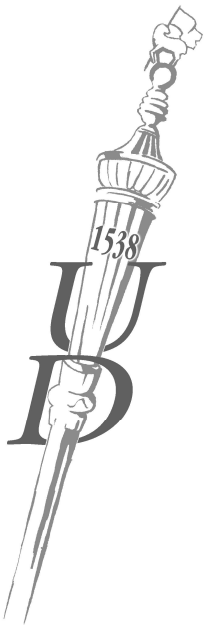
Theses of Philosophiae Doctor (PhD) dissertation

INVESTIGATION OF HUMAN PAPILLOMAVIRUS DNA IN HEAD AND NECK EPITHELIAL TUMOURS

Tamás Major

Supervisors: Judit Czeglédy† PhD, Krisztina Szarka PhD



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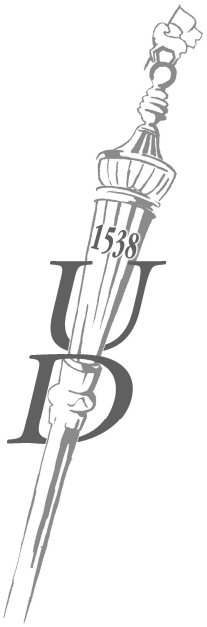

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1. INTRODUCTION

1.1. Taxonomy, general morphological description

Papillomaviruses (PV) are DNA viruses belonging to the *Papillomaviridae* family. The general morphological features of the family: 1. an approximately 8000 bp circular double-stranded DNA with transcription from one active strand; 2. 52-55 nm capsid with icosahedral simmetry, consisting of 72 capsomers. Papillomaviruses are characterized by a pronounced host species specificity. Their cellular tropism is significant, productive infection is established in stratified epithelia of the skin and mucosa (epitheliotropism).

Human papillomavirus (HPV) types detected substantially more frequently in cervical cancer in contrast to noncancerous controls are considered as „high-risk" types (HPV 16, 18, 31, 33, 35, 56, 73, etc.), while types with equal prevalence in cancers and noncancerous controls are considered as „low-risk" types (HPV 6, 11, 40, 42, 43, 44, etc.)

1.2. The HPV genome and the function of virally encoded proteins

The majority of viral genome encodes proteins (open reading frame = ORF). The ORFs are classified into two groups whether their expression is in the early (E) or late (L) phase of the viral life cycle. The E region is 4000 bp and the L region is 3000 bp in size. The genome has an approximately 1000 bp **noncoding region (NCR)**, which is termed as long control region (LCR), too, by its functions. Hereafter, the proteins significant from the viewpoint of the dissertation are described.

The **E1 protein** plays a role in the initiation and elongation of the viral replication. It binds to the origin of replication by its DNA binding domain. This binding is stabilized by the E2 protein. The E1 protein possesses a DNA dependent ATPase and a helicase activity, as well. The **E2 protein** – depending from its binding sites in the PV genome – may act either as an activator or a repressor in the transcription. The tranforming activity of high-risk **E6 and E7 proteins** is principally based on the inhibition of p53 and retinoblastoma (pRB) cellular tumour suppressor proteins, respectively. The expression of L genes is characteristic of productive HPV infection. The **L1 protein** is the major capsid protein. The immunodominant neutralizing epitopes of PVs are located on it. The **L2 protein** acts in the encapsidation of the viral genome by binding to a proportion of the L1 pentamers.

1.3. The natural history of papillomavirus infections

Productive infection. The PV life cycle is closely related to the differentiation of the stratified epithelium. The virion infects basal epithelial cells exhibiting mitotic activity. After accessing into the nucleus and decapsidation, the viral genome is initially amplified in a small copy number. This is followed by a **maintenance** phase, in which viral replication and cell cycle are synchronous (1 viral replication / cell cycle on the average). The copy number remains relatively stable during several cell generations. The **vegetative DNA replication** resulting in a large copy number is detected in terminally differentiated epithelial cells only. In addition to the replication, the E and L ORF transcription is synchronous with epithelial differentiation, too. The expression of L genes encoding structural proteins is detected exclusively in the polygonal layer and superficially. The virion assembly takes place intranuclearly. Mature virions are detectable in the *stratum granulosum* (stratified squamous epithelium) and *stratum planocellulare,* their disengagement from the cells is observed in the epithelial surface. The virion is resistant to desiccation, its infectibility is maintained for a long time in the environment. In the case of **latent HPV infection** HPV DNA is detected in histologically normal epithelium. The most obvious example of HPV latency is recurrent respiratory papillomatosis: the viral DNA persisting in histologically normal airway epithelia of the patient at remission might be the source of recurrence even several years later.

1.4. The physical state of HPV DNA

The intact, circular, extrachromosomal viral genome is designated **episomal**. This is principally characteristic of HPV associated benign dermal and mucosal lesions, and furthermore, mild cervical dysplasias. In the majority of severe dysplasias and invasive cancers the viral genome cleaves and **integrates** into the host chromosomal DNA. The predilection sites of cleavage are the E1 and E2 ORFs, and deletion of viral DNA sequences of various size might occur at integration. The consequence of integration is the cessation of the E1 and E2 ORF expression, and hence, the cessation of transcriptional regulation of E6 and E7 genes. The integration of the viral genome usually occurs into transcriptionally active non-specific regions of host DNA, and it is accompanied by the overexpression E6 and E7 genes and the increased stability of their mRNA. Integration into the vicinity of certain cellular protooncogenes (c-myc, jun-B, etc.) might lead to their overexpression. Although not an absolute prerequisite of carcinogenesis, integration can participate in it at several points.

1.5. Human papillomaviruses as tumour viruses

According to their strict epitheliotropism, HPVs generate benign proliferation in stratified epithelia of the human skin and mucosa, but they can be detected in malignant epithelial tumours at several locations, too. The close association of HPV infections with cervical cancer is best-known, which corresponds with the etiological relationship at this site. The role of HPV in cervical carciongenesis is supported by the following items: 1. over 90 % of invasive cervical cancers are HPV positive (in squamous cell cancers HPV 16, in adenocarcinomas HPV 18 is predominant); 2. the *in vitro* described key events of HPV induced carcinogenesis (the integration of viral genome, the expression of transforming E6 and E7 ORFs, the consequences of the E6-p53 and E7-pRB interactions) are confirmed *in vivo*, too.

