No evidence of XMRV provirus sequences in patients with myalgic encephalomyelitis/chronic fatigue syndrome and individuals with unspecified encephalopathy

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INTRODUCTION

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is defined as a complex disease involving profound dysregulation of the central nervous system and immune system, dysfunction of cellular energy metabolism and ion transport, and cardiovascular abnormalities (Carruthers et al., 2011). The main clinical sign is severe, unexplained, persistent chronic fatigue lasting more than six months, accompanied by at least four of the following eight symptoms: sore throat, tender cervical or axillary lymph nodes, muscle pain, impaired memory or concentration, un-refreshing sleep, post-exertional malaise and headache of new type (Fukuda et al., 1994). CFS was first defined in 1988. Later, in 1994 this definition was revised and various other definitions were created. Recently the International Consensus Panel developed criteria and suggested also using the term “myalgic encephalomyelitis” (ME), due to widespread inflammation and the multisystem-
ic neuropathology of the disease (Carruthers et al., 2011). The primer for clinical practitioners, published in 2012, gives advice on how to diagnose ME/CFS and which therapies can be used (Friedberg, 2012).

According to population-based studies, already reported 15 years ago, the estimated worldwide prevalence of ME/CFS is from 0.2 up to 2.6%. According to the latest publication reports, it affects 0.4-1% people. The disease is six times more common in women than in men (Bansal et al., 2012). The diagnosis of ME/CFS is based on clinical symptoms and differential diagnosis therefore it is important to identify specific parameters, which can be used as diagnostic markers. Still effective standardized and reproducible diagnostic tests, medical treatment and prevention strategies for ME/CFS are being sought (Albright et al., 2011). Viral infection, immune dysfunction, neurotic hypotension and depression are considered possible etiological factors for ME/CFS (Bansal et al., 2012). ME/CFS is diagnosed only for of v wspart those people suffering from this illness, affecting not only individuals, but also national economics. ME/CFS can affect everyone, irrespective of age, gender and social-economic status, therefore it is important to clarify initiation, progression and maintaining process mechanisms of the disease, as well as develop biomarkers for earlier diagnostics.

Encephalopathy is an abnormal structure or condition of the brain tissues, which can occur at any age. It can be caused by viral or bacterial infection, liver disease, metabolic or nutritional diseases, high blood pressure and hereditary diseases. Viruses are implicated in neurological diseases and neurological disorders, such as Alzheimer’s, Parkinson’s, multiple sclerosis, encephalitis and mesial temporal lobe epilepsy. It is known that encephalopathy is a common problem accompanied by such viral infections as influenza, human herpesvirus-6 and rotavirus (Hoshino et al., 2012). Still, there is a possibility that unspecified encephalopathy could be caused by xenotropic murine leukemia virus-related virus (XMRV) infection.

XMRV belongs to the Retroviridae family, Orthoretrovirinae subfamily, Gammaparetrovirus genus. It was first identified in 2006, investigating the lack of ribonuclease L-coding antiviral gene RNASEL function in patients with prostate cancer (Urisman et al., 2006). The results of studies on XMRV in patients with prostate cancer in Europe and America are controversial. Some researchers report on finding this virus in prostate cancer tissue by PCR, immunohistochemistry, fluorescence in situ hybridization and/or serological assays, but others failed to detect XMRV in patients with prostate cancer (Schlaberg et al., 2009; Switzer et al., 2011).

Observation of RNase L proteolysis in peripheral blood mononuclear cells (PBMCs) in patients with ME/CFS and viral infection-like chronic immune system activation prompted the search for XMRV in patients with these disorders. XMRV has been considered a possible trigger of ME/CFS since October 2009, when Lombardi et al. (2009) reported finding XMRV in 68/101 (67%) persons with ME/CFS and 8/218 (3.7%) healthy controls (Lombardi et al., 2009). Many subsequent studies found no evidence of XMRV in patients with ME/CFS (Groom et al., 2010; Hohn et al., 2010; Hong et al., 2010; van Kuppeveld et al., 2010). Lo and co-authors reported on murine leukemia virus (MLV)-related virus gag gene sequence in 32/37 (86.5%) patients with ME/CFS and 3/44 (6.8%) healthy donors, whereas the env gene sequence was amplified only from one patient and one donor. These sequences are more closely related to polytropic and modified polytropic MLV viruses (Lo et al., 2010). Subsequently XMRV was not detected either in patients with ME/CFS, or in apparently healthy individuals (Ali et al., 2011; Cool et al., 2011; Alter et al., 2012).

