DETECTION OF HUMAN PAPILLOMA VIRUS IN HEAD AND NECK SQUAMOUS CELL CARCINOMAS: A LITERATURE REVIEW

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DETECTION OF HUMAN PAPILLOMA VIRUS IN HEAD AND NECK CANCERS: A LITERATURE REVIEW (Abstract): Human papilloma viruses (HPV) are the most common sexually transmitted viruses. There is mounting evidence that incriminates HPV as a risk factor for malignant transformation of oropharyngeal epithelium. In 2011 the International Research Agency of Cancer and National Cancer Institute (USA) declared HPV-16 as an independent risk factor for oropharyngeal squamous cell carcinoma (OPSCC). Leaders in the field of HPV research admit that this subtype of head and neck cancer is a sexually transmitted entity and its global incidence is on the rise. In the 1980s, clinicians observed a new group of patients with head and neck squamous cell carcinoma (HNSCC) independent of tobacco smoking or alcohol use. The new HNSCC patient is a middle-aged man, non-smoker, non-drinker with higher social status and the suspected risk factors for HNSCC being related to sexual practices (oral sex, multiple sexual partners, unprotected sex and drug use). Routine HPV testing of HNSCC patients is seriously considered as HPV-positive oropharyngeal cancers comprise a distinct molecular, clinical and pathologic entity that has a markedly better prognosis than HPV-negative oropharyngeal cancers. The current treatment protocols for OPSCC include radiation, chemo-therapy and surgery alone or in combination, involving high toxicity levels. Future therapeutic concepts for OPSCC may be personalized in relation to HPV-status to avoid unnecessary toxicity. The current review summarizes the contemporary trends in the diagnosis of HPV-related head and neck cancers, presenting the advantages and disadvantages of the main methods. Keywords: HEAD AND NECK CANCER, HPV TESTING, BIOMARKERS

Papillomaviridae (reclassified in 2004) are a family of small double-stranded DNA viruses that infect warm-blooded vertebrates (1). Papillomaviruses infect different species of mammals and also distantly related hosts such as marsupials and birds (2). Traditionally HPVs has been classified in “types” based on phylogenetic analyses of the L1 gene, which is the most conserved gene in PVs. Based on their tropism the HPVs have also been grouped into mucosal or cutaneous types. Mucosal HPV types designated as “high-risk” (HR) types, isolated preferentially from malignant tumors are: 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and 70. The “low-risk” (LR) types (HPV 6, 11, 42, 43 and 44) are found in benign genital warts and other non-malignant lesions (1, 2). The causative role of HPV in cervical
cancer is well established. Approximately 70% of cervical cancers worldwide occur due to HR-HPV types 16/18 infection (3). The exact molecular mechanism responsible for malignant transformation is still unknown. The HPV oncogenic proteins E6 and E7 are incriminated in this process. Persistent infection with HR-HPV is a necessary but not sufficient cause of this cancer, which develops over a long period through precursor lesions. It may pass as much as 20 years between the initial HPV infection and clinical occurrence of cancer (4). In women with normal cervical cytology findings the estimated global HPV prevalence is 11.7% and the most common types are HPV-16 (3.2%), HPV-18 (1.4%), HPV-52 (0.9%), HPV-31 (0.8%) and HPV-58 (0.7%) (5).

Romania has the highest incidence of cancer in Europe, 23.9/100,000. There are 4,343 new cervical cancer cases each year and 1,909 cervical cancer deaths. HPV prevalence among women with or without cervical lesions in the Northeastern region of Romania is 37.4% and the most frequent types are: 16 (10.5%), 53 (5.44%), 51 (5.05%), 52 (4.08%), 18 (2.91%) and 31 (2.73%) (6, 7).

