Human papillomavirus 11 and respiratory papillomatosis: genomic and epigenetic background of different severity and response to cidofovir therapy

by Tamás Gáll

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HUMAN PAPILLOMAVIRUS 11 AND RESPIRATORY PAPILLOMATOSIS: GENOMIC AND EPIGENETIC BACKGROUND OF DIFFERENT SEVERITY AND RESPONSE TO CIDOFOVIR THERAPY

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The Examination takes place at the Library of the Department of Medical Microbiology, Medical and Health Science Center, University of Debrecen; 10:30, 06.12.2013.

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INTRODUCTION

Recurrent respiratory papillomatosis (RRP) is a chronic disease associated with human papillomaviruses (HPV), especially with HPV-6 and HPV-11. RRP is characterized by multiple papillomas of the aerodigestive tract. Although it is considered a benign disease affecting primarily the larynx, its clinical course is often unpredictable, tends to recur, and may spread throughout the airways or can undergo malignant transformation. Symptoms (stridor, hoarseness, chronic cough, recurrent pneumonia) can occur at any age, the youngest patient was 1 day old and the oldest 84 years. Although, the incidence of RRP is relatively low (1.8/100 000 in adults, 4.3/100 000 in children), it puts emotional burden on families and the economic cost of treatment (multiple surgical debulkings, adjuvant therapy) is also considerable.

Respiratory papillomatosis associated with HPV11 is considered more aggressive than HPV6-related disease, that is, HPV11-related disease is more often associated with the extralaryngeal spread of papillomas and higher frequency of surgical intervention.

For the high-risk HPV types, several distinct genetic lineages were described; some of these variants were shown to be associated with increased pathogenic potential. Associations of disease severity with sequence variants of HPV16, -18, -33, -52, -58 were reported in several studies. Differences in the severity of the disease caused by high risk HPV types can be associated with nucleotides sequence arrangements, i.e. deletions, insertions or duplications. Such mutations which can be associated with the severity of the disease caused by low-risk HPV types are also feasible, however, data illuminating the link between severity and nucleotide sequence are scant even for the diseases (i.e. RRP) which can be attributed unequivocally to low-risk HPV types. Such sequence data may be essential for the prediction of the clinical course of respiratory papillomatosis and therapy resistance.

REVIEW OF LITERATURE

HPVs belong to the *Papillomaviridae* family according to the decision of the International Committee on Taxonomy of Viruses. Classification of papillomaviruses (PV) is based on the highly conserved major capsid protein L1. A new PV isolate is described when if
the complete genome was cloned and the DNA sequence of the L1 ORF differs by more than 10% from the closest known PV type. Sequence difference of 2-10% describes a new subtype, and below 2% an intratypic variant. The genetic diversity of high-risk papillomaviruses is extensively studied, however, sequence data of low-risk HPV types are scant.

PVs are small (52-55 nm in diameter) DNA viruses with icosahedral capsid. Their DNA genome is circular, double-stranded DNA with size of approximately 8 kb. The viral genome consists of the long control region (LCR), the early (E), and late (L) genes. The LCR, which regulates oncogene (E6, E7) expression, binds viral (E1, E2) and a number of cellular transcription factors. There is an increasing evidence that the transactivation potential of LCR is influenced by CpG methylation, especially through the viral E2 protein binding sites (5’-ACCG(N)4CGGT-3). Early genes possess various functions like the regulation viral replication (E1 protein) and gene expression (E2), or some of them (E5, E6, E7) are involved in the transformation process of keratinocytes.

