資料4

XMRVに関する文献報告（続報）（平成23年6月27日）

血液事業部会運営委員会委員　岡田　義昭

文献番号	文献名	報告国	要約
1	Furuta R, Miyazawa T, Sugiyama T, et al., Retrovirology.2011, Mar 17;8:20 No association of xenotropic murine leukemia virus-related virus with prostate cancer or chronic fatigue syndorome in Japan	日本	日本の前立腺癌患者67人、慢性疲労症候群患者100人、健常人500人の血液を用いてXMRVの感染リスクを評価した。XMRVの gagのCAタンパクに対する抗体陽性者がいたが、XMRV由来の他のウイルス抗原に対する抗体陽性者はいなかった。CA抗体陽性の前立腺癌患者の末梢単核球からXMRVの遺伝子が検出されたが、再現性が乏しく、また培養法を用いてもXMRVを分離することはできなかった。一方、慢性疲労症候群患者の血漿及び末梢単核球からXMRVの遺伝子を核酸増幅法で検索したが全て陰性であった。これらの結果は、日本の前立腺癌や慢性疲労症候群の発症とXMRV感染との関連がないことを示唆している。
2	Shin H, Bateman L, Schlaberg R, et al., J Virol. 2011, May 4. [Epub ahead of print] No evidence of murine-like gammaretroviruses in CFS patients previously identified as XMRV-infected	米国	同じ地域に住む100例の慢性疲労症候群患者と200例の健常人の血液から、1）XMRV及びXMRV類似ウイルスの遺伝子、2）これらのウイルスに対する抗体、3）高感受性細胞を使用したウイルス分離、をブラインドで実施したが、全て陰性であった。また、過去２年間繰り返して陽性とされた14例のCFS患者を同様に検査したが1例もXMRV及びXMRV類似のウイルス遺伝子、抗体は検出できなかった。さらにTaq polymerase中に少量のマウスDNAがコンタミしていることを発見し、これによってNATの5%が陽性となった。
3	Knox K, Carrigan D, Simmons G, et al., Science. 2011, Jun 2 [Epub ahead of print] No evidence of murine-like gammaretroviruses in CFS patients previously identified as XMRV-infected	米国	ネバダ州の1つの医療機関から提供された過去にXMRV陽性と判断された43名を含む慢性疲労症候群患者61名の血液を核酸増幅法、血清学的検査、感染性ウイルスの検出、等を用いて再評価したところ、XMRVや他のマウス白血病ウイルス（MLV）は全く検出されなかった。以前 scienceに掲載された「慢性疲労症候群から高率にXMRVが検出された」という報告に用いられた多くの検体を提供した同一の医療機関からの検体であることから、診断や患者の集団の差からこれらの結果の不一致を説明できない。原因として、核酸増幅法に用いる酵素に添加されているマウス単クローン抗体や実験室で用いられているマウス単クローン抗体からMLVの遺伝子が検出されること、細胞で継代したXMRV（VP62DU145）は塩基配列に複数の変異が認められたが検体から検出された複数のXMRVの塩基配列はVP62や22RV1由来のものと一致したこと、等から実験室内での検体へこれらが混入したためだと思われる。さらにXMRVや異種指向性MLVは補体によって不活化されることから、実際には人では感染が成立できないことも示唆された。
4	Paprotka T, Delviks-Frankenberry K, et al., Science. 2011, Jun 2. [Epub ahead of print] Recombinant origin of the retrovirus XMRV	米国	XMRVを産生するヒト前立腺癌株のCWR22Rv1とCWR-R1はCWR22株由来である。CWR22はヌードマウスで継代された細胞株であり、継代の初期の細胞株からはXMRVは検出されなかった。一方、継代に用いたマウスからPreXMRV-1とPreXMRV-2の2つのプロウイルスが検出され、前半と後半でそれぞれXMRVと99.92%のホモロジーがあり、2つのプロウイルスが組み換えを起こし、XMRVになったと考えられる。この組み換えが別々に生じる可能性は低く、ヒトの疾患と関連したXMRVはヒトサンプルへの混入のためであることを示している。
5	Alberts B, Science. 2011, Jun 2 [Epub ahead of print] Editorial expression of concern	米国	文献3と4はLombardiらがscienceに発表した「慢性疲労症候群患者の67%からXMRVが検出された」という2009年の論文が、実験室内や解析に用いた試薬へのウイルスのコンタミだったことを強く支持している。そのためLombardiらの論文の正当性に疑念があるので彼らの論文にこれらの懸念があることを添付した。XMRVと慢性疲労症候群との因果関係の有無についてNIHの後援によって研究が行われており、 science誌としては結果を待っている。

RETROVIROLOGY

RESEARCH Open Access

No association of xenotropic murine leukemia virus-related virus with prostate cancer or chronic fatigue syndrome in Japan

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Abstract

Background: The involvement of xenotropic murine leukemia virus-related virus (XMRV) in prostate cancer (PC) and chronic fatigue syndrome (CFS) is disputed as its reported prevalence ranges from 0% to 25% in PC cases and from 0% to more than 80% in CFS cases. To evaluate the risk of XMRV infection during blood transfusion in Japan, we screened three populations–healthy donors (n = 500), patients with PC (n = 67), and patients with CFS (n = 100)–for antibodies against XMRV proteins in freshly collected blood samples. We also examined blood samples of viral antibody-positive patients with PC and all (both antibody-positive and antibody-negative) patients with CFS for XMRV DNA.

Results: Antibody screening by immunoblot analysis showed that a fraction of the cases (1.6-3.0%) possessed anti-Gag antibodies regardless of their gender or disease condition. Most of these antibodies were highly specific to XMRV Gag capsid protein, but none of the individuals in the three tested populations retained strong antibody responses to multiple XMRV proteins. In the viral antibody-positive PC patients, we occasionally detected XMRV genes in plasma and peripheral blood mononuclear cells but failed to isolate an infectious or full-length XMRV. Further, all CFS patients tested negative for XMRV DNA in peripheral blood mononuclear cells.

Conclusion: Our data show no solid evidence of XMRV infection in any of the three populations tested, implying that there is no association between the onset of PC or CFS and XMRV infection in Japan. However, the lack of adequate human specimens as a positive control in Ab screening and the limited sample size do not allow us to draw a firm conclusion.

Background

Xenotropic murine leukemia virus-related virus (XMRV), a gammaretrovirus found in humans, is possibly associated with certain diseases [1,2]. The virus was first identified in prostate cancer (PC) by using a pan-viral microarray; XMRV RNA was detected in eight of 22 R462Q homozygous patients, but in only one of 66 patients with RQ or RR (wild-type [WT]) alleles of the *RNASEL* gene [1], an important component of the innate antiviral response [3]. Schlaberg et al. [4] found XMRV proteins in nearly 25% of PC specimens and reported that XMRV infection is associated with high-grade PC. Conversely, XMRV RNA was detected in only 1.2% of PC cases in a German study [5], and neither XMRV RNA nor anti-XMRV antibodies (Abs) were detected in PC patients in another German cohort [6]. Furthermore, in a recent study, XMRV RNA was detected in the blood of 67% of patients with chronic fatigue syndrome (CFS) and 3.6% of healthy individuals [2]. Lo et al. [7] found murine leukemia virus (MLV)-related sequences in genomic DNA of peripheral blood mononuclear cells (PBMCs) in 32 of 37 (86.5%) CFS patients and three of 44 (6.8%) healthy blood donors. However, the absence of XMRV infection in CFS patients has been reported in several countries [8-12]. These conflicting results have provoked serious debates about XMRV detection methods and patient characteristics [13].

XMRV can infect many human cell lines by using XPR1 as a receptor, similar to other xenotropic murine

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retroviruses [14-16], and XMRV replication appears to be enhanced in cells with a defective interferon-gamma (IFNγ) intracellular pathway [17]. In terms of *in vivo* infection, the route of transmission, infectivity to humans, and pathogenesis of XMRV are largely unknown; therefore, its potential risk as a transfusion-transmissible infectious agent remains to be clarified.

Many blood service organizations worldwide, including those in Japan, have yet to establish a transfusion policy for XMRV, although in a few countries (e.g., Canada) blood donations are restricted from individuals previously diagnosed with CFS. To investigate the prevalence of XMRV in healthy Japanese individuals as well as in PC patients, we started screening blood samples in 2007 from donors in Osaka prefecture and PC patients in Nishiwaki City, a rural area of Hyogo prefecture close to Osaka prefecture, as a pilot study of XMRV infection. On the basis of Lombardi et al.'s results of XMRV infection in CSF patients and, to a lesser extent, in the healthy population [2], we also screened blood samples from CFS patients. We found that a proportion of the donors and patients had Abs against the XMRV Gag capsid (CA), but XMRV genes were barely detectable. These results suggest that although the presence of human infection with XMRV or XMRV-related viruses in Japan cannot be denied, such infection is likely to be limited.

Results

Study design

Our study design, summarized in Figure 1, was not standardized because the screening process for donors and PC patients was not implemented simultaneously with that for CFS patients. We employed different methods to detect XMRV nucleic acids at different stages of the study, but the same Ab-screening test was used consistently throughout. All plasma samples were screened for XMRV Abs by immunoblot assay to calculate the serological prevalence of XMRV. Plasma samples of viral Ab-positive PC patients were further screened for XMRV RNA. Moreover, PBMCs of PC patients whose plasma was positive for XMRV RNA were examined for the presence of XMRV genes and for *RNASEL* mutations in genomic DNA [1,18]. Plasma samples of CFS patients were simultaneously screened for XMRV Abs and genomic DNA according to published methods [1,2,6]. We did not examine XMRV DNA or RNA in the donor blood samples because, at present, the Japanese Red Cross Society does not have consensus for the genetic analysis of donor blood samples for research purposes, except for the analysis of blood types.

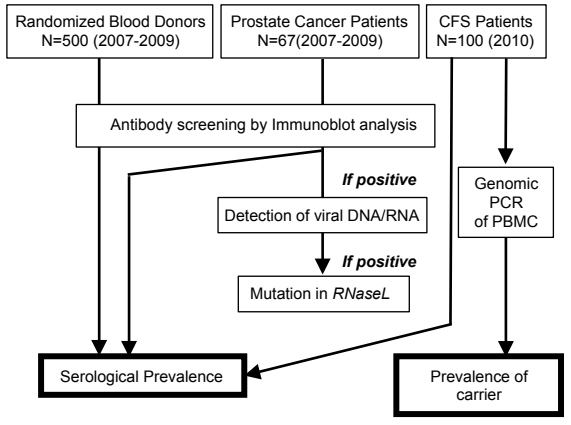

Figure 1 Study flowchart. Plasma samples randomly collected from 500 healthy donors, 67 PC patients and 100 CFS patients were screened for XMRV Abs in an immunoblot assay to estimate the serological prevalence of the virus. Viral Ab-positive PC patients were further tested for the presence of viral RNA in their plasma; genomic DNA from PBMCs of XMRV RNA-positive patients was also tested for viral DNA and *RNaseL* mutations. CSF patients were screened by genomic PCRs at three independent laboratories.

Screening for XMRV Abs

To examine Abs against XMRV by immunoblotting, concentrated viral particles were used as antigens. When the same volume of XMRV and human immunodeficiency virus (HIV)-1 lysate as a negative control was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and gel staining, we observed a comparable amount of Gag CA proteins in each preparation (Figure 2A, asterisks). The minimum amount of each virus lysate in which CA protein was detectable by gel staining with SYPRO ruby (3 μl) was used to assess sensitivity of the immunoblot assay by end point dilutions of an anti-Gag monoclonal antibody (mAb) (clone R187; Figure 2B, left) or an anti-Env rabbit polyclonal antibody (pAb) (Figure 2B, right). The detection limit of the screening assay was estimated as 6.3 ng/ml (1:640,000) for R187 mAb and 1.1 μg/ml (1:8,000) for anti-Env pAb.

In the Ab screening, we observed many nonspecific signals. Most of these reacted with both strips at the same mobility, and some weak bands were occasionally detected on either XMRV or HIV-1, or both strips at the position of the CA proteins, probably because of a large amount of CA protein on the strips. Therefore, we regarded such nonspecific signals as false positives, and considered that a band observed on the XMRV strip, but not on the HIV-1 strip, showing signal intensity comparable with that detected using the control anti-Gag mAb was positive for XMRV when the strips were blotted with 100 times-diluted plasma samples (red squares in Figure 2C-E). We identified 12 positive plasma samples: eight from the donors, two from PC patients and two from CFS patients. The prevalence of XMRV calculated from the immunoblot assay was 1.6%

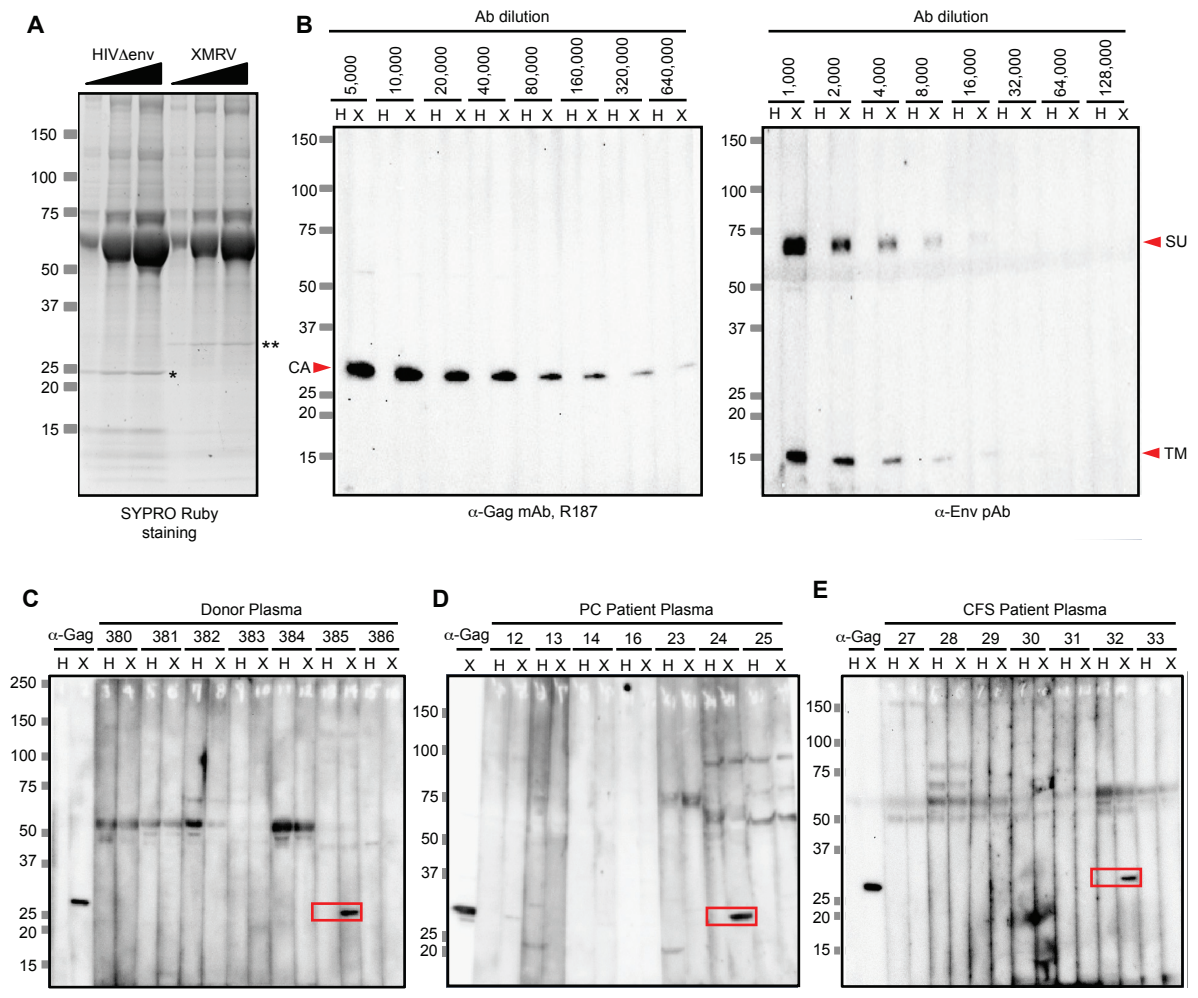

Figure 2 XMRV Ab screening. Immunoblot assay of proteins of HIV-1 Env-defective mutant (HIV Δenv) and XMRV clone VP62 for screening anti-XMRV antibodies in plasma. (A) Three different amounts of viral preparations (3, 6, or 9 μl/lane indicated by black triangles) were separated by 5-20% SDS-PAGE and stained with SYPRO Ruby. Asterisks represent Gag capsid (CA) proteins: *p24 in HIV and **p30 in XMRV. (B) Sensitivity of immunoblot assay used for screening. Viral lysates (3 μl) were detected with serially diluted control antibodies. An anti-spleen focus-forming virus (SFFV) Gag mAb (clone R187, left) and anti-XMRV Env pAb (right) was used for detection of Gag or Env proteins. Concentrations of detecting limit of each antibody were 6.3 ng/ml (1:640,000) in R187 mAb and 1.1 μg/ml (1:8,000) in anti-Env pAb. H, HIVΔenv; X, XMRV; CA, Gag capsid; SU, Env surface subunit; TM, Env transmembrane subunit. (C-E) Ab screening by immunoblot assay of blood donor samples (C), PC patients (D), and CFS patients (E) using 3 μl of each viral lysate. Pairs of strips were incubated with 1:100 diluted plasma from individuals. XMRV-specific reactivity of substantial intensity was defined as a positive reaction (red squares).

in blood donors, 3.0% in PC patients, and 2.0% in CFS patients ($p > 0.05$). Because XMRV was originally identified in PC samples [1], we analyzed whether there was a gender difference in the prevalence of XMRV; however, no significant difference between male and female subjects was noted (Table 1).

Characterization of screening-positive Abs

Because we observed Abs against only the Gag CA protein in the Ab-screening assay, we examined test plasma for reactivity against recombinant Gag and Env proteins (Figure 3A-3C). For recombinant Gag protein, we expressed glutathione S transferase (GST)-fused Gag CA protein of XMRV derived from 22Rv1 cells. The sensitivity of the immunoblot assay using the GST-CA protein was about eight times higher than that used in the screening assay (Figure 3A, 1:5,120,000 dilution corresponding to 0.78 ng/ml R187 mAb). All screening-positive plasma, but not screening-negative plasma, tested positive for GST-CA proteins (Figure 3B), suggesting that the screening-positive plasma specifically recognized XMRV CA. In the upper panel of Figure 3B, D51, P24 and C32, plasma shows some signals migrating close to that of the Env surface subunit (SU). However, these were likely to be nonspecific as we observed similar signals on the paired HIV strip at the same position

Table 1 Summary of anti-Gag Ab reactivities in study population

Population	Gender	Ab negative	Ab positive	Total	Prevalence (%)
Healthy donors	M	336	5	341	1.5
	F	156	3	159	1.9
	Total	492	8	500	1.6
Patients with PC	M	65	2	67	3.0
Patients with CFS	M	31	0	31	0
	F	67	2	69	2.9
	Total	98	2	100	2.0

No significant differences in prevalence were observed between the donors and the patients with PC and between the donors and the patients with CFS. Further, there were no significant differences in prevalence between the male and the female donors.

in the screening immunoblot assay (data not shown for D51, and Figure 2D and 2E for P24 and C32, respectively). We examined the reactivity of the test plasma against a recombinant histidine-tagged Env surface subunit protein (rSU) of a xenotropic MLV [19], in which the detection limit determined by endpoint dilutions was 1.1 μg/ml (1:8,000 dilution in Figure 3C, left), but detected no Abs against the Env SU protein in plasma samples (Figure 3C, right). An immunoblot assay after native-PAGE was also negative for Abs against Env proteins (Figure 3D). Detection limits in the native-PAGE were 6.3 ng/ml for anti-Gag mAb (R187) and 8.5 μg/ml for anti-Env pAb (data not shown).

To examine the specificity of the screening-positive plasma samples, we performed an additional immunoblot assay against proteins from Moloney murine leukemia virus (MoMLV), which has approximately 83% amino acid homology in the Gag region with XMRV. We observed multiple patterns of cross-reactivity (Figure 3E). Most screening-positive plasma samples were recognized exclusively with XMRV Gag CA (e.g., patient 24 in Figure 3E), but some showed weak cross-reactivity with Gag CA of MoMLV (donor 359 in Figure 3E). In another case, almost the same level of signal was detected against Gag CA of XMRV and MoMLV (donor 385 in Figure 3E). Plasma that predominantly reacted with MoMLV Gag was not observed. The Ab specificities are summarized in Table 2.

