No Evidence of Murine-Like Gammaretroviruses in CFS Patients Previously Identified as XMRV-Infected

Konstance Knox,[1,2] Donald Carrigan,[1,2] Graham Simmons,[3,4] Fernando Teque,[5] Yanchen Zhou,[3,4] John Hackett Jr.,[6] Xiaoxing Qiu,[6] Ka-Cheung Luk,[6] Gerald Schochetman,[6] Allyn Knox,[1] Andreas M. Kogelnik,[2] Jay A. Levy[5]*

Members of the gammaretroviruses—such as murine leukemia viruses (MLVs), most notably XMRV [xenotropic murine leukemia virus (X-MLV)–related virus—have been reported to be present in the blood of patients with chronic fatigue syndrome (CFS). We evaluated blood samples from 61 patients with CFS from a single clinical practice, 43 of whom had previously been identified as XMRV-positive. Our analysis included polymerase chain reaction and reverse transcription polymerase chain reaction procedures for detection of viral nucleic acids and assays for detection of infectious virus and virus-specific antibodies. We found no evidence of XMRV or other MLVs in these blood samples. In addition, we found that these gammaretroviruses were strongly (X-MLV) or partially (XMRV) susceptible to inactivation by sera from CFS patients and healthy controls, which suggested that establishment of a successful MLV infection in humans would be unlikely. Consistent with previous reports, we detected MLV sequences in commercial laboratory reagents. Our results indicate that previous evidence linking XMRV and MLVs to CFS is likely attributable to laboratory contamination.

Xenotropic retroviruses, first discovered in mice, have the unusual characteristic of being endogenous to animal species, i.e., integrated into the animal's genome, but not able to reinfect cells from that species. However, as the name (*xenos*, foreign) implies, these viruses can infect cells from other animal species. The xenotropic murine leukemia virus (X-MLV), for example, infects cells from several species including humans but cannot infect many mouse cells (*1*–*3*). One particular virus within this group, XMRV (xenotropic murine leukemia virus–related virus), was reported to be present in a subset of human prostate tumors (*4*) and in blood samples from patients with chronic fatigue syndrome (CFS) (*5*). Other murine-related gammaretroviruses have also reportedly been detected in CFS patients (*6*). The infection of humans with these viruses is controversial. Investigators evaluating independent cohorts of CFS patients have failed to detect XMRV or other MLVs (*7*–*12*), and contamination of human clinical material (*13*, *14*) and reagents (e.g., *Taq* polymerase) (*15*) with mouse DNA containing MLV-like sequences has been reported.

To investigate these discrepancies in a more direct manner, we performed an extensive virological evaluation of blood samples from two human populations with a clinical diagnosis of CFS (*16*), many of whom had been diagnosed previously as XMRV-infected. The first (P1) consisted of 41 CFS patients ranging in age from 5 to 73 years who came from a private medical practice (Sierra Internal Medicine, Incline Village, Nevada). Twenty-six of the CFS subjects (63%) were female, and 15 (37%) were male; the female median age was 52 years (range 5 to 72 years), and the male median age was 49 years (range 20 to 73 years). These patients were an unselected, sequentially enrolled population submitted for diagnostic testing to the Wisconsin Viral Research Group (WVRG) and were therefore a true cross section of the patients in the medical practice. Thirty-seven of these 41 patients had been tested previously for XMRV infection by the following assays: whole-blood polymerase chain reaction (PCR), serum PCR, or viral XMRV culture with PCR (*17*). These evaluations were performed by a commercial (VIPDx, Reno, Nevada) or research laboratory [Whittemore Peterson Institute (WPI), Reno, Nevada]. Twenty-six were reported as being XMRV-positive, and 11 were reported as being negative. Blood samples used from this patient cohort were archived diagnostic specimens and, therefore, exempt from Institutional Review Board (IRB) consideration [46.101 (b)(4), Code of Federal Regulations].

