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Review:

Detection of human papillomavirus in oropharyngeal squamous cell carcinoma*

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Abstract: Worldwide there has been a significant increase in the incidence of oropharyngeal squamous cell carcinoma (OPSCC) etiologically attributed to oncogenic human papillomavirus (HPV). Reliable and accurate identification and detection tools are important as the incidence of HPV-related cancer is on the rise. Several HPV detection methods for OPSCC have been developed and each has its own advantages and disadvantages in regard to sensitivity, specificity, and technical difficulty. This review summarizes our current knowledge of molecular methods for detecting HPV in OPSCC, including HPV DNA/RNA polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP), p16 immunohistochemistry (IHC), and DNA/RNA in situ hybridization (ISH) assays. This summary may facilitate the selection of a suitable method for detecting HPV infection, and therefore may help in the early diagnosis of HPV-related carcinoma to reduce its mortality, incidence, and morbidity.

Key words: Human papillomavirus (HPV); Molecular detection; Oropharyngeal squamous cell carcinoma (OPSCC)
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1 Introduction

Oropharyngeal squamous cell carcinoma (OPSCC) originates from the non-keratinizing stratified mucosal epithelium in the upper aerodigestive tract (Suciu et al., 2014). OPSCC is caused primarily by exposure to tobacco and alcohol, yet there is a significant increase in the incidence of OPSCC worldwide, etiologically attributed to oncogenic human papillomavirus (HPV). The relationship between HPV and head and neck cancer has been discussed by many researchers reporting the presence of HPV DNA in head and neck cancer patients and in healthy oral mucosa. The proportion of HPV-related OPSCC worldwide ranges from 32% to 73%. This variation is due to several factors such as sexual habits, geographic differences, and variation in the molecular techniques used to define HPV in OPSCC (Lin et al., 2011; Oji and Chukwuneke, 2012; Mehanna et al., 2013; Gupta and Johnson, 2014).

Based on its carcinogenic potential, HPV can be classified into high-risk and low-risk types. Low-risk types such as HPV6, 11, 42, 43, and 44 usually result in benign lesions, while high-risk types such as HPV16, 18, 31, 33, 35, 45, 51, 52, 56, 58, 59, 68, 73, and 82 can cause malignant tumors. The diameter of the virus is about 52–55 nm. It is characterized as non-enveloped

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double-stranded DNA and consists of 8000 bp-long circular DNA molecules (Muñoz et al., 2003; Gupta and Gupta, 2015). The genome consists of six "early" regions (E1, E2, E4, E5, E6, and E7), which specifically encode viral DNA replication proteins and transformation, and two "late" regions (L1 and L2) that encode structural proteins of the virus (Zheng and Baker, 2006). Four messenger RNA (mRNA) isoforms of the E6 protein have been detected in epithelial cells infected by HPV16. These isoforms, E6*I, FLE6, E6*II, and E6*X, are evidence that other viral proteins could have additional important functions. Two other mRNA isoforms have been observed in cells infected by HPV18 (Graham, 2010). Among the listed genes, *E6* and *E7* genes are responsible for severe infection leading to cancer (de Villiers et al., 2004). *E6* targets tumor protein 53 (p53), and *E7* targets retinoblastoma protein (pRb) tumor suppressors. Changes in apoptosis, DNA repair mechanisms, and cell cycle control due to earlier alteration eventually lead to overexpression of tumor suppressor gene (*p16*) (Babiker et al., 2013).

Several HPV detection methods for OPSCC samples have been developed at the DNA, mRNA, and protein levels. Each technique has its own strengths and limitations. Based on a recent review article, p16 immunohistochemistry (IHC), polymerase chain reaction (PCR), DNA in situ hybridization (ISH), and the loop-mediated isothermal amplification (LAMP) assay are the techniques most commonly used for HPV testing in OPSCC (Qureishi et al., 2017). It is important to have a reliable and accurate identification and detection method for HPV in OPSCC as it is relevant to prognosis and treatment strategies for patients (Bhargava et al., 2010). Information on the HPV status of patients will aid clinicians in patient management. The objective of this review was to summarize current knowledge on the molecular detection methods of HPV in OPSCC. The mechanism, strengths, limitations, sensitivity, and specificity of each method are summarized in Table 1.

2 Molecular detection assays

2.1 PCR

During recent decades, there have been huge advances in nucleic acid diagnostic tests. Molecular tests are preferred because of their rapidity, high sensitivity and specificity. They are able to pick up low copy numbers or low concentrations of sequences, and specificity is provided through a probe that is designed specifically to complement the target gene (Sigma-Aldrich, 2018). These assays have been used extensively to detect the presence of HPV in a broad range of clinical samples, such as oral rinse, plasma, fine needle aspirates, fresh tissue, and formalin-fixed paraffin-embedded (FFPE) tissue (Zarei et al., 2007). PCR is a selective target amplification assay able to increase the HPV sequences present in biological specimens exponentially and reproducibly. Theoretically, after 30 amplification cycles, the amplification process can produce one billion copies of a single double-stranded DNA molecule. The sensitivity and specificity of PCR-based methods can vary, depending on the DNA extraction procedures, site and type of clinical sample, sample transportation and storage, primer set, and performance of the DNA polymerase used in the reaction.