The serological prevalence of XMRV calculated using only the highly specific Ab was 1.0% in the donors, 1.5% in PC patients, and 1.0% in CFS patients. Again, there were no statistically significant differences in prevalence between blood donors and patients with either PC or CFS. We are unable to determine whether the anti-Gag CA Abs we identified would indicate XMRV infection or not, until panel plasma or serum samples collected from human subjects definitely infected with XMRV become available. Therefore, we tentatively regard those individuals who retain these Abs as suspicious cases.

Detection of XMRV RNA in the plasma of PC patients

In April 2008, we examined XMRV RNA from the plasma of two screening-positive PC patients (P24 and P28) by nested RT-PCR: only one patient (P24) had positive results for XMRV RNA with Gag-specific primers (Figure 4A). The sequence of the amplified PCR product was 99.8% (412/413), identical to that of XMRV VP62 (data not shown). However, we could not conclude that the PCR product was derived from XMRV infection because this fragment did not contain an XMRV-specific 24 nucleotide deletion in the *gag* region [1]. The patient's malignant prostate tissue was not available because it had already been removed and was not deposited in the hospital.

In August 2008, we collected whole blood from this patient to examine *RNASEL* mutations at amino acid positions 462 [1,18] and 541 [20], and found a WT residue at 462 and a low-risk amino acid residue (Glu) at 541 (data not shown). We tried to isolate infectious or full-length XMRV from PBMCs of this patient, but were unsuccessful. We also found that the test results of the nested PCR assay, in which detection limit was approximately 1.5 cell equivalents of genomic DNA from 293T cells infected with 22Rv1 cell-derived XMRV (Figure 4B), using PBMC-extracted genomic DNA were not reproducible (Figure 4C). In November 2009, the whole blood of P24 became available again and was tested for XMRV DNA and RNA. Although the plasma still tested positive for Abs against XMRV Gag CA, neither XMRV RNA nor DNA was detected with the same method used in April 2008 (data not shown). We further examined XMRV RNA from plasma and supernatants of co-cultured P24 PBMCs with LNCap-FGC cells using one-step RT-PCR, but both tested negative for the XMRV Gag gene (Figure 5A). We performed real time PCR on genomic DNA extracted from PBMCs, which is capable of amplifying a fragment of the Env gene with a detection limit of four copies/reaction, but the additional PCR tests of P24 were negative for the XMRV gene (Figure 5B and 5C). These data suggested that the amount of XMRV in the blood of the Ab-positive PC patient was limited, if the virus still existed. Alternatively, it remains possible that the results of the original P24 PCR tests were false positive.

Detection of XMRV DNA in PBMCs of CFS patients

To examine the prevalence of XMRV in CFS cases, we screened CFS patients for XMRV DNA in PBMCs at three independent laboratories. Figure 6 shows the representative results with two primer sets. The

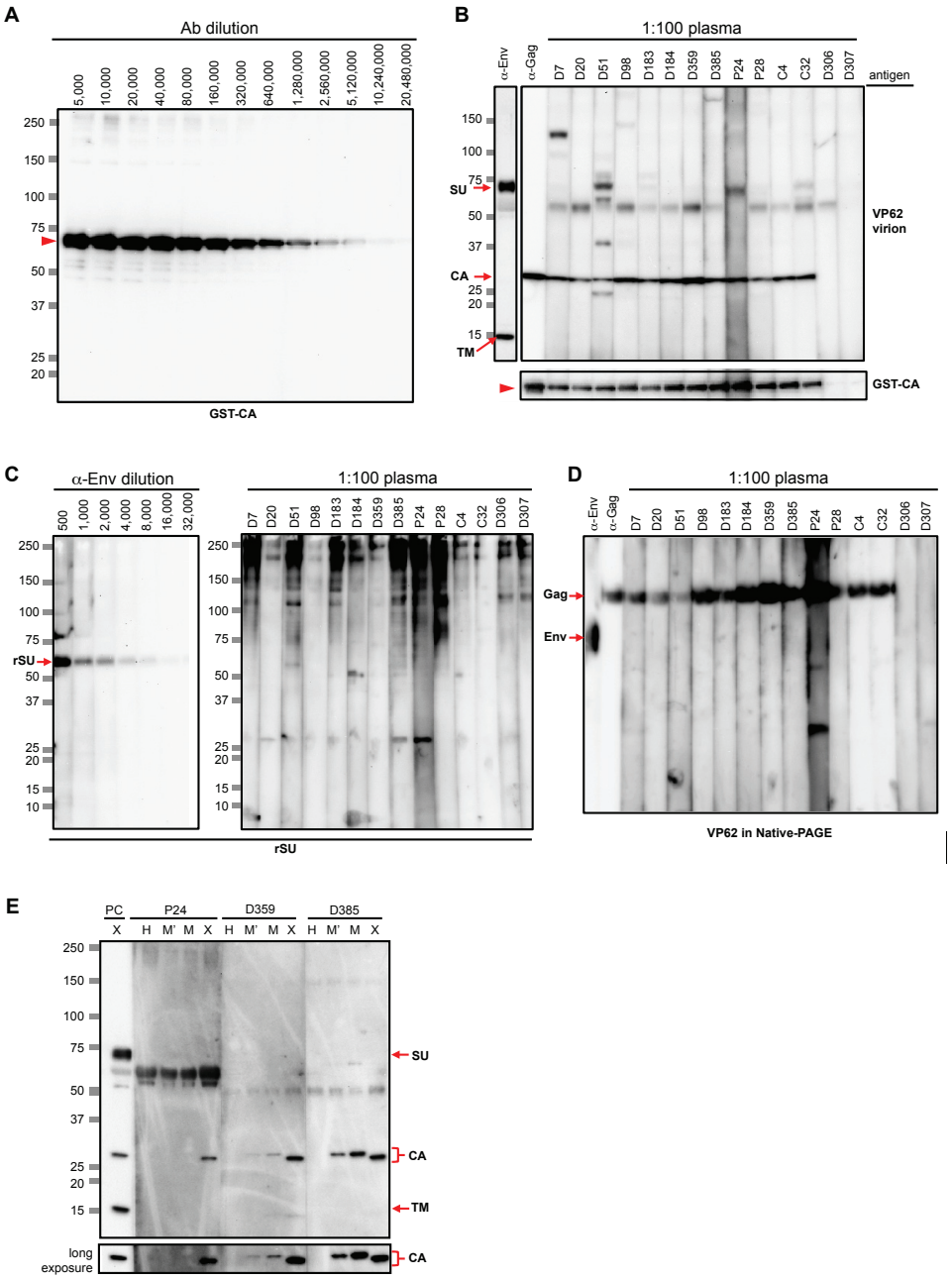

Figure 3 Characterization of Gag CA-positive plasma samples. (A) Sensitivity of immunoblot assay with GST-fused recombinant Gag CA (GST-CA) protein. GST-CA protein (300 ng per lane) was analyzed by 5-20% SDS-PAGE and detected with serially diluted R187 anti-Gag mAb. The concentration of the detection limit was 0.78 ng/ml (1:5,120,000). (B) Immunoblot assay of plasma samples that tested positive (D7, D20, D51, D98, D183, D184, D359, D385 in blood donors; P24 and P28 in PC patients; C4 and C32 in CFS patients) or negative (D306 and D307 in blood donors) for the screening immunoblot assay with 3 μl of VP62 virus lysate (upper panel) or 300 ng of the GST-CA recombinant protein (lower panel). For positive control, 8.5 μg/mL (1:1,000) of anti-Env pAb and 0.8 μg/ml (1:5,000) of anti-Gag mAb, R187, were used. (C) Immunoblot assay using recombinant Env SU (rSU) protein of xenotropic MLV. The detection limit of 300 ng of rSU protein was 1.1 μg/ml (1:8,000) by anti-Env pAb (left). One hundred diluted plasma samples tested positive for the screening assay were negative for rSU protein (right). (D) Immunoblot assay in a native-PAGE using 5 μl of the concentrated VP62 lysate in native sample buffer. Plasma samples testing positive (D7 to C32) and negative (D306 and 307) for the screening assay were examined. α-Env, anti-Env pAb (1:200, 42.5 μg/ml); α-Gag, R187 mAb (1:80,000, 50 ng/ml). (E) MoMLV particles with (M) or without (M') amphotropic Env were produced and subjected to an immunoblot assay to examine their cross-reactivity with XMRV-positive plasma. PC, a mixture of anti-Gag mAb (R187, 0.4 μg/ml) and anti-Env pAb (8.5 μg/ml) as the positive control. Arrow head, GST-fused Gag Capsid protein; SU, Env surface subunit; rSU, recombinant Env surface subunit of xenotropic MLV; TM, Env TM subunit; CA, Gag capsid protein.

Table 2 Cross-reactivities with MoMLV proteins

Population	(-)	(+)
Healthy donors	5	3
Patients with PC*	1	
Patients with CFS	1	1
Total	7	4

The XMRV Ab-positive cases were categorized as having (+) or not having (-) cross-reactivities with Gag proteins of MoMLV.

*Cross-reactivity was not examined in one Ab-positive patient with PC (P28) because additional plasma from this patient was not available.

sensitivities of our PCR tests with primer sets indicated in Figure 6A were determined using genomic DNA extracted from 293T cells infected with 22Rv1 cell-derived XMRV (Figure 6B and 6C). The detection limit of both PCR tests was calculated as approximately 1.5 cell equivalents of genomic DNA from 293T cells infected with 22Rv1 cell-derived XMRV. In screening PCR tests, we observed several nonspecific bands but the XMRV gene was not amplified as shown in Figure 6D. Although bands of a similar size to that expected were occasionally observed, sequencing analysis indicated that they contained human genomic DNA rather than XMRV genes (data not shown).

In the Japanese Red Cross Osaka Blood Center, we performed nested RT-PCR analysis of the *gag* region by using plasma RNA (Figure 5A), and a real-time TaqMan PCR assay of genomic DNA to amplify the *env* region (data not shown) if the patients tested positive for Abs. We observed no positive results from the PCR assays performed at the three independent laboratories or this additional PCR test, indicating that there were no detectable amounts of XMRV DNA in the blood of CFS patients, although two of 100 patients tested positive for the XMRV Gag Ab (Figure 2E, 3B, and 3D, and Table 1).

Discussion

In this study, we identified a small number of people who possessed Abs against XMRV Gag CA, regardless of gender or disease condition (PC and CFS), but none of the individuals in the three tested populations retained strong Ab responses to multiple XMRV proteins. We were unable to isolate XMRV from the blood of PC patients and detected no XMRV genes in the blood of any CFS patients.

We screened blood donors and patients with PC and CFS for XMRV Abs using a similar method to that developed as our in-house confirmatory test for human T-lymphotropic virus (HTLV)-1 infection in Japanese blood donors in the late 1980s, as no XMRV-positive human plasma was available to validate XMRV Ab tests.

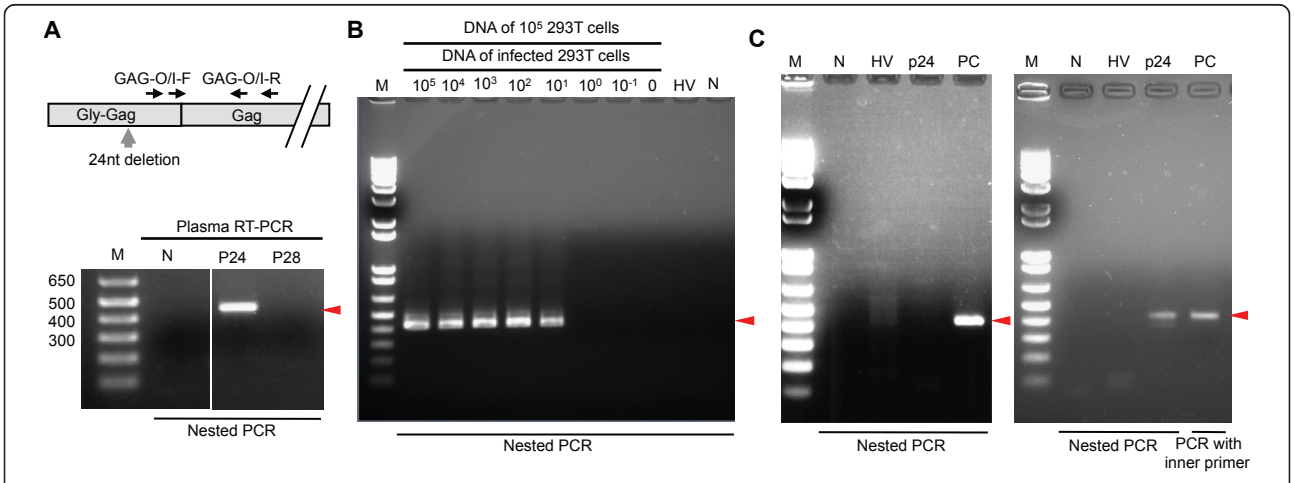

Figure 4 Detection of XMRV genes from viral Ab-positive PC patients. (A) Primer positions used in the PCR assay (upper panel). Gly-Gag; homologous region to glycosylated Gag of MLVs at the NH_2 terminus of Gag. RNAs purified from the plasma of viral Ab-positive PC patients (P24 and P28) were used in a nested RT-PCR with primers GAG-O-F/R and GAG-I-F/R. Unnecessary lanes between the negative control without template RNA (N) and P24 have been removed from the original image (lower panel). (B) The detection limit of nested genomic PCR. Genomic DNA extracted from serially diluted 293T cells infected with 22Rv1 cell-derived XMRV (indicated as 10^5~0) was mixed with genomic DNA extracted from 10^5 293T cells. For one reaction of PCR with a volume of 20 μl, 100 ng of each DNA mixture was used. The final concentration of viral genome contained in a PCR reaction was calculated as 7610.5-0.152 cell equivalents of genomic DNA from 293T cells infected with 22Rv1 cell-derived XMRV (corresponding to the lanes indicated as 10^5-10^{-1} of infected 293T cells). The detection limit of the nested PCR was calculated as approximately 1.5 cell equivalents (indicated as "10^1"). (C) Inconsistent results of nested genomic PCR tests for XMRV using genomic DNA extracted from PBMCs. In a 20 μL volume, 100 ng genomic DNA were used for amplification. Nested genomic PCRs were performed on September 17 (left) and September 18 (right), 2008. M, molecular size marker; N, negative control without nucleic acids; P24 and P28, nucleic acids purified from PBMCs of P24 or P28; HV, genomic DNA of healthy volunteer; PC, diluted XMRV VP62 plasmid; arrow head, amplified band using inner primer pair.

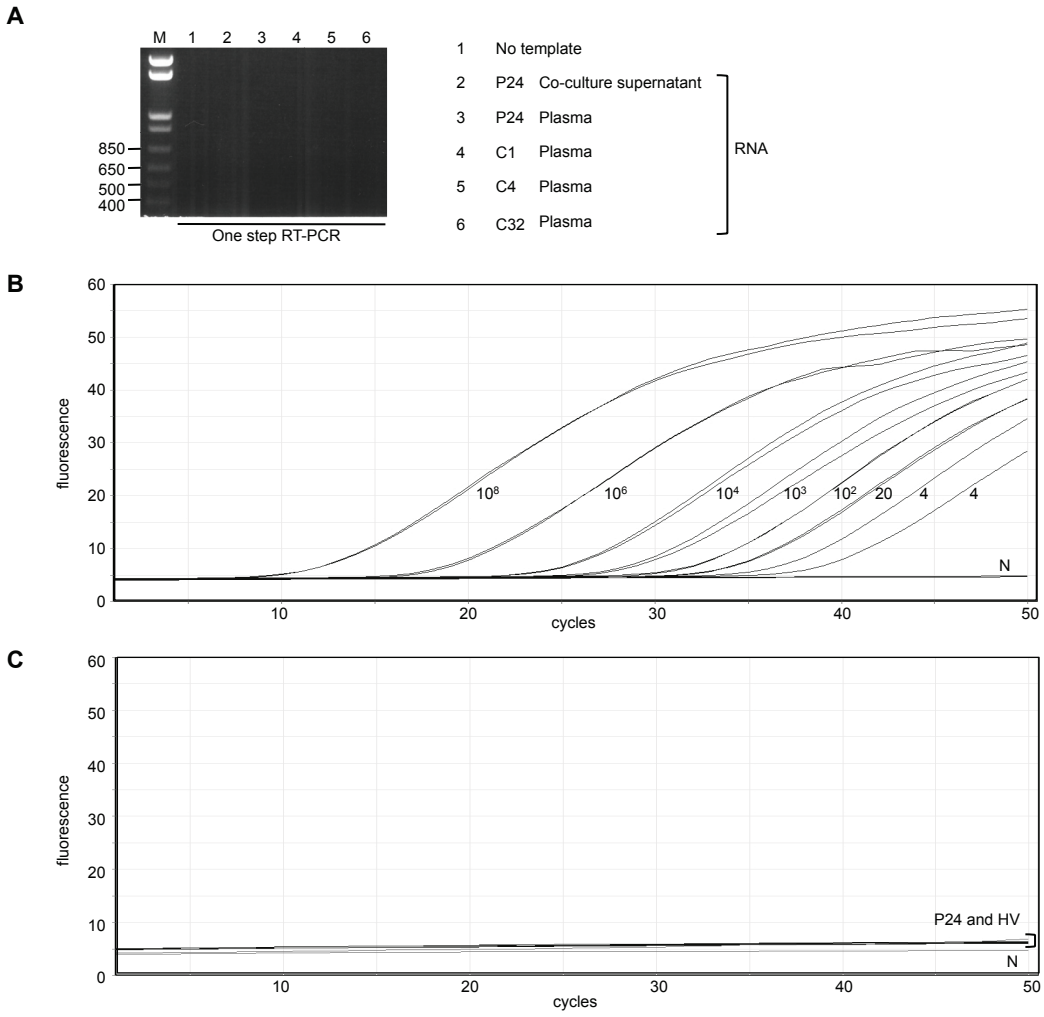

Figure 5 Detection of XMRV RNA and DNA in viral Ab-positive samples. (A) RNA was purified from 1 mL of coculture supernatant of activated PBMCs and LNCap-FGC cells (lane 2) or 1 ml plasma (lanes 3-6). For one-step RT-PCR, 15 μl of 60 μl eluted RNA was amplified in a 25 μl volume. CFS patients C4 and C32 tested positive for XMRV Abs but C1 was negative. (B) Detection of XMRV *env* by TaqMan real-time PCR assay. Duplicated test samples of diluted XMRV plasmid (VP62) were amplified. The detection limit of the TaqMan real-time PCR was 4 copies/reaction determined by VP62 plasmid. (C) Duplicated test samples without template DNA in negative control (N) or with genomic DNA extracted from PBMCs of a viral Ab-positive PC patient (P24) and healthy volunteers (HV) were amplified as for (B).

