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Background. Chronic fatigue syndrome (CFS) is a complex, heterogeneous disease characterized by debilitating fatigue that is not improved with bed rest and worsens after physical activity or mental exertion. Despite extensive research into a cause of CFS, no definitive etiology has been determined; however, a large percentage of CFS patients note an acute infectious event that triggers their fatigue.

Methods. Blood and saliva were collected from 39 CFS cases and 9 healthy control subjects. Peripheral blood mononuclear cells (PBMCs) were tested for human endogenous retrovirus-K18 (HERV-K18) env transcripts using a TaqMan quantitative polymerase chain reaction (qPCR). In addition, viral copy number of human herpesvirus-6 (HHV-6) and human herpesvirus-7 (HHV-7) were measured in both saliva and PBMCs using TaqMan qPCRs. Transcript levels and viral copy number were compared to patient CFS symptom severity.

Results. HERV-K18 env transcripts were not significantly different between healthy control subjects and CFS patients. Also, HERV-K18 env transcripts did not correlate with HHV-6 viral copy number or HHV-7 viral copy number in either PBMCs or saliva. HHV-6 viral copy number and HHV-7 viral copy number in both PBMCs and saliva were not significantly different between healthy control subjects and CFS patients. HERV-K18 env transcripts, HHV-6 viral copy number, and HHV-7 viral copy number did not correlate with CFS symptom severity.

Conclusions. We fail to demonstrate a difference in HERV-K18 env transcripts, HHV-6 viral copy number, and HHV-7 viral copy number between CFS patients and healthy controls. Our data do not support the hypothesis of reactivation of HHV-6 or HHV-7 in CFS.

Keywords. CFS; HERV-K18; HHV-6; HHV-7.

Chronic fatigue syndrome (CFS) is a complex, heterogeneous disease affecting more than 1 million Americans [1]. Patients with CFS have overwhelming fatigue that is not improved with bed rest and worsens after physical activity or mental exertion. Other symptoms of CFS include impaired memory, sore throat, tender cervical or axillary lymph nodes, muscle pain, pain in joints, and headaches [2]. Despite extensive research into a cause of CFS for over the past 3 decades, no definitive cause of CFS has been determined; however, there is evidence supporting an infectious etiology.

A large percentage of CFS patients note an acute infectious event that triggers their fatigue [3–6]. Previous reports have attempted to link the ubiquitous β-herpesviruses, human herpesvirus-6 (HHV-6) and human herpesvirus-7 (HHV-7), to CFS. Many investigators have reported that reactivation of HHV-6 is
found more frequently in CFS patients than in healthy control subjects [7–18], as reflected by higher immunoglobulin G (IgG) antibody titers, by immunoglobulin M (IgM) antibody, by the presence of viral nucleic acid in peripheral blood mononuclear cells (PBMCs) or plasma, by viral load measurements in PBMCs, or by observing the viral cytopathic effect in lymphocytes from patients. However, some other studies have not observed such differences between CFS patients and healthy control subjects [6, 19–22]. These contradictory results are difficult to interpret because HHV-6 and HHV-7 infections are widespread in the population at large with viral-specific IgG antibodies detected in more than 90% of adults [23]. Furthermore, the symptoms of the triggering infection are usually cleared before patients are diagnosed with CFS. One explanation to this quandary could be the involvement of human endogenous retroviruses (HERVs).

HERVs are ancient retroviruses that infected germ line cells and became permanently integrated into the genome. About 8% of the entire human genome is believed to be HERVs; however, most of these proviral genes are silenced or only expressed in response to an environmental factor, such as an infecting virus that can activate them in susceptible cells [24]. Recently, it was shown that the HERV-K18 env gene can be activated in B cells by Epstein-Barr virus (EBV), interferon-α (IFN-α), HHV-6A, and HHV-6B [25–28]. This envelope protein comprises a superantigen (SAg), which is recognized by the human Vβ13 T-cell receptor (TCR) gene product [26]. SAgS are microbial proteins that greatly overstimulate the immune system by directly interacting with the Vβ segment of the TCR, unlike conventional peptide antigens that are recognized by a specific hypervariable region of the TCR, which is different in every T-cell clone. This strong immune response could lead to immune dysfunction and the symptoms of CFS.

