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Human Papillomavirus Detection in Head and Neck Squamous Cell Carcinomas and Its Clinical Implications

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1. Introduction

Squamous cell carcinomas of the head and neck are a biologically heterogeneous group of cancers with a variable clinical course (Tran et al., 2007).

Human tumor viruses account for approximately one-fifth of all cancers worldwide (Psyrri & Tsiodoras, 2008). The first association between human papillomavirus and head and neck cancer was observed during the 1960s (Rabbett, 1965). A possible role for human papillomavirus in the etiology of cancers at other sites within the head and neck was first suggested by Löning et al., in 1985. Since then, mounting epidemiological, molecular, and clinical evidence indicates that high-risk human papillomavirus (especially human papillomavirus-16) account for the development of head and neck carcinoma in some individuals who do not have the classical risk factors for this disease (Psyrri & Dimaio, 2008).

Distinguishing human papillomavirus positive from human papillomavirus negative head and neck squamous cell carcinoma can provide prognostic information, because different studies have shown better clinical outcome among patients with human papillomavirus positive head and neck squamous cell carcinoma. Although there are innumerable options for human papillomavirus detection in head and neck squamous cell carcinoma, there isn´t any standardization of procedures to use in clinical practice. Several authors propose a testing algorithm of first screening for human papillomavirus using p16 immunohistochemistry, after positive p16 results confirmatory testing with polymerase chain reaction or similar technique is carried out (Pannone et al., 2012; Smeets et al., 2007).

The demonstration that human papillomavirus have a role in human carcinogenesis has allowed the development of preventive and therapeutic strategies aimed at reducing the incidence and mortality of human papillomavirus-associated cancers (Psyrri & Dimaio, 2008).

This chapter reviews the human papillomavirus detection in head and neck squamous cell carcinoma and its clinical implications. Our search strategy included an electronic search of MEDLINE (pubmed), to identify all published articles about this issue. We use the key words "Human papillomavirus", "head and neck neoplasm". We checked the titles and abstracs retrieved. Each author independently assessed the full text of studies relevant to this review.

2. Risk factors in head and neck cancer

The main risk factors for head and neck cancer globally are tobacco and alcohol (Dobrossy, 2005). These agents act by inducing mutations in key genetic pathways that govern normal cell turnover such as p53 and the product of the retinoblastoma gene (pRb) (Pfeifer et al., 2002)(figure1).

Approximately 20% of head and neck cancers occur in people lacking these established risk factors (Wiseman et al., 2003). There is strong epidemiologic and experimental evidence indicating that human papillomavirus accounts at least partly for this subset of cancers (Shanta et al., 2000), and it has suggested that human papillomavirus may be an independent risk factor for oropharyngeal carcinoma, as well as a modulator the malignancy process in some tobacco-and alcohol-induced oropharynx tumors (Turner et al., 2011).
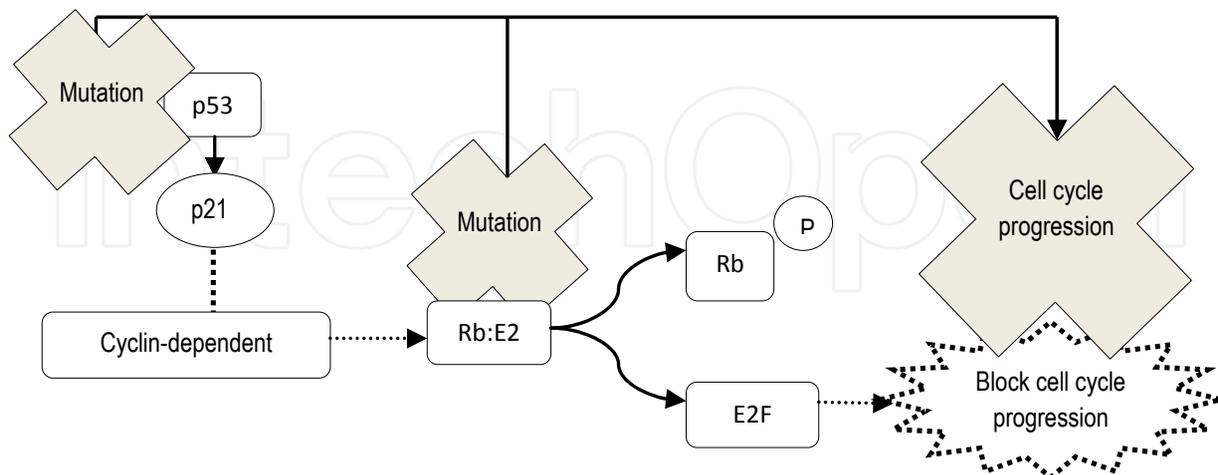
Figure 1. Inactivation of p53 and pRb by mutation by carcinogen agents. The p53 tetramers induce the expression of p21, which inhibits (dotted line) several cyclins. These cyclins induce the hyperphosphorylation of Rb, which normally binds to and inactives the E2F. The hyperphosphorylated form prevents the binding of E2F, which can then initiates uncontrolled cell division.

2.1. Human papillomavirus: Concept

Human papillomavirus is a member of the papillomaviridae family. They are small, non-enveloped, DNA viruses. They may be found integrated into the host genome, non-integrated or episomal, or as a combination or mixture of these types in infected tissue (Turner et al., 2011) [figure 2].