E1 protein interacts with the DNA replication machinery of the cell (α-primase subunit of the human DNA polymerase, topoisomerase I) and has a paramount role in the replication of viral DNA, which is performed by the DNA polymerase of the host cell. E2 protein binds to the consensus, well-conserved (5’-ACCG(N)4CGGT-3) sequence localized at the viral LCR. Genital PVs possess four E2 binding sites, which can bind E2 protein with different affinity. E2 protein, depending on its concentration, can either transactivate (at low concentration) or repress (at high concentrations) the HPV promoter. Moreover, it can be associated with various human proteins, like Brd4, TFIIB. E6 protein of HPVs binds to the p53 protein and promotes its degradation. The E6 protein of high-risk HPV types binds p53 with high affinity, while low-risk HPV E6 with low affinity. The E6 proteins of low-risk types have been shown to be associated with different human proteins like Gps2 and Bak and has an important role at genome maintenance. The E7 protein of PVs binds to the Retinoblastoma protein family (pRb) and promotes their degradation resulting in the inhibition of their ability to modulate the function of E2F transcription factors. The E7 proteins of low-risk HPVs have been shown to be able to bind pRb but with a 10-fold lower affinity than high-risk HPV types. E5 proteins of HPV-11 and HPV-16 are known to have a role in keratinocya transformation by inhibiting p21/CIPI expression in fibroblasts and keratinocytes. There is an increasing evidence that E5, E6 and E7 proteins are all involved in keratinocya transformation. Data for the function of E4 protein are scarce. E4 protein may have a role in the collapse of the cytoskelton of the cells promoting virion release from cells.
The late proteins are the major capsid protein L1 and the minor one L2. These proteins play an important role in the immune response of the host.

Papillomaviruses infect epithelial cells through microtraumas and their life cycle is dependent on the differentiation of keratinocytes. After uncoating HPV DNA is translocated to the nucleus and it exists in an episomal form. HPV DNA is replicated parallel with the genome and the average copy number of HPV virions is 100 per cell. The expression of viral proteins is strictly regulated parallel with migration of the infected basal cell towards the epithelial surface. E6 and E7 protein of PVs drive the cells in the lower epithelial layers into S-phase, which enables the viral genome to replicate and the infected cells to proliferate. The expression of E1, E2, E4 and E5 proteins raise the copy number of HPV genome from 100 to several thousands per cell in the upper cell layers, then L1 and L2 capsid protein start to express and viral genome will be encaspidated.

RRP is a chronic disease associated with HPVs, especially with HPV-6 and HPV-11. RRP is characterized by multiple papillomas of the aerodigestive tract. Although it is considered a benign disease affecting primarily the larynx, its clinical course is often unpredictable showing extralaryngeal dissemination, and rarely malignant transformation. HPV11-related RRP is generally considered more aggressive with higher rates of surgical debulking and adjuvant therapy. RRP can be divided into two groups depending on the onset of the disease, adult-onset RRP (AO-RRP) and juvenile-onset RRP (JO-RRP). Patients suffering from JO-RRP are infected during birth, while the mode of infection in AO-RRP is presumably orogenital contact.

The symptoms of RRP are primarily stridor, hoarseness, and multiple papillomas in the airways. Extralaryngeal dissemination of papillomas can sometimes occur, especially if the causative agent is HPV11.

The routine diagnosis for respiratory papillomatosis is endoscopy and histological examination. Histology often shows acanthosis, koilocytosis and dysplasia.

The goals of the therapy of respiratory papillomatosis are the complete removal of papillomas relievement of airway obstruction and the improvement of voice quality. The primary treatment for respiratory papillomatosis is surgical debulking which involves microdebrider or carbon dioxide laser. Tracheostomy may be necessary if significant airway obstruction occurs. Although the primary treatment of respiratory papillomatosis is surgical,
20% of the respiratory papillomatosis cases necessitate adjuvant therapy. Adjuvant therapy is needed when rapid growth of papillomas and distal spreading can be detected. The most widely used chemotherapeutic agents for respiratory papillomatosis are interferon-α (IFN-α) and cidofovir (CDV). IFN-α presumably inhibits HPV oncogene expression while the exact effect of CDV on HPV replication or oncogene expression remained elusive to date. CDV is primarily used for the treatment of herpesvirus infections, in which it inhibits viral DNA polymerase. However, HPVs do not possess their own DNA polymerase, HPV DNA is replicated by cellular DNA polymerase. Several authors showed that CDV treatment on HPV infected cells resulted in an increase of p53 and retinoblastoma proteins, moreover, apoptotic markers (induction of caspase-3 protease activity, disintegration of the nuclear matrix protein and DNA fragmentation). The first administration of CDV in respiratory papillomatosis was reported by Van Cutsem et al. in 1995. To date, numerous studies and case reports were published analyzing the effects and the potential side effects of CDV therapy in respiratory papillomatosis. Chadha and James reviewed 17 studies including 158 patients and concluded that 90 patients (57%) showed complete resolution, 55 patients (35%) a partial response, and 13 patients (8%) showed no improvement. The explanation why 43% of patients showed only partial or no response remained elusive to date. Potential side effects of CDV are nephrotoxicity, sometimes edema and local inflammation.

