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Phylogenetic Analysis of Murine Leukemia Virus Sequences from Longitudinally Sampled Chronic Fatigue Syndrome Patients Suggests PCR Contamination Rather than Viral Evolution▽‡

Aris Katzourakis,[1]† Stéphane Hué,[2]† Paul Kellam,[2,3] and Greg J. Towers[2]*

Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, United Kingdom[1]; MRC Centre for Medical Molecular Virology, Division of Infection and Immunity, University College London, 90 Gower St., London WC1E 6BT, United Kingdom[2]; and Wellcome Trust Sanger Institute, Hinxton, Cambridge CB10 1SA, United Kingdom[3]

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Xenotropic murine leukemia virus (MLV)-related virus (XMRV) has been amplified from human prostate cancer and chronic fatigue syndrome (CFS) patient samples. Other studies failed to replicate these findings and suggested PCR contamination with a prostate cancer cell line, 22Rv1, as a likely source. MLV-like sequences have also been detected in CFS patients in longitudinal samples 15 years apart. Here, we tested whether sequence data from these samples are consistent with viral evolution. Our phylogenetic analyses strongly reject a model of within-patient evolution and demonstrate that the sequences from the first and second time points represent distinct endogenous murine retroviruses, suggesting contamination.

Detection of murine leukemia virus (MLV) DNA in patient samples has suggested that the human population may be infected with murine gammaretroviruses (4). A particular xenotropic MLV named xenotropic murine leukemia virus-related virus (XMRV) has been cloned from human prostate cancer tumors as well as blood samples from individuals suffering from chronic fatigue syndrome (CFS) (1, 18, 29, 39). XMRV detection in a small percentage of samples from healthy controls suggested widespread infection (7, 18, 29). Controversy has since surrounded XMRV detection, partly because many laboratories have been unable to detect XMRV in patient samples (2, 5, 6, 8–11, 14, 16, 19–21, 28, 30, 35, 37, 40) and partly because an almost identical virus has been found infecting a common prostate cancer cell line called 22Rv1 (12, 15, 23). These data strongly suggest that XMRV in patient material is the result of DNA contamination from laboratory cell lines or mouse DNA. Importantly, a recent study demonstrated that XMRV arose by recombination during the experiments in which the 22Rv1 cell line was developed by xenografting prostate tumors in mice (22). This observation confirms a date for XMRV genesis in the cell line at between 1990 and 1996 and rules out any human XMRV infection before this time. These observations have raised concerns that previous XMRV detection in humans is likely to be artifactual (3).

An important study in support of MLV infection in humans is that by Lo, Alter, and colleagues (17). These authors suggested that they could confirm human infection of MLV by PCR amplifying a variety of MLV sequences from the blood of CFS patients as well as healthy controls. A PCR test for mouse mitochondrial DNA was used to control for contamination with mouse DNA and found to be negative, but recently, more sensitive intracisternal type A particle (IAP)-based PCR tests for murine contamination reveal that in some cases mouse contamination is not detected by amplification of mitochondrial DNA (26). Surprisingly, Lo et al.'s study did not find XMRV but found a set of MLV sequences almost identical to known endogenous nonecotropic gammaretroviruses of mice. These MLV sequences were characterized as type 1 (18 patients), type 2 (2 patients), and type 3 (1 patient), based on their *gag* gene sequences. Importantly, the authors suggested that evolution of patient viruses could be demonstrated by the accumulation of significant sequence variation over time. Longitudinal samples were taken from eight individuals apparently infected with type 1 viruses 15 years after the first sampling. Seven of these had detectable MLV *gag* at the second time point (28). The sequences derived from six of these longitudinal samples have been deposited in GenBank under accession numbers HQ601957 to HQ601962. Here, we used phylogenetic analyses to consider whether MLV sequences described in this study are consistent with viral infective evolution, a conservative test of whether they are likely to represent genuine human MLV infections.

The shape of a phylogenetic tree reflects the evolutionary processes under which it has grown. The expectation for longitudinally sampled retroviral sequences from the same patient, or from a population of infected patients, is that they cluster with the initial sequences and to the exclusion of all other sequences in the data set. Phylogenetic analysis of the human-derived MLV sequences as well as a variety of known MLV sequences (see the supplemental material for details)

* Corresponding author. Mailing address: MRC Centre for Medical Molecular Biology, Division of Infection and Immunity, University College London, Cruciform Building, 90 Gower St., London WC1E 6BT, United Kingdom. Phone: 020 3108 2112. Fax: 020 7679 9555. E-mail: g.towers@ucl.ac.uk.