The second population (P2) came from the same medical practice, and subjects were selected largely on the basis of a previous positive diagnosis for XMRV infection. This patient cohort included 29 CFS patients, 26 of whom (89.6%) had tested positive for XMRV in at least one of the three virus assays listed above and/or had

[1]Wisconsin Viral Research Group, Milwaukee, WI 53226, USA. [2]Open Medicine Institute, Mountain View, CA 94040, USA. [3]Blood Systems Research Institute, San Francisco, CA 94118, USA. [4]Department of Laboratory Medicine, University of California, San Francisco, San Francisco, CA 94143, USA. [5]Department of Medicine, Hematology/Oncology Division, University of California, San Francisco, San Francisco, CA 94143, USA. [6]Abbott Laboratories, Abbott Park, IL 60064, USA.

*To whom correspondence should be addressed. E-mail: jay.levy@ucsf.edu

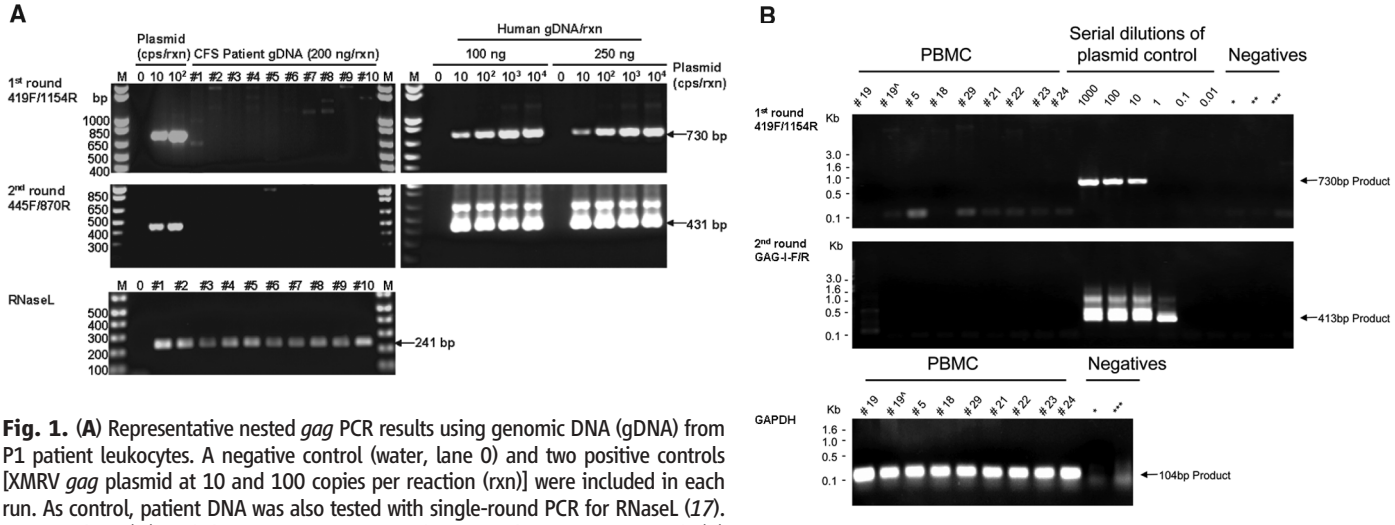


Fig. 1. (**A**) Representative nested *gag* PCR results using genomic DNA (gDNA) from P1 patient leukocytes. A negative control (water, lane 0) and two positive controls [XMRV *gag* plasmid at 10 and 100 copies per reaction (rxn)] were included in each run. As control, patient DNA was also tested with single-round PCR for RNaseL (*17*). DNA markers (M) and the positions of expected PCR products are annotated. (**B**) Representative nested RT-PCR results on P2 PBMC samples. Positive and negative controls are shown. Ten-fold serial dilutions of XMRV *gag* plasmid control start at 1000 copies per reaction. Negative controls for each reaction step were tested in triplicate: *RNA/DNA extraction negative control, **RT control, and ***PCR control.

antibodies to XMRV detected in a commercial (VIPDx) or research laboratory (WPI) (*5*) (table S1). Twenty of the patients (69%) were female and nine (31%) were male with a median age of 52 years. Nine of these subjects were also part of P1 (table S1). Fresh blood samples were used for viral culture and testing (*17*). For the serum inactivation studies, seven healthy University of California, San Francisco (UCSF) laboratory workers, ranging in age from 21 to 72 years, served as controls. These volunteers were afebrile without signs of any illness. This research received approval of the Human Subjects Committee at the UCSF. All participants signed IRB–approved consent forms.

