Xenotropic and polytropic murine leukemia virus-related sequences are not detected in the majority of patients with chronic fatigue syndrome

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SUMMARY

XMRV and polytropic MLV-related virus have been controversially associated with chronic fatigue syndrome (CFS). Subsequent reports failed to detect XMRV and MLV-related virus in CFS patients, and the previous results have been interpreted as a massive laboratory contamination by mouse DNA sequences. Among 12 sequential CFS patients, two were positive for XMRV/MLV sequences. In contrast, 40 selected control subjects were negative. CSF patients and controls were negative for mitochondrial mouse-specific DNA sequences. These findings do not confirm the high frequency of MLV-related viruses infection in CFS patients, but also contrast the widespread laboratory contamination previously suggested.

Key words: Chronic fatigue syndrome, XMRV, MLV-related virus, Polytropic MLV, Ecotropic MLV.

Received February 9, 2012 *Accepted* April 24, 2012

Chronic fatigue syndrome (CFS) is an uncommon clinical condition of unknown etiology characterized by persistent fatigue in association with malaise, multiple joint and muscle pain, unrefreshing sleep and severe mental and physical exhaustion. Recently, the xenotropic murine leukemia virus (MLV)-related virus (XMRV) genome sequence was identified in prostate tissue of 40% of patients with prostate cancer (Urisman *et al*., 2006) and in peripheral blood mononuclear cells (PBMC) of 67% of patients with CFS (Lombardi *et al*., 2009). The XMRV sequences detected in these patients were almost identical, whereas they differed from those of the related ecotropic MLV detected in prostate cancer patients (Urisman *et al*., 2006). More recently, an MLV-related virus, more closely related to the polytropic MLV than to XMRV, was identified in CFS patients (Lo *et al*., 2010). However, subsequent studies failed to detect XMRV and MLV-related virus in tissues or blood samples of CFS patients (Erlwein *et al*., 2010; Switzer *et al*., 2010; Simmons *et al*., 2011; Shin *et al*., 2011), and the previous positive findings were interpreted as the result of contamination by a laboratory-derived virus (Paprotka *et al*., 2011). Finally, the original papers by Lombardi and Lo (Lombardi *et al*., 2009; Lo *et al*., 2010) had been retracted and no correlation between the presence of XMRV/MLV and CFS has been recognized (Karafin and Stramer, 2012). However, in several papers denying the association between XMRV/MLV and human diseases, a subset of samples showed positive PCR (Arredondo *et al*., 2011; Williams *et al*., 2011) or serologic (Arredondo *et al*., 2011; Qiu *et al*., 2012) signals.

During 2010 the presence of XMRV and polytropic MLV-related provirus was investigated in 12 sequential CFS patients. As a control, matching numbers of randomly selected individuals with different clinical conditions (HIV-infected individuals, n=10, transplant recipients, n=10,