To improve sensitivity and specificity, the one-step PCR has been modified. Nested PCR is a combined PCR assay that uses a combination of two consensus primers. For example, MY09/11, $GP5^+$/$GP6^+$, PGMY09/11, and SPF10 LiPA primers target consensus sequences of variable lengths (different primer sets target sequences of different lengths) within the HPV *L1* gene (Mirghani et al., 2014). In nested PCR, two primer sets are used in two rounds of PCR amplification. The first PCR amplification yields a larger amplicon size (464 bp), while in the second PCR amplification, the amplicon size is reduced to 155 bp. This short target increases the specificity of the assay and can reduce false-negative results caused by short DNA fragments originating from fixation-induced DNA degradation in FFPE samples, for example. FFPE samples are easily degraded due to extensive cross-linking of proteins to DNA. Thus, to overcome this problem, the use of a primer that yields a small amplicon will increase the chances of it being detected (Candotto et al., 2017). In a parallel study by Jalouli et al. (2015), nested PCR showed an increase in HPV detection in FFPE samples in terms of positivity rate, efficiency rate, and sensitivity compared to a single PCR assay. Multiplex PCR allows simultaneous detection in one amplification tube. Simultaneous detection could minimize the use of reagents

Table 1 Summary of HPV detection methods for OPSCC

Method	Mechanism	Strength	Weakness	Sensitivity (%)	Specificity (%)
HPV DNA PCR	Use Taq DNA polymerase and specific primers in a standard PCR reaction to amplify the target DNA fragment in the sample	Highly sensitive and specific	Unable to distinguish "clinically significant" HPV infections (i.e., HPV transcriptional active infection in tumor cells with expression of *E6* and *E7* genes)	94–100	74–92
qPCR	The amount of the nucleic acid present in the sample is quantified using: (1) fluorescent dyes that non-specifically intercalate with double-stranded DNA, and (2) sequence-specific DNA probes consisting of fluorescently labelled reports (Abreu et al., 2012)	Able to detect transcriptionally active HPV; providing an estimation of HPV viral load; highly sensitive and specific; no post-PCR processing	Laborious; instrument is too costly; multiplexing is still limited	>95	100
Droplet digital PCR	Based on water-oil emulsion droplet technology for measuring the absolute copy number of nucleic acid targets without external standards	Highly specific and sensitive; requiring minimal amounts of nucleic acids for detection; providing absolute target quantification without reference to the standard/calibration curve; able to detect rare mutations and quantify gene mutations; technically feasible; lower sensitivity to PCR inhibitors; more precise and reproducible data compared to qPCR; cost-effective	Need further research	100	100
LAMP	Rely on auto-cycling strand displacement DNA synthesis *Bst* DNA polymerase enzyme with high strand displacement activity and a set of two specially designed inner and outer primers (Notomi et al., 2000)	Highly specific and sensitive; cost-effective; rapid; robust	False-positive results in subsequent reactions	99	>90
p16 IHC	Involve alteration of *p16* mRNA expression resulting from *p16* gene methylation of the promoter region. Methylation results in cascade events of pRb and E2F followed by *p16* upregulation, which is highly associated and is unique for HPV-positive oropharyngeal carcinoma (Lewis et al., 2012)	Highly sensitive; cost-effective; widely available	Lack of specificity (false positive results); time-consuming; result interpretation requires a pathologist for confirmation; different $p16^{INK4a}$ cut-offs to detect HPV-transformed OPSCC	91–97	78–88
DNA ISH	Labeled probes hybridize to target HPV DNA sequences in the tumor cell nucleus, and the probes may be either HPV-specific or cocktail-specific to detect different high-risk HPV types simultaneously	Highly specific; cost-effective; allowing a reliable identification of viral physical state	Limited sensitivity (false negative results are particularly likely with low HPV copy numbers); signal interpretation can be subjective due to lack of clean, clear staining signals; time-consuming; requiring a large amount of purified DNA; uncertain performance in detection of less common high-risk HPV subtypes	76–92	78–96
HPV *E6*/*E7* mRNA ISH	Involve reverse transcription of mRNA into cDNA and subsequent amplification of target DNA sequences of interest using PCR	Highly specific and sensitive; allowing detection of transcriptionally active HPV	Require dedicated equipment and workflow; cost-prohibitive for some clinical laboratories; require technical expertise that is not routinely available in pathology laboratories; interpretation is somewhat subjective based on amplification curve analysis	>93	>90

cDNA: complementary DNA; E2F: E2 factor; HPV: human papillomavirus; IHC: immunohistochemistry; ISH: in situ hybridization; LAMP: loop-mediated isothermal amplification; OPSCC: oropharyngeal squamous cell carcinoma; PCR: polymerase chain reaction; qPCR: quantitative PCR; pRb: retinoblastoma protein

and reduce errors compared to a single-plex assay. Wasserman et al. (2017) showed that multiplex PCR was able to detect a high prevalence (79%) of high-risk HPV in HPV-positive saliva samples.

Overall, PCR offers high sensitivity (>95%) and specificity (>85%) in HPV detection in OPSCC (Schache et al., 2011; Tawe et al., 2018). PCR is one of the simplest and most direct methods to use. The PCR amplicons also can be used for downstream techniques like sequencing and cloning. However, the assay has a few drawbacks. It can easily be affected by contamination of the sample by even trace amounts of DNA, which can produce misleading results. In addition, PCR amplification requires post-amplification analysis either by gel electrophoresis, restriction-fragment length polymorphism (RFLP), direct sequencing or hybridization with type-specific oligonucleotide probes using various hybridization formats such as dot blot, Southern blot, microtiter enzyme-linked immunosorbent assay (ELISA) plate, reverse line blot strip assays, and microchip format assays (Coser et al., 2011). PCR machines are quite expensive and PCR is incapable of distinguishing transcriptionally active or inactive HPV in HPV-positive samples (Schache et al., 2013).

