Journal of Clinical Virology 91 (2017) 58–61

Contents lists available at ScienceDirect

Journal of Clinical Virology

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

Short communication

Human pegivirus-1 in the CSF of patients with HIV-associated neurocognitive disorder (HAND) may be derived from blood in highly viraemic patients

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ARTICLE INFO

Keywords:
Human pegivirus-1
Cerebrospinal fluid
HIV-associated neurocognitive disorder

ABSTRACT

Background: Human pegivirus-1 (HPgV-1) infection in the brain has not been extensively examined and its association with disease remains unconfirmed. In a high throughput sequencing study to look for infectious agents that could play a role in HIV-associated neurocognitive disorder (HAND), this virus was detected in 3 of 8 CSF samples.

Objectives: To determine the significance of this finding, additional patients were screened and the viral load and viral diversity in blood and CSF were examined.

Study design: Nested PCR of the viral 5′NCR region was performed on blood and CSF pairs from 16 HAND patients. PCR products were cloned, sequenced and analysed to determine viral diversity in blood and CSF. HPgV-1 viral loads were determined in paired blood and CSF of 2 patients by digital droplet PCR. Nested PCR was also performed on CSF samples from patients with other brain disorders.

Results: Virus was detected in both blood and CSF in 3 of 16 HAND patients. Viral loads were very high in blood (8.81 and 10.56 log copies/ml) and 4–5 logs lower in CSF (4.68 and 5.84 log copies/ml). Sequence analysis of 5′NCR clones in blood and CSF showed limited variation. The dominant viral variant (based on clonal sequence identity) in blood and CSF was usually identical. HPgV-1 was detected in CSF from patients with other brain disorders at a similar frequency (15% versus 18.75% in HAND patients).

Conclusion: While several studies have reported HPgV-1 detection in CSF of patients with brain disease, this is the only study that has examined both blood and CSF compartments simultaneously. Our findings show that virus in CSF always coincided with viraemia and levels were 4–5 logs higher in blood. While a rare, but specific brain tropism cannot be excluded, blood is the more probable source of virus in HAND patients.

1. Background

High throughput sequencing (HTS) is a powerful technique for identifying microbial and viral nucleic acids in clinical samples in a sequence independent manner. This methodology has been used to discover several novel viruses of medical importance, including a novel arenavirus associated with fatal infection in transplant patients [1] and Lujo arenavirus from patients with haemorrhagic fever in South Africa [2] and a new cyclovirus associated with acute central nervous system (CNS) disease in Viet Nam [3], However, when nucleic acids from common viruses with no known disease association such as anelloviruses, pegiviruses or human parvovirus-4 (PARV4) are detected, it can be challenging to determine their clinical significance.

A study to monitor clinical responses to an alternative anti-retroviral therapeutic (ART) regimen in HIV patients with neurocognitive disease (HAND) provided an opportunity to screen CSF for opportunistic pathogens by Illumina HTS. A surprising finding was that human pegivirus-1 (HPgV-1) sequences were present in the cerebrospinal fluid (CSF) of 3/8 HAND patients.

HPgV-1, formerly GB virus C/hepatitis G virus, is a lymphotropic virus belonging to the *Flaviviridae* family, genus Pegivirus. HPgV-1 is considered to be non-pathogenic. In immunocompetent individuals viraemia is usually cleared within 2 years with appearance of antibody to viral glycoprotein E2 [4]. There are 6 genotypes with distinct geographical distribution [5]. The prevalence of infection varies around the world. In developed countries 1–4% of blood donors have viraemia,

Abbreviations: HAND, HIV-associated neurocognitive disorder; HTS, high throughput sequencing; CSF, cerebrospinal fluid; HPgV1, human pegivirus-1; ART, antiretroviral therapy; CNS, central nervous system; ddPCR, digital droplet PCR; 5′NCR, 5′ non coding regione

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http://dx.doi.org/10.1016/j.jcv.2017.04.007
Received 5 October 2016; Received in revised form 6 March 2017; Accepted 5 April 2017

Table 1
Characteristics of HPgV-1-positive HAND patients.

Patient	Age/ gender	CD4 count Cells/μl	CSF cell count	HPgV-1 viral load CSF log copies/ml	HPgV-1 viral load blood log copies/ml
004*	43F	875	0 cells	4.68	8.81
005	40F	306	0 cells	5.84	10.56
4117*	29F	285	72 RBCs	not done	No blood sample
0427*	43M	419	0 cells	not done	not done

*CSF samples which were positive in Illumina HTS experiment.

but prevalence is higher in developing countries, especially in HIV infection (reported to be > 16%) [4]. HPgV-1/HIV co-infection is associated with an ameliorated course of HIV disease, possibly due to reduced immune activation [6]. HPgV-1 has occasionally been detected in the CNS- in the brain of patients with multiple sclerosis [7] and in CSF of patients with encephalitis [8,9], but it remains to be determined whether it is a pathogen, or merely a bystander.

2. Objectives

The aim was to investigate the significance of HTS findings and confirm the results using a nested HPgV-1 PCR assay to test additional HAND patients and to determine viral kinetics in blood and CSF.