Unlike HTLV and HIV infection, XMRV-positive plasma bound only to Gag CA proteins in our study. However, in feline gammaretrovirus infections, immune responses are not always strong enough to induce a detectable amount of Abs [21]. In an animal study of XMRV infection, Qiu and colleagues [22] found that rhesus macaques intravenously inoculated with 3.6 $\times 10^6$ 50% tissue culture infective dose of XMRV showed good Ab responses against Env SU, Env transmembrane subunit (TM), and Gag proteins. In this animal model, transient viremia was observed for less than 2 weeks, but the Ab responses prolonged over 100 days post-inoculation and declined thereafter without boosting, despite high-dose viral inoculation [22]. These data suggest that XMRV replication is relatively limited *in vivo* to induce lasting immune responses compared with HIV and HTLV infection. Alternatively, the anti-Gag CA Abs we observed could account for cross-reactivity with other immunogens, although seven of 11 Ab-positive plasma samples showed high specificity to XMRV Gag (Figure 3E and Table 2). In addition, Western blotting of 2262 blood donors by Qiu and colleagues identified two blood donors positive for anti-p30 (CA) Ab and one positive for anti-gp70 (Env SU) [22]. These Ab-positive blood donors showed no multiple reactivities to viral antigens, as observed in the present study, but the prevalence of the single antigen-reactive donor was much lower than that in our current result (0.13% vs. 1.6%,

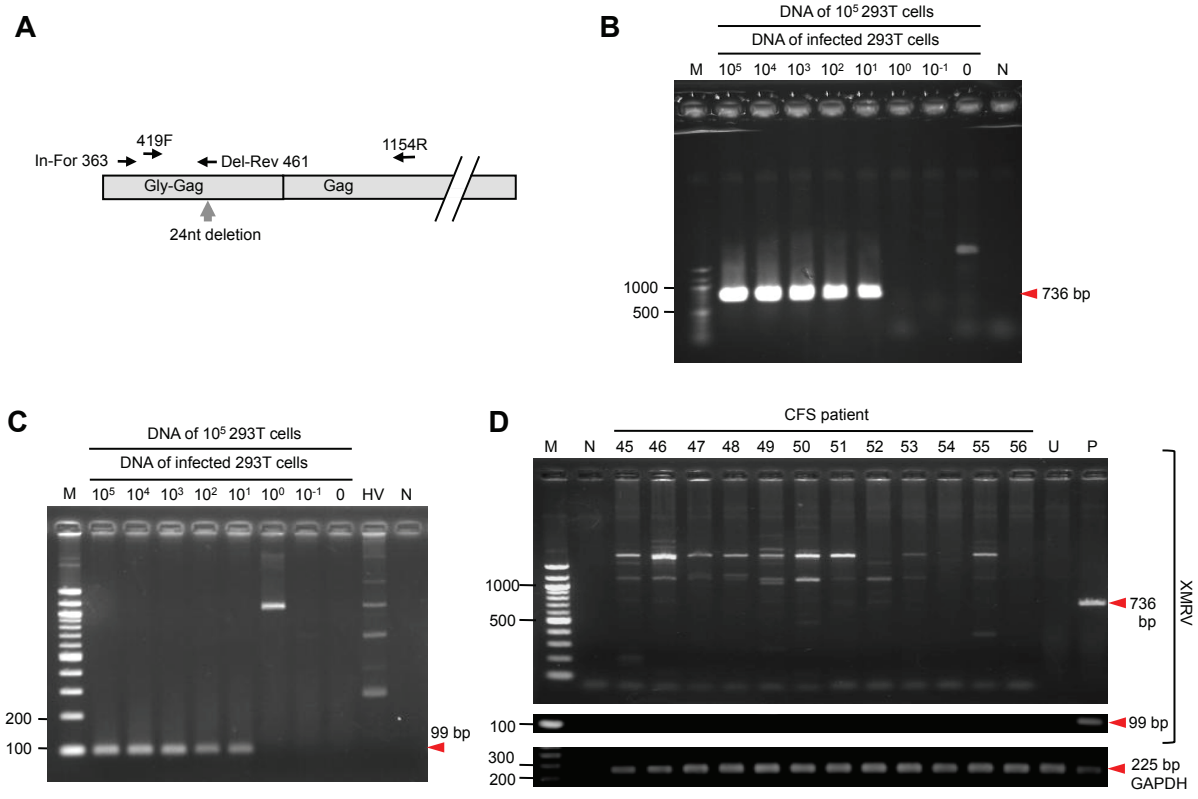

Figure 6 Screening of CFS patients using genomic PCR. (A) Primer positions used in the PCR assay. Gly-Gag; homologous region to glycosylated Gag of MLVs at the NH_2 terminus of Gag. The detection limits of the genomic PCR assays with primers 419F and 1154R, and In-For 363 and Del-Rev 461 are shown in (B) and (C), respectively. Genomic DNA extracted from serially diluted 293T cells infected with 22Rv1 cell-derived XMRV was mixed with genomic DNA extracted from 1.0×10^5 293T cells. For one reaction with a volume of 20 μl, 100 ng of each DNA mixture was used. The final concentration of the detected viral genome was calculated as 7610.5-0.152 copies (corresponding to the lanes of 10^5-10^{-1} infected 293T cells, respectively) in a reaction. The detection limit of both PCR tests is approximately 1.5 cell equivalents of genomic DNA from 293T cells infected with 22Rv1 cell-derived XMRV indicated as "10^1"of infected 293T cells. (D) Representative results of PCR assay with primers indicated in (B) (upper) and (C) (middle). The human GAPDH gene was examined as an internal control (bottom). M, molecular size marker; HV, genomic DNA of healthy volunteers; N, no template; U, genomic DNA of uninfected 293T cells; P, genomic DNA of infected 293T cells.

respectively). It is possible that the positive reaction to CA protein might include more cross-reactivity in our study. Further investigation of human plasma collected from individuals clearly infected with XMRV is required to verify our Ab screening results.

At the beginning of our study, the presence of XMRV in the blood of PC patients had not been reported; however, we speculated that XMRV might infect blood cells similar to the infection of PBMCs by other gammaretroviruses [23]. We obtained positive nested RT-PCR results on plasma collected from the Ab-positive PC patient only with extensive PCR conditions of 50 cycles using outer and inner primer pairs (Figure 4A, P24). We were, however, unable to consistently detect the XMRV gene in the same patient 4 and 15 months later using freshly collected blood samples. Co-cultivation of activated PBMCs by Concanavalin A and IL-2 with the LNCap-FGC cell line, which is highly susceptible to XMRV [17], gave rise to devastating LNCap-FGC cell death (data not shown), and we were unable to detect XMRV genes in the cell culture (Figure 5A). Our data suggest that P24 was perhaps infected with XMRV or some related viruses, but viral replication in the blood was somewhat limited. If this is the case, the prevalence of XMRV in PC patients (one of 67 patients) would be relatively close to that previously reported [5]. We cannot, however, exclude the possibility that the positive P24 signal in the PCR assays was caused by contamination, as discussed recently [24-26]. We did not PCR-amplify mouse-derived genetic materials [24,25] because of the lack of remaining P24 test sample that tested positive for XMRV PCR, although we did use a hot start Taq polymerase that is inactivated not by anti-Taq mouse mAbs but by chemical modification in our RT-PCR test [26].

We were unable to detect XMRV DNA or RNA in CFS patients, in accordance with the results of some previous studies [8-12]. It is unlikely that our detection

procedures caused such a big difference from those studies that reported a prevalence of 67% or 86.5% [2,7], because all studies employed highly sensitive PCR methods. The difference may instead be explained by the characteristics of patient populations. All CFS patients in our study met the Centers of Disease Control and Prevention (CDC) diagnostic criteria [27]; however, the currently employed diagnosis of CFS is not based on objective and quantitative measures but on the claims of patients and some authorized criteria.

Although our results of Ab screening are ambiguous, we conclude that XMRV infection is not involved in the onset and/or progression of PC and CFS in the population we screened. Even if the Abs we detected, or at least the XMRV-specific ones, were caused by XMRV infection, there was no statistically significant difference in the serological prevalence of XMRV among the three populations of the study. Moreover, the negative or inconsistent PCR results in the Ab-positive patients can be explained by the limited replication of XMRV *in vivo*. Alternatively, by assuming that the Ab reaction is attributable to cross-reactivity, the negative PCR results likely indicate the absence of XMRV infection in patients. In either case, our results do not support an association between XMRV and CFS, in line with previous findings [8-12].

Retroviral integration is theoretically harmful to the host cell because it disrupts the host genome. To reduce the risk of XMRV infection during blood transfusion, a reliable screening strategy should be established. The implementation of such a screening or inactivation protocol for blood products, however, will be influenced by the evaluation of the prevalence of XMRV by a universal test with high sensitivity and specificity, which must be urgently developed.

Conclusions

Our data for Japanese blood donors, PC patients and CFS patients imply that there is no association between the onset of PC or CFS and XMRV infection, although the lack of adequate human specimens as a positive control and the limited sample size do not allow us to draw an ultimate conclusion.

Methods

Sample collection

Plasma samples randomly collected from healthy donors (n = 500) at the Japanese Red Cross Osaka Blood Center between December 2006 and May 2009 were subjected to XMRV Ab screening. All donors had negative results in the routine tests at the Center: antigen testing of hepatitis B virus (HBV) and human parvovirus B19; Ab testing against HBV, hepatitis C virus (HCV), HIV-1, HIV-2, HTLV-1, and syphilis; nucleic acids of HIV-1, HIV-2, HBV, and HCV. All procedures in the donor screening study were performed according to the guidelines of the Japanese Red Cross Society, which do not permit the detection of nucleic acids from unapproved viruses.

All patients with PC enrolled in this study (n = 67) received medical treatment at Nishiwaki City Hospital (Hyogo Prefecture, Japan) between December 2007 and December 2009, when plasma samples were collected, and provided written informed consent. Whole blood samples in ethylenediaminetetraacetic acid (EDTA) were separated by centrifugation, and the plasma was stored at -80°C until use. PBMCs of the patients who tested positive for XMRV Abs and RNA were used for *RNA-SEL* sequencing and viral isolation. This study was approved by the ethical committee of Nishiwaki City Hospital.

CFS patients in this study fulfilled the 1994 CDC Fukuda criteria [27] and received medical treatment at the Fatigue Clinic Center, Osaka City University Graduate School of Medicine, Osaka, Japan between April and August 2010. Most of the patients were female (69%) with an age distribution of 17-62 years (mean, 38 years). The mean interval from disease onset to blood collection was 126.5 months (11-337 months). Duplicated tubes of 4 ml of whole blood in EDTA were used for Ab screening and genomic PCR assay. Whole blood samples were also collected into sodium heparin tubes (Becton Dickinson, Franklin Lakes, NJ) for cell culture. All blood samples were conveyed to the Japanese Red Cross Osaka Blood Center and genomic DNA was purified from them on the same day. Three aliquots of genomic DNA purified from one patient were independently analyzed at three laboratories. This study was approved by the Ethics Committee of Osaka City University Graduate School of Medicine and all blood samples were collected with written informed consent.

Cell lines and culture

Human 293T and 22Rv1 cells were obtained from the American Type Culture Collection (CRL-1537 and CRL-2525, respectively; ATCC, Manassas, VA). Human prostate cancer cell line LNCap-FGC was obtained from the RIKEN Cell Bank (Tukuba, Japan), and the GP293 packaging cell line was purchased from Clontech Laboratories (Mountain View, CA). These cells were grown in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. Rat hybridoma cell line R187 was obtained from ATCC (CRL-1912) and maintained in RPMI-1640 medium supplemented with 50 nM 2-mercaptoethanol, 10% FBS, and antibiotics. Before collecting the culture supernatant, the growth medium was replaced with CD Hybridoma medium (Invitrogen, Carlsbad, CA)

supplemented with 8 mM l-glutamine. For recombinant Env production, Sf9 and High Five cells (Invitrogen) were maintained in Sf-900 III SFM and Expressed Five medium (Invitrogen), respectively.

Control antibodies

IgG proteins in culture supernatants from R187 cells, prepared against SFFV Gag and able to react with Gag capsid proteins from a wide variety of gammaretroviruses [28], were purified using a protein G affinity column (MabTrap Kit; Amersham Biosciences, Piscataway, NJ). For anti-Env Abs, rabbits were immunized with a mixture of two peptides (PRVPIGPNPV[C] of Env SU and [C]QFEQLAAIHTDLG of Env TM; [C] indicates an additional cysteine residue for peptide purification), and their antisera were collected and purified after five immunization steps with a Protein A affinity column (GE Healthcare, Buckinghamshire, UK). Concentrations of the purified R187 mAb and anti-Env pAb were 4.0 mg/ml and 8.5 mg/ml, respectively.

Antibody screening

An infectious XMRV molecular clone, pcDNA3.1-VP62, was provided by Dr. R. H. Silverman. To produce the viral particles, 293T cells were transfected with pcDNA3.1-VP62 by a liposome method (Lipofectamine LTX; Invitrogen). Two days after transfection, the culture supernatant was collected, filtered, and concentrated 20 times by centrifugation at 20,000 × *g* for 4 h at 4°C. The concentrated virus was suspended in a Laemmli SDS sample buffer. As a negative control, we prepared an env-defective HIV-1 virus (pNLΔenv, provided by Dr. A. Adachi) by using the same method as for XMRV. A MoMLV-derived retrovirus vector was produced using the GP293 cell line, with or without transfection of an amphotropic Env expression vector (provided by Dr. D. R. Littman). Viral proteins were separated by 5-20% gradient SDS-PAGE and either stained with SYPRO Ruby (Bio-Rad, Hercules, CA) or transferred to a polyvinylidene difluoride membrane (Wako Pure Chemical Industries, Osaka, Japan) cut into strips. After blocking with 5% skimmed milk in Tris-buffered saline (TBS), the strips were incubated with 1:100 diluted donor or patient plasma samples at 4°C overnight. After washing with TBS containing 0.05% Tween-20, the strips were incubated with 1:5,000 diluted horseradish peroxidase (HRP)-conjugated anti-human IgG Ab (GE Healthcare), and detected by ECL Plus (GE Healthcare). For endpoint dilutions, a pair of strips was blotted with 0.8 μg/ml-6.25 ng/ml (1:5,000-1: 640,000) R187 mAb and detected using 1:5,000 diluted HRP-conjugated anti-rat IgG (H+L) secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) for Gag, or blotted with 8.5 μg/ml-66.4 ng/ml (1:1,000-1:128,000) anti-Env pAb and detected using 1:2,500 diluted HRP-conjugated anti rabbit IgG (GE Healthcare).

Other immunoblot assays

To produce GST-fused XMRV Gag CA protein, a 789-bp fragment of the CA gene was amplified using genomic DNA of 293T cells infected with XMRV derived from 22Rv1 cells, and cloned into the pET-42b(+) vector (Merck KGaA, Darmstadt, Germany). The GST-CA protein was purified by a Glutathione-Sepharose 4B column (GE Healthcare) from bacterial lysate of BL21 Star (DE3) (Invitrogen) transformed by the GST-fused CA expression plasmid. To produce His-tagged recombinant Env SU of xenotropic MLV [19], a PCR-amplified *env* SU region was cloned into pcDNA3.1myc/His (Invitrogen) followed by subcloning of an env-His DNA fragment into the Bac-to-Bac Baculovirus Expression System (Invitrogen). The supernatant of Sf9 cells transfected with the bacmid was used for infection of HighFive cells. Recombinant Env proteins collected from the culture supernatant of infected cells were purified using a HisTrapHP column (GE Healthcare). In the native-PAGE, concentrated viruses were suspended with native sample buffer (Native Sample Buffer; Bio-Rad) and separated on a 5-20% gel in a Tris-glycine buffer (25 mM Tris-Cl, 192 mM glycine, pH 8.4). The subsequent procedures were for the Ab-screening immunoblot assay.

Detection of viral nucleic acids

For RT-PCR analysis of Ab-positive PC patient samples (Figure 4A), RNA was isolated from 500 μl of plasma using the PureLink Viral RNA/DNA Kit (Invitrogen), and 8 μl of the 10 μl eluted RNA was reverse-transcribed using Superscript III (Invitrogen) with random hexamer primers in a total reaction volume of 10 μl. In the nested PCR assay, 3 μl cDNA or 100 ng genomic DNA of PBMCs was amplified in a 20 μl volume with primer pairs GAG-O-F/R and GAG-I-F/R [1] and AmplyTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) for 50 cycles. The PCR cycling conditions were as follows: activation at 95°C for 5 min; then 50 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 60 s (30 s in the second-round PCR); with a final extension at 72°C for 7 min.

To extract genomic DNA from CFS patients, 4 ml of whole blood in EDTA were centrifuged at 1500 × *g* for 10 min at room temperature, and 200 μl of the buffy coat were transferred to a 2 ml tube for DNA purification using the QIAamp Blood Mini Kit (Qiagen GmbH, Hilden, Germany). We divided 180 μl of eluted DNA equally into three tubes for analysis at three independent laboratories: Department of Research, Japanese Red Cross Osaka Blood Center, and the Laboratories of Signal Transduction and Viral Pathogenesis, Institute for

Virus Research, Kyoto University, Japan. PCR of 1 μg genomic DNA in a 50 μl reaction was performed with primer pairs GAG-O-F/R and GAG-I-F/R [1] for nested genomic PCR (data not shown) or 419F and 1154R [2] and In-For363 and n-Rev536 [6] for single PCR. In the genomic PCRs, we used PrimeSTAR GXL DNA polymerase (Takara Bio, Shiga, Japan) with the following conditions: activation at 98°C for 2 min; then 45 cycles of 98°C for 10 s, 63°C for 15 s, and 68°C for 45 s; and a final step at 68°C for 2 min. For one-step RT-PCR (Figure 5A), RNAs were purified from 1 ml of 4-day culture supernatants of P24 PBMCs activated with 10 ng/ml concanavaline A (J-Oil Mills, Tokyo, Japan) and 100 U/ml IL-2 (e-Bioscience, San Diego, CA) and maintained with LNCap-FGC cells or patient plasma using a QIAamp Ultrasense Virus Kit (Qiagen). One-step RT-PCR was performed using 15 μl of 60 μl eluted RNA and a 419F and 1154R primer pair [2] and the following conditions: reverse transcription at 50°C for 30 min; activation at 95°C for 15 min; then 45 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min.

TaqMan real-time PCR tests were performed with 200 ng of genomic DNA, Universal ProbeLibrary, and FastStart TaqMan Probe Master (Roche, Basel, Switzerland) in a total reaction volume of 20 μl with a Rotor-Gene Q thermal cycler (Qiagen). Primer and probe sequences are as follows: 5'-cctagtggccaccaaacaat-3' (Env forward), 5'-ggccccaaggtctgtatgta-3' (Env reverse), and 5'-FAM-gctccagg-3' (Env probe, #1 of Universal ProbeLibrary). The following condition was used: 1 cycle of 95°C for 10 min, and 50 cycles of 95°C for 15 s and 60°C for 45 s.

RNASEL mutation

In patients whose serum tested positive for XMRV RNA, mutations of *RNASEL* at amino acid positions 462 [18] and 541 [20] were examined as previously described [1,20]. PCR-amplified genomic DNA fragments were sequenced using an ABI PRISM 3100 genetic analyzer (Applied Biosystems).

Statistics

Non-parametric analysis was performed with the Mann-Whitney *U*-test to determine any statistical significance in the data. A p value of less than 0.05 was considered to be significant.

Abbreviations

Ab: antibody; ATCC: American Type Culture Collection; CDC: Centers of Disease Control and Prevention; CFS: chronic fatigue syndrome; EDTA: ethylenediaminetetraacetic acid; FBS: fetal bovine serum; HBV: hepatitis B virus; HCV: hepatitis C virus; HIV: human immunodeficiency virus; HRP: horseradish peroxidase; HTLV: human T-lymphotropic virus; IFNγ: interferon-gamma; MLV: murine leukemia virus; PAGE: polyacrylamide gel electrophoresis; PBMC: peripheral blood mononuclear cell; PC: prostate cancer; SDS: sodium dodecyl sulfate; TBS: Tris-buffered saline; XMRV: xenotropic murine leukemia virus-related virus; WT: wild-type.

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Authors' contributions

RAF conceived and designed the study, coordinated the collaboration, carried out the Ab screening and PCR tests, and drafted the manuscript. TM designed the study, coordinated the collaboration for studies of XRMV infection in CFS patients and attempted to isolate XMRV. TS recruited PC patients and carried out immunohistochemical testing of prostate tissues (data not shown). HK helped in designing the study and recruiting CFS patients. YI developed the real-time PCR test. ES conducted the Ab screening and PCR tests of CFS patients and attempted to isolate XMRV. NM conducted the PCR tests of CFS patients. YN and KY helped in designing the study and recruiting CFS patients. RS participated in the development of the real-time PCR test. KY participated in the Ab screening. FH helped in designing the study and drafting the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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文献２

Absence of XMRV and other MLV-related viruses in patients with Chronic Fatigue Syndrome

Running Title: Absence of XMRV and other MLV-related viruses in CFS

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Abstract

Chronic fatigue syndrome (CFS) is a multi-system disorder characterized by prolonged and severe fatigue that is not relieved by rest. Attempts to treat CFS have been largely ineffective primarily because the etiology of the disorder is unknown. Recently CFS has been associated with xenotropic murine leukemia virus-related virus (XMRV) as well as other murine leukemia virus (MLV)-related viruses, though not all studies have found these associations. We collected blood samples from 100 CFS patients and 200 self-reported healthy volunteers from the same geographical area. We analyzed these in a blinded manner using molecular, serological and viral replication assays. We also analyzed samples from patients in the original study that reported XMRV in CFS. We did not find XMRV or related MLVs, either as viral sequences or infectious virus, nor did we find antibodies to these viruses in any of the patient samples, including those from the original study. We show that at least some of the discrepancy with previous studies is due to the presence of trace amounts of mouse DNA in the Taq polymerase enzymes used in these previous studies. Our findings do not support an association between CFS and MLV-related viruses including XMRV and off-label use of antiretrovirals for the treatment of CFS does not seem justified at present.

