資料4

# XMRVの疫学に関する主な文献一覧

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 前立腺がん | 慢性疲労症候群 | 健康人 | 検出法(組織) | 報告国 | 要約 |
|---|---|---|---|---|---|---|---|
| 1 | Urisman A, et al., PLoS Pathog. 2006 Mar;2(3):e25.<br>Identification of a novel Gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant. | 9/86 10.5%<br>(遺伝子の型による内訳)<br>QQ 8/20 40%<br>RQ 0/14 0%<br>RR 1/52 1.9% | - | - | RT-PCR<br>(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<br>Prevalence of human gammaretrovirus XMRV in sporadic prostate cancer | 1/87 1.2%<br>(非家族性) | - | 1/70 1.42% | RT-PCR<br>(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.<br>Lack of evidence for xenotropic murine leukemia virus-related virus(XMRV) in German prostate cancer patients | 0/589 0%<br>(PCR)<br>0/146 0%<br>(抗体) | - | 0/5 0%<br>(抗体) | PCR、RT-PCR<br>(前立腺)<br>ELISA(血清) | ドイツ | 589例(76例のRNaseLホモ型変異(QQ)を含む)の前立腺がん組織から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<br>XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors | 14/233 6.2%<br>PCR<br>54/233 23%<br>ウイルス抗原 | - | 2/101 2%<br>PCR<br>4/101 4%<br>ウイルス抗原 | PCR<br>(前立腺)<br>組織染色<br>(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。 |
| 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<br>Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome | - | 68/101 67% | 8/218 3.7% | PCR<br>(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.<br>Failure to detect the novel retrovirus XMRV in chronic fatigue syndrome | - | 0/186 0% | - | PCR<br>(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<br>Absence of xenotropic murine leukaemia virus-related virus in UK patients with chronic fatigue syndrome | - | 0/136 0%<br>DNA<br>0/140 0%<br>RNA | 0/95 0%<br>DNA<br>0/141 0%<br>RNA | PCR(全血)<br>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, Swaninк CM, Bleijenberg G, Netea MG, Galama JM, van der Meer JW. BMJ. 2010 Feb 25;340:c1018<br>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%<br>RNA | 0/43 0%<br>RNA | RT-PCR<br>(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。 |

文献1

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PLoS PATHOGENS

# Identification of a Novel Gammaretrovirus in Prostate Tumors of Patients Homozygous for R462Q *RNASEL* Variant

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**Ribonuclease L (RNase L) is an important effector of the innate antiviral response. Mutations or variants that impair function of RNase L, particularly R462Q, have been proposed as susceptibility factors for prostate cancer. Given the role of this gene in viral defense, we sought to explore the possibility that a viral infection might contribute to prostate cancer in individuals harboring the R462Q variant. A viral detection DNA microarray composed of oligonucleotides corresponding to the most conserved sequences of all known viruses identified the presence of gammaretroviral sequences in cDNA samples from seven of 11 R462Q-homozygous (QQ) cases, and in one of eight heterozygous (RQ) and homozygous wild-type (RR) cases. An expanded survey of 86 tumors by specific RT-PCR detected the virus in eight of 20 QQ cases (40%), compared with only one sample (1.5%) among 66 RQ and RR cases. The full-length viral genome was cloned and sequenced independently from three positive QQ cases. The virus, named XMRV, is closely related to xenotropic murine leukemia viruses (MuLVs), but its sequence is clearly distinct from all known members of this group. Comparison of *gag* and *pol* sequences from different tumor isolates suggested infection with the same virus in all cases, yet sequence variation was consistent with the infections being independently acquired. Analysis of prostate tissues from XMRV-positive cases by in situ hybridization and immunohistochemistry showed that XMRV nucleic acid and protein can be detected in about 1% of stromal cells, predominantly fibroblasts and hematopoietic elements in regions adjacent to the carcinoma. These data provide to our knowledge the first demonstration that xenotropic MuLV-related viruses can produce an authentic human infection, and strongly implicate RNase L activity in the prevention or clearance of infection in vivo. These findings also raise questions about the possible relationship between exogenous infection and cancer development in genetically susceptible individuals.**

Citation: Urisman A, Molinaro RJ, Fischer N, Plummer SJ, Casey G, et al. (2006) Identification of a novel gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant. PLoS Pathog 2(3): e25.

## Introduction

Type I interferons (IFNs) are rapidly mobilized in response to viral infection and trigger potent antiviral responses. One such response is the induction by IFN of a family of 2′5′ oligoadenylate synthetases (OAS); upon activation by virally encoded dsRNA, these enzymes produce 5′-phosphorylated 2′-5′ linked oligoadenylates (2–5A) from ATP [1]. 2–5A, in turn, is an activator of ribonuclease L (RNase L) [2], which degrades viral (and cellular) single stranded RNAs [3]. In vivo evidence for the antiviral role of the 2–5A system was provided by studies with RNase $L^{-/-}$ mice, which have enhanced susceptibility to infections by the picornaviruses, encephalomyocarditis virus, and Coxsackievirus B4 [4,5]. Ultimately, sustained activation of RNase L triggers a mitochondrial pathway of apoptosis that eliminates virus-infected cells [4,6–8]. Genetic lesions in RNase L impair this apoptotic response, which has raised interest in the possibility that such mutations might also contribute to malignancy [9].

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Abbreviations: 2-5A, 5′-phosphorylated 2′-5′ oligoadenylate; aa, amino acid; FISH, fluorescence in situ hybridization; H&E, hematoxylin and eosin; HPC, hereditary prostate cancer; IFN, interferon; IHC, immunohistochemistry; LTR, long terminal repeat; MCF, mink cell focus-inducing murine leukemia virus; MTCR, murine type C retrovirus; MuLV, murine leukemia virus; nt, nucleotide(s); NZB-9-1, New Zealand Black 9-1 xenotropic retrovirus; ORF, open reading frame; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; QQ, *RNASEL* homozygous R462Q; QR, *RNASEL* heterozygous R462Q; RNase L, Ribonuclease L; RR, *RNASEL* homozygous wild-type; SFFV, spleen focus-forming virus; VR, variable region; XMRV, xenotropic MuLV-related virus; XPR1, xenotropic and polytropic retrovirus receptor

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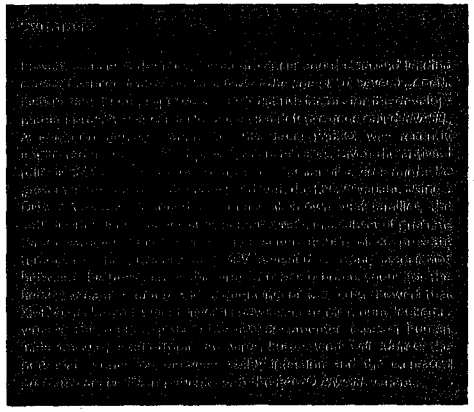
☯ These authors contributed equally to this work.



In this context, several recent studies have linked germline mutations in RNase L to prostate cancer susceptibility [10–13]. Prostate cancer has a complex etiology influenced by androgens, diet, and other environmental and genetic factors [14]. While sporadic prostate cancer displays an age-related increase in prevalence, familial prostate cancer kindreds often display early-onset disease. Such kindreds, defined by having more than three affected members per family, account for 43% of early onset cases (<55 years old) and 9% of all cases [15]. The genetics of hereditary prostate cancer (HPC) is complex, and several genes have been proposed as susceptibility factors in this syndrome. Interestingly, one of these, *HPC1*, is linked to *RNASEL* [10,11]. Several germline mutations or variants in *HPC1/RNASEL* have been observed in HPC [10–13] (reviewed in [16]), including a common (35% allelic frequency) missense variant of RNase L, in which a G to A transition at nucleotide (nt) position 1385 (G1385A) results in a glutamine instead of arginine at amino acid position 462 (R462Q). Remarkably, a large, controlled sib-pair study implicated the R462Q RNase L variant in up to 13% of unselected prostate cancer cases [11]. One copy of the mutated gene increased the risk of prostate cancer by about 50%, whereas individuals that were homozygous for the mutation had a 2-fold increased risk of prostate cancer. The R462Q RNase L variant had a 3-fold decrease in catalytic activity compared with the wild-type enzyme [9,11]. However, while several case-controlled genetic and epidemiologic studies support the involvement of *RNASEL* (and notably the R462Q variant) in prostate cancer etiology [10–13], others do not [17–19], suggesting that either population differences or environmental factors may modulate the impact of *RNASEL* on prostatic carcinogenesis.

While the antiapoptotic phenotype of RNase L deficiency has dominated previous discussions of its possible linkage to cancer, RNase L is also a key effector of the antiviral action of interferons. This led us to consider the possibility that the putative linkage of RNase L alterations to HPC might reflect enhanced susceptibility to a viral agent. To test this hypothesis, we have examined RNA derived from wild-type and RNase L variant (R462Q) prostate tumors for evidence of viral sequences, by hybridization to a DNA microarray composed of the most conserved sequences of all known human, animal, plant, and bacterial viruses [20,21]. Here we report that 40% (eight of 20) of all tumors homozygous for the R462Q allele harbored the genome of a distinct gammaretrovirus closely related to xenotropic murine leukemia viruses (MuLVs). In contrast, retroviral sequences were present in <2% of tumors bearing at least one copy of the wild-type allele (one of 66). In addition, virus-harboring cells were detected within infected prostatic tumor tissues by fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC). These findings represent the first detection of xenotropic MuLV-like agents in humans, and reveal a strong association between infection with the virus and defects in RNase L activity. The relation of retroviral infection to prostate cancer will require further study, but a cofactor role is not excluded.

## Results

### Detection of XMRV by Microarray-Based Screening

To search for potential viruses in prostate cancer tumors, we employed a DNA microarray-based strategy designed to screen for viruses from all known viral families [20,21]. Total or polyadenylated RNA extracted from tumor tissue was first amplified and fluorescently labeled in a sequence-nonspecific fashion. The amplified and labeled fragments, which contained host as well as potential viral sequences, were then hybridized to a DNA microarray (Virochip, University of California San Francisco, San Francisco, United States) bearing the most conserved sequences of ~950 fully sequenced NCBI reference viral genomes (~11,000 70-mer oligonucleotides).

The Virochip was used to screen RNA samples isolated from prostate tumors of 19 individuals (Figure 1). A positive hybridization signal suggestive of a gammaretrovirus was detected in seven of 11 tumors from patients homozygous for the R462Q *RNASEL* variant (QQ). In contrast, no virus was detected in three tumors from RQ heterozygotes, and only one of five tumors from RR individuals was positive. Clustering of the microarray oligonucleotide intensities (Figure 1) revealed a similar hybridization pattern in all positive cases. Furthermore, a computational analysis using E-Predict, a recently described algorithm for viral species identification [22], suggested that the same or similar *mammalian gammaretrovirus* was present in all positive tumors (Table S1). Thus, the Virochip detected the presence of a probable gammaretrovirus in half of the QQ tumor samples and in only one non-QQ sample.

### Characterization of XMRV Genome

To further characterize the virus, we recovered its entire genome from one of the tumors (VP35) (Figure 2). To obtain viral clones, we first employed a direct microarray recovery technique described previously [21]. Briefly, amplified nucleic acid from the tumor tissue, which hybridized to viral microarray oligonucleotides, was eluted from two specific spots. The eluted DNA was re-amplified, and plasmid libraries constructed from this material were screened by colony hybridization using the spots' oligonucleotides as probes. The array oligonucleotides used in this case derived from the LTR region of murine type C retrovirus (MTCR) and spleen focus-





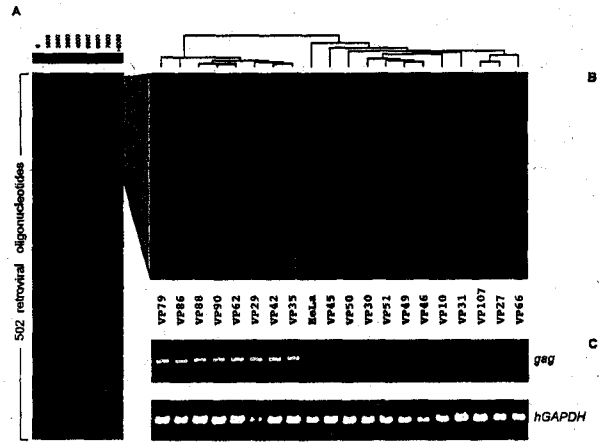



**Figure 1.** XMRV Detection by DNA Microarrays and RT-PCR

(A) Virochip hybridization patterns obtained for tumor samples from 19 patients. The samples (*x*-axis) and the 502 retroviral oligonucleotides present on the microarray (*y*-axis) were clustered using hierarchical clustering. The red color saturation indicates the magnitude of hybridization intensity.
(B) Magnified view of a selected cluster containing oligonucleotides with the strongest positive signal. Samples from patients with QQ *RNASEL* genotype are shown in red, and those from RQ and RR individuals as well as controls are in black.
(C) Results of nested RT-PCR specific for XMRV *gag* gene. Amplified *gag* PCR fragments along with the corresponding human *GAPDH* amplification controls were separated by gel electrophoresis using the same lane order as in the microarray cluster.
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forming virus (SFFV) [23]. The largest recovered fragment was 415 nt in length, and had 96% nt identity to the LTR region of MTCR, a MuLV identified in the genome of a mouse myeloma cell line (T. Heinemeyer, unpublished data). These findings established that the virus in question was indeed a gammaretrovirus, and likely a relative of MuLVs. To clone and sequence the rest of the viral genome from sample VP35, we used tumor cDNA to PCR-amplify overlapping segments using primers derived from MTCR; gaps were closed using primers from earlier recovered clones (Figure 2B and Table S2). Using a similar strategy, we have also determined the full sequence of the virus from a second tumor, VP42. Finally, a complete viral genomic sequence from a third tumor case, VP62, was obtained by PCR amplification of two ~4 Kb-long overlapping fragments jointly spanning close to the entire length of the virus (Figure 2B). The three sequenced genomes share >98% nt identity overall and >99% amino acid (aa) identity for predicted open reading frames (ORFs), and thus represent the same virus.

The full genome of the virus (Figures 2 and S1) is 8,185 nt long and is distinct from all known isolates of MuLV. The genome is most similar to the genomes of exogenous MuLVs, DG-75 cloned from a human B-lymphoblastoid cell line [24], and MTCR, with which it shares 94% and 93% overall nt sequence identity, respectively. The genome also shares up to 95% nt identity with several full-length *Mus musculus* endogenous proviruses (Figure 2C). Phylogenetic trees constructed using available mammalian type C retroviral genomes and representative full-length proviral sequences from the mouse genome (Figures 3 and S2) showed that the newly identified virus is more similar to xenotropic and polytropic than to ecotropic genomes. Based on these findings we propose the provisional name Xenotropic MuLV-related virus (XMRV) for this agent.

Translation of the XMRV genomic sequence using ORF Finder [25] identified two overlapping ORFs coding for the full-length Gag-Pro-Pol and Env polyproteins. No exogenous coding sequences, such as viral oncogenes, could be detected in the XMRV genome. The predicted Gag polyprotein is 536 aa long and is most similar to a xenotropic provirus on *M. musculus* Chromosome 9, with which it shares 97% aa identity (Figure S2A). The Pro-Pol polyprotein is 1,197 aa long and has the highest aa identity with MuLV DG-75 and a xenotropic provirus on *M. musculus* Chromosome 4, 97% and 96%, respectively (Figure S2B). An amber (UAG) stop codon separates the Gag and Pro-Pol coding sequences, analogous to other MuLVs in which a translational read-through is required to generate the full-length Gag-Pro-Pol polyprotein (reviewed in [26]).