We initially assessed the peripheral blood leukocytes from the 41 subjects in P1 for XMRV DNA using nested PCR targeting *gag* (primers 419F/1154R and 445F/870R) and *env* (primers 5922F/6273R and 5937F/6198R). The sensitivity of these PCR assays is at least 10 XMRV genomes per reaction (table S3). No XMRV DNA was detected in any sample (see Fig. 1A for representative data). Notably, a chart review of the 41 patients revealed that 19 had two blood samples drawn on the same day by the same phlebotomist, with one sample submitted to VIPDx and the other to WVRG. For XMRV analysis, VIPDx used diagnostic technologies identical to those utilized in previous studies on XMRV and CFS (*5*). The chart review indicated that 53% (10 out of 19) of the blood samples were reported by the commercial laboratory as being positive for XMRV DNA. This difference in our results (0 out of 19) versus the chart review results (10 out of 19) was statistically significant ($P < 0.0004$, two-sided Fisher's exact test).

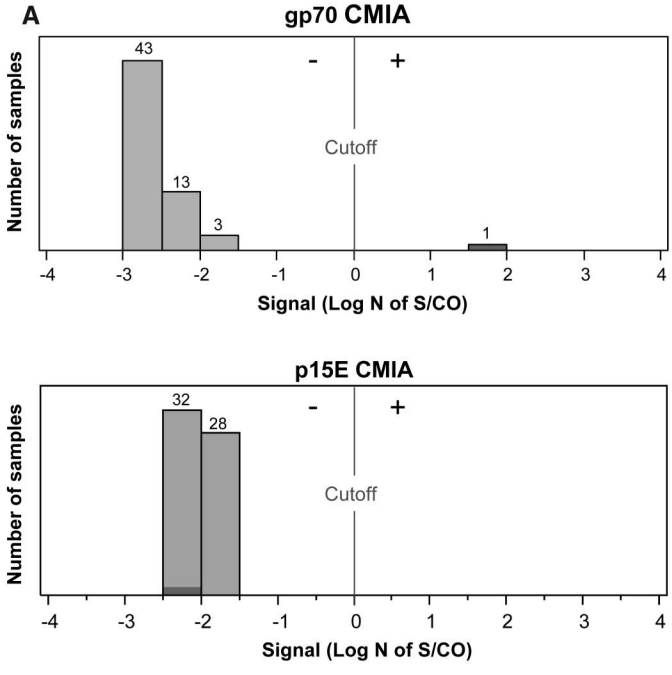
Our failure to detect XMRV DNA in patient population P1 prompted us to undertake a more extensive study of patient population P2. We used multiple methodologies to evaluate P2 blood samples for the presence of (i) nucleic acids derived from XMRV or MLV; (ii) infectious XMRV and MLV; and (iii) XMRV-specific antibodies (*17*). Ficoll-Hypaque–purified peripheral blood mononuclear cells (PBMCs) were evaluated by reverse transcription PCR (RT-PCR) procedures directly or after activation with phytohemagglutinin (PHA; 3 μg/ml for 3 days) with primers and protocols described by others (*6*) and previously demonstrated to be highly sensitive for detection of XMRV and MLVs (*6*, *18*). In addition, plasma was evaluated by RT-PCR in a similar manner. No MLV was found in the PBMCs or plasma of these 29 CFS patients (Table 1 and Fig. 1B). The positive control consisted of a 730–base pair fragment of XMRV amplified from the prostate cancer cell line, 22R*v1*. The assay was able to detect at least 10 copies of XMRV *gag* DNA per reaction; second-round PCR detected 1 to 10 copies per reaction (table S3).

We also investigated whether infectious XMRV or MLV was detectable in the P2 blood samples. The patients' PBMCs were added to duplicate plates of early-passaged mink lung cells to enhance detection of X-MLV and maintained for 5 days (*2*, *19*, *20*). The PBMCs were then removed, and the mink lung cells were passaged weekly for 3 weeks. Culture fluids were then evaluated for infectious XMRV or MLV by monitoring the induction of focus formation in the mink S+L– cell line (*19*, *20*), by measuring RT activity in the cell culture fluids (*21*), and by PCR

Table 1. Summary of assays used to evaluate blood samples from CFS patients in P2. Information about the CFS patients is provided in table S1. Two subjects were studied twice within a 3-month period (table S1) and gave the same results.