Introduction

Chronic fatigue syndrome, a disorder characterized by severe debilitating fatigue along with variable presence of post-exertion malaise, joint and muscle aches, headache, sore throat, tender lymph nodes, unrefreshing sleep and cognitive deficits, has had an uncertain etiology since its recognition. An estimated 0.4 to 4% of the US population suffers from this disease (9, 17, 18). While a series of infectious agents and environmental toxins have been proposed to be linked with CFS, none have been universally associated (2). In late 2009, XMRV, a recently discovered retrovirus was detected in the blood of 68% of patients with CFS (12). More recently, another study detected sequences related to XMRV, viz. those belonging to a polytropic murine leukemia virus (PMV) in 86.5% of CFS patients and in only 6.8% of healthy controls (11). There have also been studies that failed to detect XMRV in CFS patients in the US (6, 21, 24), in Europe (3, 5, 25) and in China (7). However, there were several confounding factors with many of these studies including differences in patient characterization, differences in geographical locations of patients vs. controls, differences in samples (whole blood vs. leukocytes vs. plasma), and many differences in methods used to detect virus. For example, both studies that found a retroviral association in CFS selected their patients and controls from completely different geographical regions (11, 12). This approach could result in a spurious association if regional differences among prevailing viruses result in detection of virus from one region but not from another. Control populations were often small, as few as 43 in one study (25), and patient and control samples were often collected at different times, sometimes several years apart (11), leaving open the possibility that patient samples might have been handled more – and thus possibly contaminated more easily than controls. Furthermore, in all except a subset of samples from one study (12), investigators were not blinded to the identity of samples. In all but two studies that

failed to detect virus in association with CFS (5, 24), only PCR-based assays were used, thus relying heavily on conservation of retroviral sequences. The limits of detection, reproducibility and precision of the assays used in different studies were not known, making it difficult to distinguish the lack of ability to detect XMRV from a genuine absence of XMRV from samples. Furthermore, tests that had resulted in more frequent detection of XMRV, such as growth of virus in cultured cells (14), were not used in subsequent studies. Adequate controls for each step of the analysis, such as controls that would flag contamination occurring during the nucleic acid extraction process, were mostly lacking. Furthermore, the number of negative controls should equal or exceed the expected prevalence of the virus in the control population. It is not clear if any of the studies employed more than one negative control per experiment, which would be important for the detection of a low incidence of sample contamination. Finally, none of the studies tested samples from the same patients that were found to be positive in the original study by Lombardi *et al* (12). In line with our own recommendations for an accurate study (23) we incorporated all of these factors in the design of the investigation reported here, and have performed what we believe is the most comprehensive study to date on the proposed association of XMRV and other related viruses with CFS.

We enrolled 105 CFS patients, including 100 who fulfilled both the 1994 case definition of the CDC (4) as well as the criteria defined by the Canadian consensus document on myalgic encephalomyelitis ME/CFS (1). Patients and 200 healthy volunteers were all from the greater Salt Lake City area. Blood samples from both patients and healthy volunteers were prospectively collected and processed in parallel. In conjunction with a third-party phlebotomy service, we also collected, in a blinded manner, samples from 14 patients in the cohort used in the original CFS-

XMRV study performed at the Whittemore Peterson Institute (WPI) (12). For virus detection, we utilized four different TaqMan qPCR assays, PCR assays that had resulted in detection of XMRV or MLV-like sequences in previous studies (11, 12) and an ELISA, to look for XMRV sequences and antibodies in all of our samples. A subset of samples was analyzed by Western blots. From some samples we also attempted to grow virus in cell culture, a technique outlined in the original study (12) and though labor-intensive, has been proposed to be the most sensitive method for viral detection (14). All samples were processed and tested in a blinded manner.

Materials and Methods

Patient and participant selection

We initially identified 150 patients from a clinic that specializes in the diagnosis and management of CFS and fibromyalgia. All patients had been diagnosed with CFS using the CDC-Fukuda criteria (4) in a clinical setting by a board certified clinician (LB). The vast majority of these patients had been serially assessed and managed by LB for many years to verify the CFS diagnosis as primary and to treat symptoms and any comorbid conditions. Each patient was subjectively assigned a severity score by the clinician (1 = severely ill, dependent on help; 2 = moderate to severely ill, not able to sustain a regular schedule of part-time work or school; or 3 = moderately ill, able to sustain at least part-time work or school). All subjects were recruited for the study by telephone contact within a one-week period, starting with the more impaired, until 105 patients had successfully enrolled. At enrollment, subjects were screened to determine if they met the Canadian consensus criteria (1), and all but 5 qualified for both case definitions. Subjects were administered the Rand SF-36 (26). The participants were 68% female which is consistent with females being afflicted with CFS in greater numbers.

Controls consisted of 100 healthy males and 100 healthy females by self report, all employed at Salt Lake City. Participants were recruited via email and enrolled after informed consent under University of Utah IRB protocol #7740.

CFS onset, duration and life impact:

CFS onset was reported to be associated with viral-like symptoms in 72% of patients. Seventeen patients had participated in the Phase III Clinical trial of Ampligen (AMP516), which required viral-like onset inclusion criteria. Age of CFS onset was under 50 years in 92% of all enrolled, with 16% under age 20, 76% age 20-50, and 8% over 50. Average duration of illness was 16 years, ranging from 2 to 60 years. The patients had been under the care of the CFS clinician for an average of 6 years, with 80% from 5-10 years, the newest under care for 2 months and the longest 16 years. Patients had been sick an average of 9 years before initial consultation at the clinic.

To assess life impact of CFS, the subjects were administered the Rand SF-36 on the day of the blood draw. Developed as a 36 item self-report instrument for the Medical Outcomes Study, the SF-36 assesses overall health status through 8 subscale domains: 1) health-related limitations in physical functioning, 2) limitations in social functioning, 3) limitations of usual life role activities due to physical factors, 4) limitations of usual life role activities due to emotional factors, 5) pain, 6) emotional well-being/psychological distress, 7) energy/fatigue, and 8) general health (26). Using the Rand scoring method, scores on these 8 subscales range from 0 for most severe symptoms to a best score of 100. In our sample of patients, 78% of participants scored

<50 on the Physical Function subscale, 88% of participants scored 0 on the Role Functioning due to Physical Factors subscale, and 92% of participants scored <25 on the Energy/Fatigue subscale. These scores indicate a high level of physical disability and limitations on ability to work, to care for home or family, or perform self-care due to physical factors in this sample.

Participant characteristics

The average age of females was 34.6 yr (median 30 yr) and average BMI (Body Mass Index) 23.7. Twenty percent had a family history of prostate cancer, 18 of which included blood-relatives (4% did not know about family history of prostate cancer). One female reported a diagnosis of fibromyalgia, and 17% reported a family history of CFS/fibromyalgia, 14 of which included blood-relatives (4% did not know about family history of CFS/Fibromyalgia). The average age of males was 34.6 yr (median 33 yr) and the average BMI was 27.6. No participant had ever been diagnosed with prostate cancer. Fourteen percent had a family history of prostate cancer, 12 of which included blood-relatives (1% did not know family history of prostate cancer). No males reported a diagnosis of fibromyalgia; however 13% reported a family history of CFS/fibromyalgia, 10 of which were blood-relatives.

Blood sampling protocol

Immediately after arrival at the clinic, subjects were given full details about the study verbally and in writing, had all questions answered, and provided informed consent in writing according to a protocol approved by the University of Utah IRB. For 15 min on average, they sat quietly and completed self-report questionnaires, then had blood drawn. The clinical research division of ARUP Laboratories, Salt Lake City, Utah, collected blood samples from all 300 individuals

158 within a period of 3 weeks. Blood was collected into 8.5ml vacutainer tubes (Becton-Dickinson):
159 2 EDTA and 1 serum separator. After allowing the blood to clot at room temperature for 30 min,
160 the serum separator tube was spun for 10 min at 3,000 rpm. Serum aliquots of 1 ml were frozen
161 in cryovials at -80°C. From the EDTA tubes, 1ml of whole blood was removed and stored at -
162 80°C in cryovials (Nunc). The remaining volume was spun for 10 min at 3,000 rpm and plasma
163 aliquots of 1ml were frozen in cryovials at -80°C. The buffy coats were removed and combined
164 into a 15ml falcon tube for each individual and 7ml of ACK lysis buffer (28) was added to the
165 tube to clear red blood cells. The tube was inverted 5 times, incubated at room temperature for
166 10 minutes, and centrifuged at 3,000 rpm for 5 min The supernatant was discarded and the pellet
167 resuspended in 10ml of wash buffer (PBS, 2mM EDTA). After spinning for 5 min at 3,000 rpm,
168 the pellet was divided into three aliquots: one in 1ml of fetal calf serum containing 10% v/v
169 DMSO, another in 1ml of RLT buffer containing guanidine isothiocyanate and 1% beta-
170 mercaptoethanol (Qiagen), and the third without any buffer. All aliquots were stored in cryovials
171 at -80°C.
172
173 **Nucleic acid extraction from buffy coat and whole blood**
174 Nucleic acid from buffy coat was extracted using the DNeasy Blood and Tissue Kit (Qiagen)
175 following manufacturer's directions. One extraction control was included for every 7 samples
176 that were extracted.
177
178 **PCR – quantitative and nested**
179 All qPCRs were done using the TaqMan probe system on a 7900HT Real Time PCR System
180 with a standard 96-well block module (Applied Biosystems). Each 20µlreaction contained 1x

TaqMan Universal PCR Master Mix, 900nM forward and reverse primers, 250mM TaqMan probe, and 400-1,000 ng of DNA or 5µlof water. Thermocycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min Four XMRV amplicons were used, 63bp in LTR, 157bp in *gag*, 65bp product in *pol*, and 86bp product in *env*. The 63bp LTR product was composed of 47F (5'- AATAAAGCCTTTTGCTGTTTGCA-3'), 109R (5'- GAGGAGACCCTCCCAAGGAA-3'), and 74MGB (5'-6FAM-AAGCGTGGCCTCGC-MGB-3'). The 157bp *gag* product was composed of 505F (5'-GAATTTTTGCTTTCGGTTTTACG-3'), 663R (5'-TCCCCAGTGCTGCAAGGT-3'), and 618MGB (5'-TET- ACAGACCGTAACTACC-MGB-3'). The 65bp *pol* product was composed of 4552F (5'- CGAGAGGCAGCCATGAAGG-3'), 4616R (5'-GCGTATACGGGGTTGAGTCC-3'), and 4572MGB (5'-6FAM-AGTTCTAGAAACCTCTACACTC-MGB-3'). The 86bp *env* product was composed of 6356F (5'- GGATGCCCCCAAAACATG-3'), 6441R (5'- GGACCTGGCGGGTCAGA'3'), and 6393MGB (5'-6FAM- TCCACTGGGGCCGAC-MGB-3'). VAMP assay to assess DNA integrity was composed of 3613F (5'- CCCCACACTTCTGGTTTTCTG-3'), 3690R (5'-CAGCATCTCTCCTACCCTTTCAC-3'), and 3645MGB (5'-TET-AGCAGGGATATCTAAGC –MGB-3'). IAP assay to look for mouse DNA contamination was composed of IAP-F (5'- GCCGCGCCCACATTC-3'), IAP-R (5'-CGCAGATTATTTGTTTACCACTTAGAA-3'), and IAP-MGB (5'-TET-CCGTTACAAGATGGTGCTGA-3'). Mlu1 assay to look for XMRV plasmid contamination was composed of Mlu1-F (5'- GGTGGCCCCCTTGCC-3'), Mlu1R (5'-AGTTAGCTTGCCTGCATCCTTT-3'), and Mlu1-MGB (5'-6FAM-CGTGGTAGCAGCCAT-MGB-3').

For the nested PCR, we made 2 modifications to the original protocol (11). We used 1.0 U of Platinum Taq instead of 0.5 U, and added dUTP to the mastermix to prevent subsequent PCR contamination with amplicons.

XMRV SU recombinant protein

Forward primer with XhoI site 5'-ATTATCCTCGAGCAACGTGACAGCCCTCAC-3' and reverse primer with HindIII site 5'-ATTATCAAGCTTCTTTTCAAACTGGCCATAAA-3' were used to PCR amplify SU from pXMRV1 (22). Forward primer with NheI site 5'-ATTATCGCTAGCTACTGAATGGCGCGTTCA-3' and reverse primer with XhoI site 5'-ATTATACTCGAGGGAGCCGGGCGAAGCAGTA-3' were used to PCR amplify the signal peptide (SP) from pNCA. PCR products were purified and digested with their respective restriction enzymes: XhoI and HindIII for SU fragment, NheI and XhoI for SP fragment. Mammalian expression vector pcDNA3.1myc-His(-)A (Invitrogen) was digested with NheI and HindIII. Digested SU fragment, SP fragment, and vector were ligated to make pXSUSP and transformed into bacteria, DNA prepared and verified by sequencing, and transfected into 293T cells with Lipofectamine 2000 (Invitrogen). Supernatant was collected after 72 hr, tested by western blot assays, and purified using His-Pur Cobalt columns (Pierce) following manufacturers' directions.

ELISA with human sera

150ng of purified XMRV SU protein diluted in PBS was absorbed onto 96-well Maxisorp Flat-Bottom Immuno plates overnight at 4°C (Nunc). Plates were washed five times with detergent-free high salt wash buffer (0.030M Potassium Phosphate, 0.080M Sodium Phosphate, 2.90M

NaCl, pH 7.2), and blocked with Seablock (Thermo) for 1 hr at RT. Human sera was then added at a 1:150 dilution in Seablock with 0.05% Tween20. Following incubation for 2 hr at RT, plates were washed five times with high salt wash buffer with 0.05% Tween20 and a 1:15,000 dilution of HRP-conjugated AffiniPure F(ab')$_2$ fragment goat anti-human IgG antibody was added (Jackson ImmunoResearch). The plates were then incubated for 1 hr at RT, and washed again five times with high salt wash buffer with 0.05% Tween20. TMB substrate was added and allowed to incubate for 30 min at RT. Development was stopped with 1N sulfuric acid and the absorbance was measured at 450nm and 650nm. Results were expressed as OD450-OD650 to correct for irregularities in the plate.

Western blot assays with human sera

5µg of purified XMRV SU protein or 5µg of uninfected 293T cell lysate was diluted in 2x sample buffer (2% SDS. 50mM Tris [pH 6.8, 10% glycerol] and heated for 5 min at 99°C. Proteins were loaded into 4-20% gradient Precise Protein Gels (Pierce) in 1x Tris/HEPES/SDS running buffer (Pierce). The gel was run at 150V for 50 min Transfer to PDVF Immobilon-FL membrane (Millipore) occurred in chilled transfer buffer (25mM Tris, 192mM Glycine, 20% methanol) at 20V for 40 min in semi-dry apparatus (Bio-Rad). Membrane was blocked in 5% milk (1xPBS, 0.01% Tween-20) for 1 hr. Membranes probed with human serum diluted 1:50 in 5% milk overnight at 4°C. Membranes were washed 4 times with PBST (1x PBS, 0.01% Tween-20) and probed with goat-anti-human-IR-700 (Rockland) at 1:10,000 in 5% milk for 2 hr at RT. Membranes were washed 4 times with PBST before imaging on Odyssey Scanner (Licor).

Viral replication assay using spin inoculation

This protocol was adapted from the one used in the original study that found XMRV in CFS (12) with extensive help from Dr. Frank Ruscetti (Leukocyte Biology Section, NCI). LNCaP cells (15,000 cells/well of 6-well tray with 300 µl RPMI) were inoculated with 100 µl of plasma from patients or controls. Trays were centrifuged at 900xg for 5 min, overlaid with 500µlof RPMI and incubated overnight at 37°C in a 5% CO2- air mix. An additional 2 ml of RPMI was added the following morning. When cells became confluent, they were trypsinized and transferred to a T-25 flask, and when that became confluent, the cells were transferred into a T-75 flask. Cells were passaged for at least 6 weeks, and at week 2, 4 and 6, cells were lysed into RIPA buffer (Tris 50mM pH 7.4, NaCl 150mM, SDS 0.1%, sodium deoxycholate 0.5%, TritonX100 1% + Roche Complete Protease Inhibitor Cocktail) for analysis by Western blots, and into guanidium isothiocyanate containing AL buffer (Qiagen) for extraction of DNA and qPCR analysis.

Western blot on spin inoculation samples

Similar to western blot protocol above except 20µg to 30µg of whole cell lysate was electrophoresed, membranes were probed with anti-XMRV rabbit anti-serum (22) at 1:10,000 and rat anti-tubulin (Millipore) at 1:10,000 overnight at 4°C, followed by goat-anti-rabbit-IR-700 (Rockland) at 1:10,000 and goat-anti-rat-IR-800 (Rockland) at 1:10,000 for 2hr at room temperature.

Results

Patient selection and study design

We enrolled 105 patients from a Salt Lake City clinic that specializes exclusively in the diagnosis and management of CFS and fibromyalgia. All patients had been diagnosed with CFS

in a clinical setting using the CDC-Fukuda criteria (4) by a board certified internal medicine specialist, a clinician with 20 years of experience with CFS (LB). At enrollment, subjects were screened to determine if they also met the Canadian consensus criteria (1), and all but 5 qualified for both case definitions. The patients were 68% female, with an average age of 48 years (range 20-70), and 90% of them lived within a 50-mile radius of Salt Lake City, Utah. For details on onset, severity, and duration of illness, *see Materials and Methods*. Controls consisted of 100 males and 100 females, all healthy by self-report, average age 35 years, and living in the greater Salt Lake City area. The participants completed a questionnaire to assess their health, height, weight, personal and family histories of prostate cancer and CFS (see *Materials and Methods*). Both the healthy volunteers and patients were primarily Caucasian, reflecting the local population. All healthy volunteers were employed full time. Among the CFS patients, a total of 43% were unable to work or attend college even part time, including 13% who were on disability.

The Clinical Research department of ARUP Laboratories, Salt Lake City, Utah, collected blood samples from all 300 individuals within a period of 3 weeks (*see Materials and Methods* and Fig. 1). Anticoagulated whole blood was separated into white blood cells (for DNA isolation and qPCR assays) and plasma (for inoculation of cultured cells to assay for viral replication). Whole blood was also allowed to clot and the serum used for ELISA and Western blots assays designed to detect anti-XMRV antibodies.

qPCR assays for XMRV are sensitive to at least five viral copies.

In order to be confident of detecting XMRV in clinical samples, we developed our PCR assays to the robust and reliable standards of clinically used assays. We developed four distinct qPCR (TaqMan) assays that target different regions of the XMRV proviral sequence. One targeting the *pol* gene region, has been used extensively by us (22) and others (15, 21, 25), and of all the published PCR-based tests for XMRV, has been shown to be the most specific (8). We improved on the sensitivity of this assay so that it could reliably detect at least 5 viral copies of XMRV DNA (see Fig. 2A). To allow for possible variations in viral sequence in our subjects, we developed three additional qPCR tests that targeted the LTR, *gag* and *env* regions of XMRV proviral DNA. We characterized each of these assays in detail to determine their limits of detection, specificity and reproducibility. Assay characteristics for the LTR qPCR are shown in Fig. 2B. We could reliably detect less than 5 copies of XMRV plasmid DNA in a background of 400 ng of human placental DNA, and the assay was linear over a large range, viz. 5000 to 5 copies of viral DNA. This sensitivity was matched by the assays targeting the *env* and *pol* regions. The *gag* assay was also able to reliably detect at least 5 viral copies with an average of 3 cycles delay in crossing the threshold (threshold cycle, C_t, see Fig. 2C). We also demonstrated that the assays had good precision and reproducibility as demonstrated by the R^2 values of C_ts being close to 1. To determine intra-run precision, 4 different amounts of XMRV plasmid DNA, ranging from 5 copies to 5000 copies, were amplified in 3 different reactions in the same run. To determine inter-run precision, the 4 different levels of XMRV DNA were amplified in 3 different runs on 3 different days. The assays had good intra-run precision with a mean CV of 0.99%, and also good inter-run precision, with a mean CV of 1.36%. We also verified that the tests were specific for XMRV and did not detect other common human pathogens, including other human retroviruses (Fig. 2C).

