactions with other parameters, such as tobacco exposure. This is an important and rapidly evolving field and is likely to become pivotal to staging and choice of treatment of oropharyngeal carcinoma in the future.

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Introduction

The role of high risk human papilloma virus (HR-HPV) in the pathogenesis of head and neck squamous cell carcinoma (HNSCC) was first suspected in 1983 when histo-pathological features, consistent with HPV infection, were identified in oral cancers [1]. Since then, strong evidence has confirmed this hypothesis and in 2009 the International Agency for Research on Cancer recognized HPV16 as a causal agent in a subset of oropharyngeal squamous cell carcinomas (OPSCC) [2].

Between 18% and 72% of OPSCC are related to HR-HPV and particularly to the HPV16 genotype [3]. Their incidence is increasing, whereas the incidence of cancers induced by tobacco and alcohol is stabilizing or falling, in Western countries, because of a drop in consumption [4–6]. Furthermore, it has recently been suggested that HR-HPV-related OPSCC should outnumber uterine cervical cancers in the next 15–20 years, in the United States of America (USA) [4]. If this hypothesis proves correct, then HPV-related OPSCC may be a major public health concern. These tumors are biologically distinct from those related to the traditional risk factors. Most studies suggest that patients affected with HR-HPV-related OPSCC have a better prognosis and some argue that a specific therapeutic approach is needed [7–9]. Indeed, traditional OPSCC therapies involve high doses of toxic radiation/chemotherapy, which may prove unnecessary for HPV-positive OPSCC. Therefore de-escalation of established treatment is currently being assessed by several clinical trials [10]. The demonstration of HPV in a tumor may have critical consequences in the near future.

In this context, an increasing number of expert working groups (the National Comprehensive Cancer Network and the College of American Pathologists for example) have recommended routine HPV screening in all OPSCC [10,11]. It has also been suggested that HPV status should be considered for inclusion into the official staging system of OPSCC [12]. However, there is no consensus

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regarding the type of tests that are required to reliably identify HR-HPV-related tumors.

As HPV cannot be cultured in vitro and serological assays are still ineffective, a wide range of methods have been developed that vary in their concept, target, performance and above all provide different information. Strategies vary considerably, reflecting laboratory facilities, resources and investigators' personal preferences.

Until a consensus is reached, physicians should be familiar with the most commonly used assays that we have summarized in this report. Our goal is to increase awareness of the strengths and limitations of these techniques.

HR-HPV DNA detection with polymerase chain reaction (PCR)

Amplification of target DNA sequences by PCR followed by hybridization with dedicated probes is the most widely used tool in the detection and genotyping of HPV [3,7,13–15]. These methods are easy to perform, highly sensitive, widely available, and cost-effective.

Several PCR screening assays are commercially available with CE-marking (Table 1). Most of them use consensus primer sets that are designed to bind to highly conserved regions (Fig. 1), allowing simultaneous identification of a large range of HPV types, whereas others target a single HPV genotype by amplifying a type-specific DNA sequence.

Although PCR-based methods are reproducible and highly effective for the detection of HPV with the ability to detect less than one viral genome copy per cell [15–20], there is strong evidence that these assays differ in their performance. These discrepancies are related to several factors which include: the choice of primer sets, PCR protocols, and the type of tissue analyzed (fresh frozen or paraffin-embedded formalin-fixed tissue (FFPE)).

Few studies have been performed to compare the effectiveness of the most commonly used primer sets in detecting HPV in OPSCC, and available data comes mainly from uterine cervical cancer studies. In general, the sensitivity of DNA detection by PCR is inversely related to the size of the amplimer [17]. Remmerbach et al. [18] and Chaiwongkot et al. [19] have compared the MY09/11 and GP5+/6+ primer sets and have concluded that GP5+/6+ was more sensitive, especially in low viral load samples. Kleter et al. [17] have assessed GP5+/6+ and a former version of SPF10 primers in a follow up study of women previously treated for cervical dysplasia. A total of 160 out of 534 (30%) cervical smear samples were found to be positive for the SPF-PCR, which was significantly higher than the 113 (21%) positive samples detected by GP5+/6+ PCR. Similarly, when combining the data from various studies, Snijders et al. [20] concluded that SPF10 had the highest analytical sensitivity. Additional gains in sensitivity have been sought by using nested PCR [16,19,21]. This method involves an initial amplification step followed by a second amplification of the initial PCR products. For specificity, the second amplification is carried out using primers that sit internal to the first primer pair.

