Follow-up of HPV DNA Copy Number in Cidofovir Therapy of Recurrent Respiratory Papillomatosis

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Abstract. *Background: Cidofovir is a cytosine nucleoside analogue antiviral drug given as an adjuvant threapy in recurrent respiratory papillomatosis (RRP). Materials and Methods: Intralesional cidofovir therapy was given to a 14-year-old male patient. The papilloma severity score (PSS) of Derkay et al. was used for follow-up. Serial fresh-frozen biopsies were taken from the lesions in the larynx and soft palate prior to therapy and during its course. After human papillomavirus (HPV) typing and the determination of the genomic physical state, the HPV DNA copy number was estimated with real-time PCR. Results: All the papillomas harboured HPV 11 DNA in episomal form. Prior to therapy, the HPV copy number fluctuated with time. In the initial treatment period with 2-week-intervals both the viral load and the PSS decreased and a transient complete remission was observed. Subsequently, when the injections were given at longer intervals, the viral load returned to the initial values or greated, fluctuations reappeared and the RRP recurred at a controlled rate. Conclusion: The initial treatment period was successful, as the viral load decreased, and long-term effects of cidofovir might account for the controlled disease as the injection intervals were prolonged.*

Recurrent respiratory papillomatosis (RRP) is the most common benign neoplasm of the pediatric airways characterized by warty exophytic lesions anywhere in the airways (1). The aetiology is productive low-risk human papillomavirus (HPV) 6 and 11 infection, where the HPV DNA genome exists in episomal form. The natural history of RRP is unpredictable due to recurrent growth. The mainstay of therapy is repetitive endoscopic removal (2). The recurrent nature of RRP with the necessity for repetitive surgery indicates the need for adjuvant therapies.

Cidofovir is a cytosine nucleoside analogue. The active intracellular metabolite cidofovir diphosphate is a competitive inhibitor of viral DNA polymerases and thereby prevents replication of a number of DNA viruses (3). Van Cutsem *et al.* (4) reported the first successful intralesional use of cidofovir in the head and neck region. They achieved complete remission of a squamous papilloma in the hypopharynx and esophagus in an adult by serial administration of cidofovir. The first use of cidofovir in RRP with promising results was reported by Snoeck *et al.* (5). Since then a number of authors have decribed their experiences with the intralesional use of cidofovir, either as a monotherapy or in conjuction with surgical debulking of papillomas (6-13). The efficacy remains unknown; 40-50% of patients achieve durable remission and, in contrast, 20% are non-responders (14).

In the current study, the HPV DNA type, physical state and copy number were investigated during intralesional cidofovir therapy in an RRP patient.

Materials and Methods

The patient was a 14-year-old male who had been suffering from RRP since the age of 18 months (1996). Before the intralesional cidofovir protocol, he had undergone endoscopic papilloma debulking and CO_2 laser evaporisation of his laryngeal RRP 31 times. In August 2001, papillomas appeared on his soft palate. Since then his RRP has been bifocal. As an adjunctive therapy he received interferon alfa-2a in the five years preceding the cidofovir therapy. Despite the combined therapy his disease became aggressive according to Doyle's criteria (15). In August 2004, he underwent an urgent tracheotomy for symptoms of airway obstruction. His tracheostoma was maintained until the start of cidofovir therapy in March 2005.

A modification of the scheduled protocol reported by Chetri *et al.* was applied and the first four treatments were scheduled every two weeks (week 0, 2, 4 and 6) (8). In the original protocol, the subsequent treatment intervals were each increased by one week (week 9, 13, 18, 24, *etc.*). The subsequent treatments for our patient were performed at week 8, 12, 16, 24, 42 and 55.

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Key Words: Human papillomavirus 11, cidofovir, laryngeal neoplasms, polymerase chain reaction.

