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**HUMAN PAPILLOMAVIRUS - ASSOCIATED HEAD AND NECK
SQUAMOUS CELL CARCINOMAS IN NORTH-EAST ITALY.**

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To my Family

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I. Summary

Background: Specific oncogenic types of human papillomaviruses (HPV), most frequently HPV16, are causally associated with a subset of head and neck squamous cell carcinomas (HNSCC). HPV DNA associated tumors appear to be heterogeneous in prevalence over time and geographically, in the oncogenic activity (direct and indirect viral markers) and clinical behavior. However, it remains unclear which biomarkers can reliably determine which HNSCC are truly driven by HPV transformation.

Aims: In the present thesis, the first aim was to determine the prevalence of the truly HPV-associated HNSCC tumors in North-East Italy. The second aim, was to investigate the association of HPV DNA positivity with other viral (viral load, oncoE6 protein, HPV antibodies) and cellular (p16^{INK4a}, pRb) markers. The third aim, was to evaluate the prognostic significance of HPV-association tumors for clinical outcome (i.e. survival).

Materials and Methods: Overall, 247 fresh frozen and 53 (21%) formalin-fixed paraffin-embedded (FFPE) tumor tissue biopsies and 102 (41%) sera were collected from 247 newly detected HNSCC patients from North-East Italy. Clinical parameters for each patient were obtained from the clinical database.

HPV DNA was determined by polymerase chain reaction (PCR) with consensus MY09/MY11 primers and Restriction Fragment Length Polymorphism (RFLP) analysis and/or BSGP5+/6+-PCR/Multiplex Papillomavirus Genotyping (MPG) capable of detecting all known 51 mucosal HPV types.

The HPV DNA+ tumor tissues were further analyzed for i) viral load by HPV16 qPCR and quantitative BSGP5+/6+-PCR/MS; ii) detection of HPV E6*I transcripts by RT-PCR; iii) expression levels of cellular protein p16^{INK4a} and pRb by IHC; and iv) presence of HPV E6 protein for types 16 and 18 by the commercial OncoE6TM Oral Test; Antibodies to HPV early and late proteins of the eight most frequent high-risk HPV types were determined in all available sera by bead-based multiplex serology.

Results: Overall, HPV DNA+ was 8.5% (21/247), type 16 was detected in 95% (20 cases) and type 58 in 5% (1 case). No multiple infections were detected. The HPV RNA+ was 6% (14/244). Oropharynx was the site with the highest HPV prevalence by DNA (27%) and RNA (20%). In the other anatomic sites, HPV prevalence was < 8%.

Among the HPV DNA+ RNA+ tumors, i) 93% of the HPV16+ tissues (13/14) showed high viral load; ii) 60% (6/10) showed both up-regulation of p16^{INK4a} and down-regulation of pRb; iii) and in 100% (8/8) HPV16 E6 oncoprotein was detected. All sera of 7 HPV-driven tumors showed strong positive antibody reactions with HPV E6 and E7 proteins, 6 for type 16 and 1 for type 58, type-concordant with the related tumor. Another single serum HPV16 DNA+ in the tumor, showed positivity for all early HPV16 proteins suggestive of an HPV-driven tumor. Kaplan-Meier analyses for the oropharynx showed a trend for better survival in the HPV-associated group than in the HPV negative ones.

Conclusions: A low HPV prevalence was found in HNSCC of the population living in the North-East of Italy. Oropharynx was the preferential site for HPV infection while the HPV prevalence in the other anatomic sites appeared negligible. We observed that the HPV DNA+ RNA+ samples showed a good correlation with the other markers like high viral load, presence of the E6 oncoprotein, and HPV E6 and E7 seromarkers. In contrast to recent reports we did not find a good correlation between HPV DNA+ RNA+ and the up-regulation of p16^{INK4a} and down-regulation of pRb. Survival analyses showed a better prognosis in the HPV-driven patients with tumors occurring in the oropharynx.

II. Sommario

Il papillomavirus umano (HPV), più frequentemente il tipo 16, sono causalmente associati agli tumori squamosi di testa collo (HNSCC). Questi tumori sono caratterizzati da un'elevata eterogeneità geografica e una migliore risposta alla terapia.

L'obiettivo di questo studio è di valutare la prevalenza e l'attività biologica di HPV in HNSCC nel nord dell'Italia. La genotipizzazione *per se* non è sufficiente a definire il ruolo del virus nella patogenesi HNSCC. E' necessario analizzare e verificare la presenza di altri marker diretti come i trascritti virali, la carica virale, oncoE6 proteine e anticorpi HPV e dei marker indiretti come l'espressione delle proteine cellulari p16^{INK4a} e pRb. I risultati ottenuti sono stati alla fine correlati con la sopravvivenza.

Nel presente studio sono stati arruolati 247 pazienti del Nord-Est dell'Italia. Sono stati raccolti biopsie tumorale congelate per tutti i pazienti, e per un sottogruppo dei blocchetti di paraffina e del plasma.

La presenza del DNA virale è stato determinato con i) reazione a catena della polimerasi con primer consenso MY09/MY11 e tipizzazione con digestione enzimatica e/o ii) BSGP5+/6+ -PCR/Multiplex Papillomavirus Genotype (MPG). I casi HPV DNA positivi sono stati ulteriormente analizzati per: i) carica virale (quantitative PCR); ii) presenza dei trascritti virali (E6*I method); iii) l'espressione delle proteine cellulari p16^{INK4a} e pRb (immunohistochimica, IHC); iv) espressione dell'oncoproteina E6 (OncoE6TM kit, AVC); v) anticorpi anti HPV (Multiplex HPV serology).

La prevalenza basata sulla positività del DNA virale era del 9% (21/247). HPV16 è stato trovato nel 95% (20/21) dei casi, 1 HPV58 è stato identificato come infezione singola. La prevalenza basata sul HPV DNA+RNA+ era del 6% (14/244). L'orofaringe era il sito con la più elevata prevalenza di HPV (HPV DNA+ = 27%, HPV DNA+ RNA+ = 20%). 86% (12/14) dei campioni aveva un'alta carica virale per il tipo analizzato; ii) over espressione p16^{INK4a} nel 90% (9/10), down-regulation pRb nel 55% (6/11); iii) la presenza dell'oncoproteina E6 era presente nel 100% (8/8) dei casi testati. La presenza di anticorpi anti HPV è stata valutata in 102 plasmi; 8 su 102 erano positivi per anticorpi HPV, con elevata correlazione con lo status HPV dei relativi tessuto tumorale. Le analisi di Kaplan-Meier per l'orofaringe hanno mostrato un trend di migliore sopravvivenza nei pazienti con tumori HPV positivi per DNA e RNA rispetto ai pazienti HPV negativi.

Bassa prevalenza di HPV nei tumori testa collo nel nord dell'Italia confrontato ad altri paesi. L'orofaringe rimane il sito prediletto dell'infezione per l'HPV (27 %). HPV16 era il principale tipo trovato (95%). Migliore sopravvivenza dei pazienti con tumori HPV positivi.

1. INTRODUCTION

1.1 Papillomaviruses

Papillomaviruses (PV) are small viruses, belonging to the Papillomaviridae family. They are widespread in nature, and have been identified in many animal species ¹. PV are small, non-enveloped DNA (deoxyribonucleic acid) viruses that measure 55 nanometers (nm) in diameter and comprise an icosahedral capsid (Figure 1) composed of 72 pentameric capsomers of the major capsid protein L1, partly associated with the minor capsid protein L2. Enclosed within the capsid is the viral genomic DNA which is packaged as a minichromosome ^{2,3}.

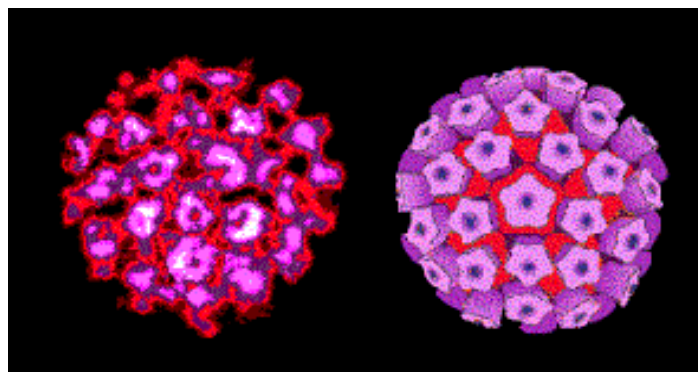


Figure 1. Crystallographic structure of HPV capsid (left) and computer-generated model (on the right) (From: <http://hpvstudy.bol.ucla.edu/hpvbug.htm>).

1.1.1 Papillomavirus taxonomy

PV phylogenetic classification is based on the nucleotide sequence of the open reading frame (ORF) encoding the major structural protein L1, as specified by the International Committee on Taxonomy of Viruses (ICTV). PV of different genera share less than 60% identical L1 nucleotide sequences, PV within a genus share 60 to 70% identity, while an identity between 70% and 90% defines a species. PV subtypes show 90 to 98% and variants more than 98% L1 nucleotide sequence identity. The Papillomaviridae family presently consists of 189 PV types spread over 29 genera (Figure 2) ⁴. The Human Papillomavirus (HPV) types are grouped in 5 genera; the

alpha (α) genus contains the HPV types most frequently associated with human diseases. Based on their tropism, HPV can be separated into cutaneous (skin) and mucosal (genital) types ⁵. Mucosal HPV are mainly sexually transmitted, and the risk of infection increases by increasing number of sexual partners ⁶. Genital HPV infections are among the most common sexually transmitted infections ⁷. Mucosal HPV types are also classified on the basis of their oncogenic potential; according to the last evaluation by the International Agency for Research on Cancer (IARC) expert group, types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 are carcinogenic to humans (Group 1A) and designated “high-risk” (hr)-types; the types 26, 53, 66, 67, 68, 70, 73 and 82 are defined as probable/possibly carcinogenic (Groups 2A and 2B) ⁸. Types found in benign warts, papillomas or non-malignant lesions include HPV6, 11, 42, 43 and 44 amongst others, and are defined as “low-risk” (lr)- types ^{4,9}.

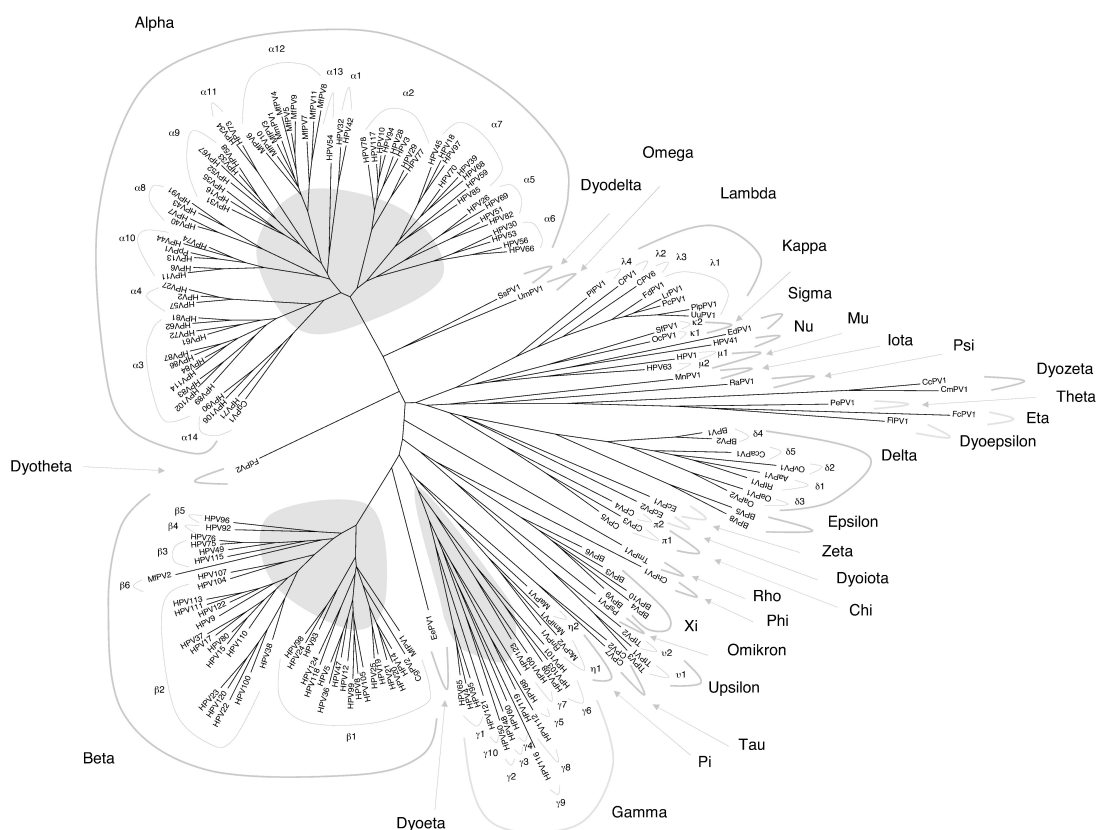


Figure 2. Phylogenetic tree of 189 PV types. The phylogenetic tree was constructed on the basis of L1 nucleotide sequences. Numbers at the end of each branch represent HPV types. See the text for detailed explanation ⁴.

1.1.2 Human papillomavirus

1.1.2.1. Genome organization

The genome organization among mucosal HPV is highly conserved (Figure 3). All HPV types contain a single double-stranded circular DNA molecule of around 8,000 base pairs (bp) replicating as an extrachromosomal plasmid in the nucleus of infected keratinocytes. Up to nine ORF are encoded from the same DNA strand and three functional areas have been identified: i) the long control region (LCR) or upstream regulatory region (URR), that contains numerous regulatory sequence elements, such as transcription factor binding sites, viral E2 binding sites, and the origin of viral DNA replication with binding sites for the viral E1 protein; ii) the “early” region (E), which consists of E1, E2, E4, E5, E6 and E7 ORF, that are involved in viral replication and cell transformation; iii) the “late” region which encodes the L1 and L2 structural proteins^{6, 10, 11}.

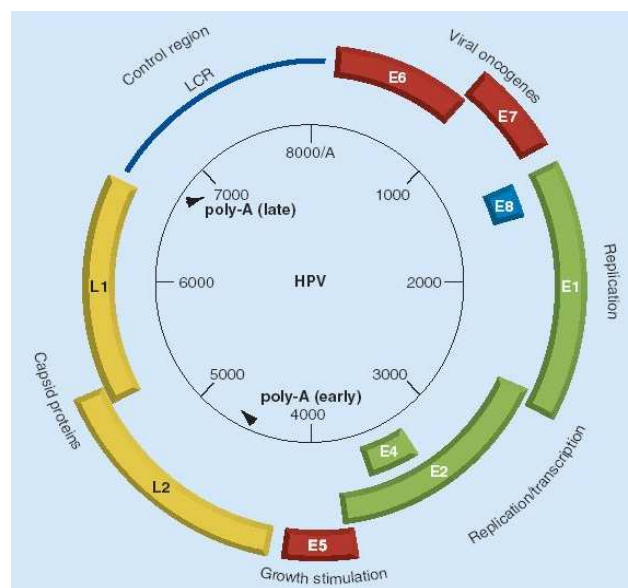


Figure 3. Genome organization of mucosal HPV. Early transcripts utilize an early polyadenylation signal (poly-A early), (downstream of the E5 ORF). Late transcripts use the late poly-A signal (downstream of the L1 ORF). Early proteins E1, E2 and E4 are indicated in green, E5, E6 and E7 viral oncoproteins in red and late structural proteins L1 and L2 in yellow (<http://img.medscape.com/fullsize/migrated/585/223/erm585223.fig2.jpg>).

1.1.2.2. HPV life cycle

Papillomaviruses display an unusual life cycle. Unlike most viruses that infect a target cell and produce progeny virus from that same cell, in papillomavirus infections the initially infected basal cell must undergo mitosis and differentiate to produce new virions. Moreover, they do not encode polymerases or other enzymes necessary for viral replication, relying on the host cell replication proteins to mediate viral DNA synthesis. Two modes of viral DNA replication are recognized, i) the reproductive replication, where the virus enters the lower portion of the epidermis and the plasmid is maintained by low level replication in synchrony with the cell and the ii) vegetative replication, which occurs in the more differentiated epithelia cells in the absence of cellular DNA synthesis.

The mechanisms regulating the switch from plasmid maintenance to vegetative viral DNA replication are not known, and may involve changes in cellular and/or viral factors occurring in differentiating keratinocytes ¹².

Infection by HPV requires the virus particles to gain access to and enter in the cells of the epithelial basal layers (Figure 4). Binding and entry of the virus are mediated through the viral coat proteins (L1 and L2) and heparin sulfate proteoglycans (HSPGs) and/or $\alpha 6\beta 4$ integrins (way not conserved among the HPV types) ^{13, 14}. Virus uptake mostly occurs by clathrin-coated endocytosis (not conserved among all HPV types) ¹⁵, disassembling of the viral particle in the late endosome/lysosome and transfer of the viral DNA to the cell nucleus (facilitated by L2 protein) ¹⁶.

Expression of the early proteins E1 and E2 establishes the viral genome as an episome and leads to the expression of the other early proteins (E4, E5, E6 and E7). This phase is characterized by a low HPV copy number (approximately 10 to 200 viral copies per cell) ¹⁷⁻¹⁹.

Upon cellular differentiation, a late viral promoter is activated and drives expression of late proteins ^{20, 21}. Expression of L1 and L2 is restricted to cells of the granular layer with viral particle assembly taking place in the cornified layer. In this phase the viral copy number reaches 1000 viral genomes per cell ².