Similar to other MuLVs [23,24,27–31], the Env polyprotein of XMRV is in a different reading frame compared with Gag-Pro-Pol. The Env protein sequence is 645 aa long, and has the highest amino acid identity with the Env protein of an infectious MuLV isolated from a human small cell lung cancer line NCI-417 [32] and MuLV New Zealand Black 9–1 xenotropic retrovirus (NZB-9–1) [28]), 95% and 94%, respectively. The XMRV Env protein also shares similarly high identity with several murine xenotropic proviruses

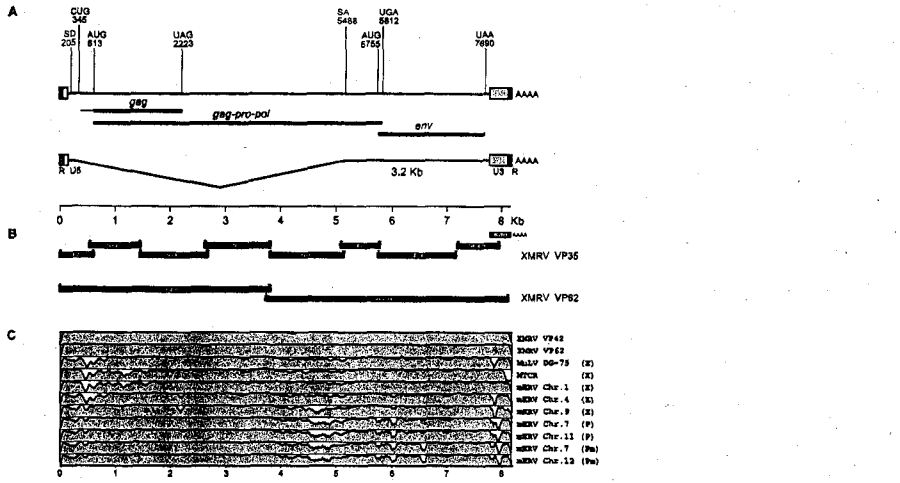


**Figure 2.** Complete Genome of XMRV

(A) Schematic map of the 8185 nt XMRV genome. LTR regions (R, U5, U3) are indicated with boxes. Predicted open reading frames encoding Gag, Gag-Pro-Pol, and Env polyproteins are labeled in green. The corresponding start and stop codons (AUG, UAG, UGA, UAA) as well as the alternative Gag start codon (CUG) are shown with their nt positions. Similarly, splice donor (SD) and acceptor (SA) sites are shown and correspond to the spliced 3.2-Kb Env subgenomic RNA (wiggled line).

(B) Cloning and sequencing of XMRV VP35 and VP62 genomes. Clones obtained by probe recovery from hybridizing microarray oligonucleotides (blue bar) or by PCR from tumor cDNA (black bars) were sequenced. Primers used to amplify individual clones (Table S2) were derived either from the genome of MTCR (black arrows) or from overlapping VP35 clones (blue arrows).

(C) Genome sequence similarity plots comparing XMRV VP35 with XMRV VP42, XMRV VP62, MuLV DG-75, MTCR, and a set of representative non-ecotropic proviruses (mERVs) (see Materials and Methods). The alignments were made using AVID [81], and plots were generated using mVISTA [82] with the default window size of 100 nt. *Y*-axis scale for each plot represents percent nt identities from 50% to 100%. Sequences are labeled as xenotropic (X), polytropic (P), or modified polytropic (Pm).

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(Figure S2C). Conserved splice donor (AGGTAAG, position 204) and acceptor (CACTTACAG, position 5,479) sites involved in the generation of *env* subgenomic RNAs [33] were found in the same relative locations as in other MuLV genomes. A multiple sequence alignment of XMRV Env and corresponding protein sequences of other representative MuLVs (Figure 4) showed that within three highly variable regions (VR), VRA, VRB, and VRC, known to be important for cellular tropism [34–36], XMRV has the highest aa identity with xenotropic envelopes from MuLVs NZB-9-1, NFS-Th-1 [37], and DG-75. Although unique-to-XMRV aa are present in each of the three VRs, based on the overall similarity to the known xenotropic envelopes, we predict that the cellular receptor for XMRV is XPR1 (SYG1), the recently identified receptor for xenotropic and polytropic MuLVs [38–40].

The long terminal repeat (LTR) of XMRV is 535 nt long and has the highest nt identity with the LTRs from xenotropic MuLVs NFS-Th-1 (96%) and NZB-9-1 (94%). The XMRV LTRs contain known structural and regulatory elements typical of other MuLV LTRs [33,41]. In particular, the CCAAT box, TATAAAA box, and AATAAA polyadenylation signal sequences were found in U3 at their expected locations (Figure S3A). U3 also contains a glucocorticoid response element sequence AGA ACA GAT GGT CCT. Essentially identical sequences are present in genomes of other MuLVs. These elements have been shown to activate LTR-directed transcription and viral replication in vitro in response to various steroids including androgens [42–45]. In addition, presence of an intact glucocorticoid response element is thought to be the determinant of higher susceptibility to FIS-2 MuLV infection in male compared with female NMRI mice [46,47]. Despite these similarities, single nt substitutions unique to XMRV and an insertion of an AG dinucleotide immediately downstream from the TATA box are present in U3 (Figure S3A). Consistent with these findings, a phylogenetic analysis based on U3 sequences from XMRV and from representative xenotropic MuLV provirus groups [48,49] showed that XMRV U3 sequences formed a well-separated cluster most similar to the group containing NFS-Th-1 and NZB-9-1 (Figure S3B).

The 5′ *gag* leader of XMRV, defined as the sequence extending from the end of U5 to the ATG start codon of *gag*, consists of a conserved non-coding region of ~200 nt, containing a proline tRNA primer binding site as well as sequences required for viral packaging [50,51] and the initiation of translation [52,53]. The non-coding region is followed by a ~270-nt region extending from the conserved CTG alternative start codon of *gag*. This region represents the





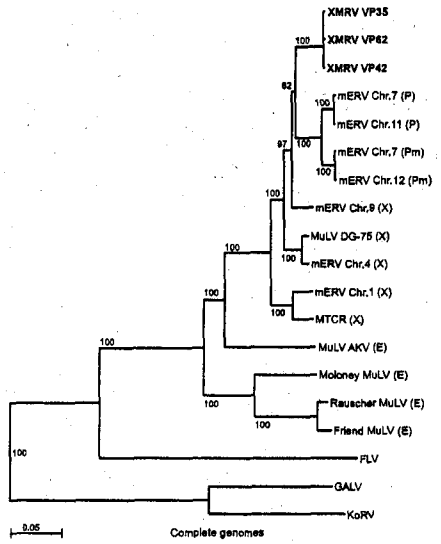


**Figure 3.** Phylogenetic Analysis of XMRV Based on Complete Genome Sequences

Complete genomes of XMRV VP35, VP42, and VP62 (red); MTCR; MuLVs DG-75, AKV, Moloney, Friend, and Rauscher; feline leukemia virus (FLV); koala retrovirus (KoRV); gibbon ape leukemia virus (GALV); and a set of representative non-ecotropic proviruses (mERVs) were aligned using ClustalX (see Materials and Methods). An unrooted neighbor-joining tree was generated based on this alignment, excluding gaps and using Kimura's correction for multiple base substitutions. Bootstrap values ($n$ = 1000 trials) are indicated as percentages. Sequences are labeled as xenotropic (X), polytropic (P), modified polytropic (Pm), or ecotropic (E).

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most divergent segment of the genome compared with other MuLVs (Figures 5 and 2C). Unlike ecotropic MuLVs, where translation from this codon adds an ~90 aa N-terminal leader peptide in frame with the rest of the Gag protein, thus generating a glycosylated form of Gag [54], XMRV has a stop codon 53 aa residues downstream from the alternative start. Interestingly, both MuLV DG-75 and MTCR *gag* leader sequences are also interrupted by stop codons, and therefore are not expected to produce full-length glyco-Gag. Furthermore, a characteristic 24-nt deletion was present in this region of the XMRV genome, which is not found in any known exogenous MuLV isolate. However, a shorter deletion of nine nt internal to this region is present in the sequences of several non-ecotropic MuLV proviruses found in the sequenced mouse genome (Figure 5). In cell culture, expression of intact glyco-Gag is not essential for viral replication [55,56]. However, lesions in this region have been associated with interesting variations in pathogenetic properties in vivo [57–61]. For example, an alteration in ten nt affecting five residues in the N-terminal peptide of glyco-Gag was found to be responsible for a 100-fold difference in the frequency of neuroinvasion observed between CasFrKP and CasFrKP41 MuLV strains [62]. In addition, insertion of an octanucleotide resulting in a stop codon downstream of the CUG start codon prevented severe early hemolytic anemia and prolonged latency of erythroleukemia in mice infected with Friend MuLV [58]. While we do not yet know the pathogenetic significance of the lesions in XMRV glyco-Gag, the high degree of sequence divergence suggests that this region may be under positive selective pressure and therefore may be relevant to the establishment of infection within the human host.

## Association of XMRV Infection and R462Q *RNASEL* Genotype

To further examine the association between presence of the virus and the R462Q (1385G->A) *RNASEL* genotype, we developed a specific nested RT-PCR assay based on the virus

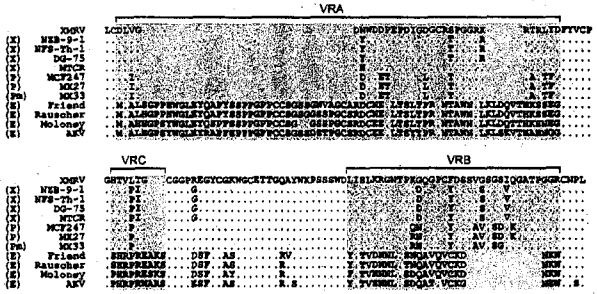


**Figure 4.** Multiple-Sequence Alignment of Protein Sequences from XMRV and Related MuLVs Spanning SU Glycoprotein VRA, VRB, and VRC, Known to Determine Receptor Specificity

Env protein sequence from XMRV (identical in VP35, VP42, and VP62; red); MTCR; MuLVs DG-75, NZB-9-1, NFS-Th-1, MCF247, AKV, Moloney, Friend, and Rauscher; and polytropic proviruses MX27 and MX33 [77] were aligned using ClustalX. Sequences are labeled as xenotropic (X), polytropic (P), modified polytropic (Pm), or ecotropic (E). VRs are boxed. Dots denote residues identical to those from XMRV, and deleted residues appear as spaces.

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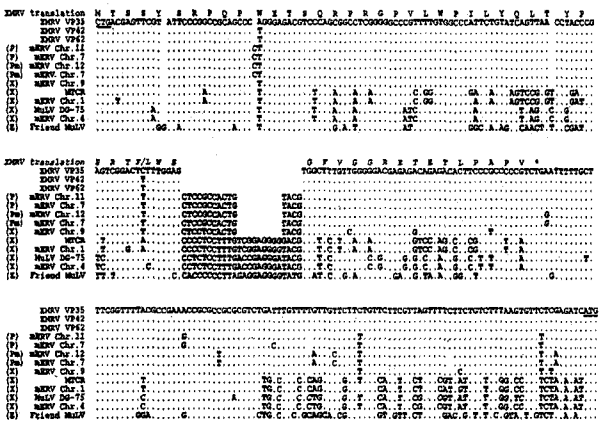

**Figure 5.** Multiple-Sequence Alignment of 5′ *gag* Leader Nucleotide Sequences from XMRV and Related MuLVs

Sequences extending from the alternative CUG start codon to the AUG start codon (underlined) of *gag* derived from XMRV VP35, VP42, and VP62 (blue); MTCR, MuLVs DG-75, and Friend; and a set of representative non-ecotropic proviruses (mERVs) were aligned with ClustalX (see Materials and Methods). Predicted amino acid translation corresponding to the VP35 sequence is shown above the alignment (red); asterisk indicates a stop. Sequences are labeled as xenotropic (X), polytropic (P), modified polytropic (Pm), or ecotropic (E). Dots denote nt identical to those from XMRV, and deleted nt appear as spaces.

DOI: 10.1371/journal.ppat.0020025.g005

sequence recovered from one of the tumor samples (VP35, see above). The primers in this assay (Figure S1) amplify a 380-nt fragment from the divergent 5′ leader and the N-terminal end of *gag*. The RT-PCR was positive in eight (40%) of 20 examined tumors from homozygous (QQ) individuals. In addition, one tumor from a homozygous wild-type (RR) patient was positive among 52 RR and 14 RQ tumors examined (Figure 1 and Table 1). Interestingly, this case was associated with the highest tumor grade among all XMRV-positive cases (Table S3). PCR specific for the mouse *GAPDH* gene was negative in all samples (unpublished data), arguing strongly against the possibility that the tumor samples were contaminated with mouse nucleic acid. Collectively, these data demonstrate a strong association between the homozygous (QQ) R462Q *RNASEL* genotype and presence of the virus in the tumor tissue ($p < 0.00002$ by two-tail Fisher's exact test).

**Table 1.** XMRV Screening by *gag* Nested RT-PCR

| PCR | *RNASEL* Genotype[a] | | | Total |
|---|---|---|---|---|
| | QQ | RQ | RR | |
| PCR + | 8 | 0 | 1 | 9 |
| PCR − | 12 | 14 | 51 | 77 |
| Total | 20 | 14 | 52 | 86 |

[a]*RNASEL* genotypes are as follows: QQ, homozygous R462Q variant; RQ, heterozygous; RR, homozygous wild-type.

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## XMRV Sequence Diversity in Samples from Different Patients

To examine the degree of XMRV sequence diversity in different patients, we sequenced the amplified fragments from all nine samples, which were positive by the nested *gag* RT-PCR. The amplified *gag* fragments were highly similar (Figure 6A) with >98% nt and >98% aa identity to each other. In contrast, the fragments had <89% nt and <95% aa identity with the most related exogenous sequence of MuLV DG-75. Several corresponding endogenous non-ecotropic sequences were more similar to the XMRV fragments, including the xenotropic provirus from *M. musculus* Chromosome 9, which was <98% identical on the nt level. Nevertheless, all XMRV-derived fragments were more similar to each other than they were to any other sequence.

In addition to the *gag* gene, we also examined the same patient samples for sequence variation in the *pol* gene. We sequenced PCR fragments obtained with a set of primers targeting a 2500-nt stretch in the *pol* gene (Figure S1). Similar to the *gag* fragments, the amplified *pol* fragments were highly similar (Figure 6B) and had >97% nt and >97% aa identity to each other. In contrast, the fragments had <94% nt and <95% aa identity with the most related sequence, that of MuLV DG-75. Interestingly, XMRV-derived *pol* sequences were less similar to and approximately equidistant from the examined representative xenotropic and polytropic endogenous sequences.

Close clustering of the sequenced *gag* and *pol* fragments (Figure 6) indicates that all microarray and RT-PCR positive cases represent infection with the same virus. On the other hand, the degree of sequence variation in the examined





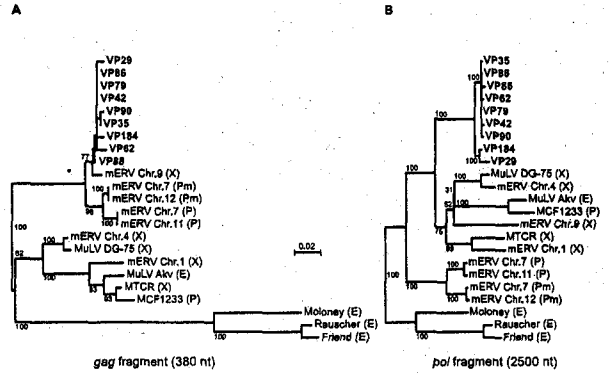


**Figure 6.** Comparison of XMRV Sequences Derived from Tumor Samples of Different Patients

(A) Phylogenetic tree based on the 380 nt XMRV *gag* RT-PCR fragment from the nine positive tumor samples (red) and the corresponding sequences from MTCR; MuLVs DG-75, MCF1233, Akv, Moloney, Rauscher and Friend; and a set of representative non-ecotropic proviruses (mERVs). The sequences were aligned using ClustalX, and the corresponding tree was generated using the neighbor-joining method (see Materials and Methods). Bootstrap values ($n = 1000$ trials) are indicated as percentages. Sequences are labeled as xenotropic (X), polytropic (P), modified polytropic (Pm), or ecotropic (E).
(B) Phylogenetic tree based on a 2500-nt *pol* PCR fragment from the 9 XMRV-positive tumor samples. The tree was constructed as described in (A).

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fragments is higher than that expected from errors introduced during PCR amplification and sequencing. The frequency of nt misincorporation by Taq polymerase has been estimated as $10^{-6} - 10^{-4}$ ([63] and references therein), compared with the observed rate of up to 2% in the *gag* and *pol* fragments. These findings suggest that the observed XMRV sequence variation is a result of natural sequence diversity, consistent with the virus being independently acquired by the affected patients, and argue against laboratory contamination as a possible source of XMRV.