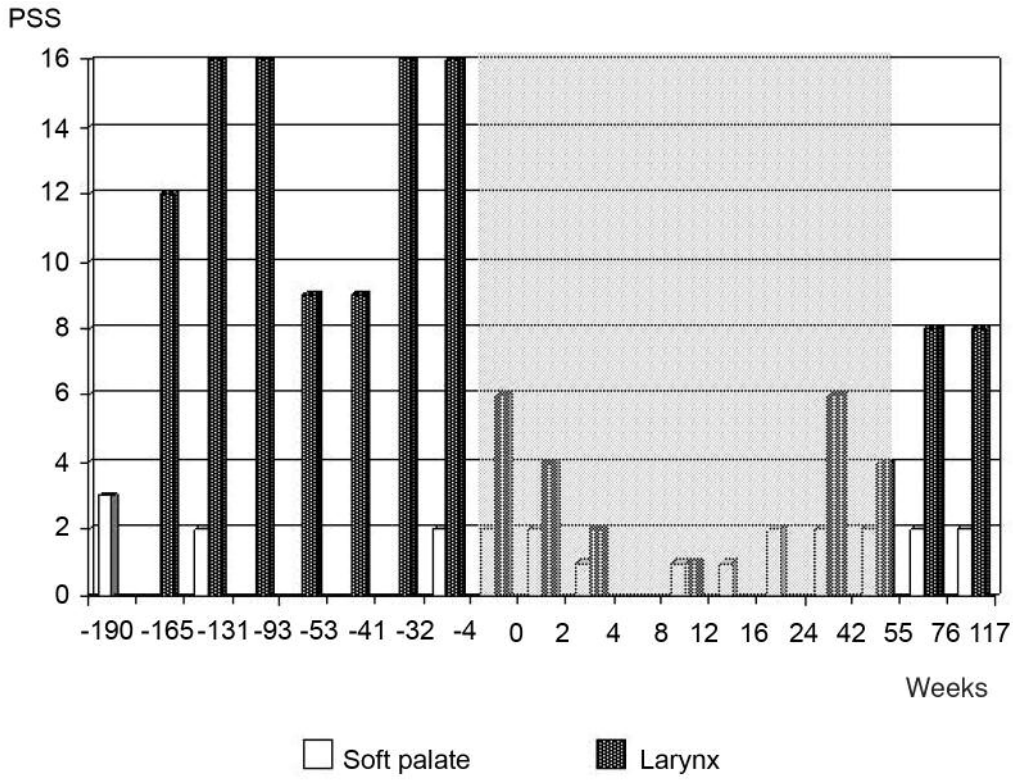

Figure 1. *Changes of papilloma severity score (PSS) over time (weeks) in the two affected sites, 0 refers to the initiation of cidofovir therapy. The light grey area represents the duration of cidofovir therapy. Negative numbers of weeks refer to the pre-treatment period.*

Microsuspension direct laryngoscopy was performed under general anesthesia (endotracheal intubation was performed *via* the tracheostoma as it was maintained until the achievement of durable laryngeal remission by the 7th treatment). After assessment of the RRP stage as described by Derkay *et al*. [papilloma severity score, PSS (16)], the lesions were removed with cupped forceps followed by laser ablation of the base both in the larynx and the soft palate. Cidofovir (Vistide; Gilead Sciences, Foster City, CA, USA) was diluted to a concentration of 10 mg/ml for the larynx (to prevent airway obstruction) and 5 mg/ml for the soft palate. A total dose of 1 mg/kg was given at each treatment. At remission, injections were continued at the former localisations in the larynx and soft palate.

Histological examination was routinely performed at each debulking, except when a site was in complete remission. Fresh-frozen tissue samples derived from the centre of the lesions or the former place of the papillomas at remission were collected for HPV DNA examination.

The MY09/MY11 and GP5+/GP6+ nested polymerase chain reaction (PCR) methods for HPV detection and E1-, E2- and E1E2-specific PCRs for determination of the HPV DNA physical state have been published elsewhere in detail (17). Briefly, the HPV DNA may exist either in episomal form or integrated into the host's genome. In the episomal form, the circular double-stranded papillomavirus DNA is intact. In contrast, integration results in disruption of the circular viral genome, mainly in the E1E2 open reading frames (ORF). The product of the E2 ORF has a regulatory role on transcription of transforming E6 and E7 viral genes. The lack of E1, E2 or E1E2 amplimers supposes the integration of HPV DNA into the host genome.

Real-time PCR for the assessment of the 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). PCR amplification was conducted in 20 μl, containing 1× ABI SYBR Green PCR Master Mix, 5 pmol of each MY primer and 10 ng template DNA. The amplification ramp included an initial hold step of 10 min at 95˚C followed by repeated three-step cycles (45 cycles total) consisting of denaturation at 95˚C for 20 s, annealing at 52˚C for 20 s and extension at 60˚C for 1 min. 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 by increasing from 60˚C to 95˚C at a transition rate of 0.1˚C/s. The virus copy numbers were calculated by a standard curve (10^{11}-10^{0} copies per μl) derived from HPV11 plasmid DNA.