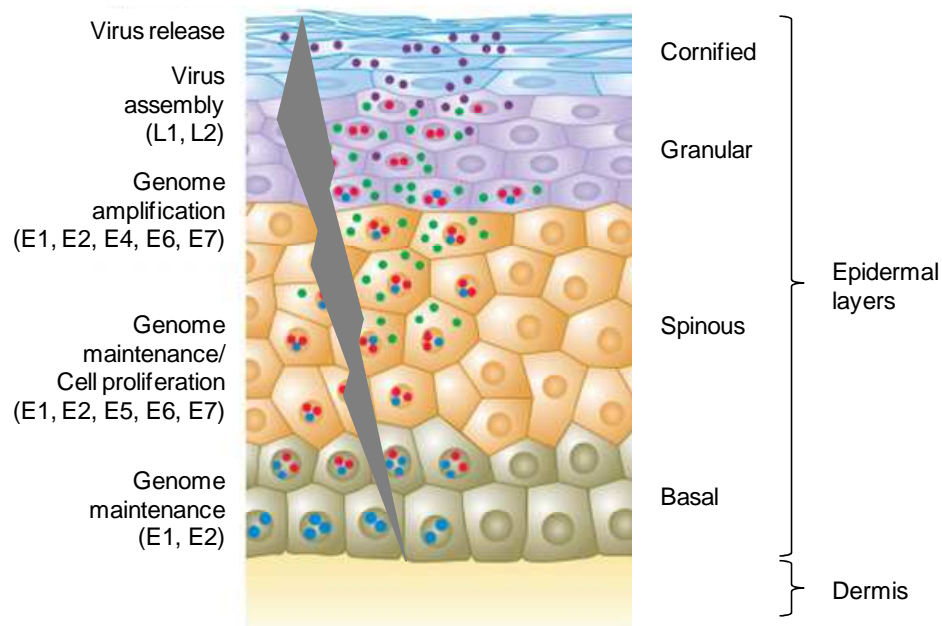


Figure 4. HPV life-cycle organization and viral protein expression in the cervix. Modified from ²². Blue circles, cells expressing early E1 and E2 proteins. With cell proliferation expression of viral oncoproteins E5, E6 and E7 increases (red circles) and later also of E4 protein (green circles). Purple circles represent viral L1 and L2 particles required for assembly of infectious viral particles (courtesy of G. Halec).

1.1.2.3. HPV transcription, transcripts and proteins function

Regulation of HPV transcription is complex and differs between genotypes. Since HPV16 is the most studied and the leading type in HPV-carcinogenesis, its transcription mechanisms are presented (Figure 5).

HPV16 gene expression is regulated by at least two promoters (p97 and p670) ^{23, 24}, multiple splice donor and acceptor sites, two polyadenylation signals and further by post-transcription mechanisms. All transcripts are polycistronic and synthesized from the same DNA strand. Depending on splicing events, up to three different reading frames can be employed for translation. The early and late poly-A signals are located at nucleotide (nt) 4215 and 7321, respectively. Maturation of viral RNA involves complex splicing. The HPV16 transcriptome exhibits several splice donor giving rise to at least 11 different splice junctions ²⁴⁻²⁸. The detailed functions of the various spliced transcripts are still under investigation. Spliced transcripts can be translated into truncated proteins exhibiting important functions for the viral life cycle regulation ^{29, 30}.

In the hr-HPV types, differential expression of E6 and E7 is mediated through a single primary transcript that is alternatively spliced in the E6 gene. While the E6 full-length (fl) transcript encodes for both E6 and E7 proteins, the spliced transcripts E6*I and E6*II encode for E7 only and truncated E6* proteins. Since E7 is the most prominent HPV oncoprotein^{31, 32} these transcripts have been intensely studied. Increased levels of E6/E7 mRNA correlate with cancer progression³³. To better understand the role of each E7 coding transcript during cancer progression, quantification of expression profiles from E6*I, E6*II, and E6/E7 fl transcripts has been described for pre-malignant lesions³⁴⁻³⁷, cervical cancer³⁷ and tumor-derived cell lines^{34, 38, 39}. Spliced E6 transcripts might play a role in HPV type carcinogenicity; they have been described for the carcinogenic but are not found in non-carcinogenic HPV types⁴⁰⁻⁴².

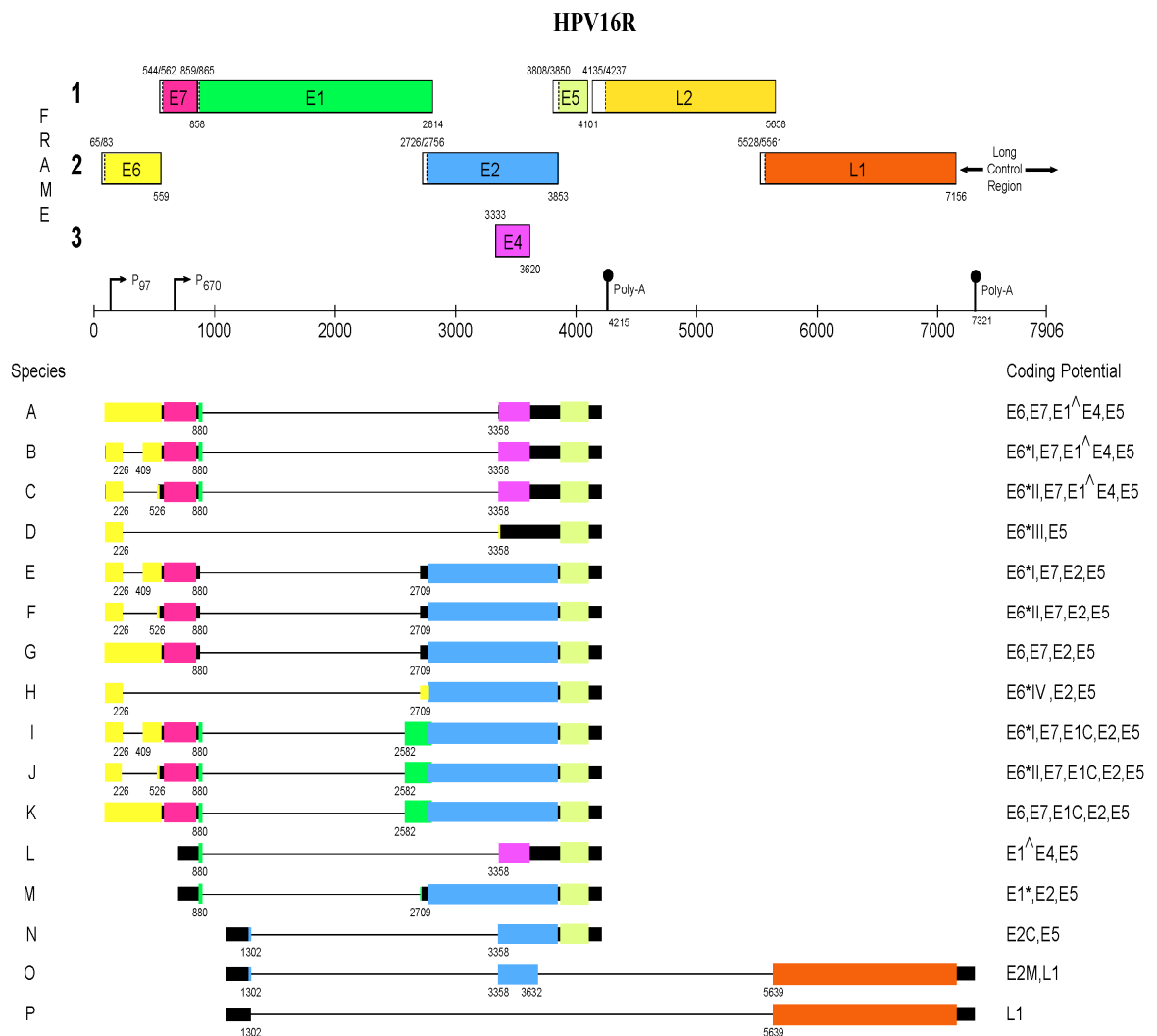


Figure 5. Transcription map of HPV16R (RNA). ORF of each gene are shown in their proper reading frames as colored rectangles (top of the figure). The first number at the upper left end of the rectangles corresponds to the nucleotide (nt) position of the ORF start, *i.e.* the first nt following a stop codon. The second number is the nt position of the first ATG, which is also indicated by a dotted line within the rectangle. The position of the last nt in the stop codon of each ORF is printed at the lower right corner of the rectangles. Located below the genome scale are diagrams of spliced mRNA species and their coding potential at the right. Non translated exons are illustrated by black rectangles, while intervening introns are indicated by black hairlines. Colored rectangles indicate in-frame sequences that can potentially code for proteins. Numbers printed below the lines indicate the 5' and 3' splice junction positions. The promoter for the last three transcript *i.e.* species N-O has not been mapped. Transcripts encoding full-length E1 and L2 protein are not depicted. Potential truncated gene products of E6 and E1 are indicated by asterisks (*). The fusion product of the E1 and E4 protein is indicated as E1^{*}E4. Source ⁴³. (from <http://pave.niaid.nih.gov/images/transcript/HPV16.png>)

The E1 protein is required for viral DNA replication, has a helicase activity and binds to cellular proteins, including RPA (replication protein A) and DNA polymerase α primase⁴⁴⁻⁴⁷.

The E2 protein is involved in the viral DNA replication and segregation⁴⁸. E2 is a DNA-binding protein recognizing a palindromic motif in the non-coding region of the viral genome. Via a protein-protein interaction, E2 recruits E1⁴⁹. E2 can also be active as a transcription factor that regulates the viral early promoter p97 causing auto-regulation of E2 expression and regulation of the expression of viral oncogenes (E6 and E7). At low level, E2 is a transcriptional activator, whereas at high level, E2 represses oncogene expression⁵⁰.

The E4 ORF is located in the early region and its gene product, E1^E4 is only expressed in differentiated, upper epithelial layers and plays a role by binding to proteins of the keratin cytoskeleton during virus assembly and virus release⁵¹. It was proposed that E1^E4 and E5 (see below) oncoprotein may cooperatively foster viral activities since E5- and extracellular signal-regulated kinase (ERK) phosphorylation lead to an increase in E1^E4 abundance and stability in infected cells⁵².

The E5 protein is one of the three oncoproteins encoded by the virus but its biochemical role and precise contribution to human cell transformation are still unclear. Is a transmembrane protein that predominantly remains in the endoplasmic reticulum (ER). E5 can delay the process of endosomal acidification⁵³⁻⁵⁵, and this leads to an increase in EGF (epidermal growth factor)-mediated receptor signaling and the maintenance of a replication competent environment in the upper epithelial layer⁵⁶, by affecting the recycling of growth factor receptor on the cell surface. It has been shown that E5 expression is lost during the integration of HPV DNA into the cellular genome⁵⁷. Since in cervical cancer viral integration occurs during progression, E5 is probably not necessary in the late events of HPV-mediated carcinogenesis.

E6 and E7 are the major oncoproteins in HPV cell transformation.

The E6 protein is translated only from RNA containing the full E6 ORF (Figure 5, species A, G, K). HPV16 E6 protein promotes proliferation of infected cells leading to their resistance to apoptosis and can induce cell immortalization. E6-driven cell immortalization and p53 reduction was demonstrated also for BPV-1 (Bovine papillomavirus type 1) and non-carcinogenic HPV6 but with lower efficiency and requiring a longer time period in comparison to HPV16 E6⁵⁸. Four spliced RNA species all using the splice donor at nt 226 and splice acceptors at nt 409 (E6*I), nt 526 (E6*II), nt 3358 (E6*III) or nt 2709 (E6*IV)^{42, 59, 60} can generate two internally truncated E6 protein variants (E6*I and *II) or truncated forms fused to short non-E6 sequences (E6*III or *IV). E6*I transcripts give rise to the truncated E6*I protein and the full E7 protein and have been considered the characteristic of carcinogenic versus non-carcinogenic HPV types⁴² because E6*I transcripts have been described for 14 HPV types that occur in cervical cancer as a single infection^{24, 61-64} but are not generated by mucosal HPV types not associated with cervical cancer^{41, 42}. E6*I protein is translated from 226-409 in HPV16 E6 ORF.

The fundamental action of E6 protein (158 amino acids) of transforming mucosal HPV types is its ability to degrade p53, through combined activity of an ubiquitin ligase called E6-associated protein (E6AP)⁶⁵ (Figure 6). This is achieved by formation of E6-E6AP-p53 protein complex that shortens the p53 half life from 3 hours to 20 minutes and more importantly, blocks apoptosis. The ability to bind and degrade p53 was demonstrated for majority of the HPV types from the hr-clade (hr HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59⁶⁶⁻⁶⁹). E6 proteins from lr-HPV types do not bind p53 at detectable levels and have no effect on p53 stability in vitro⁷⁰. An important feature of hr-E6 proteins is the presence of a PDZ-binding motif at the C-terminus, the feature that is not shared with lr-HPV types. Multiple PDZ-binding partners have been described, all of which are degraded by proteasomes (*e.g.* Dlg, Scribble, MAGI-1, 2, 3). The primary functions of PDZ proteins involve the regulation of cell growth, cell polarity, and cell adhesion in response to cell contact. It remains to be determined, however, which of E6-PDZ interactions are functionally important in vivo⁷¹.

The E6 protein also activates the protein telomerase in differentiated cells resulting in an increased life-span of the affected cells. The precise mechanism by which this occurs is still unclear. E6 interacts with several other proteins, such as SRC kinases⁷² and the pro-apoptotic BAK⁷³, thereby enhancing its proliferative and anti-apoptotic effect.

E7 protein is translated from both the full E6/E7 mRNA^{24, 74, 75} and from alternatively spliced E6*I transcripts (species B, E, I)^{42, 59, 60}. The E7 protein is considered to be transcribed also from E6*II mRNA (species C, F, J) but with a lower efficiency⁷⁶. E6*II transcripts were found in cell lines⁷⁷⁻⁷⁹ and precancerous lesion^{80, 81} and cervical cancer⁸¹ of HPV types 16 and 18 only. Of other HPV types, E6*II of HPV33 have been identified but in tonsillar carcinoma⁸².

The E7 oncoprotein is the crucial HPV protein in a transgenic mouse model leading to cervical cancer development by inducing cell immortalization and transformation⁸³. This nuclear, 100 amino acids protein promotes G₁-to-S phase (Figure 6) progression in HPV infected cells by targeting numerous cellular substrates for proteasome-mediated degradation⁸⁴. One of the most eminent functions of E7 is binding to the retinoblastoma tumor suppressor family members: p105 (pRb) and its related “pocket proteins” p107 and p130⁸⁵. The pRb proteins regulate G₁-to-S phase transition in the cells by binding to the E2F transcription factors⁸⁶. Many genes that are involved in regulation of the cell cycle progression, mitosis, and apoptosis contain E2F binding sites in their promoters⁸⁷. The E7 protein binds pRb over its LXCXE motif in the N-terminal half of the protein which results in E2F release and constitutive expression of E2F-responsive genes. Upon E7-pRb binding, pRb protein is targeted for proteasomal degradation. Moreover, E2F responsive genes, cyclin A (CyA) and E, (CyE) complex with cyclin-dependent kinase 2 (CKD2) and further promote pRb phosphorylation and E2F release.

In addition to pRb binding E7 can also directly interact with E2F factors *e.g.* E2F1 and E2F6 in order to maintain the S phase environment conducive for viral replication^{88, 89}. Another important action of E7 is binding of histone deacetylases (HDACs) and therewith facilitating activation of transcription⁹⁰⁻⁹². Moreover, E7-pRb-HDAC interaction is shown to be important for episomal maintenance and maintenance of S

phase environment, and therefore is considered a crucial event for successful viral replication in suprabasal cells^{90,92}.

Further E7 cellular targets, tumor suppressor proteins p21 and p27, bind to the carboxy-terminus of hr-E7 oncoproteins which efficiently neutralizes their inhibitory effects on CyE- and CyA/CDK2 pRb phosphorylating activities and promotes cell growth and proliferation^{93,94}.

The hr-E7 expressing tumor cells disrupt anoikis, a form of programmed cell death, through interaction of E7 with the pRb-associated protein p600 that functions as an ubiquitin ligase⁹⁵. P600 is a cytoplasmic protein, suggesting that E7 can target cellular factors in the cytoplasm and in the nucleus.

The lr-E7 proteins *e.g.* HPV-6 E7, bind to pRb family members with an affinity that is about 10-fold lower than that of hr-E7 proteins⁹⁶.

L1 is encoded from transcripts initiated at p670, either unspliced or spliced between splice donor nt 1302 and splice acceptor nt 5639 (species P) (described in W12 cells²⁸) or nt 3632 (species O). Cell binding studies with non-infectious virus-like particles (VLP) that self-assembled from overexpressed L1 or both L1 and L2, showed similarly binding characteristics, implying that L1 contains the major determinants for initial attachment^{97,98}.

L2 protein is produced only from unspliced late transcripts. During late stages of the productive infection the major capsid protein, L1, and the minor capsid protein, L2, are expressed in differentiated cells near the top of the epithelium. They form the viral capsids in the granular layer and virions are believed to be released in a non-cytolytic manner. L1 protein has been found only in the upper layers of the epithelium. However, L1 and L2 RNA can also be detected in lower epithelial layers^{99,100} indicating that their protein expression is also regulated post-transcriptionally.