AIMS

1. To analyze phylogenetically HPV11 sequences available in GeneBank.
2. To examine the virological background of the course of respiratory papillomas with different aggressivity.
3. To examine the virological background of virological failure of CDV treatment in a case of aggressive RRP
4. To monitor accidental occurring mutations in the HPV11 genome during CDV therapy and examine the stability of viral genome during a 7-year-follow-up period (1999-2006).
MATERIALS AND METHODS

Patients

HPV11 isolates from six patients with minimally aggressive solitary papillomas (Patients 1 and 2), moderately (Patients 3 and 4) and highly (Patient 5 and Patient 6) aggressive respiratory papillomatoses were investigated. Patient 6 underwent CDV treatment because of the high recurrence rate of papillomas. The severity was defined based on clinical data, that is, the frequency of surgical interventions, vertical spreading in airways and the need for tracheotomy. For patients with moderately or highly aggressive papillomatoses (Patients 3, 4 and 5), the complete genomes of HPV11s from two temporally distant samples were determined, while five sequential samples collected from the papillomas of Patient 6 before, during and after intralesional cidofovir therapy leading to virological failure after initial response were analyzed. CDV treatment was performed according to the modified protocol reported by Chetri et al.

Patient 1 and 2 with minimally aggressive solitary papillomas showed only one episode of papillomatosis localized only on the larynx. Patient 3 had nine episodes, while six episodes of papillomatosis occured in Patient 4, both patients recieved IFN as adjuvant treatment, and the solely affected site was the larynx. Patient 5 and Patient 6 had multiple papillomas; larynx, pharynx, maxillary sinus, nasal cavity, and auditory duct were affected by papillomas in Patient 5, while larynx and soft palate in Patient 6. Patient 5 was treated with IFN while patient 6 underwent IFN and CDV treatment as well. Tracheostomy was performed in the case of Patient 2 (emergency intervention), Patient 5 and Patient 6.

DNA preparation, HPV DNA detection and typing

Fresh-frozen tissue samples were homogenized in TNE buffer (10 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA). DNA isolation was performed with phenol/chloroform/isoamylalcohol extraction. The integrity of DNA was examined with β-globin PCR using GoTaq DNA polymerase. HPV DNA detection and typing was performed with MY/GP nested PCR followed by RsaI/TruI digestion.

Amplification and sequencing of HPV11 genomic DNA fragments

Genomic regions of HPV11 were performed using 500 ng of total DNA with specific primers with GeneAmp High Fidelity PCR System according to the manufacturer’s guide.
Amplimers from two independent PCRs were run on 1.5% agarose gel in 0.5× TBE (Tris/Borate/EDTA), stained with Et-bromide, and purified with QIAquick Gel Extraction Kit. Sequencing was performed with the Sanger method directly from both directions with the ABI BigDye terminator v3.1 Cycle kit in an ABI 3100 Genetic Analyzer. For the whole genome analysis of HPV11 isolates, sequences were assembled and analysed (CLC Gene Workbench 4.1.2; CLC Bio, Aarhus, Denmark) against the reference HPV11 genome derived from a highly aggressive JO-RRP reported by Gissmann et al.