As a control for the effect of cidofovir, virological studies were also performed from five pre-protocol biopsies.

The patient was followed up with outpatient rigid 70˚ video-laryngoscopy (at week 76) and microsuspension laryngoscopy (at week 117).

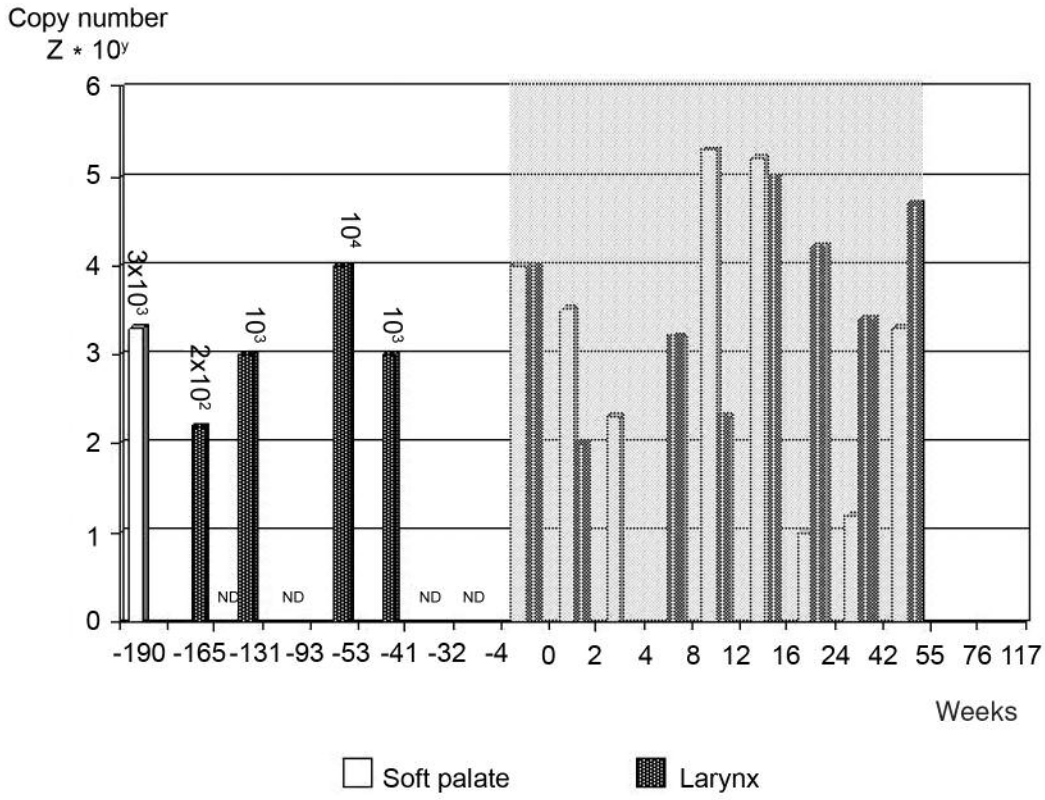

Figure 2. *Changes of HPV DNA copy number over time (weeks) in the two affected sites, 0 refers to the initiation of cidofovir therapy. The light grey area represents the duration of cidofovir therapy. Copy number of 1 μl DNA is expressed by Z * 10y, where y is the integer (whole number) scaled on the vertical axis, Z is the number of decimal places between two consecutive y whole numbers. Exact copy numbers are also displayed at the top of columns in the pre-treatment period. For exact copy numbers of the two affected locations during therapy see Figures 3 and 4. ND = not done.*

Results

The intralesional administration of cidofovir was carried out without systemic side-effects. Moderate scarring was seen in the larynx prior to treatment, which remained constant during the course of therapy.

Histology confirmed the diagnosis of RRP at each surgical debulking except one case of chronic laryngitis at the third injection (see below). At the fourth injection when the patient was disease-free, routine histology was not performed. Mild dysplasia was seen in some slides, but neither severe dysplasia nor malignant degeneration were observed.