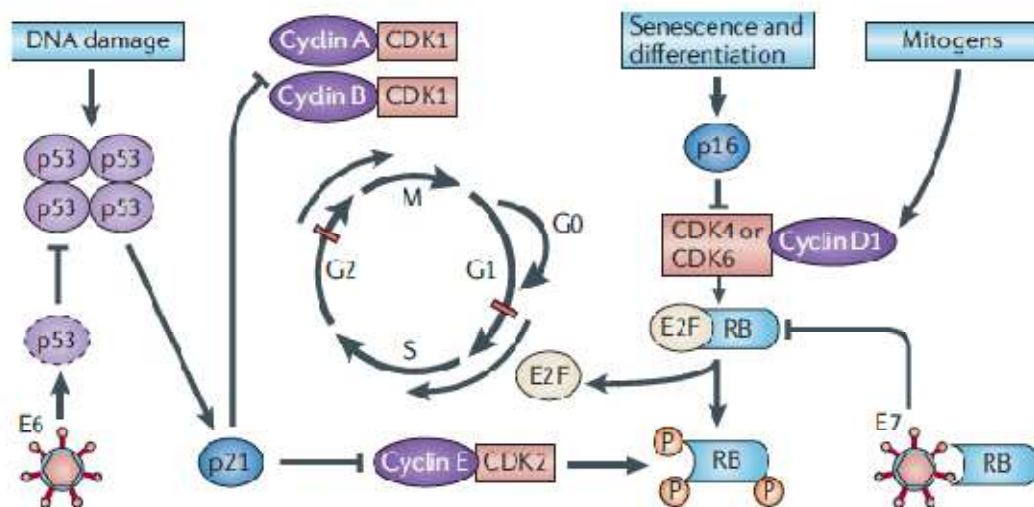


Figure 6. Cell cycle deregulation by HPV infection. The cell cycle is regulated by complexes of cyclins and CDKs. Those complexes are in turn regulated by CDK inhibitors, such as p16 and p21. The two cell cycle restriction points are depicted as red lines. The HPV16 protein E6 binds cellular p53 leading to its degradation. In contrast, cellular pRb is inactivated by HPV16 protein E7. Thus, viral oncoproteins lead to entry into S-phase resulting into cell proliferation. In addition, p53-mediated apoptosis is inhibited making viral replication possible ¹⁰¹.

1.2 Head and neck carcinomas

1.2.1 Epidemiology

Head and neck cancers (HNC) include tumors which arise from oral cavity, nasopharynx, oropharynx, larynx and hypopharynx.

Worldwide, HNC represent the sixth most malignant tumor. Yearly, 500.000 new cases are estimated, where approximately 50% of the patients die due to the disease ¹⁰².

The incidence of HNC varies considerably throughout the world as illustrated in Figure 7 ¹⁰³ with incidence peaks in Southeast Asia, parts of Central and south-western Europe (Spain, France) and Brazil, and low incidence figures in Japan, China and West Africa.

The observed incidence variation of HNSCC appears mainly to be due to a variation of carcinogen exposure intensity (tobacco smoking, alcohol, HPV infection, betel quid, oral hygiene).

In the developed world, high incidence figures of HNC in certain regions including Western Scotland and Eastern Europe/Russia are attributed to heavy tobacco and alcohol consumption¹⁰⁴, while consumption of Betel nut/Betel quit (beside tobacco and alcohol) is an important contributor to the high incidence figures in the developing world, particularly South East Asia (up to 75% of the population).

In addition to geographic variation, the incidence of HNC has been characterized by significant temporal variation over the last 50 years. Overall incidence rates show a declining trend in both sexes in India, Hong Kong, Brazil and USA whites¹⁰⁵, while an increasing trend is observed in most other populations, particularly in Central and Eastern Europe, Scandinavia, Canada, Japan and Australia.

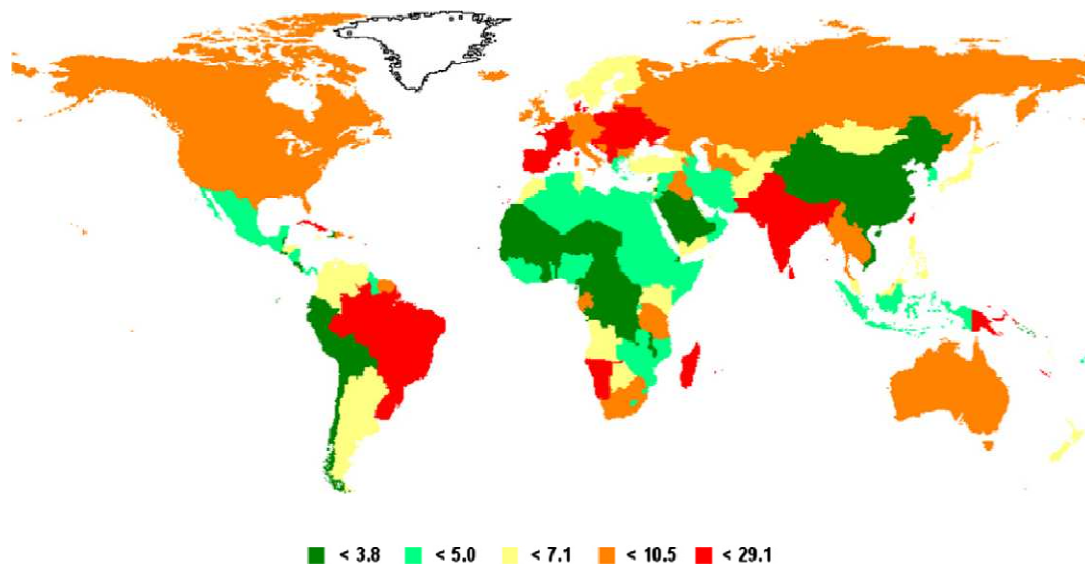


Figure 7. Geographic distribution of head and neck cancer. Estimated age-standardized incidence rates per 100,000, both sexes, all ages, Globocan 2008.

In Italy, head and neck tumors represent about 5% of all malignant tumors, and rank 5th infrequency. Each year about 12,000 new cases are diagnosed. The incidence rate (standardized to the European population) is 16 cases per 100,000 Italians per year, while in Europe (EU countries) it is equal to 18 per 100,000. The incidence rates are higher in the northern regions of Italy than the central and southern areas and islands.

In Italy, the risk of developing HNC is 7 times higher in males than in females; the incidence is particularly high in Veneto with a rate of 48 per 100,000 in men and 8 per 100,000 in women (ratio 6:1). The most affected age group is between 50 to 70 years old ¹⁰⁶.

1.2.1 HPV related to head and neck carcinomas

1.2.1.1 Incidence and risk factors

A worldwide systematic review from Kreimer and collaborators, reported an overall HNSCC HPV prevalence of 25.9%. HPV prevalence was significantly higher in oropharyngeal squamous cell carcinoma (OSCC) (35.6%) than oral SCC (23.5%) or laryngeal SCC (24%) ¹⁰⁷. However, a wide variation of HPV-positive OPSCC is reported in the literature, varying from 4.4% up to 93% ^{108, 109}.

While HN-tumors showed a decline/unchanged incidence in the last decades, an increase of the HPV-associated head and neck cancer, particularly for the oropharynx was observed ¹¹⁰⁻¹¹².

In the US population the yearly increase of the new-OSCC was estimated to be 5% ¹¹³. In a recent publication from Denmark, a 6-fold increase of incidence rates for tonsillar carcinoma were reported in male patients for the time between 1978-2007 ¹¹⁴. Norway between 1991 and 2005 reported increasing rates of 5% for males and 4.2% for females ¹¹⁵. Spain, reported an increase of 4.3% from 2000 to 2009 ¹¹².

In the healthy population, the oral HPV prevalence was estimated to 4.5% overall, where HPV type 16 had the highest prevalence (1.3%) among all the other hr-HPV type found ¹¹⁶.

This incidence increase is probably in line with the change of sexual practices. Even though data are still lacking, Herrero et al. ¹¹⁷ showed that oral sex was reported 3 times more often by the HPV-positive group than the HPV negative group in the oropharyngeal cancer. Other risk factors are the number of sexual partners ¹¹⁸, an early beginning of sexual life and homosexual contacts ¹¹⁹. Controversial are the data regarding the association of HPV and smoking in relation to OPSCC carcinogenesis. HPV is an established risk factor for OPSCC in non-smokers but there are a significant

number of HPV positive patients that are ex/light-smokers, and smoking might act as a promoter¹²⁰.

1.2.1.2 Molecular markers in the HPV-associated HNSCC

HPV DNA: most of the techniques in use for HPV DNA detection, are PCR-based techniques, most of them use general (or consensus) primers targeting highly conserved regions of L1¹²¹⁻¹²⁷. However, depending on the material biopsy (fresh frozen tissues and FFPE) a different sensitivity is achieved. For e.g. the MY09/11-RFLP method, which gives a product of 450 bp is approximately 10-fold less sensitive than the BS-L1-MPG₅₁)^{126, 127} or 87.9% sensitivity compared to the HPV type-specific PCR¹²⁸.

HPV RNA: only a subset of HPV DNA positive tumors display carcinogenic activity in the tumor tissue, defined as HPV-driven. Thus, HPV DNA alone is not sufficient to determine the causal involvement of any HPV found in the tumoral tissue. The oncogenic potential of HPV types is related to their ability to express the viral E6 and E7 oncoproteins that are translated from bicistronic or polycistronic mRNAs in infected cells^{42, 60, 76}. Splicing of mRNA transcribed from the E6 open reading frame (ORF) is considered a biological feature of hr-HPV, but not lr-HPV^{41, 42}. Splice sites within the E6 ORF, giving rise to the most abundant E6*I transcript, have been described for all 12 hr-HPV, and for probable hr-HPV types 66 and 68^{24, 61, 63, 64, 82, 129}. Continuous E6/E7 expression is required for the maintenance of the transformed phenotype of HPV positive tumoral cells. Recently, an ultra-short amplicon, E6*I mRNA RT-PCR assay has been described for HPV16^{130, 131}. Advantages of such an assay are applicability to FFPE material and absolute RNA specificity by using a splice-site as identification target.

Cellular markers HPV tumor related: as described (see section 1.1.2.3), the E6 and E7 oncoproteins bind to, and regulate steady state levels of cell cycle proteins p53 and pRb. As a consequence, increased p16^{INK4a} expression occurs as an indirect consequence of E7-induced pRb inactivation (down-regulated)¹³²⁻¹³⁵. Therefore, p16^{INK4a} and pRb are considered surrogate markers of HPV involvement. Their expression in the cells can be easily analyzed by IHC methods. Analyses require FFPE materials.

Other potential markers are HPV-induced antibodies against the HPV proteins. Initial HPV infection induces both systemic and local humoral immune responses^{136, 137}. The serum antibodies (Abs) to L1 capsid protein are induced in 50–70% of infected patients months after HPV infection and are detectable for years after clearance of the infection; they represent past and/or present HPV exposure^{138, 139}. Antibodies (Abs) to HPV16 E6 and E7, were reported as the best markers in invasive cancers^{117, 140-142}. The frequency of virus-like capsid particle (VLP), E6, and E7 seropositivity is directly associated with the presence of HPV DNA in the tumour^{107, 143}. Seropositivity for HPV16 E6 or E7 is strongly associated with the odds of OPC (64% of cases; OR: 58)¹⁴⁴, and predicts an improved prognosis¹⁴⁵. Humoral immunity to other HPV early gene (E1, E2 and E4) products has not been well evaluated, and the relationship of early gene Abs to the pathogenesis and prognosis of OPC and other HPV-related.

1.2.3 Clinical characteristics and diagnostic implication

HPV-induced HNSCC have a peculiar clinical presentation with regard to both tumor and neck stage and characteristics.

Patients with HPV positive HNSCC tend to be younger by approximately 5 years, on average, when compared with HPV-negative HNSCC patients¹⁴⁶⁻¹⁴⁸. With regard to sex, men appear to be at equal risk to women. It is clear that the majority of HPV positive tumors arise largely from the lingual and palatine tonsils in the oropharynx, compared with other anatomic sites of the head and neck^{105, 149}. Compared to HPV-unrelated tumors, HPV-induced carcinomas are generally diagnosed in earlier T-category with a trend for a more advanced N-category¹⁵⁰. Extreme clinical manifestation of HPV-related HNSCC is a neck metastasis from occult primary tumor^{151, 152}. Histopathologically, HPV positive tumors tend to have a poorly differentiated and frequently basaloid histology¹⁵³.

It has been reported a better response of HPV-associated tumors, particularly to radiotherapy (RT). The possible mechanisms underlying immune-enhancing like an increased uptake of antigen-containing dead cells killed by radiation, normalization of tumor vasculature that enhances immune system cells to the tumor bed, production of induces pro-inflammatory cytokines (*like* TNF α), also, the tumor microenvironment, may contribute to the greater radiosensitivity¹⁵⁴.

1.3 Aim of this thesis

Only a subgroup of the head and neck squamous cell carcinomas is truly caused by HPV transformation. It is now well established that HPV DNA alone is not sufficient to determine HPV-causality in HPV-infected cancer tissues. Thus, detection of transcriptional activity is a primary and necessary condition to define HPV carcinogenicity, which also has a relevant impact on detecting truly HPV-associated (or driven) tumors. The first aim of this thesis was to determine the prevalence of the HPV-driven tumors in patients affected by HNSCC living in North-East of Italy.

The RNA material to detect the presence of viral HPV transcripts is not always available (i.e. FFPE blocks), therefore the expression of cellular markers should be analyzed for HPV involvement. The second aim, was to analyze additional viral load, oncoE6 protein, HPV antibodies) and cellular (p16^{INK4a}, pRb) markers, and investigate their association with HPV DNA positivity.

Several studies have reported a better survival of HPV positive HNSCC patients, particularly when the anatomic site involved was the oropharynx. So, the third aim of the project was to evaluate the prognostic significance of HPV-association with clinical outcome (i.e. survival).

2. MATERIALS

2.1 Consumables

Adhesion glass slides (Super Frost® Plus)	Menzel-Gläser, Braunschweig (Germany)
Cover glasses (24x50 mm)	Knittel, Bielefeld (Germany)
Centrifuge tubes (15 ml)	Thermo Scientific, Wilmington (USA)
Centrifuge tubes (CELLSTAR® Tubes; 50 ml)	Greiner Bio-One, Frickenhausen
Filter tips	Starlab GmbH, Ahrensburg (Germany)
Filter washplates (MultiScreen® HTS; 96-well)	Millipore, Bedford (USA)
Glass slideboxes	Neolab, Heidelberg (Germany)
Glass cuvettes (for staining)	Neolab, Heidelberg (Germany)
Glass shuttles (for staining)	Neolab, Heidelberg (Germany)
Microtome blades (Leica 819)	Leica Microsystems, Wetzlar (Germany)
Oligonucleotide beads (SeroMap™ beads)	Luminex Corp., Austin (USA)
Output plates (MagNA Pure 96 Output Plate)	Roche Applied Science, Mannheim (Germany)
PCR plates (µultra Amp Skirted 96 PCR Plates; nuclease-free; 96-well)	Sorenson, Bioscience Inc., Utah (USA)
PCR plates (LightCycler®480 Multiwell Plate96)	Roche Applied Science, Mannheim (Germany)
Reaction tubes (Safe-Lock Reaction Tubes 1.5 ml; nuclease-free)	Eppendorf, Hamburg (Germany)
Reaction tubes (Quali-Low Retention; nuclease- & pyrogen-free)	Kisker Biotech, Steinfurt (Germany)
Sealing foils (MagNA Pure 96 Sealing Foil)	Roche Applied Science, Mannheim (Germany)
Sealing foils (LightCycler® 480 Sealing Foil)	Roche Applied Science, Mannheim (Germany)

Sealing foils (for PCR plates)	Roche Applied Science, Mannheim (Germany)
Thermomix (Vortemp 56) RledImmInkrels	Labnet, Laborsysteme, (Austria)

2.2 Chemicals

Acetic acid (glacial, 100%)	Merck, Darmstadt (Germany)
Acetone absolute	Sigma-Aldrich, Steinheim (Germany)
β -Mercaptoethanol	Sigma-Aldrich, Steinheim (Germany)
Casein (from bovine milk)	Sigma-Aldrich, Steinheim (Germany)
DAB Substrate Kit for Peroxidase	Vector Laboratories Inc., Burlingame (USA)
DAKO-buffer	DakoDenmar
Distilled water (nuclease-free)	GIBCO Life Technologies, Paisley (Scotland)
dNTPs (each 25 mM)	Carl Roth, Karlsruhe (Germany)
EDTA-Disodium (RNAse-free)	Applichem, Darmstadt (Germany)
EDTA (0.5 M)	Applichem, Darmstadt (Germany)
Eosin G (C.I. 45380, formicroscopy)	Carl Roth, Karlsruhe (Germany)
Ethanol absolute	Sigma-Aldrich, Steinheim (Germany)
Eukitt	O. Kindler GmbH, Freiburg (Germany)
Mounting medium (Eukitt®)	Kindler, Freiburg (Germany)
Haemalaun (Mayer´s Haemalaun Solution, ready-to-use solution)	Applichem, Darmstadt (Germany)
Magnesium chloride	Merck, Darmstadt (Germany)
RNAse Away Spray	Molecular Bioproducts, San Diego (USA)

TE buffer, RNase-free, 1x	Acros Organic, Geel (Belgium)
Tetramethylammoniumchloride (TMAC)	Aigma-Aldrich, Steinheim (Germany)
Tris-HCl, 1 M, pH 8.0, RNase-free	Jena Bioscience, Jena (Germany)
Tween® 20	Serva Electrophoresis, Heidelberg (Germany)
xMAP™ Sheath Fluid	Luminex Corp., Austin (USA)
Xylene	Sigma-Aldrich, Steinheim (Germany)
Phenol/Chloroform Carl	Roth, Karlsruhe
Sodiumacetate (NaAc)	J.T. Baker, Deventer (The Netherlands)
N,N,N',N'-Tetramethylethylendiamin (TEMED)	Merck, Darmstadt

2.3 Prepared Buffers

2.3.1 DNA Extraction Buffer

DDL Buffer	45 mM Tris-HCl 0.9 mM Na ₂ EDTA 0.45% Tween® 20 pH 8.0
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2.3.2 Solution and Buffers for Luminex Bead Detection