2.2 Quantitative PCR

Quantitative PCR (qPCR) provides an assessment of HPV detection and quantification of viral load (Coser et al., 2011). A tumor with a high viral load could be a favorable prognostic factor. If the viral load is higher than the median value, it may indicate that there is virus replication in the tumor cells (Mellin et al., 2002). This assay uses the accumulation of a fluorescent reporter molecule to monitor the PCR progression and DNA concentration. It offers a shorter time and fewer manual steps than conventional PCR due to the elimination of post-amplification detection procedures. qPCR is more reproducible, rapid, and applicable to clinical samples than other PCR-based assays (Wang et al., 2014). In addition, qPCR offers a sensitive and specific detection and quantification of HPV subtypes in a wide range of samples including fresh tissue, frozen tissue, FFPE tissue, and cellular samples. Several researchers have demonstrated the presence of HPV DNA in $p16^{INK4a}$-positive salivary oral rinse samples using qPCR with high sensitivity (>90%) and specificity (100%) (Ritari et al., 2012; Chai et al., 2015; Zile-Rubab et al., 2018). The improved sensitivity and specificity reported in their studies could be due to the use of strain-specific primers instead of primers that target the conserved *L1* open reading frame (ORF) which contains degenerate primer sequences that may lower sensitivity (Fontaine et al., 2007; Chai et al., 2015). Ahn et al. (2014) reported a sensitivity of 52.8% for saliva, 67.3% for plasma, and 76.1% when using combined saliva and plasma samples, for detection of HPV16 *E6*/*E7* DNA using qPCR in pre-treatment patients. Dang et al. (2015) demonstrated that 33 of 100 cancer patients were positive for any type of HPV in oral rinse samples using qPCR.

qPCR can determine how much of a specific DNA sequence or gene is present in the sample. It allows for both detection and quantification of the viral load in real time, while it is being synthesized (Arney and Bennett, 2010). The two common methods used to detect and quantify the product include fluorescent dyes that non-specifically intercalate with double-stranded DNA and sequence-specific DNA probes consisting of fluorescently-labelled reports (Abreu et al., 2012). The drawbacks of this method are that the detection probe is expensive, and although post-amplification analysis is not required, the real-time machine is very expensive (Duncan et al., 2013).

2.3 Quantitative reverse-transcription PCR

HPV *E6*/*E7* mRNA detection by quantitative reverse-transcription PCR (RT-qPCR) is highly correlated with improved patient survival in OPSCC and is considered to be the "gold standard" test for classifying an OPSCC as being positive for transcriptionally active high-risk HPV (Mirghani et al., 2014; Bishop et al., 2015). These transcripts are associated with cellular genotoxic damage and gene expression changes that cause cancer (Robinson et al., 2012). Several studies have reported the detection of HPV *E6*/*E7* mRNA in OPSCC using this technique. Deng et al. (2013) demonstrated that HPV *E6*/*E7* transcripts were detected in 27.8% (15/54) of HPV-positive tumor samples, while Holzinger et al. (2012) detected HPV *E6*/*E7* transcripts in 50% of OPSCC tumor samples. HPV *E6*/*E7* mRNA PCR is less favorable for routine screening as it is technically demanding,

needing fresh-frozen tissue which is fragile and is of limited availability (Yu et al., 2012). The RNA isolation process for RT-qPCR is laborious as it requires additional sample preparation steps and more tumor cells than HPV ISH and p16 IHC (Mirghani et al., 2014).

2.4 Droplet digital PCR

Droplet digital PCR (ddPCR) is the latest technique for the detection of oncogenic HPV. It is currently the most accurate, rapid, and sensitive method to quantify HPV viral load in OPSCC (Biron et al., 2016; Albano et al., 2017; Antonsson et al., 2018; Stevenson et al., 2020). ddPCR involves partitioning a single nucleic acid sample into up to 20000 discrete water-in-oil droplets and performing PCR analysis on each droplet independently, using custom-designed HPV16 *L1* or *E6*-specific primers and probe sets, with the results reported digitally and quantitatively. This technique offers better precision, accuracy, and reproducibility than RT-qPCR. It also allows quantification of the absolute amount of target present in samples, while dealing with competing targets and DNA degradation. It can be used to detect rare mutations, to quantify gene expression even with a low copy number, and can be multiplexed for higher efficiency. ddPCR quantification indicated that a higher viral load correlates with improved survival in HPV-positive oropharyngeal tumors (Stevenson et al., 2020; Veyer et al., 2020).

Several studies have made use of ddPCR for detection of oncogenic HPV *E6*/*E7* mRNA in OPSCC (Isaac et al., 2017; Stevenson et al., 2020; Veyer et al., 2020). HPV *E6*/*E7* mRNA has been detected in fresh tissues of OPSCC and oropharyngeal swab specimens, and was found to have 100% and 98% sensitivity, respectively, compared to p16 IHC using target RNA 20%–50% lower concentration than reported for RT-qPCR. Because of its high sensitivity and specificity, this technique is suitable for assessing the HPV16 viral load in more sample types, including oral/oropharyngeal swabs as opposed to fresh tissue, compared to assessments based on the commonly used IHC p16 marker (Carcopino et al., 2012). Researchers found that ddPCR analysis of oropharyngeal swabs is a quantitative, rapid, and cost-effective tool for minimally invasive oncogenic detection of HPV (Isaac et al., 2017). In the available literature, it is the most sensitive and reliable tool for detection of HPV in OPSCC without a tissue biopsy, and has many potential applications for both diagnosis and disease control (Ahn et al., 2014). A schematic diagram of PCR-based assays including conventional PCR, qPCR, RT-qPCR, and ddPCR for detection of HPV in OPSCC is shown in Fig. 1.

