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Detection of HHV-6B in post-mortem central nervous system tissue of a post-bone marrow transplant recipient: a multi-virus array analysis

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Abstract

Background: HHV-6 has been implicated in a number of neurological disorders. Recent evidence has suggested high incidence of HHV-6 infection in patients (46%) undergoing allogeneic bone marrow transplant (BMT).

Objective: To investigate whether HHV-6 plays a role in the development of fatal encephalopathy in an allogeneic post-BMT patient using an unbiased approach.

Results: Detection of HHV-6 viral DNA sequence and RNA expression were demonstrated in fresh frozen post-mortem autopsy material derived from the insular cortex using a multi-virus array platform. In addition, PCR analysis by real-time quantitative TaqMan demonstrated high viral burden in multiple brain regions tested. Sequencing analysis of PCR product confirmed the virus to be HHV-6 variant B.

Conclusions: Active infection as demonstrated by expression of viral RNA and high viral load in the CNS suggest a possible pathogenic role of HHV-6 in development neurologic complications post-BMT.

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1. Introduction

In recent years, bone marrow (BMT) and peripheral blood stem cell (SCT) transplantation are used as treatments for a number of disorders and have been associated with a wide spectrum of neurologic complications including encephalopathy, seizures, psychiatric symptoms, and cerebral hemorrhage. Early clinical manifestations of these central nervous system (CNS)-associated complications are often non-specific and patients commonly present with altered mental status or delirium with or without seizures (Rosenfeld and Pruitt, 2006). CNS infections in these patients have been suggested to play a role in development of neurologic disorders (Donati et al., 2003; Rosenfeld and Pruitt, 2006) and were more frequently observed in allogeneic transplants cases most likely related to prolonged immune suppression. A wide spectrum of pathogens associated with CNS-related complications including both bacterial and viral species have been described. Some of these opportunistic agents include staphylococci, Pneumocystis carinii, Listeria monocytogenes, adenovirus, Coxsackievirus B4, Cytomegalovirus (CMV) and recently Human Herpesvirus 6 (HHV-6). Consequences associated with CNS infections in allogeneic post-BMT or SCT can be fatal due to their inability to mount sufficient inflammatory responses as a result of immunosuppressant medication. In addition, the classic findings of fever and meningismus may be absent rendering early diagnosis difficult.

While hundreds of organisms are known to infect human and induce pathology, clinical manifestations are rarely specific for a single agent. Conventional laboratory methods used for identification of such pathogens are time consuming and labor intensive and often require large quantities of clinical samples.

Therefore, to aid diagnosis, assays are needed for simultaneous consideration of multiple agents. We had previously reported on the development and use of a multi-virus array containing eight genetically and pathologically
2. Materials and methods

2.1. DNA isolation and PCR analysis

Genomic DNA was isolated from fresh frozen or formalin-fixed paraffin embedded material as previously described (Donati et al., 2003). HHV-6B DNA viral load was determined using real-time quantitative TaqMan PCR with HHV-6B strain specific primers as previously described (Nitsche et al., 2001). A standard curve was generated using a known concentration of variant-specific HHV-6 plasmids. Results were plotted using the Sequence Detector System (PerkinElmer). Normalization was performed using a human genomic β-actin calibration curve. Absolute viral and β-actin DNA copy number was assessed and final viral DNA load per 10^6 cells was calculated by the following formula:

\[
\text{HHV-6 DNA copy number} = \frac{\text{β-actin DNA copy number}^2 \times 10^6}{2}
\]

Conventional PCR was performed using DNA extracted from various regions of the CNS with HHV-6 specific primers for U67, U57, and U69 using the following PCR condition: 94°C hold, 3 minutes; 94°C hold, 30 s, 60°C hold (annealing), 30 s; 72°C hold (extension), 30 s (35 cycles); 72°C hold, 7 minutes. For sequencing analysis, PCR products were purified using MinElute PCR Purification Kit (Qiagen, CA). Sequencing was performed with Genetic Analyzer 3100 (Applied Biosystems).