Detection solution Luminex DNA/RNA	2 M TMAC 75 mM Tris-HCl, pH 8.0 6 mM EDTA, pH 8.0 1.5% Sarcosyl 1 mg/ml Casein
Hybridization solution Luminex DNA/RNA	0.15 mM TMAC 75 mM Tris-HCl, pH 8.0 6 mM EDTA, pH 8.0 1.5% Sarcosyl
Hybridization wash buffer Luminex	0.02% Tween® 20 1 x PBS, pH 7.4

PBS (Phosphate-buffered saline), 1x, pH 7.4	124 mM NaCl 22 mM Na ₂ HPO ₄ 10 mM KH ₂ PO ₄
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2.3.3 Enzymes

Proteinase K (recombinant, PCR grade)	Roche Diagnostics, Mannheim (Germany)
DNaseI (RNase-free)	Qiagen, Hilden (Germany)
Rsa I	Roche Diagnostics, Mannheim (Germany)
Hae	Roche Diagnostics, Mannheim (Germany)
Dde III	Roche Diagnostics, Mannheim (Germany)

2.3.4 Commercial Kits

LightCycler® 480 Probes Master	Roche Applied Science, Mannheim (Germany)
LightCycler® 480 RNA Master	Roche Applied Science, Mannheim (Germany)
Hydrolysis Probes	Roche Applied Science, Mannheim (Germany)
MagNA Pure 96 DNA and Viral NA	Roche Applied Science, Mannheim (Germany)
Large Volume Kit	Roche Applied Science, Mannheim (Germany)
PureLink™ FFPE Total RNA	Invitrogen, Karlsruhe (Germany)
Isolation Kit, Qiagen Multiplex PCR Kit	Qiagen, Hilden (Germany)

2.4 Oligonucleotides and Oligonucleotide probes

PCR primers for BSGP5+/6+-PCR/Multiplex HPV Genotyping^{126, 127} as well as the probes for subsequent hybridization and PCR primers for the viral load determination (qPCR) were established by Dr. Markus Schmitt (DKFZ). All primers were ordered in

HPLC purification quality and all probes had a 5'-amino C₁₂-spacer modification. Primers and probes were obtained from MWG Biotech AG (Ebersberg, Germany). PCR primers and probes for the HPV E6*I mRNA analysis (ultrashort RT-PCR)^{64, 155, 156} were obtained from Sigma-Aldrich (Hamburg, Germany). Backward primers were biotinylated to allow subsequent Luminex-based detection of hybridized amplicons. PCR primers and probes for HPV16 RNA pattern assay were obtained by Roche Molecular Diagnostics (Pleasanton, USA) and Sigma-Aldrich (St. Louis, USA) in HPLC purification quality.

To protect intellectual property, oligonucleotide primers and probe sequences are not shown.

2.5 Laboratory devices

Centrifuge (Haraeus Fresco 17)	Thermo Scientific, Wilmington (USA)
Cooling plate (EG 1150 C)	Leica Microsystems, Wetzlar (Germany)
Horizontal shaker (Unimay 1010)	Heidolph Instruments, Schwabach (Germany)
Light cycler (cobas z 480)	Roche Applied Science, Mannheim (Germany)
Light microscope	Leica Microsystems, Wetzlar (Germany)
Luminex analyzer (Luminex® 200TM)	Luminex Corp., Austin (Germany)
Luminex sheath fluid delivery system (Luminex® SDTM)	Luminex Corp., Austin (Germany)
MagNA Pure 96 System	Roche Applied Science, Mannheim (Germany)
Microtome (RM 2245)	Leica Microsystems, Wetzlar (Germany)
Mini centrifuge (UltraCruzTM)	Santa Cruz Biotechnology, Dallas (USA)
Mini platespinner (MPS 1000)	Labnet Laborsysteme, Ried im Innkreis (Austria)
Multichannel pipette, 8-channel, 20-200 µL	Brand, Roskilde (Denmark)
Pasteur Pipettes	Sigma-Aldrich, Steinheim (Germany)
PCR thermocycler (Mastercycler®)	Eppendorf, Hamburg (Germany)
PCR workstation (captair® bio)	Erlab, Köln (Germany)
Pipettes (PipetmanNeo®)	Gilson-Ambimed, Düsseldorf (Germany)
Pipetting robot (Qiagility)	Qiagen, Hilden (Germany)
Thermomixer (Vortemp 56)	Labnet Laborsysteme, Ried im Innkreis (Austria)

Vaccumwashstation	Millipore, Bedford (USA)
Vortexmixer (Vortex-Genie 2)	ScientificIndustries, Bohemia (USA)

2.6 Software

Luminex 100 IS 2.3 SP1 Software	LuminexCorp., Austin (TX, USA)
Microsoft Office	Microsoft Corp., Unterschleißheim
Microsoft Windows XP	Microsoft Corp., Unterschleißheim
GraphPadPrism® 6	GraphPad Software, Inc. La Jolla, (CA, USA)
SAS 9.3	SAS Institute Inc., Cary (NC, USA)
LightCycler Probe Design software 2.0	Roche MolecularDiagnostics (CA, USA)

2.8 Study participants

A total of 247 samples collected from patients from Northern-Italy were recruited in the present study. Any patient who visited the ENT (Ear Nose and Throat) Unit from 2003-2012, and was diagnosed for the first time with a head and neck squamous cell carcinoma (HNSCC) was enrolled. In the first 3 years (2003-2006) of the project only 1 ENT was involved (Treviso Regional Hospital Treviso), subsequently other 2 ENT Units joined (Hospital of Mirano, Mirano and Hospital of Cattinara, Trieste).

Prior to joining the project the patient was informed from the clinician about the study and signed a written informed consent adapted from D'Souza 2007¹⁴⁴. From each patient a fresh tumor biopsy was collected from the ENT surgeon, before the start of any treatment, A part of the tumor biopsy to, after embedded in formalin and was used for histological evaluation from the the Pathology Department of each ENT units participating the study , while the other part of the biopsy was used as a frozen biopsy for molecular analyses. For a subgroup of the patients serum samples were collected within the same day of tumor biopsy collection.

Four anatomical sites from head and neck region were included, the oral cavity, oropharynx, larynx and hypopharynx. Anamnesis data regarding age, gender, and tobacco and alcohol consumption, treatment and follow-up were collected for each of the patients.

2.9 Ethical clearance

The study, for all the 3 ENT units, was approved by the regional ethic committee for clinical experimentation (CEP) of Treviso with the study codes 345/AULSS9 and 421/AULSS9.

3. METHODS

3.1 Sectioning of tissues

3.1.1 Cryosectioning of fresh frozen tissues

Sections were cut from frozen biopsies using a cryostat (Leica CM1815) as described¹⁵⁷. For each biopsy the first 16µm section was discarded in order to remove surface contaminations and obtain a flat surface. A 4µm section was taken for hematoxylin-eosin (H&E₁) staining, followed by 16µm thick tissue sections (yielding 5 mg of tissue) for DNA analyses and a second section for H&E (H&E₂). The sections were collected in DNaseRNase free 1.5 ml Eppendorf homogenized with a single-used pestle and stored at -80°C until further use. The above sectioning described the sectioning protocol performed at the DKFZ, in Heidelberg, while the sectioning protocol used in Padua, was different regarding the model of the cryostat (Leica CM 1915) and the homogenization with the single-used pestle which was not performed. The HPV DNA positive tumor tissues were re-sectioned for RNA extraction.

To avoid cross-contamination different precautions were taken. Before starting, and after each tissue the cryostat was thoroughly cleaned with acetone and absolute ethanol. For each biopsy a new disposable microtome blade was used. To confirm cross-contamination free sectioning mouse brain tissue was included after each 5th human tissue.

3.1.2 Sectioning of FFPE tissues

All FFPE tissue blocks were sectioned using the Leica microtome as described¹³⁵. The sectioning procedure is described in section 3.1.1.

Prior to sectioning tissue blocks were cooled down by placing them in a -20°C refrigerator for approximately 60 minutes. Tissue sections for DNA and RNA analyses were placed into DNaseRNase free 1.5ml Eppendorf tubes. For H&E staining and immunohistochemistry (IHC) sections heated to 36°C in a water bath were placed on microscope slides and dried in a 37°C oven for 24 hours prior to staining. Contamination controls (rat liver) were constantly included after each 5th tissue block.

3.2 H&E staining

To confirm the histology and to determine the tumor content of the fresh frozen and FFPE tissue blocks biopsies, 4µm sections were stained with H&E. Hematoxylin stains the cell nuclei in blue, while the eosin stains the cytoplasm in red. After the sections were mounted on slides, they were dried for at least 24 hours at room temperature and then placed for 10 minutes in the hematoxylin staining solution. Thereafter, the sections were rinsed with tap water, and bathed for 10 minutes in 1% eosin solution, briefly washed with ddH₂O, dehydrated in an ascending ethanol series (70%, 96% and 100%, x min each step), bathed in xylene and finally covered with a coverslip embedded with Eukitt.

The slides were evaluated by D.H. and L.B. Samples with no tumor cells present in the H&E stained slides, defined as “absent neoplastic cells”, were excluded from the study.

3.3 DNA extraction

3.3.1 Phenol-Chloroform (PC) DNA extraction of frozen tissue sections

The method is based on liquid-liquid extraction, which separates mixtures of molecules based on their differential solubility¹⁵⁸. The PC extraction method was performed only for the samples sectioned in Padua, which protocol did not include the pestle homogenization (see section 3.1.1).

The sections were digested overnight at 37°C after adding 500 µl of TE buffer, 10 µl Proteinase K (10 mg/ml), 25 µl SDS 25%. Following, 500 µl of phenol-chloroform (phenol-chloroform mixture consisting of 100 ml of phenol (Sigma), 10 ml 50 mM TRIS HCl, pH 9.0, 100 ml chloroform) was added to the lysed samples. Subsequent centrifugation of the mixture yields 2 phases: the lower organic phase (containing proteins) and the upper aqueous phase (containing the nucleic acids). The upper phase was transferred to another DNase RNase free Eppendorf tube and 1 volume of Chloroform (Sigma) was added, in order to remove residual phenol. DNA was precipitated by adding 50 µl of Sodium Acetate (3M p.H. 5.2) and 100 µl of 99% cold ethanol, washed once with 70% ethanol and resolved in ddH₂O. The DNA samples were stored at -20°C.

3.3.2 DNA extraction from fresh frozen tissues with MagNAPure 96 kit

DNA extraction was performed according to the manufacturer's recommendations for the *MagNA Pure 96 DNA and viral NA Large Volume Kit*¹⁵⁹. The technique's principle is based on the MagNA Pure Magnetic Glass Particle (MGP) Technology. This DNA extraction method was applied only to the fresh tumor biopsies sectioned at the DKFZ. After the sections were lysed, the DNA were released and the nucleases were denatured. The binding between NA and silica surface of the MGP is favored by the chaotropic salt conditions and the high ionic strength of the lysis/binding buffer (guanidine thiocyanate). MGP with bound DNA were magnetically separated from the residual lysed samples. Unbound substances were removed by several washing steps. Purified DNA was resolved in 200 µl of elution buffer provided in the kit and stored at -20°C.

3.3.3 DNA extraction from FFPE tissue sections

DNA extracted from FFPE samples is usually of lower molecular weight than DNA from fresh frozen samples. The degree of fragmentation depends on the type and age of the FFPE sample and the conditions used for fixation. Isolation of DNA was performed according to the DDL protocol (Delft Diagnostic Laboratory) with some modifications¹⁶⁰. Tissue sections were treated with Proteinase K solution consisting of 1mg/ml Proteinase K dissolved in an *in house*-prepared DNA extraction buffer (DDL buffer) prepared as described in section 2.3.1.

For preparation of Proteinase K solution 25mg of lyophilized Proteinase K was dissolved in 25ml DDL buffer, resulting in a final concentration of 1 mg/ml Proteinase K.

Approximately, 300 µl of Proteinase K solution was added to the samples followed by vortexing, centrifugation and overnight incubation in a thermomix at 56°C (50 rpm, round per minute). Proteinase K was inactivated at 72° for 10 min and samples were spun (8000 x g, 2 min) in order to separate the light liquid paraffin from the dense aqueous phase.

Paraffin-free lysates were transferred into new 1.5ml DNaseRNase free Eppendorf tubes. When no distinct paraffin layer was formed in the lysates, samples were reheated (72°C, 2 min) centrifuged and transferred for a second time. At least one buffer control,

comprising Proteinase K solution without tissue, was included per run (24 tissue samples). The obtained lysates were stored at -20°C until use.

3.4 RNA extraction methods

3.4.1 RNA extraction from fresh frozen tissues

The RNA extraction from homogenate sections was performed using the RNeasy Mini Kit and QIAshredder (Qiagen) following the manufacturer's instructions.

Briefly, 350 μl of lysis buffer (Buffer RTL) were added to the homogenized sections. The lysate was transferred to the QIAshredder column (used for further tissue homogenization) and spun. The lysate was transferred to a new reaction tube, mixed with 350 μl of 70% ethanol, and transferred to the QIAamp spin column where it bound to the silica membrane, followed by a first washing step. To ensure only RNA amplification 10 μl of DNaseI stock solution and 70 μl buffer RDD were gently mixed and applied to the QIAamp column and incubated for 15min at RT. Afterwards, 350 μl of a washing buffer (RW1 buffer) was added, centrifuged at 8000xg, 15s, and the flow-through was discarded. The washing step was repeated twice. To remove ethanol residuals, the QIAamp columns were centrifuged with open lids at maximum speed for 1 minute. The RNA was eluted in 50 μl RNase-free water and stored at -80°C .

3.4.2 RNA extraction from FFPE tissue sections

Total RNA was prepared from FFPE tissue sections using the PureLink FFPE Total RNA Isolation Kit (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions slightly modified by adding DNase I (RNase-Free DNase Set, Qiagen, Hilden, Germany) treatment on the RNA purifying columns during sample processing.

Briefly, 300 μl of Melting Buffer were added to each Eppendorf tube with tissue sections followed by incubation at 72°C to melt the paraffin. After spinning the samples to collect any condensation, 20 μl of Proteinase K (20 mg/ml) was added to each sample followed by incubation at 60°C for 3 – 5 hours until complete lysis. The lysate was transferred into new DNaseRNase free 1.5 ml eppendorf tube (see section 3.3.3). Selective RNA binding to the silica membrane was ensured by prior addition of Binding Buffer to the sample. Unspecific binding to the spin column membrane was

removed by three washing steps with Wash Buffer. DNase treatment (for conditions see section 3.4.1) was carried out between 2nd and 3rd washing step. Extracted RNA was eluted in 50µl of RNase-free water and stored at - 80°C until further use.

3.5 HPV DNA analyses

3.5.1 HPV amplification with MY09/11 PCR followed by RFLP

The MY09/11-Restriction Fragment Length Polymorphism (RFLP) assay can identify 49 different mucosal HPV types^{161, 162}.

The MY09/11 primers amplify L1 region sequences generating an amplicon of 450 base pair (bp) long. In a separate PCR, the sample DNA validity was tested by amplifying cellular sequences (β-globin gene) which gives an amplified product of 268 bp. Sample positivity/negativity, for both PCRs, were defined by visual inspection after PCR product separation in a 2% agarose gel. HPV-positive MY09/11 PCR products were digested with type II restriction endonucleases. These enzymes hydrolyze phosphodiester bonds of double-stranded DNA in a sequence-dependent manner leaving blunt, or 5'-or 3'-protruding ends. Ten µl of the amplified product was digested with 3 different restriction enzymes: RsaI (extracted from the microorganism *Rhodospseudomonasphaeroides*), Hae III (extracted from *Haemophilus aegyptius*) and DdeI (extracted from *Desulfovibrio desulfuricans*) (Roche Diagnostics, Germany). Each of the enzymes gives a specific DNA fragment pattern that after gel electrophoresis of the digested PCR products allows identification of the HPV type/s¹⁶².

3.5.2 BSGP5+/6+-PCR/Multiplex HPV Genotyping

The BSGP5+/6+-PCR/MPG₅₁ assay comprises the BSGP5+/6+-PCR, which homogeneously amplifies ~150 bp from the L1 region all known 51 mucosal HPV types, sequences of the β-globin gene and internal standards¹²⁷ and the MPG hybridization assay with bead-based LuminexxMAP suspension array technology, able to simultaneously detect the 51 HPV types and the β-globin gene^{126, 163, 164}. Briefly, amplification was performed using the Multiplex PCR Kit (Qiagen, Hilden, Germany) using 0.2 – 0.5µM of each BSGP5+ and 5'-biotinylated BSGP6+ primers and 0.15µM of each β-globin primer MS3 and 5'-biotinylated MS10. Following PCR amplification,

10µl of each reaction mixture was hybridized to bead coupled probes as described¹²⁷. Bound biotinylated amplicons were quantified with the Luminex 100 analyzer. For each PCR product the median reporter fluorescence intensity (MFI) of at least 100 beads was computed for each bead set. As described earlier¹⁶⁵, a cut-off value of 5 net MFI to define HPV positivity was applied.

3.6 HPV viral load analysis

3.6.1 HPV viral load from BSGP5+/6+ - PCR/MPG analyses

Quantification of HPV signals was implemented with the MFI values obtained by the BSGP5+/6+ - PCR/MPG₅₁ assay. In detail, for each positive reaction, the relative HPV MFI signal (%) was computed by dividing the measured HPV MFI value with the maximum value detected of this HPV type using saturating amounts of PCR product obtained from direct PCR of plasmid-containing bacterial colonies. Finally, the relative MFI (%) was divided by the measured β -globin MFI value to form a non-descriptive viral load value (%HPV MFI/ β -globin MFI). High viral load was assessed for all HPV types by a HPV type-independent high viral load cut off (0.0007 units) correlating to 0.5 HPV copies per cell, as recently described^{163, 166}.