Assay	Percent XMRV-positive (*n*)
PCR analysis of PBMC-derived DNA	0 (0/31)
RT-PCR analysis of patient plasma	0 (0/31)
PBMC culture fluids*	0 (0/19)
Reverse transcriptase assay of supernatants from mink lung cells passaged after PBMC coculture*	0 (0/30)†

*Infectious virus assay: Fluids were tested for infectious virus production by reverse transcriptase and the mink S+L– cell assays (see text) (*17*). †Insufficient cells were available for these studies from subject no. 24.



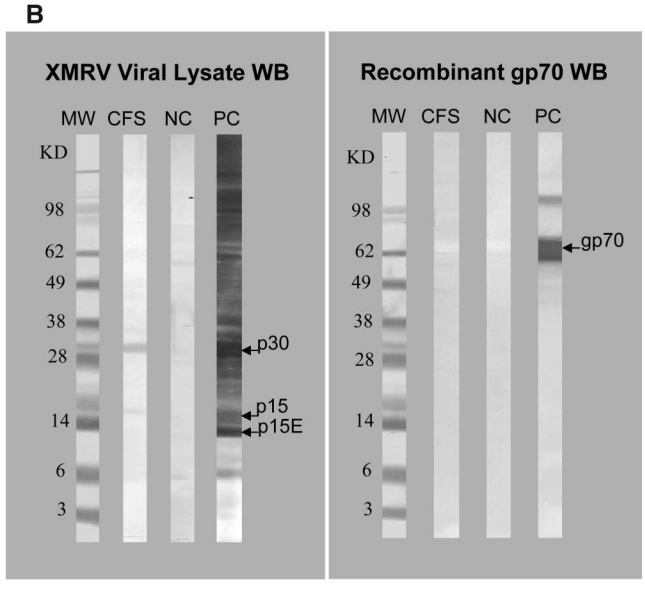


Fig. 2. Evaluation of 60 CFS plasma samples for the presence of XMRV antibodies. (**A**) Two recombinant protein–based CMIAs were used to detect specific antibodies to XMRV gp70 and p15E proteins (*17*). The *x* axis represents the CMIA signal in a unit of natural log–transformed ratio of sample signal to the cutoff signal (log N of S/CO). (**B**) Western blot analysis of gp70 CMIA reactive CFS sample using native XMRV viral proteins and mammalian-expressed recombinant gp70 protein. Sample keys: the gp70 CMIA-reactive (CFS) sample 09-7571, positive control (PC) of XMRV-infected macaque plasma, negative control (NC) of normal blood donor, and molecular mass (MW) markers in kilodaltons (kD).

analysis (*11*, *18*). We also looked for infectious virus in culture fluids from 19 patient PBMCs that had been cultured for 1 to 3 weeks after PHA stimulation. As summarized in Table 1, we did not detect XMRV or MLV in any of the patient samples.

A previous study reported that 50% (9 out of 18) of patients with CFS had plasma antibodies reactive with XMRV (*5*). We evaluated 60 plasma samples from P1 and P2 patients for the presence of XMRV-specific antibodies by means of two direct-format chemiluminescence immunoassays (CMIAs), using either transmembrane p15E or envelope gp70 recombinant proteins of XMRV (*22*). These assays can detect antibodies to other MLVs. None of the 60 plasma samples from these CFS patients was reactive in the p15E CMIA (Fig. 2A). One of the 60 samples was weakly reactive in the gp70 CMIA with a sample/cut-off (S/CO) value of 5.4 (log N of S/CO = 1.68). However, the plasma was not positive by Western blot (WB) assay with purified XMRV viral lysate as well as recombinant gp70 protein (*22*) (Fig. 2B). It was therefore considered negative.