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† These authors contributed equally.

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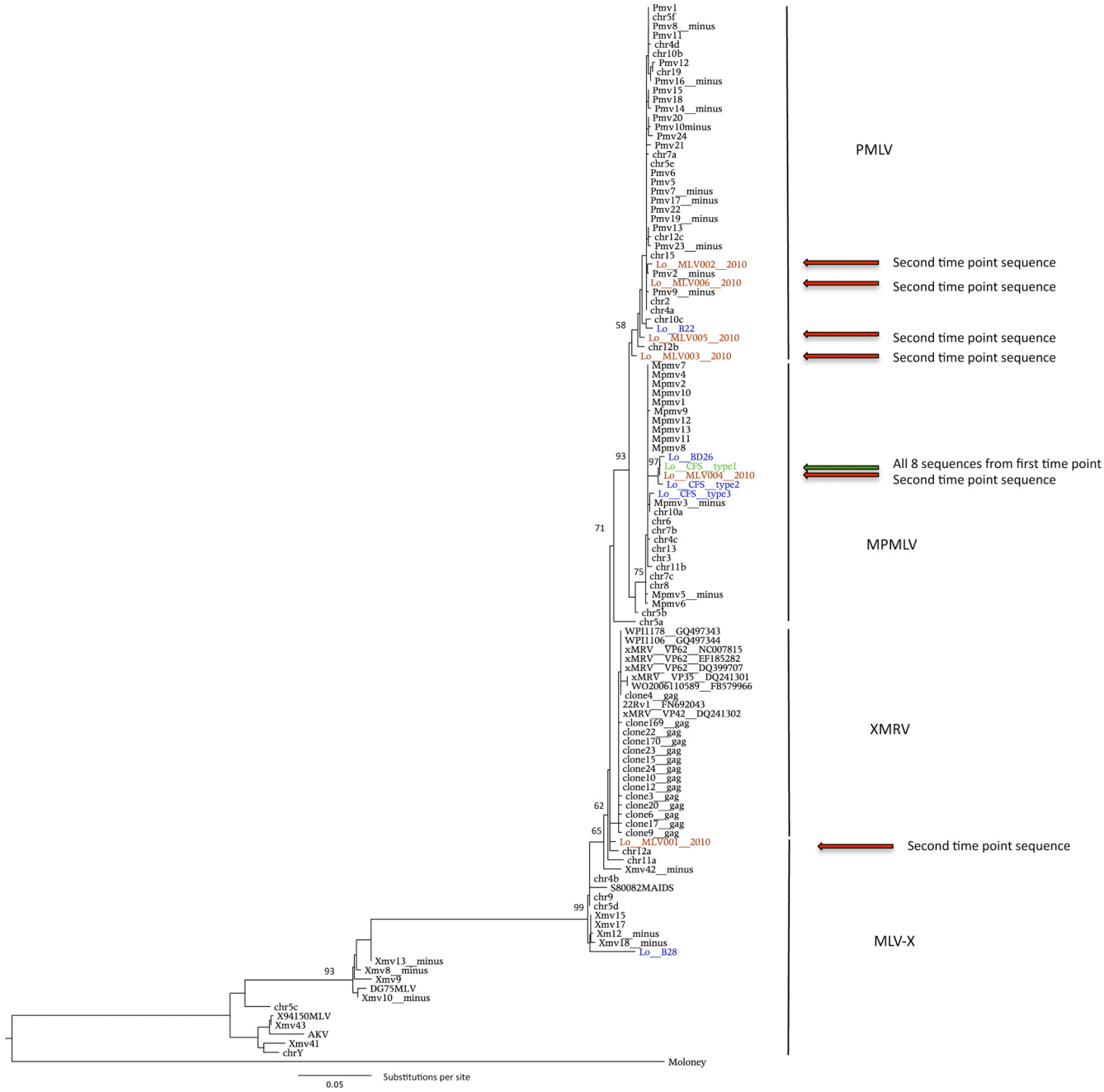