Mucosal human papillomavirus can be categorized in 2 major groups based on oncogenic potential: "low-risk" and "high-risk". Human papillomavirus 16 and 18 are the major ''high-risk'' types, which are associated with precancerous lesions (Tran et al., 2007; Psyrri & Tsiodoras, 2008; Psyrri & Dimaio, 2008; Snow & Laudadio, 2010).
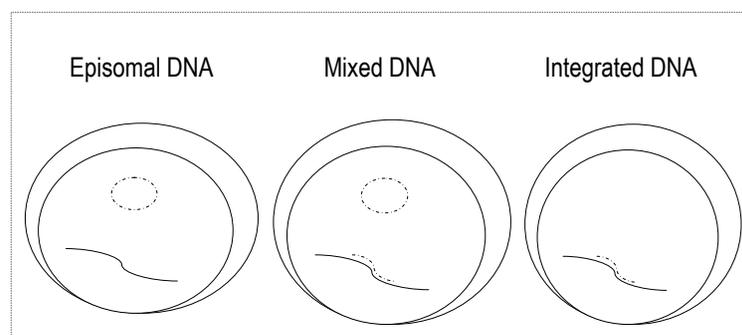

Figure 2. DNA viruses (dotted) and the host genome

2.2. Human papillomavirus life cycle and its role in the pathogenesis of head and neck squamous cell carcinoma (head and neck squamous cell carcinoma)

Through wounds or abrasions, the papillomaviruses infect basal epithelial cells. The viral DNA is maintained in the nuclei of infected epithelial cells (Stubenrauch & Laimins, 1999). human papillomavirus-DNA replicates to a high copy number only in terminally differentiated cells near the epithelial surface (Stubenrauch & Laimins, 1999). The late viral genes, which encode the L1 and L2 proteins that constitute the virus particle, are expressed only in the highly differentiated cells (Bedell et al., 1991).

Replication of the human papillomavirus genome is critically dependent on the host-cell DNA replication machinery (Cheng et al., 1995). The papillomavirus E1 and E2 proteins are required for viral DNA replication and papilloma formation (Wu et al., 1994). E1 is an ATP-dependent helicase that initiates viral replication in cooperation with the E2 protein. In addition, the E2 protein can function as a transcriptional repressor of E6 and E7 oncogene expression among other functions (Psyrri & Dimaio, 2008). E2 loss of function allows up-regulation of E6 and E7 oncoproteins (Pannone et al., 2012).

Transcription of human papillomavirus-16 E6/E7 mRNA in tonsillar carcinomas is not necessarily dependent on viral DNA integration, and the viral DNA is predominately in episomal form (Mellin et al., 2002). It has been also demonstrated that high risk human

papillomavirus episomal DNAs up-regulate the activity of E6/E7 promoter, which in turn gives rise to elevated E6 and E7 protein expression in cancer cell (Pannone et al., 2012). Mellin et al (Mellin et al., 2002) concluding that in oropharyngeal carcinomas human papillomavirus is almost exclusively not integrates and its carcinogenic activity is due to E6/E7 oncoproteins expressed from episomal viral sequences. It is unknown whether the physical state of the virus influences tumor biology (Tran et al., 2007; Koskinen et al, 2003). However, the data suggested that a higher viral load cloud be a favourable prognostic indicator and that tumours with episomal DNA had larger tumours than patients with mixed or integrates forms of viral DNA. Higher copy number of episomal viral DNA was able to induce more rapid growth, perhaps by higher expression of the viral oncogenes (Pannone et al., 2011).

Human papillomavirus encode E6 and E7 proteins that create a state competent for DNA replication. The E6 protein of the high-risk human papillomavirus binds and induces the degradation of the p53 tumor suppressor protein via an ubiquitin-mediated process. E6 also activates telomerase allowing the regenesis of the ends of chromosomes after cell division. While, the human papillomavirus-E7 protein binds and destabilizes the retinoblastoma (Rb) tumor suppressor protein and related proteins. The molecular consequence of the expression of these viral oncoproteins is cell cycle entry and inhibition of p53-mediated apoptosis (figure 3). The E6 and E7 proteins also interact with other cellular targets. Together, these effects promote cell-cycle progression and viral DNA replication in differentiated keratinocytes (Tran et al., 2007; Leemans et al., 2011; Hobbs et al., 2006).
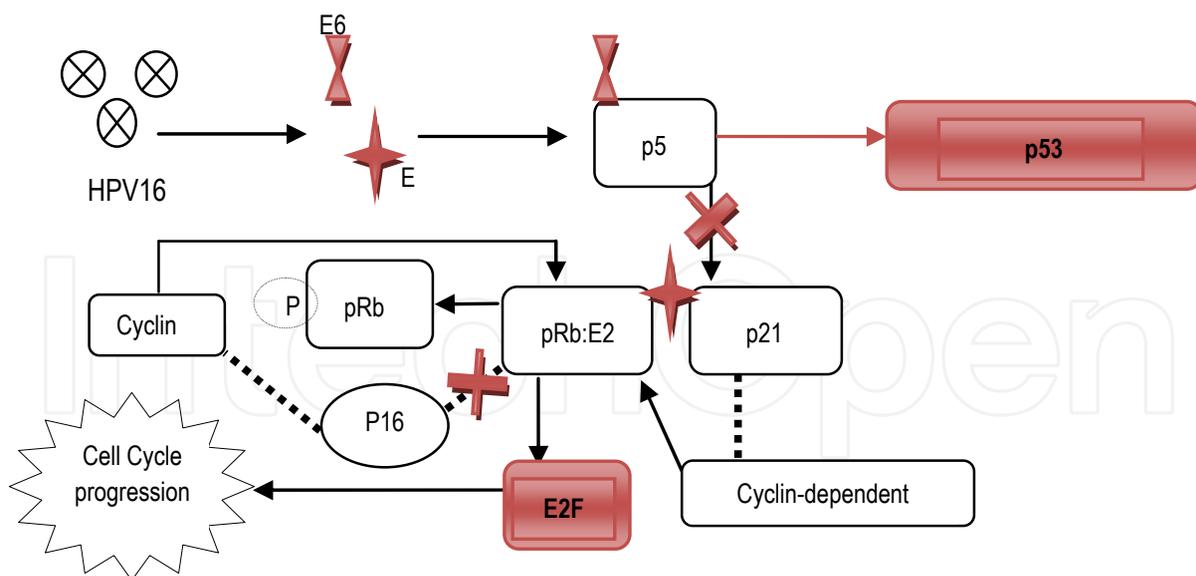

