Detection of HHV-6-specific mRNA and antigens in PBMCs of individuals with chromosomally integrated HHV-6 (ciHHV-6)

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Abstract

After inheritance of chromosomally integrated HHV-6 (ciHHV-6), viral DNA is found in every nucleated cell. The prevalence of ciHHV-6 is estimated to be 0.2–5% of humans. There are conflicting data on the potential for replication, possibly leading to clinical implications. We analysed peripheral blood mononuclear cells (PBMCs) from individuals with ciHHV-6 proven by fluorescence in situ hybridization (FISH) for HHV-6-specific mRNA (U94, U42, U22) and antigens by means of reverse transcription PCR and an indirect immunoperoxidase staining. U94 transcripts indicative of latent infection were detected in six (54.5%) out of 11 individuals at least once. Transcripts indicative of lytic infection (i.e. U42 and U22) were detected in four (36.4%) out of 11 individuals at least once. HHV-6 antigen was detected in seven (70%) out of 10 individuals at least once. The presence of viral mRNA and proteins supports virus gene expression from ciHHV-6, which may lead to virus replication. Considering the properties of active HHV-6 infection together with obvious replicative activity in individuals with ciHHV-6, pathophysiological effects leading to clinical consequences of chromosomally integrated viral DNA might be considered.

Keywords: Chromosomal integration, herpesvirus, HHV-6, lytic infection, mRNA, viral antigen, viral replication
Original Submission: 8 January 2014; **Revised Submission:** 29 March 2014; **Accepted:** 31 March 2014
Editor: D. Raoult
Article published online: 3 April 2014
Clin Microbiol Infect 2014; **20**: 1027–1032
10.1111/1469-0691.12639

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Preliminary data on antigen detection in four of the reported patients were selected for oral presentation at the 19th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), which was held in Helsinki in May 2009. Preliminary data on antigen detection and mRNA detection have been presented at the 7th International HHV-6 Conference (2011, Reston, USA) and 8th International HHV-6 Conference (2013, Paris, France). The manuscript containing complete data on antigen detection and mRNA has neither been presented nor submitted or accepted for publication elsewhere.

Introduction

Like other herpesviruses, HHV-6A and HHV-6B persist in the host after primary infection [1,2]. This persistence comprises a true latent state without replication and a low-level chronic replication occurring at the same time in different cell types. While latency is typically seen in peripheral blood mononuclear cells (PBMCs) and bone marrow progenitor cells, chronic replication at a low level is attributed to the salivary glands contributing to viral shedding [1]. Furthermore, HHV-6 can be reactivated by a variety of mechanisms (e.g. other infections, endotoxins, endocrine stimulation, certain cytokines and immune deficiency) leading to lytic infection [1,2]. In addition, chromosomal integration of HHV-6 DNA (ciHHV-6) into the human genome is described in individuals with different diseases (meningitis, encephalitis, myocarditis, convulsions, liver dysfunction, haemolytic and aplastic anaemia, various lymphomas and multiple sclerosis) for both species (HHV-6A and HHV-6B) [3–10]. However, ciHHV-6 is also found in healthy individuals [3]. It is inherited, and its prevalence is estimated to be 0.2–5% [3]. It might be speculated that the high expression of the HHV-6 receptor CD46 on the inner

acrosomal membrane of spermatozoa [11], leading to a high affinity of HHV-6 to spermatozoa [12], might facilitate chromosomal integration into germ line cells and therefore its inheritance.

After stem cell transplantation (SCT) from a donor with ciHHV-6, ciHHV-6 is found in the recipient in every cell derived from haematological stem cells [10].

CiHHV-6 results in persistently high levels of HHV-6 DNA in blood and tissue, which might be interpreted as persistent active HHV-6 infection [5,7,8,10]. Up to now, it is unclear whether there is transcription of the integrated DNA, translation into viral proteins or even full replication of viral particles [3]. Hence, the clinical consequences of ciHHV-6 remain unclear [3,8].

We investigated 12 individuals out of six families with proven ciHHV-6 for the presence of HHV-6-specific mRNA and antigens, which both have been shown to be able to discriminate active infection from latency.