FIG. 1. Maximum likelihood phylogeny of XMRV, xenotropic MLV (MLV-X), polytropic MLV (PMLV; Pmv), and modified polytropic MLV (MPMLV; Mpmv) *gag* gene sequences (710 nt). The initial chronic fatigue syndrome patient-derived XMRV sequences from Lo et al. (17) are indicated in blue. The eight sequences taken from the first time point are represented by a single branch, colored green. XMRV sequences sampled 15 years later from the same patients (GenBank accession numbers HQ601957 to HQ601962) are colored in red, while other sequences that do not have corresponding second-time-point sequences from Lo et al. are colored blue. The tree is rooted with Moloney MLV (GenBank accession number AF033811). Bootstrap scores of >50% are indicated on the corresponding branches. The scale bar represents the number of nucleotide substitutions per site.

indicates that the three patient-derived sequence types, CFS types 1 to 3, fall within the modified polytropic MLV clade. The overall shape of the phylogenetic tree, including the three main groupings (polytropic, modified polytopic, and xenotropic) and the relationships between them, is consistent with previous studies based on full-length proviruses (Fig. 1) (12, 13). Sequences described in Lo et al.'s original report are colored blue and green. While the type 3 sequence is clearly separated from types 1 and 2, the latter sequences form a strongly supported monophyletic cluster (97% bootstrap support) together with the sequence BD26, derived from a healthy donor, and the sequence MLV004_2010, a longitudinal CFS

TABLE 1. Comparison of maximum likelihood phylogenetic tree with hypotheses consistent with within-patient viral evolution[a]

Constraint	Log likelihood	Decrease in likelihood	Standard deviation	*P* value		Expected likelihood wt
				SH	AU	
Best tree	−3,658.76	0.00	NA	NA	NA	0.99
All 5 MLV 2010	−3,721.22	62.46	14.92	<0.001	<0.0001	<0.0001
MLV001 2010	−3,689.93	31.17	9.62	0.001	<0.0001	<0.0001
MLV002 2010	−3,696.66	37.90	10.80	0.002	<0.0001	<0.0001
MLV003 2010	−3,679.73	20.96	9.31	0.022	0.002	0.0086
MLV005 2010	−3,687.62	28.86	10.15	0.004	0.0003	0.0004
MLV006 2010	−3,701.63	42.87	12.90	0.002	<0.0001	<0.0001

[a] The most stringent constraint involved all 5 MLV sequences from the second time point, while each of the 5-s time point sequences was also constrained individually to cluster with the CFS type 1 sequence from the first time point. The trees were compared using pairwise Shimodaira-Hasegawa (SH) tests, the approximately unbiased (AU) test, and expected likelihood weights. All of the constrained trees were significantly worse than the maximum likelihood tree. NA, not applicable.

patient sample (Fig. 1). All the resampled patients originally yielded identical polytropic MLV CFS type 1 sequences (S. C. Lo, personal communication); in other words, all eight sequences derived from patients at the first time point are represented by a single branch in the phylogeny. Under a model of within-patient viral evolution, we would expect all of the 2010 daughter sequences to branch from the parental CFS type 1 sequence with longer but approximately equidistant branches. This is true for all other longitudinally sequenced viruses (reviewed in reference 24).

We assessed the fit of the data to this model by inspection of the phylogenetic tree and by maximum likelihood-based model testing. The phylogenetic placement of the longitudinal sequences does not fit this expected model. When the sequences from the second time point were examined, we found that 5 of 6 are phylogenetically distinct from the parental CFS type 1 sequence and from each other. The more recent longitudinally sampled sequences are shown in red in Fig. 1. While the originating CFS type 1 sequences belong to the modified polytropic clade, longitudinally sampled sequences from the same CFS type 1-infected patients are derived from three strongly supported and distinct regions of the tree, namely, the polytropic, modified polytropic, and xenotropic clades. Surprisingly, 3 of the new sequences (MLV002_2010, MLV005_2010, and MLV006_2010) do not even form sister taxa within the polytropic clade. In fact, the two most distantly related sequences from these longitudinal patient samples are about as different from each other as the biggest distance possible within the polytropic clade. Another 2010 (MLV001_2010) sequence is placed within the xenotropic clade, at the base of the XMRV cluster. It also contains a large deletion in *gag*, which would be expected to inactivate the virus. Although this sequence is basal, aside from the deletion, it is very similar to other XMRV sequences, differing at only a single nucleotide position across its 330-nucleotide (nt) length from the prostate cancer patient sequence VP62 (39). It is therefore substantially more similar to XMRV than to either the polytropic or modified polytropic MLV sequences previously reported by Lo et al. (17). There is no evidence for hypermutation mediated by APOBEC proteins in the patient-derived sequences as might be expected during an infection, given the susceptibility of MLV to mutation by these proteins (23). Thus, these new patient-derived MLV sequences show tremendous variation from the parental CFS type 1 sequence and as such are extremely unlikely to have evolved from the CFS type 1 sequence.