For the sequential analysis of HPV11 genomes derived from the CDV-treated patient five specimens were selected: one laryngeal sample collected prior to cidofovir or interferon therapy (Sample 1, from 1999), one laryngeal (Sample 2, from 2005) and one palatal samples (Sample 3, from 2005) collected during the initial response to CDV therapy as well as one laryngeal (Sample 4, from 2006) and one palatal samples (Sample 5, from 2006) collected after the failure of CDV regimen were analyzed.

Sequence data of the HPV11 regions compared to the reference clone M14119 isolated by Gissmann et al. and sequenced by Dartmann et al.

**Single strand conformational polymorphism (SSCP)**

In order to exclude the presence of infection with multiple HPV11 strains, SSCP analysis of the viral E1, E2, E6, E7 and LCR was performed. Each regions subjected to SSCP analysis was amplified with specific primers and digested with different restriction endonucleases (NdeI for E6, SspI for E7, AluI and SspI for E1A, HaeIII and SspI for E1B, DraI for E2, and bsh1235I for LCR), and an aliquot of the digested amplimers were run on 3% agarose gel to verify the restriction digestion. Three microliter of the verified digestion was diluted in 1:10 in 95% formamide containing 0.04% bromophenol blue and 0.05% xylene cyanol, then denaturated at 95°C for 5 minutes and placed immediately on ice. DNA was run on 15% polyacrilamide gel and stained with silver staining.

**LCR methylation analysis during CDV therapy**

The same five samples of the CDV-treated patient applied for complete genome sequencing was subjected to bisulfite modification carried out as described by Kalantari et al. using 1000 ng DNA. Modified DNA was amplified by methylation-specific PCRs and amplimers were sequenced as described above.
Plasmids and cloning of LCRs

The LCRs of the different strains were cloned into the BamHI-KpnI sites of the luciferase reporter vector pALuc. Ligation products were transformed into *Escherichia coli* XL1 according to the protocol reported by Chung et al. Transformed cells were plated on LB agar containing 100 µg/mL of ampicillin and incubated at 37˚C, overnight. Isolated colonies were cultured in LB Broth containing 100 µg/mL of ampicillin and at 37˚C, 250 rpm, overnight. Plasmids were isolated from overnight cultures and verified by sequencing. Plasmid midipreps for transfection were isolated with Wizard Plus Midiprep Kit according to the manufacturer’s guide.

Site directed mutagenesis

All polymorphisms (except those corresponding to probable sequencing errors; see below) were individually reproduced in the pHPV11-LCR-pALuc plasmid construct containing the whole LCR of the reference HPV11 sequence. Site-directed mutagenesis were performed using 15 ng of plasmid DNA as template. Amplifications were performed with Velocity DNA polymerase (Bioline, London, UK) according to the manufacturer’s guidelines. Amplification products were digested overnight with DpnI, then transformed into *Escherichia coli* XL1 according to the protocol reported by Chung et al. All constructs were verified by sequencing.

Cell culture, transient transfection and luciferase assay

HEp-2 (ATCC Number CCL-23) laryngeal cancer cells were cultured in DMEM (Sigma, St. Louis, MO, USA) supplemented with 10 % foetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 lg/mL streptomycin. Cells were transfected with 2 µg of the reporter vector pALuc and 1 µg of pRSV-βGal as an internal control for transfection efficiency. Cells were transfected in 6-cm-diameter dishes (106 cells/dish) with Lipofectamine 2000 according to the manufacturer’s guide. Cells were harvested 48 h posttransfection by the addition of 500 µL Reporter Lysis Buffer and lysed by a freeze–thaw cycle; the luciferase activities of the cell extracts were measured with the Luciferase Assay System according to the manufacturer’s recommendation. Standardization for transfection was performed with the β-galactosidase assay. All assays were run in triplicates. Average
luciferase activities were compared by means of independent samples T-tests using the software SPSS 15.0 for Windows.

**Phylogenetic analysis**

Sequence data were analyzed using CLC Gene Workbench 4.1.2. Phylogenetic analysis included all available (40) HPV11 whole genomes, 49 L1, 47 E6 and 47 LCR sequences from GeneBank.