3. Study design

3.1. Samples

HTS had detected HPgV-1 sequences in CSF of three individuals (indicated with an asterisk in Table 1). To determine the significance of this finding, blood and CSF from these and an additional 9 HAND patients were examined. One additional HPgV-1 positive patient was identified (4/17).

Paired blood and CSF was available from 16/17 patients and only CSF in the remaining case. Further, in 2 HPgV-1 positive cases (004 and 005) a 6 month blood/CSF pair was also available for analysis.

Table 1 shows details of the 4 HPgV-1 positive patients.

3.2. Nucleic acid extraction

Total nucleic acid was extracted from 500 μl serum or CSF using the NucliSENS easyMag (bioMerieux).

3.3. 5′non coding region (5′NCR) PCR

cDNA was generated with random primers using the RevertAid First Strand cDNA synthesis kit (Thermo Scientific, Lithuania), as per manufacturer's instructions. A nested PCR amplified a 366 bp region of the 5′NCR (−518 to −152 of U36380) using primers from [10]. PCR was performed in a 50 μl reaction containing 5 μl cDNA, 15 mM Tris-HCL (pH 8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (ABgene, Epsom, UK), 50 pmol of primers and 1.5U Supertherm Taq polymerase (JMR Holdings, Kent, UK). Amplification was as follows: 95 °C for 3 min, 40 cycles of 95 °C for 15s, 50 °C for 25 s and 72 °C for 35 s followed by 72 °C elongation for 7 min. Nested amplification was the same, but with the annealing temperature 55 °C.

3.4. ddPCR

Digital droplet PCR (ddPCR) was used to quantify virus in blood and CSF. Analyses were performed in duplicate. cDNA from blood was diluted 10^{-2},but CSF was undiluted. The 5′NCR outer primers [10] were used. Fragmentation of cDNA with restriction enzyme HindIII was done to enhance ddPCR efficiency. The QX200 ddPCR EvaGreen reaction mix was prepared as per manufacturer's instructions using 5 μl cDNA. (Bio-Rad Laboratories, Hercules, CA,USA). 20 μl of the reaction was loaded into the DG8 Cartridge and 70 μl of Droplet Generation Oil. Droplets were generated and 40 μl transferred to a 96 well plate for PCR. Cycling conditions were: 1 enzyme activation cycle of 95 °C for 5 min, 40 cycles of 95 °C 30s, 58 °C 1 min, signal stabilization cycles of 4 °C for 5 min and 90 °C for 5 min and hold at 4 °C. The plate was transferred to the Droplet Reader and viral load determined using QuantaSoft software.

3.5. Cloning

The 366 bp 5′NCR PCR products from blood and CSF were cloned into pGEM-T vector (Promega Corporation, Madison, WI, USA).

3.6. Sequencing

Clones were sequenced with the BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City CA, USA) using PCR-specific primers. Sequences were aligned with reference sequences using BioEdit version 7.2.5. Phylogenetic trees were constructed in MEGA 6.06 using the maximum- likelihood algorithm with 1000 bootstrap re-sampling [11]).

3.7. Screening of CSF samples from patients with other brain disorders for HPgV-1

Nested PCR (as above) was performed on CSF samples from patients with brain disorders of known aetiology including: 18 with viral meningitis (enterovirus or mumps PCR positive), 16 with progressive multifocal leuco-encephalopathy (PML), 9 with sub-acute measles encephalitis (cases characterised in [12]), 11 with varicella-zoster-related brain disease and also 46 patients with meningitis where no pathogen had been identified.

4. Results and discussion

The presence of HPgV-1 in brain has not been extensively examined and its association with disease remains unconfirmed. In an HTS study to look for infectious agents that could play a role in HAND, this virus was detected in 3/8 CSF samples. As its significance was unknown, a nested PCR targeting the 5′NCR was performed on paired blood and CSF samples of these and additional HAND patients. HPgV-1 was detected by nested PCR in both blood and CSF in 3 of 16 patients and in the CSF in an additional patient on whom there was no blood sample available. It was also present in paired samples collected 6 months later in 2 patients (004 and 005), indicating persistence in both compartments.

Viral loads were substantially lower in the CSF relative to blood for all positive sample pairs: ddPCR of the 5′NCR target quantified virus in blood and CSF of patients 004 and 005. In 004 the blood load was 8.81 log copies/ml and 4.68 in CSF, while in 005 levels were 10.56 log copies/ml in blood and 5.84 in CSF. (Table 1) (We were not able to obtain accurate readings on the other samples for technical reasons).

Phylogenetic analysis of the 366 bp region of the 5′NCR confirmed that HAND patients were infected with common South African genotypes [13], namely genotype 5 in patients 0427 and 4117 and genotype 2 in patients 004 and 005 (Fig. 1). Each patient's virus was distinctly different, with limited quasispecies (clones differing by 1–3 single nucleotide polymorphisms) in both blood and CSF. All patients had a dominant viral variant in blood, based on sequence identity of 5′NCR clones (17/21 clones in 004; 21/22 in 005 and 3 of 4 in 0427). Similarly in CSF, one variant was dominant. This was usually identical to the dominant variant in blood (13/20 clones for 004; 4/8 in 005; 5/5 for 0427). But the 6 month CSF of patient 004 had a dominant variant distinct from that in the contemporary blood (and historic blood and