Figure 3. Inactivation of p53 by E6, inactivation of pRb by E7, and p16 over-expression. The E6 protein binds p53 and targets the protein for degradation, whereas the E7 protein binds and inactivates the Rb protein. pRB family proteins negatively regulate p16 gene expression. When E7 binds to pRB, this protein is inactivated, thus, p16 expression increase. Although p16 levels rise, normal feedback is by-passed, as human papillomavirus (HPV)-mediated cell proliferation is not dependent on cyclinD/Cdk4/6 (Dotted line = inhibition)

As a result, somatic mutation in TP53 (encoding p53), cyclin D1, and deletion or silencing CDKN2A (encoding p16) are established cancer genes in human papillomavirus-negative head and neck squamous cell carcinoma. In contrast, human papillomavirus-associated tumors are less likely to harbor TP53 mutation and the genes encoding the Rb family are established cancer genes in human papillomavirus-positive head and neck squamous cell carcinoma. In addition, human papillomavirus-positive head and neck squamous cell carcinoma has strong expression of p16 (as a component of the retinoblastoma tumor suppressor pathway) (Snow & Laudadio, 2010; Leemans et al., 2011). In the other hand, p16 expression loss defines a subgroup of head and neck squamous cell carcinoma patients with human papillomavirus-negative tumors.

So, the etiology of head and neck cancer is complex. Human papillomavirus, tobacco and alcohol represent three independent risk factors for head and neck carcinoma in the oral cavity and oropharynx.

The different risk factors can be combined. Smith et al (Smith et al., 2012) found that cancer in oral cavity or oropharyngeal risk was different among patients with several risk factors (Table 1). This investigation suggests that while risk of head and neck squamous cell carcinoma by tumor site is both different between oral cavity and oropharynx, both sites are nonetheless associated with independent effects for each of the three major head and neck squamous cell carcinoma risk factors.

The association between tobacco/alcohol, human papillomavirus, and tumor site is complex.

Oral Cavity/Oropharynx	*Human papillomavirus-positive*	*Human papillomavirus-negative*
Heavy alcohol user	**OR=3,5**/OR=4,7	**OR=1,4**/OR=11
Heavy tobacco user	**OR=9,8**/OR=8,5	**OR=3,1**/OR=24,3

Table 1. Risk of oral cavity and oropharyngeal carcinoma (Smith et al., 2012). OR=Odds Ratio

3. Epidemiologic and experimental evidence of an etiologic role for human papillomavirus in head and neck squamous cell carcinoma

Certain subsets of head and neck squamous cell carcinoma have fallen in parallel with the reduction in smoking, rates of oropharyngeal squamous cell carcinomas have risen by 2.1% and 3.9% among men and women respectively, from 1973 to 2001, particularly tongue and tonsillar cancers (Shiboski et al., 2005). Similarly, the incidence of tonsillar cancer increased by approximately 2–3% per year among men younger than 60 years from 1975 through 1998 (Canto & Devesa, 2002). In addition, the incidence of human papillomavirus-associated oropharyngeal cancer has increased between 1973 and 2004 (Chaturvedi et al., 2008).

These data suggest that human papillomavirus has emerged as an increasingly important cause of oropharyngeal cancer not only because tobacco-associated head and neck squamous cell carcinoma have decreased, but also because the incidence of human papillomavirus-associated oropharyngeal cancer is increasing (D´Souza & Dempsey, 2011).

This increase in the incidence of oropharyngeal cancer was paralleled by an increase in certain sexual behaviors. This change in the demographics of patients with head and neck squamous cell carcinoma is consistent with a role for genital human papillomavirus in the pathogenesis of oropharyngeal squamous cell carcinoma in individuals whose sexual practices are typically associated with sexual transmission of the virus (Psyrri & Dimaio, 2008). An elevated risk of oropharyngeal cancer has been associated with increasing number of sexual partners, younger age of first sexual intercourse, the practice of oral sex, and a history of genital warts (Trans et al., 2007).

One of the most important studies establishing the causal relationship between human papillomavirus and head and neck cancer was a multi-center case control study conduced by the International Agency for Research into Cancer (IARC) (Herrero et al., 2003). Findings confirmed that human papillomavirus-positive tumors cluster among non-smokers and nondrinkers.

There has been wide variation in human papillomavirus positivity rates in cancers at different sites within the head and neck. Approximately 25% of oropharyngeal cancers have tested human papillomavirus-positive, with rates in tonsillar cancer considerably higher (Trans et al., 2007). In fact, tonsillar crypts seem particularly susceptible to transformation by human papillomavirus, which is similar to the transformation zone of the uterine cervix, the location in which most cervical cancers originate (Psyrri & Dimaio, 2008).

4. Human papillomavirus detection

Since Syrjänen´s initial observations in 1983 (Syrjänen et al., 1983), there have been numerous reports on human papillomavirus-DNA detection in head and neck squamous cell carcinoma with rates varying from 0% to 100% of tumors studied (Clifford et al., 2003; Campisi et al., 2007). These differences in detection rate are due to at least two principal factors (Pannone et al., 2012):

1. Differences in the epidemiological distribution of oncogenic high risk human papillomavirus in the world
2. Different analytical methods utilized

So, there are nearly innumerable options for human papillomavirus detection in head and neck squamous cell carcinoma and no standardization of procedures to be used in clinical practice. The method choice depends greatly upon the desire information (test directed at identifying a broad group of high risk human papillomavirus or targeted at specific human papillomavirus genotypes), available tissue type (fresh tissue, fixed tissue, incision biopsy, brush cytology, saliva, serum, fine needle aspiration biopsy), the ubiquity and preservation of the candidate target molecule (DNA, RNA, and protein), and resources in (Snow & Laudadio, 2010; Robinson et al., 2010)

The **Southern blot** has long been considered the gold standard for detection of specific DNA sequence, however, with its technical demand, necessity for large quantities of DNA… Its use in clinical applications for human papillomavirus detection is rare (Snow & Laudadio, 2010).