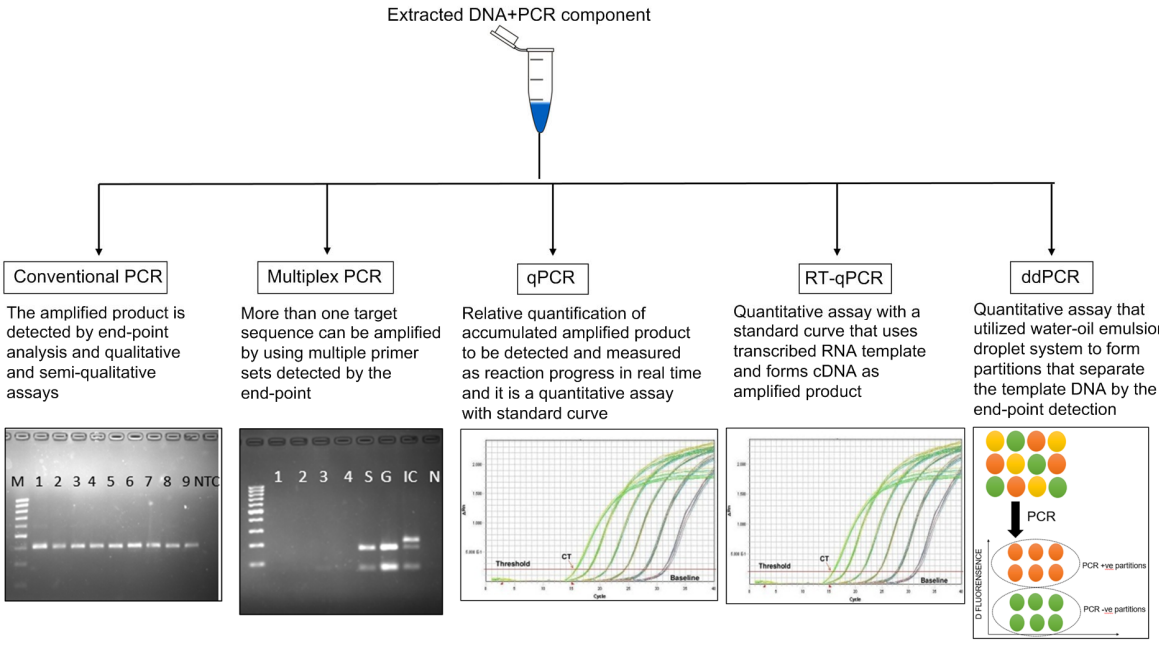

Fig. 1 A schematic diagram of PCR-based amplification assays for detection of HPV

HPV: human papillomavirus; PCR: polymerase chain reaction; qPCR: quantitative PCR; RT-qPCR: quantitative reverse-transcription PCR; ddPCR: droplet digital PCR

2.5 Loop-mediated isothermal amplification assay

LAMP is a technique that applies isothermal conditions for amplification of the target DNA. LAMP was introduced 20 years ago (Notomi et al., 2000) and since then has been used extensively in nucleic acid research and in clinical applications as a screening tool. It is a single tube technique that amplifies a few copies of DNA into a billion copies within an hour. Since its establishment, the LAMP assay has proved to be a simple, time-efficient, and cost-effective method for the detection of high-risk HPV. LAMP applies four to six primers specially designed to recognize six to eight distinct regions of a target gene, hence its high efficiency and precision. The use of four compulsory primers which are a forward primer (F3), backward primer (B3), forward inner primer (FIP), and a backward inner primer (BIP) increases specificity. An additional loop primer can increase the amplification speed and efficiency, thereby reducing the reaction time of the original LAMP reaction (Mori et al., 2013). The LAMP amplification technique is very simple and straightforward to perform. The amplification can be completed within an hour using simple and inexpensive equipment, such as a water bath and heating block, which is available in most laboratories (Dhama et al., 2014). A semi-skilled person provided with comprehensive protocols can effectively perform the assay (Abdullahi et al., 2015). In addition, LAMP is more sensitive than PCR, with a 10- to 100-fold higher sensitivity and a detection limit of 0.01–10.00 plaque forming units (PFUs) of virus (Parida et al., 2008). One of the unique features of LAMP is that this technique has an ability to amplify genes in a poorly processed or non-processed sample, eliminating the DNA extraction step. *Bst* polymerase enzyme has high resistance to some PCR inhibitors that are often present in saliva and blood. This enzyme also helps to eliminate the need for extensive sample purification (Hamzan et al., 2018).

The amplified target can be visualized using the naked eye because of the large amount of magnesium pyrophosphate by-product produced during the reaction, and can be followed by agarose gel electrophoresis to confirm the result (Mori et al., 2001). The presence or absence of the target DNA can be clearly observed by color changes, and later determined by its optical density using a spectrophotometer at 650 nm. Naked eye detection is extremely simple, inexpensive, and reliable, and can prevent cross-contamination between samples. In addition, real-time monitoring of LAMP can be accomplished through spectrophotometric analysis using a real-time turbidity meter. A real-time turbidity meter was first developed and commercialized by Mori et al. (2004). This machine is capable of maintaining the LAMP reaction solutions in commercially available 0.2-mL PCR tubes at the optimum temperature and continuously measuring the turbidity of multiple samples (Notomi et al., 2015). The highly efficient and sensitive LAMP assay has some drawbacks. For example, it can cause false-positive results in subsequent reactions due to a small amount of aerosolized amplification product. Fig. 2 shows the LAMP process for detection of HPV in OPSCC.

Several LAMP assays have been reported for the detection of HPV in OPSCC (Chen et al., 2015; Livingstone et al., 2016; Rohatensky et al., 2018). Livingstone et al. (2016) reported LAMP sensitivity of 99.4% and specificity of 93.2%, which were highly comparable to those of PCR for the detection and subtyping of clinical OPSCC samples. A recent study showed that the LAMP reaction was able to detect viral DNA down to a copy number of 10^5 for HPV16, 10^3 for HPV18, 10^4 for HPV31, and 10^5 for HPV35, with 100% specificity and without DNA purification (Rohatensky et al., 2018).