## Detection of XMRV in Tumor-Bearing Prostatic Tissues Using FISH

To localize XMRV within human prostatic tissues, and to measure the frequency of the infected cells, XMRV nucleic acid was visualized using FISH on formalin-fixed prostate tissues. A SpectrumGreen fluorescently labeled FISH probe cocktail spanning all viral genes was prepared using cDNA derived from the XMRV isolate cloned from patient VP35 (Materials and Methods). Distinct FISH-positive cells were observed in the tumors positive for XMRV by RT-PCR (e.g., VP62 and VP88) (Figure 7). To identify cell types associated with the positive FISH signal, the same sections were subsequently stained with hematoxylin and eosin (H&E). Most FISH-positive cells were stromal fibroblasts (Figure 8A), including those undergoing cell division (Figure 8B). In addition, occasional infected hematopoietic cells were also seen (Figure 8C). XMRV FISH with concurrent immunostaining for cytokeratin AE1/AE3 to achieve specific labeling of epithelial cells [64] showed no XMRV-infected cells that also had the epithelium-specific staining, confirming their non-epithelial origin (Figure 8C). While the XMRV nucleic acid was usually present within nuclei (Video S1), suggesting integrated proviral DNA, some cells showed cytoplasmic staining adjacent to the nucleus, suggestive of viral mRNA and/or pre-integration complexes in non-dividing cells (Figure 8A).

We also used FISH to obtain a minimal estimate of the frequency of XMRV-infected prostatic cells. For this purpose we employed a tissue microarray containing duplicates of 14 different prostate cancer tissue specimens (Table 2). FISH with DNA probes derived from XMRV VP35 showed five to ten XMRV/FISH-positive cells (about 1% of prostate cells observed) in each of five homozygous RNase L 462Q (QQ) cases: VP29, 31, 42, 62, and 88. Patient sample VP79, also a QQ case, contained two positive cells (0.4% of total cells examined). All of the XMRV/FISH-positive cells observed were stromal cells. In contrast, three RR tissue samples and two RQ tissue samples showed one or no (<0.15%) FISH-positive cells. Two of the QQ cases, VP35 and VP90, positive by *gag* RT-PCR, showed only one FISH-positive cell each (Table 2). Conversely, one case, VP31, was FISH-positive, but *gag*-RT-PCR negative. As expected, Chromosome 1–specific probes used as a positive control specifically labeled nearly every cell from the examined case VP88, whereas a KSHV-specific probe used as a negative control did not label any cells in sections from cases VP88 and VP51, but did efficiently label 293T cells transfected with KSHV DNA (unpublished data). Thus, consistent with the microarray and RT-PCR data, detection of XMRV by FISH was associated primarily with QQ cases. In addition, in samples where XMRV was detected, all positive cells were stromal and did not account for more than 1% of all prostatic cells. Finally, differences in the numbers of XMRV-positive cells detected in the different samples could be due to heterogeneity in virus copy numbers between different patients and/or specific regions of the prostate sampled.

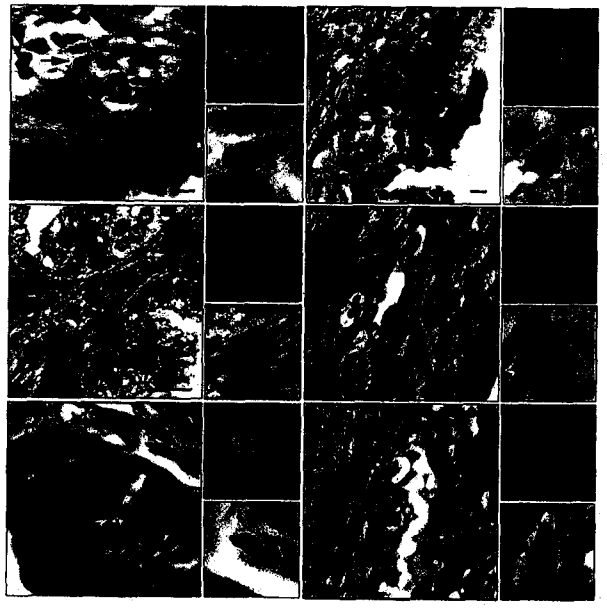

**Figure 7.** Detection of XMRV Nucleic Acid in Prostatic Tissues Using FISH

Prostatic tumor tissue sections from QQ cases VP62 (A–C) and VP88 (D–F) were analyzed by FISH using DNA probes (green) derived from XMRV VP35 (top right enlargements). Nuclei were counterstained with DAPI. The same sections were then visualized by H&E staining (left panels). Scale bars are 10 μm. Arrows indicate FISH positive cells, and their enlarged images are shown in the bottom right panels.

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### Detection of XMRV in Tumor-Bearing Prostatic Tissues Using IHC

To identify cells expressing XMRV proteins, we assayed for the presence of Gag protein using a monoclonal antibody against (SFFV); this antibody is reactive against Gag proteins from a wide range of different ecotropic, polytropic, and xenotropic MuLV strains [65]. Using this antibody, positive signal by IHC was observed in prostatic tissues of XMRV-positive cases VP62 and VP88, both QQ (Figure 9). An enhanced alkaline phosphatase red detection method allowed Gag detection in the same cells with both fluorescence (Figure 9A–9D, left) and bright field (Figure 9A–9D, middle) microscopy. The Gag-expressing cells were observed in prostatic stromal cells with a distribution and frequency similar to that detected by FISH (Figure 9 and unpublished data). In contrast, no Gag-positive cells were observed in VP51 prostatic tissue, which is of RR genotype (Figure 9E).

## Discussion

The results presented here identify XMRV infection in prostate tissue from approximately 40% of patients with prostate cancer who are homozygous for the R462Q variant (QQ) of RNase L, as judged by both hybridization to the Virochip microarray and by RT-PCR with XMRV-specific primers. Parallel RT-PCR studies of prostate tumors from wild-type (RR) and heterozygous (RQ) patients revealed evidence of XMRV in only one of 66 samples, clearly demonstrating that human XMRV infection is strongly linked to decrements in RNase L activity. This result supports the view that the R462Q RNase L variant leads to a subtle defect in innate (IFN-dependent) antiviral immunity.

As its name indicates, XMRV is closely related to xenotropic murine leukemia viruses (MuLVs). Unlike ecotropic MuLVs, such as the canonical Moloney MuLV, which grow only in rodent cells in culture, xenotropic MuLVs can grow in non-rodent cells in culture but not in rodent cell lines. Xenotropic viruses have been isolated from many inbred as well as wild mouse strains. Studies of the distribution of non-ecotropic sequences in different mouse strains show that the diversity of xenotropic proviral sequences in wild mice is greater than that found in the inbred laboratory strains [49,66]. This finding led to the conclusion that these endogenous elements were independently and relatively recently acquired by different mouse species as a result of infection rather than inheritance [49]. Unlike ecotropic MuLVs, which can only recognize a receptor (CAT-1) specific to mouse and rat species [67–69], xenotropic





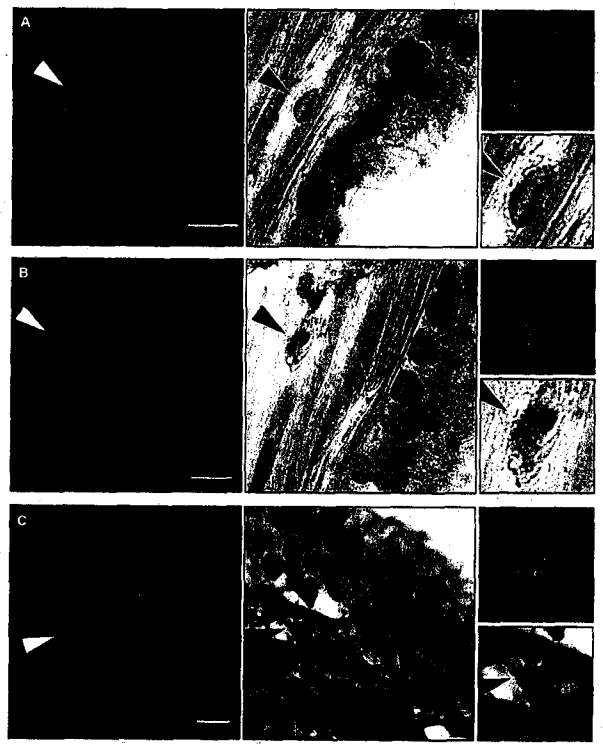


**Figure 8.** Characterization of XMRV-Infected Prostatic Cells by FISH and FISH/Immunofluorescence

Using a tissue microarray, prostatic tumor tissue sections from QQ case VP62 were analyzed by FISH (green) using DNA probes derived from XMRV VP35 (left panels). Nuclei were counterstained with DAPI. The same sections were then visualized by H&E staining (middle panels). Arrows indicate FISH-positive cells, and their enlarged FISH and H&E images are shown in the top right and bottom right panels, respectively. Scale bars are 10 μm.
(A) A stromal fibroblast.
(B) A dividing stromal cell.
(C) A stromal hematopoietic cell. The section was concomitantly stained for XMRV by FISH (green) and cytokeratin AE1/AE3 by immunofluorescence (red).

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viruses recognize a protein known as XPR1 or SYG1. XPR1 is expressed in all higher vertebrates, including mice, but polymorphisms in the murine gene render it unable to mediate xenotropic MuLV entry [38–40]. Thus, xenotropic MuLVs have a potential to infect a wide variety of mammalian species, including humans.

Xenotropic MuLVs have occasionally been detected in cultured human cell lines. For example, MuLV DG-75 was cloned from a human B-lymphoblastoid cell line [24], and an infectious xenotropic MuLV was detected in a human small cell lung cancer line NCI-417 [32]. Although laboratory contamination, either in culture or during passage of cell lines in nude mice, cannot be ruled out as a possible source in these cases, such contamination cannot explain our results. The evidence for this is as follows: (i) XMRV was detected in primary human tissues; (ii) no murine sequences (e.g., GAPDH) could be detected in our materials by PCR; (iii) infection was predominantly restricted to human samples with the QQ *RNASEL* genotype; (iv) polymorphisms were found in the XMRV clones recovered from different patients consistent with independent acquisition of the virus by these individuals; and (v) viral nucleic acids and antigens could be detected in infected QQ prostate tissue by FISH and IHC, respectively. Taken together, the above evidence argues strongly against laboratory contamination with virus or cloned DNA material as the source of XMRV infection in the analyzed samples. To our knowledge, this report represents the first published examples of authentic infection

**Table 2.** Frequency of XMRV-Infected Prostatic Cells Determined by FISH

| Patient | *RNASEL* Genotype[a] | Number of Cells Counted[b] | Number of FISH-Positive Cells (%) | XMRV FISH[c] | XMRV *gag* RT-PCR |
|---|---|---|---|---|---|
| VP 88 | QQ | 408 | 5 (1.23) | ++ | + |
| VP 31 | QQ | 526 | 6 (1.14) | ++ | − |
| VP 42 | QQ | 530 | 6 (1.13) | ++ | + |
| VP 62 | QQ | 904 | 10 (1.11) | ++ | + |
| VP 29 | QQ | 659 | 7 (1.06) | ++ | + |
| VP 79 | QQ | 464 | 2 (0.43) | + | + |
| VP 10 | QQ | 872 | 1 (0.12) | +/− | − |
| VP 35 | QQ | 849 | 1 (0.12) | +/− | + |
| VP 90 | QQ | 843 | 1 (0.12) | +/− | + |
| VP 45 | RQ | 987 | 0 (0) | − | − |
| VP 46 | RQ | 794 | 0 (0) | − | − |
| VP 30 | RR | 661 | 1 (0.15) | +/− | − |
| VP 50 | RR | 787 | 1 (0.13) | +/− | − |
| VP 51 | RR | 842 | 0 (0) | − | − |

[a]*RNASEL* genotypes are as follows: QQ, homozygous R462Q variant; RQ, heterozygous; RR, homozygous wild-type.
[b]*All types of prostatic cells are included.*
[c]+/− = 0.1%–0.2%; + = 0.2%–1%; ++ = >1%.
DOI: 10.1371/journal.ppat.0020025.t002

of humans with a xenotropic MuLV-like agent. Although our efforts to clone the sites of XMRV integration into the host genome have been limited by the small amounts of prostate tissue available for this purpose, our work to clone such sites is ongoing and will provide an important additional piece of evidence for XMRV infection in humans.

The XMRV sequence is not found in human genomic DNA, and none of the human endogenous retroviruses, including the only known gammaretrovirus-like human endogenous sequences (*hERVs E and T*) [70], bare any significant similarity to the XMRV genome. This indicates that XMRV must have been acquired exogenously by infection in positive subjects. From what reservoir and by what route such infections were acquired is unknown. It seems unlikely that direct contact with feral mice could explain the observed distribution of infection in our cohort, since there is no reason to believe that rodent exposure would vary according to *RNASEL* genotype. It is possible that infection is more widespread than indicated by the present studies, especially if, as seems likely, individuals with the wild-type RNase L clear infection more promptly than those with the QQ genotype. But if so, a cross-species transfer model of XMRV infection would require improbably high levels of rodent exposure for a developed society like our own. Thus, although the viral sequence suggests that the ultimate reservoir of XMRV is probably the rodent, the proximate source of the infection seems unlikely to be mice or rats. Provisionally, we favor the notion that the XMRV infections we have documented were acquired from other humans, i.e., that XMRV may have been resident in the human population for some time. This speculation will, however, require direct epidemiologic validation. It also remains to be determined if RNase L R462Q homozygotes are more sensitive to the acquisition of infection, or are simply less likely to clear infection once acquired. This is an important issue, since if the latter model is correct, it would imply that in younger humans, XMRV prevalence may be higher than what is observed in our prostate cancer cohort (mean age 58.7 y). We are currently developing serologic assays for use in population-based studies that should shed light on these matters.

While presented work documents a clear link of XMRV infection to RNase L deficiency, we emphasize that the data we have accumulated does not mandate any etiological link to prostate cancer. Furthermore, our finding that XMRV infection is targeted to stromal cells and not to carcinoma cells and the fact that the XMRV genome harbors no host-derived oncogenes rule out two classical models for retroviral oncogenesis: direct introduction of a dominantly acting oncogene and insertional activation of such a gene. However, more indirect contributions of the virus to the tumor can certainly be envisioned. Recent work has shown that stromal cells have an active role in directly promoting tumorigenesis of adjacent epithelial cells by producing various cytokines and growth factors that serve as proliferative signals [71] or indirectly by modifying the tumor microenvironment by promotion of angiogenesis or recruitment of inflammatory mediators leading to oxidative stress [72]. In particular, cancer-associated fibroblasts stimulate growth of human prostatic epithelial cells and alter their histology in vivo [73]. It is conceivable that XMRV-infected prostatic stromal cells could produce and secrete growth factors, cytokines or other factors that stimulate cell proliferation or promote oxidative stress in surrounding epithelia. Such a paracrine mechanism could still function quite efficiently even with the relatively small number of XMRV-infected cells that characterize the lesion.

Finally, we note that the identification of an exogenous infection such as XMRV could help explain why not all genetic studies have consistently identified RNase L as a prostate cancer susceptibility factor. If such an infection were linked, however indirectly, to prostate cancer risk, and if the prevalence of infection is not uniform in different populations, populations with low XMRV prevalence might be expected to show no association of *RNASEL* lesions to prostate cancer.

Clearly, resolution of these issues will require much further investigation. We need to determine the prevalence of XMRV infection in the general population, understand its routes of transmission and tissue tropism, explore its associations with pre-maligant and other prostatic conditions, and define the biochemical interactions of the virus with the 2–5A/RNase L system. The availability of molecular clones, infectious virus stocks, and susceptible cell culture systems should greatly enhance our ability to probe these and other questions in the near future.