Several **amplification techniques** (polymerase chain reaction [PCR]) have been developed for human papillomavirus type–specific using a specific primer set or for wide-spectrum human papillomavirus detection. Some of them adequately and equivalently amplify the target of interest, as L1 (late gene that encodes the viral capsid). However, multiple portions of the human papillomavirus genome, including L1, may be deleted in the process of integration to false negative results. For this reason, assays have been developed, which amplify portions of E6 and E7 (Snow & Laudadio, 2010). Many studies have shown reproductible results and high sensitivity with RNA-based assays (*reverse transcriptase polymerase chain reaction*) when using frozen tissue, but this material is not always available for testing. Multiple studies have compared RNA extraction from fresh or frozen tissue with that from formalin-fixed-paraffin-embedded tissue. The greatest decrease in RNA quality occurs immediately after fixation and processing (Snow & Laudadio, 2010).

Consensus polymerase chain reaction and genotyping is applicable to formalin-fixed-paraffin-embedded material and it has high sensitivity, however, it can detect of biologically irrelevant human papillomavirus, and the sample can be contaminated during biopsy acquisition. *Type specific polymerase chain reaction* has similar characteristics to consensus polymerase chain reaction. *Real time polymerase chain reaction* is applicable to formalin-fixed-paraffin-embedded material, it has high sensitivity and specificity, and it gives an estimate of the viral load, however, it requires tissue microdisecction and DNA extraction (Robinson et al., 2010).

The human papillomavirus-DNA test may be used in head and neck pathology departments with the following diagnostic and prognostic purposes (Reimers et al., 2007):

1. Distinguish human papillomavirus positive from human papillomavirus negative head and neck squamous cell carcinoma and providing prognostic information
2. Distinguish human papillomavirus positive metastases to the loco-regional lymph nodes derived from oropharyngeal cancers versus metastases of other origins
3. Furnish potentially useful indications for cancer treatment options
4. Contribute to the differential diagnosis of rhino-pharynx undifferentiated carcinoma (Worl Health Organization type I potentially related to human papillomavirus infection whereas Type II and III potentially related to Epstein Barr Virus)
5. Provide valuable information for head and neck cancer research

Table 2 shows the different types of primers (Hunsjak et al., 2000; Gravitt et al., 2003; Snow & Laudadio, 2010; Micalessi et al., 2012). Figure 4 shows the genomic structure of human papillomavirus.

Immunohistochemistry for the expression of viral human papillomavirus proteins as p16, E5, E6, E7 as surrogate markers of human papillomavirus infection. In the case of p16, human papillomavirus independent pathways of oncogenesis can lead to increased expression of p16 and the specificity is only 79% (Snow & Laudadio, 2010; Pannone et al., 2011). In fact, the immunohistochemistry detection of p16 protein has been proposed as surrogate marker of human papillomavirus infection in head and neck squamous cell carcinoma (Reimers et al., 2007).
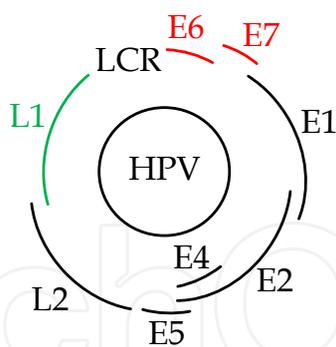
Figure 4. Genomic structure of human papillomavirus

	Degenerated	*Not degenerated*	
Consensus Target: L1	MY09, MY11 (450 base pairs) GP5+, GP6+ (140 base pairs)	SPF (65 base pairs)	*Advantages*: They have a larger spectrum of human papillomavirus detection. *Disadvantages*: during the integration into the host DNA, parts of the L1 region may be deleted, contributing to false negative results. *SPF:* it has higher sensitivity compares to MY and GP because of its shorter amplification product (65 base pairs).
Specific Target: (for example: E6/E7)	Polymerase chain reaction directed at a single human papillomavirus The use of several specific primer pairs combined is called multiplex reaction		*Advantages*: they are available for target detection and type discrimination. *Disadvantages:* they require a polymerase chain reaction for each human papillomavirus type. *Multiplex primer sets* are directed at high or low-risk, but not specific human papillomavirus .

Table 2. Different types of primers.

In situ hybridization is a reproducible technique applicable to detection of a wide of human papillomavirus types particularly from formalin-fixed-paraffin-embedded tissues. However, in situ hybridization is considered method with a low sensitivity, because the low applicability in clinical routine for the long and hard technical word required in (Snow & Laudadio, 2010; Pannone et al., 2011).

A recent study (Pannone et al., 2012) has tested the reliability of a triple method which combines evaluation of p16 expression of viral human papillomavirus proteins by immunohistochemistry, human papillomavirus-DNA genotyping by polymerase chain reaction, and viral integration into the host by in situ hybridization. All the head and neck

squamous cell carcinoma confirmed human papillomavirus positive by polymerase chain reaction and/or in situ hybridization were also p16 positive by immunohistochemistry. So immunohistochemistry showed a very high level of sensitivity as single test but lower specificity level. The double method, in situ hybridization and polymerase chain reaction increased significantly the specificity, but reduced the sensitivity. They observed different levels of p16-immunohistochemistry accuracy in the different cancer subpopulation studied. So, in a cohort of prevalently alcohol/tobacco associated cancers, p16-immunohistochemistry test showed a lower level of specificity in detecting human papillomavirus positive cases. In addition, a recent literature report demonstrates different p16 accuracy according to different anatomical sub-sites of head and neck region (Doxtader & Katzenstein, 2011). In this context, the p16-immunohistochemistry test alone could be used only as a screening method and need to be associated with molecular tests in order to detect human papillomavirus-DNA and to assess its integration status.