Methods

Clinical samples

Twelve individuals with suspected ciHHV-6 (six patients and six relatives) were analysed. One of these individuals had become HHV-6 DNA positive after SCT [10]. CiHHV-6 was proven by fluorescence in situ hybridization (FISH) in all 12 individuals, as described elsewhere [13]. HHV-6A and HHV-6B were differentiated by a nested PCR, resulting in different sized amplicons for HHV-6A and HHV-6B [14]. In the seven persons who were repeatedly tested for mRNA and/or antigen expression, the mean interval between blood drawings was 5.3 (1.6–11.3) months.

mRNA Analyses

HHV-6 transcriptional activity was analysed by nested qualitative PCR and real-time quantitative PCR (qPCR) after retrotranscription (RT-PCR, RT-qPCR), determining the presence of lytic (U42, U22) or latent (U94 in the absence of U42) mRNAs, as previously reported [15]. Briefly, total RNA was extracted from cell pellets by the RNA Easy isolation kit (Qiagen, Hilden, Germany), according to manufacturer's instructions, and DNA contamination was eliminated by digestion with RNase-free DNase (Roche Rotkreuz, Risch, Switzerland). After reverse transcription using AMV reverse transcriptase (Ambion Inc., Austin, TX, USA), PCR amplifications were performed using an amount of cDNA corresponding to 200 ng of total RNA. All RNA preparations were devoid of DNA, as assured by multiple DNase digestions and lack of amplification in PCR reactions where retrotranscription had been omitted [16]. Amplifications of the house-keeping human β-actin and RNase P genes were used as controls, respectively, in qualitative and quantitative PCR amplifications. qPCR reactions were carried out in triplicate on a 7500 PCR System (Applied Biosystems, Foster City, CA, USA), as already described [15,17]. In each assay the cDNAs obtained from uninfected and HHV-6-infected JJhan T cells were also included as negative and positive controls, respectively. All clinical samples were analysed in a randomized and blinded fashion. Qualitative PCR amplification products were visualized on ethidium bromide-stained agarose gel after electrophoresis migration.

Antigen analyses

For the HHV-6 antigen test, PBMCs were acetone fixed on microscope slides and stored at −70°C. HHV-6 antigens were demonstrated in PBMCs by means of an indirect immunoperoxidase staining as described elsewhere [18]. Two monoclonal antibodies were used: an antibody against a 101 kDa virion protein of HHV-6B (MAB 8535; Chemicon, Inc., Temecula, CA, USA) [19], which is a late antigen, usually not expressed in the absence of virus replication [20], and an antibody recognizing both HHV-6A and HHV-6B (Ref-11-242, Argene Biosoft, Varilhes, France). This latter antibody detects a 70 kDa protein of HHV-6A and a 62 kDa protein of HHV-6B. A peroxidase-conjugated rabbit anti-mouse (Dako, Copenhagen, Denmark) and peroxidase-conjugated goat anti-rabbit antibody (Zymed, San Francisco, CA, USA) were used as second and third antibody. Positive and negative control specimens were included in every series of immunostaining. The reaction was revealed by 3-amino-9-ethyl carbazole solution containing hydrogen peroxidase; Mayer's hemalum was used for counterstaining.

Ethics statement

The study has been approved by the ethics committee of the Medical University Graz. Participants or their caregivers have provided written informed consent.

Results

mRNA analyses

Out of 27 samples analysed for HHV-6-specific mRNA, nine (33.3%), five (18.5%) and one (3.7%) samples were positive for U94, U42 and U22 transcripts, respectively (Fig. 1, Table 1). U94 transcripts indicative of latent infection were detected in six (54.5%) out of 11 individuals at least once. Transcripts indicative of lytic infection (i.e. U42 and U22) were detected in four (36.4%) out of 11 individuals at least once (Table 1).

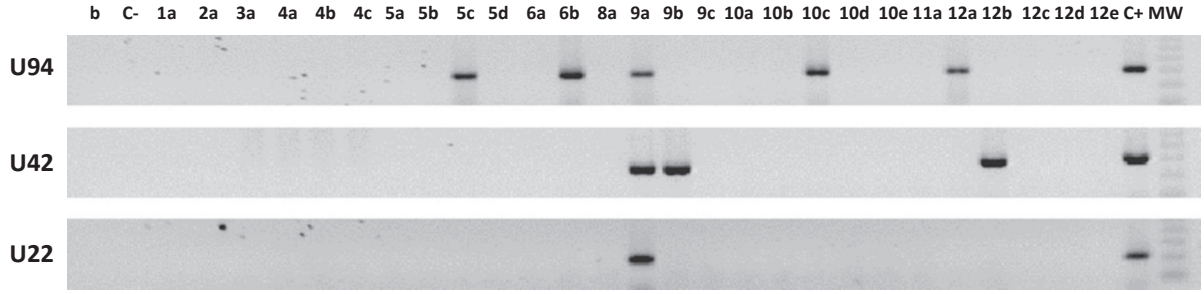

FIG. 1. Results of HHV-6 mRNA search by nested qualitative PCR. PCR amplifications were performed using an amount of cDNA corresponding to 200 ng of total RNA, using primers and conditions specific for genes U94, U42 and U22 of HHV-6. The cDNAs obtained from uninfected or infected JJhan T cells were also included as negative (C−) and positive (C+) controls. PCR products were visualized on ethidium bromide-stained agarose gel after electrophoresis migration. Note: Some specimens had a positive result only in nested qPCR. For details see Table 1.