**RESULTS**

**Investigation of occurrence of multiple HPV11 infections in respiratory papillomas of different aggressivity**

SSCP analysis of E1, E2, E6, E7 and LCR always excluded the feasibility of infection with different HPV11 strains, and samples of the same patient always harboured identical HPV11 genomes.

**Complete genome analysis of HPV11 strains derived from respiratory papillomatosis of different aggressivity**

On the whole, 41 nucleotide sequence alterations were detected in the analyzed HPV11 strains compared to the reference HPV11 isolate M14119. Four probable sequencing mistakes were identified in the reference HPV11 genome: nucleotides 1783 and 1784 should read GC instead of CG in the original publication, which corresponds to arginine instead of alanine in amino acid residue 318. The sequencing mistake localized to the LCR at positions (7719-7720 GC) reported by Heinzel et al. was also confirmed.

Six of the 37 alterations were found in the LCR, two of these were detected in all six genomes. The alteration localized to the non-coding region was also identical in all complete genomes sequenced examined. Thirteen of the 30 alterations detected in the coding regions were observed in all six complete genome sequences.

Eleven alterations resulted in amino acid changes; A45S in E7, A72E in E1; Q86K and K308R in E2, Q46R, G61E, S76L in E2/E4, but these latter three led to amino acid changes merely in the E4 protein; I28F and V41L in E5A; A476V and S486F in the L1. Six of the eleven alterations were uniformly observed in all six genomes, one (Q46R in the E4
ORF) was found in all but one (Patient 6) genomes. The four remaining nucleotide changes were unique, that is, alterations A72E in E1 and Q86K in E2 proteins were only detected in the sequence from Patient 4 (with a moderately aggressive disease), while L1 ORF sequence polymorphisms were unique to Patient 5 with a highly aggressive papillomatosis. These alterations were not shown in any sequences deposited in the GenBank either. Genomes from papillomas of different severity did not varied in the translated amino acid sequence of ORFs E5/E6/E7 involved in the pathogenesis of papillomatosis.

The six alterations detected in the LCRs consisted of four nucleotide changes, one insertion and one deletion. Two of the four nucleotide changes were unique; one of these was located immediately next to the third E2 binding site upstream from the promoter and was exclusively present in the HPV11 of Patient 6 (with a highly aggressive disease). The insertion was localized to the common region of the putative binding sites for transcription factors USF and Sp1, and was detected in all complete genomes. The deletion was found in five of the six genomes. Thus, only two of the LCRs were identical, those of Patient 1 and Patient 2 with solitary papillomas; all LCRs determined were different from the LCR of the reference genome sequenced from a patient with a highly aggressive papillomatosis as well.

**Phylogenetic analysis**

All available complete HPV11 genomes were relatively closely related; however, the two proposed lineages could be distinguished. The six Hungarian sequences identified by us belonged to lineage A2 and clustered closely together, regardless of severity of the disease caused, except the HPV11 sequence derived from Patient 6. Three sequences from Thailand, one Slovenian sequence and that of Patient 6 formed a distinguishable subgroup within lineage A2.

E6 and L1 ORFs were relatively well conserved; 82/94 and 62/95 sequences were identical, respectively. All E6 and L1 determined in the present work fell within this identical group of sequences, except the L1 ORF of the HPV11s of Patients 5 and 6. HPV11 of Patient 5 showed two unique SNPs, both resulting in missence mutations, while in the case of Patient 6, the alterations were silent.

The LCR showed the highest diversity, 55/94 sequences formed the main cluster of identical sequences. Interestingly, merely one of our sequences, that is from Patient 5 belonged to the main cluster. Sequences from the two minimally aggressive papillomatosis showed identical sequences and from any other LCR sequences from the GeneBank. LCR
sequence of HPV11 strain from Patient 3 with moderately aggressive papillomatosis was identical with eleven LCR sequences isolated from anal samples from Slovenia. LCR from Patient 4 with moderately aggressive papillomatosis was unique, while the LCR of the HPV11 strain derived from Patient 6 with highly aggressive papillomatosis was identical to two HPV11 sequences from Thailand.