318

319 **Blood from CFS patients and healthy volunteers is negative for XMRV by qPCR**

320 Using our four qPCR assays, we looked for XMRV and related viral sequences in DNA made
321 from white blood cells of 100 CFS patients and 200 healthy volunteers. We did not find any
322 positive samples even when reactions were carried out to 45 cycles. Positive control reactions
323 were reliably positive for 50 and 5 copies of XMRV plasmid DNA. To verify that the DNA
324 extracted from samples was of adequate and comparable quality, each sample was also tested by
325 a qPCR targeting a single copy gene, VAMP2 (vesicle associated membrane protein 2) (22), and
326 was found to be positive at a C_t of 21 to 23 cycles. Water controls that were subjected to the
327 same extraction method as samples were consistently negative. Each plate of 96 PCRs also
328 contained 12 wells with water instead of template DNA; these were always negative, as
329 expected.

330

331 **Absence of XMRV anti-SU antibodies in serum of CFS patients and healthy volunteers**

332 Infection of Rhesus macaques with XMRV has shown that the most prominent antibody
333 response is to the XMRV Env protein, gp70 (SU) (16). We tested serum from CFS patients and
334 healthy volunteers for reactivity to recombinant XMRV-SU protein in an ELISA that we
335 developed. Rabbit anti-XMRV antisera were used as controls. We found no difference in the
336 reactivity to XMRV-SU protein between patients and healthy volunteers ($p=0.541$, Kruskal-
337 Wallis test) (Fig. 3). Samples with reactivity higher than 2 SD from the mean were tested by
338 Western blotting against recombinant His-tagged XMRV-SU protein. For controls on the
339 Western blots, we used a His-tagged protein that is unrelated to XMRV, as well as uninfected

cell lysates. While we saw a good response with the XMRV antisera, no reactivity was seen with any of the human sera.

Absence of infectious XMRV in plasma of CFS patients and healthy volunteers

Inoculating cultured cells with patient plasma and monitoring for evidence of XMRV replication has been proposed to be the most sensitive method for XMRV detection in plasma samples from CFS patients (14). Because of the labor-intensive nature of this method, we decided to perform this procedure on a subset of our samples (n=65) chosen by a random number generator. We inoculated LNCaP cells with 100 µl of plasma from 31 patients and 34 healthy volunteers, and passaged the cells weekly for 6 weeks. 13 negative controls and 2 positive controls were also included. Only one culture was handled at a time to prevent any cross-contamination. After weeks 2, 4 and 6, cultures were lysed and analyzed by Western blots (Fig. 4) and by qPCR for XMRV. No XMRV protein or DNA was detected in any of the cultures.

Mouse DNA is present in a reagent used in previously published nested PCR assay

Since we were unable to find any evidence of XMRV using our sensitive qPCR assays, serological methods or viral growth assays, we decided to test our samples using the PCR assay first used in the original study that found XMRV in CFS (12). Another study utilized a modified, nested version of this assay to discover the presence of polytropic murine leukemia virus-like sequences in CFS (11). Using this assay, we found approximately 5% of our samples to be positive for products of the expected size, regardless of whether they were patients or healthy volunteers. It was possible that our samples were contaminated with XMRV plasmid DNA, even though work with the plasmid is done in a separate laboratory. We decided to test for this

possibility with qPCR primers flanking a restriction site for endonuclease MluI that had been introduced during the construction of our infectious clone (22). Thus, the laboratory plasmid can be distinguished from a wild isolate of XMRV by a qPCR assay where the probe would bind to the MluI site. Fig. 5A shows that the peak fluorescence from the reporter for plasmid pAO-H4, which does not have the MluI site, is consistently lower than the MluI containing plasmid pXMRV1 at varied copy numbers. Using this assay, we determined that none of the positive reactions were due to contamination with the XMRV infectious clone.

We next checked if our samples were contaminated with mouse DNA, since that has been shown to be a potential source of XMRV-like sequences (15, 19). Based on an assay first introduced by Oakes *et al* (15), we developed a qPCR TaqMan assay to detect small amounts of contaminating mouse DNA. This assay targeted the sequences coding for intracisternal A-type particles (IAP), which are present in approximately 2000 copies per diploid genome of many mouse strains (13). As seen in Fig. 5B, our qPCR assay was linear down to as little as 62.5fg of C57BL/6 mouse DNA and could reproducibly detect as little as 625 ag of mouse DNA per reaction, making it a remarkably sensitive method for detection of contaminating mouse DNA. Using this assay, we determined that our samples did not contain any mouse DNA, and the nested PCRs could not be positive due to mouse DNA in the samples.

When repeating the nested PCR assays, we noticed that the initially positive samples were not consistently positive in subsequent nested assays. However, the proportion of positive reactions remained constant at approximately 5%. Even though extraction and amplification controls (1 per 7 samples) were consistently negative, we suspected that contamination of a PCR reagent

might cause the lack of reproducibility and the consistent positivity rate of 5%. We tested 36 replicates of genomic DNA from uninfected LNCaP cells with the nested PCR and found that 2 produced a positive result (Fig. 5C for a subset of the data). Sequencing these products revealed MLV-related sequences that ranged from 95 to 100% in similarity to those published previously (11, data not shown). In contrast to the nested PCR results, this DNA tested consistently negative with all our XMRV qPCR assays and the IAP qPCR assay for detection of trace amounts of mouse DNA. We next tested each component of the nested PCR using the IAP qPCR assay in replicates of 8. We discovered that both recombinant Taq polymerase (Invitrogen) and the Platinum Taq polymerase (Invitrogen) tested positive for IAP sequences. Furthermore, adding increasing amounts of both Taq polymerases resulted in progressively lower C_t (Fig. 5D). Along similar lines, when increasing amounts of both Taq polymerases were used as a template for the *gag* qPCR, positivity also increased. Positive reactions were obtained with four different batches of Taq polymerase. ABI's Amplitaq Gold Taq polymerase contained in the mastermix of all of our qPCR TaqMan assays did not contain any IAP sequences (Fig. 5D), indicating that it was free of mouse DNA. When adding additional Amplitaq Gold Taq polymerase as template for the IAP qPCR assay as was done with the other polymerase preparations, all reactions remained negative. Contamination of Taq polymerase preparations with mouse RNA has been reported in an independent study (20). Taken together, our analysis shows that certain Taq preparations contain very small amounts of mouse DNA that can cause false-positive reactions when used in highly sensitive assays for XMRV.

Blinded subset of samples from the original XMRV-CFS study was negative for XMRV

To test if we could detect XMRV in samples that had previously tested positive or negative for XMRV, we obtained a subset of samples from the original cohort that was used to make the association of XMRV with CFS (12). Using a third party phlebotomy service that collected blood samples in home visits, we obtained blinded whole blood and serum samples from 14 individuals. These individuals had repeatedly tested positive in the last two years when tested by the labs at the WPI, though this information was not available to us till the completion of our study. The Clinical Research department at ARUP Laboratories received these specimens and processed the blood using the same protocols as for our healthy volunteers and CFS patient samples. Thus the samples were never opened in a research lab where XMRV might be present – until they reached us. We tested these samples using all of the assays we developed – four qPCR assays, ELISA and Western blots. None of the samples contained any evidence of XMRV. Serologically, there was no difference in the reactivity to XMRV-SU between healthy volunteers and the WPI cohort (p-value = 0.467, Kruskal-Wallis test), indicating that there was no detectable antibody response that was specific to XMRV in the WPI cohort. Furthermore, we also analyzed the WPI samples using tests utilized in the two studies that found XMRV or XMRV-like viruses in CFS, viz. a PCR assay for *gag* sequences, both in single-round (12) and nested formats (11), and a test for viral growth in cultured cells (12). Neither of these tests revealed any evidence of XMRV.

Discussion

We examined blood samples from 100 CFS patients and 200 regionally matched healthy volunteers. The patients met both CDC-Fukuda and Canadian criteria for CFS/ME, and over 70% reported the association of a flu-like illness with the onset of their disorder. All blood

431 samples had been freshly collected, blinded, processed, and analyzed identically. Special care
432 was taken to avoid contamination using proper controls during DNA extraction, spin inoculation,
433 and PCR analysis. Despite using a number of carefully characterized tests that were capable of
434 detecting small amounts of XMRV and related MLVs, we did not detect XMRV in any of our
435 samples. These tests consisted of sensitive qPCR assays, ELISA and Western blots that we
436 developed. We also performed PCR assays for *gag* sequences used in the studies that found
437 XMRV or XMRV-like viruses in CFS (11, 12). In addition, we used a viral replication assay
438 used in the original study that made the association between XMRV and CFS (12), and was
439 claimed to be the most sensitive assay for XMRV detection. Extending these negative findings,
440 and adding more validity to our study was our inability to detect any XMRV in samples from
441 patients that had tested positive for XMRV in the original study. We report here a repeat testing
442 of samples obtained from CFS patients that were recruited, diagnosed and defined as positive
443 exemplars of XMRV infection by the investigators who performed the original WPI-based study.
444 This testing was performed in an independent laboratory (ours), using many of the same
445 techniques as in the original study. To our knowledge, this is the first study to report negative
446 findings after a full repetition of all assay methods in patients who have previously tested
447 positive for XMRV.
448
449 Our experience has taught us that the detection of XMRV in blood is fraught with difficulties. In
450 our own laboratory, starting with aliquots of samples from the same patients that we report here,
451 we initially found some samples to be positive for XMRV. DNA from these aliquots had been
452 extracted on a biorobot (Qiagen) in a 96-well format. Twelve wells spread throughout the plate
453 served as negative extraction controls, and a few of these also tested positive. It turns out that a

few months prior to extracting our blood samples, the same biorobot had been used to extract DNA from tissue culture cells that had been infected with XMRV. Despite the several months interval between the two extractions and the use of sterile, disposable reagents in the biorobot, we obtained false positives in our negative extraction controls and some patient samples. Once we abandoned the biorobot, and used new aliquots of samples to extract DNA manually, we did not find any patient or healthy volunteer samples to be positive. We continued this process of extreme care not to contaminate samples in all of our techniques, especially the viral replication assay. Because the viral replication assay consists of passaging cells inoculated with patient samples and controls inoculated with infectious XMRV, every week for 6-8 weeks, this assay is especially vulnerable to contamination. We prevented this by handling only one set of cultures in the biosafety cabinet at a time, and meticulously decontaminated the cabinet between cultures with 70% ethanol and UV irradiation. This made the viral replication assay very time-consuming and labor-intensive, and we could perform it only on a subset of our samples. But it is easy to see how the sample extraction and tissue culture processes might be the most vulnerable to contamination, providing a possible explanation for why the 14 samples from individuals tested repeatedly by the WPI over a period of two years were positive in their hands and negative in ours. Our early false positive findings did have one benefit: they confirmed beyond a doubt that our assay methods were highly sensitive to even tiny quantities of XMRV, and thus we would have every expectation of detecting it if it had been present in any of the samples we tested.

The presence of mouse DNA in PCR reagents emphasizes the critical importance of proper controls and carefully chosen, sensitive assays to detect trace amounts of mouse DNA. Sato *et al.* (20) found that Platinum Taq polymerase (Invitrogen) contained RNA from polytropic

endogenous MLV using a sensitive RT-PCR kit. This is not too surprising because the mouse monoclonal antibody used to prevent enzyme activity prior to heat activation might be the source of mouse DNA in the enzyme. What was surprising, however, was our finding mouse sequences in Invitrogen's recombinant Taq polymerase that is expressed in E. coli; we are not sure what the source of mouse DNA is, in this case. We did confirm, however, that Applied Biosystems' Amplitaq Gold polymerase that was used in all of our qPCRs, both here and in previous studies (5, 22, 25) did not contain any detectable mouse DNA. Lo *et al.* used the finding of negative results with the mouse mitochondrial semi-nested PCR assays to support the assertion that their samples were free of mouse DNA. Like others (15, 19), we propose the detection of IAP sequences instead of mouse mitochondrial DNA as a better approach to look for contamination. We demonstrate that our qPCR assay for IAP sequences is exquisitely sensitive and can detect attogram quantities of mouse DNA.

The question remains how mouse DNA in the Taq polymerase could lead to a disproportionate number of positives in patients versus controls in the two studies linking XMRV to CFS. It is possible, as has been suggested before (27), that patient samples were handled more than control samples and thus had a higher likelihood of contamination. In our study, both patient and control samples were handled in the same manner with the same frequency, in a blinded manner. We also suggest that any planned studies proposed to screen for XMRV carefully check their reagents, equipment, and all possible - and seemingly not possible - sources of contamination with exogenous XMRV and mouse DNA. Obviously, all such studies should be conducted with careful blinding of investigators and staff to prevent unintended experimental bias.

Unlike molecular and cell culture amplification assays, serological assays have the advantage of being difficult to contaminate. However, serological assays are still susceptible to false positives because of non-specific binding of antibodies to related antigens. Serologically, our patient samples appear indistinguishable from controls, as do the samples from the WPI cohort. It is possible that assays that have found anti-XMRV reactivity in CFS patients are due to cross-reactivity to related antigens.

Given the lack of evidence for XMRV or XMRV-like viruses in our cohort of CFS patients, as well as the lack of these viruses in a set of patients previously tested positive, we feel that that XMRV is not associated with CFS. We are forced to conclude that prescribing antiretroviral agents to CFS patients is insufficiently justified and potentially dangerous. It is also vital to state that there is still a wealth of prior data (2, 10) to encourage further research into the involvement of other infectious agents in CFS, and these efforts must continue.

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qPCR assay.

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615

Figure Legends

Fig. 1. Study scheme showing collection, processing, and analysis of blood samples from CFS patients and healthy volunteers.

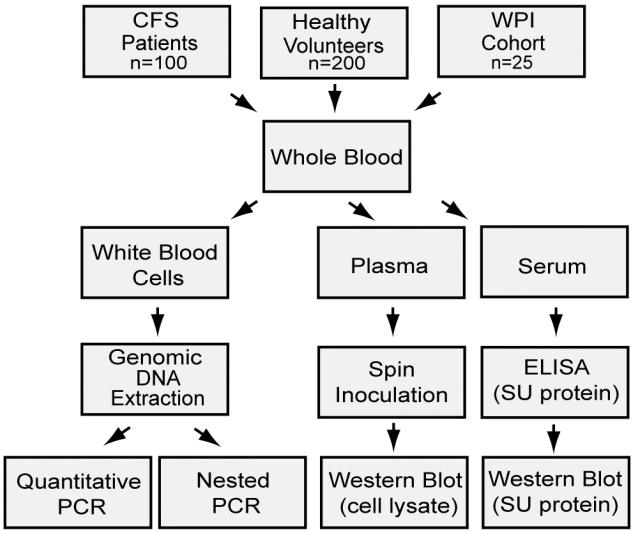
Fig. 2. Defining qPCR assay characteristics. A. Reproducibility of all XMRV qPCR assays (*pol*, *env*, LTR, *gag*), performed in triplicate with pXMRV1 template amounts of 500, 50 and 5 copies. R^2 values show high reproducibility for each assay. B. Sensitivity of XMRV LTR qPCR assay with a FAM reporter showing that the assay was linear with as low as 5 copies of pXMRV1 template added in a background of 400 ng of human placental DNA. R_n is the difference in fluorescence between the FAM reporter and the standard reference ROX dye. C. Testing for cross reactivity (or specificity) of XMRV qPCR assays against other common human pathogenic viruses. A positive clinical sample or plasmid DNA from a variety of common pathogenic viruses was amplified using the LTR and *pol* (shIN) qPCR assays. No significant cross-reactivity was seen with any of the following: BK virus, Cytomegalovirus (CMV), Epstein Barr virus (EBV), Enterovirus (EV), Human Herpes virus 6 Variant A (HHV6-A), Human Herpes virus 6 Variant B (HHV6-B), Human immunodeficiency virus (HIV), Human metapneumovirus (HMPV), Influenza A virus (FLUA), and Influenza B virus (FLUB). Each sample was extracted with an exogenous internal control plasmid IC2, containing *C. elegans* pax1/9 gene fused to GFP, added to each aliquot of whole blood prior to sample extraction. This internal control plasmid was co-amplified with each sample to identify potential inhibitors of PCR and to monitor extraction efficiency. Extraction was efficient as seen from the IC C_ts ranged between 33.5 and 35.9.

Fig. 3. ELISA to measure reactivity of human sera against recombinant XMRV-SU protein. Sera from CFS patients (black bars), healthy males (light grey bars) and healthy females

(dark grey bars) in reaction with gp70 Env (SU) fragment tested in three replicates. Arrow indicates average OD values using XMRV anti-serum at 1:10,000 (22).

Fig. 4. Western blot analysis on a subset of CFS patients (P) and controls (C) six weeks after spin inoculation of plasma onto cultured LNCaP cells. Cell lysates from XMRV infected cells probed with rabbit anti-XMRV antisera are shown in lane (+). Lane (–) represents cell lysates from uninfected cells probed with rabbit anti-XMRV antisera. M = molecular weight marker. To indicate loading amounts, the same gel was probed with anti-tubulin antibody (bottom).

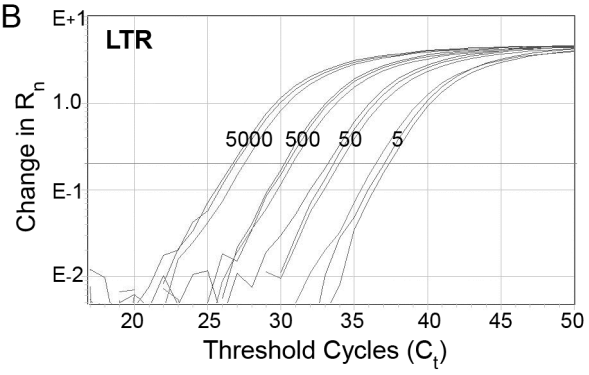
Fig. 5. Nested PCR assay. A. MluI qPCR assay to detect pXMRV1 contamination. The 5' end of the probe spans the MluI restriction site that was introduced to create pXMRV1. pAO-H4, which does not have the MluI restriction site has lower peak fluorescence as well as a delay in C_ts for the same copy numbers of plasmid. B. Sensitivity of the IAP qPCR assay for different amounts of C57BL/6 mouse DNA ranging from 62.5 ng to 625 ag, all in the presence of 400 ng of human placental DNA. C. Nested PCR on a small set of samples, showing ~5% samples to be positive for MLV *gag* sequences using NP116/NP117 (11). LNCaP genomic DNA show in lanes G. Lane (–) represents negative control. Lane l shows 100bp ladder. D. Detection of mouse DNA in Platinum Taq (IP Taq, Invitrogen) and Recombinant Taq (IR Taq, Invitrogen), but not in Amplitaq Gold (AAG Taq, ABI). For each qPCR assay, the left column shows number of replicates that are positive, and the right column shows the average C_t at which positivity occurs. The more XMRV-specific *pol* qPCR assay (in triplicates) was consistently negative, but IAP and *gag* assays (eight replicates each) were both positive as more Platinum or Recombinant Invitrogen Taq was used as template.