In the current study, we compared the viral copy number of HHV-6 and HHV-7 in the saliva, and PBMCs of 39 CFS patients and 9 healthy controls, in order to determine whether high viral titers of these ubiquitous viruses were associated with CFS. Furthermore, we measured the transcript levels of HERV-K18 env to look for a possible association with CFS. We found no statistically significant differences in viral copy numbers or HERV transcript levels between healthy controls and CFS patients in our cohort.

MATERIALS AND METHODS

Patient Cohort
Forty CFS cases and 10 healthy control subjects were recruited by one of us (A. L. K.) who has been a CFS clinician and investigator for 30 years. The cases were taken from a cohort that has been systematically followed for up to 25 years. All cases met the Centers for Disease Control and Prevention (CDC) criteria for CFS [29] at study entry. (Reanalysis revealed 1 case may not have met the CDC criteria, but removal of that patient’s data did not change the results). Healthy controls were solicited from advertisements posted at Brigham and Women’s hospital. Each healthy control completed a questionnaire to verify a state of good health and that they did not have the symptoms of CFS. Each control subject was matched by gender and age (within 5 years) to a CFS case. (One of the healthy controls was removed from the study due to being suspected of having chromosomally integrated HHV-6).

Blood and saliva were collected from cases and control subjects in the outpatient practice of Brigham & Women’s Hospital (BWH). Saliva samples were collected using a previously developed protocol involving mouth rinse and gargling with 5 mL of phosphate-buffered saline (PBS) [30]. Samples were coded so that cases and controls could not be determined by laboratory personnel. All samples were transported to the Tufts University laboratories on the day of collection. On arrival, blood samples were processed immediately. Saliva samples were immediately frozen at −80°C until processing. At the time of sample collection, patients were interviewed to determine the severity of their fatigue, as described in more detail subsequently. All participants gave their informed consent in accordance with the protocols approved by the Institutional Review Board for research with human subjects at BWH.

Blood Processing
PBMCs were isolated from blood with Ficoll (GE Healthcare) using previously published lab protocols [31]. After isolation, PBMCs were washed with PBS at a 1:1 ratio and counted using a light microscope and a hemocytometer. PBMCs were aliquoted to 5 × 10⁶ cells per tube, spun down and resuspended in 350 μL of Buffer RLT Plus (1% β-mercaptoethanol; Qiagen). Samples were stored in this lysis buffer at −80°C.

DNA and RNA Isolation
DNA and RNA were isolated from PBMCs using the procedures provided by the AllPrep DNA/RNA Mini Kit (Qiagen). In total, 350 μL of PBMC lysate (5 × 10⁶ cells in RLT buffer) were placed on the DNA spin column. Samples were spun down at 10 000 rpm for 30 seconds in an Eppendorf centrifuge 5417C. The column was then transferred to a new collection tube and put aside until after RNA was isolated. The flow-through was used to isolate RNA. In sum, 350 μL 70% ethanol was added to the flow-through, mixed well, and placed on an RNA spin column. After completing the manufacturer’s procedures for both RNA and DNA columns (Qiagen), isolated RNA and DNA was stored on ice until concentration was determined using a Thermo Scientific Nanodrop 2000 Spectrophotometer. RNA was stored at −80°C.
and DNA was stored at −20°C until polymerase chain reaction (PCR) testing.

DNA was isolated from the saliva wash using the QIAGEN supplementary protocol to the QIAamp DNA Blood Mini Kit spin procedure (Qiagen). The saliva wash was spun down at 1800 g for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 180 µL PBS. Twenty µL QIAGEN Protease and 200 µL buffer AL were added and mixed immediately by vortexing. The entire sample was then incubated at 56°C for 10 minutes. After the 10 minutes incubation, 200 µL 100% ethanol was added to the sample and mixed by vortexing. The entire sample was then added to a QIAamp Spin column. After completing the manufacturer’s procedures for the QIAamp Spin column, isolated DNA was kept at −20°C until PCR testing.