Further studies of antiviral responses in the P2 population assessed whether serum samples from these patients could inactivate X-MLV and XMRV. Previous work (*23*) had indicated that X-MLV is sensitive to inactivation by sera from healthy individuals, most likely by human complement (*24*–*26*); conceivably, CFS patient sera are deficient in this activity. X-MLV and XMRV were mixed with unheated or heated human sera from 7 healthy subjects and 19 CFS patients (*17*). Both viruses were susceptible to inactivation by unheated, complement-containing sera from both groups; over a 2-log reduction in virus infectivity was noted in several cases. XMRV was less susceptible to inactivation than X-MLV (Fig. 3), most likely a reflection of the passage of XMRV through human cells, which renders the virus less sensitive to human complement (*24*–*26*). These results, as well as other reports showing restriction of XMRV replication in human cells (*27*, *28*), suggest that an established MLV infection in humans is unlikely.

Because neither XMRV or MLV sequences or infectious virus could be detected in the blood of the 61 CFS patients in our P1 and P2 populations, we explored whether XMRV and MLV sequences might be present in research reagents used to detect these viruses. While our own studies were under way, other investigators considered the same possibility (*29*) and reported that mouse DNA and MLV sequences were detectable in reagents and tissues used for RT-PCR (*13*–*15*), particularly the mouse monoclonal antibodies (MAbs) in *Taq* polymerase preparations (*15*). Notably, we detected MLV sequences not only in 3 out of 5 *Taq* polymerases that utilize MAbs, but also in 9 out of 17 other MAbs-containing reagents used in research laboratories (table S2), including antibodies to CD4, CD8, and CD14. Sequencing of these PCR products revealed a high degree of sequence homology with known MLV sequences from laboratory strains; they most closely resembled the MLV sequences reported by others in the blood of CFS patients (*6*) (figs. S1 and S2).

Bioreagent contamination, however, does not adequately explain the detection of XMRV by Lombardi *et. al.* (*5*). In this regard, we have found that the DNA sequences of three XMRV proviruses they described are identical to that of VP62, which is the prototype XMRV cloned from prostate cancer tissue (*4*). Long-term passage of VP62 led to proviruses with accumulated multiple point mutations (fig. S3). As suggested by others (*30*), independently derived XMRV DNA sequences should show increased genetic diversity compared with the VP62 clone sequence. Therefore, the remarkable conservation of the WPI-XMRV sequences is most consistent with laboratory contamination with the original infectious VP62.

In conclusion, we have found no evidence that XMRV or other murine-like gammaretroviruses are present in blood samples from 43 CFS patients who were previously reported to be infected by XMRV (*5*, *6*). Notably, over a period of several months, seven of our subjects were studied on two occasions; two subjects on three occasions. Because our blood samples were obtained from CFS patients from the same clinical practice that provided the majority of patients described in the early XMRV report (*5*), differences in the patient cohort or clinical diagnosis cannot account for the discrepancies between our findings and the previous observations. We believe that the detection of MLV in human blood in previous studies (*5*, *6*) reflects contamination of reagents used to assess their presence and/or contamination of human samples during laboratory manipulation of the infectious XMRV clone, VP62 (*5*). In addition, our studies indicate no antibodies to XMRV and that X-MLV and XMRV are fully or partially inactivated by human serum. The latter finding suggests that these viruses could not readily establish a human infection. Because an activated immune system has been observed in CFS patients (*31*), the possibility of another infectious agent(s) being associated with this illness merits continued attention.