**HPV INVOLVEMENT IN HEAD AND NECK CANCER**

The involvement of HPV in head and neck cancers was suggested for the first time in 1983 by Syrjanen et al. (8). The numerous studies dedicated to test this theory supported HPV involvement on the basis of: morphological similarities between genital and oropharyngeal epithelia, broad epithelial tropism of HPV, detection of HR-HPV genotypes in samples of oropharyngeal squamous cell carcinoma (OP-SCC), and the HPV-induced immortalization of human oral keratinocytes *in vitro*. In 2011 the International Research Agency of Cancer and National Cancer Institute (USA) declared HPV-16 as an independent risk factor for oropharyngeal squamous cell carcinoma (OPSCC) (9). Experts in the field of HPV research admit that this subset of head and neck cancers is a sexually transmitted entity with an increasing global incidence. A growing body of evidence is supporting that HPV is responsible for approximately 20% of oral cancers and 60-80% of them are squamous cell carcinomas (10, 11).

In the 1980s, clinicians observed a new group of patients with HNSCC independent of tobacco smoking or alcohol use (12). The new HNSCC patient was a middle-age man, non-smoker, non-drinker with higher social status and the suspected risk factors were related to sexual practices (oral sex, multiple sexual partners, unprotected sex and drug use) (13-16).

HNSCC is the sixth most common malignant tumor worldwide with approximately 650,000 new cases diagnosed each year. While the reported incidence rate for all head and neck carcinomas is decreasing and the rates of coincident identification of oncogenic HPV as a new risk factor for OPSCC is increasing, it is obvious that a virus-related cancer epidemic is discussed (16-18). The involvement of oncogenic HPV types was clearly demonstrated only in cancers arising from the oropharynx (especially palatine and lingual tonsils). Romania is lacking HPV-related OPSCC studies. HR-HPV can be found in the oropharynx of healthy subjects and it is very important to differentiate between high-risk and low-risk HPV-types. Indeed, oral infection with HR-HPV types increases the odds ratio for developing oropharyngeal
cancer. In a recent systemic review of the literature the pooled prevalence of oral HPV in healthy individuals was found to be 4.5% with 3.5% of them having oncogenic HPV types (19). However, in the tonsils of healthy people the incidence of oncogenic HPV was only 1% (20).

Very often these tumors are diagnosed at an advanced stage, with an enormous impact on 5-year survival rate (80% for early-stage disease and only 20% for late-stage disease). In contrast to skin cancer screening where visual examination has high sensitivity (93%) and specificity (98%) the detection of oral cancers and premalignant lesions by conventional visual and tactile examination is problematic due to the complex anatomy of the oropharynx. Therefore, adjunctive screening tests are desperately needed (21).

**MAJOR DIFFERENCES BETWEEN HNSCC BY HPV STATUS**

HPV-positive oropharyngeal cancers comprise a distinct molecular, clinical and pathologic disease entity that has a markedly improved prognosis compared to HPV-negative oropharyngeal cancers. In HPV-negative OPSCC genetic alterations lead to mutations in p53 and retinoblastoma (pRb) tumor suppressor genes and also loss of heterozygosity. In contrast, HPV-positive cancers harbor the wild type of p53 gene, p16 is upregulated and pRb is downregulated (22, 23). Many researchers are looking for a predictive transcriptomic signature of oropharyngeal cancer according to HPV status in order to develop new reliable biomarkers for screening and early detection of OPSCC (24). Also, HPV-positive OPSCCs are strongly infiltrated by lymphocytes, are predominantly non-keratinizing squamous carcinomas and are described as poorly differentiated or basaloid carcinomas. The contrary facts are available for HPV-negative OPSCC (25). Also, the clinical response to therapy and overall survival are improved (a 28-58% reduction in the risk of death) for HPV-positive compared to HPV-negative cancers (26).

**DIAGNOSIS OF HPV-POSITIVE HNSCC**

At the moment, there is no consensus on the optimal algorithm to identify HPV-positive HNSCCs (Table I). Common practices include the detection of p16 protein through immunohistochemistry as well as HPV genetic material. Accurate HPV detection and typing is based on nucleic probe technology as HPVs cannot be cultured (27). At present HPV detection tests are included in three large categories: nucleic acid-hybridization, signal amplification and nucleic-acids amplification assays. In this review we will not address HPV serology tests (inconsistent seroconversion rates) and immunohistochemical detection of E6 and E7 proteins (expressed at very different levels) since these methods are lacking sensitivity and therefore their routine implementation is of limited value (28, 29).