Quantitative Real-time HHV-6 Quantitative Polymerase Chain Reaction (qPCR)

Primers and probe, designed by Karlsson et al [32], were ordered from Applied Biosystems (see Table 1 for sequences). The target sequence for the HHV-6 qPCR was chosen from a conserved region of the HHV-6 U67 gene, which was a perfect match for both HHV-6A and HHV-6B. The analytical sensitivity and linear detection range of the assay corresponds to 0.5–5 × 10^5 HHV-6 genome copies per PCR reaction. This was determined using a quantitated viral DNA control given to us by D. Ablashi of the HHV-6 Foundation. The reaction mix for the Taqman qPCRs contained 1× Gene Expression Master Mix (Applied Biosystems), 900 nM forward and reverse primers, 250 nM probe, and 200 ng of DNA in a reaction volume of 20 µL. A standard curve using the quantitated HHV-6 viral DNA was run on each plate as a positive control and water as a negative control. Thermocycler conditions were 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds and then 60°C for 1 minute using 96-well optical reaction plates (Applied Biosystems) on a 7300 real-time PCR system by Applied Biosystems. All reactions were performed in triplicate. Quality of DNA was assessed, using a Taqman qPCR for the 18s gene in the same reaction (Applied Biosystems).

Subtype Specific PCR for HHV-6A and HHV-6B

A subtype-specific, probe-based, real-time PCR for detection and typing of HHV-6 was developed by Lou et al [33]. The DNA polymerase genes (U38) were selected as target genes. Three nucleotides differ between the strains in the region selected (see Table 1). The 5’ end of the probes for HHV-6A and HHV-6B was labeled with the fluorescent reporter dye VIC and 6-carboxyfluorescein (FAM), respectively, whereas the 3’ end was quenched with 6-carboxytetramethylrhodamine (TAMRA). Primers and probes were synthesized by Applied Biosystems. The reaction mix and the thermocycler conditions were the same as the HHV-6 qPCR (see above). All samples were tested in triplicate. Quality of DNA was assessed, using a Taqman qPCR for the 18s gene in the same reaction (Applied Biosystems). HHV-6A and HHV-6B standard DNA was used as positive controls.

Quantitative Real-time HHV-7 qPCR

A TaqMan qPCR for HHV-7 was developed by Fernandez et al [34]. The primers and probe were chosen in the conserved U100 gene (sequences shown in Table 1). The 5’ end of the probe was labeled with the fluorescent reporter dye 6-carboxyfluorescein (FAM). Primers and probe were synthesized by Applied Biosystems. The reaction mix and the thermocycler conditions were the same as the HHV-6 qPCR (see above). All samples were tested in triplicate. Quality of DNA was assessed using a Taqman qPCR for the 18s gene in the same reaction (Applied Biosystems). Quantitated HHV-7 standard DNA (Advanced Biotechnologies, Inc) was used to make a standard curve for each plate. The threshold of sensitivity of this assay is 10 viral copy numbers.

Real-time RT-PCR for HERV-K18 env

Complementary DNA (cDNA) was generated from 500 ng of total RNA using iScript reverse transcriptase (Bio-Rad) per the manufacturer’s instructions. The Taqman probe and primers specific for the read-through transcript of HERV-K18 env were synthesized by Applied Biosystems. The reaction mix and the thermocycler conditions were the same as the HHV-6 qPCR (see above). All samples were tested in triplicate. Quality of DNA was assessed using a Taqman qPCR for the 18s gene in the same reaction (Applied Biosystems). Quantitated HERV-K18 standard DNA (Advanced Biotechnologies, Inc) was used to make a standard curve for each plate. The threshold of sensitivity of this assay is 10 viral copy numbers.

Table 1. TaqMan Primers and Probes Used for Quantitative Polymerase Chain Reactions

<table>
<thead>
<tr>
<th>Primer/Probe Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HHV-6 forward</td>
<td>5′-CCC GAG CGC TAG GTT GAG-3′</td>
</tr>
<tr>
<td>HHV-6 reverse</td>
<td>5′-AAC GCT CGT CAC AAA CAT AAA ATT C-3′</td>
</tr>
<tr>
<td>HHV-6 probe</td>
<td>5′-FAM-ATG ATC GAA ACG CCT ACA C-MGB-3′</td>
</tr>
<tr>
<td>HHV-6AB forward</td>
<td>5′-CTA AGG TGA GCC AGA TTC G-3′</td>
</tr>
<tr>
<td>HHV-6AB reverse</td>
<td>5′-GAA TAC CCA CAG GCA CTC C-3′</td>
</tr>
<tr>
<td>HHV-6A probe</td>
<td>5′-VIC-TGC AGC CAT TTC TTT GGA AAG C-TAMARA-3′</td>
</tr>
<tr>
<td>HHV-6B probe</td>
<td>5′-FAM-TGC AGC CAC CTC CTT GGA AAG-TAMARA-3′</td>
</tr>
<tr>
<td>HHV-7 forward</td>
<td>5′-AGA GCT TGC GTT GTG CAT GTT-3′</td>
</tr>
<tr>
<td>HHV-7 reverse</td>
<td>5′-ATG TAC CAA TAC GTT CCC ACT TG-3′</td>
</tr>
<tr>
<td>HHV-7 probe</td>
<td>5′-FAM-CACGCAATAACTCTAG-MGB-3′</td>
</tr>
<tr>
<td>HERV-K18 forward</td>
<td>5′-CCG CCT TTT GAG CAG AAC AAG A-3′</td>
</tr>
<tr>
<td>HERV-K18 reverse</td>
<td>5′-CAG TAA TGG CAA TGG CTG CTA TG-3′</td>
</tr>
<tr>
<td>HERV-K18 probe</td>
<td>5′-FAM-TAA GTC CTA CAG ACA AAC TT-MGB-3′</td>
</tr>
</tbody>
</table>