PCR amplification is more efficient on frozen tissue. Several authors reported difficulties in reproducing the results obtained in frozen tissue when testing FFPE samples [13–16]. The fixation process leads to DNA fragmentation [13–16] into sequences that are often shorter than 200 base pairs [22]. Therefore, it is preferable to target short DNA sequences in FFPE samples as this results in higher sensitivity. Consequently, SPF10 and GP5+/GP6+ primer sets are more frequently used with FFPE.

However, the main problem with PCR-based methods is the interpretation of results. Indeed, these methods are extremely sensitive and therefore analytic (laboratory) sensitivity should be distinguished from clinical relevance as proposed by several authors [20]. Despite the use of stringent procedures and proper controls (5), previously amplified material can potentially contaminate negative specimens. Moreover, it is not possible to determine if viral DNA arises from the population of cancer cells, or the surrounding non-neoplastic tissue (not clinically relevant) in HPV-positive samples unless laser-assisted microdissection is performed which is technically cumbersome and not used routinely. Indeed, analyses of healthy oral and oropharyngeal mucosa have shown HPV infection in at least 5–14% of cases [23,24], but rates exceeding 50% have also been reported [25–27]. Another potential problem with the PCR-based method is related to the lack of tissue context and the possibility of testing a tumor-free sample, resulting in a false-negative result.

In contrast to cervical cancers, which are virtually all related to HR-HPV [28], only a subset of OPSCC are HR-HPV induced. Consequently, the question is not only whether HR-HPV is present in the tumor, but also if the virus is implicated in the initiation and maintenance of the cancer phenotype. Indeed, previous analyses have reported that 14–50% of HPV DNA-positive OPSCC are negative for E6/7 mRNA expression [13,29,30], the gold standard for clinically relevant HPV infection [13,31,32].

Viral load (VL) quantification, with HPV-type-specific Real time PCR, may help to answer this question. Jung et al. [32] and Jordan et al. [29] have measured the VL of HNSCC that tested positive for HPV16-DNA. Both have noted that only tumors with a high-VL express E6/E7 mRNA. Cohen et al. [33] and Mellin et al. [34] have reported that among HPV-positive OPSCC only those with a high-VL have an improved clinical outcome relative to HPV-negative OPSCC. These observations cast doubt on the oncogenic role of HPV16 in low-VL tumors and highlight that the amount of HPV in a sample is likely to become important in distinguishing clinically-relevant HPV infections [31–35]. To date, there is no critical threshold of HPV-VL that has been determined to cause HPV related tumors. Most of the protocols and calibration ranges vary between laboratories and are for research purposes.

Detection of viral transcripts

The final goal of any HPV detection strategy, in OPSCC, lies in its ability to recognize the presence of HPV and above all its implications in oncogenesis.

E6 and E7 viral oncogenes, by inhibiting TP53 and pRb respectively, play a key role in the abrogation of cell cycle control, apoptosis and promotion of genetic instability that contributes to the development of cancer [36,37]. In vitro studies have demonstrated that their expression induce keratinocyte immortalisation and that their inhibition in HPV-induced cancer cell lines results in the loss of the transformed phenotype [38,39].

Several authors have demonstrated that tumors containing transcriptionally active HPV (E6/E7 mRNA) represent a specific subgroup. These tumors are characterized by the absence of TP53 mutation [40–42], a significantly decreased number of chromosomal abnormalities [43–45], and a specific gene expression profile [46,47] when compared to HPV-negative and transcriptionally inactive HPV-positive OPSCC (those tumors that contain HPV DNA but do not express viral oncogenes). Additionally, survival analyses have shown that, among HPV-positive OPSCC, only transcriptionally active tumors have significantly better survival [32]. Transcriptionally inactive and HPV-negative tumors have poorer survival.

These data strongly support the concept that HPV is implicated in tumorgenesis only when viral oncogenes are expressed. In other words, among HPV-positive OPSCC only those expressing E6 and E7 behave differently from tobacco/alcohol-related OPSCC. Consequently, RT-PCR measurement of E6/E7 mRNA, in high quality fresh frozen material, is considered the gold standard in assessing if the virus is etiologically involved [13,31,32,40,48–50]. Such

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