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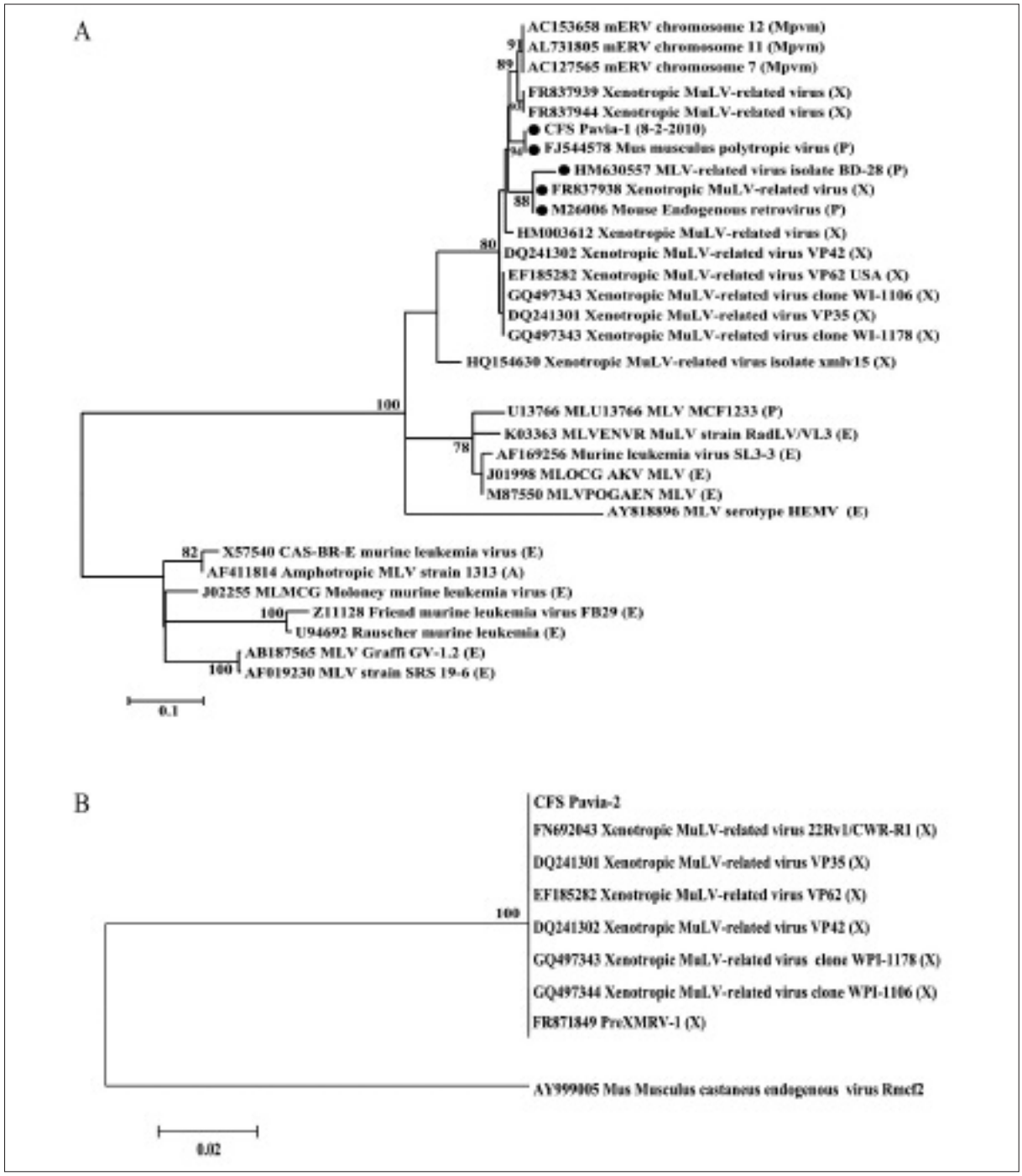

FIGURE 1 - *A) Phylogenetic analysis of gag nucleotide sequences (ranging from nt 63 to nt 378 of XMRV isolate 5956, accession number HM003612) in a patient with chronic fatigue syndrome (CFS Pavia-1). The tree was generated by using the maximum likelihood method with tamura 3-parameters and Gamma distribution (5 categories) as an evolutionary model. Boostrap values >80 are reported at each branch node. Sequences of the most relevant MLVs were included in the tree and referred to as amphotropic (A), ecotropic (E), polytropic (P), xenotropic (X) or modified polytropic (Mpmv). All reference strains are reported with the accession number. Sequences with a deletion are indicated with black dots. B) Phylogenetic analysis of integrase nucleotide sequences (80 nt) in a patient with chronic fatigue syndrome (CFS Pavia-2). Abbreviations are the same as those in Figure 1A.*