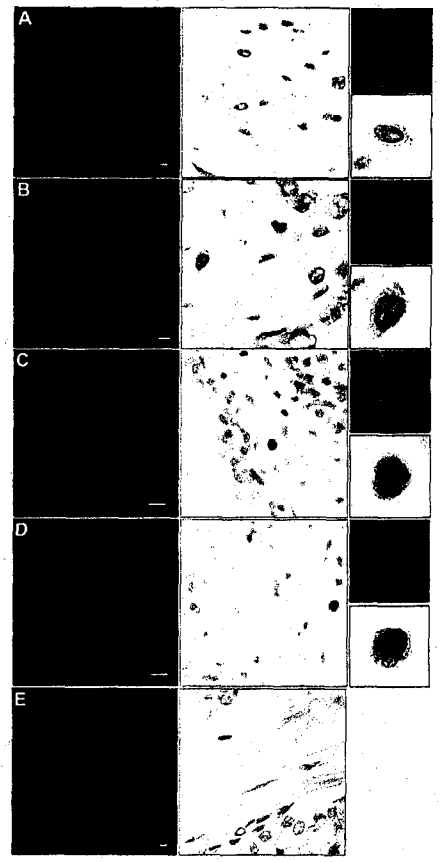


**Figure 9.** Detection of XMRV Protein in Prostatic Tissues Using *Immunostaining*

Prostatic tumor tissue sections from QQ cases VP62 (A and B) and VP88 (C and D), as well as an RR case VP51 (E) were stained, then visualized by immunofluorescence (left) or bright field (middle) using a monoclonal antibody to SFFV Gag protein. Nuclei are counterstained with hematoxylin. Enlarged images corresponding to the positive cells are shown on the right. Scale bars are 5 μm in (A), (B), and (E) and 10 μm in (C) and (D).
DOI: 10.1371/journal.ppat.0020025.g009

## Materials and Methods

**Genotyping of patients, and prostate tissue processing.** All human samples used in this study were obtained according to protocols approved by the Cleveland Clinic's Institutional Review Board. Age, clinical parameters, and geographical locations of XMRV-positive prostate cancer cases are provided in Table S5. Men scheduled to undergo prostatectomies at the Cleveland Clinic were genotyped for the R462Q (1385C->A) *RNASEL* variant using a premade TAQMAN genotyping assay (Applied Biosystems, Foster City, California, United States; Assay c__935591__1) on DNA isolated from peripheral blood mononuclear cells. Five nanograms of genomic DNA were assayed according to the manufacturer's instructions, and analyzed on an Applied Biosystems 7900HT Sequence Detection System instrument. Immediately after prostatectomies, tissue cores were taken from both the transitional zone (the site of benign prostatic hyperplasia, BPH) and the peripheral zone (where cancer generally occurs), snap-frozen in liquid nitrogen, and then stored at −80 °C. Remaining prostate tissue was fixed in 10% neutral buffered formalin, processed, and embedded in paraffin for later histological analyses. Frozen tissue cores were transferred from dry ice immediately to TRIZOL reagent (Invitrogen, Carlsbad, California, United States), homogenized with a power homogenizer or manually using a scalpel followed by a syringe, and total RNA was isolated according to the manufacturer's instructions. The prostate tissue RNA was then subjected to RNase-free DNase I (Ambion, Austin, Texas, United States) digestion for 30 min at 37 °C. The sample was then extracted with phenol and the RNA was precipitated with isopropanol overnight at −20 °C followed by centrifugation at 12,000 g for 30 min at 4 °C. Poly-A RNA was isolated from the DNase digested total RNA using the Oligotex mRNA Midi Kit (Qiagen USA, Valencia, California, United States) as instructed by the manufacturer. The poly-A RNA concentration was measured using the RIBOgreen quantitation kit (Molecular Probes, Invitrogen), and the samples were stored at −80 °C.

**Microarray screening.** Virochip microarrays used in this study were identical to those previously described [20–22]. Prostate tumor RNA samples were amplified and labeled using a modified Round A/B random PCR method and hybridized to the Virochip microarrays as reported previously (Protocol S1 in [21]). Microarrays were scanned with an Axon 4000B scanner (Axon Instruments, Union City, California, United States) and gridded using the bundled GenePix 3.0 software. Microarray data have been submitted to the NCBI GEO database (GSE3607). Hybridization patterns were interpreted using E-Predict as previously described [22] (Table S1). To make Figure 1, background-subtracted hybridization intensities of all retroviral oligonucleotides (205) were used to cluster samples and the oligonucleotides. Average linkage hierarchical clustering with Pearson correlation as the similarity metric was carried out using Cluster (version 2.0) [74]. Cluster images were generated using Java TreeView (version 1.0.8) [75].

**Genome cloning and sequencing.** Amplified and labeled cDNA from the VP35 tumor sample was hybridized to a hand-spotted microarray containing several retroviral oligonucleotides, which had high hybridization intensity on the Virochip during the initial microarray screening. Nucleic acid hybridizing to two of the oligonucleotides (9628654__317__rc derived from MTCR: TTC GCT TTA TCT GAG TAC CAT GTG TTC TTG GCC GTG AGC CGG GGC CCA GGT GCT CGA CCA CAG ATA TCC T; and 9626955__16__rc derived from SFFV: TCG GAT GCA ATC AGC AAG AGG CTT TAT TGC GAA CAC GGG TAC CCG GGC GAC TCA GTC TGT CGG AGG ACT G) was then individually eluted off the surface of the spots and amplified by PCR with Round B primers. Preparation of the hand-spotted array, hybridization, probe recovery, and PCR amplification of the recovered material were carried out according to Protocol S1. The recovered amplified DNA samples were then cloned into pCR2.1-TOPO TA vector (Invitrogen), and the resulting libraries were screened by colony hybridization with the corresponding above oligonucleotides as probes. Hybridizations were carried out using Rapid-Hyb buffer (Amersham, Piscataway, New Jersey, United States) according to the manufacturer's protocol at 50 °C for 4 h. Eight positive clones were sequenced, of which two (one from each library; clones K1 and K2R1 in Figure 2A) were viral and had 94–95% nt identity to MTCR.

To sequence the remainder of the VP35 genome as well as the entire genome from the VP42 tumor, we amplified fragments of the genome by PCR using either amplified (Round B) or unamplified

(Round A) cDNA prepared for original Virochip screening. This was accomplished first using a combination of primers derived from the sequence of MTCR and earlier recovered clones of XMRV. The two overlapping fragments from VP62 were amplified by PCR from cDNA generated by priming poly-A RNA with random hexamers. All PCR primers are listed in Table S2. The amplified fragments were cloned into pCR2.1-TOPO TA vector (Invitrogen) and sequenced using M13 sequencing primers. Genome assembly was carried out using CONSED version 13.84 for Linux [76].

**PCR.** Screening of tumor samples by *gag* nested RT-PCR was carried out according to Protocol S2. PCR fragments in all positive cases were gel-purified using QIAEX II gel extraction kit (Qiagen), cloned into pCR2.1-TOPO TA vector (Invitrogen), and sequenced using M13 sequencing primers.

*Pol* PCR was carried out using amplified cDNA (Round B material) as the template.

Sequence of the primers used for amplification (2670F, 3870R, 3810F, and 5190R) is listed in Table S2. Amplified products were gel-purified using QIAEX II gel extraction kit (Qiagen), and purified products were directly used for sequencing.

**Phylogenetic analyses.** Xenotropic mERV Chromosome 1, xenotropic mERV Chromosome 4, and xenotropic mERV Chromosome 9 were chosen by BLAST querying the NCBI nr database with the complete XMRV genomes and selecting the most similar full-length proviral sequences, all of which happened to have xenotropic envelopes (Figure S2C). Polytropic mERV Chromosome 7 and polytropic mERV Chromosome 11 were chosen by selecting NCBI nr full-length proviral sequences with envelopes most similar to a prototype polytropic clone MX27 [77]. Similarly, modified polytropic mERV Chromosome 7 and modified polytropic mERV Chromosome 12 were selected on the basis of similarity to a prototype-modified polytropic clone MX33 [77]. U3 analysis was performed using previously described reference sequences: Mcv18, Mcv3, Mxv2, Mcv11, Mxv11, and HEMV18 [49]; CWM-T-15, CWM-T-15-4, CWM-T-25a, and CWM-T-25b [48].

To generate the neighbor-joining tree of complete genomic sequences (Figure 3), the sequences were first manually edited to make all genomes the same length, i.e., R to R. The edited sequences were then aligned with ClustalX version 1.82 for Linux [78,79] using default settings. The tree was generated based on positions without gaps only; Kimura correction for multiple base substitutions [80] and bootstrapping with $n = 1000$ were also used.

All other trees were generated as above, except sequences were first trimmed to the same length, gaps were included, and Kimura correction was not used, as using these parameters did not have any significant effect on the trees.

**Antibodies.** Monoclonal antibody to SFFV Gag protein was produced from R187 cells ([65]; ATCC: CRL-1912) grown in DMEM (Media Core, Cleveland Clinic Foundation, Cleveland, Ohio, United States) with 10% ultra-low IgG FBS (Invitrogen) until confluent. Conditioned media was collected every three days from confluent cultures. Five ml of conditioned media per preparation was centrifuged at 168 × g for 5 min at 4 °C. Supernatant was filtered through a 0.22-μm syringe filter unit (Millipore, Billerica, Massachusetts, United States) and concentrated 16-fold in an Amicon ultrafiltration unit with a 100-kDa molecular weight cutoff membrane (Millipore). Sodium azide was added to a final concentration of 0.02%. Concomitant XMRV FISH/cytokeratin immunofluorescence was performed using a mouse anti-cytokeratin AE1/AE3 (20:1 mixture) monoclonal antibody (Chemicon International, Temecula, California, United States) capable of recognizing normal and neoplastic cells of epithelial origin.

**FISH.** The XMRV-35 FISH probe cocktail was generated using both 2.15-kb and 1.84-kb segments of the viral genome obtained by PCR with forward primer-2345, 5′ ACC CCT AAG TGA CAA GTC TG 3′ with reverse primer-4495, 5′ CTG GAC AGT GAA TTA TAC TA 3′ and forward primer-4915, 5′ AAA TTG GGG CAG GGG TGC GA 3′ with reverse primer-6755, 5′ TTG GAG TAA GTA CCT AGG AC 3′, both cloned into pGEM-T (Promega, Madison, Wisconsin, United States). The recombinant vectors were digested with *EcoR*I to release the viral cDNA fragments, which were purified after gel electrophoresis (Qiagen). The purified viral cDNA inserts were used in nick translation reactions to produce SpectrumGreen dUTP fluorescently labeled probe according to manufacturer's instructions (Vysis Inc., Des Plaines, Illinois, United States). Freshly baked slides of prostatic tissues or tissue microarray arrays with ~4-μm thick tissue sections were deparaffinized, rehydrated, and subjected to Target Retrieval (Dako, Glostrup, Denmark) for 40 min at 95 °C. Slides were cooled to room temperature and rinsed in $H_2O$. Proteinase K (Dako) at 1:5000 in Tris-HCl (pH 7.4) was applied directly to slides for 10 min at room temperature. Adjacent tissue sections were also probed with SpectrumGreen dUTP fluorescently labeled KSHV-8 DNA (nts 85820–92789) as a negative control or, as a positive control with SpectrumGreen and SpectrumOrange labeled TelVysion DNA Probe cocktail (Vysis), specific for subtelomeric regions of the P and Q arms of human Chromosome 1 as a positive control to ensure the tissue was completely accessible to FISH. FISH slides were examined using a Leica DMR microscope (Leica Micro-Systems, Heidelberg, Germany), equipped with a Retiga EX CCD camera (Q-Imaging,Vancouver, British Columbia, Canada). FISH images were captured using a Leica TCS SP2 laser scanning confocal with a 63× oil objective numerical aperature 1.4 (Leica Micro-Systems) microscope. XMRV nucleic acids were visualized using maximum intensity projections of optical slices acquired using a 488-nm argon-laser (emission at 500–550 nm). TelVysion DNA Probes were visualized using maximum intensity projections of optical slices acquired using a 488-nm argonlaser (emission at 500–550 nm) and 568-nm krypton-argon-laser (emission at 575–680 nm). DAPI was visualized using maximum intensity projections of optical slices acquired using a 364-nm UV-laser (emission at 400–500 nm). Slides were subsequently washed in 2× SSC (0.3 M sodium chloride and 0.03 M sodium citrate, [pH 7.0]) to remove coverslips, and H&E stained for morphological evaluation.

**IHC.** IHC on human tissues was performed on a Benchmark Ventana Autostainer (Ventana Medical Systems, Tucson, Arizona, United States). Unstained, formalin-fixed, paraffin-embedded prostate sections were placed on electrostatically charged slides and deparaffinized followed by a mild cell conditioning achieved through the use of Cell Conditioner #2 (Ventana Medical Systems). The concentrated R187 monoclonal antibody against SFFV p50 Gag was dispensed manually onto the sections at 10 μg per ml and allowed to incubate for 32 min at 37 °C. Endogenous biotin was blocked in sections using the Endogenous Biotin Blocking Kit (Ventana Medical Systems). Sections were washed, and biotinylated ImmunoPure Goat Anti-Rat IgG (Pierce Biotechnology, Rockford, Illinois, United States) was applied at a concentration of 4.8 μg per ml for 8 min. To detect Gag protein localization, the Ventana Enhanced Alkaline Phosphatase Red Detection Kit (Ventana Medical Systems) was used. Sections were briefly washed in distilled water and counterstained with Hematoxylin II (Ventana Medical Systems) for approximately 6 min. Sections were washed, dehydrated in graded alcohols, incubated in xylene for 5 min, and coverslips were added with Cytoseal (Microm International, Walldorf, Germany). Negative controls were performed as above except without the addition of the R187 monoclonal antibody.

Concomitant XMRV FISH/cytokeratin IHC was performed on slides of prostate tissue from patient VP62. First, sections were immunostained for cytokeratin AE1/AE3 using the Alexa Fluor 594 Tyramide Signal Amplification Kit (Molecular Probes, Invitrogen). Briefly, unstained, formalin-fixed, paraffin-embedded sections cut at ~4 μm were placed on electrostatically charged slides, baked at 65 °C for at least 4 h, deparaffinized in xylene, and rehydrated through decreasing alcohol concentrations. Slides were incubated in Protease II (Ventana Medical Systems) for 3 min at room temperature and washed in phosphate-buffered saline (PBS) in peroxidase quenching buffer (PBS + 3% $H_2O_2$) for 60 min at room temperature, then incubated with 1% blocking reagent (10 mg/ml BSA in PBS) for 60 min at room temperature. The slides were incubated with cytokeratin AE1/AE3 antibody diluted in 1% blocking reagent for 60 min at room temperature and rinsed 3× times in PBS. Goat anti-mouse IgG-horseradish peroxidase (Molecular Probes, Invitrogen) was added and incubated for 60 min at room temperature. The slides were rinsed 3× in PBS. The tyramide solution was added to the slides for 10 min at room temperature and the slides were rinsed 3× in PBS. Slides were then placed in Target Retrieval solution (Dako) for 40 min at 95 °C. FISH for XMRV was performed as described above except in the absence of proteinase K treatment. After FISH, the slides were mounted with Vectashield Mounting Medium plus DAPI (Vector Labs, Burlingame, California, United States) and examined using fluorescence microscopy. Immunofluorescence images were captured using a Texas red filter with a Leica DMR microscope (Leica Micro-Systems), equipped with a Retiga EX CCD camera (QImaging).

## Supporting Information

**Figure S1.** Complete Nucleotide Sequence of XMRV VP35

Numbers to the left indicate nt coordinates relative to the first nt. Predicted open reading frames for Gag, Gag-Pro-Pol, and Env polyproteins are shown below the corresponding nt. Characteristic 24-nt deletion in the 5′ *gag* leader is indicated with a triangle. Other genome features as well as primers used in the nested *gag* RT-PCR are shown as arrows.

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**Figure S2.** Phylogenetic Analysis of XMRV Based on Predicted Gag, Pro-Pol, and Env Polyproteins

Predicted Gag (A), Pro-Pol (B), and Env (C) sequences of XMRV VP35, VP42, and VP62 (red) as well as the correspondihg sequences from MTCR; MuLVs DG-75, MCF1233, Akv, Moloney, Friend, and Rauscher; feline leukemia virus (FLV); koala retrovirus (KoRV); gibbon ape leukemia virus (GALV), and a set of representative non-ecotropic proviruses (mERVs) were aligned using ClustalX. The resulting alignments were used to generate unrooted neighbor-joining trees (see Materials and Methods). Sequences are labeled as xenotropic (X), polytropic (P), modified polytropic (Pm), or ecotropic (E).

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**Figure S3.** Comparison of XMRV U3 Region to Representative Non-Ecotropic Sequences

(A) Multiple sequence alignment of U3 sequences from XMRV VP35, VP42, and VP62; MuLVs NZB-9-1 and NFS-Th-1; and from representative non-ecotropic proviruses [37,48,49]. The sequences were aligned using ClustalX (see Materials and Methods). Only sequences most similar to XMRV are shown. Glucocorticoid response element (GRE), and TATA and CAT boxes are indicated by lines. Direct repeat regions (boxed) are numbered according to the existing convention [37,49]. Triangle indicates a 190 nt insertion in polytropic proviruses [37]. XMRV-specific AG dinucleotide insertion is shown in red. Dots denote nt identical to those from XMRV, and deleted nt appear as spaces.