2.2. RNA Isolation and in vitro transcription synthesis of anti-sense RNA

Total RNA was isolated from control and infected cell lines with the RNaseasy Mini Kit (Qiagen, CA) according to manufacturer's instructions. Subsequently, anti-sense messenger RNA (mRNA) was amplified for microarray and RT-PCR analysis using in vitro transcription with MessageAmp aRNA kit (Ambion, CA) from 1μg of total RNA starting material, according to manufacturer's protocol.

2.3. cDNA synthesis and CyDye coupling of anti-sense mRNA

Sample labeling and microarray hybridization were performed as described (Ghedin et al., 2004). Briefly, 2μg of amplified anti-sense were reverse transcribed into cDNA using 6μg of random hexamers (Invitrogen, CA) with 2μL of Superscript II Reverse Transcriptase (Invitrogen), 6μL of 5X First Strand Buffer (Invitrogen), 3μL of 0.1M DTT (Invitrogen) and 0.6μL 50X a-dNTP with a dCTP:dTTP ratio of 2:3. The reverse transcription reaction was carried out in a final volume of 30μL at 42°C overnight in a water bath. RNA template was hydrolyzed with 10μL 1M NaOH after completion of cDNA synthesis. Ten microliters of EDTA (Invitrogen, CA) were added followed by 15 minutes heating at 65°C to inactivate all enzymatic reactions and 25μL of 1M Hepes pH=7.0 was subsequently added. cDNA was then purified with Micron-30 Spin Columns (Millipore, CA) according to manufacturer's instructions and samples were dried under vacuum centrifugation. Each sample was re-suspended in 4.5μL of 0.1M carbonate (Na2CO3, pH=9.0) and 4.5μL of either NHS-Cy3 or NHS-Cy5 (Amersham Pharmacia, NJ) to fluorescently label the cDNA probe. Incubation at room temperature proceeded for one hour in the dark. Post CyDye coupling, 35μL of 100nmM NaOAc (pH=5.2) was added to assist in cDNA precipitation. Purification was performed using the QIAquick PCR Purification Kit (Qiagen, CA) according to manufacturer's instructions and samples were eluted in 100μL of RNase/DNase free H2O.
Quality of CyDye labeling was assessed with a Beckman spectrophotometer and samples were again concentrated in a vacuum centrifuge.

2.4. Hybridization of fluorescently labeled cDNA samples on multi-virus array

Concentrated infected sample was resuspended in 6.15 µL of RNase/DNase free H2O, and combined with uninfected sample. Salmon sperm (0.75 µL per reaction) was added for blocking, followed by denaturation at 95°C for 5 minutes and cooling on ice for 30 seconds. A hybridization solution (50% formamide, 5X SSC, 0.1% SDS, and 0.2 mg/ml bovine serum albumin) was prepared and 23.1 µL was added to each reaction. The probes were incubated for 20 minutes at 42°C and applied to multi-virus arrays that had previously been rinsed in 0.1% SDS and denatured in boiling water. Hybridization reaction was carried out for 12–16 hours overnight at 42°C. Hybridized arrays were sequentially washed in 2X SSC, 0.1% SDS (42°C, 5 minutes); 0.1X SSC, 0.1% SDS at RT for 10 minutes; 0.1X SSC at RT for 2 minutes twice; 0.01X SSC at RT for 5–10 seconds. Arrays were dried by centrifugation at 600rpm (Sorvall 6000) and scanned with a GenePix 4000A Microarray Scanner (Axon Instruments) and images saved as paired single-color TIFF images. Analysis of pixel intensity were performed as previously described (Ghedin et al., 2004; Yao et al., 2006) using TMEV package obtained from TIGR.

3. Results

3.1. Detection of HHV-6 viral DNA sequence in the CNS

Fluorescently labeled DNA obtained from the insular cortex was co-hybridized with DNA derived from control (non-malignant) HHV-6 negative brain material obtained from a tumor patient and analyzed for presence of viral genome. As shown in Fig. 1 (top panel), normalized fluorescence intensity calculated as the geometric mean of all replicates (12) was elevated for HHV-6 viral sequences compared to control DNA fluorescence signal. In contrast, no significant hybridization to other viruses could be detected. HHV-6 sequences U47, U53, U76, U83 were specifically detected as indicated in the bottom panel. To confirm this observation with the multi-virus array, we determined by real-time quantitative TaqMan PCR the HHV-6 viral loads in various regions of the brain using fresh frozen or formalin-fixed paraffin embedded specimen. As shown in Fig. 2, a significant amount of virus could be detected from all brain regions tested while viral loads in serum, CSF, and PBMC were not significantly elevated, suggesting a localized viral reactivation confined to the CNS. Consistent with our previous reports estimating the sensitivity limit of this assay to be approximately $1 \times 10^6$