The hybrid capture technique is used extensively among pathology labs to detect 13 high-risk human papillomavirus genotypes in cervical cytology specimens. The use of this method is limited for head and neck squamous cell carcinoma human papillomavirus testing, but the technique has potential for screening oral brushings. However, at this time, oral brush cytology has not achieved a sensitivity or specificity sufficiently competitive with surgical biopsy for diagnosis and prospective studies are necessary to determine the clinical use of screening in (Snow & Laudadio, 2010; Pannone et al., 2011).

Luminex system combines PCR with hybridization to fluorescence-labeled polystyrene bead microarrays. This technology provides a new platform for high-throughput nucleic acid detection and is being utilized with increasing frequency. It is a sensitive, reproducible technique for the simultaneous genotyping of all clinically relevant genital HPV types. However, these Luminex assays have shown low ability for type-specific genotyping and have missed variants with the type-specific probes. Multiple infections may occur in 20-40% of specimens. Luminex-based HPV genotyping can be used to differentiate between newly acquired HPV types and pre-existing infections when applied over time. Nevertheless, a limitation of the assay is the reduction of signal that occurs for a plasmid target in low abundance when it is amplified with another target that is 2 or 3 logs higher in abundance. This technology has been tested in cervical samples (Oh et al., 2007; Lowe B et al., 2010).

Human papillomavirus serology. The immune to human papillomavirus infection involves both the cell-mediated and humoral responses. Human papillomavirus seropositivity is potentially indicative not only of current oral infection but also of any past infection not limited to the oral cavity or oropharynx (Pannone et al., 2011). Antibodies to human papillomavirus E6 and E7 proteins are markers for an invasive human papillomavirus-associated cancer. The use of human papillomavirus viral load in conjunction with serological markers may serve to identify a subset of human papillomavirus-associated head and neck tumors in which human papillomavirus is biologically active (Ragin et al., 2007).

Table 3 resumes the characteristics of different methods for human papillomavirus detection (Dobrossy, 2005).

Method	Detect	Characteristics	Sample
Southern Blot	Specific DNA sequence	It needs large quantities of DNA. It don´t use in clinical practice. Low sensitivity	Frozen tissue
Polymerase chain reaction	Amplify particular DNA sequence	There are several sets. - *Consensus polymerase chain reaction*: high sensitivity, but it can detect biological irrelevant human papillomavirus. - *Type specific polymerase chain reaction*: as above - *Real time polymerase chain reaction*: high sensitivity and specificity, but it requires tissue microdissection and DNA extraction - *Reverse transcripase polymerase chain reaction*: high sensitivity and specificity, but adequate performance is limited to frozen tissue	Formalin-fixed-paraffin-embedded More accuracy in fresh frozen tissue for reverse trancriptase Polymerase chain reaction
Immuno-histochemistry	Viral human papillomavirus proteins	High sensitivity in screening. Specificity is low.	Formalin-fixed-paraffin-embedded
In situ hybridization	Specific DNA or RNA sequence	It has a low sensitivity	Formalin-fixed-paraffin-embedded
Hybrid Capture	High-risk human papillomavirus genotypes	It has potential for screening oral brushing. Lower sensitivity and specificity than surgical biopsy	Oral brush cytology
Serology	Cell-mediated and humoral responses	Minimally invasive test. It indicates human papillomavirus infection but not limited to the oral cavity or oropharynx. Low sensitivity and specificity.	Blood

Table 3. Characteristics of different methods for human papillomavirus detection

Establishing a diagnosis of head and neck cancer requires the acquisition of adequate biopsy material. Typically, tissue samples are fixed in formalin, processed in the laboratory and formalin-fixed-paraffin-embedded, whereas fine needle aspiration biopsy samples are usually treated with an alcohol-based fixative. So, for an human papillomavirus test to be useful it should be capable of reliably classifying 'human papillomavirus related' cancers in fixed cell and tissue samples. The techniques used should be reproducible, subject to standardization and quality assurance and be economically viable (Robinson et al., 2010).

The presence of the viral DNA does not establish causality, since the majority of human papillomavirus infections may be transient rather than persistent (Ragin et al., 2007). The important issue is that human papillomavirus is transcriptionally active (Trans et al., 2007; Hobbs et al., 2006). In fact, cases that are human papillomavirus positive but negative for p16 expression (or negative for E6/E7 mRNA) are molecularly more similar to human papillomavirus negative cases suggesting that in these instances human papillomavirus is not directly involved in carcinogenesis (Snow & Laudadio, 2010; Weinberger et al., 2006).

Detection of high-risk E6/E7 mRNA or protein would be the ideal test for classifying a tumor as truly human papillomavirus-associated, while it´s possible to perform quantitative polymerase chain reaction on formalin-fixed-paraffin-embedded samples the maximum accuracy is found using fresh frozen tissue (Pannone et al, 2012). Determination of p16 expression status by immunohistochemistry could serve as a reasonable surrogate marker for biologically relevant high-risk human papillomavirus infection (Psyrri & Dimaio, 2008).

Smeets et al (Smeets et al., 2007) propose a testing algorithm of first screening for human papillomavirus using p16 immunohistochemistry, after positive p16 results confirmatory testing with polymerase chain reaction is carried out. This approach had almost 100% sensitivity and specificity, with 2% risk of false positive. Others authors as Westra (Westra, 2009) propose confirmatory testing by in situ hybridization (Robinson et al., 2010). The majority of pathology laboratories have the capability of delivering the first algorithm.