A

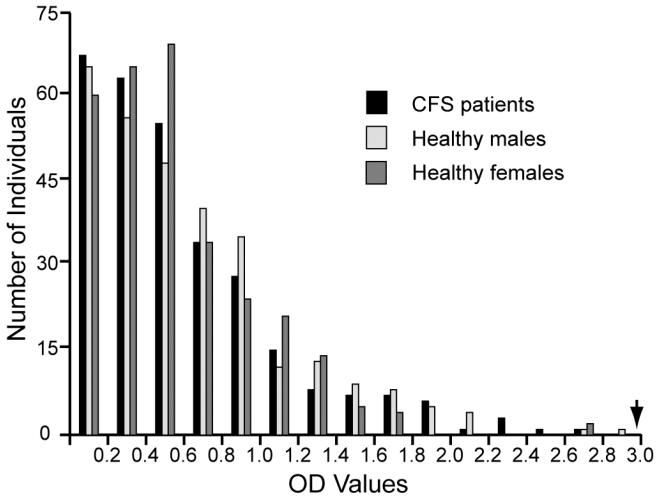
qPCR assay	pXMRV1 Template Amounts 500 copies	50 copies	5 copies	R^2
pol	30.1	33.5	36.3	0.989
env	29.5	33.1	36.3	0.993
LTR	30.4	33.6	36.9	0.992
gag	32.6	35.8	39.9	0.989

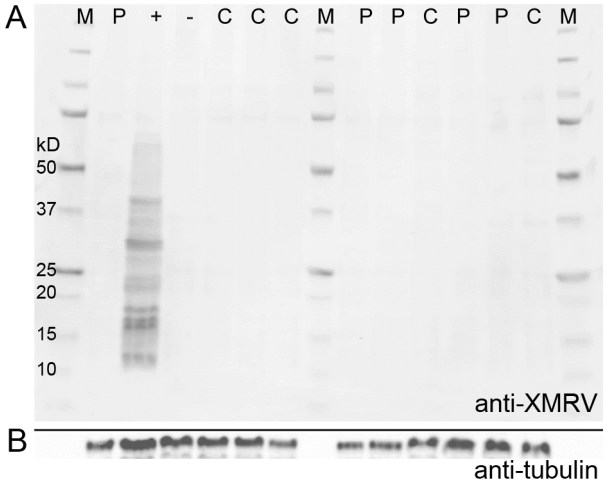
B



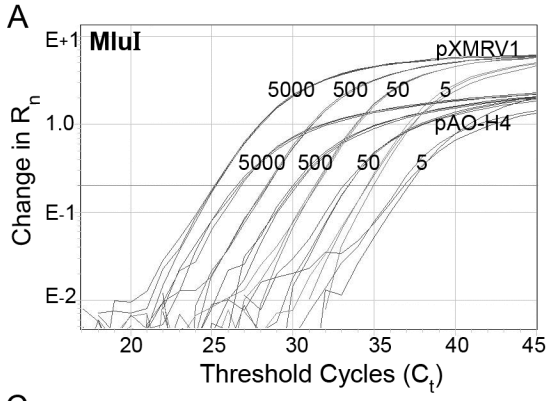
C

Template	*pol* C_t	IC C_t	LTR C_t	IC C_t
BK	0	33.5	0	34.5
CMV	0	34.5	0	34.4
EBV	0	34.4	0	34.8
EV	0	34.9	0	35.9
HHV6-A	0	34.7	0	35.4
HHV6-B	0	34.6	0	33.7
HIV	0	34.9	0	34.5
HMPV	0	34.7	0	34.3
FLUA	0	35.3	0	34.9
FLUB	0	34.4	0	34.9





A



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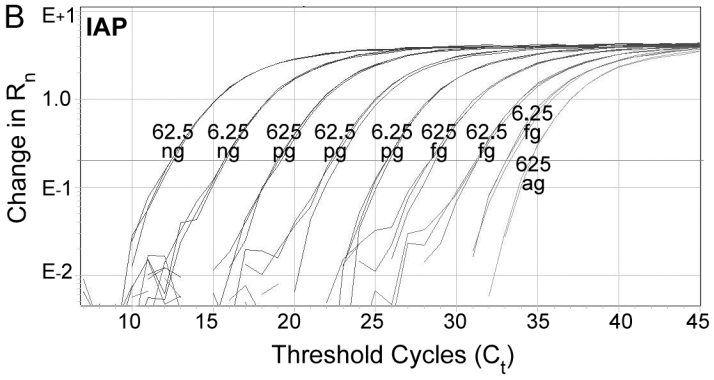
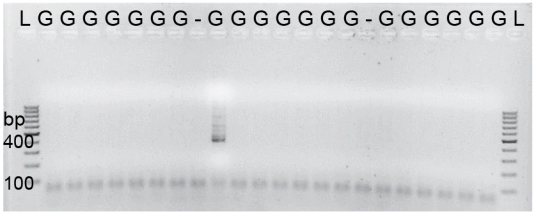


C



D

	pol qPCR		IAP qPCR		*gag* qPCR	
	+	C_t	+	C_t	+	C_t
IP Taq 0.5U	0/3	0	8/8	35.4	0/8	0
IP Taq 1.0U	0/3	0	8/8	34.7	1/8	39.3
IP Taq 2.0U	0/3	0	8/8	34.2	2/8	37.9
IR Taq 0.5U	0/3	0	8/8	37.9	0/8	0
IR Taq 1.0U	0/3	0	8/8	36.8	1/8	48
IR Taq 2.0U	0/3	0	8/8	36.3	2/8	42.4
AAG Taq 0.5U			0/8	0		
AAG Taq 1.0U			0/8	0		
AAG Taq 2.0U			0/8	0		

Sciencexpress Report

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

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Murine-like gammaretroviruses (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 re-infect 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), has been 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 (MLVs) 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 MLV (*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 years 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 male median age was 49 years (range 20 to 73 years). These patients were an unselected, sequentially enrolled population submitted for diagnostic testing to 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 PCR, serum PCR, or viral XMRV culture with PCR (*17*).

These evaluations were performed by a commercial (VIPDx, Reno, NV) or research laboratory (Whittemore Peterson Institute [WPI], Reno, NV). 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 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 antibodies to XMRV detected in a commercial (VIPDx) or research laboratory (WPI) (*5*) (table S1). Twenty of the patients (69%) were female and 9 (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 (see supporting online material). For the serum inactivation studies, seven healthy 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 University of California, San Francisco. 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% (10/19) of the blood samples were reported by the commercial laboratory as being positive for XMRV DNA. This difference in our results (0/19) versus the chart review results (10/19) 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 (PBMC) were evaluated by RT– PCR procedures directly or after activation with phytohemagglutinin (PHA; 3 μg/ml for 3 days) using 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 PBMC or plasma of these 29 CFS patients (Table 1, Fig. 1B). The positive control, consisting of the 730 bp fragment of XMRV amplified from prostate cancer cell line, 22Rvl, was able to detect at least 10 copies of XMRV *gag* DNA per reaction; second-round PCR detected 1-10 copies/reaction (table S3).

We next 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 passed 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 analysis (*11, 18*). We also looked for infectious virus in culture fluids from 19 patient PBMCs that had been cultured for 1-3 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% (9/18) 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 using 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 from 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 reflecting 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 underway, 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 3/5 Taq polymerases that utilize MAbs, but also in 9/17 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*). We have found that the DNA sequences of 3 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 to the VP62 clone sequence. Therefore, the remarkable conservation of the WPI-XRMV 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, 7 of our subjects were studied on two occasions; 2 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 that X-MLV and XMRV are fully or partially inactivated by human serum, suggesting that these viruses could not readily establish a human infection. Since an activated immune system has been observed in CFS patients (*31*), the possibility of another infectious agent(s) being associated with this illness merits continual attention.

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Supporting Online Material

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

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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/reaction) 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/reaction. Negative controls for each reaction step were tested in triplicate: *RNA/DNA extraction negative control, **RT control, and ***PCR control.

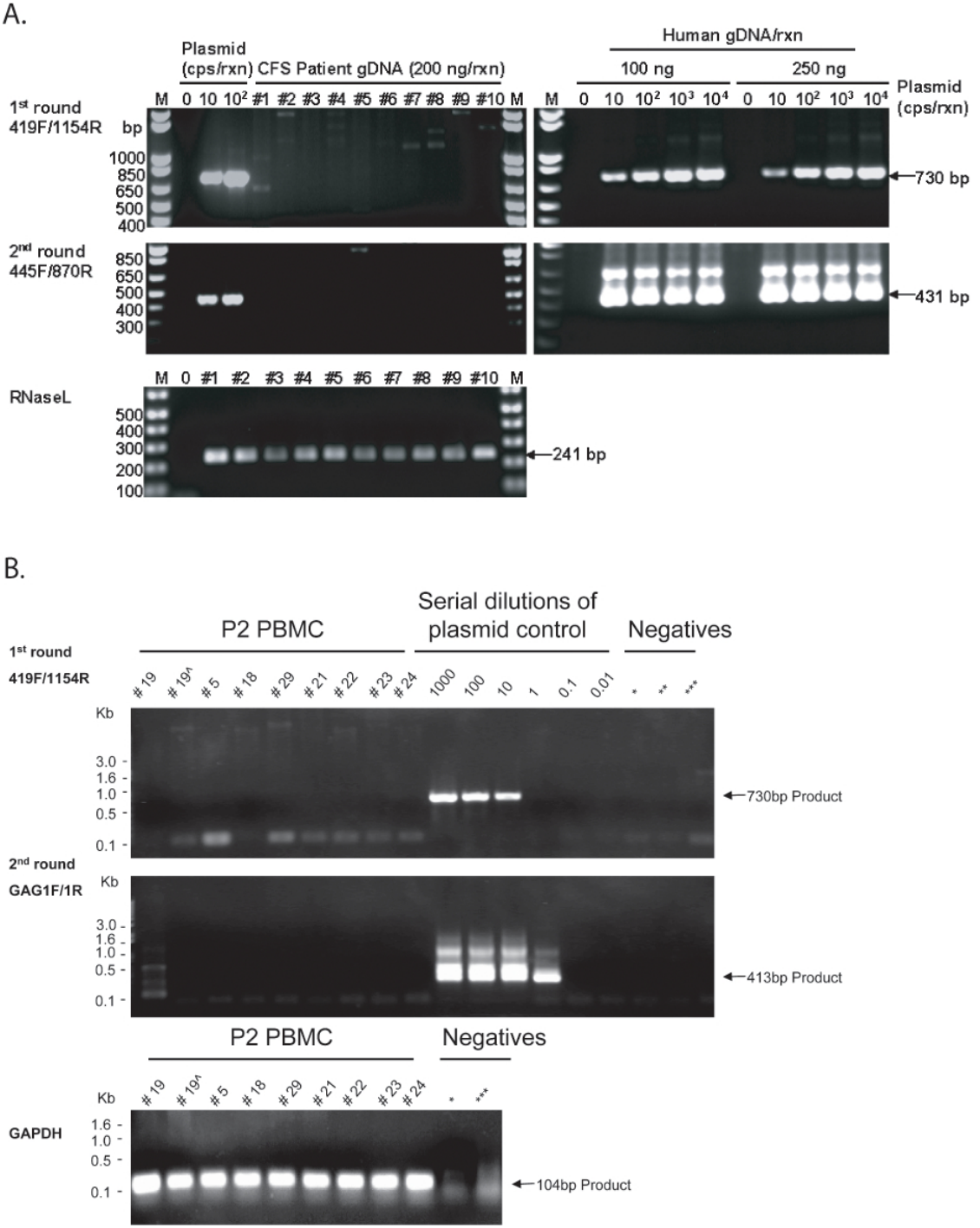
Fig. 2. Evaluation of 60 CFS plasma samples for the presence of XMRV antibodies. (**A**) Two recombinant protein-based CMIAs were used to detect specific antibodies to XMRV gp70 and p15E proteins (*17*). The X axis represents the CMIA signal in a unit of natural log-transformed ratio of sample signal to the cutoff signal (Log N 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 antisera of XMRV-infected macaque, negative control (NC) of normal blood donor and Molecular weight (MW) markers in kilodaltons (KD).

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 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. X-MLV was obtained from NZB mouse cells and propagated in mink lung cells (*20*). XMRV was obtained from the human prostate cell line (22Rv1). For the five studies conducted, the control virus titers measured as focus formation in mink S+L- cells were 126, 430, 168, 246, 208 foci (X-MLV); 84, 376, 208, 284, 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.

Table 1. Summary of assays used to evaluate blood samples from CFS patients in population 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 passed after PBMC co-culture*	0 (0/30)†

*Infectious virus assay: Fluids were tested for infectious virus production by reverse transcriptase (RT) and the mink S+L- cell assays (see text) (*17*). †Insufficient cells were available for these studies from subject #24.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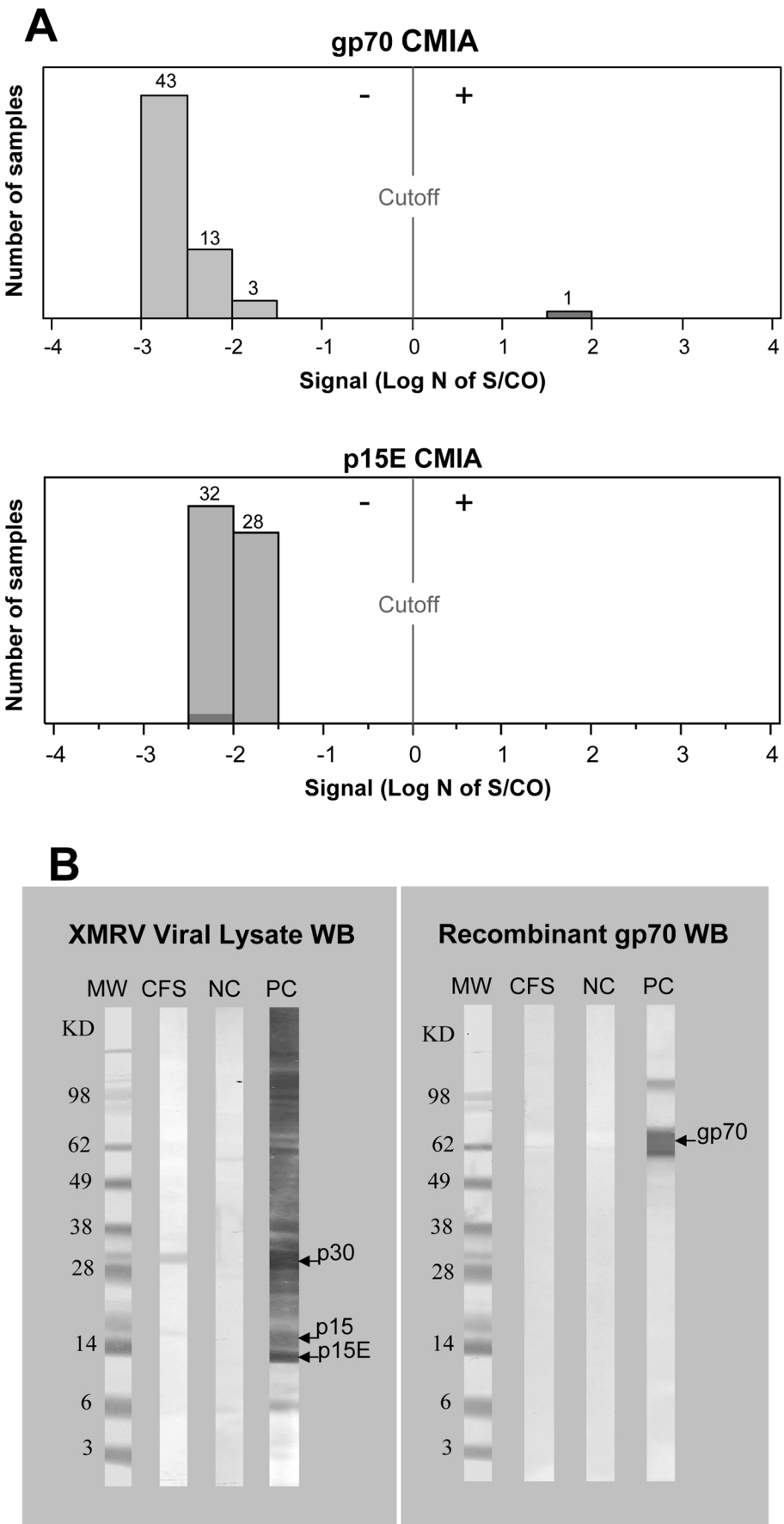
CFS Patient gDNA (200 ng/rxn)
Human gDNA/rxn
100 ng
250 ng
Plasmid (cps/rxn)
1st round 419F/1154R
bp
730 bp
2nd round 445F/870R
431 bp
RNaseL
241 bp
B.
Serial dilutions of plasmid control
P2 PBMC
Negatives
1st round 419F/1154R
730bp Product
2nd round GAG1F/1R
413bp Product
GAPDH
104bp Product
Kb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gp70 CMIA
Number of samples
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13
3
1
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+
Cutoff
-4
-3
-2
-1
0
1
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3
4
Signal (Log N of S/CO)
p15E CMIA
Number of samples
32
28
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+
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-4
-3
-2
-1
0
1
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4
Signal (Log N of S/CO)
B
XMRV Viral Lysate WB
MW
CFS
NC
PC
KD
98
62
49
38
28
14
6
3
p30
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p15E
Recombinant gp70 WB
MW
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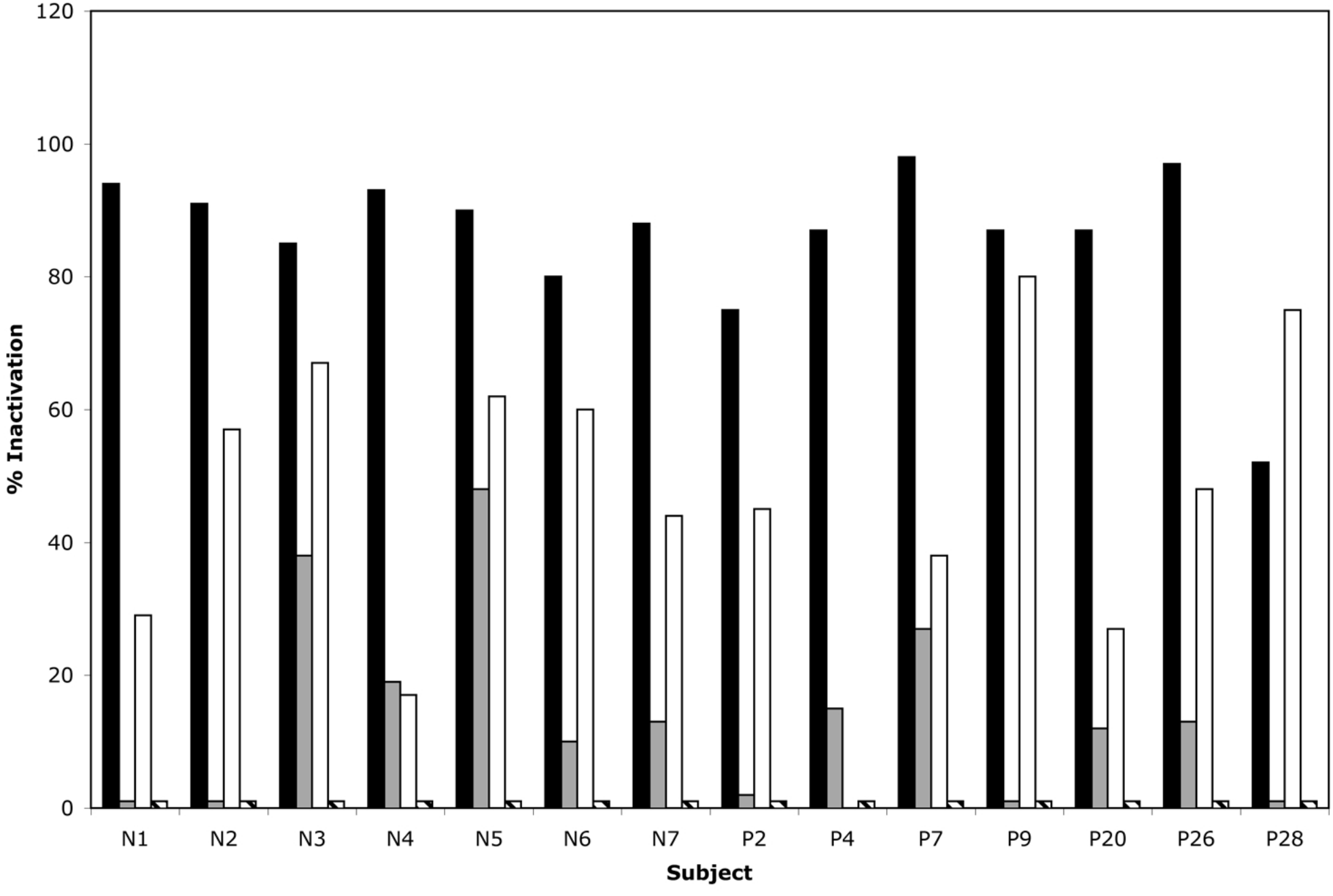
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100
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% Inactivation
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P2
P4
P7
P9
P20
P26
P28
Subject

Sciencexpress Report

Recombinant Origin of the Retrovirus XMRV

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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*), making 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/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*). Fig. 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, 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 7th 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 (STR) analysis at 7 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 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/cell and used the 22R*v1* DNA to generate a standard curve. The CWR22 xenografts had significantly fewer copies of XMRV *env* (<1–3 copies/100 cells) compared to the 22R*v1* cells (2000 copies/100 cells). The CWR-R1 cell line had ~3000 copies/100 cells, and the NU/NU and Hsd nude mice, thought to have been used to passage the CWR22 xenograft, had 58 and 68 copies/100 cells, respectively. Since xenograft tumors are expected to contain a mixture of human and mouse cells, we quantified the amount of mouse DNA by analyzing mouse intracisternal A-type particle (IAP) DNA as previously described (*18*, *19*). Approximately 0.3–1% of the total DNA from all 6 xenografts consisted of mouse DNA (Fig. 1D); this result is consistent with the <1–3 XMRV *env* sequences/100 cells detected in the same samples (Fig. 1C).