TABLE 1. Results of mRNA and antigen detection in 12 individuals with proven ciHHV-6

Family (virus) chromo-somal site	Individual, clinical details	Year of birth	Sex	Drawing	Nested PCR/qPCR U94	Nested PCR/qPCR U42	Nested PCR/qPCR U22	Antigen detection Antibodies used[a] (proportion of positive cells)
A (HHV-6B) 17p ter	1 transient arterial hypertension with RPLE	1998	M	a	–	–	–	AB (0%), B (0%)
	2 healthy, mother of no. 1	1965	F	a	–	–	–	B (15%)
B (HHV-6B) 9p ter	3 psychogenic seizure	1992	F	a	–	–	–	B (20%)
	4	1948	M	a	+[b]	–	–	AB (10%), B (20%)
	healthy, grandfather of no. 3			b	+[b]	–	–	AB (10%), B (20%)
				c	–	–	–	n.d.
	5	1968	M	a	+[b]	–	–	B (20%)
	healthy, father of no. 3			b	–	–	–	AB (10%), B (10%)
				c	+	–	–	AB (40%), B (40%) Fig. 1
				d	–	+[b]	–	n.d.
C (HHV-6A) 17p ter	6 AML, ciHHV-6 via SCT	1993	F	a	+[b]	–	–	AB (10%), B (0%)
				b	+	–	–	n.d.
D (HHV-6B) 9p ter	7 CMP, unclear aetiology	2003	F	a	n.d.	n.d.	n.d.	B (0%)
	8 healthy, father of no. 7	1965	M	a	–	+[b]	–	B (0%)
E (HHV-6A) 17p ter	9 healthy, mother of no. 10	1964	F	a	+	+	+	AB (0%)
				b	–	+	–	AB (15%), B (0%)
				c	–	–	–	n.d.
	10 chronic parotitis, unclear aetiology	1990	F	a	–	–	–	AB (60%)
				b	–	–	–	AB (60%)
				c	+	–	–	AB (60%), B (10%) Fig. 2
				d	–	–	–	n.d.
				e	–	–	–	n.d.
F (HHV-6A)	11 healthy, father of no. 12	1960	M	a	–	–	–	
	12 Hodgkin disease	1993	F	a	+	–	–	n.d.
				b	–	+	–	n.d.
				c	–	–	–	n.d.
				d	–	–	–	n.d.
				e	–	–	–	n.d.

AML, acute myeloid leukaemia; SCT, stem cell transplantation; CMP, cardiomyopathy; RPLE, reversible posterior leukoencephalopathy syndrome; n.d., not done.
[a]Antibodies used: B, against HHV-6B (101p); AB, against HHV-6 A and B (see text).
[b]Samples had a positive result only in nested qPCR.

Antigen analyses

Virus antigen expression was detected in 12 out of 16 samples (75%) (Figs 2 and 3, Table 1). Positivity for one or both of the tested HHV-6 antigens was detected in seven out of 10 individuals with ciHHV-6 (70%) at least once. Six individuals refused further blood drawings and were tested only once. Interestingly, we found HHV-6B antigen in one individual with integrated HHV-6A (individual no. 10). The amount of cells positive for HHV-6B was much lower than that positive with the anti-HHV-6A+B antibody (Fig. 3). The signal achieved with the anti-HHV-6B antibody resembles the picture seen in HHV-6 reactivation under immunosuppression [18,21–23].