Some authors are searching for other defining molecular characteristics. There is evidence that human papillomavirus positive head and neck squamous cell carcinoma tends to contain normal copies of the p53 gene (Braakhuis et al., 2004). However, p53 mutations have been described in these tumours (Westra et al., 2008). This presence of mutant p53 along with human papillomavirus infection in the same tumour raises the possibility that human papillomavirus infection is simultaneous and has no influence on pathogenesis (Robinson et al., 2010). Human papillomavirus viral oncoproteins are known to have epigenetic effects. They can silence the expression of key tumour suppressor genes by promoter methylation (Henken et al., 2007). The emergence of global genome methylation assays represents novel ways of refining the molecular classification of head and neck cancers in the future (Robinson et al., 2010).

5. Clinical implications

Several lines of clinical evidence also suggest that human papillomavirus-associated head and neck squamous cell carcinoma could be biologically distinct from classical head and neck squamous cell carcinoma (Table 4).

CHARACTERISTICS		HUMAN PAPILLOMAVIRUS +	HUMAN PAPILLOMAVIRUS -
MOLECULAR & HISTOPATHOLOGICAL	TP53 y RB1	Inactivation	Mutation
	p16	↑	↓
	Cyclin D1	↓	↑
	Histological grading	↑ Poorly differentiated tumours	Any
	Histotype	Often basaloid	Basaloid uncommon
CLINICAL	Sex	♂ y ♀ [or ♂ > ♀]	♂ > >♀
	Age	Younger [< 40 yrs]	Older
	Tobacco y alcohol	Generally NO	Generally YES
	Stage at diagnosis	Advanced and Later	Earlier
	Prognostic	Better	Worse

Table 4. Molecular & clinical characteristics in human papillomavirus + and human papillomavirus - head and neck squamous cell carcinoma

Several experiments delineated three biologically and clinically distinct types of oropharyngeal tumors (Weinberger et al., 2006) (Table 5):

		HUMAN PAPILLOMAVIRUS	
		Negative	Positive
P16 expressing	No	Class I	Class II
	Yes	Class IV	Class III

Table 5. Biologically and clinically distinct types of oropharyngeal tumors

- Class I, human papillomavirus-negative/p16 non expressing. Conventional head and neck squamous cell carcinoma with no evidence of human papillomavirus infection, typically exhibiting inactivation of p16, with p53 mutations and probably caused by tobacco and alcohol abuse.
- Class II, human papillomavirus-positive/p16 non expressing. Conventional head and neck squamous cell carcinoma that acquire simultaneous human papillomavirus infection late in its pathogenesis, with no consequences for p16 expression

- Class III, human papillomavirus-positive/ p16 expressing. "human papillomavirus related" head and neck squamous cell carcinoma that contains human papillomavirus DNA and shows evidence of oncogenic human papillomavirus protein expression, using p16 as a surrogate marker.
- Class IV, human papillomavirus-negative/p16 expressing. Small number of apparently human papillomavirus negative carcinomas that over express p16. There are two major reasons for this entity: a] misclassification as human papillomavirus negative, because the human papillomavirus test chosen lacks sensitivity, b] tumours where accumulation of p16 has been caused by perturbation of other cellular signaling pathways, or due to possible an as yet unidentified infectious agent.

Class III had the highest viral loads. The 5-year survival in class III was 79%, significantly higher than in the other two classes (20% and 18%, $P = 0.0095$). Disease free survival for class III was 75% compared with 15% and 13% for classes I and II, respectively ($P = 0.0025$). The 5-year local recurrence was 14% in class III compared with 45% and 74% ($P = 0.03$). Multivariate survival analysis confirmed the prognostic value of the three class model. It is clear that head and neck squamous cell carcinoma human papillomavirus-positive and p16 expressing is different from classic head and neck squamous cell carcinoma, but it is not clear whether head and neck squamous cell carcinoma human papillomavirus-positive and p16 non expressing (probably, tobacco/alcohol-related tumors that are infected by high-risk human papillomavirus) represents a group biologically distinct from human papillomavirus-negative tumors (Cheng et al., 1995). Other studies in (Smith et al., 2012; Harris et al., 2011) have confirmed better disease-specific and recurrence-free survival in human papillomavirus and/or p16 positive tumors.

About class IV, several studies (Reimers et al., 2007; Harris et al., 2011; Shah et al., 2009; Weinberger et al., 2004) have shown that patients with human papillomavirus negative and p16 positive tumors had better outcomes than patients with p16 negative tumors. So, p16 status could be the truly important prognostic marker in head and neck squamous cell carcinoma, independent of human papillomavirus infection.

For all this, p16 positivity has been proposed to be a more reliable and reproducible prognostic marker in head and neck squamous cell carcinoma (Harris et al., 2011).

On the basis of these results, we can refine a model for human papillomavirus-associated oropharyngeal cancer. The favorable outcome of human papillomavirus-induced oropharyngeal cancers might be attributable to the absence of field cancerization or enhanced radiation sensitivity (Lindel et al., 2001). In 1953, the term "field cancerization" was proposed to explain the high propensity to develop local recurrence after treatment of head and neck squamous cell carcinoma and the high likehood that multiple independent tumours will develop in the head and neck mucosa. This phenomenon is due to the presence of carcinogen induced early genetic changes in the epithelium from which multiple independent lesions arise (Slaughter et al., 1953).

Disrupting E6 and E7 in oropharyngeal cell lines results in increased levels of p53 and pRB and increased levels of p53-activated genes (Rampias et al., 2009). These findings indicate that in human papillomavirus-induced carcinoma the p53 and pRB pathways remain intact.