The changes in the PSS before and during cidofovir therapy are shown in Figure 1. The patient achieved complete remission by the fourth course (8 weeks) of therapy. As the interval between injections was prolonged, RRP recurred at a controlled degree below the PSS of the one-year pre-treatment period. One year and three months after the initiation of the cidofovir therapy, the PSS was 2 for the soft palate and 8 for the larynx. The changes of the scores for the two distinct locations were slightly different in time, but the initial four injections proved to be very successful.

Each histologically verified papilloma biopsy harboured HPV 11 DNA in episomal form (positive results for E1, E2 and E1E2 PCRs). In one case where the HPV DNA copy number was not determinable with the real-time PCR, the MY09/MY11 – GP5+/GP6+ nested PCR alone showed the presence of HPV DNA, which was presumably in an extremely low copy number (soft palate, fourth injection). Figure 2 shows the fluctuation of HPV-11 DNA copy number before and during the course of cidofovir therapy. Figures 3 and 4 illustrate the PSS and the HPV DNA copy number simultaneously at the two affected sites. At the initial three and four injections in the larynx and soft palate, respectively, the decrease of PSS was accompanied by a decrease in HPV DNA copy number. By the fourth injection, when the patient was in complete remission, the HPV copy number could not be determined in the soft palate, but a copy number of 2×10^3 was detected in the papilloma-free larynx.