1.6. Human papillomaviruses in head and neck cancers

In head and neck cancers, intranuclearly located HPV antigenes were first detected in 1983. Though several authors detected HPV DNA in cancers of the oral cavity, pharynx and larynx by hybridization methods (in situ and Southern-blot hybridization) in the following years, the widespread advent of polymerase chain reaction (PCR) as a sensitive and specific DNA detecting method gave a new impulse to the researches in the nineties. HPV 16 is the most prevalent type in each head and neck site. HPV DNA is detected most frequently in the cancers of the oropharynx and the palatine tonsils within it. The **physical state** of HPV genome in head and neck cancers might be either episomal or integrated, and furthermore, their simultaneous presence has been described. Relatively few studies are concerned with the **copy number** of HPV DNA in head and neck cancers. In general, the HPV DNA copy number is less in head and neck cancers in contrast to cervical cancer. Tonsillar cancer might be an exception, as its HPV DNA copy number is commensurable with cervical cancer.

1.7. Recurrent respiratory papillomatosis (RRP)

RRP is the most frequent benign tumour of the upper airways. The exophytic, pendulated or sessile, warty lesions might occur at virtually any part of the airways. The predilection site is the larynx, which is affected in more than 90 % of all RRP cases. The first symptoms usually develop between 1 and 5 years of age and in the 3rd decade of life, therefore *juvenile-onset* (J-RRP) and an *adult-onset* (A-RRP) forms are distinguished.

The main etiological agents of RRP are HPV 6 and 11. By applying adequately sensitive DNA detection methods, the HPV positivity of airway papillomas approximates or even achieves 100 %. HPV DNA is detectable in macroscopically normal tissues next to papillomas and at distant locations, and furthermore, in the airway mucosa of patients in complete remission. The presence of viral DNA in these normally appearing tissues might be the source of frequent recurrences, and on the other hand, it might explain the common failure of surgical monotherapy of RRP.

In J-RRP cases HPV infection presumably occurs at labour, at passage through the infected birth canal (**maternofetal transmission**). In the case of A-RRP the presumed way of transmission is orogenital sex, but the reactivation of previously acquired latent infection (even at birth) might not be excluded, either.

The **predilection sites** of lesions are the natural squamociliary junctions at several locations of the upper airways.

The **symptoms** are depending from the location and the patient age (e.g. the diameter of airways). The leading symptom in the case of the most common vocal cord location is progressive hoarseness. The second most common symptom is stridor. Especially in infancy, the rapid growth of papillomatosis in the narrow airways might lead to airway obstruction.

The unpredictable **natural history** is characterized by frequent recurrences. J-RRP usually represents a spontaneous remission at puberty. After several decades of disease, malignant degeneration occurs in 3-5 % of RRP cases.

If RRP is suspected by physical examination or fiberoscopy, a tissue biopsy is needed by laryngomicroscopy (LMC), in general anaesthesia. The **diagnosis** of RRP is histological: the benign proliferation of the stratified non-keratinizing squamous epithelium constitutes fingerlike projections with the fibrovascular stroma. The specific cytopathic effect of HPV infections is koilocytosis, but abnormal keratinization (primarily parakeratosis) is observed frequently, too.

The **therapy** of RRP is based on the s**urgical excision** of lesions: the most frequently affected laryngeal location is explorated by LMC and lesions are removed with either CO_2 laser, microdebrider or cold steel instruments. In infants, in the case of severe acut airway distress urgent **tracheotomy** might be necessary. **Adjuvant therapy** is performed in approximately 10 % of all RRP cases, with alpha-interferon (IFN-α) and cidofovir being the two most suggested drugs nowadays.

Due to the unpredictable natural history and the potential unfavourable outcome, desperate attempts are aimed at the prediction of **prognosis**. In general, the low initial age at onset and type 11 are considered as negative prognostic factors (complete duration of symptoms, number of surgeries, necessity of tracheotomy, distal spread into the lower airways, etc.).

1.8. Cidofovir therapy of recurrent respiratory papillomatosis

The (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)-cytosine (cidofovir, CDV), belonging to the group of acyclic nucleoside phosphonates, is an inhibitor of the replication of several DNA viruses. Nowadays it is one of the most frequently given „off-label" drugs in the adjuvant therapy of RRP. In the past 15 years several studies describe experiences with the CDV therapy of RRP: the low number of cases and the intralesional administration given at some weekly intervals are common in them.

As the bioavalability of CDV is rather low (<5%), therefore it is given locally in HPV associated lesions (intralesionally in RRP). In the most frequently affected laryngeal site, the intralesional administration is performed by LMC, in general anesthesia. Though the half life of the active intracellular metabolite is 17-65 hours, the 2-week-intervals of intralesional CDV injections efficiently decrease the bulk of papillomatosis in the practice .

There is no uniform **dose and protocol** for the serial intralesional CDV therapy of RRP. Carcinogenesis and nephrotoxicity described in animal experiments have not been observed in intralesional CDV therapy, yet.

2.OBJECTIVES

Our objective is to evaluate the HPV DNA status in the patients of the Clinic of Otorhinolaryngology and Head & Neck Surgery, University of Debrecen: head and neck cancers, RRP cases and some lesions habouring papilloma and dysplasia at onset and showing malignant degeneration at follow-up are investigated. Our experiments are performed in f**resh-frozen tissue biopsies**, which, according to our expectations, contain sufficient amounts of DNA lacking autolysis and the effects of histological processing. We are designing a **prospective study**, with the intention of the serial virological analysis of tissue samples to better understand the natural history of HPV infections.

Our main **investigation points** are as follows.