So, unlike tobacco associated oropharyngeal cancers that harbor mutant TP53, the apoptotic response of human papillomavirus-associated tumors to radiation and chemotherapy might be intact. Some authors have proposed that p16-expressing cells are less hypoxic and respond with less accelerated repopulation when irradiated (Lassen et al., 2009).

Given that the rate of p53 mutation is quite low in human papillomavirus-associated tumors, the addition of p53 mutation sequencing could have added valuable information had sufficient tissue been available (Harris et al., 2011). There have been conflicting data on p53 expression in human papillomavirus positive head and neck squamous cell carcinoma tumor cells. Some studies have observed high expression of nuclear p53 in some human papillomavirus-containing tumors with wild-type p53 (Hafkamp et al., 2003), and other studies demonstrating low p53 expression (Wilczynski et al., 1998). Mechanism of over-expression of wild-type p53 in the presence of the virus is known (Tang et al., 2011).

It has been suggested that less intensive treatment modalities should be examined in order to decrease treatment-toxicities. For that, the National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology (USA) (Pfister et al., 2011) recommend that oropharyngeal squamous cell carcinoma is tested for high risk oncogenic human papillomavirus.

Furthermore, epidermal growth factor receptor expression has been suggested to be correlated with human papillomavirus status (Almadori et al., 2001). There are data suggesting a direct link between human papillomavirus-encoded proteins and epidermal growth factor receptor expression (Kim et al., 2006). Kumar et al (Kumar et al., 2008) reported the phenotype human papillomavirus positive and epidermal growth factor receptor high to be associated with poorer survival after chemotherapy and radiation than human papillomavirus positive and epidermal growth factor receptor low tumors.

Bonner et al (Bonner et al., 2010) tested the combination of cetuximab, a monoclonal antibody directed against epidermal growth factor receptor, and radiotherapy in head and neck squamous cell carcinoma. They demonstrated improved patient survival compared with radiation alone. The use of this combination increased skin irritation, but otherwise it had the same side effects as radiotherapy alone. An analysis of patients in this trial revealed that those with oropharyngeal squamous cell carcinoma who were male and younger, a group that mirrors the human papillomavirus-positive population, benefited most from the combination therapy. These results suggested that radiation plus cetuximab, instead of cisplatin-based chemotherapy, may reduce treatment toxicity without compromising cancer control for patients with human papillomavirus-positive oral squamous cell carcinoma. For this reason, Radiation Therapy Oncology Group (RTOG) has initiated a phase III randomized study of radiotherapy with cisplatin or cetuximab in patients with human papilloma virus-associated oropharyngeal cancer (RTOG-1016).

So, epidermal growth factor receptor, and p53 are also relevant markers that modify the prognostic effect of human papillomavirus and may help guide the development of targeted therapy in head and neck squamous cell carcinoma.

However, not all patients with human papillomavirus positive tumors respond well to therapy and the reasons for failure in some cases are not known (Maxwell et al., 2010). The variability of high risk human papillomavirus containing cell lines enhances our ability to study the role that human papillomavirus plays in head and neck squamous cell carcinoma development and response or resistance to therapy (Tang et al., 2011). Combination of several risk factors could explain this. A positive tobacco history in patients with human papillomavirus positive tumors may represent a distinct group of head and neck squamous cell carcinoma when all head and neck squamous cell carcinoma are divided by etiologic factors: human papillomavirus negative smokers, human papillomavirus positive never smokers, human papillomavirus positive ever smokers, etc... TPV status likely provided an additive and possibly synergistic effect with others risk factors (Tang et al., 2011).

In addition, in the patient with metastatic head and neck carcinoma of unknown origin, the presence of human papillomavirus in a fine needle aspiration biopsy sample can be used to direct the search to the oropharynx (Zhang et al., 2008).

All of this could lead to a new diagnosis and treatment algorithm (Figure 5).

Others implications are the preventions actions:

1. **Screening studies** have been performed in healthy adults using biopsy samples or less invasive saliva and oral lavage-based testing methods to identify human papillomavirus. These techniques revealed prevalence rate between 0-25% (Turner et al., 2011).

Detection of high risk human papillomavirus DNA may help identify individuals, including those with: a) any genetic predisposition to acquire high risk human papillomavirus infection and/or b) a limited immunologic ability to eliminate the virus. Whether oral exfoliated high risk human papillomavirus status is predictive of cancer before invasion or progression in patients with head and neck squamous cell carcinoma is unknown.

Quantitative measurement of salivary human papillomavirus16 DNA can be promise for surveillance and early detection of recurrence. Detection of high risk human papillomavirus in oral exfoliated cells may serve as clonal markers to monitor the presence of residual tumor after surgery or radiation, cancer recurrence, and progression (Pannone et al., 2011).

A recent study (Turner et al., 2011) recruited patients and screened saliva samples for high risk human papillomavirus using quantitative polymerase chain reaction. They confirmed human papillomavirus16, but not human papillomavirus18 in a small subset of the healthy adult patients. These patients were female and minority (2.6%).

2. **Prophylactic vaccines** that prevent persistent cervical human papillomavirus-16 infections might be effective in preventing these cases of head-and-neck cancer as well, either indirectly by eliminating an anogenital source of virus or directly by protecting the oropharyngeal epithelium itself from infection (Psyrri & dimaio, 2008).

In the U.S, two vaccines are currently available. The quadrivalent vaccine, Gardasil® (human papillomavirus4), protects against infection with human papillomavirus types -6, -11, -16, and 18. The second human papillomavirus vaccine, Cervarix® (human papillomavirus2), is a

bivalent vaccine that provides protection against human papillomavirus types -16 and -18 in (D´Souza & Dempsey, 2011).