(B) Phylogenetic tree based on U3 nt sequences. Multiple sequence alignment from (A) was used to generate an unrooted neighbor-joining tree (see Materials and Methods). Bootstrap values ($n = 1000$ trials) are shown as percentages. U3 sequences from XMRV are shown in red.

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**Protocol S1.** Probe Recovery from Hand-Spotted Microarrays by "Scratching"

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**Protocol S2.** XMRV *gag* Nested RT-PCR

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**Table S1.** Computational Viral Species Predictions Using E-Predict for the Virochip Microarrays Shown in Figure 1

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**Table S2.** PCR Primers Used for Sequencing of XMRV Genomes

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**Table S3.** Age, Clinical Parameters, and Geographical Locations of XMRV-Positive Prostate Cancer Cases

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**Video S1.** Confocal Optical Image Planes of a Representative XMRV FISH Positive Cell

Optical image planes (0.5 μm step-size) of the XMRV FISH positive cell from Figure 1A acquired using a Leica TCS SP2 laser scanning spectral confocal microscope (Leica, Heidelberg, Germany) were reconstructed into a 3D volume set using Volocity 3.5 (Improvision, Lexington, Massachusetts, United States). Using Volocity's movie sequence editor, each volume was rotated along horizontal and vertical axes, adjusting nuclei stained DAPI (blue) channel brightness to visualize underlying XMRV FISH (green) nucleic acid signal. The resulting image frames were exported as a movie sequence. Underlying grid represents glass slide to which tissue was placed for FISH analysis. Each square unit within grid represents 4 μm in height and width.

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### Accession Numbers

Accession numbers from Gen Bank (http://www.ncbi.nlm.nih.gov/Genbank) are: AKV MuLV (J01998), feline leukemia virus (NC_001940), Friend MuLV (NC_001372), gibbon ape leukemia virus (NC_001885), koala retrovirus (AF151794), modified polytropic mERV Chromosome 7 (AC127565; nt 64,555–72,720), modified polytropic mERV Chromosome 12 (AC155658; nt 85,452–93,817), Moloney MuLV (NC_001501), MTCR (NC_001702MuLV DG-75 (AF221065); MuLV MCF 1233 (U13766), MuLV NCI-417 (AAC97875), MuLV NZB-9-1 (K02730), polytropic mERV Chromosome 7 (AC167978; nt 57,455–65,805), polytropic mERV Chromosome 11 (168–229,176,580), polytropic clone MX27 (M17327), Rauscher MuLV (NC_001819), xenotropic mERV Chromosome 1 (AC083892, nt 158,240–166,448), xenotropic mERV Chromosome 4 (AL627077; nt 146,400–154,635), xenotropic mERV Chromosome 9 (AC121813; nt 37,520–45,770), XMRV VP35 (DQ241301), XMRV VP42 (DQ241302), and XMRV VP 62 (DQ399707).

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**Author contributions.** AU, RJM, NF, DG, RHS, and JLD conceived and designed the experiments. AU, RJM, NF, SJP, KM, CMG, and RRT performed the experiments. AU, RJM, NF, SJP, GC, EAK, KM, CMG, RRT, DG, RHS, and JLD analyzed the data. AU, RJM, NF, SJP, GC, EAK, DG, RHS, and JLD contributed reagents/materials/analysis tools. AU, RJM, NF, DG, RHS, and JLD wrote the paper.

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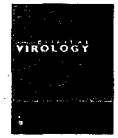

# Prevalence of human gammaretrovirus XMRV in sporadic prostate cancer

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ABSTRACT

*Background:* We previously identified a novel exogenous gammaretrovirus (xenotropic murine leukemia virus-related gammaretrovirus (XMRV)) using a pan-viral microarray. XMRV is the first MLV-related virus found in human infection. Forty percent (8/20) of familial prostate cancer patients homozygous for a mutation in RNase L (R462Q) were positive for XMRV, while the virus was rarely (1/66) detected in familial prostate cancer patients heterozygous for R462Q or carrying the wild type allele.
*Objectives:* To determine the presence of XMRV in non-familial prostate cancer samples.
*Study design:* RNA from prostate tissue was analyzed for XMRV using nested RT-PCR. In all samples, RNase L (R462Q) genotyping was performed using an allele-specific PCR.
*Results:* XMRV-specific sequences were detected in one of 105 tissue samples from non-familial prostate cancer patients and from one of 70 tissue samples from men without prostate cancer. The two XMRV-positive patients were wild type or heterozygous for the R462Q mutation and thus carried at least one fully functional RNase L allele.
*Conclusions:* XMRV was rarely detected in non-familial prostate cancer samples from Northern European patients. The homozygous mutation R462Q (QQ) was significantly underrepresented (<6%) in this cohort when compared to other studies (11–17%).



## 1. Introduction

Prostate cancer is the most frequent cancer of men in North America and Europe. Well known factors contributing to the risk of prostate cancer are age, androgens, environmental and genetic factors.[1] Sporadic (non-familial) prostate cancer is the most common form of prostate cancer (80–90%) and its incidence increases with age. Familial prostate cancer, which accounts for 10–20% of all prostate cancer cases, occurs much earlier in life and is defined as prostate cancer occurring in individuals with three or more first degree relatives who had prostate cancer.

Recent work emphasizes that prostate cancer is frequently associated with chronic prostatic inflammation. A lesion called proliferative inflammatory atrophy is often found in the premalignant stages of the disease.[1] Viral infections may be triggers for the inflammatory process. However, epidemiological studies designed to detect links between specific viral infections and prostate cancer have been inconclusive.[2–9]

Recently, a new gammaretrovirus, xenotropic murine leukemia virus-related gammaretrovirus (XMRV), was discovered in prostatic tissue from patients with familial prostate cancer;[10] specifically in patients homozygous for a missense mutation in the RNase L gene, R462Q. Fluorescence in situ hybridization revealed that prostatic stroma cells were infected at low frequency (0.5–1.2%).

RNase L, an endoribonuclease of the antiviral defense pathway, was one of the first prostate cancer susceptibility genes recognized. The missense mutation R462Q has been linked to hereditary prostate cancer [11–13] and has been implicated in up to 13% of all prostate cancer cases in some studies.[11] Not all studies have confirmed this finding, perhaps because of differences in population genetics or environmental factors.[14–16]

In the present study, we analyzed 105 RNA samples from the prostate tissue of 87 sporadic prostate cancer patients and also biopsy samples from 70 healthy men without prostate cancer for the presence of XMRV.

*Abbreviations:* PCR, polymerase chain reaction; PCA, prostate cancer; SNP, single nucleotide polymorphism; XMRV, xenotropic murine leukemia virus-related virus; PSA, prostate specific antigen; PIA, proliferative inflammatory atrophy; HPC, hereditary prostate cancer.

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**Table 1**
Clinical and pathological data of all patients

| Sample | Age | Group | PSA (ng/ml) | Free PSA (%) | Tumor-stage | Gleason-score |
|---|---|---|---|---|---|---|
| 1 | 36 | B | 0.33 | 51.52 | NA | NA |
| 2 | 45 | B | 0.25 | 84 | NA | NA |
| 3 | 48 | B | 0.6 | 53.33 | NA | NA |
| 4 | 51 | B | 0.45 | 37.87 | NA | NA |
| 5 | 34 | B | 0.2 | 95 | NA | NA |
| 6 | 33 | B | 0.22 | 50 | NA | NA |
| 7 | 46 | B | 0.57 | 54.39 | NA | NA |
| 8 | 45 | B | 0.81 | 38.27 | NA | NA |
| 9 | 43 | B | 0.6 | 53.33 | NA | NA |
| 10 | 40 | B | 0.44 | 52.27 | NA | NA |
| 11 | 57 | B | 0.2 | 30 | NA | NA |
| 12 | 56 | B | 0.35 | 25.71 | NA | NA |
| 13 | 57 | B | 0.49 | 30.61 | NA | NA |
| 14 | 63 | B | 0.75 | 24 | NA | NA |
| 15 | 56 | B | 1.01 | 50.5 | NA | NA |
| 16 | 57 | B | 1.12 | 31.25 | NA | NA |
| 17 | 55 | B | 0.81 | 24.69 | NA | NA |
| 18 | 56 | B | 0.42 | 35.71 | NA | NA |
| 19 | 52 | B | 0.63 | 49.21 | NA | NA |
| 20 | 57 | B | 0.82 | 21.95 | NA | NA |
| 21 | 55 | B | 0.83 | 24.1 | NA | NA |
| 22 | 65 | B | 0.5 | NA | NA | NA |
| 23 | 63 | B | 0.69 | 34.78 | NA | NA |
| 24 | 64 | B | 0.86 | 27.91 | NA | NA |
| 25 | 55 | B | 1.1 | 25.45 | NA | NA |
| 26 | 80 | B | 20.54 | 12.37 | NA | NA |
| 27 | 64 | B | NA | NA | NA | NA |
| 28 | 63 | B | 6.53 | 20.52 | NA | NA |
| 29 | 52 | B | 8.15 | 18.53 | NA | NA |
| 30 | 66 | B | 6.28 | 30.89 | NA | NA |
| 31 | 65 | B | 13.39 | 29.42 | NA | NA |
| 32 | 70 | B | 27.54 | 10.64 | NA | NA |
| 33 | 65 | B | 3.05 | 31.15 | NA | NA |
| 34 | 57 | B | 5.32 | 22.37 | NA | NA |
| 35 | 77 | B | NA | NA | NA | NA |
| 36 | 71 | B | 3.05 | 9.18 | NA | NA |
| 37 | 70 | B | 7.52 | 23.94 | NA | NA |
| 38 | 71 | B | 10.46 | 18.36 | NA | NA |
| 39 | 60 | B | 6.4 | 19.69 | NA | NA |
| 40 | 60 | B | 6.36 | 17.45 | NA | NA |
| 41 | 66 | A | 5.6 | 21.79 | pT2c | 3+3 |
| 42 | 65 | A | 2.85 | 21.05 | pT2c | 3+3 |
| 43 | 58 | A | 4.99 | 11.82 | pT2c | 3+3 |
| 44 | 67 | A | 4.07 | 23.83 | pT2c | 3+3 |
| 45 | 68 | A | 6.1 | 15.9 | pT2a | 3+3 |
| 46 | 62 | A | 3.14 | 14.01 | pT2c | 3+4 |
| 47 | 63 | A | 8.53 | 8.79 | pT2c | 3+4 |
| 48 | 65 | A | NA | NA | NA | 3+3 |
| 49 | 62 | A | 7.84 | 23.85 | pT2c | 3+3 |
| 50 | 50 | A | 6.42 | 12.31 | pT2a | 3+3 |
| 51 | 51 | A | 7.86 | 14.5 | pT2c | 3+3 |
| 52 | 50 | A | 3.69 | 25.75 | pT2c | 3+3 |
| 53 | 68 | A | 5.91 | 19.8 | pT3b | 4+3 |
| 54 | 69 | A | 3.67 | 16.08 | pT2a | 4+3 |
| 55 | 65 | A | 9.81 | 9.68 | pT3a | 4+3 |
| 56 | 68 | A | 11.35 | 8.11 | pT3a | 5+4 |
| 57 | 63 | A | 9.26 | 18.79 | pT2c | 4+3 |
| 58 | 54 | A | 5.8 | 8.1 | pT2c | 3+3 |
| 59 | 64 | A | NA | NA | pT2c | 3+4 |
| 60 | 52 | A | 3.61 | 13.57 | pT2c | 3+2 |
| 61 | 65 | A | 6.02 | 10.3 | pT3a | 3+4 |
| 62 | 64 | A | 2.68 | 23.88 | pT2a | 3+3 |
| 63 | 71 | A | 9.67 | 11.48 | pT3a | 4+3 |
| 64 | 62 | A | 6.4 | 6.72 | pT3a | 4+3 |
| 65 | – | A | 9.32 | 4.4 | pT2c | 3+2 |
| 66 | 60 | A | NA | NA | pT2c | 3+4 |
| 67 | 52 | A | 8.77 | 8.32 | pT2c | 3+4 |
| 68 | – | A | 3.34 | 15.27 | pT3a | 3+3 |
| 69 | 57 | A | 5.46 | 20.88 | pT2c | 3+3 |
| 70 | 57 | A | 5.33 | 14.45 | pT3b | 4+3 |
| 71 | 67 | A | 11.91 | 9.15 | pT2c | 4+3 |
| 72 | 53 | A | 3.04 | 15.46 | pT2c | 3+4 |

**Table 1** (*Continued*)

| Sample | Age | Group | PSA (ng/ml) | Free PSA (%) | Tumor-stage | Gleason-score |
|---|---|---|---|---|---|---|
| 73 | 62 | A | 9.11 | 9.77 | pT2c | 3+3 |
| 74 | 63 | A | 9.38 | 6.18 | pT2c | 3+3 |
| 75 | 67 | A | 5.13 | 15.01 | pT2c | 3+3 |
| 76 | 66 | A | 12.27 | 14.83 | pT3b | 3+4 |
| 77 | 57 | A | 24.17 | 28.71 | pT2c | 3+3 |
| 78 | 66 | A | 3.7 | 12.16 | pT2c | 3+4 |
| 79 | 65 | A | 6.2 | 4.52 | pT2c | 3+4 |
| 80 | 54 | A | 9.53 | 5.14 | pT2a | 4+3 |
| 81 | 58 | A | 17.98 | 13.24 | pT3a | 4+3 |
| 82 | 53 | A | 5.52 | 13.22 | pT2c | 3+4 |
| 83 | 62 | A | 4.48 | 7.81 | pT2a | 3+4 |
| 84 | 70 | A | 3.56 | 18.82 | pT2c | 3+3 |
| 85 | 61 | A | 3.15 | 21.9 | pT3a | 3+4 |
| 86 | 58 | A | 6.48 | 6.48 | pT2c | 3+4 |
| 87 | 65 | A | 10.28 | 10.21 | pT2c | 3+4 |
| 88 | 54 | A | 22.62 | 4.02 | pT3a | 3+4 |
| 89 | 65 | A | 16.71 | 6.1 | pT2a | 4+4 |
| 90 | 71 | A | 6.19 | 16.16 | pT2c | 3+3 |
| 91 | 54 | A | 7.31 | 7.8 | pT2c | 3+4 |
| 92 | 62 | A | 7.85 | 6.62 | pT2c | 3+4 |
| 93 | 64 | A | 5.75 | 9.91 | pT2c | 3+3 |
| 94 | 63 | A | 5.04 | 8.5 | NA | 3+3 |
| 95 | 73 | A | 10.13 | 20.24 | NA | 3+4 |
| 96 | 69 | A | 6.22 | 9.8 | NA | 3+4 |
| 97 | 60 | A | 9.18 | 13.2 | NA | 4+4 |
| 98 | 69 | A | 17.98 | 1.7 | NA | 3+4 |
| 99 | 73 | A | 29.76 | 5.7 | NA | 3+4 |
| 100 | 57 | A | 7.09 | 14.1 | NA | 3+3 |
| 101 | 60 | A | 4.02 | 17.7 | NA | 3+3 |
| 102 | 75 | A | 6.75 | 21 | NA | 3+3 |
| 103 | 64 | A | 4.54 | 29.7 | NA | 3+3 |
| 104 | 72 | A | 6.94 | 1.2 | NA | 4+3 |
| 105 | 65 | A | 10.59 | 18.1 | NA | 3+3 |
| 106 | 59 | A | 8.67 | 18.2 | NA | 4+3 |
| 107 | 71 | A | 2.79 | 17.6 | NA | 3+3 |
| 108 | 49 | A | 1.12 | 24.1 | NA | 3+3 |
| 109 | 66 | A | 8.17 | 7.6 | NA | 5+3 |
| 110 | 65 | A | NA | NA | pT3a | 3+4 |
| 111 | 71 | A | NA | NA | pT2c | 4+3 |
| 112 | 48 | A | NA | NA | pT2c | 3+3 |
| 113 | 67 | A | NA | NA | pT3b | 4+3 |
| 114 | 62 | A | NA | NA | pT3b | 4+3 |
| 115 | 76 | A | NA | NA | pT3b | 3+4 |
| 116 | 58 | A | NA | NA | pT3b | 3+4 |
| 117 | 63 | A | NA | NA | pT2c | 3+4 |
| 118 | 59 | A | NA | NA | pT3b | 3+4 |
| 119 | 69 | A | NA | NA | pT3b | NA |
| 120 | 67 | A | NA | NA | pT2b | 3+3 |
| 121 | 70 | A | NA | NA | pT3a | 3+4 |
| 122 | 60 | A | NA | NA | pT3b | 3+4 |
| 123 | 70 | A | NA | NA | pT3b | 4+3 |
| 124 | 67 | A | NA | NA | pT3b | 3+4 |
| 125 | 67 | A | NA | NA | pT2c | 3+3 |
| 126 | 44 | A | 2.77 | 13 | NA | NA |
| 127 | 65 | A | 10.46 | 12.05 | NA | NA |
| 128 | 67 | B | 4.63 | 13.6 | NA | NA |
| 129 | 62 | B | 3.54 | 13.37 | NA | NA |
| 130 | 68 | B | 14.78 | 17.48 | NA | NA |
| 131 | 55 | B | 4.78 | 22.18 | NA | NA |
| 132 | 70 | B | 10.52 | 19.1 | NA | NA |
| 133 | 67 | B | 3.49 | 24.64 | NA | NA |
| 134 | 44 | B | 3.74 | 14.44 | NA | NA |
| 135 | 69 | B | 9.14 | 18.2 | NA | NA |
| 136 | 59 | B | 5.33 | 12.4 | NA | NA |
| 137 | 63 | B | 4.1 | 14.2 | NA | NA |
| 138 | 57 | B | 1 | 35 | NA | NA |
| 139 | 61 | B | 9.72 | 21.71 | NA | NA |
| 140 | 60 | B | 4.58 | 34.7 | NA | NA |
| 141 | 66 | B | 4.6 | 30.65 | NA | NA |
| 142 | 67 | B | 5.42 | 35.1 | NA | NA |
| 143 | 62 | B | 4.1 | 17.6 | NA | NA |
| 144 | 75 | B | 3.89 | 28.3 | NA | NA |