1. What is the proportion of HPV DNA positivity in the three patient groups?
2. Which HPV types are detectable?
3. What is the physical state of HPV DNA?
4. How much is the copy number of HPV DNA?
5. Is there any relationship between the HPV DNA status of cancers and the clinical (age, gender, smoking, alcohol consumption), pathological (pT, pN, grade, location) parameters and outcome?
6. Does the HPV DNA status possess any prognostic value?
7. How does the adjuvant intralesional CDV therapy of RRP influence the HPV DNA status?

3. PATIENTS AND METHODS

3.1. Patient follow-up

Our investigations were performed among the inpatients of the Clinic of Otorhinolaryngology and Head & Neck Surgery, University of Debrecen between March, 2001 and October, 2006. Virological samples were taken simultaneously with histological biopsies during the curative surgeries of patients, under sterile operative conditions. At patient follow-up we considered the outpatient and inpatient visits in the insitutes of the Medical and Health Science Center, University of Debrecen, acquired from the MEDSOLUTION 97 database. The follow-up period lasted from the first virological sampling until the last visit of the patients.

3.1.1. Cancer group (n=33). In the dissertation the cancers of the oropharynx, hypopharynx and larynx are termed as head and neck cancers, as each tissue sample in our investigations originated from any of these three organs. At the enrollment into the study, the patient gender, age, location of tumour, patological TNM compared to the clinical, histological type and grade (G) of the tumour were each recorded. The mean age of the 31 males and 2 females was 54.5 (37-71) years. The **histological diagnosis** was squamous cell cancer in 32 cases and adenocarcinoma in one case. The differentiation of tumour was G-I in 9 cases, G-II in 8 cases, and G-III in 16 cases. The **location** was laryngeal in 15 cases, oropharyngeal in 5 cases, hypopharyngeal in one case, laryngo-hypopharyngeal in 8 cases, laryngo-oropharyngeal in 3

cases and laryngo-tracheal in one case. The ***pathological*** **T stage of primary tumours** was T1-2 in 9 cases and T3-4 in 24 cases. The distribution of ***pathological*** **N stages** was as follows: N0: 18 cases; N1: 9 cases; N2: 6 cases. None of the patients exhibited **distant metastases** at the enrollment. In two cases virological sampling was performed from cancer recurrences following surgeries with partial organ resection.

In the follow-up period the overall follow-up time, the disease-free survival time (calculated until the last disease-free visit, in tumour-free patients this corresponded with the overall follow-up time), local tumour recurrences, regional lymph node recurrences and distant metastases were recorded. The mean **overall follow-up time** in the cancer group was 29.9 (0-78) months. The mean **disease-free survival time** was 24.9 (0-78) months.

Twelve patients remained tumour-free in the overall follow-up time. **Local tumour recurrences** were developed in 10 cases, r**egional lymph node recurrences** in 8 cases and **distant metastases** in 2 cases. Nine patients **died** of local tumour recurrence or regional lymph node recurrence. One patient died of causes other than the head and neck tumour.

3.1.2. The RRP group (n=14). At the enrollment into this group the histological diagnosis of papilloma without dysplasia was a prerequisite. The majority of patients has already had RRP in their case history. Two patients were enrolled with RRP suspected from the preoperative clinical examinations, and histology performed simultaneously with virological tests confirmed the RRP. At enrollment the age, gender, location and anamnestic data were recorded.

In the **J-RRP group (n=6)** the mean age at onset was 4.78 (1.9-12) years, and larynx was the most frequently affected site. The time elapsed from the age at onset until the enrollment into this study was 0-50 years, and patients underwent a mean of 5.67 (0-20) surgeries. Two patients received adjuvant IFN-α therapy. In the J-RRP group two patients were adults at the enrollment (ages 26 and 62, respectively). In the **A-RRP group (n=8)** the diagnosis of RRP was set up at a mean 34.75 (18-71) years of age. Larynx was the most frequently affected site in this group, too, with two exceptions (a soft palate and a nasal cavity site, respectively). The time elapsed from the age at onset and the enrollment to the present study was 2 years in mean (0-5 years), and patients underwent a mean of 1.75 (0-5) surgeries. One patient received adjuvant IFN-α therapy. The **prospective study period** started from the first (and in some cases the only) fresh surgical sampling for virology.

3.1.3. Tissues initially harbouring papilloma and dysplasia and exhibiting malignant degeneration at follow-up (n=5). Five patients were enrolled to this patient group, in whom

the histology of the surgeries in the case history initially harboured papilloma only or papilloma with different degrees of dysplasia. The benign and the premalignant lesion is observed simultaneously in the subsequent biopsies. Finally, the lesion showed malignant degeneration in each case, either at the enrollment into the prospective period or in the prospective follow-up. The initial lesion site was the larynx in all cases, with glottic spread in 4 cases and glottic and supraglottic spread in one case. The **mean age** of the 4 males and one female at the *initial diagnosis of the mixed lesion* was 51 (44-60) years, and the time elapsed *until the first diagnosis of cancer* was 41.6 (13-71) months. The mean *follow-up time after the diagnosis of cancer* was 31.2 (5-58) months. By the end of the follow-up period one patient died of local tumour recurrence and distant metastases, one patient exhibited regional lymph node recurrences and the remaining 3 patients were disease-free. During the total follow-up time patients underwent a mean of 5.6 (4-7) surgeries. The average number of tissue biopsies necessary for the diagnosis of cancer was 4 (3-6). In the prospective period we performed 1-3 surgeries per patient, in two cases only from the already malignant lesion.

3.2. Virological sampling

At the sampling for virological studies a resection of an at least 3x3x3 mm tissue from the centre of the lesion was a prerequisite. Another prerequisite was not to influence the exact histological diagnosis. Care was taken to the sufficient mass of tissue in smaller papillomas and the evasion of the resection margins in cancers. The histological biopsy was performed prior to the CO_2 evaporisation in papillomas, and on the other side, immediately after the resection of the affected organ in cancers, before placing it into the fixative. The resected tissues were stored at -70 °C until further investigations.