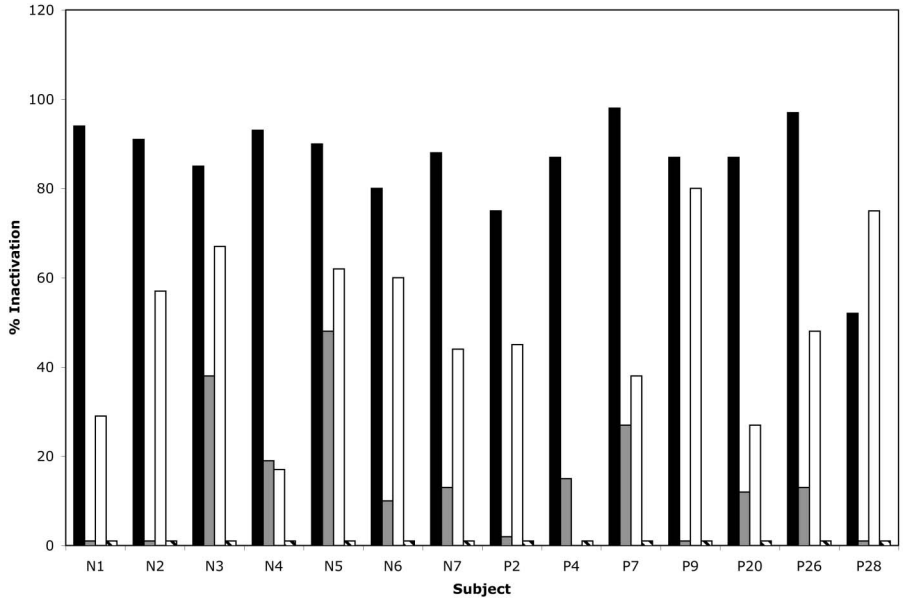


Fig. 3. Effects of human serum on xenotropic MLV and XMRV. Shown is the percent serum inactivation of virus, as measured by induction of focus formation in mink S+L– cells by control untreated X-MLV and XMRV (*17*). Representative results are shown. Unheated sera from 12 other CFS patients gave similar findings with nearly complete inactivation of X-MLV and partial to high inactivation of XMRV. The X-MLV was obtained from New Zealand Black (NZB) mouse cells and propagated in mink lung cells (*20*). XMRV was obtained from the human prostate cell line (22R*v1*). For the five studies conducted, the control virus titers measured as focus formation in mink S+L– cells were 126, 430, 168, 246, and 208 foci (X-MLV); 84, 376, 208, 284, and 206 foci (XMRV). N, control; P, CFS patient (see table S1); black bars, X-MLV unheated sera; shaded bars, X-MLV heated sera; white bars, XMRV unheated sera; hatched bars, XMRV heated sera.

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Acknowledgments: We thank D. Peterson for the referrals of all the subjects evaluated, J. Weismann for coordinating subject participation, I. Livinti for assistance with data management, E. Delwart and J. Fiddes for comments on the manuscript, and K. Peter for help in preparation of the manuscript. These studies were conducted with support of private funds to the investigators. Patent applications have been filed by Abbott Laboratories relating to detection of XMRV using immunoassays and molecular-based assays.

Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1204963/DC1
Materials and Methods
Figs. S1 to S3
Tables S1 to S3
References

1 March 2011; accepted 16 May 2011
Published online 31 May 2011;
10.1126/science.1204963

Recombinant Origin of the Retrovirus XMRV

Tobias Paprotka,[1]* Krista A. Delviks-Frankenberry,[1]* Oya Cingöz,[3,4]* Anthony Martinez,[5] Hsing-Jien Kung,[5,6] Clifford G. Tepper,[5] Wei-Shau Hu,[2] Matthew J. Fivash Jr.,[7] John M. Coffin,[3,4] Vinay K. Pathak[1]†

The retrovirus XMRV (xenotropic murine leukemia virus–related virus) has been detected in human prostate tumors and in blood samples from patients with chronic fatigue syndrome, but these findings have not been replicated. We hypothesized that an understanding of when and how XMRV first arose might help explain the discrepant results. We studied human prostate cancer cell lines CWR22R*v1* and CWR-R1, which produce XMRV virtually identical to the viruses recently found in patient samples, as well as their progenitor human prostate tumor xenograft (CWR22) that had been passaged in mice. We detected XMRV infection in the two cell lines and in the later passage xenografts, but not in the early passages. In particular, we found that the host mice contained two proviruses, PreXMRV-1 and PreXMRV-2, which share 99.92% identity with XMRV over >3.2-kilobase stretches of their genomes. We conclude that XMRV was not present in the original CWR22 tumor but was generated by recombination of two proviruses during tumor passaging in mice. The probability that an identical recombinant was generated independently is negligible (~10^{-12}); our results suggest that the association of XMRV with human disease is due to contamination of human samples with virus originating from this recombination event.