Transfection and measurement of LCR transactivating potential

All LCRs studied showed significantly higher transactivating potential compared to the baseline, but showed significantly decreased activities compared to activity of the reference HPV11 LCR (M14119). HPVs from the two solitary lesions (Patient 1 and Patient 2) as well as from the two moderately aggressive papillomatoses (Patient 3 and Patient 4) showed activities comparable to each other; LCR activities of the two minimaly aggressive papillomatoses was significantly lower than any other activities measure (p=0.049 to p=0.001). Interestingly, luciferase activities of the LCRs from the two highly aggressive respiratory papillomatoses were different (p=0.004); that of Patient 6 demonstrated the highest, though still significantly lower activity than the activity of the LCR of the reference plasmid (p=0.025). In contrast, the LCR activity of the HPV11 strain derived from Patient 5 showed an activity comparable to one of the moderately aggressive diseases (Patient 3; p=0.575) and was even significantly lower than that of the other patient with moderately aggressive disease (Patient 4; p=0.022). The latter activity was comparable to that detected for Patient 6 (p=0.105).

Site directed mutagenesis

Out of the six polymorphisms reproduced by mutagenesis, one strong attenuator (T7546C), two potentially but not significantly attenuator (7509 T deletion and A7413C), and two potentially but not significantly enhancer (T7904A and C7479T) mutations were identified. The sixth mutation in the LCR (7584 C insertion) has no effect (p=0.919) on transactivating potential. T7546C change detected in all six genomes studied showed significant attenuating effect (p=0.003) on LCR transactivating potential, and it was detected in 83 of 88 HPV11 LCR sequences in the GenBank.; T deletion at position 7509 present in five genomes (except from Patient 4) and an A7413C change present merely in the genome from Patient 5 demonstrated detectable but not statistically significant attenuator effect (p=0.111 and p=0.472, respectively). These polymorphisms were present in 81/88 and 58/88
of LCRs from the GenBank, respectively. T7904A located immediately next to an E2 binding site was present in the genome from Patient 6, as well as in two additional GenBank LCRs, and showed enhancer effect, which was statistically not significant (p=0.551). C7479T polymorphism was detected in genomes from both highly aggressive papillomatoses and from Patient 3 with moderately aggressive disease as well as in 81/88 of LCRs available in the GenBank and was associated with the not statistically significant enhancement (p=0.372) of the activity of the LCR.

Investigation of multiple HPV11 infections during CDV therapy

All analyzed samples prior to, during and after the CDV therapy (14 laryngeal and 10 palatal) uniformly showed HPV11 infection based on the RsaI/Tru fragment analysis of MY/GP nested PCR products. The E6, E7, E1, E2 and the hypervariable LCR showed identical SSCP patterns for all samples analyzed during the 8-year-long follow-up period.

Complete genome analysis of HPV11 isolates before, during and after intralesional cidofovir therapy

The five complete genomes analyzed were identical, genetic alterations were not detected during and after the CDV therapy compared to the HPV11 genomes derived from the samples prior to the CDV therapy. Compared to the HPV11 reference genome (M14119), 24 nucleotide alterations (three in the LCR, one in the non-coding region at nucleotide position 3832, and 20 in protein coding regions) have been detected. Seven of the single nucleotide polymorphisms/mutations in the coding regions were missense resulting in amino acid alterations, while the remaining thirteen nucleotide changes in the protein coding regions were silent. Sequencing data confirmed the results of SSCP analysis, that is, identical HPV11 genome was present during the 8-year-long follow-up period.

Methylation analysis of E2 binding sites during CDV therapy

All eight CpG islands of the four E2 binding sites responsible for transcriptional regulation were uniformly unmethylated in all five samples investigated.
DISCUSSION

There is a solid evidence that HPV16 intratypic variants possess characteristic geographical distribution pattern and markedly differ in their pathogenic (oncogenic) potential.