![Fig. 1. Multi-virus array detection of HHV-6 sequence from CNS material. Equimolar (2.8 µg) of DNA isolated from the insular cortex and HHV-6 negative control brain were fluorescently labeled with Cy3 and Cy5, receptively. Normalized pixel intensity was calculated as the difference in pixel intensity between test and control samples. Top panel represents intensity of open reading frames from all the viruses on the multi-virus platform. Bottom panel shows only open reading frames from HHV-6 region.

![Fig. 2. Real-time quantitative analysis of viral load from various regions of the CNS using fresh frozen and formalin-fix paraffin embedded samples. DNA extracted from either fresh frozen or formalin-fix paraffin embedded samples as indicated were subjected to quantitative TaqMan PCR analysis for determination of viral load. As shown, significant amounts of virus could be detected in almost all regions of the CNS. By contrast, DNA samples from the CSF or periphery did not indicate high viral burden. (A) denotes TaqMan quantitation performed using fresh tissue; (B) denotes quantitation performed with formalin-fixed material.]}
copies/million cells, viral load as measured by TaqMan PCR in the insular cortex, which demonstrated specific hybridization with HHV-6 sequence using the multi-virus array analysis, harbored $8 \times 10^5$ copies of HHV-6/million cells. It is worth noting that the quality of DNA extracted from formalin-fix tissue versus those obtained from fresh tissue may differ; thus viral burden obtained from these two sources should not be compared directly.

3.2. Detection of HHV-6 RNA indicative of an active infection

HHV-6 contains DNA as its genomic composition; therefore, detection of DNA sequence alone is not a conclusive indicator of an active viral infection despite a high level of viral load as HHV-6 chromosomal integration has been described in a rare population of individuals (Ward et al., 2006). To investigate and confirm the presence of an active infection from autopsy material derived from this post-BMT recipient, we also performed analysis of viral messenger RNA expression using the multi-virus array. As there is significant overlap on the nucleic acid sequence level between HHV-6 DNA and RNA, to ensure specific detection of viral gene expression, we linearly amplified all messenger RNA from total RNA derived from the left insular cortex using previously described methods (Conejero-Goldberg et al., 2005; Feldman et al., 2002). Similarly, messenger RNA was also amplified from the control brain and used for normalization of background signals. As shown in Fig. 3, up-regulated HHV-6 gene expression could be observed for the following gene transcripts: DR1, LJ1, U24, U33, U43, U77 and U89. This finding is consistent with our recent report demonstrating detection of HHV-6 p41 RNA by RT-PCR and viral protein expression by immunohistochemistry in various areas of the CNS obtain from this patient (Fotheringham et al., 2006). Collectively these results suggest an active viral infection process in the CNS.

3.3. PCR and sequence analysis for sub-typing of HHV-6 from brain materials

To further confirm and substantiate the observation of HHV-6 infection in the CNS of this patient, we performed PCR analysis using HHV-6 specific primers that span various segments of the HHV-6 genome on multiple brain regions including lateral temporal lobe, hippocampus, insular cortex, and mesial temporal lobe. HHV-6 U67, U57, and U69 were successfully amplified from all available areas examined while the control non-malignant brain sample was negative for all HHV-6 genes tested (Fig. 4). As we have recently reported (Fotheringham et al., 2006), the variant B of HHV-6 is most frequently observed in neurologic complications associated with post-BMT. Therefore, we sub-typed the virus with sequencing analysis of the U57 (major capsid protein) gene of HHV-6 amplified from the lateral temporal lobe. BLAST analysis against the NCBI non-redundant nucleotide database demonstrated 100% identity between sequence amplified from this patient's autopsy brain sample and the annotated Genbank sequence of the HHV-6 variant B, HST strain (data not shown). The homology between HHV-6 variants A and B is ~92% for the U57 region of HHV-6. Together, these data support the finding of the multi-virus array analysis regarding the specific of HHV-6 B detection in the CNS.
4. Discussion