The probability that the data are consistent with a model of within-patient evolution can be explicitly tested by comparing the maximum likelihood phylogenetic tree directly derived from the data with a tree in which sequences from the second time point are constrained to cluster with sequences from the first time point. The difference in likelihood of these two topologies was determined by using the Shimodaira-Hasegawa test (33), the approximately unbiased test (31), and expected likelihood weights (34) as implemented in CONSEL (32). We can reject the hypothesis of clustering of sequences from the second time point with those from the first (Table 1). In order to explore the robustness of this test to the possibility that any one individual sequence is consistent with within-patient evolution, and that the *P* value may be unduly influenced by any of the other sequences, we also explored the relative fit of each sequence from the second time point to a model consistent with within-patient evolution (i.e., constrained to cluster with sequences from the first time point). We compared the likelihood of five additional trees, where each patient sample was constrained in turn to cluster with the CFS type 1 sequence while the other patients were allowed to assume the most likely position within the tree. For each patient individually, the tree that is consistent with within-patient evolution was rejected (Table 1). The use of these likelihood-based tests is robust to the short sequences used in the alignment, which account for the low bootstrap support scores for many nodes in the phylogenetic tree.

To calculate the chance of a modified polytropic virus evolving into a polytropic virus, we reconstructed the common ancestors of the polytropic and modified polytropic clades by taking a consensus of each sequence set. We then estimated the probability of a virus making the specific changes required to evolve from one clade to the other by running 10,000 simulations in Seq-Gen and counting the number of times the mutations arose (25). The hypothesis that the characteristic mutations could have arisen by chance, given that a number of mutations equivalent to the distance between these groups had occurred, can be rejected with a *P* value of <0.0001. Indeed, the fact that 3 of the newly sampled viruses appear to have independently made these specific changes underlines the fact that these sequences represent different viruses rather than CFS type 1 descendants. The last of the longitudinal sequences is 100% identical to the CFS type 1 sequence identified in 18 patients at the initial time point, 15 years earlier. This observation indicates that in this case, there has been no viral evolution throughout 15 years of infection. Some slowly

TABLE 2. Comparison of patient-derived MLV sequences and known MLV sequences within the mouse genome[a]