However, these vaccines do not alter the prognosis of established human papillomavirus infection. Therapeutic vaccines based on the viral oncogenes are still in the developmental stage, but they may eventually prove beneficial if used in association with conventional approaches for the management of advanced disease (Tran et al., 2007).
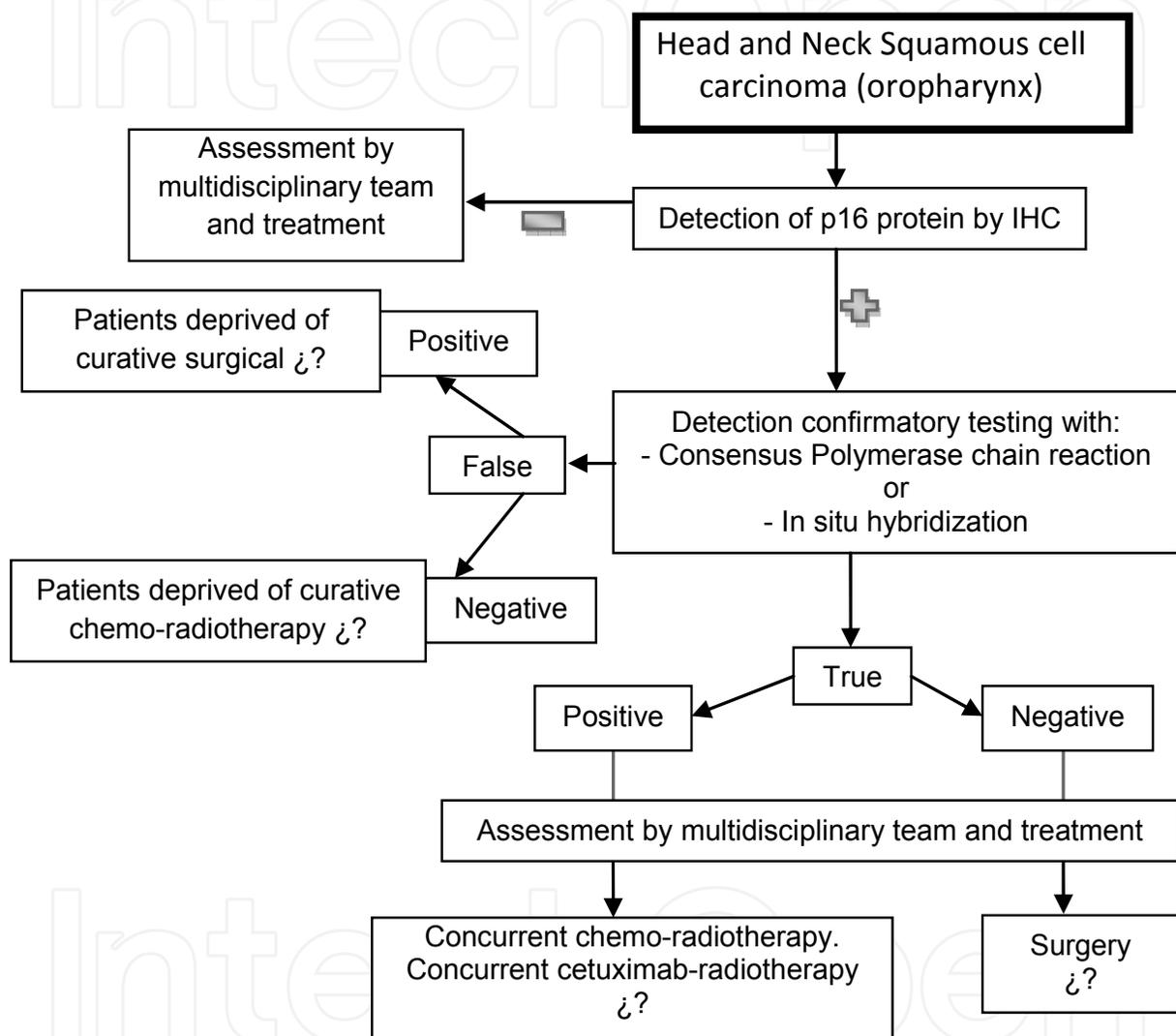

Figure 5. Diagnosis and treatment algorithm

6. Conclusion

Emerging evidences suggest that human papillomavirus-associated head and neck squamous cell carcinoma is a separate subgroup and biologically distinct from classical head and neck squamous cell carcinoma.

Human papillomavirus positive head and neck squamous cell carcinoma is typically found in the oropharynx and have been associated with younger patients who are less

likely to be smokers or drinkers and with improved response to therapy and overall survival (Harris et al., 2011).

So, recognition that human papillomavirus has an etiologic role in head and neck squamous cell carcinoma has important implications for prognosis, treatment, disease prevention, and screening tests which are still being developed. Several authors have suggested that patients with human papillomavirus positive head and neck squamous cell carcinoma can be treated with chemo-radiotherapy or cetuximab-radiotherapy instead surgery.

Although high risk human papillomavirus detection is of utmost importance in clinical setting of head and neck squamous cell carcinoma, there is no agreement about the "golden standard" considering the number of molecular methods or combinations available.

There is evidence that detection of high risk human papillomavirus by consensus polymerase chain reaction alone is insufficient to accurately classify tumours, however, there is convincing evidence that the detection of p16 protein by immunohistochemistry can be used as a surrogate marker for the elaboration of oncogenic human papillomavirus proteins (Robinson et al., 2010). So, this is feasible as part of a routine diagnostic process using either a combination of p16 immunohistochemistry and in situ hybridization (Westra, 2009) or p16 detection and consensus polymerase chain reaction (Smeets et al., 2007).

Several studies have shown p16 expression status as a predictor of prognostic marker in head and neck squamous cell carcinoma, independent of human papillomavirus infection. In addition, epidermal growth factor receptor, and p53 are also relevant markers that modify the prognostic effect of human papillomavirus and may help guide the development of targeted therapy in head and neck squamous cell carcinoma.

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