**Table 1** (*Continued*)

| Sample | Age | Group | PSA (ng/ml) | Free PSA (%) | Tumor-stage | Gleason-score |
|---|---|---|---|---|---|---|
| 145 | 56 | B | 9.23 | 21.9 | NA | NA |
| 146 | 65 | B | 4.94 | 13.3 | NA | NA |
| 147 | 64 | B | 15.58 | 10.14 | NA | NA |
| 148 | 71 | B | 7.52 | 17.6 | NA | NA |
| 149 | 71 | B | 6.17 | 23.82 | NA | NA |
| 150 | 72 | B | 2.79 | 34.4 | NA | NA |
| 151 | 47 | B | 2.76 | 17 | NA | NA |
| 152 | 67 | B | 2.87 | 35.5 | NA | NA |
| 153 | 53 | B | 5.5 | 18.9 | NA | NA |
| 154 | 65 | B | 5.84 | 36.5 | NA | NA |
| 155 | 62 | B | 4.94 | 21.1 | NA | NA |
| 156 | 65 | B | 9.02 | 21.1 | NA | NA |
| 157 | 54 | B | 5.97 | 13.1 | NA | NA |

NA: "not analyzed".

## 2. Methods

### 2.1. Tissue sampling and RNA isolation

We studied histological tumor-free prostate biopsies from 87 patients (Group A; samples 41–127) with confirmed cancer undergoing radical prostatectomy at the Urology Department of the University Hospital Hamburg-Eppendorf, and from 70 control donors (Group B). Group B samples 1–40 were from men defined as healthy according to the following parameters: serum PSA <1 ng/ml; no family history of prostate cancer; normal transrectal ultrasound or negative digital rectal examination. Group B samples 128–157 were from men with multiple negative biopsy series.

In some patients with a large prostate cancer, biopsies were taken from the cancerous region (T) as well as from the region without signs of cancer (N) as confirmed by histology.

Tissue specimens were collected strictly from the peripheral zone of the prostate by ultrasound-guided transrectal biopsy. Samples were fixed in RNAlater (Qiagen) and RNA was isolated using RNeasy-columns (Qiagen) followed by RNA quality control using an Agilent 2100 bioanalyzer (Agilent Technologies, Inc.). Clinical and pathological data for all individuals are shown in Table 1. The study was approved by the local ethics committee (*no. OB-052-04*).

### 2.2. XMRV RT-PCR; sequencing and clustering of XMRV gag sequences

XMRV-specific RT-PCR was performed as described previously.[10] Briefly, total RNA extracted from tissue obtained by needle biopsy was analyzed in a nested RT-PCR reaction using XMRV specific primers. PCR fragments were gel purified using QIAEX II gel extraction kit (Qiagen) cloned into pCR2.1-TA vector (Invitrogen) and sequenced. For phylogenetic tree analysis, a published sequence set was used.[10] The gag sequences were aligned with ClustalX version 1.82 [17,18] using default settings.

### 2.3. RNase L genotyping and quantitative real-time PCR

Allele-specific PCR to detect single nucleotide polymorphism R462Q was performed as described.[11] Briefly, this allele-specific PCR utilized two primers, each with the 3′ terminal base complementary to one of the alleles to be identified. Two separate PCR amplification reactions using the same reverse primer were performed to detect each allele. For the quantitative real time PCR, 100 ng of total RNA was reverse transcribed in a total volume of 25 μl using random primers. 5 μl of cDNA were amplified using the Qiagen SyBr Green Master Mix on a Biorad iCycler according to manufacturer's instructions. Each experiment was performed in triplicate. The primers used were described recently.[19]

DU145 and LNCaP, two prostate cancer cell lines (American Type Culture Collection), were cultured in RPMI medium supplemented with 10% FCS.

## 3. Results

### 3.1. Low frequency of XMRV in sporadic prostate cancer

The gammaretrovirus XMRV was originally identified in RNase L-deficient prostate cancer tissue of patients with familial prostate cancer. In the absence of epidemiological data for XMRV, the present study was initiated to extend the search for XMRV-specific sequences to include patients with sporadic prostate cancer independent of the RNase L status. Only one sample of 105 obtained from the sporadic prostate cancer patients was positive for XMRV (0.95%) by RT-PCR. Additionally, XMRV sequences were detected in one of the 70 (1.42%) RNA samples from prostate tissue of healthy donors (Table 2).

To examine the relationship between the amplified sequences and those previously published for XMRV,[10] the gag region from both samples was amplified by nested RT-PCR and sequenced. The sequences were highly similar (Fig. 1), showing 98–99% sequence identity within a 390-bp region of gag, suggesting that the amplified sequences are indeed from the same virus, XMRV.

### 3.2. RNase L genotyping

Data from our earlier studies provide evidence that functional mutations in RNase L might be important for the acquisition of XMRV. In the present study, an allele-specific PCR [11] for the SNP R462Q within RNase L was performed (Fig. 2). Neither of the two XMRV-positive samples was homozygous for the R462Q mutation. The prostate cancer sample (sample 57) was heterozygous (QR) and the control sample (sample 6) displayed a wild type (RR) RNase L genotype. The results obtained by PCR were confirmed by sequencing (Fig. 2B). Table 3 summarizes the results of the RNase L SNP R462Q genotyping of all samples included in the study. Only a few of the samples (<6%) showed the homozygous QQ genotype previously reported to be present in 13–15% of control cases, sporadic prostate cancer samples and familial prostate cancer samples.[11,12,15,16,20–22] The distribution of wild type and heterozygous mutations was concordant with published results.

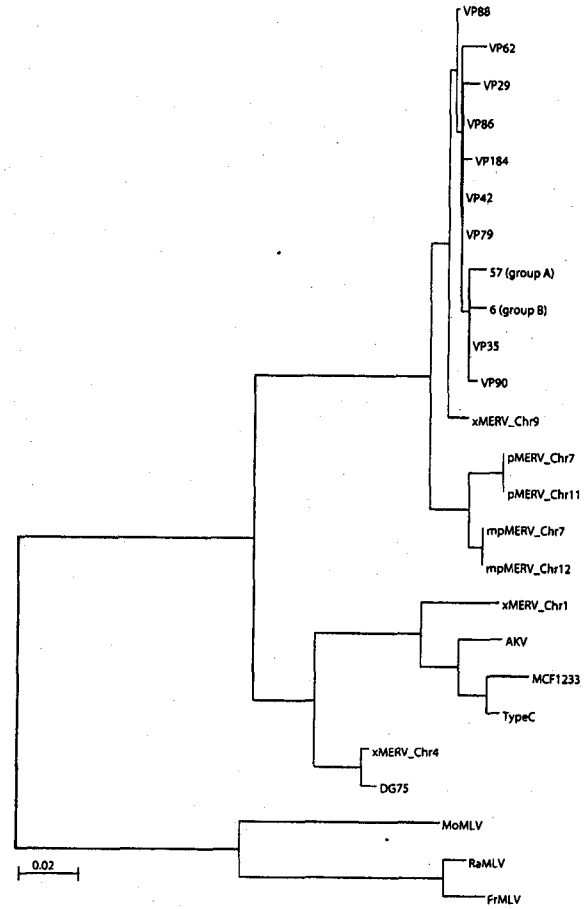


**Fig. 1.** XMRV gag sequences derived from sporadic and familial prostate cancer samples. Phylogenetic tree comparing a 390-nt RT-PCR gag fragment amplified from sporadic tumor samples (57 in Group A and 6 in Group B) with recently published XMRV sequences from familial prostate cancer patients.[10] The sequences were aligned using ClustalX and the tree was generated using the neighbor-joining method. Sequences are labeled as xenotropic (X), polytropic (P), modified polytropic (Pm), or ecotropic (E).

### 3.3. RNase L expression

Relative RNase L mRNA expression levels were assayed in XMRV-positive samples using quantitative real time RT-PCR. LNCaP cells, which have an inactivating deletion mutation in one allele of *RNASEL*,[20] These cells had a 20-fold reduction in RNase L expression levels compared to DU145 cells (Fig. 3). In contrast, the two XMRV-positive samples (6 and 57) did not have reduced RNase L expression when compared to DU145 cells. Randomly selected samples from our cohort representing Group A and Group B did not show major differences in RNase L expression. However, we observed a 50-fold difference in RNase L mRNA expression when comparing RNA from tumor cells (T) with normal tissue (N) from patient 117. Two other samples, 118 and 119, did not show major differences in RNase L expression between tumor cells and normal cells.

**Table 2**
XMRV detection using nested gag RT-PCR

| | PCA (Group A) | Control (Group B) |
|---|---|---|
| XMRV RT (+) | 1/105 | 1/70 |
| Median age (yrs) | 61.1 | 58.5 |

Total RNA isolated from prostate tissue from patients with prostate cancer (PCA) was analyzed for the presence of XMRV sequences using an RT-nested PCR. GAPDH was amplified in parallel as an internal control.



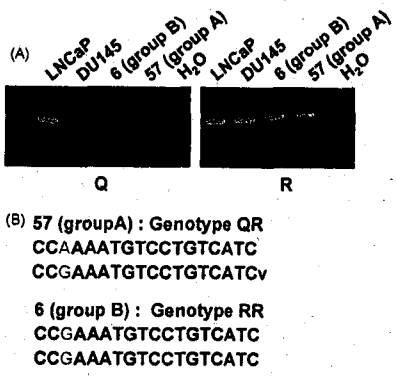


**Fig. 2.** Allele-specific PCR for the genotyping of the R462Q (A1385G) mutation in the RNase L gene. Ethidium bromide stained agarose gel showing PCR-positive fragments of RNase L using allele-specific forward primers Q (left) or R (right) in separate PCR reactions with a common reverse primer R. RNA from prostate cancer cell lines LNCaP (Genotype QR) and DU145 (Genotype RR) were used as controls. (B) Nucleotide sequence of the two XMRV positive PCR fragments from tumor samples shown in (A). Nucleotide exchange at position 1385 is shown in light grey.

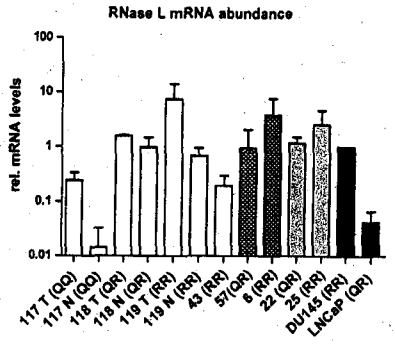


**Fig. 3.** RNase L mRNA expression in prostate tissue samples. RNase L mRNA expression was measured by quantitative real time PCR as described in Section 2. Standard deviations from three independent experiments (four replicates each) are shown. Prostate cancer cell lines DU145 and LNCaP (shown as black bars) were used as controls. RNase L mRNA levels from healthy control patients (Group B) are shown in light grey, samples from PCA patients (Group A) are shown as white bars. T indicates RNA from tumor region, N stands for RNA from normal tissue. The two XMRV-positive cases are indicated as speckled bars. The RNase L genotype of all samples is shown in brackets.

**Table 3**
RNase L genotyping of SNP R462Q

| Study group | Number screened | RNASEL (SNP 462) | | | Sample type |
|---|---|---|---|---|---|
| | | RR | RQ | QQ | |
| PCA (Group A) | 87 | 51 | 29 | 7 | Tissue DNA |
| Control (Group B) | 70 | 42 | 24 | 4 | Tissue DNA |

DNA from prostate tissue from patients with prostate cancer (PCA) was analyzed for the presence of a single nucleotide polymorphism (SNP) at amino acid position 462 within the RNase L gene. RR, RQ, QQ: wild type, heterozygous and homozygous genotype, respectively.

## 4. Discussion

XMRV, a novel gammaretrovirus, was recently identified in familial prostate cancer samples using a pan-viral microarray.[10] Our earlier studies suggested that functional mutations in RNase L might be important for the acquisition of XMRV. Almost all XMRV-positive prostate cancer cases described so far carry a mutation within RNase L (R462Q), resulting in reduced RNase L activity.[10]

However, the current study found only a low prevalence of XMRV in non-familial prostate tissue of men in Northern Europe.

RNase L, an endoribonuclease of the antiviral defense pathway, was one of the first susceptibility genes discovered in prostate cancer. The HPC1 (hereditary prostate cancer) locus was linked to prostate cancer in several genetic linkage studies performed in North America and Finland.[11,12,20,23] This finding was not be confirmed in a large case control study recently conducted in Germany.[16] RNase L is implicated in the interferon-mediated antiviral defence pathway and has been shown to play a role in several models of viral infection including influenza A, West Nile virus and herpes simplex virus.[21,22,24–26]

In our previous study, XMRV was detected in familial prostate cancer patients homozygous for the R462Q variant (QQ) of RNase L.[10] Overall, 40% (8/20) of patients homozygous for the SNP R462Q (QQ) has XMRV infection, whereas only 1.5% (1/66) patients heterozygous (QR) or carrying the wild type allele (RR) were XMRV-positive. Subsequent in vitro experiments demonstrating that XMRV replication increases with reduced RNase L activity further corroborated our previous results.[27]

So far, there are no epidemiological data regarding the prevalence of XMRV in prostate tissue, independent of the RNase L status. The prevalence of XMRV in our cohort was low (1.14%). The two XMRV-positive patients were heterozygous (HR) or wild type (RR) genotype and showed no deficiency in RNase L expression. These results are in accord with our previous observation that XMRV sequences are predominantly found in prostate cancer patients with a deficiency in RNase L and only rarely found in prostate cancer patients with at least one fully functional RNase L allele.

Genotyping for the SNP R462Q in the C-terminal domain of RNase L revealed that homozygosity of R462Q (QQ) is a relatively rare event (<6%). These results are in contrast to previous studies that have reported homozygous R462Q (QQ) mutations in 11–17% of cases, independent of the genetic background of the population. At present, we do not have an explanation for this observation, since all of our patients are of Caucasian background and live in Northern Europe.

We were not able to look for SNP R462Q in the germline cells of our cohort, as such material was not available. However, a recent study comparing germline with somatic mutations of RNase L observed a similar distribution of homozygous, heterozygous or wild type allele frequency in both tissue types.[28]

In conclusion, our results suggest that XMRV is not associated with sporadic prostate cancer in Northern Europe. The availability of an XMRV antibody-screening test should greatly enhance epidemiological studies of the prevalence of XMRV in larger cohorts of prostate cancer patients as well as in the general population.