3 Detection using hybridization techniques

3.1 DNA in situ hybridization

ISH is another widely used technique to detect the presence of HPV, particularly in fixed tumor tissue samples. This technique uses an antisense probe to bind to a complementary HPV DNA target on tumor cells. The presence of HPV DNA in ISH during the hybridization process is evaluated using a microscope to search for punctate dot-like hybridization signals within the nuclei of epithelial cells (Snijders et al., 2010). Determination of the integration status of HPV DNA by ISH has been a routine practice because of its cost effectiveness and feasibility. DNA ISH allows a reliable identification of the physical state of the virus. The appearance of HPV DNA either in integrated or episomal form can be differentiated through the appearance of punctuate or diffuse signals (Anneroth

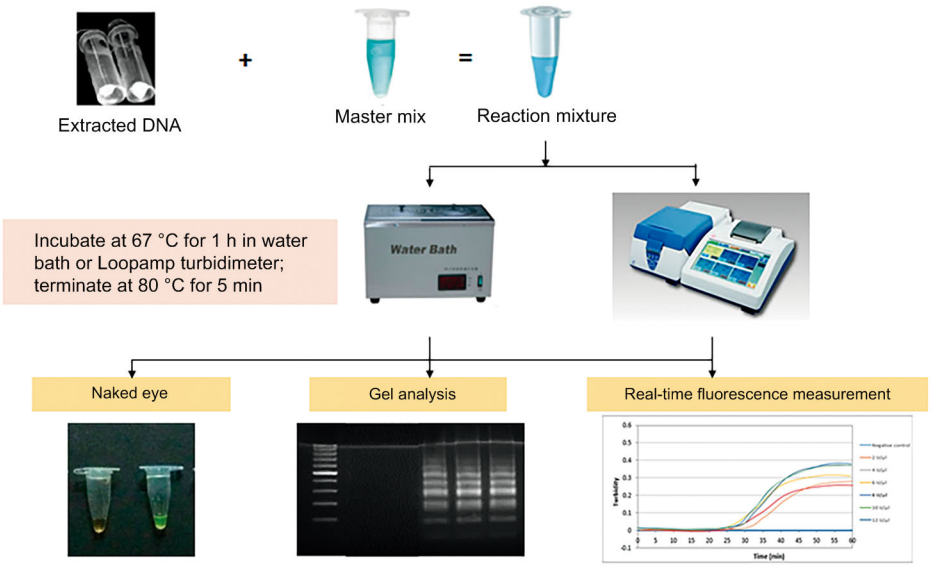

Fig. 2 A schematic diagram of the LAMP process for detection of HPV in OPSCC
LAMP: loop-mediated isothermal amplification; HPV: human papillomavirus; OPSCC: oropharyngeal squamous cell carcinoma

et al., 1987). Generally, sample tissues are treated for hybridization using histochemistry to fix the target transcripts in place and to increase probe access. The probe hybridizes to the target sequence at an elevated temperature and then the excess probe is washed away (in the case of an unhybridized excess RNA probe, following previous hydrolysis using RNase). The probe, which is labeled with either radio-, fluorescent-, or antigen-labeled bases (e.g., digoxigenin), is then identified and quantified in the tissue using either autoradiography, fluorescence microscopy, or IHC, respectively. ISH can also use two or more radioactively labeled probes or other non-radioactive labels to detect two or more transcripts simultaneously.

The effectiveness of this assay is limited by its low sensitivity at low viral loads. The differentiation of HPV DNA by its appearance is often difficult to interpret and can be time-consuming. This can happen because of unusual staining characteristics or when the samples are small, such as small core biopsies and cell blocks which need extra interpretation time. Subsequently, these problems could present a false-positive result, especially if the observation is done under low magnification. It was suggested to examine the DNA ISH slides at high magnification to avoid missing weak or focal staining. Automated DNA ISH could be used to reduce the time consumption of manual DNA ISH. Another drawback of this procedure is that it is relatively insensitive. The sensitivity was once reported to be 63.6% for DNA ISH, compared to 81.8% in p16 IHC (Khor et al., 2013). Nevertheless, the specificity is high. DNA ISH also requires relatively large amounts of purified DNA. A low DNA copy number could cause weak staining in a DNA ISH assay (Bray et al., 2018).

3.2 RNA in situ hybridization

RNA ISH uses probes that are complementary to *E6*/*E7* mRNA as the gold standard for HPV detection in tumor tissue (Schache et al., 2011). At present, HPV mRNA ISH has a great advantage compared with DNA ISH because of its ability to detect the presence of transcriptionally active HPV, which indicates the existence of HPV-related oncogenesis. RNA ISH was demonstrated to have higher sensitivity than DNA ISH (Bishop et al., 2012; Schache et al., 2013; Mirghani et al., 2015). It correlated more strongly with p16 immunostaining, produced results with strong signals, and was easily interpreted due to a brighter and more diffuse signal than that generally seen with the DNA ISH assay. Another study reported its sensitivity as 97% and specificity as 93%, and proved that this assay could untangle the HPV status in 88% of negative DNA ISH cases. RNA ISH allows direct visualization of viral transcripts from a processed tissue section. The improvement of viral detection due to natural target amplification by viral mRNA transcription has also been identified. Standardization can

be enhanced by the use of an automated staining platform that can reduce turnaround time and enhance reproducibility (Saetiew et al., 2011).

RNA ISH could serve as a reliable standalone test to clarify the presence of HPV due to a strong association with the HPV-associated OPSCC biomarker, the p16 protein (Bishop et al., 2012). ISH for high-risk HPV *E6*/*E7* mRNA is a highly specific, highly sensitive tool for HPV detection in OPSCC. Because of HPV-independent mechanisms, the p16 protein may be overexpressed. Therefore, all p16 IHC-positive OPSCCs should be considered for retesting using mRNA ISH to check transcriptionally active HPVs. This is particularly relevant when considering de-escalated treatment strategies for patients with HPV-positive tumors and providing favorable results for this subgroup of patients (Randén-Brady et al., 2019). The disadvantages of RNA ISH are that the probe is expensive and is not readily available because it requires extensive space, infrastructure, and equipment, and the process requires more technical expertise (Garibyan and Avashia, 2013). PCR requires nucleic acid extraction from FFPE samples and more specific techniques to proceed for fragmented or ruptured RNA after FFPE sample processing. The use of frozen material is technically demanding and labour-intensive for a routine process (Mirghani et al., 2013).