3.6.2 HPV16 ultra-short quantitative PCR (viral load determination)

Viral load of HPV16 DNA positive samples was determined by TaqMAN probes-based quantitative real-time PCR (qPCR) assay (Schmitt et al. manuscript in preparation). In intact probes the reporter fluorescence signal is suppressed, due to closeness of the reporter to the quencher (Figure 8). During PCR the hybridized probe is hydrolyzed by the 5' nuclease activity of the polymerase resulting in separation of quencher and reporter dye. Hereby the quencher cannot repress the reporter activity anymore, leading to fluorescence emission after excitation with a particular wavelength. Generated signals are expressed as crossing point (Cp) values indicating the particular cycle with fluorescence above cut off.

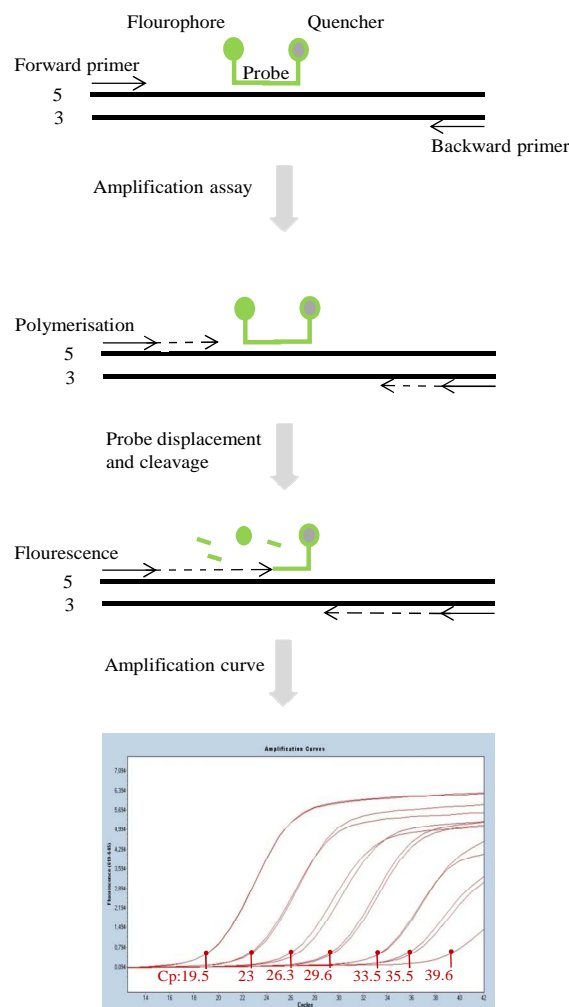


Figure 8. Schematic principle of quantitative PCR using TaqMan probes and the sigmoidal profile of fluorescence accumulation across cycles during amplification (courtesy of D. Höfler).

The HPV16 qPCR assay used to determine the viral load, involves a multiplex detection of a 104 bp amplicon of the HPV16E6 and a 110 bp amplicon of the human β -globin sequence.

The PCR reactions were performed in a 10 μ l volume containing 5 μ l of 2x Light Cycler Probes master mix (Roche Diagnostics, Mannheim, Germany), 0.5 μ M of each respective primer, 0.2 μ M of each probe, and 1 μ l DNA template. The amplification conditions were 10 min at 95 $^{\circ}$, followed by 45 cycles of 95 $^{\circ}$ C for 10 s, 60 $^{\circ}$ C for 30 sec and 70 $^{\circ}$ C for 1 sec.

Standard curves for HPV16E6 were obtained by amplification of a dilution series of 1×10^6 to 1 copy of a plasmid clone containing the HPV16 full-length genome in 50ng/ μ l of human placenta DNA.

The standard curve for β -globin was obtained by a dilution series, ranging from 100 to 0.01 ng of human placenta DNA.

Absolute quantification of genome copy numbers was achieved by linear regression analysis comparing the C_p values of the unknown sample against the standard curve with known copy numbers. The predefined cut-off for high viral load was 0.5 HPV genome copies/cell, and samples with less than 100 cells (cut off) were considered as invalid samples and were not evaluated for statistical analyses. The detection limit of the assay is less than 100 HPV16 genome copies.

3.7 HPV RNA analysis

3.7.1 E6*I mRNA RT-PCR assay

The ultra-short E6*I mRNA reverse transcription (RT)-PCR assay was performed using the QuantiTect® Virus Kit (Qiagen, Hilden, Germany) as described previously¹³¹. The kit offers highly sensitive RT-PCR analyses of viral nucleic acids and internal controls using sequence-specific probes.

Primers that amplify an ultra-short 65-75 bp sequence of cDNA across the E6*I splice site for 20 HPV types, allowing the detection of 12 high-risk and 8 possibly high-risk HPV types. As internal quality control, splice-specific detection of cellular housekeeping-gene ubiquitin C cDNA was included.

One μ l of extracted RNA was applied for cDNA synthesis. Biotinylated primers generated by PCR (HPV E6*I and ubiquitin C) were hybridized to type- and splice site-specific oligonucleotide probes coupled to fluorescence-labeled polystyrene beads (Luminex suspension array technology) detectable with Luminex readers. In total 2 different bead sets were used; one for E6*I splice site specific probe for each HPV type tested, and one for the ubiquitin C probe. Results were expressed as MFI of at least 50 beads per set. To define positive results a cut-off value of 5 net MFI was applied. Analytical sensitivity determined by a 10-fold in vitro transcript dilution series, was at or below 100 transcript copies per reaction per 18 HPV (16, 18, 31, 33, 35, 39, 45, 51,

52, 56, 58, 59, 26, 53, 66, 68, 73 and 82) types and Ubiquitin C; specifically for the types 16 and 58 sensitivity was 10 transcript copies per reaction.

3.8 Multiplex serology

Multiplex serology (MS) is a high-throughput technology that allows the quantification of specific antibodies against up to 100 different antigens in parallel. The method is a glutathione S-transferase (GST) capture immunosorbent-based assay in combination with fluorescent bead technology as previously described¹⁶⁷⁻¹⁶⁹. The method was performed for the early (E6, E7) and late (L1) proteins of high-risk HPV types 16, 18, 31, 33, 45, 52 and 58 and low-risk types 6 and 11 and for the E1, E2, and E4 proteins of types 16 and 18.

Briefly, the method employs beads derivatized with glutathione, thereby permitting bead-mediated *in situ* affinity purification of viral antigens bacterially expressed as GST fusion proteins. Spectrally distinct bead sets carrying different viral antigens were individually washed and subsequently mixed. Each diluted serum in preincubation buffer and mixed beads were combined and incubated. Bound antibodies were detected with biotinylated anti-human secondary antibody (goat anti-human IgA, IgM, IgG (H+L), Dianova) and fluorescent detection conjugate (streptavidin-R-phycoerythrin, Molecular Probes). Reporter fluorescence of the beads was determined by a Luminex-100 analyzer, and expressed as median fluorescence intensity (MFI) of at least 100 beads per set per serum. For background determination beads were loaded with GST alone.

The cut-off was determined as described in^{170, 171}. Briefly, a reference set of samples belonging to a self-declared sexually naive and genital HPV DNA negative group, was used. For each of the HPV seromarkers the cut off was defined as the mean of the MFI values from the reference samples + 5 standard deviations.

3.9 Immunohistochemistry

The expression level of cellular proteins p16^{INK4a} and pRb was analyzed by immunohistochemistry (IHC) using monoclonal antibodies. The staining principle is shown in Figure 9.

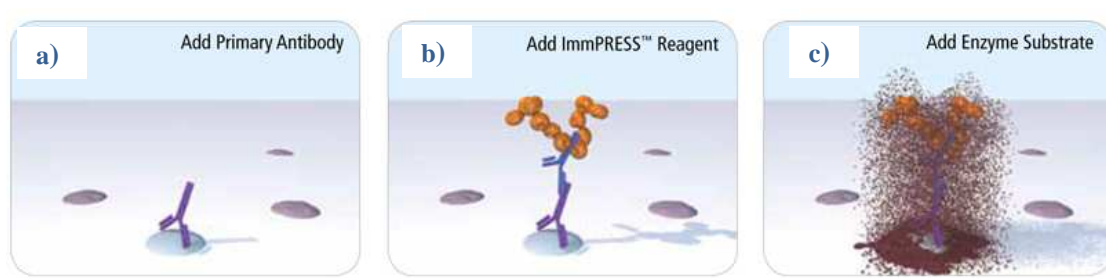


Figure 9. Immunohistochemistry staining procedure. a) sections are incubated with the primary antibody after previous blocking with normal horse serum; b) after washing steps the sections are incubated with a secondary antibody peroxidase-conjugated; c) development with a peroxidase substrate (adapted from Vectorlab website ¹⁷²).

The IHC staining was processed manually as follows: sections were deparaffinized in xylene, rehydrated in a descending alcohol series, boiled in 10 mmol/l of citrate buffer for 30 min to unmask antigens, blocked in normal serum (ImmPRESSKit ready to use) for 20 min, and then incubated with the primary antibody. Further, sections were incubated with the secondary antibody (Vector: ImmPRESS Kit mouse, ready to use) for 20 min at room temperature, developed with 3,3'-diaminobenzidine (DAB) from 3 to 5 min, counterstained with hematoxylin, dehydrated in an ascending alcohol series, and covered with a coverslip embedded with Eukitt (section 3.2). Primary antibodies used were: CINtec for p16^{INK4a} (V-kit, MTM laboratories, Heidelberg, Germany) incubation for 75 min at room temperature; pRb (NCL-RB, Novocastra, Newcastle upon Tyne, United Kingdom) incubated for 60 min at room temperature in dilution 1:35.

Stained sections were evaluated by two investigators (L.B. and D.H.) by scoring the percentage of stained tumor cells over the whole tumor section. Discordant cases were discussed together at the microscope and a consensus was reached. For the simplicity of evaluation only two categories of deviation from normal expression were assigned; protein down-regulation or up-regulation.

The cut-off for protein up- or down-regulation was determined by evaluation of the staining intensity and stain distribution in healthy uvula analyzed in parallel with cervical carcinoma (CxCa) tissues¹³⁵. Nuclear and cytoplasmic p16^{INK4a} was absent in the healthy uvula and up-regulated in the CxCa. Nuclear pRb was abundant in the healthy uvula, and expressed in < 25% of the tumor cells of the CxCa. Tumor sections with CxCa-like staining pattern and intensity were classified as down-regulated pRb.

3.10 OncoE6 kit (Arbor Vita)

The OncoE6TM Oral Test allows the detection of the E6 oncoprotein of high-risk HPV types 16 and 18 in different types of samples (like, saliva, cervical smears, swab, homogenized fresh tissues)^{173, 174}.

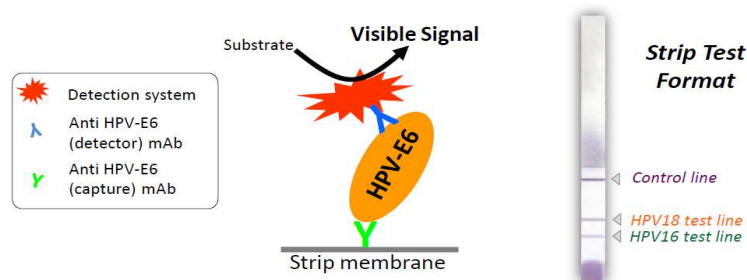


Figure 10. Principle of the assay (source: Arbor Vita Corporation)

The assay was applied to fresh frozen tumor sections (see section 3.1.1). Figure 13 shows the kit's principle assay. The specimen was prepped sequentially by treating the swab with a lysis solution (15 minutes), a condition solution (15 seconds), and then clarifying the specimen solution using a table-top microcentrifuge (10 minutes at 8000 rpm). A 200 μ l aliquot of the clarified specimen solution is then transferred into a vial with lyophilized detector monoclonal antibody alkaline-phosphatase conjugate. The test strips with immobilized capture monoclonal antibodies are inserted into specimen-conjugate mixture and the solution is permitted to migrate up the strip by capillary action. After 55 minutes, the tests are washed for 12 minutes and then immersed into the developing solution containing the alkaline-phosphatase substrate (NitroblueTetrazolium). After 15 to 25 minutes (depending on the ambient temperature), the test unit is removed from the developing solution vials and placed on

a reading guide, allowing for visual inspection. Appearance of one or more test lines indicates E6 oncoprotein of the corresponding HPV type present in the initial cervical swab specimen.

The analytical sensitivity for E6 protein for HPV type 16 was determined by using dilution series (from 10^5 to 10^0 cells) of the cell line MRI-H186 (HPV16 positive) combined with 10^6 cells from the cell line HEK293T (HPV negative), to mimic the tumor sample condition. One diploid cell contains 6.66 pg of DNA. The mean DNA concentration from our samples measured by a spectrophotometer (Nanodrop ND1000 formula $50 \times OD_{260}$) was 50 ng/ μ l (50 ng in 1 μ l). Therefore in 50 ng (5×10^4 pg) of DNA contains approximately 7575 cells in 1 μ l volume. The assay uses a lysate of 200 μ l aliquot thus, we expect approximately 1.5×10^6 cells (= 7575 cells x 200 μ l).

3.11 Statistical analyses

Patient and tumor characteristics were evaluated in relation to their HPV DNA and RNA status. Follow-up was determined as the time-difference between the date of the last clinical examination and the diagnosis date.

Overall survival (OS) was measured as the time from the date of primary tumor diagnosis to the date of death. Survival times of patients who were alive at date of last follow-up were censored.

Progression-free survival (PFS) time was calculated from the date of primary tumor diagnosis to the date of the first local recurrence, new lymph node or distant metastasis, second primary carcinoma. Survival times of patients who were alive at the date of last follow-up or dead due to HNSCC-unrelated tumor were censored. The method of Kaplan and Meier was used to estimate survival distributions. HPV-associated and HPV negative groups were compared using the log-rank test. In all statistical tests a P value of 0.05 or below was considered as statistically significant. The statistical analyses were carried out using SPSS, version 17, as well as the software Sigmaplot.

4. RESULTS

Two-hundred-forty-seven (247) consecutive patients with newly detected head and neck cancer were enrolled in the North-East area of Italy between 2003 and 2012. Tissue samples (247 fresh tumor biopsies and 53 FFPE blocks) were available for all patients and serum samples for only 102 (41%) since serum collection was possible only in the last three years of the project (2010-2012). All tumor samples of the patients were firstly analyzed for HPV DNA status. HPV DNA positive tumors and some HPV DNA negative tumors as negative controls were further characterized for viral load, the presence of viral transcripts (E6*I), up-regulation of p16^{INK4a} and down-regulation of pRb, presence of the oncoprotein E6. HPV seromarkers were determined for all available sera.

4.1 Clinicopathologic characteristics of the study population

Of the enrolled 247 head and neck tumor patients 76% were males, in a ratio 3:1 compared to females (24%) and a median age at diagnosis of 66 years (range 27-95) (Table 1).

More than 50% of the patients were current and another 15% were past tobacco smokers. Current alcohol consumption was reported by 53% of patients and another 12% had this habit in the past.

Of the tumors 39% were in the larynx, 26% each in the oropharynx and the oral cavity, and 10% in the hypopharynx. Small-sized (T1-T2) tumors were present in 50% of the patients and lymph nodes were involved in 52%. Only 1% of patients had distant metastases.

The patients received treatment with various modalities: 35% underwent surgery alone, 29% received radio- and/or chemo-therapy without surgery and 35% in combination with surgery.

Table 1. Clinicopathologic characteristics of all study participants and stratified by HPV DNA status

Characteristics		Total N= 247	HPVDNA ⁻ N= 226	HPVDNA ⁺ N= 21	
Age (Median, y)		65	65	68	
		N (%)	N (%)	N (%)	P-value
Gender	Male	188 (76)	173 (76)	15 (71)	0.3
	Female	59 (24)	53 (24)	6 (29)	
Tobacco	Never	79 (32)	69 (31)	10 (48)	0.2
	Former	34 (15)	30 (13)	4 (19)	
	Current	124 (50)	118 (52)	6 (28)	
	Missing	10 (4)	9 (4)	1 (5)	
Alcohol	Never	75 (30)	69 (30)	6 (28)	0.6
	Former	29 (12)	26 (11)	3 (14)	
	Current	131 (53)	120 (53)	11 (53)	
	Missing	12 (5)	11 (5)	1 (5)	
Localization	Oral cavity	63 (26)	62 (28)	1 (5)	0.0001
	Oropharynx	63 (26)	46 (20)	17 (81)	
	Larynx	96 (39)	95 (42)	1 (5)	
	Hypopharynx	25 (10)	23 (10)	2 (9)	
Tumor size	T1-T2	124 (50)	113 (50)	11 (52)	
	T3-T4	123 (50)	113 (50)	10 (48)	
Lymph nodes	N0	128 (52)	123 (54)	5 (24)	0.011
	N+	119 (48)	103 (46)	16 (76)	
Metastasis	M0	244 (99)	223 (99)	21 (100)	
	M+	3 (1)	3 (1)	0	
Therapy	Surgery only	87 (35)	84 (37)	3 (14)	
	RT and/or CT	71 (29)	61 (27)	10 (48)	
	Surgery and RT and/or CT	87 (35)	79 (35)	8 (38)	
	Missing	2 (1)	2 (1)	0	

4.2 HPV DNA prevalence and genotyping

H&E₁ stained slides showed presence of >80% tumor cells in all tumor biopsies. Directly neighboring sections were analyzed for presence of mucosal HPV DNA genotypes by two different methods (see section 3.4.1-2), MY09/11-RFLP and BSGP5+/6+-PCR/MPG₅₁. Both techniques target the L1 region of HPV and amplify a broad spectrum (49 and 51 types, respectively) of HPV types^{127, 162}.