Immunosuppressant therapy used in transplant recipients can interfere with their ability to clear infections (Rosenfeld and Pruitt, 2006). Approximately half of the allogeneic BMT or SCT transplant recipients reactivate latent HHV-6 post transplantation (Ihira et al., 2002) and in some cases are associated with potentially fatal consequences (Fotheringham et al., 2006). Thus, early detection of viral infection prior to extensive CNS damage may improve prognosis of HHV-6-associated neurologic complications. In this study, we demonstrated a feasible alternative method for rapid, simultaneous detection for multiple pathogens in clinical specimens using a multi-virus array. Comparing conventional laboratory techniques such as serology or PCR amplification, which often require large quantities of starting materials when multiple pathogenic agents are considered, the multi-virus array approach offers a number of advantages over current accepted methods. In the case of allogeneic transplant patients, serological testing for antibody against suspected agents may not be informative due to immune suppression. However, with multi-virus array analysis, in a single hybridization, all potential agents are screened without the need of extensive manipulation of a limited amount of clinical samples. Additionally, less starting material is required as all potential candidates are tested simultaneously as opposed to PCR amplification for individual candidate pathogens separately. Moreover, as studies of viral gene expression often require a large amount of RNA samples that are rarely attainable with clinical samples, the linear amplification of messenger RNA coupled with multi-virus array analysis opens the possibility to investigate the mechanism of viral gene expression using \textit{ex-vivo} tissue.

Two variants of HHV-6 have been described, HHV-6A and HHV-6B. Although sharing over 95\% sequence homology, these two viruses have different biological properties, cellular tropism and clinical associations (Ahlgqvist et al., 2005; Donati et al., 2003, 2005; Soldan et al., 1997). The HHV-6 B variant is exclusively associated with development of post-bone marrow encephalitis (Gentile, 2000). The ability of our multi-virus array to distinguish and subtype variant specific viruses further highlights its potential for use in clinical settings. As demonstrated in Fig. 3, detection of HHV-6 variant B specific transcript U89 is consistent with the role of HHV-6B in development of post-BMT encephalitis. Interestingly, of the HHV-6 transcripts detected, the U24 glycoprotein which shares an immunodominant peptide sequence with brain myelin basic protein has been shown to elicit immune response from T cells derived from multiple sclerosis patients, suggesting an immune mediated pathogenic mechanism of HHV-6 in CNS disorders (Tejada-Simon et al., 2003). Pharmacologically, several case reports have also supported the involvement of HHV-6 in post-BMT neurological complications as administration of ganciclovir resulted in resolution of symptoms (Rapaport et al., 2002; Yoshida et al., 2002). Therefore, rapid and specific identification of pathogens likely to be responsible for particular clinical manifestations may increase chances of survival of these patients. As evident in this study, extensive viral infection was already found in the CNS shortly after the initial presentation of mental status change at autopsy, which further stresses the need for development of rapid, sensitive assays for identification of pathogenic agents.

Of note, the high viral burden observed in this patient may not be characteristic of all patients suffering from post-BMT complications (Ihira et al., 2002). Specifically, as shown in Fig. 3, only a subset of HHV-6 gene transcripts were detected despite a high viral load, possibly because of varying hybridization efficiency of different open reading frames and the overall sensitivity limit associated with virus array analysis as we had previously demonstrated (Ghedin et al., 2004). Therefore, continual development of multi-virus array detection with enhanced sensitivity is critical as only low viral burden may be associated with initial phase of the disease process. Additionally, extending the multi-virus array used in this study, which contains eight genetically and pathologically diverse viruses, to include other known CNS-related pathogens for detection of infections in the brain represents a novel, “unbiased” approach to study a number of neurologic complications with unknown etiology. This approach not only would define the agent(s) that may be associated with a disease but could also identify potential pathogenic viral gene products. As pathogen-arrays have been instrumental in the discovery of other human pathogens such as SARS-coronavirus (Wang et al., 2003), the possibility of using a multi-virus array to aid clinical diagnosis may provide insight into the microbial flora of the CNS and mechanisms of viral–host interaction.

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