4 p16 immunohistochemistry

Overexpression of p16 is accepted as a surrogate diagnostic biomarker of transcriptionally active oncogenic HPV infection in OPSCC (Ang et al., 2010). HPV oncoproteins (E6 and E7) are responsible for causing genetic alterations in HPV+OPSCC (Akagi et al., 2014). E6 contains zinc-binding motifs, which form complexes with the host cell p53 tumor suppressor protein that causes p53 degradation (Hewitt et al., 2014). Degradation of p53 leads to a decrease of p21, a downstream target in the pathway. Since p53 functions in regulating cell growth and tumor suppression, the loss of p53 results in deregulation of the cell cycle and promotes mutation, chromosomal instability, and carcinogenesis of the host genome. On the other hand, E7 oncoprotein forms complexes with proteins in the pRb gene family, which are negative regulators of cell growth. *pRb1* regulates cell cycle progression by binding and disrupting the function of transcription factors, such as E2 factor (E2F) (Suresh, 2016). Hypo-phosphorylated forms of pRb are thought to block cell cycle progression. HPV E7 binds to hypo-phosphorylated forms of pRb and releases free E2F transcription factors and others, such as p16, into the cell, which consequently leads to transcriptional activation of several genes involved in cell proliferation. The release of these transcription factors causes a cascade of events in the cell as part of its progression through the cell cycle (Singhi and Westra, 2010). Downstream upregulation of p16 is seen when pRb is made ineffective by E7. Once HPV is integrated, E6 and E7 work in concert to effectively transform epithelial cells and this action enables the progression to cancer. The degradation of pRb automatically enhances expression of $p16^{INK4a}$, which can be a hallmark marker for HPV-related oncogenic activity and malignant transformation in OPSCC (Weiss et al., 2012). Fig. 3 shows the mechanisms of E6 and E7 proteins involved in the development of HPV-associated cancers.

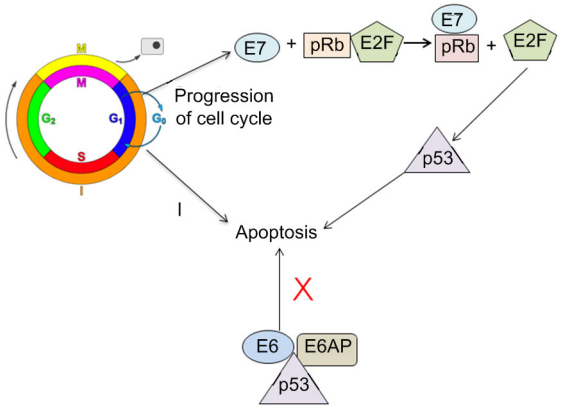

Fig. 3 Mechanisms of HPV E6 and E7 proteins in the development of HPV-associated cancers
E7 and E6 react with the tumor suppressor gene products pRb and p53 in host cell proteins, respectively, resulting in carcinogenesis. E2F: E2 factor; E6AP: E6-associated protein; HPV: human papillomavirus

Detection of the $p16^{INK4a}$ surrogate marker by p16 IHC is commonly used as a standalone test for the diagnosis of OPSCC. IHC typically detects antigens in tumor tissue sections by immunological and chemical reactions. Several studies have reported a strong correlation between p16 overexpression and HPV-associated OPSCC and prognosis (Schache et al., 2011; Lewis et al., 2012). Another study reported that the presence

of HPV DNA in tumors correlated well with the expression of $p16^{INK4a}$ (κ=0.80; 95% confidence interval (CI), 0.73–0.87) (Ang et al., 2010). A pooled analysis of p16 data reported a sensitivity of 94%, specificity of 83%, and positive predictive value of 93% for high-risk HPV *E6*/*E7* expression (Goot-Heah et al., 2012).

The low cost and feasibility of p16 testing on FFPE tissue have made p16 IHC the most favored test compared to ISH and PCR-based assays (Lewis et al., 2012). This method demonstrated very good agreement with the gold standard HPV *E6*/*E7* mRNA expression (Kim et al., 2018). However, it is moderately specific and could lead to a false-positive result, since p16 overexpression that represents the loss of pRb could also occur through mechanisms other than oncogenic HPV *E7* expression (Fischer et al., 2010). Few studies have reported that HPV DNA is not present in 8% to 20% of p16-positive OPSCCs (Rietbergen et al., 2014; Mirghani et al., 2015). Thus, they have recommended that HPV status in all p16-positive cases should be confirmed by another method (Mirghani et al., 2016; Volpi et al., 2018; Randén-Brady et al., 2019). Thus, using p16 IHC as a standalone test for HPV detection can mislead diagnostic and treatment approaches, particularly when considering de-escalation (Rietbergen et al., 2014). Furthermore, p16 requires additional cost and could lead to diagnostic lags in health care systems with limited pathologists, because of the need for additional human resources (Hewitt et al., 2014).

5 Diagnostic algorithms: why is detection of HPV in OPSCC important?

OPSCC containing transcriptionally active high-risk HPV in its tumor cells (HPV-positive OPSCCs) is classified as a distinct clinical entity according to the recent World Health Organization (WHO) classification of head and neck tumors (El-Naggar et al., 2017). Its risk factors, demographic, morphological, molecular, and clinical profiles differ substantially from other types of head and neck squamous cell carcinomas (HNSCCs) (Lewis et al., 2018). Apart from the prevailing evidence of HPV16 as a causative factor in more than 90% of cases, this subset of patients is less likely to contain smokers and alcohol users, and more likely to be associated with oral sex as a major risk factor. They are typically younger (50–56 years old) white males of higher economic standing (Gillison et al., 2008). Histopathological results of these cases commonly show non-keratinizing squamous cell carcinoma as opposed to HPV-negative tumors, with a site predilection for tongue and palatine tonsils.