HCV-positive patients, n=10, and healthy subjects, n=10) were analyzed in parallel during the same period. Following digestion of 10^6 PBMC with proteinase K, DNA was extracted using the easy MAG™ automatic extractor (Biomerieux, Lyon, France). Nested PCR of the XMRV gag region was performed using previously described primer pairs (Urisman *et al.*, 2006; Lombardi *et al.*, 2009). A second XMRV gene was amplified in parallel by a real-time PCR assay detecting a fragment of the XMRV integrase gene (Schlaberg *et al.*, 2009). The XMRV VP62 clone (obtained from the NIH AIDS Research and Reference Reagent Program, Rockville, MD, USA) was used as a positive control. β_2-microglobulin was amplified in all samples to normalize real-time PCR results. To prevent potential carry-over contamination, samples were processed in three separate rooms, multiple negative controls were included in each assay and real-time PCR assays were performed using the TaqMan Universal PCR Master Mix including the UNG (Applied Biosystems, Foster City, CA, USA). To exclude potential contamination by mouse DNA, mouse-specific mitochondrial DNA sequences were searched for as reported (Lo *et al.*, 2010). PCR amplicons were sequenced using the BigDye Terminator Cycle Sequencing Ready kit (Applied Biosystems, Foster City, CA). Viral sequences were analyzed by the Blast program (http://blast.ncbi.nlm.nih.gov). Multiple sequence alignment was performed with the MEGA version 5.0 software for the phylogenetic analysis.

Two out of 12 (16.6%) CFS patients were positive for gag PCR and integrase PCR respectively. In contrast, no evidence of XMRV or other MLV-related viruses was detected in blood cells from HIV-positive patients, HCV-positive patients, transplant recipients or healthy donors. Nucleotide sequencing of the gag amplicon from the first positive patient (CFS Pavia-1) showed that the retrovirus was more closely related to the polytropic MLV rather than to XMRV (identity 100% vs. 96%) (Figure 1A). In particular, a specific deletion of seven amino acids between codon 45 and codon 51 of the gag gene observed in our strain and five additional virus strains reported in GeneBank characterized the similarity of these retroviruses to the polytropic MLV-related virus. Sequencing of the integrase gene amplicon from the second positive patient (CFS Pavia-2) confirmed the similarity with the XMRV sequence (Figure 1B).

The finding of two related, but different, virus sequences (polytropic MLV and XMRV) in two CFS patients, does not confirm the high frequency of MLV-related virus infection in CFS patients reported in previous studies (Lombardi *et al.*, 2009). On the other hand, our results are in contrast with the reported massive and widespread laboratory and reagents contamination (Robinson *et al.*, 2010; Oakes *et al.*, 2010; Tuke *et al.*, 2011). In fact:

i) all samples were negative for mitochondrial mouse-specific DNA sequences;
ii) all the several negative reaction controls were consistently scored as negative;
iii) all blank reagent controls were consistently scored as negative.

These results would exclude the presence of contaminating mouse or plasmid DNA in reagents. In addition, the two positive amplicons showed different retrovirus sequences, thus excluding amplicon carry-over contamination. The data here reported are relevant to a small group of CFS patients, and studies in larger patient cohorts have not attributed an etiologic role for XMRV or MLV-related viruses in CFS (Erlwein *et al.*, 2010; Switzer *et al.*, 2010; van Kuppeveld *et al.*, 2010; Simmons *et al.*, 2011). It is however intriguing that the only positive results were obtained in these patients. On the other hand, in both cases the positivity could not be confirmed by the amplification of a different virus gene. The proviral DNA amount was very low in our patients, which might explain the stochastic amplification of a single virus gene in each of the two positive patients. Another possibility is that only fragment of virus DNA might be present in biologic samples. In conclusion, while it appears established that XMRV/MLV sequences are not detectable in a significant proportion of CFS patients, the frequency and the role of evolutionary relic retrovirus sequences potentially detectable in the human chromatin remain to be further elucidated.

ACKNOWLEDGEMENT

We thank Daniela Sartori for manuscript editing and Laurene Kelly for revision of the English.

This work was supported by the Ministero della Salute, Ricerca Corrente (grant 80207) and Programma Nazionale AIDS (grant 40H55).

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