Burk et al. reported that two lineages (A1 and A2) are also feasible among HPV11 strains, and that observation raised the possibility that intratypic variants may also exist among HPV11s. Though, the genetic distance between these two lineages is relatively small, the pattern of the genome polymorphisms characteristic to the lineages is similar to that described in the case of HPV16 intratypic variants. Assessment of the differences in pathogenic potential of these lineages is challenging, as relevant clinical data on the course of the disease and the severity are frequently unreported together with the GenBank sequence data.

According to our results, E5/E6/E7 oncogenes have no or only a minor role in the differences of pathogenic potential of HPV11 sequences in respiratory papillomas, since all of the E5/E6/E7 ORFs sequenced were identical regardless severity. This curiously contrast the situation of HPV16, since intratypic variants of HPV16 differ in the sequences of the E6 ORF, amino acid sequences of the E6 oncoprotein and the oncogenic activity. In contrast, the E7 sequences of high-risk HPV types show high conservation.

Unique polymorphism resulting in amino acid change was detected in the E1 and another in the E2 ORF in the case of Patient 4 (from moderately aggressive papillomatosis), both alterations in the region responsible for binding of E2 to E1. Since the E1–E2 complex plays a crucial role in viral replication, these polymorphisms may be associated with the virulence of the virus. However, this assumption needs confirmation by parallel cases.

In the HPV11 genome from Patient 5, two unique amino acid alterations were identified in the L1 ORF, both in the C-terminal end of the protein, which is responsible for the self-assembly of the virus capsid. These changes may increase the virulence of the virus by enhancing the assembly and virion production (this patient had an extensive extralaryngeal dissemination) and/or feasibly affecting the display of the neutralizing epitope by modifying the quaternary structure of the capsid protein and consequently may contribute to the evasion of the immune recognition. Such differences in the L1 ORF were also reported among HPV16 intratypic variants, but their importance, together with the importance of the reported changes in case of HPV11, remains to be determined.
The LCR undeniably plays a paramount role in the viral replication as well as in the cell proliferation mediated by the virus. The activity of the LCR of intratypic variants of HPV16 correlates well with the pathogenic potential of the variants. In the present study, viruses producing one episode without recurrences showed the lowest LCR activities. A probable explanation is that these LCRs contained not only the alteration significantly decreasing LCR activity, but also an additional nucleotide change, which also seems to decrease transactivating potential further and they lack alterations associated with higher LCR activity. This may also explain the mild clinical course of the disease. Moderate activities were measured for the moderately aggressive viruses, which carried either the main attenuator polymorphism together with a polymorphism with neutral effect (Patient 4) or besides the main attenuator, one potential attenuator and one potential enhancer polymorphisms (Patient 3). We hypothesize that in the former case, the polymorphisms found in the E1 and the E2 ORFs, while in the latter, the potential enhancer alteration may have partially compensated the effect of the main attenuator polymorphism in the LCR. Curiously, one of the HPV11s associated with severe disease (Patient 5) also showed moderate luciferase activity. This LCR contained the main attenuator, plus one potential enhancer and two additional potential attenuator alterations. The severe clinical course may be explained by the unique L1 polymorphisms (see above). High activities were measured for Patient 6, whose virus carried LCR with the alterations seen for Patient 3, but in contrast to the case of Patient 5, with an additional potential enhancer alteration. This additional alteration (T7904A) was also found in two sequences from Thailand; one from a severe papillomatosis with lung involvement, another from a high-grade squamous intraepithelial lesion of the uterine cervix, which is mostly caused by high-risk HPV genotypes rather than HPV11. These parallel data also point to the possibility that this alteration may have an enhancer effect.

The highest activity was measured for the reference genome, which can be explained by the lack of the strong attenuator polymorphism. It is plausible that the nucleotide alteration termed as the main attenuator (T7546C) represents the wild type, and the thymine at position 7546 found in the reference sequence is in fact an enhancer alteration. All six studied HPV genomes were unique, suggesting that virulence in most cases is not a direct consequence of a single mutation with a strong effect, but is determined by the combined effect of different changes in the genome, even if these effects are not statistically significant when tested individually.