Murine leukemia viruses (MLVs) are retroviruses belonging to the genus *Gammaretrovirus* that cause cancers and other diseases in mice, and they are divided into the ecotropic, amphotropic, polytropic, and xenotropic classes on the basis of their receptor usage. Xenotropic MLVs cannot infect cells from inbred mice but can infect cells from other species, including humans. Xenotropic murine leukemia virus–related virus (XMRV) was isolated from a human prostate cancer (PC) in 2006 and has been reported to be present in 6 to 27% of human PCs (*1*, *2*) and in the peripheral blood of 67% of chronic fatigue syndrome (CFS) patients (*3*). The assertion that XMRV is circulating in the human population has been challenged by several studies that have failed to detect XMRV in multiple cohorts of PC and CFS patients or healthy controls [reviewed in (*4*)]. Endogenous xenotropic MLVs can infect human tumors during passage through nude mice (*5*), and it has been suggested that XMRV may have arisen in this manner (*5*, *6*). In addition, XMRV replication is highly sensitive to human APOBEC3s and tetherin (*7*–*11*), which makes it doubtful that XMRV replication occurred efficiently in human peripheral blood mononuclear cells of CFS patients as previously reported (*3*).

The human PC cell line CWR22R*v1* (hereafter 22R*v1*) (*12*) produces infectious XMRV essentially identical in sequence to that obtained from patients. 22R*v1* contains ≥10 proviral copies per cell (*13*) and was proposed to have been derived from an XMRV-infected tumor. This cell line was derived from a xenograft (CWR22) that was established from a primary prostate tumor at Case Western Reserve University and serially passaged in nude mice (*14*, *15*). To explore the origin of the virus in 22R*v1* cells, we analyzed various passages of the CWR22 xenograft, as well as a subline of the CWR22 xenograft (2152) from which the 22R*v1* cell line was established (*12*), and another prostate cancer cell line, CWR-R1, which was also derived from CWR22 (*16*). Figure 1A traces the timeline of the serial xenograft transplants of CWR22 up to the derivation of the cell lines 22R*v1* and CWR-R1 and indicates (bold letters) the samples that were available for analysis. Nude mouse strain(s) maintained by Charles River (NU/NU) and Harlan Laboratories [Harlan Sprague Dawley (Hsd)] are likely to have been used for in vivo passages of the xenograft (*17*). DNA samples from passage 3 (777 in Fig. 1A) and an unknown early passage (736) were obtained along with samples from a 7th passage, CWR22-9216R and CWR22-9218R. A xenograft tumor from the early seventh passage was independently propagated at the University of California, Davis, using Hsd nude mice (CWR22-8R and 8L). Total nucleic acid from relapsed androgen-independent tumors (CWR22R) 2152, 2524, 2272, and 2274 and the 22R*v1* and CWR-R1 cell lines was available for analysis (*14*).

We verified that the xenograft samples (736, 777, 9216R, 9218R, 8R, and 8L) and the 22R*v1* or CWR-R1 cell lines were all derived from the same person by performing short tandem repeat analysis at seven loci (Fig. 1B and fig. S1). The probabilities that the xenografts and the two cell lines have the same allele patterns for these loci by chance are 1.6×10^{-13} and 6.3×10^{-13}, respectively.

To quantify the amount of XMRV DNA in the CWR22 xenografts, we developed a real-time polymerase chain reaction (PCR) primer-probe set that specifically detected XMRV *env* and excluded murine endogenous proviruses present in BALB/c and NIH3T3 genomic DNA (Fig. 1C). We used quantitative PCR of 22R*v1* DNA to estimate 20 proviruses per cell and used the 22R*v1*

[1]Viral Mutation Section, HIV Drug Resistance Program, National Cancer Institute at Frederick, Frederick, MD 21702, USA. [2]Viral Recombination Section, HIV Drug Resistance Program, National Cancer Institute at Frederick, Frederick, MD 21702, USA. [3]Department of Molecular Biology and Microbiology, School of Medicine, Tufts University, 150 Harrison Avenue, Boston, MA 02111, USA. [4]Genetics Program, School of Medicine, Tufts University, 150 Harrison Avenue, Boston, MA 02111, USA. [5]Department of Biochemistry and Molecular Medicine, University of California, Davis, Sacramento, CA 95817, USA. [6]Department of Urology, University of California, Davis, Sacramento, CA 95817, USA. [7]Data Management Services, Inc., National Cancer Institute at Frederick, Frederick, MD 21702, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: vinay.pathak@nih.gov

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Science **333** (6038), 94-97.
DOI: 10.1126/science.1204963originally published online May 31, 2011