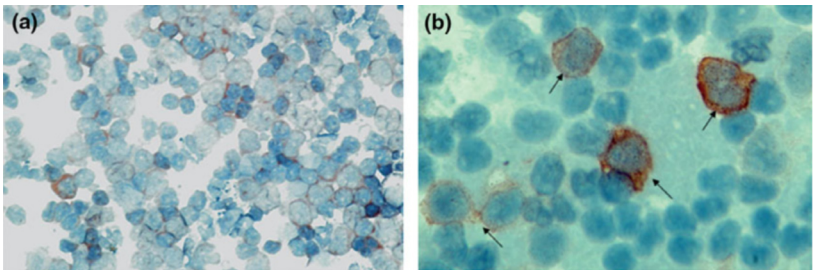

FIG. 2. HHV-6-positive lymphocytes in the cytocentrifuge preparations of peripheral blood mononuclear cells (PBMCs) of individual no. 5. (a) HHV-6-positive cells demonstrated by indirect immunoperoxidase staining with the monoclonal antibody against HHV-6A and -B types (Ref-11-242; Argene Biosoft). (Original magnification ×400.) (b) HHV-6B-positive cells (arrows) demonstrated by the monoclonal antibody against the B type (MAB 8535, Chemicon, Inc.). Again *c.* 40% of PBMCs are positive for HHV-6B in this preparation (Original magnification ×1000.)

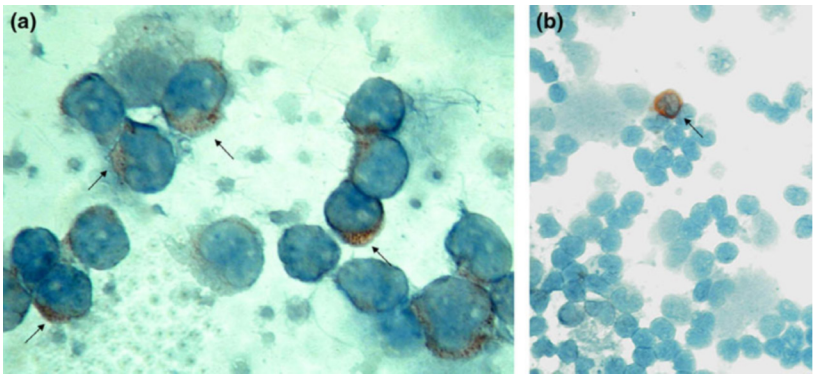

FIG. 3. HHV-6-positive lymphocytes in the cytocentrifuge preparations of peripheral blood mononuclear cells (PBMCs) of individual no. 10. (a) HHV-6-positive cells (arrows) demonstrated by indirect immunoperoxidase staining with the monoclonal antibody against HHV-6A and HHV-6B (Ref-11-242, Argene Biosoft). *c.* 60% of PBMCs are positive with this antibody. (Original magnification ×1000.) (b) indirect immunoperoxidase staining with the monoclonal antibody against HHV-6B (MAB 8535, Chemicon, Inc.), *c.* 10% of the PBMC are expressing the HHV-6B protein (arrow). (Original magnification ×400.)

This indicates exogenous infection or reactivation of HHV-6B in this individual with ciHHV-6A.

Interestingly, HHV-6 specific antigens were found in specimens without detectable HHV-6-specific mRNA and vice versa (Table 1).

Discussion

For the first time, we analysed HHV-6-specific mRNA as well as antigens in a series of persons with FISH-proven ciHHV-6. Both were detected in the majority of participants indicating intermittent replication of HHV-6. While U94 transcripts can also be detected in latently infected persons, U22 and U42 transcripts are indicative for lytic infections and are only detected during exogenous primary infection or reactivation but not in latently infected individuals [15–17]. In our series, mRNA transcripts indicative for lytic infection were detected in four of 11 tested persons.

The antibodies we used for antigen detection have been shown to be sensitive and specific for HHV-6 antigens indicating primary infection with or reactivation of HHV-6. These antibodies are successfully used in monitoring of patients after solid organ transplantation and SCT and their target antigens are not detectable in latently infected persons without reactivation [18,21–23].

In the current study, HHV-6 mRNAs and antigens were detected in most of the individuals with ciHHV-6. However, they were not detectable in all and at every time-point of testing. Positive as well as negative samples were found within the same families, within individuals with the same site of integration, in individuals with integrated HHV-6A or HHV-6B and even within the same individual at different time-points of sampling. This may indicate a differential viral activity within a time course of the same individual. *Chlamydia trachomatis* infections as well as different drugs have been shown to be able to trigger HHV-6 reactivation [8,24]. Exogenous factors might play a role even in activation of ciHHV-6.

The lack of concordance between mRNA and antigen detection in some specimens can be explained by the different dynamics and biological half-life of the analysed parameters. While mRNA is detectable for only a few days after infection [17], HHV-6-specific antigens have been shown to be detectable for several weeks to months [25].

In addition, because U94 mRNA is commonly detected during viral latency, but only intermittently and at low amounts in individuals with ciHHV-6, it might be postulated that the molecular mechanism underlying inherited ciHHV-6 and latency after primary exogenous infection are considerably different.