Abbreviations: HERV, human endogenous retrovirus; HHV-6, human herpesvirus-6; HHV-7, human herpesvirus-7.
were designed to recognize HERV-K18 env in human cells by Hsiao et al (sequences shown in Table 1) [35]. The reaction mix and thermocycler conditions were the same as the HHV-6 qPCR (see above). The housekeeping gene HPRT was used to measure the quality of DNA (Applied Biosystems). The ΔΔCt method was used to compare relative expression of HERV-K18 env. HERV-K18 env transcript levels were measured relative to a standard that was always present on every plate. All samples were tested in triplicate.

**Patient Interviews**

At the time of the sample collection, CFS patients completed a symptom questionnaire to assess the level of their fatigue. Specifically, they were asked 5 questions to determine the severity of their fatigue. These questions are as follows: (1) On a scale of 1 to 10 (10 indicating the most fatigue), how would you rate your fatigue over the last 24 hours? (2) At what % of your pre-illness level have you been functioning? (3) Which of the following statements best describes the severity of your fatigue at its worst over the past several months (1 indicating the most fatigue)? (4) Which of the following statements best describes the severity of your fatigue on an average day over the past months (1 indicating the most fatigue)? (5) Have you been so fatigued that you had to reduce your average activity level below half of what was your normal level before you became ill?

**Statistics**

Statistical analysis was performed using Microsoft Excel and SAS. Comparison of viral loads between CFS group and healthy controls was done using the Kolmogorov-Smirnov test. Correlations between viral loads and symptom severity scales were tested using the Spearman Correlation test.

**RESULTS**

Forty-eight blinded samples—39 CFS patients and 9 healthy controls—were analyzed for differences in HERV-K18 transcript levels, HHV-6 viral copy number, and HHV-7 viral copy number using real-time qPCR. HERV-K18 env transcript levels were measured in PBMCs by qRT-PCR. No difference in HERV-K18 transcript levels was detected between the CFS group and the healthy controls (Figure 1). HHV-6 viral copy number was measured in saliva samples as well as PBMCs. No difference in HHV-6 viral copy number using real-time qPCR. HERV-K18 env transcript levels were measured in PBMCs by qRT-PCR. No difference in HERV-K18 transcript levels was detected between the CFS group and the healthy controls (Figure 1). HHV-6 viral copy number was measured in saliva samples as well as PBMCs. No difference in HHV-6 viral copy number was seen in saliva or PBMCs, respectively (Figure 2A and 2B). All HHV-6 viral sequences were of the HHV-6B variant. HHV-7 viral copy number was also measured in saliva samples as well as PBMCs. No difference in HHV-7 viral copy number was seen in saliva or PBMCs, respectively (Figure 3A and 3B). There was a trend toward higher HHV-6 copy number in PBMCs and toward lower HHV-7 copy number in PBMCs. Neither trend was statistically significant, but the power of this study was limited.

CFS patients were interviewed at the time of their sample collection to determine their severity of symptoms. Interview questions were designed to measure the level of fatigue experienced by the individual on the day of the blood draw and over the past month. No correlation was seen between symptom severity and HERV-K18 transcript levels or viral copy numbers of either HHV-6 or HHV-7 (data not shown).