Good DNA quality, as defined by positivity for the cellular β -globin gene was confirmed for all the tested samples. The MY09/11-RFLP assay was performed in 223 (90%) of the tumor biopsies and identified 18 (8%) single HPV infections (HPV DNA+) of which 17 were HPV16 and 1 was HPV58.

The BSGP5+/6+-PCR/MPG₅₁, a more sensitive method, was used in 138 (60%) of the tumors and identified 10 (7%) single HPV infections of which 9 were HPV16 and 1 was HPV58.

Agreement between the two HPV genotyping methods was evaluated on 114 samples. Six cases were concordantly positive, 105 cases concordantly negative, two cases positive only with BSGP5+/6+-PCR/MPG₅₁ and one case positive with MY09/11-RFLP only, resulting in a κ -value of 0.8. The same HPV genotype was identified in each of the concordantly positive cases. The analytical sensitivity of the BSGP5+/6+-PCR/MPG₅₁ is ~10 fold higher¹²⁷ than the MY09/11-RFLP¹²⁸. Thus, the 2 cases positive only by BSGP5+/6+-PCR/MPG₅₁ were considered as true positives and included in the subsequent analyses. The single sample HPV⁺ only by MY09/11-RFLP was HPV RNA- and therefore considered as a false positive.

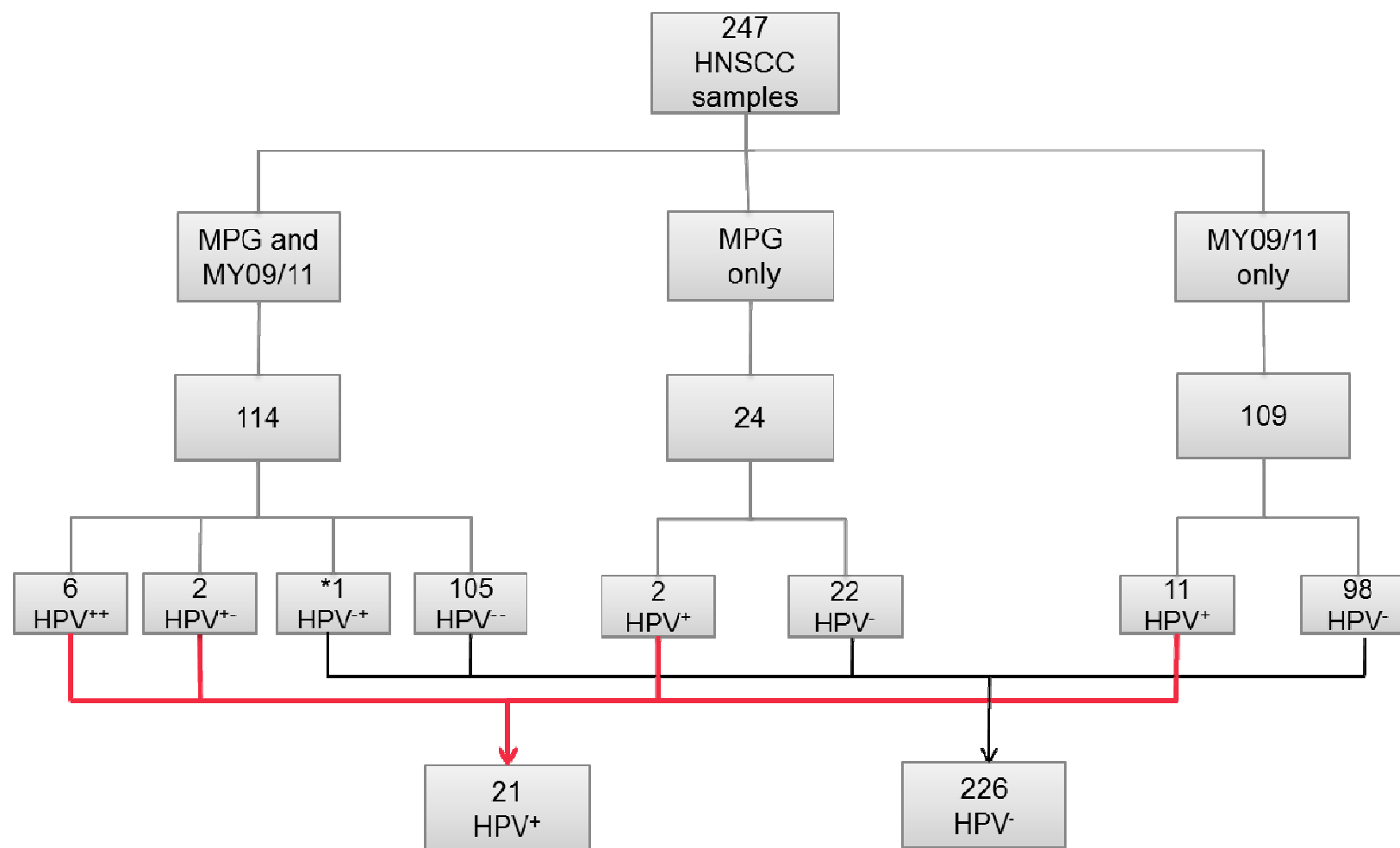


Figure 11. Distribution of HPV DNA genotyping results by BSGP5+/6+-PCR/MPG₅₁ and/or MY09/11-RFLP in tumor tissues of 247 head and neck squamous cell carcinoma (HNSCC) patients from North-Eastern Italy.

Overall HPV DNA prevalence was 8.5% (21/247) (Figure 12). No multiple infections were identified. The most frequent HPV genotype was type 16 (95%, 20/21 HPV DNA+ cases), while HPV58 was identified once (5%)

Oropharynx showed the highest HPV DNA prevalence (27%, 17/63), followed by hypopharynx (8%, 1/25), oral cavity (2%, 1/63) and larynx (1%, 1/96).

4.3 Viral load

HPV viral load was determined by HPV16qPCR (only for samples HPV16 DNA+) and by BSGP5+/6+-PCR/MPG₅₁ (for all HPV DNA+ types).

By HPV16qPCR, the viral load was evaluated in 17 samples and resulted high in 13 tumors with a median of 2.6 (range 0.6 – 1000) copies per cell, negative in 3 tumors and invalid in one tumor (no β -globin copies detected) (Table 2). The cut-off for high viral load was > 0.5 viral genome copies per cell¹⁵⁷.

For 9 HPV16 DNA positive cases with valid HPV16 qPCR results, viral load was also estimated by the BSGP5+/6+-PCR/MPG₅₁ as described previously¹²⁷. Using a normalized HPV/ β -globin MFI ratio of 0.0007 as cut-off, high viral load was estimated in 7 (6 type 16 and 1 type 58) and low viral load in two tumors. One viral load determination was discordant between the two methods with high (ratio 0.000748) in BSGP5+/6+ -PCR/MPG₅₁ assay and negative in qPCR (Table).

4.4 RNA analyses

RNA from 18 HPV DNA positive tumors was available for HPV16 E6*I and ubiquitin C transcription analysis. RNA from 27 HPV DNA negative tumors served as negative controls. The presence of HPV58 E6*I transcript was tested only in the HPV58 DNA positive sample.

All the 45 RNA samples were ubiquitin C RNA positive and thus valid. The 27 HPV DNA negative samples showed no HPV16 transcripts.

Among the 18 HPV DNA positive samples, 4 were negative and 14 (78%) showed expression of type-concordant HPV E6*I mRNA. The single case identified as HPV

DNA positive for type 58 showed presence of HPV58 and absence of HPV16 transcripts (Table 2).

Table 2. HPV DNA+ (positive) tumors, the types identified, their viral load by HPV16 qPCR (HPV16 VL) and by MPG (HPV MPG VL), presence of HPV RN+ (positive), oncoE6 protein, p16^{INK4a} up-regulation and pRb down-regulation.

Tumor ID	Anatomic site	HPV DNA+	HPV16 VL CO > 0.5 copies/cell	HPV MPG VL CO > 0.0007	HPV RNA+	oncoE6 protein	p16^{INK4a} up-regulation	pRb down-regulation
49	Oropharynx	16	high		16			no
208	Oropharynx	16	high		16		yes	yes
64	Hypopharynx	16	high		no		no	no
327	Oropharynx	16	high		16		yes	no
521	Oropharynx	16	high	high_16	16	3 ^a		
159	Oropharynx	16	high	high_16	16	3	yes	yes
14	Oropharynx	16	high	high_16	16	3		
58	Oropharynx	16	high		16		yes	yes
196	Larynx	16	high	high_16	16	4	yes	yes
12	Oropharynx	16	high	high_16	16	4	yes	no
103	Oropharynx	16	high		16		yes	no
44	Oropharynx	16	high	high_16	16	2		
498	Oropharynx	16	high	high_16	16	3		
32	Oropharynx	16	negative	high_16	no	0		
65	Hypopharynx	16	negative	low_16	no	0		
23	Oropharynx	16	negative		no		no	
399	Oropharynx	58	negative	high_58	58		yes	yes
56	Oropharynx	16						
30	Oropharynx	16						
313	Oropharynx	16						
17	Oral cavity	16	invalid		16		yes	yes

^aoncoE6 protein staining intensity: 4= very strong positive, 3= positive, 2= weak positive and 0= negative.

Assuming that all HPV DNA negative samples were negative for E6*1 mRNA, the overall prevalence of HPV DNA+RNA+ tumors was 6% (14/244), and that of HPV DNA+RNA- tumors was 1.7% (4/244) (Figure 15). Compared to the other head and neck anatomical sites, oropharynx with 20% (12/61) showed the highest prevalence of HPV DNA+RNA+ tumors, followed by 1.6% (1/63) in the oral cavity, 1% (1/95) in the larynx, and 0% (0/25) in the hypopharynx.

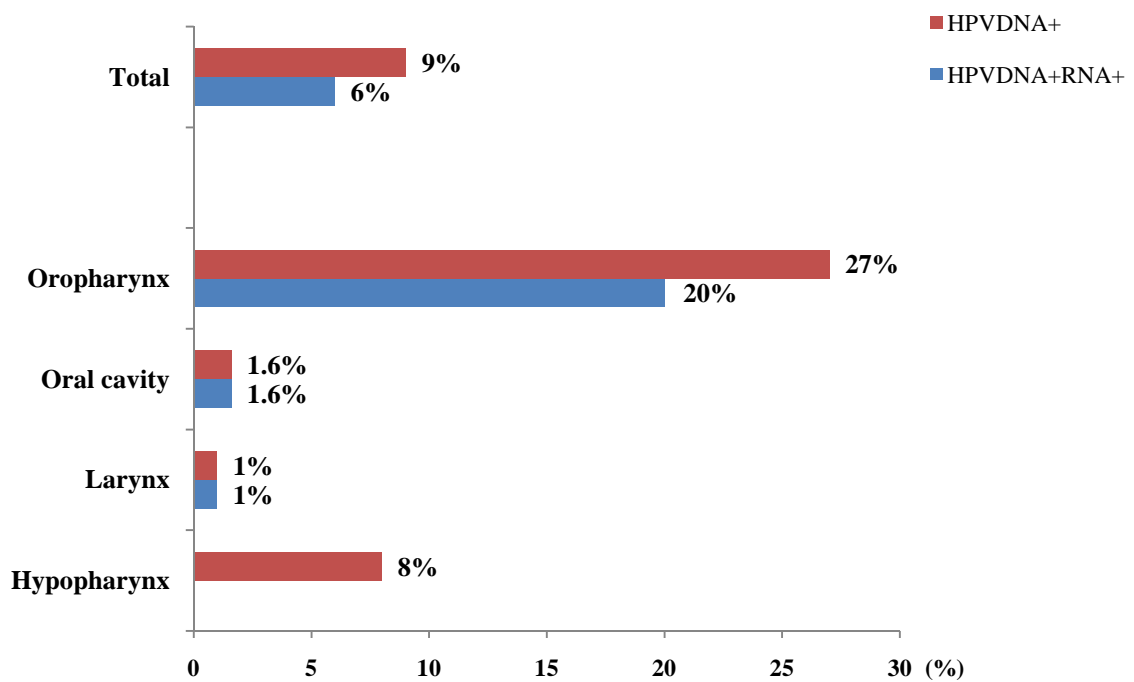


Figure 12. Overall and site specific HPV DNA and RNA prevalence among all head and neck tumors and stratified by anatomical site.

Overall, 247 tumors were analyzed for HPV DNA, 63 from the oropharynx, 63 from the oral cavity, 96 from the larynx, and 25 from the hypopharynx. Of the HPV DNA+ tumors 18 samples were analyzed for HPV RNA, while for 3 cases (2 from oropharynx and 1 from larynx) could not be analyzed.

4.5 OncoE6 protein expression

4.5.1 Analytical sensitivity of HPV16 E6 detection

The analytical sensitivity of the OncoE6 protein assay was determined in 10-fold dilution series of the HPV16 positive cervical carcinoma cell line MRI-H 186 with and without a constant amount of the HPV negative cell line HEK 293T as background. HPV16 E6 protein was detected in as little as 5×10^3 cells MRI-H 186 both with and without background HEK 293T cells (Table 3).

Table 3. HPV16 E6 detection limit of the OncoE6 assay determined in serial dilution of HPV16+ MRI-H cells (in numbers, N) without (A) and with (B) a background of HPV16- HEK 293T cells.

A)	MRI-H 186 cells (N)	HEK 293T cells (N)	Staining intensity ^b	B)	MRI-H 186 cells (N)	HEK 293T cells (N)	Staining intensity ^b
	5×10^6	0	5		5×10^6	0	5
	5×10^5	0	4		5×10^5	0	4
	5×10^4	0	2		5×10^4	0	3
	5×10^3	0	1		5×10^3	0	1
	5×10^2	0	0		5×10^2	0	0

^bStaining intensity: 5= very strong positive, 4= strong positive, 3= positive, 2= weak positive, 1= very weak positive and 0= negative.

4.5.2 E6 oncoprotein in HNSCC

HPV16 E6 protein was detected in lysates from 7 out of 9 fresh frozen HPV16 DNA positive HNSCC tissues (Table 2). The other two HPV16 DNA positive HNSCC and the one HPV negative HNSCC used as specificity control did not show HPV16 E6 protein. None of the HNSCC lysates analyzed reacted positive for HPV18 E6 protein. All 7 HPV16 E6 positive HNSCC (Tumor ID, 521, 159, 14, 196, 12, 44 and 498, in Table 2) were positive for E6*1 RNA and had high viral loads, while the two E6 protein negative but HPV16 DNA positive tumors showed no E6*I RNA and had low viral loads.

4.6 Expression levels of cellular proteins p16^{INK4a} and pRb

Expression of p16^{INK4a} and pRb was evaluated by immunohistochemistry on 54 and 48 FFPE sections, respectively.

Out of 54 cases, 10 samples showed an up-regulation of p16^{INK4a}, 42 cases showed a normal low expression and 2 cases were excluded from the analyses (1 case was not possible to evaluation and the other one the tumor cells were absent). Relating p16^{INK4a} expression to the HPV DNA status (see Table 4), 9 cases HPV DNA positive showed an up-regulation for p16^{INK4a}, while the other 2 cases showed down-regulation. Among the HPV DNA negative group, 40 cases had p16^{INK4a} down-regulated and only 1 case showed up-regulation of the protein.

pRb expression was tested in 48 slides but could be evaluated in 47 (1 case was excluded due to absent tumor cells). Seven (7) cases showed pRb down-regulated and 40 had up-regulation of the protein. Among the HPV DNA positive cases 6/11 showed pRb down-regulation, while in the HPV DNA negative group pRb up-regulation was observed in 35/36 cases.

Table 4. Expression of p16^{INK4a} in (A) and pRb in (B) related to the HPV DNA positive and negative tested samples given in numbers (N).

A)

	p16 ^{INK4a}	
	up-regulated	down-regulated
HPV DNA positive (N=11)	9	2
HPV DNA negative (N= 41)	1	40
Total= 52	10	42

B)

	pRb	
	down-regulated	up-regulated
HPV DNA positive (N= 11)	6	5
HPV DNA negative (N= 36)	1	35
Total= 47	7	40

Ten (10) cases HPV DNA positive were evaluated for expression of both p16^{INK4a} and pRb proteins (Table 2). Six (6) out of 10 cases had up-regulated p16^{INK4a} and down-regulated pRb.

4.7 HPV antibodies

All 102 sera obtained from patients with HNSCC of the oropharynx (N= 22), the larynx (N= 31), the hypopharynx (N= 10) and the oral cavity (N= 39) were analyzed by bead-based multiplex serology for antibody reactivity against the E6, E7 and L1 proteins of the 7 hr-HPV types 16, 18, 31, 33, 45, 52 and 58 and against the E1 and E2 proteins of HPV types 16 and 18. High cut-off values were applied to classify sera as antibody-positive since previous studies had shown strong associations of HPV antibodies with oropharyngeal cancer^{108, 171, 175, 176} with strong reactions to the individual HPV proteins (M. Pawlita, personal communication).

All sera from the 7 patients with HPV-driven (HPV DNA+RNA+) tumors (6 HPV16, 1 HPV58; 6 oropharynx, 1 (tumor 196) supraglottic larynx) showed strong positive antibody reactions with HPV E6 and E7 proteins, 5/7 (71%) also with E2 and L1, respectively, and still 4/7 (57%) with E1 (Table 5; Emax and L1max; Table 6). In contrast, among the 94 patients with HPV DNA- (negative) HNSCC antibody-positive reactions with these proteins were rare and weaker (Table 5), leading to prevalences of 7% or less for the early proteins and 14% for L1 (Table 6). When a universal super strong cut-off of 2000 MFI was applied, prevalences in patients with HPV-driven tumors for the early protein antibodies remained constant but in the patients with DNA-tumors declined to 3% or less.