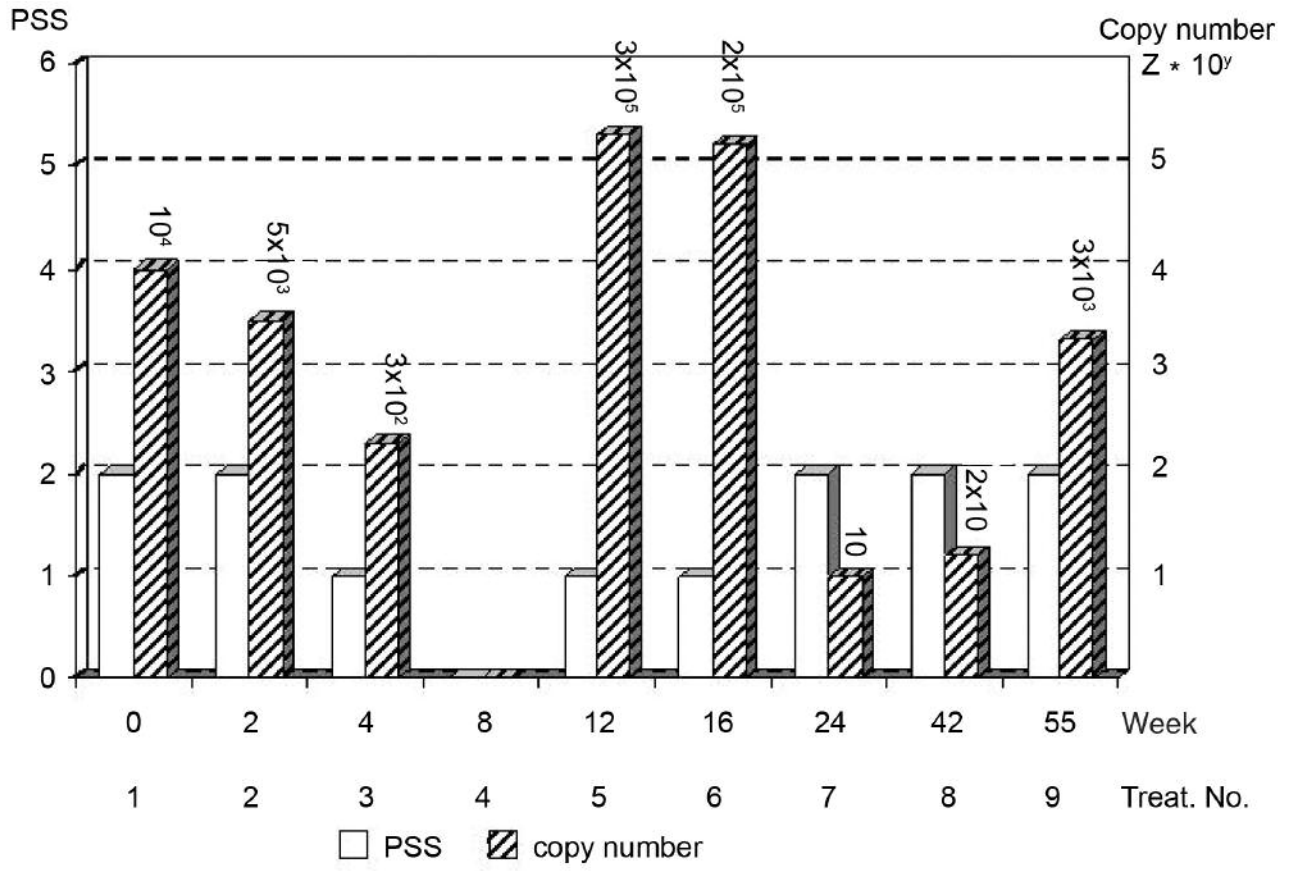

Figure 3. *PSS and HPV DNA copy number (logarithmic scale) in the soft palate during cidofovir therapy (weeks). Copy number of 1 μl DNA is expressed by Z * 10^y, where y is the integer (whole number) scaled on the vertical axis, Z is the number of decimal places between two consecutive y whole numbers. Exact copy numbers are also displayed at the top of columns.*

In contrast, the PSS and viral DNA copy number tended to fluctuate independently in the subsequent period of cidofovir therapy. At the fifth injection, a small surface lesion was observed on the soft palate accompanied by a high HPV DNA copy number (2×10^5). At the sixth and seventh injections, the larynx was in remission with copy numbers of 10^5 and 2×10^4, respectively. After the eighth injection (42 weeks), the patient had moderate PSS in both sites vith varying copy numbers.

At the third injection, surface lesions were seen in the anterior commissure and on the left vocal cord (PSS=2) though the copy number was not determinable with real-time PCR. The histology confirmed the virological finding, as it revealed chronic laryngitis without histological signs of papilloma.

Due to the durable remission in the larynx, the tracheostoma was closed at the seventh injection (week 24). However, a persistent 3-mm-diameter tracheocutaneous fistula was observed at the last two injections and in the post-treatment follow-up period.

Discussion

The long-standing recalcitrant aggressive RRP in this case prompted the cidofovir therapy.

The intralesional administration of cidofovir *via* microsuspension laryngoscopy is widely accepted nowadays as an adjuvant therapy of RRP. The relatively long intracellular half-life of cidofovir (between 17 and 65 hours) and its metabolites allows for infrequent dosing (18). Our protocol (1 mg/kg total dose/treatment, 2 week initial treatment intervals, 9 injections) based on the study of Chetri *et al*. represented a mean of the literature (8). Higher laryngeal concentrations permit lower injectable volumes to prevent airway obstruction.

Cidofovir did not affect the type or physical state of the HPV-11 DNA in this case. The uniform episomal physical state is the characteristic of papillomas. To our knowledge, this is the first report of the follow-up of HPV DNA copy number in intralesional cidofovir therapy of RRP. In the pre-