Prognosis wise, a higher three-year overall survival rate in HPV-positive OPSCCs (82.4% vs. 52.7% in HPV-negative lesions) and rates of progression-free survival (73.7% vs. 43.4%) were reported. There is 58% reduction in risk of death in the HPV-positive OPSCC group, regardless of whether these patients were treated with concurrent systemic therapy (cisplatin) combined with standard-fractionation radiotherapy or accelerated-fractionation radiotherapy. Furthermore, the cumulative incidence of second primary tumors among patients with HPV-positive tumors was significantly lower. They concluded that the HPV status of the tumor was the major determinant of overall survival rate and its higher rate reflected increased intrinsic sensitivity to radiation or better radiosensitization (Ang et al., 2010). Current guidelines by the National Comprehensive Cancer Network® (NCCN, 2020) on diagnostic workup for cancer of the oropharynx involving the base of the tongue, tonsil, posterior pharyngeal wall, and soft palate include testing newly diagnosed OPSCC patients for high-risk HPV, either from the primary tumor or from cervical nodal metastases, using p16 IHC with a 70% nuclear and cytoplasmic staining cutoff (Lewis et al., 2018; NCCN, 2020).

The incorporation of more than one testing method may further increase the sensitivity of detection results (NCCN, 2020). A combination of two diagnostic algorithms commonly used was p16 IHC as the first-line assay, followed by HPV DNA/RNA PCR or HPV DNA ISH. In an algorithm described by Chai et al. (2016), PCR was able to confirm the presence of 92.9% HPV16 DNA and 60.0% HPV16 RNA using oral fluid samples in $p16^{INK4a}$-positive tumors. This study indicated that $p16^{INK4a}$ positivity in a tumor is strongly associated with HPV16 infection. Schache et al. (2011) applied the combination of p16 IHC with DNA and RNA qPCR, which demonstrated high sensitivity (97%) and specificity (94%) of DNA qPCR when compared to the RNA qPCR. In predicting tumor p16 positivity using saliva, HPV RNA PCR has

a lower sensitivity compared to HPV DNA PCR. There are two possible reasons for these results: these may be due to the unstable nature of RNA or no *E6*/*E7* mRNA expression in patients with HPV DNA tumors. Another study compared the detection of HPV DNA in p16-positive samples in which qPCR successfully detected 54% HPV DNA, while ISH failed to detect any HPV DNA in p16-positive samples. This study suggested that p16 immunoreactivity and HPV genotyping by qPCR may be useful markers of HPV infection in OPSCC (Kouketsu et al., 2016).

Rooper et al. (2016) described a two-step technique comprising p16 IHC, followed by HPV DNA ISH and HPV RNA ISH, using tumor tissue. RNA ISH has been reported to be a highly sensitive and specific platform capable of clarifying the status of OPSCC tumor tissue HPV that is p16-positive for IHC, but HPV-negative for DNA ISH. Mirghani et al. (2011) demonstrated a false-negative result through a combination of p16 and DNA ISH where the overexpression of p16 was related to a non-viral mechanism. Later, they suggested using RNA ISH as a potential algorithm in line with p16 because of its high sensitivity (Mirghani et al., 2015). Lewis et al. (2012) successfully identified 94% of additional HPV-positive cases in samples that were negatively amplified using ISH assays, but p16-positive in IHC assays. Rietbergen et al. (2014) reported that detection of HPV by PCR in addition to p16 is crucial for a better identification of HPV-related OPSCC. Combined testing of $p16^{INK4a}$ IHC and HPV DNA PCR significantly improves specificity, while retaining high sensitivity. According to their studies, the pooled sensitivities of $p16^{INK4a}$ IHC, HPV DNA PCR, HPV DNA ISH, and $p16^{INK4a}$ IHC/HPV DNA PCR combined testing were 94% (95% CI, 91%–97%), 98% (95% CI, 94%–100%), 85% (95% CI, 76%–92%), and 93% (95% CI, 87%–97%), respectively. The pooled specificities were 83% (95% CI, 78%–88%), 84% (95% CI, 74%–92%), 88% (95% CI, 78%–96%), and 96% (95% CI, 89%–100%), respectively. The combined testing of $p16^{INK4a}$ IHC/HPV DNA PCR showed a sensitivity similar to either $p16^{INK4a}$ IHC or HPV DNA PCR alone, but was significantly more specific than either separate test (Prigge et al., 2017). Marino et al. (2020) recently established a novel multiplex HPV RNA ISH/p16 IHC assay to detect both HPV E6/E7 transcripts and $p16^{INK4a}$ overexpression simultaneously. There are advantages from combining various detection assays to achieve an accurate and reliable HPV status; however, it is technically inconvenient, may produce discordant results, and requires more cost and time. Fig. 4 shows an algorithm for the detection of HPV in FFPE tissue from head and neck biopsies.