## Conflict of interest

The authors declare they have no competing interest.

## Acknowledgement

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# Lack of evidence for xenotropic murine leukemia virus-related virus(XMRV) in German prostate cancer patients

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## Abstract

**Background:** A novel gammaretrovirus named *xenotropic murine leukemia virus-related virus* (XMRV) has been recently identified and found to have a prevalence of 40% in prostate tumor samples from American patients carrying a homozygous R462Q mutation in the RNaseL gene. This mutation impairs the function of the innate antiviral type I interferon pathway and is a known susceptibility factor for prostate cancer. Here, we attempt to measure the prevalence of XMRV in prostate cancer cases in Germany and determine whether an analogous association with the R462Q polymorphism exists.

**Results:** 589 prostate tumor samples were genotyped by real-time PCR with regard to the RNaseL mutation. DNA and RNA samples from these patients were screened for the presence of XMRV-specific *gag* sequences using a highly sensitive nested PCR and RT-PCR approach. Furthermore, 146 sera samples from prostate tumor patients were tested for XMRV Gag and Env antibodies using a newly developed ELISA assay. In agreement with earlier data, 12.9% (76 samples) were shown to be of the QQ genotype. However, XMRV specific sequences were detected at neither the DNA nor the RNA level. Consistent with this result, none of the sera analyzed from prostate cancer patients contained XMRV-specific antibodies.

**Conclusion:** Our results indicate a much lower prevalence (or even complete absence) of XMRV in prostate tumor patients in Germany. One possible reason for this could be a geographically restricted incidence of XMRV infections.

## Background

Prostate cancer (PCa) is currently the most commonly diagnosed cancer in European males and causes approximately 80,000 deaths per year [1]. Modern methods of diagnosis and extensive programs for early detection have increased the chances for successful treatment in recent

years, but there is still only limited knowledge concerning susceptibility and putative risk factors for PCa. In addition to age, the risk factors for developing PCa are thought to be diet, alcohol consumption, exposure to ultraviolet radiation [2], and genetic factors [3]. One of the first studies to investigate the hereditary factors associated with a predisposition for developing prostate cancer identified the HPC1 locus (hereditary prostate cancer locus-1) [4], which is now known to harbor the RNaseL gene. RNaseL codes for a endoribonuclease involved in the IFN-regulated antiviral defense pathway (reviewed by [5]). The significance of RNaseL gene polymorphisms for the development of prostate cancer is still under scrutiny. The R462Q (rs486907) polymorphism for example is implicated in up to 13% of all US prostate cancer cases [6] and three other variants contribute to familial prostate cancer risk in the Japanese population [7], whereas no significant association with disease risk could be found in the German population [8].

Recently, an analysis for viral sequences in prostate cancer stroma tissues using custom-made microarrays resulted in the discovery of a new gammaretrovirus named *xenotropic murine leukemia virus-related virus* (XMRV), [9,10]. XMRV was present in eight of twenty (40%) cases in patients with familial prostate cancer that were homozygous at the R462Q locus for the QQ allel. On the other hand, the virus could be detected in only 1.5% of carriers of the RQ or RR allels. In subsequent studies involving smaller cohorts of European prostate cancer patients, the prevalence and correlation of the QQ-phenotype with the presence of XMRV were either far less significant [11] or the virus could not be detected at all [12]. Very recently XMRV was recognized by immunohistochemistry in 23% of prostate cancers from US American donors, independent of the R462Q polymorphism [13].

This present study describes the development and use of sensitive PCR and RT-PCR assays to test DNA and RNA from 589 PCa tumor samples obtained from the Charité hospital in Berlin (Germany) for the presence of proviral XMRV DNA and corresponding viral transcripts. In addition, we used an ELISA based on recombinant XMRV proteins to screen 146 PCa patient sera for viral Env- and Gag-specific antibodies. Neither in the 76 specimens homozygous for the QQ allele, nor in any of the other samples could XMRV or a related gammaretrovirus be detected. Furthermore, none of the sera contained antibodies specific for the XMRV Env or Gag proteins.

## Methods

### *Patients*

Tissue samples were collected from 589 patients undergoing radical prostatectomy for histologically proven primary prostate cancer at the Department of Urology, Charité - Universitätsmedizin Berlin, between 2000 and 2006. Institutional review board approval for this study was obtained and all patients gave their informed consent prior to surgery. Tissue samples were obtained immediately after surgery, snap-frozen in liquid nitrogen and stored at -80°C. Histopathologic classification of the samples was based on the World Health Organization and 1997 TNM classification guidelines (International Union Against Cancer, 1997). The patient's median age was 63 years (range 43 - 80). The serum PSA levels were measured prior to surgery and ranged from 0.1 to 100 ng/ml (median 7.5 ng/ml). 405 of 589 patients (69%) had organ-confined disease (pT2) while the remaining 31% had non organ-confined disease (pT3 and pT4). Using the Gleason-score (GS) system, the sample population was divided into low-grade tumors (GS 2-6, n = 282), intermediate cases (GS 7, n = 175), and high-grade prostate carcinomas (GS 8-10, n = 68).

### *Nucleic acid isolation*

Frozen tissues were mechanically sliced and immediately lysed in DNA- or RNA-lysis buffer, column-purified, and eluted (50-200 µl) according to the manufacturers instructions (QIAamp DNA Mini Kit, RNeasy Mini Kit, QIAGEN GmbH, Hilden, Germany). The $OD_{260/280}$ ratio and nucleic acid concentrations were determined using the Nanodrop-1000 instrument (PeqLab Biotechnologie GmbH, Erlangen, Germany). In addition, RNA samples were checked for integrity using a Bioanalzyer-2100 (Agilent Technologies, Inc., Santa Clara CA, United States). No additional macro-/micro-dissections were performed on the prostate tissues because viral nucleic acids were expected to be present preferentially in the stromal compartments.

### *Diagnostic PCR*

A nested PCR was developed for the detection of XMRV sequences that amplifies regions upstream of the *gag* start codon, harboring the unique 24 nt deletion [10]. First, we constructed by fusion-PCR a synthetic gene representing the region from nucleotide 1 to 800 of the MLV DG-75 (Genbank acc. number AF221065). This fragment was cloned into the pCR4-TOPO vector (Invitrogen, Karlsruhe, Germany), and the identity of the fragment was confirmed by sequencing. The same procedure was used to clone a corresponding 800 nt fragment for use as a positive control of the XMRV genome (Genbank acc. num. EF185282). Conditions of first round PCR for the detection of proviral sequences were: 100 ng patient DNA, primer Out-For 5'-CCGTGTTCCCAATAAAGCCT-3', Out-Rev 5'-TGACATCCACAGACTGGTTG-3', (30 sec @ 94°C, 30 sec @ 60°C, 30 sec @ 72°C) × 20 cycles. Using $1/10^{th}$ of the first reaction and primer In-For 5'-GCAGCCCTGGGAGACGTC-3' and In-Rev 5'-CGGCGCGGTTTCGGCG-3' the second round PCR is able to detect any XMRV-like sequences, e.g. MLV DG-75. In addition, using a primer spanning the XMRV-specific deletion 5'-CCCCAACAAAGCCACTCCAAAA-3' we were able to distinguish between XMRV and DG75 sequences. Second round PCR reaction was performed at an elevated annealing temperature of 64°C for 35 cycles.

A nested-PCR strategy was used to detect XMRV-specific viral RNAs in the total RNA of prostate tissue samples in which the first round RT-PCR was performed as described above using *In-For* and *In-Rev* followed by a quantitative real-time PCR published by Dong *et al.*, 2007 [9,14]. As an internal control, a human GAPDH specific primer and probe set were included in which the primers for the outer RT-PCR were the same as for the inner PCR: forward 5'-GGCGATGCTGGCGCTGAGTAC-3' reverse 5'-TGGTCCACACCCATGACGA-3' and the probe 5'-YAK-TTCACCACCATGGAGAAGGCTGGG-Eclipse Dark quencher-3' [15].

### *RNaseL genotyping*

A real-time PCR setup designed by Olfert Landt/TIB MOLBIOL, Berlin was used for RNaseL genotyping of tumor samples which detects the single nucleotide polymorphism G1385A (rs486907) responsible for the R462Q mutation. PCR was carried out with R462Q_F CCTATTAAGATGTTTTGTGGTTGCAG, R462Q_A GGAAGATGTGGAAAATGAGGAAG and the probes R462Q_(A) YAK-ATTTGCCCAAAATGTCCTGTCATC-BBQ and R462Q_(G) FAM-ATTTGCCCGAAATGTCCTGTCATC-BBQ following a two-step protocol with 95°C for 20 sec and 60°C for 1 min. Positive controls were constructed by fusion PCR, starting with 40 mer oligonucleotides, of the two 297 bp fragments corresponding to the "R"- and "Q" versions of the RNAseL genomic region and cloning these into the pCR4-TOPO vector (Invitrogen). For each PCR, positive control plasmids containing the R- or Q-sequence were included.

### *Recombinant Proteins, Immunization*

Recombinant proteins of XMRV pr65 (Gag) and gp70 (Env/SU) were generated for immunization and for the ELISA assays. For XMRV Env/SU, a fragment containing the amino acids 1-245 of the surface unit (gp70) was amplified, cloned in pET16b vector (Novagen, Gibbstown, USA) and expressed in BL21 *E. coli*. For XMRV Gag (pr65), two fragments (amino acids 1-272 and 259-535) that overlap by 14 amino acids were constructed. The expressed proteins were affinity purified using a Ni-NTA column and eluted in 8 M urea, and proteins for immunization were subsequently dialyzed against phosphate buffered saline. BALB/c mice were immunized with the recombinant fragments of the Envelope or Gag proteins, and sera were collected throughout the period of four immunizations. All animal experiments were performed in accordance with institutional and state guidelines.

### *ELISA*

Two weeks after the last immunization the mice were bled, and serum antibodies were measured by solid phase enzyme-linked immunosorbent assay (ELISA). Briefly, bacterially expressed and purified (via His-tag) protein fragments were coated overnight on Probind-96-well plates (Becton Dickinson Labware Europe, Le Pont de Claix, France) at room temperature in equimolar amounts. The plates were blocked with 2% Marvel milk powder in phosphate buffered saline (PBS) for 2 h at 37°C, washed three times with PBS, 0.05% Tween 20 and serial diluted mouse sera or patient sera at a 1:200 dilution in PBS with 2% milk powder and 0.05% Tween20 added into each well. After incubation for 1 hour at 37°C, each well was again washed three times and a 1:1000 dilution of a goat anti-mouse IgG-HRP conjugate (Sigma Aldrich, Munich, Germany) in PBS, 2% milk powder, 0.05% Tween 20 (Serva, Heidelberg, Germany) was added. After further incubation for 1 hour at 37°C, each well was again washed three times. The chromogen orthophenylendiamin (OPD) in 0.05 M phosphate-citrate buffer, pH 5.0 containing 4 µl of a 30% solution of the hydrogen peroxide substrate per 10 ml was then added. After 5-10 minutes the color development was stopped by addition of sulphuric acid and the absorbance at 492 nm/620 nm was measured in a microplate reader.

Patient sera were tested for XMRV-Gag or -Env binding antibodies in the same way, using a goat anti-human IgG-HRP conjugate as secondary antibody (Sigma Aldrich, Munich, Germany). Out of the 146 sera samples only from 30 patients the corresponding nucleic acids were included in the 589 DNA/RNA samples.

### *Immunofluorescence microscopy*

Cells were grown on gelatine (0.3% coldwater fish gelatine in distilled water) coated glass slides in 12-well plates and 24 h after seeding were transfected using Polyfect Reagent (Qiagen) with the full length molecular clone pCDNA3.1-VP62 or with the pTH-XMRV-coEnv or pTH-XMRV-coGAG plasmids containing codon optimized synthetic full-length genes of the XMRV *env* or *gag* under control of the CMV promoter. 48 h after transfection the cells were fixed with 2% formaldehyde (Sigma) in PBS. Cells were rinsed briefly in PBS, permeabilized with 0.5% Triton X-100 in PBS for 15 min and washed 3 times with PBS. After 30 min incubation with blocking buffer (2% Marvel milk powder in PBS) cells were incubated for 60 min at 37°C with the mouse or human antisera diluted 1:200 in blocking buffer. The slides were washed extensively with PBS. The secondary antibodies conjugated to fluorophores were added for 30 min. After thorough

washing steps with PBS, the cells were mounted in Mowiol and the glass slides were placed upside-down on microscopy slides. Images were obtained on a Zeiss (LSM510) confocal laser-scanning microscope.

### Electron Microscopy

Transfected cells were fixed with 2.5 % glutaraldehyde in 0.05 M Hepes (pH 7.2) for 1 h at room temperature. Fixatives were prepared immediately before use. The samples were embedded in epoxy resin (Epon) after dehydration in a series of ethanol solutions (30%, 50%, 70%, 95%, and 100%) and infiltrated with the resin using mixtures of propylene oxide and resin followed by pure resin. Polymerization was carried out at 60°C for 48 h. Ultrathin sections (60-80 nm) were cut with an ultramicrotome (Ultracut S or UCT; Leica, Germany) and picked up on slot grids covered with a pioloform supporting film. To add contrast, sections were stained with uranyl acetate (2% in distilled water) and lead citrate (0.1% in distilled water). Sections were examined with a FEI Tecnai G2 transmission electron microscope.

## Results

### Determination of the RNaseL genotype of prostate cancer samples

The highly significant correlation between XMRV-positive prostate cancers and homozygosity for the QQ allel of the RNaseL SNP R462Q previously published [9,10] prompted us to analyze the genotypes of all 589 PCa samples included in our study. The DNA was extracted from prostate biopsies consisting of tumour cells and surrounding stromal tissue. Using a real-time PCR method that allows the underlying G1385A mutation at the DNA level to be detected, 76 specimens (12.9 %) were found to be homozygous for the QQ genotype. The RQ and RR genotypes were present at frequencies of 52.5% and 34.6%

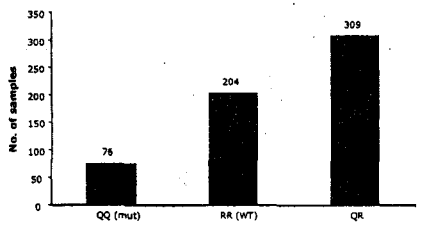


**Figure 1**
**Analysis of sample DNA with allele specific real time PCR for the R462Q genotype.** 76 of 589 samples (12.9%) are homozygous for the QQ allele, 204 samples (34.6%) are homozygous for the RR allele and 309 (52.5%) are heterozygous.

respectively (Fig. 1). All samples were screened in duplicate and gave consistent results.

### Screening for proviral XMRV sequences by nested PCRs

We developed a nested PCR able to detect and discriminate between XMRV and proviral sequences closely related to the endogenous murine gammaretrovirus DG-75 [16]. This discrimination is based on the XMRV-specific 24 nt deletion within a conserved retroviral region (Fig. 2A). To facilitate the development of the nested PCR and to evaluate its sensitivity, we constructed *de novo* the corresponding XMRV genomic region (nt 1-800 of the XMRV VP62 sequence) via fusion-PCR of oligonucleotides and cloned this fragment into the pCR4-TOPO vector to generate the pXMRV plasmid. In addition, the corresponding sequence of the DG-75 provirus was assembled and cloned in the same way to yield the pDG-75 vector.

Chromosomal DNA from a healthy human was spiked with serial 1:10 dilutions of pXMRV and used to assess the sensitivity of the nested PCRs. Following the first round that used the outer primers, two parallel second rounds with the primer pair In-For/In-Rev and In-For/Deletion-Rev were performed.

Both primer pairs allowed the specific detection of 10 or more copies of their targets. Use of the primers In-For/In-Rev with the pXMRV template resulted in a 174 nt PCR product, and a 198 nt product was produced with pDG-75 as template (Fig. 2B). Mouse tail DNA was also included as a positive control to amplify a 198 nt sequence from murine endogenous DG-75-like proviruses. As expected, no PCR signal was generated if the In-For/Deletion-Rev primer pair was used with pDG-75 or mouse tail DNA as template (Fig. 2C, lower panel, lane 17 and lane 21).