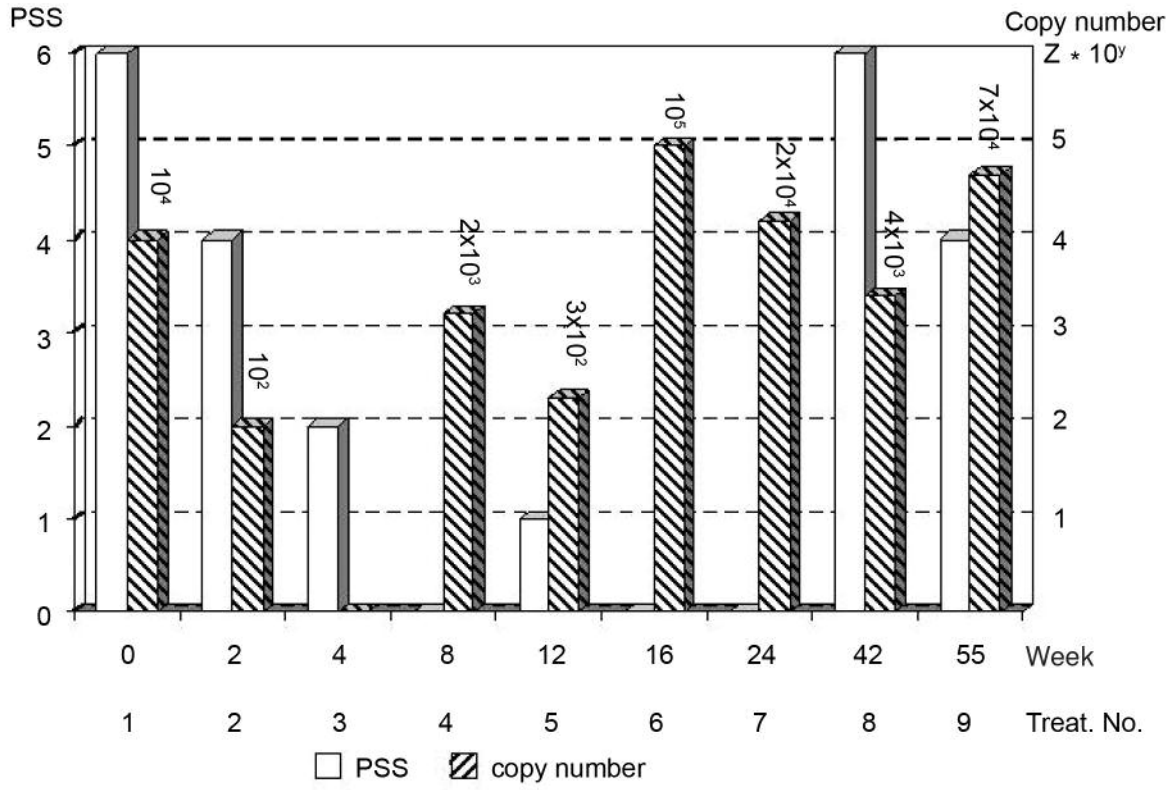

Figure 4. *PSS and HPV DNA copy number (logarithmic scale) in the larynx during cidofovir therapy (weeks). Copy number of 1 μl DNA is expressed by Z * 10y, where y is the integer (whole number) scaled on the vertical axis, Z is the number of decimal places between two consecutive y whole numbers. Exact copy numbers are also displayed at the top of columns.*

treatment period, the viral load fluctuated in the larynx. The soft palate was assessed only once. The laryngeal viral load fluctuation might be attributable to the natural history of HPV infection or to the changes of the host antiviral immunity.

The initial cidofovir treatment period proved to be successful in terms of both PSS and viral load. The viral DNA copy number decreased below the detection level of real-time PCR by week 4 in the larynx and week 8 in the soft palate. The decrease of PSS approximately followed this initial decrease of viral load. Thus, an injection interval of two weeks seems necessary for the efficient antiviral effect of cidofovir, but continuing such frequent surgery and the possible side-effects of cidofovir would be unbearable for the patient.

With the subsequent elongation of treatment intervals, the HPV copy number rose again in both sites, fluctuations reappeared and, moreover, the viral load occasionally exceeded the pre-treatment values. In contrast, RRP recurred in a controlled rate at both sites. This durable control of disease might suggest long-term effects of cidofovir on HPV infection other than the inhibition of viral replication. Van Cutsem *et al*. suggested a potential direct inhibitory effect on tumor cells or activation of host natural killer cell activity (4). Cidofovir has been shown to reduce E6 and E7 expression in head and neck HEP2 squamous carcinoma cells at the transcriptional level (19). Similar inhibition of viral DNA transcription may exist in papillomas.

The HPV DNA-positive sites of remission (the soft palate at fourth injection detected by the nested PCR, and the larynx at fourth, sixth and seventh injections) might demonstrate latent HPV infection, indicating imminent disease recurrence.

Finally, the conditions that may have affected the evaluation of cidofovir therapy, must be recognized. As the indicators of successful cidofovir therapy are the decrease of PSS and prolonged injection intervals, our intralesional cidofovir therapy was effective (20). The formerly aggressive disease turned into a non-aggressive form (15). The initial fall in laryngeal PSS might be attributable to the frequent initial surgeries since the patient underwent laryngoscopies more frequently than indicated by the natural course of the disease. Furthermore, he had a thorough surgical debulking four weeks before the start of cidofovir therapy. The preceding subcutaneous interferon alfa-2a therapy might also have affected the virological findings. In spite of these

considerations, real-time PCR might be a potential follow-up method for accessing the efficacy of intralesional cidofovir therapy.

Acknowledgements

This study was supported by the Hungarian Scientific Research Fund (OTKA grant No. K 63743).

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Received January 29, 2008
Revised April 17, 2008
Accepted April 23, 2008