3.3. HPV DNA detection with PCR

DNA was extracted from the samples with the standard phenol-chloroform-isoamylalcohol method. The amount and purity of DNA was checked with spectophotometry. The control of DNA integrity was performed with PC03/PC04 PCR amplifying a 110 bp sequence of the human β-globin gene. The first step of HPV DNA detection was the MY09/MY11 consensus PCR amplifying an approximately 450 bp sequence from the L1 ORF of many mucosotropic HPV types genome. The second step of viral DNA detection was the GP5+/GP6+ consensus (nested) PCR ensuring an approximately 145 bp amplimer of many mucosotropic HPVs. PCR

products were evaluated in ultraviolet light after agarose gel electrophoresis (the agarose gel was stained with ethidium-bromide).

3.4. HPV typing by restriction endonucleases

HPV DNA positive products were cut with *Rsa*I and *Mse*I restriction endonucleases. The resulting fragments were run on non-denaturing polyacrylamide gel, detected by silver staining and identified by the electroforetic pattern of GP amplimers deriving from reference plasmids and cut with the same restriction endonucleases. Reference plasmids containing HPV 6, 11, 16 and 18 DNA were used as positive controls.

3.5. Determination of HPV DNA physical state by PCRs specific for the E1, E2 and E1E2 genomic regions

Referring to the Introduction section, the HPV genome usually cleaves in the E1 or the E2 ORFs at integration. These regions of interest in the HPV 6, 11 and 16 DNA positive samples were investigated by PCRs specific for the E1, E2, and E1E2 ORFs of the certain types, in which amplimers were 845-2206 bp in size. After agarose gel electroforesis, PCR products were identified under ultraviolet light.

3.6. Determination of HPV DNA physical state by Southern-blot hybridisation (SBH)

SBH can identify the simultaneous integrated physical state of HPV DNA in samples positive for the E1, E2 and E1E2 specific PCRs. Viral DNA was digested by *Eco*RI (non-cut for HPV 6), *BamH*I (one cut for HPV 6), *Bgl*II (non-cut for HPV 11) and *BamH*I (one cut for HPV 11) restriction endonucleases, and following agarose gel electroforesis, DNA was blotted onto positively charged nylon filter by capillary transfer. Type specific probe DNA was used for the identification of HPV specific sequences. Due to its relatively lower sensitivity and higher quantitative requirement of sample DNA compared to PCR techniques, the routinish application of SBH was ceased later in the study.

3.7. Comparison of the sensitivities of the E1, E2, E1E2 and MY09/MY11 specific PCRs

The sensitivities of the E1, E2, E1E2 and MY09/MY11 PCRs were compared by performing them on tenfold serial dilutions of DNA prepared from a HPV 6 positive papilloma.

3.8. Comparison of copy numbers of HPV DNA positive papillomas and cancers

The copy numbers of some cancers and papillomas positive for HPV 6 or 11 DNA with the nested PCR were compared with tenfold serial dilutions of pBR322 plasmid harbouring the HPV 6 or 11 genome. Following the MY09/MY11 PCR on the calibrating serial dilutions and 1 μg of sample DNA (as measured by spectrophotometry), the copy number of samples were estimated on the agarose gel of amplimers by the densitometric comparison of the sample bands to the calibration bands.

3.9. Real-time PCR for the assessment of HPV DNA copy number

Real-time PCR for the assessment of HPV DNA copy number was performed with Applied Biosystems 7500 Real Time PCR System and ABI SYBR Green PCR Master Mix (Applied Biosystems, PN, USA). The PCR amplification mix contained 10 ng of template DNA. The fluorescence spectra were recorded during the elongation step of each PCR cycle. To identify and control the PCR product generated in the presence of SYBR Green, a melting point analysis was performed. The virus copy numbers were calculated by a standard curve derived from HPV 6 and 11 plasmid DNA.

3.10. Correspondence of the HPV DNA positivity with the clinicopathological parameters and outcome of cancers patients

In cancer patients the correspondence of HPV positivity with the **clinical** (age, gender, smoking and alcohol consumption) and **pathological** (pT, pN, location, histological grade) parameters at the initial diagnosis was investigated with logistic regression by the SPSS 15.0 for Windows statistic software, determining the crude odds ratio (OR) and p values. The effect of the aforementioned clinicopathological parameters and HPV positivity on the poor **outcome** was investigated with logistic regression, too. Poor outcome denotes local tumour recurrence, regional lymph node recurrence or distant metastases at any time during the follow-up.

3.11. Follow-up of HPV DNA status in cidofovir therapy of RRP

A male patient participating in the RRP group was born in 1994. After a persistent hoarseness period RRP was diagnosed at the age of 18 months with LMC and histological biopsy. Prior to CDV therapy he underwent cold steel or CO_2 laser papilloma excision 28 times. He has

been the patient of the Clinic of Otorhinolaryngology and Head & Neck Surgery, University of Debrecen since June, 1999. Subcutaneous IFN-α therapy started at this time, and lasted for over five years, until the onset of CDV therapy. In August, 2001 papillomas appeared on his soft palate. In August, 2004 he underwent an urgent tracheotomy for acute airway obstruction. Due to the fulminant recurrences, the tracheostoma was maintained until the start of CDV therapy in March, 2005.