**DISCUSSION**

CFS is a complex heterogeneous disease. Patients do not receive a CFS diagnosis unless other explanations for their symptoms have been excluded, they have unexplained fatigue lasting >6 months, and they have 4 of these 8 symptoms: impaired memory, sore throat, tender cervical or axillary lymph nodes, muscle pain, pain in several joints, new headaches, unrefreshing sleep, or malaise after exertion [2, 3, 5, 29]. With a heterogeneous population of individuals classified as having CFS, it is not difficult to postulate that there could be multiple causes leading to immune dysfunction and the diagnosis of CFS. Although our patient cohort was diagnosed by an expert in CFS (A. L. K.), it is still possible that CFS patients could be divided into greater subsets, as some studies have performed focusing on post viral fatigue [4, 20]. Furthermore, our patient population was restricted to the Boston, Massachusetts, area, which may not account for regional differences in infectious agents that may be linked to CFS.
Some studies have reported higher HHV-6 viral copy number in PBMCs, or other evidence of reactivated HHV-6 infection, in patients with CFS [7, 8, 10–12, 14–18]. However, other studies have not [19, 21, 22]. In our study, there was a trend toward higher levels of HHV-6 copy number in PBMCs; the difference between CFS patients and healthy control subjects was not statistically significant, although the power of our study was limited. There was no difference in the HHV-6 copy number in saliva between CFS patients and healthy controls.

There was a trend toward lower levels of HHV-7 copy number in PBMCs in our study, although the difference between CFS patients and healthy controls was not significant. Similarly, we could not distinguish the HHV-7 viral copy number between CFS patients and healthy controls. Therefore, it is not surprising that the CFS patient’s symptom severity scores show no correlation with either HHV-6 or HHV-7 viral copy number.

Early studies have shown the potential of HERV-K18 to encode a SAg that could disrupt the immune system [24, 25].
SAGs are microbial proteins that greatly overstimulate the immune system by directly interacting with the Vβ segment of the TCR. Conventional peptide antigens are recognized by a specific hypervariable region of the TCR, which is different in every T-cell clone. Since there are only 49 functional human Vβ genes and infinite amounts of hypervariable regions, a vast number of T cells can be activated by a single SAg. It was previously shown that the HERV-K18 SAg can be activated by EBV, IFN-α, and both HHV-6 variants [25–28]. In this study, we hypothesized that HERV-K18 env transcripts would be higher in CFS patients than in healthy controls. However, we see no difference in HERV-K18 transcripts between CFS patients and healthy controls. Not surprisingly, we demonstrate no correlation between CFS symptom severity and HERV-K18 transcript level.

Most CFS patients note that an infectious-like syndrome characterized by flu-like symptoms heralded the onset of chronic fatigue [3–6, 9]. The trigger, by the definition of CFS, must have occurred at least 6 months prior to a CFS diagnosis; therefore, by the time a patient with CFS comes to medical attention, the opportunity to conduct studies of etiologic agents at the time of acute infection has been lost. There are instances, however, in which an acute viral or bacterial infection has been well documented at the time of fatigue onset, and the development of chronic fatigue syndrome has been confirmed by longitudinal studies [4, 36–38].

Both HHV-6 and HHV-7 are ubiquitous in our population, with >90% of the population becoming infected asymptotically as young children. Active infections of HHV-6 and HHV-7 in adults are usually only found in immunocompromised individuals such as transplant recipients or patients with human immunodeficiency virus, showing that these are “opportunistic” infectious agents [39]. Although some past studies have shown an association of these viruses with CFS [8–12], this does not mean that these viruses are causing CFS. HHV-6 and HHV-7 could be bystanders that are taking advantage of the immune dysfunction seen in CFS patients.

At the same time, there is considerable evidence of central nervous system (CNS) dysfunction in CFS [40], and most of the infectious agents linked to CFS are neurotropic. Many investigators have postulated that the symptoms of CFS may be caused by an immunologic attack on infectious agents in the CNS. If that were true, a study examining saliva or PBMCs for viral load and endogenous retroviral SAg expression might not be measuring the central pathology.

In summary, the purpose of this study was to assess the possible association of HERV-K18 to CFS in an already defined population of CFS patients. Although we failed to demonstrate a difference in HERV-K18 env transcripts between CFS patients and healthy controls in this cohort, a more defined cohort of CFS patients with documented cases of post infective fatigue may yield a different result. Our data do not support the hypothesis of reactivation of HHV-6 or HHV-7 in CFS. Viral copy number of HHV-6 and HHV-7 in both saliva and PBMCs was not different between our CFS patients and our healthy controls.

Notes

Acknowledgments. We thank all the participants in our study as well as Dr Albert Tai for technical advice and support.

Financial support. This work was supported by the HHV-6 Foundation.

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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