We characterized XMRV and related sequences in the xenografts, cell lines, and nude mouse strains by PCR and DNA sequencing (Fig. 2). Using primers previously used to clone and sequence XMRV from 22R*v1* cells (*8*), we determined that all the XMRV proviruses in the CWR-R1 and 22R*v1* cell lines are identical in sequence, with the exception of some rare hypermutated proviruses (Fig. 2A and figs. S2 and S3). Next, we developed several primer sets to specifically amplify XMRV sequences and exclude endogenous murine retroviruses (fig. S2). Primers that specifically amplified XMRV were used to perform PCR on DNA from the late-passage xenografts 2152, 2524, 2272 and 2274; sequencing confirmed the presence of these XMRV sequences in these tumors (Fig. 2A and fig. S3A; boxed in Fig. 1A).

We used the same XMRV-specific primer sets to amplify and sequence DNA from early passage xenografts (736, 777, 8L, 8R, 16R, and 18R; Fig. 2B); the results showed that XMRV *env*, but not *gag* sequences were present (sequencing coverage summarized in fig. S3), indicating that the early xenografts did not contain XMRV. However, we did find that early xenografts contained a previously undescribed XMRV-related provirus that we have named PreXMRV-1 (Fig. 2B). The complete sequence of PreXMRV-1 was determined from the early passage xenografts, the NU/NU and Hsd strains, and the CWR-R1 cell line. PreXMRV-1 and consensus XMRV differed by only one base in a 3211-nt stretch of the genome encoding the 3' half of *pol* and the 5' 2/3 of *env*. In addition, the LTRs were nearly identical; PreXMRV-1 had a single adenine deletion relative to XMRV in a run of 6 adenines. The two genomes differed by 10% over the remaining 3.5-kb stretch of *gag-pro-pol* and by 9% in a 600-nt stretch at the 3' end of *env*. PreXMRV-1 is replication defective because of a 16-nt deletion in *gag* and a +1 frameshift mutation in *pol*. Late-passage xenografts 2524 and 2274, but not 2152 and 2272, also contained PreXMRV-1. The detection of low levels of XMRV *env* sequence in the early xenografts (Fig. 1C) can be attributed to the PreXMRV-1 proviruses present in the contaminating mouse DNA. Overall, these results indicate that PreXMRV-1 is an endogenous murine provirus that is present in the NU/NU and Hsd strains, but neither of these strains contains XMRV (the PCR and sequencing coverage are detailed in fig. S3, A and B).

To screen for the presence of endogenous XMRV in mouse strains, we developed an XMRV-specific PCR assay based on sequence differences in the LTR and *gag* leader regions that excluded all known endogenous murine retroviruses (fig. S2). A survey of 45 laboratory mouse strains and 44 wild mice failed to detect XMRV (fig. S4). In a search for proviruses that might contain XMRV-specific sequence features, we found a second previously undescribed endogenous provirus that we named PreXMRV-2 (Fig. 2C). A portion of PreXMRV-2 corresponds to an 1124-nt sequence of an endogenous provirus from the 129X1/SvJ mouse genome (Acc. No. AAHY0159188.1) (*6*, *20*). The sequence of PreXMRV-2 revealed that *gag*, *pol*, and *env* reading frames are open and can potentially express functional proteins. A 3.6-kb stretch encompassing the *gag* leader region and *gag-pro-pol* differs by one base from the consensus XMRV (99.9% identity); in addition, a ~700-nt region of *env* is 99% identical to XMRV; however, the LTRs and the remaining viral genome differ by 6–12% from consensus XMRV. Phylogenetic analysis indicates that PreXMRV-1 groups with xenotropic viruses whereas PreXMRV-2 appears to be a recombinant, grouping with polytropic and modified polytropic viruses for certain stretches of its genome (fig. S5).

We screened 15 mouse strains, which included 12 nude mice, for the presence of XMRV, PreXMRV-1, and PreXMRV-2 using XMRV-specific primers, primers that

amplified XMRV or PreXMRV-1, and PreXMRV-2-specific primers (Fig. 2D and fig. S2). None of the mouse strains contained XMRV and only the Hsd and the NU/NU outbred nude strains contained PreXMRV-1 (Fig. 2D and fig. S6). Six of the 15 mouse strains contained PreXMRV-2, but only the NU/NU and Hsd mice contained both PreXMRV-1 and PreXMRV-2 (Fig. 2D and fig. S6). It should be noted that since the Hsd and the NU/NU are outbred strains, individual mice differ in their endogenous proviruses. NU/NU mice showed variation in the presence of these two endogenous proviruses, and two out of five animals tested contained both (fig. S6). The 22R*v1* cell line contained only XMRV as confirmed by sequence analysis; however the CWR-R1 cell line contained both XMRV and PreXMRV-1. The CWR-R1 cell line has been reported to contain contaminating mouse cells (*21*) (and see IAP signal, Fig 2D), which is likely to be the source of the PreXMRV-1 sequences.

We used the same specific primer sets to determine the distribution of XMRV, PreXMRV-1 and PreXMRV-2 in early and late xenografts (Fig. 2E). None of the early xenografts (736, 777, 9216R, 9218R, 8R and 8L), but all of the late xenografts (2152, 2524, 2272, and 2274) and both cell lines were positive for XMRV. The primers used to detect PreXMRV-1 could also detect XMRV; sequencing analysis of the PCR products from all of the early xenografts detected only PreXMRV-1, but both XMRV and PreXMRV-1 were detected from the late xenografts 2524 and 2274 (Fig. 2B). Amplification with PreXMRV-2-specific primers revealed the presence of this provirus in early xenografts 736, 777, 8R and 8L, and late xenografts 2272 and 2274 (Fig. 2, C and E, and fig. S3C). The variable detection level of PreXMRV-2 in the late xenografts could be due to individual differences in the outbred mice, and by extension, in the mouse DNA in these samples.

Comparison of the PreXMRV-1 and PreXMRV-2 sequences revealed that the regions of near identity to XMRV are reciprocal and largely non-overlapping. We therefore hypothesized that recombination between these two retroviruses resulted in the formation of XMRV. As shown in Fig. 3A, reverse transcriptase template switching events during minus-strand DNA synthesis can form a recombinant that is essentially identical to the sequences of all of the XMRVs reported to date, and differing from the consensus XMRV by only 4 nucleotides. The six switching events occurred in 20–73 nucleotide stretches that are identical between PreXMRV-1 and PreXMRV-2 (Fig. 3A, red numbers; fig. S7A). Of the four nucleotide differences between the predicted recombinant and consensus XMRV, only the A>G change at position 790 results in a conservative valine-to-isoleucine amino acid substitution; the other 3 substitutions are silent. The 22R*v1* and CWR-R1 cell lines as well as VP42 have an A at position 790, whereas all other XMRV isolates have a G at position 790. The insertion of an A at position 8092 occurred within a run of 6 adenines; frameshift mutations commonly occur in such homopolymers during retroviral replication (*22*). A comparison of the predicted recombinant to the available XMRV sequences is shown in fig. S7B. The available XMRV sequences all have the same six recombination junctions predicted in the hypothetical recombinant, and differ from the consensus XMRV by 3 – 14 nucleotides. These differences may be the result of errors during PCR or sequencing, or mutations during the passage of XMRV in another cell line. Phylogenetic analysis supports the predicted recombinant virus as the precursor of the virus in the CWR22 xenografts, the 22R*v1* and CWR-R1 cell lines, and all XMRVs isolated and sequenced from patients (Fig. 3B) (*23*).

Our findings indicate that virus derived from two previously undescribed murine endogenous retroviruses, PreXMRV-1 and PreXMRV-2, most likely underwent retroviral recombination to generate XMRV during *in vivo* passaging of the CWR22 xenograft in nude mice. The fact that both parental endogenous proviruses were present in some of the nude mouse strains used for *in vivo* passaging of the xenografts indicates that there were opportunities for this recombinant to form and spread in the tumor cells that were the progenitors of the 22R*v1* and CWR-R1 cell lines. Only 6 template switching events, which is close to the average of 4 template switches per replication cycle (*24*), are needed to generate a recombinant that is both essentially identical and ancestral to all XMRV sequences characterized to date from cell lines and patients (Fig. 3B). We have estimated the probability that the exact set of template switching events occurred independently is 1.3×10^{-12} (fig. S8) (*23*), making it very likely that contamination of human samples with XMRV originating from the relapsed CWR22 xenografts or either of the two cell lines, perhaps through other intermediate cell lines, contributed to its reported association with PC and CFS. Our results and conclusions relate to XMRV detection by isolation of virus of this specific sequence (*1*–*3*), and do not directly address detection of antibodies or viral antigens (*25*, *26*), or PCR detection of related but distinct MLV sequences (*27*). We note, however, that most "XMRV-specific" PCR assays may detect PreXMRV-1 or -2 proviruses in contaminating mouse DNA, and that specific detection of XMRV requires the use of primers that flank a crossover site.

The alternative possibility is that recombination between PreXMRV-1 and PreXMRV-2 occurred during mouse evolution, giving rise to an endogenous XMRV provirus that is present in mice and can occasionally infect humans. We think this possibility is remote because analysis of the early xenografts, which contained contaminating nude mouse DNAs, failed to detect XMRV. Furthermore, we were unable

to detect XMRV in a screen of 89 inbred and wild-derived mouse strains including 17 individual nude mice (fig. S4) (*23*).

We conclude that XMRV was generated as a result of a unique recombination event between two endogenous MLVs that took place around 1993–1996 in a nude mouse carrying the CWR22 PC xenograft. Since the probability that the same recombination event could occur independently by random chance is essentially negligible, any XMRV isolates with the same or nearly the same sequences identified elsewhere originated from this event (*23*).

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Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1205292/DC1
Materials and Methods
Figs. S1 to S8
Table S1
References

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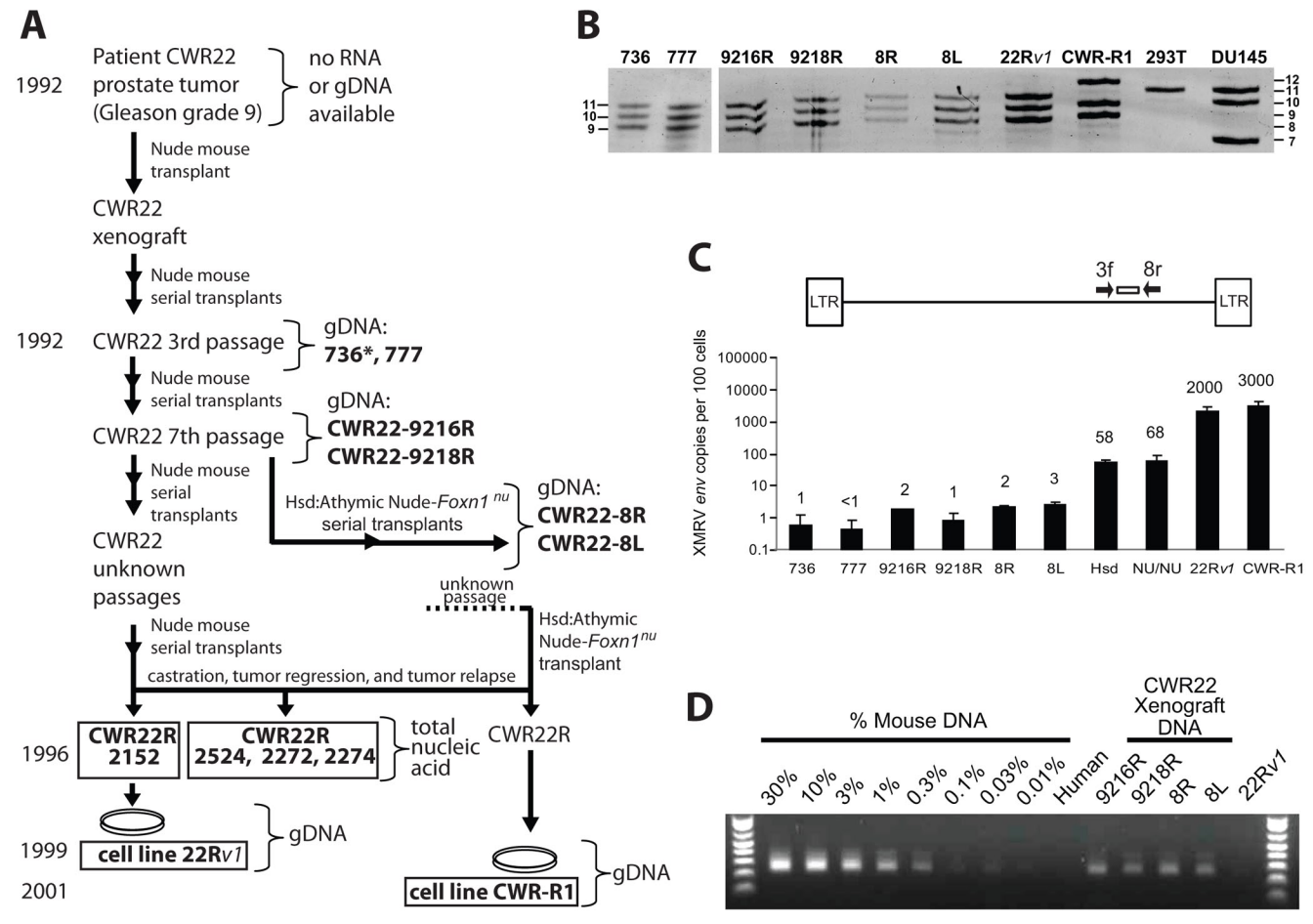
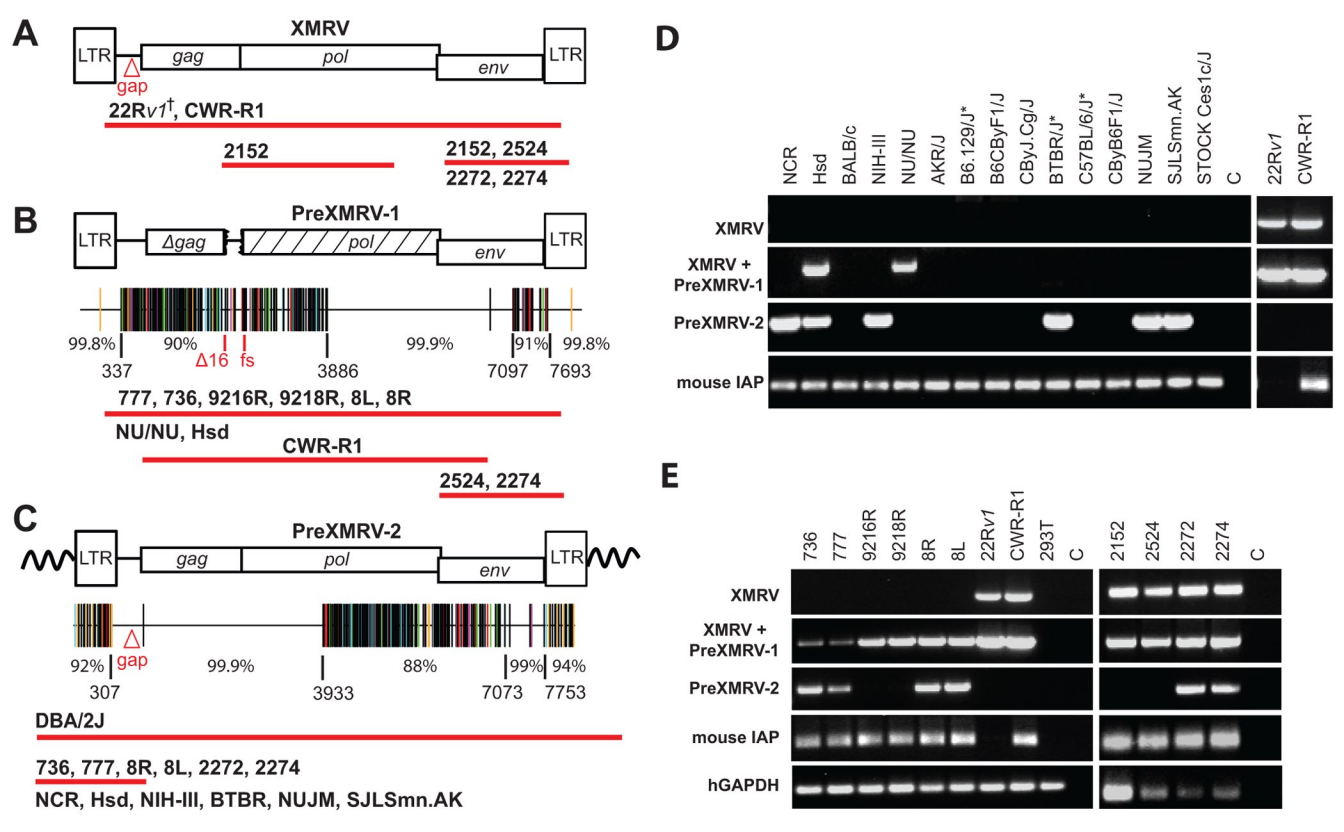
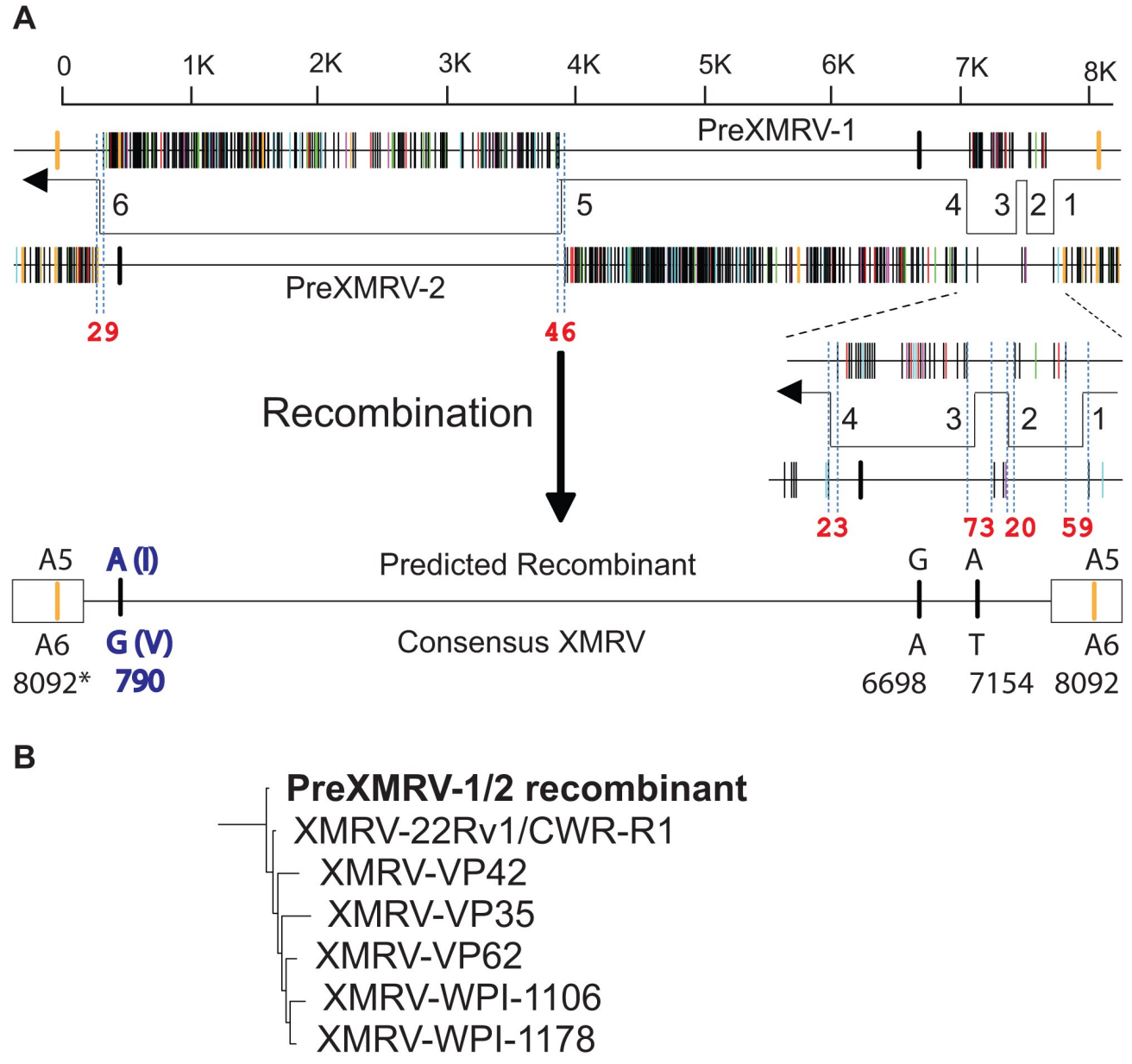
Fig. 1. Characterization of CWR22 xenografts and XMRV-related sequences. (**A**) Genesis of 22R*v1* and CWR-R1 cell lines. Bold letters indicate samples from which genomic DNA (gDNA) or total nucleic acid was available for analysis. XMRV-positive samples are boxed. *, unknown early passage. (**B**) Short tandem repeat (STR) analysis. Representative D7S280 allele pattern of xenografts, 22R*v1* and CWR-R1 cell lines, along with analysis of six additional loci (fig. S1). An allelic ladder is shown on left and right of gel. (**C**) Quantitative real-time PCR to detect XMRV *env* sequences. Calculated copies/100 cells are indicated above each bar. (**D**) IAP assay to quantitate the amount of mouse DNA present in the xenograft gDNAs.