In patients with HPV-driven HNSCC seropositivity with early proteins frequently showed cross-reactions with the homologous proteins of the 6 other HPV types, most common for E7 (mean number of E7 proteins recognized 2.9, range 1-5) followed by E6 (mean 1.7, range 1-4) while among the HPV DNA- tumor patients positive sera recognized always only the protein of one HPV type (Table 5). This multiple reactivity was also visible for the E1 and E2 proteins (data available only for HPV types 16 and 18) with means of 1.5 and 1.8 among the patients with HPV-driven tumors and 1.3 and 1.0 among the HPV DNA- tumor patients.

Patients with HPV-driven tumors also recognized more of the early proteins than those with HPV DNA- tumors. When a score of 1 was given to each positive reaction with

one of the 4 different early proteins, the cumulated early antibody score (score E-Ab) was 2 or greater for all 7 patients with HPV-driven tumors while only one of the HPV DNA negative tumor (tumor 489) patients had a E-Ab score of 2. With the super-stringent cut-offs of 2000 MFI the E-Ab scores in the HPV-driven tumor patients group did not change, as well as the E-Ab score of 2 of the HPV DNA negative tumor (tumor 489) patient.

Type concordance of the strongest serological reaction to the individual proteins with HPV type by DNA was high. All 6 patients with HNSCC driven by HPV16 showed the strongest reactions with the E6 and E7 proteins of HPV16. The patient with the HPV58-driven tumors reacted strongest with E7 of HPV58 but the reaction with HPV33 E6 was stronger than that with HPV58 E6.

The serum of the patient with the HPV16 DNA+ tumor (Tumor ID 313) for which no other molecular markers could be analyzed demonstrated strong antibody reactivities with all four early proteins of HPV 16, suggestive for the presence of an HPV16-driven tumor.

The serum of the patient with the DNA- tumor 489 was double positive with E6 and E1 of HPV16 (E-Ab score of 2), even with the super-stringent cut-off, a serological pattern suggestive of the presence of an HPV-driven tumors. It should be attempted to analyze the tumor for HPV16 E6*I RNA and the cellular surrogate markers of HPV transformation both in the currently available tumor as well as to attempt to obtain a second tissue sample of this tumor for these analyses to surely exclude a false-negative HPV-genotyping result.

In the molecularly well-characterized HNSCC patient series analyzed here, HPV serology using an E-Ab score of 2 demonstrated a sensitivity of 100% for detection of patients with HPV-driven tumors and a specificity of about 99%.

Table 5. Antibody reactivity of HNSCC patient's sera^a with early and late proteins of hr-HPV types ordered by HPV status of the tumor.

Tumor ID	HPV type DNA RNA			Serum ID	E-Ab Score	HPV protein type cut-off ^b	Antibody reactivity (Median Fluorescence Intensity, MFI)																													
							E6					E7					E1				E2				L1											
							max	max	max	max	max	max	max	max	max	max	max	max	max	max	max	max	max	max	max	max	max	max	max	max	max	max	max	max		
							800	1000	1400	1400	1000	16	18	31	33	45	52	58	16	18	31	33	45	52	58	16	18	16	18	16	18	31	33	45	52	58
327	16	16	329	2			13290 ^c	10992	151	437	531	13290	129	128	1265	88	147	618	10992	108	6806	8486	936	3225	2656	151	133	115	437	409	255	320	411	255	397	531
159	16	16	222	4			12519	4079	2780	13349	1076	12519	91	101	94	48	101	100	4079	167	267	1200	81	64	284	2780	183	13349	8000	1076	195	275	313	150	293	335
208	16	16	210	4			9219	7651	7737	8344	7471	9219	234	212	3616	986	187	1758	7651	1	53	98	11	23	1	7737	2425	6579	8344	7471	777	3720	1927	1215	2930	1927
196	16	16	198	3			8864	8859	70	5763	2143	8864	87	94	235	62	115	173	8859	1	5061	3080	155	73	1523	62	70	5763	448	2143	176	276	312	168	316	430
498	16	16	500	4			8283	4932	6016	8080	1860	8283	40	68	24	27	43	35	4932	1	3490	1326	1	22	71	6016	1094	7081	8080	1860	1060	911	284	1104	530	266
521	16	16	523	4			6332	3044	6813	7083	880	6332	239	186	112	499	298	132	3044	88	28	263	15	35	27	6813	789	7083	3688	880	119	189	234	78	310	158
399	58	58	401	2			2374	3278	107	188	2136	644	70	70	2374	38	152	1855	1138	1	2087	2020	113	435	3278	107	76	73	188	2136	323	1168	1798	860	1962	1742
313	16		315	4			7769	7475	6107	7593	587	7769	89	491	344	89	242	302	7475	1	3120	248	6	823	22	6107	1721	7593	4434	570	239	587	353	234	432	371
241	-		243	1			3367	75	54	318	317	51	66	85	68	3367	90	706	39	75	38	25	15	38	37	38	54	71	318	317	136	300	217	98	241	277
489	-		491	2			2571	65	4527	124	187	2571	27	37	55	44	40	86	65	1	19	17	1	19	18	4527	2219	41	124	187	111	145	96	126	139	109
307	-		309	1			2526	76	21	192	252	43	55	2526	56	89	69	89	25	1	30	59	1	76	65	21	17	51	192	176	252	142	152	188	176	235
342	-		344	1			1243	245	272	436	541	68	138	1243	157	105	165	104	245	35	37	39	17	43	26	272	73	328	436	479	237	505	541	236	369	504
357	-		359	1			1230	301	251	1040	1793	287	455	607	342	351	678	1230	202	106	167	76	101	301	145	64	251	437	1040	1232	1023	1431	1105	990	892	1793
187	-		189	1			1213	26	31	278	240	38	68	86	1213	83	91	670	17	1	13	11	1	26	6	30	31	60	278	195	124	188	208	118	195	240
492	-		494	1			814	1168	157	1263	684	52	71	814	75	27	77	116	1168	3	148	106	239	383	45	56	157	403	1263	307	684	513	638	592	438	505
274	-		276	1			217	3931	130	306	606	214	103	217	119	58	115	105	3931	1	49	34	16	101	31	35	130	90	306	503	223	481	366	209	606	467
277	-		279	1			143	3660	42	183	180	36	57	65	143	81	71	73	3660	80	21	52	18	26	19	31	42	89	183	163	87	174	141	87	158	180
467	-		469	1			158	1515	83	297	279	54	133	92	76	49	158	149	1515	16	30	83	13	35	17	83	81	177	297	262	168	201	215	162	226	279
190	-		192	1			103	1222	49	227	257	62	103	80	83	59	85	91	34	66	25	9	239	127	1222	34	49	81	227	186	210	247	239	125	229	257
512	-		514	1			221	1007	82	182	249	25	100	152	69	44	221	190	160	1007	51	29	62	41	16	67	82	72	182	236	200	206	249	132	225	202
211	-		213	1			97	39	6697	309	257	65	78	87	78	97	82	93	33	1	39	20	10	33	18	728	6697	112	309	241	189	243	250	140	209	257
147	-		149	1			128	64	78	6457	386	59	92	97	88	61	128	103	37	1	44	30	55	64	40	60	78	86	6457	329	136	271	386	139	282	308
289	-		291	1			136	90	2514	727	372	58	119	119	81	85	111	114	59	1	38	13	32	90	18	2514	77	727	443	345	81	301	283	223	283	372
460	-		462	0			121	49	442	217	2428	43	35	121	52	37	73	56	26	1	32	1	1	49	27	442	43	37	217	180	83	164	89	69	127	2428
298	-		300	0			168	54	47	304	1869	26	168	85	87	52	96	77	29	1	29	54	53	39	47	47	32	63	304	209	149	231	218	138	270	1869
476	-		478	0			80	58	128	183	1829	16	43	80	55	28	61	57	58	1	12	34	1	12	24	128	47	82	183	1829	98	181	137	79	125	155
509	-		511	0			63	33	55	206	1674	4	23	37	27	8	43	63	9	1	12	33	8	6	1	51	55	41	206	687	1674	698	618	85	273	429
354	-		356	0			175	84	53	455	1589	70	86	104	79	69	100	175	33	84	34	24	25	56	38	43	53	455	386	281	283	1589	262	197	293	399
381	-		383	0			300	225	112	823	1588	109	236	300	251	169	267	267	65	1	81	225	34	46	54	56	112	239	823	1588	824	511	840	682	909	626
336	-		338	0			70	256	40	332	1581	17	70	55	30	44	25	48	97	1	36	13	1	256	1	26	40	332	186	1581	418	397	190	555	373	220
263	-		265	0			249	828	67	402	1350	37	115	114	116	82	249	150	34	251	67	828	31	39	19	53	67	119	402	1350	711	820	708	1100	1020	608
235	-		237	0			121	46	75	426	1280	36	83	90	72	67	82	121	16	1	28	9	11	46	14	47	75	84	426	1072	1061	757	412	1280	577	423
178	-		180	0			456	384	332	926	1212	15	157	209	130	1	456	333	27	1	1	384	1	1	1	332	211	118	926	673	426	896	1131	715	1212	1191
202	-		204	0			739	69	347	497	1187	739	129	139	161	169	142	147	61	69	41	18	44	24	33	53	347	162	497	516	1187	396	341	220	350	367
181	-		183	0			281	550	173	563	1115	144	181	233	176	110	182	281	550	34	41	134	38	39	30	173	90	139	563	595	446	884	564	358	503	1115

^a A total of 102 sera were analyzed. In addition to the 7 sera from all HPV DNA+RNA+ patients and the serum of the single patient with a HPV DNA+ tumor (no other molecular markers analyzed) of the 94 sera from HPV DNA- tumor patients only the 27 sera with at least one positive antibody reaction are shown; ^bE-Ab score, number of E-proteins with at least one positive reaction; ^c reactions above cut-off are highlighted by colour, in blue the highest reaction for a given protein, in red other, less strongly positive reactions with the same protein from other HPV types for the HPV type.

Table 6. Antibody prevalence against HPV early and late proteins in HNSCC patients with HPV-driven and HPV DNA- tumors applying strong and super strong cut-offs for classification.

Stringent cut-off	Protein	E6		E7		E1		E2		L1	
	Cut-off (MFI)	800		1000		1400		1400		1000	
	total	N+	%	N+	%	N+	%	N+	%	N+	%
HPV-driven	7	7	100	7	100	4	57	5	71	5	71
DNA-	94	7	7	6	6	3	3	1	1	13	14

Super stringent cut-off	Protein	E6		E7		E1		E2		L1	
	Cut-off (MFI)	2000		2000		2000		2000		2000	
	total	N+	%	N+	%	N+	%	N+	%	N+	%
HPV-driven	7	7	100	7	100	4	57	5	71	3	43
DNA-	94	3	3	2	2	3	3	1	1	1	1

4.8 Definition of the HPV-driven HNSCC

Positivity for DNA and E6*I mRNA viral transcripts was primarily used to define a tumor as HPV-driven (Table 7). The other markers were then analyzed to evaluate their efficacy as additional or alternative biomarkers for HPV causality.

E6*I mRNA positivity showed concordance with high viral load (13/14, 93%), presence of E6 protein (7/7, 100%) and HPV E6+E7 type concordant seropositivity (8/8, 100%). While, concordance of E6*I mRNA positivity with up-regulated p16^{INK4a} and down-regulated pRb was observed only in 6 out of 10 tested (60%) samples.

Based on the serological results (E-Ab score of 4) we defined as HPV-driven one additional case (Table 8, Tumor ID 313) that could not be tested for RNA transcripts.

On the contrary one other case, belonging to the hypopharyngeal site (Tumor ID 64, Table 8) showed a high viral load but showed negativity for the presence of viral transcript and thus was considered as non HPV-driven.

Overall, out of 21 HPV DNA positive tumors, 15 were defined as HPV-driven, 4 as non-HPV-driven, while for the 2 remaining cases no tumor or serum specimen was available to perform the additional analyses (n.d.) (Table 7).

Table 7. HPV DNA and the other analyzed markers (HPV16 viral load, RNA, up-regulation of p16^{INK4a} and down-regulation of pRb and HPV16E6 protein).

Tumor ID	Anatomic site	HPV DNA+	HPV RNA+	HPV viral load	oncoE6 protein	up-regulated p16 ^{INK4a} and down-regulated pRb	HPV E6+E7 Ab reactivity	HPV-driven
49	Oropharynx	16	16	high_16				yes
208	Oropharynx	16	16	high_16		yes	yes	yes
327	Oropharynx	16	16	high_16		no	yes	yes
521	Oropharynx	16	16	high_16	+		yes	yes
159	Oropharynx	16	16	high_16	+	yes	yes	yes
14	Oropharynx	16	16	high_16	+			yes
58	Oropharynx	16	16	high_16		yes		yes
196	Larynx	16	16	high_16	+	yes	yes	yes
12	Oropharynx	16	16	high_16	+	no		yes
103	Oropharynx	16	16	high_16		no		yes
44	Oropharynx	16	16	high_16	+			yes
498	Oropharynx	16	16	high_16	+		yes	yes
399	Oropharynx	58	58	high_58		yes	yes	yes
17	Oral cavity	16	16	invalid		yes		yes
313	Oropharynx	16					yes	yes
64	Hypopharynx	16	no	high_16		no		no
32	Oropharynx	16	no	negative	-			no
65	Hypopharynx	16	no	negative	-			no
23	Oropharynx	16	no	negative				no
56	Oropharynx	16						n.d
30	Oropharynx	16						n.d

4.9 Statistical analyses

4.9.1 Correlation between clinicopathologic parameters and HPV-driven

Statistical analyses performed among HPV status (DNA positive and negative cases) and each of the parameters gender, age, tobacco, alcohol resulted not statistically significant ($P > 0.005$) see Table 1. The only significant correlation was observed between HPV status and the site of oropharynx ($P < 0.0001$).

4.9.2 Survival analyses

Patients were enrolled between 2003 and 2012; 95 (38.46%) were dead (53 due to the primary HN-tumor, 20 for a second malignancy, 22 not tumor related) and 152 (61.54%) were alive. Follow-up median time was 20 months (range, 1-121 months). A

negative event (local or distant recurrent HN-tumor) was recorded for 93 (37.65%) patients.

Considering all head and neck anatomical sites together, no significant difference in overall survival (OS) was observed between the HPV-driven group compared to the HPV negative (HPV DNA negative and HPV DNA positive RNA negative) ones ($p=0.67$) (Fig. 13A.). Instead, a better trend of progression free survival (PFS) was observed in the HPV-driven patients after 40 months of follow-up, but still not statistically significant (Fig. 13 B.).

Restricting the analyses to the oropharyngeal site, a trend of better survival (OS and PFS) in the HPV-driven group compared to the HPV negative patients was observed (Figure 13 C and D).

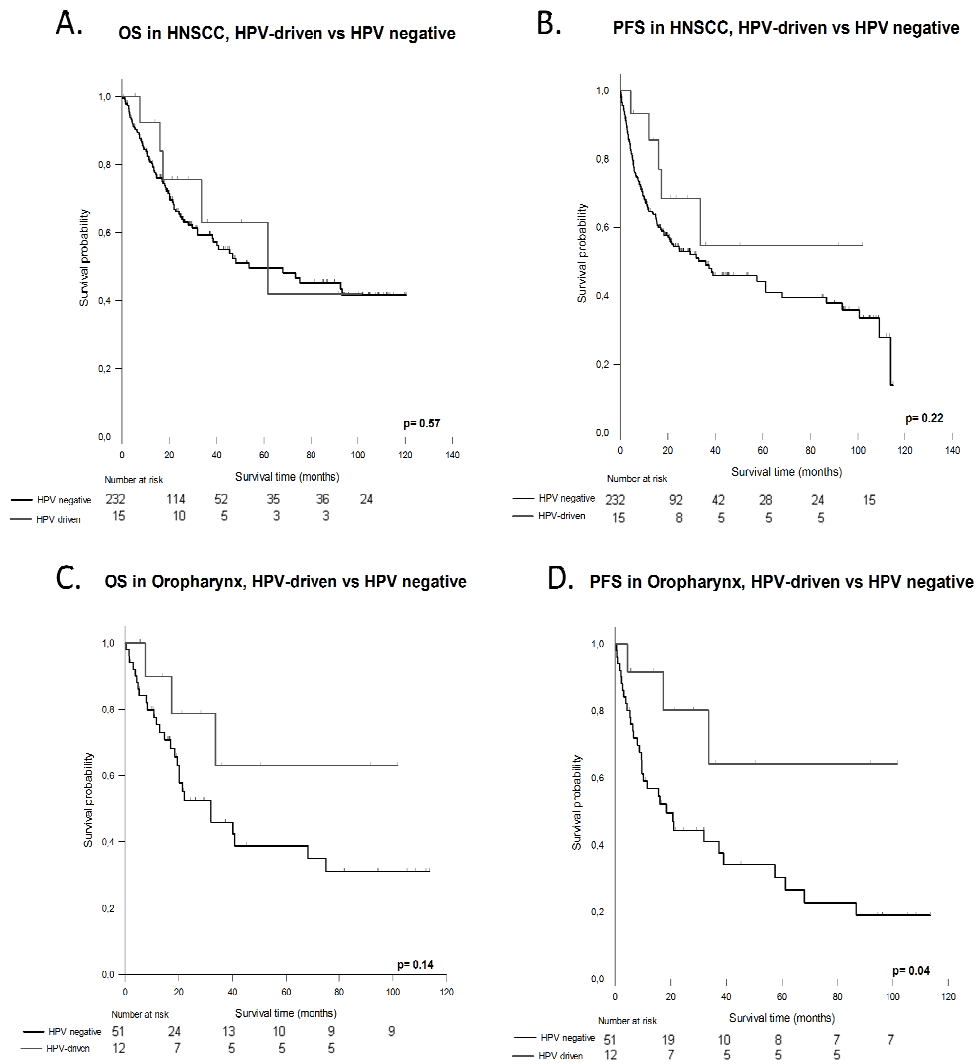


Figure 13. Overall survival (OS) and progression free survival (PFS) in head and neck anatomical sites (A. and B.) and in the oropharynx (C. and D.)