CDV therapy (Vistide®) was based on the modification of Chetri's protocol: according to the original protocol, the first 4 injections are given at every two weeks, and subsequent injection intervals are each increased by one week (weeks 0, 2, 4, 6, 9, 13, 18, 24, etc.). Our patient received injections at weeks 0, 2, 4, 8, 12, 16, 24, 42 and 55. LMC was performed in intratracheal narcosis (endotracheal intubation was performed *via* the tracheostoma maintained until the 7th treatment, and *via* a persistent tracheocutaneous fistula at the last two injections). In the case of the soft palate site, the exploration of the pharynx was performed with oral straddlers used at tonsillectomies in narcosis. Following the exploration, the first step was the **quantitative assessment of RRP severity** with the Derkay's scale, in which a papilloma severity score (**PSS**) between 0 and 3 is determined at each airway site (0 = no lesion; 1 = surface lesion; 2 = raised lesion; 3 = bulky lesion). For the better follow-up of the bifocal disease, the scores of the larynx and soft palate were assessed separately. After the recording of PSS values, lesions were removed with cupped forceps followed by laser ablation of the lesion base. The 40 mg total CDV dose per surgery (approximately 1 mg/kg) was diluted with saline to 5 mg/mL for the soft palate (2 mL each into the submucosa at the left and right side of the free margin) and 10 mg/mL for the larynx, due to the narrow airway anatomy (1 mL each into the submucosa of the left and right vocal cords). Aside from the tissue sent for routine histology, a portion of the excised sample was fast-frozen and stored until virological tests. Tissue samples for virological tests were taken from the former disease sites even at complete remission. To assess the efficacy of CDV, five former tissue samples were used as controls, these were excised 190, 165, 131, 53 and 41 weeks prior to the onset of CDV therapy, respectively.

The detection of HPV DNA, determination of physical state and copy number was performed in the aforementioned ways.

After the cessation of CDV therapy, aside from routine indirect laryngoscopy, outpatient rigid 70° videolaryngoscopy in local anesthesia and LMC in general anesthesia with excision of recurrent lesions were performed at weeks 76 and 117, respectively.

Due to frequent recurrences of laryngeal papillomatosis, a single intralesional CDV injection was performed at the 6th surgery of an adult male patient.

4. RESULTS AND DISCUSSION

4.1. HPV DNA status of cancer patients

Location	HPV	HPV type				
	pos / all	6	11	11+16	16	other
Larynx	5/15	1	2	0	2	0
Larynx + hypopharynx	5/8	1	1	1	2	0
Oropharynx	2/5	1	0	0	1	0
Larynx + oropharynx	2/3	1	0	0	1	0
Hypopharynx	0/1	0	0	0	0	0
Larynx + trachea	1/1	1	0	0	0	0
Overall	**15/33**	**5**	**3**	**1**	**6**	**0**

HPV DNA positivity and types. The 45.5 % (15/33 patients) overall head and neck cancer HPV DNA positivity rate exceeds the 25.9 % mean prevalence in a review calculated from 60 PCR studies.

The **high-risk HPV 16** was detected in 7 cases (with HPV 11 coinfection in one case), which constitutes 21.2 % of the total cancer group. This prevalence matches to the published 20 % mean value. The prevalence of HPV 16 in *cancers confined to the larynx and hypopharynx* is 20.8 % (5/24 cases) which is slightly higher than the 17 % mean prevalence calculated from 35 PCR studies. In *oropharyngeal cancers or cases involving the oropharynx* the HPV 16 prevalence is 25 % (2/8 cases) which is slightly lower than the 31 % mean prevalence calculated from 27 PCR studies. The oropharyngeal predilection of HPV 16 is not confirmed by our study, though the number of oropharyngeal cancer patients is rather low.

HPV 18 was not detected at all, although this type is considered to be the second most frequent in head and neck cancers. In contrast, the high prevalence of l**ow-risk HPV types (6 and 11)** is surprising, and unequivocally, this accounts for the high overall HPV positivity rate. The prevalence of low-risk types in the total cancer group (including the case of HPV 11 and 16 coinfection) was 27.3 % (9/33 cases), with an essentially even distribution among sites: 6/24 cases in cancers confined to the larynx and hypopharynx and 2/8 cases in cancers involving the oropharynx, and 1/1 case at other sites. The high prevalence of types 6 and 11 might be considered as a geographical speciality.

The **coinfection** of two HPV types was detected in one case (3 %): a laryngo-hypopharyngeal cancer harboured types 11 and 16. According to the literature, the prevalence of HPV coinfections is 3.6 % in head and neck cancers, and the coinfection of HPV 16 with another type is detected most frequently. Coinfection is considered to be a result of repeated HPV expositions.

At investigation of HPV distribution by sites, 41.7 % of (10/24 cases) of *cancers confined to the larynx and hypopharynx* and one half (4/8 cases) of *oropharyngeal cancers or cases involving the oropharynx* were HPV positive, although the positivity of a single case can markedly influence the results, which is attributable to the low case number. Due to the aforementioned presence of low-risk types, both values exceed the 24 % prevalence for the larynx and hypopharynx and 35.6 % prevalence for the oropharynx calculated from 35 and 27 PCR studies, respectively.

Physical state and copy number. The E1, E2 and E1E2 specific PCRs yielded assessable result in one case only: this was a HPV 6 positive T2 oropharyngeal cancer of a 60-year-old male patient, whose cancer located to the tongue base and the right tonsillolingual groove. In this case, by the positivity of E1 PCR and the negativity of E2 and E1E2 PCRs we suppose that the cleavage of viral genome took place in the E2 ORF. Anyway, the integration of HPV DNA may confirm the etiological role of the otherwise low-risk HPV 6.

The very low viral DNA copy number is suspected as a cause for the negative E1, E2 and E1E2 PCR results in the remaining HPV positive cases. To confirm this, the copy numbers of 3 cancers and 5 papillomas positive for HPV 6 with nested PCR and 3 cancers and 5 papillomas positive for HPV 11 were estimated with a tenfold serial dilution of pBR322 plasmid harbouring HPV 6 or 11 genome, after performing the MY09/MY11 PCR. According to the results, the copy number of either HPV 6 or 11 in 1 μg sample DNA in cancers was under 10. In contrast, in the majority of papillomas copy number varied in the range of 10^2-10^7. The other suspected cause for the failure of physical state determination might be the different sensitivities of our PCR methods, which was demonstated by performing them on tenfold serial dilutions of DNA prepared from a HPV 6 positive papilloma: MY09/MY11 PCR proved to be the most sensitive, followed by the E2, E1, and E1E2 PCRs.

