Antiviral Research 92 (2011) 356–358

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Short communication

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

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ARTICLE INFO

Article history:
Received 28 June 2011
Revised 23 August 2011
Accepted 11 September 2011
Available online 16 September 2011

Keywords:
Laryngeal papillomatosis
Therapeutic response
Antiviral resistance
Complete genome
LCR methylation

ABSTRACT

Five sequential human papillomavirus type 11 (HPV11) positive samples collected from an aggressive juvenile onset recurrent respiratory papillomatosis before, during and after intralesional cidofovir therapy leading to virological failure after initial response were analyzed. Sequencing of the complete genome as well as methylation analysis by bisulfate modification and sequencing of the long control region (LCR) were performed to seek for genetic and epigenetic changes as a possible background for therapy failure. Single-strand conformation polymorphism of E1, E2, E6, E7 and LCR was used to exclude the presence of multiple HPV11 infection. All five complete genomes were identical and all four E2 binding sites in the LCR were uniformly unmethylated in all five genomes. Thus the virological failure was not due to virological factors suggesting that cidofovir action may depend more heavily on the host.

Recurrent respiratory papillomatosis (RRP) is a common benign tumor of the airways (Derkay, 2001; Pransky and Kang, 2003) caused by human papillomaviruses (HPVs), principally low-risk HPV types, HPV6 and HPV11 (Dickens et al., 1991). Current standard treatment for RRP is surgical, but the recurrent nature of RRP frequently necessitates multiple surgical interventions (Reeves et al., 2003). In order to improve the outcome, adjuvant therapy is used for the treatment of RRP including cidofovir, ribavirin, interferon-α, indol-3-carbinol chemotherapy or photodynamic therapy (Avidano and Singleton, 1995).

Cidofovir (CDV) is a cytosine nucleotide analog, which, in the active cidofovir diphosphate form can inhibit DNA polymerase of herpesviruses and other DNA viruses (Safrin et al., 1997; Xiong et al., 1997).

CDV has recently been introduced into the treatment of RRP as an adjuvant therapy (Chetri and Shapiro, 2003; Pransky et al., 1999, 2000; Snoeck et al., 1998; Wilson et al., 2000). Van Cutsem et al. (1995) reported the complete remission of a squamous papilloma in the hypopharynx and the esophagus by administration of CDV directly into the tumor. However, in other cases CDV seems to be less effective (Chadha and James, 2007). The mechanism of action of CDV in HPV-infected cells presumably differs from its action against herpesviruses as HPV does not encode DNA polymerase, and its replication is mediated by the host-cell enzyme. Andrei et al. (1998) demonstrated that the growth of HPV-infected keratinocytes is inhibited by CDV considerably more as compared to non-infected cells. Infected cells seem to be trapped in the S phase indicating inhibition of DNA synthesis (Johnson and Gangemi, 1999). Interestingly, growth inhibition by CDV detected in HPV-infected keratinocytes seems to be considerably decreased after 20–30 passages as compared to early-passage cells (Johnson and Gangemi, 1999).

Putative alternative mechanisms of CDV action include apoptosis induction, and down-regulation of HPV E6/E7 oncoprotein expression at transcriptional level (Abdulkarim et al., 2002). In contrast, other authors reported that CDV increases the levels of E6 oncoproteins of low-risk and high-risk HPV types (Donne et al., 2009), and malignant degeneration of a papilloma in a patient undergoing CDV therapy was also reported (Lott and Krakovitz, 2009). Effect on the cellular DNA polymerases was also suggested as a mechanism of action of CDV against HPVs (Abdulkarim and Bourhis, 2001; Pisarev et al., 1997).

In a previous study, virological failure of intralesional CDV treatment with moderate clinical improvement was reported in a 14-year-old boy with HPV11-associated severe juvenile-onset RRP (Major et al., 2008). The aim of the present study was to examine whether the reported virological failure is associated with genetic or epigenetic alterations in the virus genome using complete genome sequencing and examination of the methylation status of CpG islands in the E2 binding sites of the long control region (LCR).

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doi:10.1016/j.antiviral.2011.09.007

Table 1
SSCP analysis of different ORFs and the long control region of HPV11.

HPV11 region	Amplimer for SSCP analysis (nucleotide position)	Restriction enzyme(s)	Resulting fragments (bp)
E6	86-533	NdeI	181, 149, 117
E7	514-842	SspI	277, 51
E1A	824-1879	AluI + SspI	464, 301, 155, 135
E1B	1861-2771	HaeIII + SspI	302, 260, 205, 143
E2	2717-3878	DraI	395, 333, 162, 150, 121
LCR	7192-160	Bsh1236I	369, 320, 212

SSCP, single stranded conformation polymorphism; bp, base pairs; E, early; L, late; LCR, long control region.

Relevant clinical information about the scheduled cidofovir therapy and the patient was published earlier (Major et al., 2008). Clinical and virological status was monitored for 5 years prior to cidofovir treatment, throughout the therapy and for 1 year after cessation of cidofovir administration. All 24 (14 laryngeal and ten palatal) samples were HPV11 positive. Five HPV11-positive samples, one laryngeal collected prior to cidofovir or interferon therapy (Sample1, from 1999), one laryngeal (Sample2, from 2005) and one palatal (Sample3, from 2005) collected during the initial good response to as well as one laryngeal (Sample4, from 2006) and one palatal (Sample5, from 2006) collected after the failure of the CDV regimen were analyzed.