5. DISCUSSION

We analyzed the tumor samples of 247 consecutive patients living in North-East Italy affected by head and neck squamous cell carcinoma in a prospective study. The correct definition of the causal role of HPV in these tumors has clinical implications. The active involvement of the virus was investigated by analyzing several markers (viral load, viral transcripts, regulation of p16^{INK4a} and pRb, presence of oncoE6 protein, and HPV seromarkers).

5.1 HPV DNA prevalence

In the present study, the overall HPV DNA prevalence was 8.5% (21/247). Oropharynx was the site with the highest frequency of HPV DNA positive cases (27%). HPV DNA prevalence in the other head and neck anatomic sites was 8% in the hypopharynx, 2% in the oral cavity and 1% in the larynx.

A systematic review published by Kreimer et al.¹⁰⁷ reported a global HPV DNA prevalence of 25.9% in HNSCC, much higher compared to what obtained from our data (overall HPV DNA prevalence 8.5%). Although the highest incidence of head and neck tumors is recorded in northern Italian regions (compared to the southern regions and the islands), the low HPV prevalence data obtained are probably due to the actual main role of tobacco smoking and alcohol consumption in this area^{106, 177} and /or a lower exposure to the virus potentially due to different sexual behavior.

Although, the prevalence of HPV-associated HNSCC in the different geographic areas varies greatly, oropharynx is always the predilected site. The literature reports a wide-ranged prevalence of HPV in the oropharyngeal tumors (OPSCC) between 20% up to 93%.

In North America the HPV positive OPSCC proportion ranges between 47% - 70%^{107, 178}, while for Asia it was estimated to be 47.5%¹⁰⁷.

Among the Northern European countries, Sweden was the country with the highest HPV DNA incidence (93%) in the tonsils¹⁰⁹. The Netherlands appears to have the lowest HPV proportion in the oropharyngeal tumors, about 20% from 1997-2002, however showed a 10% increase (overall 30%) in the last years (2010)^{179, 180}. Spain, a South European country, reported an HPV incidence of 29% in the OPSCC¹⁸¹, indicating a similar HPV behavior like in the population in study.

In our series, the most prevalent type found was HPV16 (95%), and one case was infected by type 58. In this study for the first time causal involvement of HPV58 in a HNSCC has been documented by a broad variety of viral and cellular markers of HPV transformation¹⁸².

In cervical carcinoma, worldwide, HPV type 16 is the most prevalent type (61%), the other carcinogenic types, i.e. HPV18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 (here referred to as non-HPV16 types) are responsible for approximately another 33% of CxCa, with HPV58 specifically accounting for 2% of CxCa¹⁸³.

Differently from CxCa, HPV-associated HNSCC are caused almost exclusively by infection with type 16 (89-97%) and the DNA of the other non-HPV16 types has been rarely detected^{107, 157, 184}. A recent metanalysis of HPV DNA prevalence in head and neck cancers (Ndiaye C, Alemany L et al., in preparation) identified 11 (0.8%) HPV58 DNA positives among a total of 1466 HPV DNA positive oropharyngeal cancer cases that had been analyzed for presence of HPV58 DNA.

The geographical heterogeneity might be the result of differences in the frequency of the different risk factors, HPV biology and study characteristics.

HPV presence in the oral mucosa in the north Italian regions is very low (1%). A study conducted in 81 healthy patients living in North Italy, detected a single HPV DNA positive case for type 90¹⁸⁵. Also, a systematic review of the literature (18 studies published between 1997-2009) reported a prevalence of any HPV infection of 4.5%. HPV16 prevalence was 1.3%, the highest prevalence among the other hr-HPV types detected¹¹⁶. In the US population prevalence of oral HPV in the healthy population (in 2009-2010) appeared to be higher (6.9%), however HPV16 was the most common hr

HPV type found (1%)¹⁸⁶. The other risk factors, tobacco smoking and alcohol consumption are relevantly present in our population.

Differences in composition of the patient series constitutes another source of variability; studies with a small sample size have a low statistical power, misclassification of the head and neck anatomic sites can influence the relative HPV prevalence in the subsites. Moreover, poor quality of analyzed specimens and the HPV detection methods can substantially alter the results.

In our study, fresh frozen specimens were analyzed by two different methods to genotype HPV, the MY09/11-RFLP and the BSGP5+/6+ -PCR/MPG. Both methods target conserved sequences of the L1 region of HPV and amplify 49 and 51 types, respectively. The BSGP5+/6+ -PCR/MPG is a multiplex assay which homogeneously amplify all the included mucosal types. The detection limit for each of the 51 HPV types ranges from 10-1000 copies, thus detecting HPV types also when present with a low viral load. MY09/11 primers amplify a 450 bp sequence, and have an approximately 10-fold lower sensitivity¹²⁸.

A group of samples were doubly tested. We observed a high concordance among the two methods (overall concordance 99/102, 97%). Three cases were discordant, 2 were identified as HPV DNA type 16 positive only by BSGP5+/6+ -PCR/MPG and considered positive as a result of its higher analytical sensitivity, and 1 was identified as positive by the MY09/11-RFLP method only and was considered as false positive.

It is now well established that DNA alone is not sufficient to determine HPV causality in head and neck tumors^{130, 157}.

To elucidate the biological role of the HPV DNA sequences found in the tumors, the study included other markers that combined with HPV DNA status provided the necessary information to identify the fraction of tumors with active HPV involvement.

5.2 HPV RNA prevalence

The gold standard to demonstrate the viral activity and the transforming capacity of HPV in the tissues is considered to be the detection of E6 and/or E7 viral mRNA, the two relevant oncoproteins.

An ultra-short amplicon, type and splice-site specific, E6*I mRNA RT-PCR assay, was used on fresh-frozen or FFPE samples. The analysis was performed on 18 HPV DNA positive (for 3 cases no specimens were available) and 27 HPV DNA negative specimens.

Overall, in 14 out of 18 (78%) HPV DNA positive tumors the viral transcripts were detected; 13 were positive for type 16 and one for HPV58, confirming the DNA HPV type detection. None of the HPV DNA negative samples showed positivity for HPV16 viral transcripts. The proportion of HPV DNA cases showing active viral transcription (78%) is higher compared to other studies where the HPV transformed proportion was lower (40% and 50%)^{157, 187}.

Viral transcripts analysis showed a reduced prevalence (6%) compared to that found by DNA-based (8.5%) methods. Thus, HPV DNA prevalences reported in previous studies^{107, 184, 188, 189} might have been overestimated in previously reported studies. The HPV RNA prevalence, like the DNA data, indicated oropharynx as the site with the highest HPV positivity (20%). In the other anatomic sites, differently from the DNA data, the oral cavity showed higher prevalence than the hypopharynx (1% and 0%, respectively), while the larynx maintained the same value (1%). However, due to the low number of the HPV DNA positive cases in each of the non-oropharyngeal sites analyzed the change of HPV RNA positivity does not provide any significant information.

Good quality of RNA is often difficult to obtain especially when no fresh frozen material is available. Therefore, there is a need to find other direct and/or indirect markers that can be alternatively used to define the truly HPV-associated tumors.

5.3 Additional viral and cellular markers in relation to the HPV DNA status and definition of the HPV driven group.

Viral load was measured in HPV DNA positive samples (17 cases) by an ultra-short quantitative PCR (HPV16 qPCR) and the quantitative BSGP5+/6+ -PCR/MPG₅₁. The majority of the samples (82%, 14/17) showed a high viral load (> 0.5 genome viral copies per cell), 18% (3/17) of the samples showed a negative viral load.

Comparing viral load and HPV RNA positivity, 13 cases were identified as RNA+ and high viral load, 3 cases as RNA negative and negative viral load, while one case, that was RNA negative with high viral load showed discordance to this pattern. Our data showed a good concordance (93%, 13/14) between RNA expression and viral load and are in line with what published by Jung et al., where showed that 11/12 RNA positive samples had a high viral load¹⁸⁷.

Expression of the HPV E6 oncoprotein increases the level of p16^{INK4a} protein via a negative feedback mechanism, while the expression of the E7 protein leads to pRb degradation. Therefore, in tumors truly HPV-associated p16^{INK4a} is up-regulated while pRb is down-regulated, making these 2 proteins of potential use as indirect markers.

In our study, overall p16^{INK4a} was up-regulated in 20% (10/52) while pRb was down-regulated in 18% (7/47) of the tested samples. Relating the expression of p16^{INK4a} and pRb cellular proteins (as single markers) to the HPV DNA positivity, 82% (9/11) of the cases showed up-regulated p16^{INK4a} and 54% (6/11) had down-regulated pRb.

Among the HPV DNA+ cases, both markers could be evaluated in 10 cases; up-regulated p16^{INK4a} and down-regulated pRb was observed in 60% (6/10) of the cases (Table 2).

In our series, p16^{INK4a} expression (as a single marker) was a more suitable marker compared to pRb when combined with HPV DNA status as reported also in other studies¹⁹⁰. This correlation was particularly good for tumors occurring in the oropharynx^{130, 191, 192} and larynx¹⁵⁵. Nevertheless, there is still a percentage of oropharyngeal cases that are positive for p16^{INK4a} immunohistochemistry but lack HPV DNA; these cases were shown to have a molecular profile typical of non-HPV

associated tumors¹⁸⁰. Also, p16^{INK4a} was reported to be a non suitable marker for the hypopharynx¹⁹³ and the oral cavity¹⁹⁴.

When combined, the up-regulation of p16^{INK4a} with the down-regulation of pRb did not show a strong correlation with HPV DNA+ and RNA+ (60%).

Presence of the E6 protein in cervical carcinoma¹⁹⁵ could represent a good marker for HPV-driven carcinoma, and it also represents a potential target for therapy¹⁹⁶. In the present study, the presence of HPV16 and HPV18 E6 protein was investigated by the commercial assay OncoE6 (Arbor Vita Corporation) in 7 HPV16 DNA positive tumors. Although the number of samples was low, we observed an excellent concordance (100%, 7/7) between the expression of the E6 protein and HPV DNA and RNA positivity, high viral load and overexpression of p16^{INK4a}.

In a larger set of samples, the sensitivity and specificity of the OncoE6 test were 97% and 96%, respectively, with an excellent agreement with the HPV RNA status (K=0.94) (Holzinger et al. manuscript in preparation).

In an unselected subgroup (41%) of HN-patients we tested the seropositivity for 29 HPV serological markers, i.e. antibodies to early and late proteins of the 7 most frequent hr-HPV types.

HPV L1 antibodies are considered markers of past and present infection. HPV type concordant E6 and E7 double antibody positivity has been demonstrated to be highly associated and specific for invasive HPV-associated cancers with an extremely low prevalence of about 0.1% in tumor-free individuals¹¹⁷ and to be strongly associated (odds ratios of 44 to 180) with invasive cancer of the cervix¹⁹⁷, the penis¹⁹⁸ and the upper aerodigestive tract^{117, 175}.

Among the HPV proteins assessed, all the sera from the 7 patients with HPV-driven (DNA+RNA+) tumors showed strong positive antibody reactions with HPV E6 and E7 proteins. In contrast, none of the other sera (94) from patients with HPV DNA negative tumors resulted positive for both E6 and E7 for any of the HPV types tested. Also, in patients with HPV DNA+RNA+ HNSCC, seropositivity with early proteins frequently showed cross-reactions with the homologous proteins of the 6 other HPV types, most common for E7 (mean number of E7 proteins recognized 2.9) followed by E6 (mean

1.7). Patients with HPV-driven tumors also recognized more of the early proteins than those with HPV DNA- tumors. Type concordance of the strongest serological reaction to the individual proteins with HPV type by DNA was high. All 6 patients with HNSCC driven by HPV16 showed the strongest reactions with the E6 and E7 proteins of HPV16. The patient with the HPV58-driven tumor reacted strongest with E7 of HPV58 but the reaction with HPV33 E6 was stronger than that with HPV58 E6. The observed reactivity is probably due to the low number of divergent amino acids (< 40 amino acids) present in the HPVE6 and E7 proteins (Michael K. diploma thesis 2004). Also, a case-control studies reported a higher cross-reactivity for antibodies against the E7 (<5%) than the E6 (0.8%) proteins, probably due to technical reasons ¹⁷⁵.

One serum from a patient with a HPV16 DNA+ tumor for which no other molecular markers could be analyzed demonstrated strong antibody reactivities with all four early proteins of HPV16, suggestive for the presence of an HPV16-driven tumor. Another case was double positive with E6 and E1 of HPV16, even with the super-stringent cut-off. Further analyses should be performed (HPV16 E6*I RNA and the cellular surrogate markers) to exclude false negative HPV genotyping result.

One of the limitations of the serologic assays is the non site-specificity, therefore infections outside the head and neck region might influence the estimates. Also, not all the individuals exposed to HPV seroconvert ¹⁹⁹ or maintain detectable antibody levels over time. Seroconversion could be missed due to waning of antibodies or host genetic background ²⁰⁰. HPV variants might, also, enhance a different immunogenicity ²⁰¹.

From our study and also in line with what is published, the double HPVE6 and E7 seropositivity was the best marker identifying the HPV DNA+RNA+ cases related to the tumor, with a good sensitivity (100%) and specificity (99%).

Based on the correlations found for each of the analyzed markers, we defined as HPV-driven all the samples that showed HPV DNA and RNA positivity (14/18 cases), as well as the single case that could not be tested for RNA expression but was HPV DNA positive and seropositive for both oncoproteins E6 and E7.

5.4 Presence of the risk factors in the HPV DNA+ group and survival analysis

Patients with HPV-associated tumors have been described to exhibit unique characteristics, including a younger age at diagnosis, less exposure to tobacco and alcohol, lower T-classification, higher N-classification and a better prognosis¹⁹¹. In our HN-series, we did not observe in the HPV-positive group statistical significant differences related to age (68 years old in the HPV+ vs 65 years old in the HPV-), or smoking (48% never vs 28% current), or alcohol consumption (28% never vs 53% current), or tumor size T (52% T1-T1 size vs 48% T3-T4). The majority (76%) of the HPV-positive HN-cases had a nodal involvement.

Among patients with all head and neck tumors, overall and progression free survival were not significantly better in the HPV-positive group compared to the HPV-negative one.

Considering that the majority of the HPV-driven cases belong to the oropharynx we restricted the analyses to this anatomic site.

Most of the HPV-driven patients were non smokers while most of the HPV negative one were smokers (p-value < 0.05). We could not demonstrate the same significance regarding the alcohol consumption (p-value > 0.05), even though the alcohol consumption was declared by a higher proportion of patients with HPV-related tumors. Statistical analyses for Overall Survival (OS) and Progression Free Survival (PFS) showed a trend of better survival in the HPV-driven group (p-value = 0.14 and 0.04, respectively). Still, the low number of the HPV-driven cases influenced the statistical power of all the performed analyses.

5.6 Conclusions

In the present study, in samples of patients affected by head and neck squamous cell carcinoma living in Northern Italy, collected from 2003-2012, a very low HPV prevalence was detected. Oropharynx was the predominant site for HPV infection.

We highlighted the importance of a better classification of the HPV-driven tumors by analyzing several markers, and a trend for a better survival of the HPV-driven group in the oropharyngeal site.

6. ABBREVIATIONS

Abs	Antibodies
bp	base pair
bp	base pairs
BS	broad-spectrum
CT	chemotherapy
Cp	crossing point
Cy	cyclin
CKD	cyclin-dependent kinase
°C	degree Celsius
DAB	3,3'-Diaminobenzidine
DNA	deoxyribonucleic acid
E6AP	E6-associated protein
ER	endoplasmic reticulum .
EGF	epidermal growth factor
e.g.	<i>Exempli gratia</i>
ERK	extracellular signal-regulated kinase
FFPE	formalin-fixed paraffin-embedded
fl	full-length
GST	glutathione S-transferase
HNC	Head and neck cancer
HNSCC	head and neck squamous cell carcinoma
H&E	hematoxilyn and eosin staining
HPSGs	heparin sulfate proteoglycans
hr-	high-risk types
HDACs	histone deacetylases
HPV	Human Papillomavirus
HC2	Hybrid Capture 2
i.e.	id est
IHC	immunohistochemistry
IARC	International Agency for Research on Cancer
	International Committee on Taxonomy of
	Viruses
ICTV	
l	liter
LCR	long control region
lr-	low-risk” types
MFI	Median Fluorescence Intensity
mRNA	messenger-RNA

µg	microgramm
µl	microliter
mg	milligram
ml	milliliter
mmol	millimolar
min	minute
M	Molar
MPG	Multiplex Papillomavirus Genotyping
TEMED	N,N,N',N'-Tetramethylethylenediamine
ng	nanogram
nm	nanometers
nt	nucleotide
ORF	open reading frame
OD	Optical Density
OSCC	oropharyngeal squamous cell carcinoma
OS	Overall survival
PV	Papillomaviruses
PC	Phenol-Chloroform
PE	Phycoerythrin
PCR	Polymerase Chain Reaction
PFS	Progression-free survival
qPCR	quantitative real-time PCR
RT	radiotherapy
RPA	replication protein A
RFLP	restriction fragment length polymorphism
pRB	Retinoblastoma tumour
RT-PCR	reverse transcription
RNA	ribonucleic acid
SDS	sodio dodecyl sulfate
URR	upstream regulatory region
vs	versus
VL	viral load
VLP	virus-like capsid particle

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