Correspondence of the HPV DNA positivity with the clinicopathological parameters of cancers patients. As the number of patients positive for the individual HPV types was low, the overall HPV DNA positivity was correlated with clinicopathological parameters. The relationship between the **clinical** parameters and HPV positivity was not significant. Among

pathological parameters pathological T stage was the only to show correlation close to significance with HPV DNA positivity (p = 0,115): the larger the primary tumour, the more frequent the HPV DNA in it.

The effect of clinical, pathological, and virological parameters on the outcome of cancer patients. Despite the low case number, it is evident that **smoking** and **alcohol consumption** adversely affect the outcome, although the correlation is not significant, presumably due to the few available data. Among the **pathological** parameters, the effect of **lymph node metastasis** at onset on the unfavourable outcome is unequivocal (p = 0.025). The effects of pT and location on unfavourable outcome was not significant. In the total head and neck cancer group, HPV positivity slightly enhanced the poor outcome. The **prognostic role of HPV positivity** is equivocal in the literature, although several studies suggest that HPV positivity improves the 3-year disease-free survival in oropharyngeal (including tonsillar) cancer.

The correlation with clinicopathological parameters and outcome was investigated with the high-risk HPV 16 separately, but significant relationship was not recorded for this type, either.

4.2. HPV DNA status of RRP patients

HPV DNA positivity and types. Twenty-six tissue samples of the 14 patients were analysed. The mean follow-up time was 31.7 (0-86) months, zero when a patient was lost to follow-up after the first (and single) surgery. In the follow-up period the overall number of surgeries was 33, while the mean number of surgeries per patient was 2.35 (1-10) (omitting the elective surgeries of CDV therapy).

Each RRP patient was positive for HPV DNA. HPV 6 and 11 were detected in 6 and 8 cases, respectively. The 100 % HPV DNA positivity is not surprising in the aware of investigating fresh and abundant tissues in the majority of cases with a highly sensitive technique.

At the follow-up of the individual patients, the presence of the same HPV type DNA was detected. This confirms the role of types 6 and 11 in the etiology of RRP. HPV typing of similar serial biopsies is rather rare in the literature.

No coinfections were detected in the RRP group. Literature is equivocal with regard to coinfections, even the presence of several HPV types has been described.

In the **juvenile-onset** RRP group the mean follow-up time was 48 (0-86) months with a number of 16 total surgeries. The mean number of surgeries per patient was 2.7 (1-10). HPV

11 (5 out of 6 patients) was the more frequent type. Three patient required urgent tracheotomy of acute airway obstruction, all of them were HPV 11 positive.

In the **adult-onset** RRP group the mean follow-up time was 19.5 (0-78) months with a number of 17 total surgeries. The mean number of surgeries per patient was 2.1 (1-3). HPV 6 (5 out of 8 patients) was the more frequent type. The only patient requiring urgent tracheotomy of acute airway obstruction was HPV 11 positive.

In the **HPV 6 positive** (juvenile and adult) group the mean follow-up time was 29 (0-78) months, with a total number of 12 surgeries. The mean number of surgeries per patient was 2 (1-3).

In the **HPV 11 positive** (juvenile and adult) group the mean follow-up time was 33.5 (0-86) months, with a total number of 21 surgeries. The mean number of surgeries per patient was 2.63 (1-10).

The literature is equivocal in the concern of HPV 6 and 11 distribution. In two studies with relatively high case numbers, type 11 was predominant in J-RRP, while type 6 was predominant in A-RRP cases. In contrast, in other studies the prevalence of types 6 and eleven is equal in J-RRP.

In our study – although the case number is rather low – the follow-up time of HPV 6 and 11 positive cases was virtually equal (29 versus 33.5 months). HPV 11 positive cases required slightly more surgeries per patient than HPV 6 positive cases (2.63 versus 2). Each patient requiring tracheotomy was HPV 11 positive. Our results confirm the negative prognostic role of HPV 11.

In the J-RRP group a 62-year-old male patient must be emphasized, whose laryngeal RRP was diagnosed at the age of 12 years, and furthermore, he underwent surgeries of recurrent disease at the age of 22 and 27 years. The subsequent duration of complete remission until a fulminant relapse at enrollment to the present study was 35 (!) years. Similar duration of complete remission is sporadically reported in the literature. Otherwise, a „secondary primary" HPV infection *via* orogenital contact can not be excluded, either.

Neither tracheal nor bronchopulmonary distal spread were observed.

Physical state. The E1, E2 and E1E2 PCRs for the determination of viral DNA physical state were each positive in all RRP samples, referring to intact E1 and E2 ORFs. As the presence of the E1 and E2 region itself can not exclude neither a simultaneous integrated physical state nor a multicopy tandem integration, we performed the SBH. SBH yielded assessable results in 9 samples of 7 patients, and confirmed the exclusive episomal state in each case. In the

remaining patients we have no data concerning a simultaneous integrated physical state. The exclusive episomal physical state presumably indicates a good prognosis to evade a malignant degeneration, but further monitoring is required.

Copy number. The copy number of HPV DNA was investigated by real-time PCR. Melting point analysis was performed to check PCR products. Unfortunately, in many cases – after the HPV typing and determination of physical state – the sufficient amount of sample DNA for real-time PCR was lacking. Copy number showed fluctuations in several orders of magnitude among both HPV 6 and 11 positive cases (HPV 6: $10 – 2x10^5$, HPV 11: $10^2 – 7x10^4$ in 10 ng of template DNA). These fluctuations were observed at the follow-up of individual patients, and furthermore, among samples deriving from the same surgery of one patient.

To our knowledge, the follow-up of HPV DNA copy number is RRP has not been investigated, yet. The fluctuations of copy number might correspond to the natural history of RRP, which is determined by the balance of HPV infection and host cellular immunity.Due to the very low number of cases measured by real-time PCR, the correlation between copy number and clinical parameters (age, gender, PSS, prior adjuvant therapy, remission time following the certain sampling) was not investigated further.

By the comparison of the copy numbers of HPV 6 and 11 positive cancers and papillomas, in RRP viral DNA copy number tends to be higher with several orders of magnitude than in cancers, confirming the key role of human papillomaviruses in the etiology of RRP.