In the literature, there are conflicting data on the possible viral replication in individuals with ciHHV-6. While culturing HHV-6 from specimens of individuals with ciHHV-6 has not succeeded [3], *in vitro* experiments have shown the capability of cells with ciHHV-6A [26] or ciHHV-6B [27] to infect other cells, clearly indicating the replicative potential of ciHHV-6 at least *in vitro*. Hall *et al.* reported transient DNA detection in blood and saliva of two unrelated newborns without ciHHV-6 born to mothers with ciHHV-6. DNA sequences of maternal ciHHV-6 and of DNA detected in the ciHHV-6-negative neonates were identical. Beside maternal microchimerism (caused by small amounts of maternal DNA crossing the placenta), replication of maternal ciHHV-6 as a source of congenital infection might be an explanation for this observation [28,29]. On the other hand, Pantry *et al.* [30] demonstrated HHV-6-specific mRNA of four patients with ciHHV-6 and chronic fatigue syndrome (CFS) with differences in the nucleotide sequences between the integrated and the transcribed DNA, indicating an exogenous infection as the source of replication.

While our study comprises the largest series of individuals with ciHHV-6 (most of them without specific symptoms) reporting mRNA expression and the first report of *in vivo* antigen detection, our data do not, of course, allow distinguishing reactivation of endogenous ciHHV-6 from exogeneous super-infection. If our observations reflect lytic infections/reactivations not derived from the chromosomally integrated virus DNA, this might indicate that individuals with ciHHV-6 are prone to exogenous HHV-6 re-infection or reactivation, as proposed by Pantry *et al.* [30]. This hypothesis is further substantiated by our observation of a documented infection with or re-activation of HHV-6B in a person with integrated HHV-6A. Of course, this is speculative and the origin of viral transcripts and antigens in individuals with ciHHV-6 should be determined in further studies of larger numbers of individuals with and without symptoms to clarify this aspect (actually, the effects may not be different at all).

The presence of markers specific for lytic infection as well as of viral antigens may tempt speculation regarding possible pathophysiological effects leading to clinical consequences of chromosomally integrated viral DNA. It might be speculated that intermittent or permanent replication of viral structures alters the immune response to an additional exogenous infection with HHV-6 [8]. This might lead to a decreased response with subclinical courses or with difficulties in clearing the virus in individuals with ciHHV-6, leading to severe and/or protracted courses. The observed replication might also reflect the consequences of an altered immune status.

In individuals without ciHHV-6, HHV-6 wild-type infection and persistent activity has been reported to be associated with different types of diseases such as malignant lymphomas, encephalitis, multiple sclerosis, brain tumours, myocarditis, Hashimoto's thyroiditis, hepatitis and collagen vascular diseases [1,2,15]. *In vitro* studies have shown that HHV-6 enhances the replication of EBV, HPV and HIV, influences the activity of interferons, interleukins, TNF-alpha and different cytokine receptors and activates NF-κB [1]. Furthermore, expression of HLA class I and II molecules and of CD46 was seen to be altered by HHV-6. The proliferative response of stimulated T cells was shown to be markedly reduced after HHV-6 infection [1], indicating an HHV-6-induced immune suppression. HHV-6 was also reported to induce apoptosis of CD4+ lymphocytes [1].

Considering all these properties of active HHV-6 infection together with obvious replicative activity in individuals with ciHHV-6 observed in our study, a pathophysiological impact of this phenomenon might be assumed, irrespective of the origin of the transcripts (replicated ciHHV-6 or persistent exogenous super-infection). Furthermore, if replication occurs frequently in persons with ciHHV-6, it might serve as a (permanent) source of infection.

Our results demonstrate the presence of HHV-6-specific mRNA and proteins in individuals with ciHHV-6. This indicates virus gene expression and translation in individuals with ciHHV-6, which may lead to virus replication. Especially in individuals expressing U42/U22 mRNA and p101 protein, there is evidence of virus replication because U42/U22 transcription is only detected in lytic infection [17] and p101 is a late gene whose expression is dependent on virus replication [19,20].

So far, ciHHV-6 is not linked to specific clinical conditions but, based on the evidence of HHV-6 transcriptional activity, a possible clinical impact might be considered. Epidemiological and detailed clinical-molecular studies are needed with a large number of individuals with ciHHV-6 to identify the implications and clinical consequence of this phenomenon.

Acknowledgements

This work was partly supported by 'Fondi Ateneo per la Ricerca' (University of Ferrara).

Transparency Declaration

Nothing to declare.

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