DNA was isolated from fresh-frozen tissue samples by phenol/chloroform/isoamylalcohol extraction. HPV detection and typing were performed by MY/GP consensus PCR followed by restriction enzyme analysis (Major et al., 2008).

Specific primers were designed and applied to amplify the whole genome (Supplementary Table S1). Annealing temperatures for the different primer pairs are listed in Supplementary Table S1. PCRs were performed with GeneAmp High Fidelity PCR System (Applied Biosystems, Foster City, CA, USA).

To exclude the presence of multiple HPV11 strains, single-strand conformational polymorphism (SSCP) analysis of the genomic regions E1, E2, E6, E7 and the long control region (LCR) was performed on all 24 samples using the amplimers described above. Amplimers were digested with the restriction enzymes NdeI, Bsh1236I (Fermentas, Vilnius, Lithuania), AluI, SspI, HaeIII, and DraI (Promega, Madison, WI, USA) according to the manufacturer's guidelines (Table 1). Each digested amplimer (3 μl) was diluted tenfold in 95% formamide containing 0.04% bromophenol blue and 0.05% xylene cyanol. Samples were denatured at 95 °C for 5 min, then immediately placed on ice. Each sample was run in 15% polyacrylamide gel with 1 × TBE at 5 °C and 250 V for 6 h. DNA fragments were visualized by silver staining.

For sequencing, amplimers derived from two independent PCR reactions were purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced directly from both directions with the ABI BigDye terminator v3.1 Cycle kit (Applied Biosystems, Foster City, CA, USA) in an ABI 3100 Genetic Analyzer. Sequences were assembled and analyzed against the reference HPV11 genome (GenBank Accession Number: M14119) using the software CLC Gene Workbench 4.0 (CLC Bio, Aarhus, Denmark).

The same five samples used for complete genome sequencing was subjected to bisulfite modification carried out as described by Kalantari et al. (2004) using 1000 ng DNA. Modified DNA was amplified by methylation-specific PCRs (Supplementary Table S1) and amplimers were sequenced as described above.

All analyzed regions showed uniform SSCP patterns for all 24 samples. The five complete genomes analyzed were identical (GenBank Accession Number: FR872717), genetic alterations were not detected. Compared to the HPV11 reference genome (GenBank Accession Number: 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. Additional C and GC insertions as well as a T deletion were also found in the LCR. 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 (Supplementary Table S2).

All eight CpG islands of the four E2 binding sites responsible for transcriptional regulation were uniformly unmethylated in all five samples tested (Fig. 1).

CDV is the first synthetic chemotherapeutical used against HPVs, which may potentially target viral processes. However, the

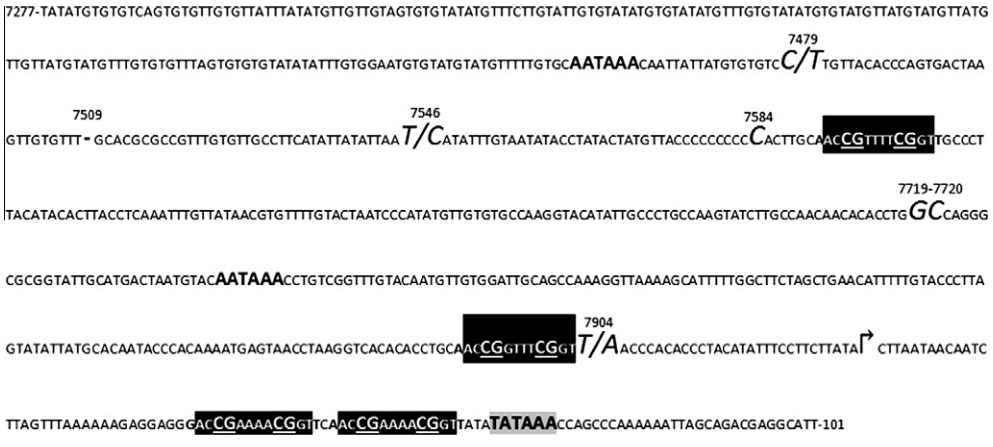

Fig. 1. Structure of the long control region of the five identical human papillomavirus 11 sequences. Broken arrow shows nucleotide position 1. Numbered nucleotides in bold italics show single nucleotide polymorphisms compared to the reference genome (M14119, first nucleotide represents the reference sequence) and insertions; the numbered dash indicates the deletion; binding sites for the regulatory E2 viral protein are shown by black background, regulatory CpG islands within are highlighted by underlining and larger font; bolded regions indicate poly-A sites and the TATA box is shaded gray.

mechanism of CDV action against other viruses is not applicable for HPVs and the exact mechanism of action remains unclear. Most studies report only clinical response and do not investigate virological factors in the background of the outcome reported (Akst et al., 2003; Tanna et al., 2008).

CDV is used against HPVs mostly in the treatment of RRP as an adjuvant therapy. Approximately 60% of the patients show good response to the drug but 40% of them show partial or no response (Chadha and James, 2007), however, the causes in the background of poor response have never been examined to our knowledge.

Studies on HPV variant analysis in consecutive samples are infrequent. Only a few studies were carried out in this field of HPV research and these studies focus on high-risk HPV types isolated from the anogenital regions (Mayrand et al., 2000; Steinau et al., 2010; Xi et al., 2010). 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; 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 E1, E4, E5 and E7 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 sequenced shows 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.

Acknowledgments

The authors thank Tamás Major for the sample collection. The study was partially supported by the Hungarian National Research Grant (OTKA K73145). The funding source had no role in the study design, experimental work or interpretation of results.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2011.09.007.

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