4.3. HPV DNS status of lesions initially harbouring papilloma and dysplasia and exhibiting malignant degeneration at follow-up

HPV DNA positivity and types. At the initial diagnosis of the mixed lesions the mean age of patients approximately 16 years exceeded the A-RRP group (51 versus 34.75 years). In the prospective study period the 5 patients underwent 17 surgeries, of which we were able to perform virological sampling at 10 times. In two cases the HPV DNA test were performed both from the premalignant and malignant lesions. One patient was tested only from the premalignant lesion, while two patients were tested only from the already malignant lesions.

Among **lesions harbouring papilloma and dysplasia** 4 samples were negative for HPV DNA, while HPV 16 was detected in one case. In the **cancer stage** 5 samples were tested, 4 of them were HPV DNA negative, and the remaining sample harboured HPV 16 DNA. In the two patients the presence of HPV 16 DNA (in a premalignant mixed lesion and in a cancer) was preceded by HPV negative result (from a premalignant mixed lesion in both patients).

Physical state and copy number. E1, E2 and E1E2 PCRs were each negative in both HPV 16 positive samples, presumably as a consequence of very low copy numbers. Types 6 or 11 in high copy numbers characteristic of RRP were not detected at all, despite of the histological diagnosis of papilloma. Accordingly, the persistence of low risk HPV infection playing a key role in the etiology of RRP or the presence of the same HPV type in papillomas and subsequent cancers were not detected, either. Otherwise, both the numbers of cases and samples were limited. There are sporadical case reports or case studies in which the etiological role of HPV in malignant degeneration is established: the presence and the integrated physical state of HPV 11 in RRP and in subsequent invasive cancer arising from RRP is an example. The other way of HPV associated malignant degeneration of RRP might be the appearance of high-risk types near the antecedent low-risk types. In the two patients HPV 16 DNA in low copy numbers in a dysplasia and a cancer were detected after HPV negative samples. Unfortunately we were not able to determine the physical state by our PCRs, and furthermore, there were no virological samplings from the subsequent surgeries. Therefore, due to the insufficient data, we can not evaluate the etiological role of HPV 16.

The literature data concerning the HPV DNA status of laryngeal squamous intraepithelial lesions (SIL) must be emphasized. Laryngeal SIL is a widely interpreted histological definition, covering the spectrum from squamous hyperplasia through atypic hyperplasia with an increased risk of malignant degeneration (the moderate and severe degree of dysplasia system corresponds with this) to carcinoma in situ. According to the literature, the mean prevalence of HPV DNA in laryngeal SIL is 12 % and HPV 16 is predominant. Thus, the HPV status of our 5 cases principally resembles SILs (and corresponding dysplasias).

4.4. Follow-up of HPV DNA status in cidofovir therapy of RRP

A review of intralesional cidofovir therapy. Several authors report their experiences with intralesional CDV therapy. The objective comparison of these studies is practically impossible. The number of cases is rather low (between 3 and 26). The course of J-RRP and A-RRP is different. The indications for CDV therapy are different, too. In many reports patients received other adjuvant treatment modalities prior to CDV or concurrently. Surgical techniques also differ: in some studies intralesional CDV injections were given alone, while in others, CDV injections were preceded by the surgical excision of macroscopic lesions. The dose and concentration of CDV varied. Most protocols possess an induction phase with some (4-6) injections given at intervals of 2-8 weeks, and a maintenance phase with injections given

at longer intervals. The total number of injections (1-19) and the duration of therapy depend on the objectives which usually denotes complete remission or stable residual disease with low PSS values. According to a review of 17 studies (158 patients), 57 % of patients achieved complete and 35 % achieved partial remission, while 8 % were non-responders. Thus, intralesional CDV therapy is considered successful in general.

The parameters of our CDV therapy (9 injections in 55 weeks, 1 mg/kg dose per injection, 5-10 mg/mL concentration depending from location) represent a mean of literature data. No systemic side effects were observed.

Evaluation of histology. Histology – with one exception - revealed papillomatosis, with concomitant mild dysplasia at the last three injections. Neither severe dysplasia nor malignant degeneration were observed by the pathologist. The single exception was a chronic laryngitis at the 3rd injection of the larynx. Invasive malignant tumours have not been diagnosed so far during CDV therapy of RRP and even dysplasia is condsidered as a rarity.

The effect of cidofovir therapy on the severity (PSS) of RRP . In the period preceding CDV therapy laryngeal PSS showed fluctuations between 9 and 16, despite the combined CO_2 laser and IFN-α treatment. The soft palate disease was less severe (PSS was maximum 3), and furthermore, this site had been in remission for over 2 years. Four weeks prior to CDV therapy papillomas involving the posterior edge of the soft palate and virtually the entire larynx were removed.

The recurrent nature of the disease is represented by a PSS=6 in the larynx and PSS=2 in the soft palate hardly four weeks later (at the onset of CDV therapy). At the initial 4 injections given at 2-week-intervals, PSS reduced in both sites, and by the 4th injection (8 weeks), patient achieved the complete remission. Subsequently, with the elongation of the injection intervals, RRP recurred at a controlled rate below the laryngeal PSS of the pre-treatment period (with PSS max. 6 for the larynx and 2 for the soft palate). This partial remission remained stable in the 62 weeks of post-treatment follow-up.

The effect of cidofovir on the HPV DNA status. Each biopsy harboured HPV 11 DNA. To our knowledge, the physical state and copy number of HPV DNA in intralesional CDV therapy have not been investigated before. When copy number achieved the sensitivity of E1, E2 and E1E2 PCRs, we detected the intact E1 and E2 ORFs referring to episomal **physical state**. In the pre-treatment period, laryngeal copy number showed fluctuations in several orders of magnitude ($2x10^2$ - 10^4 copies / 10 ng template DNA), while soft palate copy number was assessed only once (3×10^3). The natural history of the HPV infection and

changes of host immunity might account for the fluctuation of laryngeal copy number (even at combined surgical and IFN-α therapy).