Studies on HPV variant analysis in consecutive samples are infrequent and data regarding the stability of HPV genome in human cells are scarce, only a few studies were
carried out in this field primarily focusing on high-risk HPV types isolated from the anogenital regions. Moreover, most studies concentrate merely on the long control region, the most variable region of HPVs. Based on SSCP and sequence data we found no evidence for the existence of a multiple HPV11 infection in the CDV-treated patient; during the monitored period in the course of the disease (1999–2007) the same HPV11 was present in all samples regardless of localization, excluding the existence of strains with differential sensitivity to CDV in the background of the virological failure observed. The data also demonstrate the marked stability of HPVs during an infection. HPV detection and typing also excluded coinfection with other HPV types. Alterations in the amino acid sequence detected in the viral proteins as well as LCR nucleotide alterations as compared to the reference genome were present in all samples sequenced. Though one or several of these differences may explain the therapy resistance and/or the aggressivity of the HPV11, they cannot explain the course of the therapeutic response. In summary, total identity of all five complete genomes derived from the patient underwent CDV therapy showed that resistance mutations could not be responsible for the virological failure. It cannot be explained by alterations in the gene expression pattern either, as all regulatory CpG sites were uniformly unmethylated in all samples. The response followed by late virological failure and clinical recurrence may have been determined by host factors, at least in the examined case, suggesting that CDV action may depend more heavily on the host than on viral factors.
SUMMARY

Previous studies on HPV-16 isolates described five distinct variants of HPV-16. Some of these variants were associated with increased oncogenic potential. Several studies revealed that the differences in the severity of HPV-16 and other high-risk HPV type-related (HPV-18, -33, -52) diseases can be attributed to single nucleotide polymorphisms or deletions in the HPV genome. These findings suggest that such nucleotide polymorphisms in the HPV genome may influence the clinical course of low-risk HPV-related diseases such as recurrent respiratory papillomatosis (RRP). Two recently published papers reported that two HPV-11 genetic lineage may exist based on the sequence analysis of HPV-11 isolates from Slovenia. Unfortunately, clinical data are not reported, therefore, the link between the severity of the HPV-11 related diseases and HPV-11 sequence polymorphism remained unclear. Cidofovir is one of the most frequently applied antiviral agent in RRP therapy. However, authors reported that that 60% of CDV-treated RRPs show partial or no response to the treatment. Present study examined the role genetic alterations in the virological failure of CDV therapy in the case of a highly aggressive papillomatosis. In addition, we studied the feasible association of nucleotide alterations with the pathogenic potential of HPV-11 isolates from two weakly aggressive solitary papillomas, two moderately and two highly aggressive RRPs.

Consistent changes in the virus genome explaining the clinical behaviour of RRPs with different severity associated with HPV11 were not identified; virulence seems to be determined by interaction of multiple genetic differences. LCR activities corresponded well to severity, except one case with L1 alterations. The variability of oncoproteins does not seem to play a role; this finding contrasts the situation of the high oncogenic risk types HPV16 and HPV18, where intratypic variants differ in the sequences of the E6/E7 ORFs. All Hungarian sequences belonged to phylogenetic lineage A2 and clustered closely together, excepting that of Patient 6 from highly aggressive RRP, regardless of severity of the disease caused.

Our data showed that virological failure of CDV therapy was not associated with genetic or epigenetic changes in the HPV-11 genome suggesting that cidofovir action may depend more heavily on the host.
List of publications related to the dissertation

   DOI: http://dx.doi.org/10.1007/s00430-013-0297-y
   IF:3.545 (2012)

2. Gáll, T., Kis, A., Fehér, E., Gergely, L., Szarka, K.: Virological failure of intralesional cidofovir therapy in recurrent respiratory papillomatosis is not associated with genetic or epigenetic changes of HPV11: Complete genome comparison of sequential isolates.
   DOI: http://dx.doi.org/10.1016/j.antiviral.2011.09.007
   IF:4.301

List of other publications

   DOI: http://dx.doi.org/10.1016/j.taap.2013.02.014
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Total IF (publications related to the dissertation): 7.846

The Candidate’s publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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