No signs of XMRV were found either in patients with ME/CFS and a control group, but also from patients with rheumatoid arthritis and transplant recipients (Henrich et al., 2010). Patients with spondyloarthritis, multiple sclerosis, as well as pediatric patients with idiopathic and respiratory diseases have been shown to be XMRV negative (Jeziorski et al., 2010; Maric et al., 2010). Likewise, XMRV has not been found in human immunodeficiency virus (HIV)-positive patients (Henrich et al., 2010).

Independent studies show that XMRV could be detected due to contamination of commercially available reagents for PCR or human DNA samples contaminated with mouse DNA that
contains MLV-related virus genomic sequences (Sato et al., 2010; Oakes et al., 2010). The finding of XMRV could also emerge from the infected prostate cancer cell line 22Rv1 and cloned or amplified XMRV DNA (Hue et al., 2010; Smith, 2010; Kearney et al., 2012).

After several years of efforts to replicate the finding of XMRV in patients with ME/CFS, previous publications by Lombardi et al. (2009) and Lo et al. (2010) were retracted because of insufficient quality of experiments and the subsequent inability to detect XMRV in ME/CFS cases (Alberts, 2011; Lo et al., 2012).

We investigated the involvement of β-herpesviruses human herpesvirus (HHV)-6 and HHV-7 and parvovirus B19 infection in the development of ME/CFS, post-infection and unspecified origin encephalopathy. Active viral infection including concurrent infection is found in 64.8% (70/108) of patients and in 13.3% (12/90) of practically healthy persons (Chapenko et al., 2012). It is known that gammaretrovirus-like sequences occur in most vertebrate genomes and murine leukemia virus-like retroviruses (MLLVs) are a subset, which may be pathogenic and spread across species (Blomberg et al., 2011). Taking into account the immunomodulating properties of human β-herpesviruses HHV-6 and HHV-7 and parvovirus B19 as well as the fact that activation of one virus could lead to activation of another we examined XMRV in Latvian ME/CFS patients and individuals with unspecified origin encephalopathy. The aim of this study was to analyse the frequency of XMRV proviral sequences in DNA extracted from peripheral blood leukocyte (PBL) samples of patients with ME/CFS and apparently healthy individuals, as well as in PBL and brain tissue DNA isolated from individuals with and without unspecified encephalopathy in Latvia.

**MATERIAL AND METHODS**

One hundred and fifty patients [52 (35%) male and 98 (65%) female, mean age 37.8±9.5] with clinically diagnosed ME/CFS according to the Centers for Disease Control and Prevention diagnostic criteria and 30 age and gender-matched apparently healthy individuals, as well as 61 [41 (67%) male and 20 (33%) female, mean age 56.6±10.5] individuals post-mortem (30 with and 31 without unspecified encephalopathy) were involved in this study. The cohort was established with the approval of the Ethics Committee of Riga Stradins University. DNA from PBL and brain tissue samples was isolated using a phenol-chloroform extraction method, DNA quality assured by β-globin gene polymerase chain reaction (PCR). Potential carry-over contamination was prevented, processing samples in separate rooms and multiple negative controls were included in each assay. The presence of XMRV provirus gag and env sequences was detected by nested PCR (nPCR) using previously described primer sets. The sensitivity of the nPCR was five copies per reaction (Lombardi et al., 2009; Lo et al., 2010). XMRV VP62 plasmid (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: XMRV VP62 cDNA from Drs Robert H. Silverman and Beihua Dong) was used as a positive control (Urisman et al., 2006; Dong et al., 2007). Electrophoretic analysis of amplification products was carried out in 2% agarose gel.

**RESULTS**

Using a PCR gradient method positive control DNA was tested by nPCR assays with modified oligonucleotide primer melting temperatures for hybridisation. An optimal result was obtained at temperatures 59.8ºC, 65.6ºC, 66.6ºC and 67ºC, while a weaker band was visible at 57ºC and 64.2ºC. By contrast, a result was practically impossible to detect using 57.4ºC, 58.4ºC, 61.3ºC and 62.7ºC hybridisation temperatures (Figure 1).