Sequence	GenBank accession no.	Sampling yr(s)	Closest relative in the C57BL/6J mouse genome (July 2007 assembly)						
			Chr.	Strand	Span (nt)	Start (nt)	End (nt)	% identity	Changes[b]	Gaps (length in nt)
BD22	HM630560	Mid-1990s	10	−	697	8269377	8270073	99.30	5/697	0
			10	−	696	50145707	50146402	99.30	5/696	0
			X	+	696	15052167	15052862	99.30	5/696	0
BD26	HM630561	Mid-1990s	6	−	339	73242571	73242909	98.30	6/339	0
			2	−	339	15949590	15949928	98.30	6/339	0
			10	−	339	4627251	4627589	98.30	6/339	0
BD28	HM630557	Mid-1990s	9	+	331	62288048	62288378	98.10	6/318	1 (21)
			5	+	331	23722164	23722494	98.10	6/318	1 (21)
			4	+	331	133716363	133716693	98.10	6/318	1 (21)
CFS type 1	HM630562	Mid-1990s	8	+	697	125689652	125690348	99.30	5/697	0
			11	+	697	102946013	102946709	99.30	5/697	0
			6	−	696	73242413	73243108	98.90	5/696	0
CFS type 2	HM630558	Mid-1990s	8	+	698	125689652	125690349	99.00	7/698	1 (1)
			11	+	698	102946013	102946710	99.00	7/698	1 (1)
			6	−	697	73242412	73243108	98.60	7/697	1 (1)
CFS type 3	HM630559	Mid-1990s	2	−	697	15949431	15950127	99.90	1/697	0
			13	+	697	21905315	21906011	99.80	2/697	0
			6	−	696	73242413	73243108	99.30	1/696	1 (1)
MLV001	HQ601957	2010	12	+	340	19250254	19250593	99.70	1/276	2 (63; 1)
			9	+	339	62288048	62288386	99.00	2/276	1 (63)
			5	+	339	23722164	23722502	99.00	3/276	1 (63)
MLV002	HQ601958	2010	4	−	339	107826090	107826428	99.80	1/339	0
			2	−	339	57074273	57074611	99.80	1/339	0
			15	−	339	76395902	76396240	99.80	1/339	0
MLV003	HQ601959	2010	6	−	339	73242571	73242909	99.20	3/339	0
			4	−	339	107826090	107826428	99.20	3/339	0
			2	−	339	57074273	57074611	99.20	3/339	0
MLV004	HQ601960	2010	6	−	339	73242571	73242909	98.60	5/339	0
			2	−	339	15949590	15949928	98.60	5/339	0
			10	−	339	4627251	4627589	98.60	5/339	0
MLV005	HQ601961	2010	4	−	339	107826090	107826428	99.50	2/339	0
			2	−	339	57074273	57074611	99.50	2/339	0
			15	−	339	76395902	76396240	99.50	2/339	0
MLV006	HQ601962	2010	4	−	339	107826090	107826428	100.00	0/339	0
			2	−	339	57074273	57074611	100.00	0/339	0
			15	−	339	76395902	76396240	100.00	0/339	0

[a] Due to the short lengths of the patient-derived sequences, several mouse sequences are equally similar. Thus, three sequences are shown. The span column refers to the total length of the best match in the murine chromosome (Chr.).
[b] Number of nucleotides different/total number of nucleotides.

evolving retroviruses (e.g., simian foamy virus) can remain virtually identical over many years (36); however, this patient sample contrasts markedly with all the other samples from the second time point. In summary, in some patients, viral diversity is as vast as the diversity that the whole set of known nonecotropic MLVs allows, and yet from another patient, an identical sequence was amplified 15 years later. The repeat samples could not have been derived from the initial samples via a process of viral evolution. In fact, they represent different endogenous murine viruses. It is theoretically possible that the 6 patients were originally coinfected with diverse viruses, but this possibility is rather undermined by the fact that the original samples each gave rise to the same identical type 1 sequence from 18 independent patients. An alternative possibility is that all but one of the patients that retested positive for MLV were superinfected with a distinct MLV prior to the samples being taken at the second time point and that in each of these patients, the viral infection from the first time point was cleared.

Examination of the viral sequences amplified reveals that they are almost identical to known sequences in mouse genomic DNA. The numbers of differences between the pa-

tient amplified samples and their nearest relatives in the published mouse C57BL/6J genome are shown in Table 2. For example, MLV002 is 1 nucleotide out of 339 nucleotides different from polytropic MLV on the C57BL/6J mouse chromosomes 4, 2, and 15, most likely due to a single nucleotide polymorphism within the mouse population. The only realistic explanation that could account for these observations is that all of the MLV-positive patient samples, or the PCRs performed using patient DNA as template, were contaminated with mouse DNA. This would act as a source for amplification of the diverse viruses found and could perhaps have occurred as a result of repeated handling of patient-derived samples (see reference 41 for a discussion). There is no credible hypothesis that could explain these observations in the absence of PCR contamination. It appears to be extremely difficult to do mouse-free PCR, and we note that other studies in which contamination has been demonstrated have also amplified a diverse range of MLVs (27, 38). We propose that the detection of murine virus in human samples be more rigorously controlled using IAP PCR (26) to rule out murine DNA contamination and robust phylogenetic analysis to rule out random amplification of endogenous proviruses (12), which can exist at a high copy number in the genomes of mice or in cell lines that become infected with mouse viruses during routine experimentation.

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