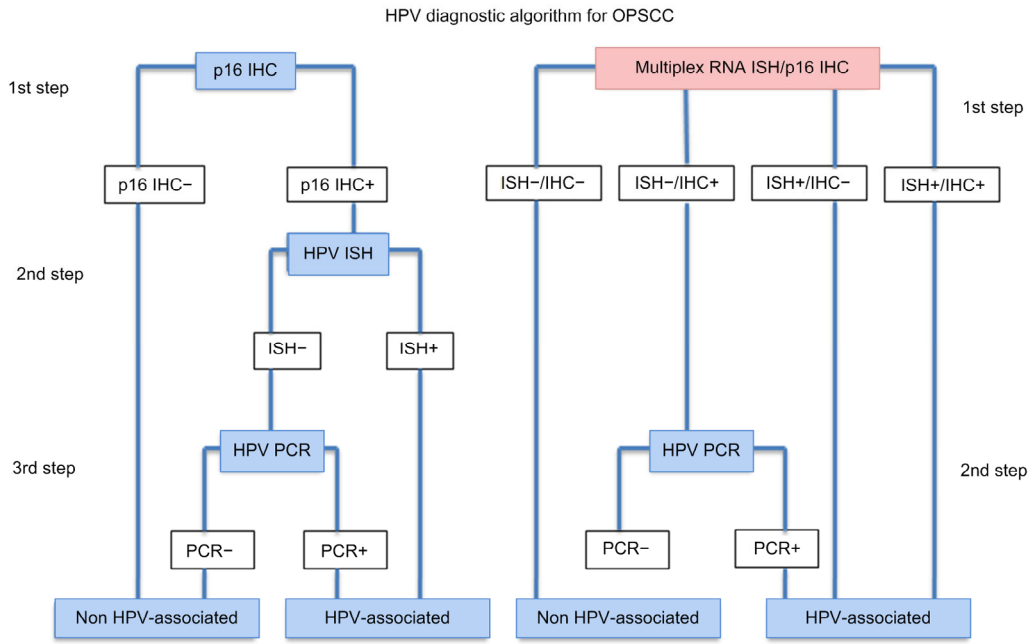

Fig. 4 HPV diagnostic algorithm for OPSCC

Reprinted from Marino et al. (2020), with permission from Springer Nature via Copyright Clearance Center's RightsLink service. HPV: human papillomavirus; IHC: immunohistochemistry; ISH: in situ hybridization; OPSCC: oropharyngeal squamous cell carcinoma; PCR: polymerase chain reaction

According to the NCCN (2020), p16 IHC staining and PCR-based assays are highly reliable in terms of sensitivity, while ISH has the highest specificity. Thus, they recommended integration of several testing methods including both PCR (higher sensitivity, lower specificity) and ISH (less sensitivity, higher specificity) for an equivocal p16 or uncertain clinical scenario (Singhi and Westra, 2010; Snow and Laudadio, 2010; Lewis et al., 2018; NCCN, 2020). Despite significant heterogeneity in patient populations, sample size, HPV detection methods, tumor stage and treatment, comorbidity, and the inclusion of various other prognostic factors in the analysis, the survival benefit of HPV-positive OPSCC has been maintained across almost all studies. For HPV-positive tumors, significant decreases in the risk of progression and disease-related death were confirmed in large prospective studies where OPSCC patients were uniformly staged and treated (Lewis et al., 2018).

6 Conclusions

The HPV status of a primary or metastatic OPSCC may have consequences for treatment, staging, and even for therapy. The recommendation for regular HPV testing currently reflects its role as an important prognostic predictor for OPSCC patients. The choice of a suitable method for HPV detection has become increasingly complex. Detection can be accomplished either by PCR, qPCR, LAMP, IHC, ISH, or a combination of these methods. Despite the availability of various techniques, molecular tests have always been the gold standard and are the most commonly used. Detecting the presence of HPV oncogene *E6*/*E7* mRNA transcripts is regarded as the gold standard, and p16 as a surrogate biomarker in clinical settings. However, each of the listed methods has its pros and cons. To choose the best techniques for detection, it is important to consider the type of specimen that will be used and the availability of equipment and skilled personnel. Strong financial resources are needed as some of the reagents and probes are very expensive. The guidelines for the identification of HPV in cervical carcinoma are widely available, but no specific consensus on the gold standard for HPV testing in OPSCC has yet been reached. None of the developed assays seems to have both very high specificity and sensitivity. Therefore, multimodal testing that integrates two HPV detection methods could help to reliably identify patients with transcriptionally active high-risk HPV-positive OPSCC and to avoid the possibility of false-positive and false-negative cases (Marino et al., 2020). The use of highly sensitive, specific, and accurate methods is critical, especially when considering de-escalation treatment approaches for HPV-positive OPSCC patients.

Contributors

Fatin Hazwani FAUZI and Nurul Izzati HAMZAN analyzed the literature and prepared the first draft of the manuscript. Nurhayu Ab RAHMAN and Siti SURAIYA edited and checked the final version. Suharni MOHAMAD designed, revised, edited, and checked the final version. All authors have read and approved the final manuscript and, therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

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Compliance with ethics guidelines

Fatin Hazwani FAUZI, Nurul Izzati HAMZAN, Nurhayu Ab RAHMAN, Siti SURAIYA, and Suharni MOHAMAD declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题　目：口咽鳞状细胞癌组织中人乳头瘤病毒的检测

概　要：在世界范围内，人乳头瘤病毒（HPV）的致癌性

导致口咽鳞状细胞癌（OPSCC）的发病率显著增加。随着 HPV 相关癌症发病率的上升，寻找可靠且准确的识别和检测 HPV 的工具变得非常重要。目前，虽然在 OPSCC 组织中检测 HPV 的方法已有多种，但是在灵敏度、特异性和技术难度等方面各有优缺点。本文综述了近年来应用于 OPSCC 组织中检测 HPV 的方法，包括 HPV DNA/RNA 聚合酶链反应（PCR）、环介导等温扩增（LAMP）、肿瘤蛋白 16（p16）免疫组化（IHC）和 DNA/RNA 原位杂交分析（ISH），对选择合适的 HPV 检测方法具有指导意义，从而有助于 HPV 相关癌症的早期诊断，降低其死亡率和发病率。

关键词：人乳头瘤病毒（HPV）；分子检测；口咽鳞状细胞癌（OPSCC）