**Nucleic acid-hybridization assays** include techniques such as Southern blotting, in situ hybridization (ISH) and dot-blot hybridization. The advantage of ISH is its high specificity, the detection of HPV DNA is site-specific and has the ability to differentiate between episomal and integrated DNA. ISH is time-consuming, requires large amount of purified DNA and the sensitivity is low (83%) with an estimated 13-14% false-negative rate. All these makes ISH improper for routine clinical use (29).
TABLE I
Sensitivity and specificity of main diagnostic methods used in HPV infection detection, related with OPSCC

<table>
<thead>
<tr>
<th>Authors</th>
<th>Year (reference)</th>
<th>Sample size</th>
<th>Detection method</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schache et al (41)</td>
<td>2013</td>
<td>79 FFPE</td>
<td>RNA Scope (RNA ISH) and DNA qRT-PCR</td>
<td>97%</td>
<td>91%</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>93%</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>87%</td>
</tr>
<tr>
<td>Chaudhary et al (30)</td>
<td>2010</td>
<td>222 OSCC</td>
<td>Hybrid capture 2 Ts-PCR E6 primers</td>
<td>DR=32,4%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DR=31,4%</td>
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<td></td>
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<tr>
<td>Thavaraj et al (42)</td>
<td>2011</td>
<td>142 tonsil SCC</td>
<td>P16+HPV DNA ISH P16+PCR GP5+/6+ P16+ISH+PCR</td>
<td>Dr=53%</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dr=61%</td>
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<td></td>
<td></td>
<td>Dr=63%</td>
<td></td>
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<tr>
<td>Ahn et al (44)</td>
<td>2014</td>
<td>81OPSCC saliva + plasma</td>
<td>Q-RT-PCR saliva Q-RT-PCR plasma</td>
<td>S+P=76</td>
<td></td>
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<td>S or</td>
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<td>S+P=100%</td>
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<td></td>
<td></td>
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<td>+p.v=100%</td>
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</tbody>
</table>

Abbreviations: FFPE=formalin fixed paraffin embedded, ISH=in situ hybridization, qPCR= quantitative polymerase chain reaction, Ts=type-specific, OSCC=oral squamous cell carcinoma, SCC=squamous cell carcinoma, S=saliva and P=plasma, Dr=detection rate

Signal amplification. At the moment two signal amplification assays are commercially available and are approved by the Food and Drug Administration (FDA). These kits are: Digene HPV test using Hybrid Capture 2 (hc2) technology and the Cervista HR HPV assay (29).

Hc2 detects 13 HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) and 5 low-risk types (6, 11, 42, 43 and 44) based on the hybridization of the target HPV-DNA to labeled RNA probes in solution. This assay was not designed for genotyping single HPV type and distinguishes only between high-risk and low-risk groups. Also this test uses full-genome RNA probes complementary to HPV DNA, specific antibodies, signal amplification and chemiluminescent detection (28, 29). Chaudhary et al. found a slight difference between the positivity rates of HR-HPV infection detected by hc2 and PCR method in OSCC, with a better sensitivity attributed to hc2 assay (30).

The Cervista HPV signal amplification assay (FDA approval in 2009) detects 14 HR-HPV types as a group (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) in addition to simultaneous HPV-16/18 detection. Also, this method demonstrates a lower false-positive rate, high sensitivity and specificity to genotyping HPV-16/18 (28, 29, 31).

PCR-based techniques can be consensus or type specific detection methods. Although it is very important to discriminate between HR and LR HPV types, genotyping of a single HPV type has a greater significance not only for research purposes but also for clinical findings since in persistent infections with HPV-16/18 the risk of a precancerous lesion is estimated at 10-15% and 3% for all other HPV strains combined (29, 32).