Fig. 2. PCR and sequencing analysis of XMRV and XMRV-related sequences from xenografts, cell lines, and nude mouse strains. Using specific primer sets (fig. S2), cloned PCR products from the xenografts, 22R*v1*, CWR-R1, or mouse strains were sequenced. Approximate length and location of sequences determined from samples that were positive for XMRV (**A**), PreXMRV-1 (**B**) and PreXMRV-2 (**C**) are shown as red bars beneath each provirus. Details of primers and numbers of cloned products sequenced are shown in figs. S2 and S3. Hypermut plots (see fig. S3 for details), which indicate nucleotide mismatches relative to XMRV as color-coded vertical lines, are shown for PreXMRV-1 (**B**) and PreXMRV-2 (**C**), together with the percent identity to consensus XMRV for different regions of each provirus (nucleotide numbers refer to the 22R*v1* XMRV sequence [FN692043]). PreXMRV-1 has a 16-nt deletion (Δ16) in *gag* and a frameshift (fs) in *pol* making it replication defective while PreXMRV-2 *gag, pol, and env* reading frames are open. Mouse strains (**D**) and xenograft and PC cell lines (**E**) were analyzed by PCR for the presence of XMRV, PreXMRV-1 and PreXMRV-2. Mouse IAP and human GAPDH serve as positive controls for the presence of mouse and human DNA, respectively. For both (**D**) and (**E**) the primer set used to detect PreXMRV-1 can also detect XMRV. For ease of comparison, the 22R*v1* and CWR-R1 gel lanes from (**E**), which were run in parallel, are duplicated in (**D**). DNAs in (**D**) and (**E**) were all amplified with the same PCR primer master mix. †We previously determined the full-length sequence of XMRV from 22R*v1* cells (*8*). Δgap refers to the 24-bp deletion in the *gag* leader characteristic of XMRV. All mouse strains shown in (**D**) are nudes except for those indicated with *.

Fig. 3. Predicted recombinant between PreXMRV-1 and PreXMRV-2 is nearly identical to XMRV. (**A**) Alignment of Hypermut plots of PreXMRV-1 and PreXMRV-2 reveals the reciprocal and largely nonoverlapping regions of near identity to XMRV. The direction of minus-strand DNA synthesis catalyzed by reverse transcriptase, and predicted template switching events (numbered 1–6) are shown. The lengths of nucleotide identity within the presumed template switching regions are indicated in red numbers. The predicted recombinant and the 4 nucleotide differences with consensus XMRV are shown. The nucleotide numbers refer to numbers of the 22R*v1* XMRV (Acc. No. FN692043). Note that nucleotide 8092 is within the U3 region, and is present in both LTRs (boxes). A5 and A6 refer to homopolymeric runs of 5 and 6 adenines, respectively. The A>G change at 790 results in an isoleucine (I) to valine (V) substitution. (**B**) Phylogenetic tree of all full-length XMRV sequences to date and the predicted recombinant implicates the predicted recombinant as the ancestor of all sequenced XMRV isolates. The tree shown is an enlargement of the XMRV-specific portion of the complete endogenous MLV tree (fig. S5A) (*23*).


A
1992
Patient CWR22 prostate tumor (Gleason grade 9)
no RNA or gDNA available
Nude mouse transplant
CWR22 xenograft
Nude mouse serial transplants
1992
CWR22 3rd passage
gDNA: 736*, 777
Nude mouse serial transplants
CWR22 7th passage
gDNA: CWR22-9216R CWR22-9218R
Nude mouse serial transplants
Hsd:Athymic Nude-Foxn1 nu serial transplants
gDNA: CWR22-8R CWR22-8L
CWR22 unknown passages
unknown passage
Hsd:Athymic Nude-Foxn1nu transplant
Nude mouse serial transplants
castration, tumor regression, and tumor relapse
1996
CWR22R 2152
CWR22R 2524, 2272, 2274
total nucleic acid
CWR22R
1999
cell line 22Rv1
gDNA
2001
cell line CWR-R1
gDNA
B
736 777 9216R 9218R 8R 8L 22Rv1 CWR-R1 293T DU145
11 10 9
12 11 10 9 8 7
C
3f 8r
LTR LTR
XMRV env copies per 100 cells
100000 10000 1000 100 10 1 0.1
1 <1 2 1 2 3 58 68 2000 3000
736 777 9216R 9218R 8R 8L Hsd NU/NU 22Rv1 CWR-R1
D
% Mouse DNA
CWR22 Xenograft DNA
30% 10% 3% 1% 0.3% 0.1% 0.03% 0.01% Human 9216R 9218R 8R 8L 22Rv1


A
XMRV
LTR
gag
pol
env
LTR
gap
22Rv1†, CWR-R1
2152
2152, 2524
2272, 2274
B
PreXMRV-1
LTR
Δgag
pol
env
LTR
99.8%
90%
Δ16
fs
99.9%
91%
99.8%
337
3886
7097
7693
777, 736, 9216R, 9218R, 8L, 8R
NU/NU, Hsd
CWR-R1
2524, 2274
C
PreXMRV-2
LTR
gag
pol
env
LTR
gap
92%
99.9%
88%
99%
94%
307
3933
7073
7753
DBA/2J
736, 777, 8R, 8L, 2272, 2274
NCR, Hsd, NIH-III, BTBR, NUJM, SJLSmn.AK
D
NCR
Hsd
BALB/c
NIH-III
NU/NU
AKR/J
B6.129/J*
B6CByF1/J
CByJ.Cg/J
BTBR/J*
C57BL/6/J*
CByB6F1/J
NUJM
SJLSmn.AK
STOCK Ces1c/J
C
22Rv1
CWR-R1
XMRV
XMRV + PreXMRV-1
PreXMRV-2
mouse IAP
E
736
777
9216R
9218R
8R
8L
22Rv1
CWR-R1
293T
C
2152
2524
2272
2274
C
XMRV
XMRV + PreXMRV-1
PreXMRV-2
mouse IAP
hGAPDH


A
0
1K
2K
3K
4K
5K
6K
7K
8K
PreXMRV-1
6
5
4
3
2
1
PreXMRV-2
29
46
Recombination
4
3
2
1
23
73
20
59
A5
A (I)
Predicted Recombinant
G
A
A5
A6
G (V)
Consensus XMRV
A
T
A6
8092*
790
6698
7154
8092
B
PreXMRV-1/2 recombinant
XMRV-22Rv1/CWR-R1
XMRV-VP42
XMRV-VP35
XMRV-VP62
XMRV-WPI-1106
XMRV-WPI-1178

Sciencexpress Letter

Editorial Expression of Concern

Bruce Alberts

Editor-in-Chief

In the issue of 23 October 2009, *Science* published the Report "Detection of an infectious retrovirus, XMRV, in blood cell of patients with chronic fatigue syndrome," a study by Lombardi *et al*. purporting to show that a retrovirus called XMRV (xenotropic murine leukemia virus-related virus) was present in the blood of 67% of patients with chronic fatigue syndrome (CFS) compared with 3.7% of healthy controls (*1*). Since then, at least 10 studies conducted by other investigators and published elsewhere have reported a failure to detect XMRV in independent populations of CFS patients. In this week's edition of *Science* Express, we are publishing two Reports that strongly support the growing view that the association between XMRV and CFS described by Lombardi *et al*. likely reflects contamination of laboratories and research reagents with the virus. In the first Report, "Recombinant origin of the retrovirus XMRV" (*2*), T. Paprotka *et al*. trace the ancestry of XMRV and provide evidence that the virus originated when two mouse leukemia viruses underwent recombination during experimental passage of a human prostate tumor xenograft in mice in the 1990s. A combination of sequencing, phylogenetic, and probability analyses lead Paprotka *et al*. to conclude that laboratory contamination with XMRV produced by a cell line (22Rv1) derived from these early xenograft experiments is the most likely explanation for detection of the virus in patient samples. In the second Report, "No evidence of murine-like gammaretroviruses in CFS patients previously identified as XMRV-infected" (*3*), K. Knox *et al*. examined blood samples from 61 CFS patients from the same medical practice that had provided patient samples to Lombardi *et al*., including 43 patients who had been diagnosed previously as XMRV-positive. Comprehensive assays by Knox *et al*. for viral nucleic acids, infectious virus, and virus-specific antibodies revealed no evidence of XMRV in any of the samples.

The study by Lombardi *et al*. (*1*) attracted considerable attention, and its publication in *Science* has had a far-reaching impact on the community of CFS patients and beyond. Because the validity of the study by Lombardi *et al*. is now seriously in question, we are publishing this Expression of Concern and attaching it to *Science*'s 23 October 2009 publication by Lombardi *et al*.

The U.S. National Institutes of Health is sponsoring additional carefully designed studies to ascertain whether the association between XMRV and CFS can be confirmed. *Science* eagerly awaits the outcome of these further studies and will take appropriate action when their results are known.

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Include this information when citing this paper.

（参考）

XMRVの疫学に関する主な文献一覧(平成22年5月18日作成、平成22年11月24日改訂）

血液事業部会運営委員会委員　岡田　義昭

【前立腺癌関係】

文献番号	文献名	XMRVの陽性率			検出法（組織）	報告国	要約
		前立腺がん	慢性疲労症候群	健康人			
1	Urisman A, et al., PLoS Pathog. 2006 Mar;2(3):e25. Identification of a novel Gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant.	9/86 10.5% （遺伝子の型による内訳） QQ 8/20 40%　RQ 0/14　0% RR 1/52 1.9%	-	-	RT-PCR（前立腺）	米国	DNAアレイによって前立腺がん組織から新たなウイルス（XMRV）を発見した。RNaseLにホモ型変異（QQ)にもつ前立腺癌の40%からXMRVが検出されたが、変異がない前立腺癌(RR)では1.9%であった。
2	Fischer N, Hellwinkel O, Schulz C, Chun FK, Huland H, Aepfelbacher M, Schlomm T. J Clin Virol. 2008 Nov;43(3):277-83 Prevalence of human gammaretrovirus XMRV in sporadic prostate cancer	1/87 1.2% (非家族性)	-	1/70 1.42%	RT-PCR（前立腺）	ドイツ	非家族性の前立腺がん組織からXMRVの検出が試みられた。その結果、欧州北部においてはほとんど検出されなかった。但し、本研究において、RNaseLにホモ型変異（QQ)をもつサンプルは6%未満であったことに注意を要する。
3	Hohn O, Krause H, Barbarotto P, Niederstadt L, Beimforde N, Denner J, Miller K, Kurth R, Bannert N. Retrovirology. 2009 Oct 16;6:92. Lack of evidence for xenotropic murine leukemia virus-related virus(XMRV) in German prostate cancer patients	0/589　0% (PCR) 0/146　0% （抗体）	-	0/5　0% （抗体）	PCR、RT-PCR（前立腺） ELISA（血清）	ドイツ	589例(76例の RNaseLホモ型変異型を含む）の前立腺癌組織からDNAとRNAを抽出し、核酸増幅法を用いてXMRVの遺伝子の有無を調べたが検出できなかった。また、血清中からもXMRVに反応する抗体は検出できなかった。
4	Schlaberg R, Choe DJ, Brown KR, Thaker HM, Singh IR. Proc Natl Acad Sci U S A. 2009 Sep 22;106(38):16351-6 XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors	14/233 6.2% PCR 54/233 23% ウイルス抗原	-	2/101 2% PCR 4/101 4% ウイルス抗原	PCR（前立腺） 組織染色（前立腺）	米国	233例の前立腺癌中14例からPCR法によってXMRV遺伝子が検出できた。RNaseLの変異とは関連がなかった。XMRVのタンパクは上皮細胞に存在していた。
13	Danielson B.P.,Ayala G.E.,and Kimata J.T. JID.2010 Nov.202:1470-77 Detection of xenotropic murine leukemia virus-related virus in normal and tumor tissue of patients from the southern United States with prostate cancer is dependent on specific polymerase chain reaction conditions	32/144　22.2%	-	-	PCR（前立腺）	米国（南部）	米国の南部にある州での前立腺癌患者からXMRV遺伝子の検出を行なった。前立腺癌の生検検体から DNAを抽出し、PCRを実施（env領域）した。32例が陽性であった。内28例は正常組織と癌組織を独立に検討でき、18例は両方とも陽性であった。XMRV陽性例ではRNASEL遺伝子の変異やgleason score（病理組織分類）との間に有意な差は認められなかった。
14	Aloia AL, Sfanos KS, Isaacs WB, Zheng Q, Maldarelli F, De Marzo AM, Rein A; Cancer Res; Published OnlineFirst October 21, 2010 XMRV: A New Virus in Prostate Cancer?	0/約800 0%	-	-	PCR（前立腺） 組織染色（前立腺）		約800の前立腺検体について、リアルタイムPCRと免疫組織染色を用い、XMRVの検出を試みた。その結果、前立腺癌にXMRVは見られなかった。XMRVは実際にはヒトには感染を起こしていない可能性がある。もし感染していても、このデータは前立腺癌との因果関係を支持しない。

XMRVの疫学に関する主な文献一覧(平成22年5月18日作成、平成22年11月24日改訂)
【慢性疲労症候群関係】

血液事業部会運営委員会委員　岡田　義昭

文献番号	文献名	XMRVの陽性率			検出法(組織)	報告国	要約
		前立腺がん	慢性疲労症候群	健康人			
5	Lombardi VC, Ruscetti FW, Das Gupta J, Pfost MA, Hagen KS, Peterson DL, Ruscetti SK, Bagni RK, Petrow-Sadowski C, Gold B, Dean M, Silverman RH, Mikovits JA. Science. 2009 Oct 23;326(5952):585-9 Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome	-	68/101 67%	8/218 3.7%	PCR (末梢単核球)	米国	慢性疲労性症候群(CFS)患者の67%からXMRV遺伝子が検出され、XMRVに感染したCFS患者の細胞や血漿中に感染性ウイルスが存在した。また、一部の症例ではウイルスと抗体が共存していた。 健常人の3.7%からもXMRVが検出された。CFS由来のXMRVは塩基配列が前立腺癌由来のものとクラスターを形成していた。
6	Erlwein O, Kaye S, McClure MO, Weber J, Wills G, Collier D, Wessely S, Cleare A. PLoS One. 2010 Jan 6;5(1):e8519. Failure to detect the novel retrovirus XMRV in chronic fatigue syndrome	-	0/186 0%	-	PCR (全血)	イギリス	慢性疲労性症候群186例を対象に全血から核酸増幅法によるXMRV遺伝子の検出を行ったが、検出できなかった。
7	Groom HC, Boucherit VC, Makinson K, Randal E, Baptista S, Hagan S, Gow JW, Mattes FM, Breuer J, Kerr JR, Stoye JP, Bishop KN. Retrovirology. 2010 Feb 15;7:10 Absence of xenotropic murine leukaemia virus-related virus in UK patients with chronic fatigue syndrome	-	0/136 0% DNA 0/140 0% RNA	0/95 0% DNA 0/141 0% RNA	PCR(全血) RT-PCR(血清)	イギリス	全血及び血清から核酸を抽出し、核酸増幅法を用いてXMRVの遺伝子を検出したが、慢性疲労性症候群及び健常人から検出されなかった。
8	van Kuppeveld FJ, de Jong AS, Lanke KH, Verhaegh GW, Melchers WJ, Swanink CM, Bleijenberg G, Netea MG, Galama JM, van der Meer JW. BMJ. 2010 Feb 25;340:c1018 Prevalence of xenotropic murine leukaemia virus-related virus in patients with chronic fatigue syndrome in the Netherlands: retrospective analysis of samples from an established cohort	-	0/32 0% RNA	0/43 0% RNA	RT-PCR (末梢単核球)	オランダ	1991～1992年に凍結保存されていた末梢単核球からRNAを抽出し、核酸増幅法によってXMRV遺伝子を検出したが、慢性疲労性症候群及び健常人から1例も検出されなかった。
9	Switzer WM, Jia H, Hohn O, Zheng HQ, Tang S, Shankar A, Bannert N, Simmons G, Hendry RM, Falkenberg VR, Reeves WC, Heneine W; Retrovirology 2010, 7:57 Absence of evidence of Xenotropic Murine Leukemia Virus-related virus infection in persons with Chronic Fatigue Syndrome and healthy controls in the United States	-	0/51 0% DNA	0/56 0% DNA	PCR (末梢単核球) 免疫学的試験	米国	米国カンザス州とジョージア州のCFS患者51名とコントロール56名の血清について、PCRと抗体検査が行われた。その結果、いずれからもXMRVは検出されなかった。この結果は、XMRVとCFSの関係を支持しない。
10	Lo SC, Pripuzova N, Li B, Komaroff AL, Hung GC, Wang R, and Alter H.J.PNAS.2010,107.1470-77 Detection of MLV-related virus gene sequences in blood of patients with chronic fatigue syndrome and healthy blood donors	-	32/37 86.5% DNA (XMRVとは異なるウイルス)	3/44 6.8% DNA (XMRVとは異なるウイルス)	PCR (末梢単核球)	米国	既に報告されているgag領域のプライマーを用いて37人のCFS末梢血を解析したところ、32人からマウス白血病に類似したレトロウイルスが検出された。塩基配列からは、XMRVよりもpolytropic(多種指向性)マウス内因性レトロウイルスに類似していた。
11	Barnes E.,Flanagan P.,Brown A.,Robinson N.,Brown H.,McClure M.,Oxenius A.,Collier J.,Weber J.,Gunthard H.F.,Hirschel B.,Fidler S.,Phillips R.,and Frater J. JID.2010 Failure to detect xenotropic murine leukemia virus-related virus in blood of individuals at high risk of blood-borne viral infection	-	0/151 0% DNA 0/79 0% RNA	-	PCR (末梢単核球) RT-PCR (血漿)	英国 西ヨーロッパ	英国と西ヨーロッパの HIV-1感染者163人(慢性期84人、急性期79人)とHCV感染者67人(慢性期)において、慢性感染者からは DNA、急性期にある感染者からはRNAを抽出し、 NAT検査を実施したが、XMRVの遺伝子は検出できなかった。さらにgagに対するT細胞の反応性も63人で検討したが、反応性は認められなかった。以上から、英国や西ヨーロッパでは血液や性的感染リスクを持つヒトでのXMRV感染率は高くなかった。
12	Hnrich T.J.,Li J.Z.,Felsenstein D.,Kotton C.N.,Plenge R.M.,Pereyra F.,Marty F.M.,Lin N.H.,Grazioso P.,Crochiere D.M.,Eggers D.,Kuritzkes D.R.,and Tsibris A.M.N.JID.2010 Xenotropic mueine leukemia virus-related virus prevalence in patients with chronic fatigue syndrome or chronic immunomodulatory conditions	-	0/198 0% DNA	0/95 0% DNA	PCR (末梢単核球)	米国	ボストン周囲にある大学病院において、XMRV感染の頻度を調べるためにCFS32人、 HIV感染者43人、幹細胞及び臓器移植患者26人、関節リュウマチ(RA)患者97人、RAのコントロールの患者95人計230人から DNAを抽出し NAT検査を行なった。XMRVの遺伝子は検出できなかった。