In the induction phase of CDV therapy with 2-week-intervals, at the initial three and four injections in the larynx and soft palate, respectively, the decrease of PSS was accompanied by the decrease of HPV DNA copy number. By the fourth injection, when the patient was in transient complete remission, the HPV DNA copy number was below the sensitivity of real-time PCR (<10), but a copy number of $2x10^3$ was detected in the papilloma-free larynx. In the former case only the MY09/MY11 – GP5+/GP6+ nested PCR indicated the presence of HPV DNA, presumably in an extremely low copy number.

We must emphasize the 3rd injection of the larynx, when real-time PCR showed negative result for HPV DNA at PSS=2 (surface lesions at the anterior commissure and the left vocal cord). In accordance with this, histology confirmed chronic laryngitis and excluded papilloma. In this case, too, only the nested PCR detected HPV DNA.

In the subsequent maintenance phase of CDV therapy with the elongation of intervals, however, RRP recurred at both sites, and PSS and HPV DNA copy number fluctuated independently. At the 5th injection of the soft palate, for instance, a small surface lesion (PSS=1) was accompanied by a high HPV DNA copy number ($3x10^5$). Alhough larynx was in remission at the 6th and 7th injections, the viral genome copy number was rather high (10^5 and $2x10^4$, respectively). The copy numbers in the two sites might differ even in 4 orders of magnitude, and furthermore, the two sites show independent fluctuations in copy number. It must be emphasized, that in the late phase of therapy, although PSS decreased compared to the pre-treatment period, HPV DNA copy number occasionally even exceeded the pre-treatment values.

The tracheostoma was closed at the 7th injection, when the larynx was disease-free. Subsequently, however, a persistent 3-mm-diameter tracheocutaneous fistula indicated the recurrent papillomatosis partially obstructing the larynx, while the soft palate lesion remained stable.

Thus, an injection interval of t**wo weeks** seems necessary for the effective antiviral effect of CDV. However, this would be unbearable for the patient, and on the other side, we must not forget about the potential CDV toxicity, either. For these reasons, the majority of authors use CDV with longer intervals already from the onset, or elongate intervals after a short-interval induction phase. In the second phase with the **elongation of intervals**, both sites show a stable disease, despite the fluctuating high copy numbers. Long-term effects of CDV other

than the inhibition of viral replication might account for this control of papillomatosis. These are confirmed by *in vivo* and *in vitro* experiments (down-regulation of E6 and E7 expression on the transcriptional level, cell cycle inhibition by „S" phase arrest, induction of apoptosis, etc.). Although these studies were performed in cancer cell lines, the inhibition of viral DNA transcription by CDV might act in papillomas, too.

The evaluation of intralesional CDV therapy. Several conditions might affect the evaluation of the efficacy of CDV. The initial fall in PSS might be attributable to the frequent initial surgeries (since the patient underwent LMCs more frequently than indicated by the natural course of disease). Virological findings might have been affected by the preceding IFN-α therapy and its cessation. In the second stage of therapy the natural history of RRP (principally the beneficial effect of puberty) itself might have explained the controlled disease. Thus, the intralesional CDV therapy was effective but not curative in our case.

The long-standing RRP recalcitrant for combined (surgical + adjuvant) treatment prompted the „off-label" cidofovir therapy. Due to its relatively long injection intervals in contrast to several other drugs, local applicability (although general anesthesia is necessary), and rare side effects, CDV became one of the most widespread adjuvant treatment modalities in RRP.

One single intralesional laryngeal CDV injection was performed at the 6^{th} surgery of a HPV 6 positive male patient. In the subsequent follow-up of 33 months, he remained in complete remission. Thus, serial determination of HPV DNA copy number was impossible in this case.

5. SUMMARY

The human papillomavirus DNA status (prevalence, types, physical state and copy number) of head and neck cancers (n = 33), recurrent respiratory papillomatosis cases (n = 14), and mixed lesions initially harbouring papilloma and dysplasia and showing malignant degeneration at follow-up (n = 5) was examined in fresh-frozen tissue biopsies by polymerase chain reaction and Southern blot hybridization techniques.

Forty-five percents of cancers were positive for HPV DNA. Both low-risk (6 and 11) and high-risk (16) types were detected, regardless of cancer site. As a consequence of low copy number we failed to determine the physical state of HPV DNA except one HPV 6 positive oropharyngeal cancer, in which the viral genome was integrated and the disruption of circular DNA affected the E2 ORF. There were no relationships between the HPV positivity and the clinico-pathological features or prognoses of patients. According to these results, the etiological role of HPVs in head and neck carcinogenesis remains equivocal.

Each patient in the RRP group harboured either HPV 6 or 11 DNA. The presence of a certain low-risk HPV type is consistently demonstrable in serial samples deriving from a number of patients. HPV 11 positivity tends to predict a worse prognosis (slightly more frequent relapses, necessity of tracheotomy). In contrast to cancers, the viral genome exists in episomal physical state and in substantially higher copy numbers, with fluctuations of the latter in several orders of magnitude.

During the malignant degeneration of mixed lesions initially comprising of papilloma and dysplasia - by performing biopsy from the precancerous mixed lesion or the already invasive cancer – the majority of tissue samples were negative for HPV DNA. Two samples harboured HPV 16 DNA, presumably in very low copy numbers. Therefore the HPV status of this group is similar to those diagnosed initially as invasive cancers.

During the intralesional cidofovir therapy of a 14-year-old child with RRP, the initial phase of therapy with injections given at shorter intervals the complete remission of lesions was accompanied by the substantial decrease of HPV DNA copy number. In the maintenance phase with injections given at longer intervals, disease recurred at a controlled rate and HPV DNA copy number showed fluctuations in several orders of magnitude again, similarly to the pretreatment period. In this maintenance phase the long-term effects of cidofovir other than the inhibition of DNA replication and the natural history of RRP can account for the controlled disease.

Key words: human papillomavirus, DNA, head and neck, cancer, papilloma, cidofovir

6. PUBLICATIONS

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