At the moment there are different primer sets for HPV detection in consideration to HPV biology. The most commonly used primer pair sets are MY09/MY11, GP5/GP6 and SPF10 LiPA sets which due to the high variability of HPVs (189 types)
target the L1 open reading frame (ORF) which is highly conserved among HPV strains. Upon viral integration into the host genome, the L1 ORF of the HPV genome may be deleted, consequently resulting in decreased sensitivity of HPV detection. PCR strategies targeting sequences retained upon viral host genome integration can offer more sensitivity for HPV detection. Such sequences are E6 and E7 HPV oncogenes. This was proved by a study on HPV detection in anal carcinoma where the HPV detection rate with L1 consensus primers was found to be 16%, whereas with strain-specific E6 primers the HPV positivity was 46% (28, 33-35). Also, in the integration process of HPV genome into the host genome, very often occur breaking points at E1 and/or E2 ORF (regulators of E6 and E7 protein expression) resulting in gene disruptions. When E2 gene is disrupted, the designed primers targeting E2 gene and aiming the entire E2 amplification will fail. Therefore primer sets targeting shorter regions will enhance the sensitivity of the method (35-37).

The drawbacks of conventional PCR methods are: low specificity, not site-specific like in situ hybridization assay (the detected HPV-DNA can originate from both malignant cells and surrounding non-transformed tissue), does not differentiate episomal from integrated HPV-DNA and also implies technical difficulties in performing. At the moment, there are no standardized PCR-based techniques leading to discordances in analytical sensitivities and specificities between laboratories. Furthermore, the genetic variation of HPV types and other unknown yet genomic features of HPVs are responsible for false-negative results (27, 29, 35, 38).

Equally important is the read-out system used to detect and visualize the PCR products. Novel PCR-based methods and commercially available kits improved their performance via more powerful read-out systems and implying hybridization technology. Most read-systems use hybridization techniques of PCR products to labeled oligonucleotide probes. The reverse line hybridization technique uses a solid support with a biotin-labeled PCR product hybridized to an array of immobilized oligonucleotide probes. Ultimately the visualization is performed through colorimetric or fluorescent staining procedures. Another read-out system incriminates an enzyme immunoassay method which cannot detect single genotype. This technique imply capturing of biotinylated PCR products to streptavidin-coated microplate wells and then hybridization with labeled oligonucleotide probes specific for the HPV types of interest (28, 30, 35, 38).

The real-time PCR (RT-PCR) has more analytical power this is higher sensitivity, and also provides a quantitative analysis of viral load. The quantification of targeted DNA is done via colorimetric markers during PCR amplification. High analytical sensitivity (a few copies of HPV DNA) does not translate into more clinical relevance because of presence of latent, non-productive and transient (not uncommon) HPV infections. On the other hand, the quantification of viral load and adjusting the cut-off viral load results in increased specificity and clinical relevance of the results of RT-PCR investigation. For a cut-off viral load >0.5 copies per cell, Smeets et al estimated the sensitivity at 92% and the specificity at 97%. Therefore false-negatives still exist (28, 35, 37, 39).

Detection of HPV mRNA in tumor samples is currently considered “the gold
Detection of human *Papillomavirus* in head and neck squamous cell carcinomas: a literature review

The clinical significance of HPV E6/E7 mRNA detection is greater than that of HPV-DNA detection since this provides evidence of active gene transcription, the HPV infection is a productive one and the virus is an etiological factor. Several authors reported that 14-50% of oro-pharyngeal cancers found to be HPV-DNA-positive mainly through PCR-based techniques were negative for E6/E7 mRNA expression, meaning that the false-positive rate was 14-50% and the specificity 50-86% (35, 40). “Why is specificity important?” Even though the HPV-status does not influence the treatment plan for OPSCC, the impact of a HPV false-positive test of a cervical specimen in female patients is much bigger often leading to over treatment and psychological discomfort. A drawback of HPV E6/E7 mRNA detection is that it is not site specific, but performing RNA *in situ* hybridization proves that HPV E6/E7 mRNA originates from HPV-induced malignant cells. These techniques are time consuming, expensive, and too laborious for use in routine screening (35, 41).

**CONCLUSIONS**

Even though the relation between HPV infection and HNSCC was formulated in 1983, over the years experts in HPV field have not yet reached a clear conclusion regarding the best techniques for the detection of premalignant lesions in order to implement effective methods of treatment. RT-PCR, detection of HPV mRNA and *in situ* hybridization of mRNA seems to be the most useful methods.

Future large-scale anti-HPV vaccination program worldwide will be able to decrease the incidence of HR HPV infection and the risk of OPSCC and HNSCC as well.

**REFERENCES**