Targeting the XMRV specific gag gene sequence by nPCR with previously reported primer sets 419F, 1154R for the first round and GAG-I-F, GAG-I-R for the second round, XMRV proviral gag gene sequence was not detected either in DNA isolated from 150 patients with ME/CFS PBL or DNA extracted from PBL and brain samples of 61 individuals post-mortem (30 with and 31 without unspecified encephalopathy), or in 30 apparently healthy individuals’ DNA. Only the positive control gave amplimers of 735 bp after the first round and 410 bp after the
second round, corresponding to the expected XMRV gag gene fragment (Figure 2). Moreover, patients with ME/CFS, individuals with and without unspecified encephalopathy, and apparently healthy individuals’ DNA was found to be negative in nPCR assay, targeting the XMRV provirus specific env gene sequence with previously described primer pairs 5922F, 6273R for the first round and 5942F, 6159R for the second round. In addition, only the positive control gave amplimers of 351 bp after the first round and 218 bp after the second round, corresponding to the expected XMRV env gene fragment (Figure 3).
DISCUSSION

ME/CFS is a heterogeneous disease accompanied by a group of symptoms, often followed by viral infection or long-lasting stress. Despite intensive research, there is no consensus on the presence, form or level of immune dysfunction in this condition (Bansal et al., 2012). The disease is characterized by severe fatigue making people unable to work, and lacking a pathophysiological explanation. Different studies on ME/CFS use various diagnostic criteria for the disease, causing heterogeneity between patient groups and uncertainty in results. Therefore, it is necessary to use one common diagnostic criterion. Most patients report a sudden start of the illness with flu-like symptoms, therefore viral infections are considered as one of the possible ME/CFS causative agents. Some viruses may cause fatigue after infection and many patients with ME/CFS have immunological disturbances that could be the result of viral infection or promoted by the infection. Still possible involvement of viral infections in the etiopathogenesis of ME/CFS is discrepant (Morinet and Corruble, 2012). It is not clear whether viral infection causes ME/CFS or follows after the disease.

In 2009, Lombardi and colleagues reported on the prevalence of XMRV in 67% patients with ME/CFS and 3.7% of healthy donors (Lombardi et al., 2009). In 2010, Lo et al. also found MLV-related virus gag gene sequence in 86.5% patients with ME/CFS and 6.8% healthy donors, still the env gene sequence had one patient and one donor (Lo et al., 2010). However, the findings of the current study do not support the previous research. In this study XMRV specific gag and env gene sequences were not detected either in DNA from 150 patients with ME/CFS or 61 individuals post-mortem (30 with and 31 without unspecified encephalopathy), or in the DNA of 30 apparently healthy donors. Obtained results are in accordance with studies in Sweden, China, Netherland, Germany, United States of America, United Kingdom, Japan and Canada which found no evidence of XMRV in patients with ME/CFS or donors (Blomberg et al., 2011; Hong et al., 2010; van Kuppeveld et al., 2010; Hohn et al., 2010; Satterfield et al., 2011; Erlwein et al., 2011; Furuta et al., 2011; Steffen et al., 2011). In addition, XMRV or MLV-related sequences, infectious virus or antibodies were not detected in tested groups of patients with ME/CFS, previously tested as XMRV positive, including part of patient samples from the original study by Lombardi et al. (2009) (Shin et al., 2011; Knox et al., 2011). This retrovirus was also absent in ME/CFS patients’ cerebrospinal fluid (Schutzer et al., 2011). The presence of XMRV
infection was sought in post-mortem brains of autistic individuals, but no signs of XMRV or MLV-related viruses were found (Lintas et al., 2011). Studies published later consistently showed no association between XMRV and prostate cancer (Furuta et al., 2011; Lee et al., 2012). Furthermore, researchers were not able to detect any signs of XMRV infection markers in HIV-positive patients and patients with fibromyalgia (Arredondo et al., 2011; Gingaras et al., 2012). XMRV was not found in patients with such chronic inflammatory diseases as rheumatoid arthritis, systemic lupus erythematosus and Behcet’s disease (Ali et al., 2011). In addition, chronic hepatitis B and C, lymphoid malignancies and lymphadenopathy are proven not to be associated with XMRV (Arredondo et al., 2011; Waugh et al., 2011). The findings of the current study are consistent with other research concluding after more than two years of intensive research on XMRV that this virus is not associated with human diseases and the positive results may be due to contamination (Groom and Bishop, 2012). However, a group of researchers in Italy recently reported the detection of a gag gene sequence in two out of 12 (16.6%) patients with ME/CFS. One was closely related to polytropic MLV-related virus and other was similar to XMRV. Although the amount of XMRV proviral DNA is very low, the role of XMRV in human diseases remains obscure (Paolucci et al., 2012). This study using nPCR found no evidence of XMRV infection either in patients with ME/CFS or individuals with unspecified encephalopathy, or in apparently healthy individuals.

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