All 589 DNAs isolated from prostate biopsies were screened using the nested PCR setup and primer combinations described. The successful RNAseL genotyping of all 589 samples confirmed DNA integrity and the absence of PCR inhibitors in the samples. Specific fragments indicating the presence of XMRV (Fig. 2C upper panel) or a DG-75 related gammaretrovirus were obtained from none of the samples (Fig. 2C lower panel).

### Examination of total RNA for the presence of XMRV transcripts

To assess corresponding RNA samples, a comparable approach was used in which a first round RT-PCR for cDNA synthesis with primers amplifying XMRV and DG-75-like sequences was followed by quantitative real-time PCR for the specific detection of XMRV (Fig. 3A). Preliminary experiments performed with XMRV RNA (kindly provided by R. Silverman) indicated the ability to detect





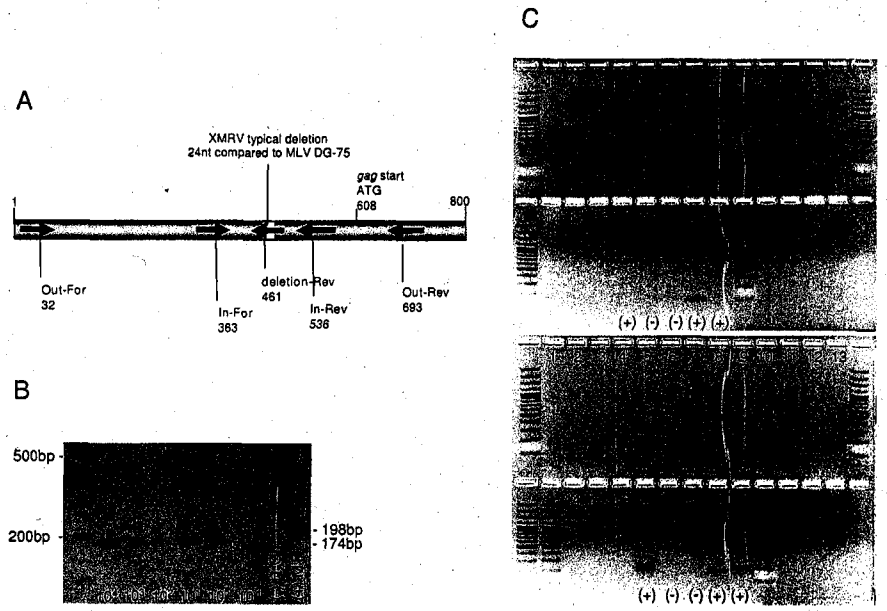


**Figure 2**
**Nested PCR for sensitive screening of patient tumor tissue DNA. (A)** A nested PCR primer setup was used as indicated for the screening of 589 PCa patient DNA isolated from prostate tumor and stroma tissue. Primer sites are numbered according to the XMRV VP62 sequence (Genbank EF185282). **(B)** The reproducible detection limit was 10 copies of plasmid DNA in human genomic DNA resulting in a 174 bp PCR product for XMRV. In the experiment shown even 1 copy could be amplified. Mouse tail DNA (MT) was used as positive control yielding a 198 bp product amplified from endogenous genomic MLV sequences. **(C)** Nested-PCR screen of the first 16 QQ patients (lane 1-16) with the In-For and Deletion-Rev primer pair (upper panel) and In-For and In-Rev primer setup (lower panel); lane 17 = mouse tail DNA, lane 18 = water control outer PCR mix, lane 19 = water control inner PCR mix, lane 20 = pXMRV, lane 21 = pDG75, marker = 100 bp marker.

as few as 10 transcripts (e.g. Fig. 3B), and the reproducible sensitivity to detect 100 transcripts. A human GAPDH primer and probe set was used in each sample as an internal control for the integrity of the RNA. Whereas all 589 samples generated a positive GAPDH signal with Ct-values between 16 and 20, no signals with the XMRV specific probe were obtained (Fig. 3C).

### XMRV antibody detection

Productive infection of humans by a murine gammaretrovirus related virus should induce an antibody response. Fragments of the cloned XMRV VP62 envelope (gp70) and the gag (pr65) protein were expressed in *E. coli* to provide a basis for an ELISA to detect XMRV-specific antibodies in the sera of prostate cancer patients. One fragment spanning the region from amino acids 1 to 245 of Env and two overlapping fragments spanning Gag were expressed and purified via an N-terminal His-tag.

Sera from immunized Balb/c mice (but not pre-immune sera) were reactive in ELISA against the recombinant proteins (data not shown). In addition, the specificity of the antibodies was confirmed by immunofluorescence microscopy using HEK 293T cells transfected with the expression plasmid pcDNA3.1-VP62 (kindly provided by R. Silverman) that carries the sequence of the replication active XMRV molecular clone (Fig. 4A and 4B). After transfection, these cells produce gammaretroviral particles vis-

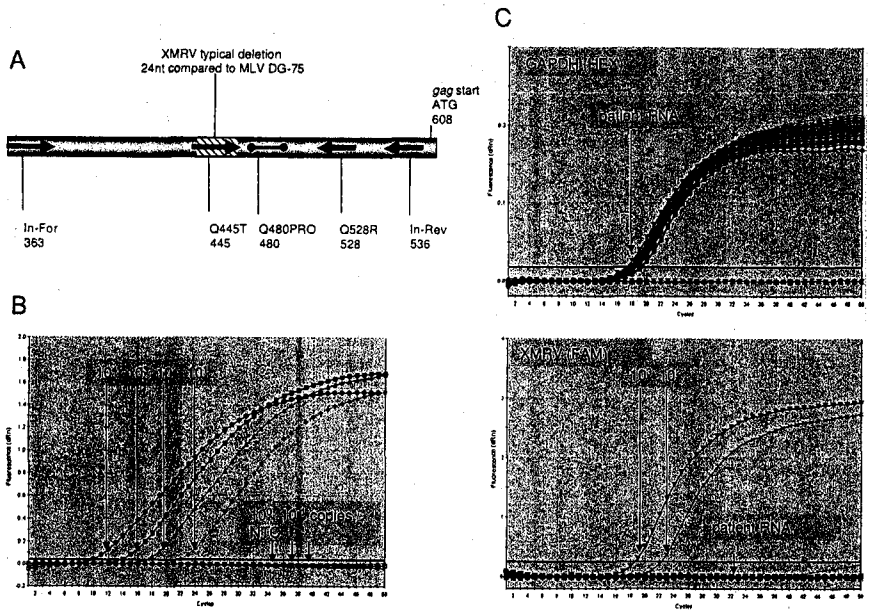


**Figure 3**
**Nested RT-PCR for sensitive and specific screening of patient tumor tissue RNA. (A)** RT-PCR for all 589 RNA samples was carried out with In-For and In-Rev primers, followed by a quantitative real-time PCR using primers and probe as indicated. Using the Q445T forward primer spanning the XMRV typical deletion ensured specific detection of XMRV sequences. Primer sites are numbered according to the XMRV VP62 sequence. **(B)** Real-time PCR curves showing the mean of triplicates. The sensitivity shown in this example was 10 copies. **(C)** Example of the first 16 QQ patients RNA screen including GAPDH control reactions as the mean value of duplicates.

ible by electron microscopy of ultrathin sections (Fig. 4C). This is, to our knowledge, the first visualisation of XMRV particles using thin section electron microscopy of transfected cells. The particles show the typical C-type budding structures and a classical morphology of MLV.

Of the 146 sera samples tested, the corresponding nucleic acids were available in 30 cases and were included in the amplification reactions as a subset of the 589 DNA/RNA samples. Of these 30 patients 2 were of the QQ genotype, 20 of QR and 8 were RR homozygous. In total, 146 sera from prostate cancer patients and 5 healthy control individuals were tested negative for antibodies binding recombinant XMRV gp70 and Gag proteins in ELISA, although postive control immunized mouse sera reacted strongly (Fig. 5A and 5B). One patient serum that reacted strongly in ELISA against the recombinant pr65 protein was subsequently tested by immunofluorescence assay using HEK 293T cells expressing XMRV and cells expressing the gp70- or pr65 proteins alone. No XMRV specific binding was seen, indicating a non-specific ELISA reaction.

## Discussion

XMRV is a recently discovered gammaretrovirus, found using RNA-based microarray techniques in tissue samples from prostate cancer patients [10]. XMRV was detected predominantly in patients who are homozygous for the QQ allele at R462Q in the RNaseL gene, which results in a reduced RNaseL activity and therefore in a diminished IFN-based antiviral defense. Later studies showed XMRV to be an infectious virus for human prostate-derived cells





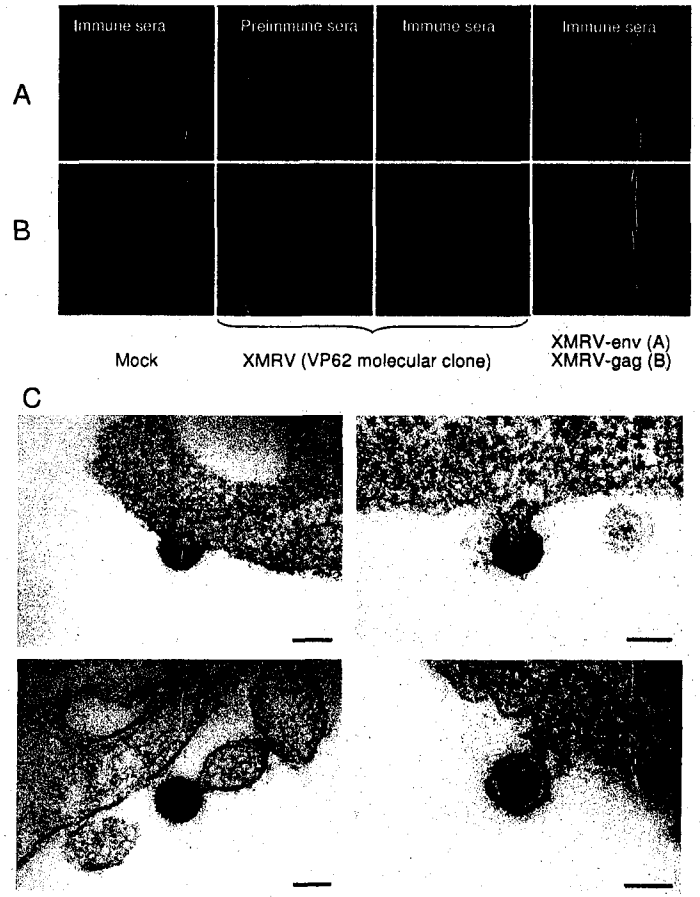


**Figure 4**
**Immunoflourescence microscopy and electron microscopy of transfected 293T cells.** Mice were immunized with recombinant gp70 or pr65 protein fragments, and sera were used for immunoflourescence microsocopy of 293T cells transfected two days earlier with the molecular XMRV clone VP62 or with gp70 and pr65 expression constructs. **(A)** A pool of sera from gp70 immunized mice showed reactivity against whole XMRV or XMRV envelope protein expressing cells. Preimmune sera showed no binding, and immune sera did not react with naive 293T cells. **(B)** A pool of immune sera from pr65 (Gag) immunized mice showed similar reactivity to whole virus or XMRV Gag expressing cells. Gag protein was expressed at higher levels in cells transfected with the CMV-driven codon-optimized *gag* construct than in those transfected with the VP62 molecular clone of XMRV. **(C)** Thin section of 293T cells 2 days after transfection with the VP62 molecular clone of XMRV. Particles budding at the cell membrane and a mature XMRV virion are shown. Scale bar = 100 nm.

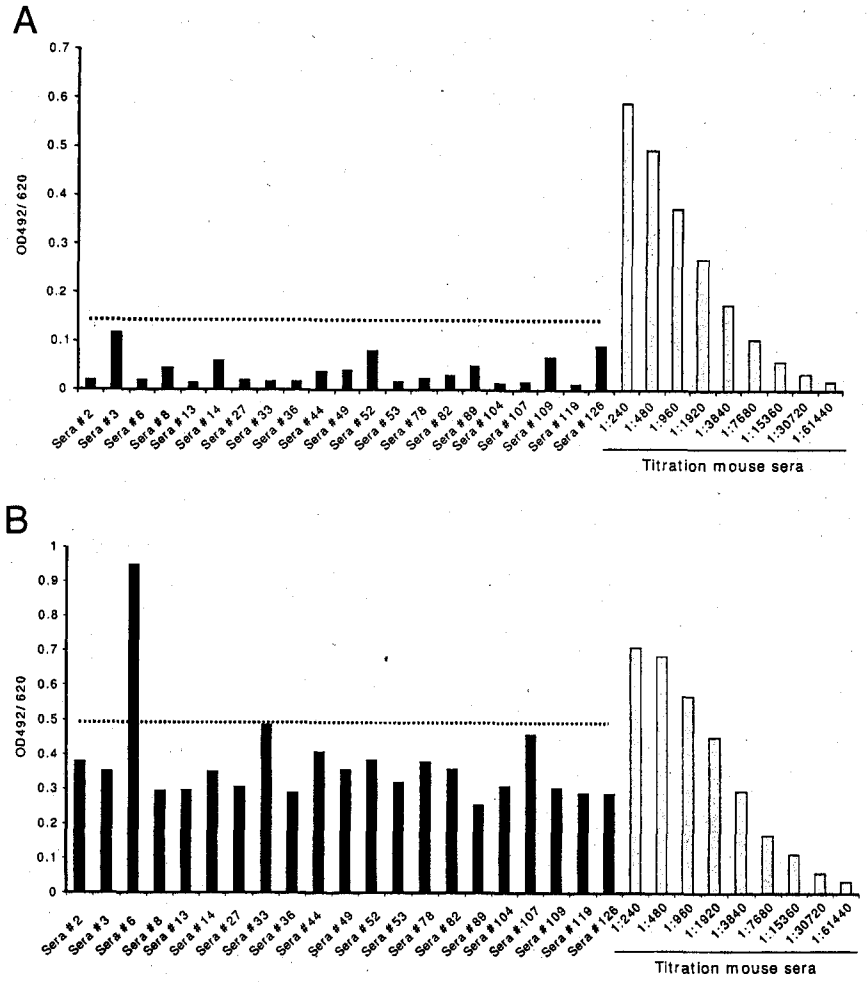


**Figure 5**
**ELISA of PCa patient's sera using recombinant XMRV proteins.** Mean ODs with two replicates of each patient sera diluted 1:200 (dark bars) and of serially diluted sera from immunized mice (light bars). Cut-off was calculated as the mean of four (gp70) and five (pr65) sera from healthy controls plus two times standard deviation. **(A)** ELISA of randomly chosen PCa patient sera using the gp70 (Env) fragment (aa 1-245). **(B)** ELISA using a mixture of both pr65 (Gag) fragments. In general there was a higher background against the pr65 proteins, seen also with the sera of healthy humans and the preimmune mouse sera.





and to be sensitive to RNaseL-mediated inhibition of replication by IFN-β [9]. The question of whether carcinogenic transformation renders the prostate epithelia cells susceptible to XMRV infection as a bystander virus or whether XMRV is itself a prostate cancer causing agent has not yet been addressed. It was very recently shown that XMRV could be detected in 22Rv1 prostate carcinoma cells originally derived from a primary prostatic carcinoma [17]. This observation further highlights the need to clarify the participation of XMRV in the etiology of human prostate carcinomas.

As known for many years in other cancers, e.g. HPV in cervical carcinoma or other cancers (reviewed by zur Hausen, 2009 [18]), infectious agents causing inflammatory (precancerous) lesions are suspected to be involved in the pathogenesis of prostate carcinoma [19,20]. An increased susceptibility of prostate epithelia cells to infection with RNA-viruses as a result of the impaired function of RNaseL, resulting in proliferative inflammatory atrophy (PIA), could be an intriguing scenario. These focal areas of epithelial atrophy are presumed to be precursors of prostatic intraepithelial neoplasia and prostate cancer [21]. A small number of other studies during the last ten years attempting to demonstrate a role for viral infections in the development of PCa have yielded rather inconclusive data [22-26].

If a real correlation between viral infection and prostate cancer exists, new therapeutic or even prophylactic treatments against the development of PCa could be developed by targeting, for example, viral antigens. In this respect, a recent observation that radiolabelled therapeutic monoclonal antibodies specific for HPV or HBV proteins can inhibit subcutaneous tumor development *in vivo* by cells expressing these antigens [27] is of particular interest.

In the present study, we report the testing of 589 DNA and RNA samples from sporadic prostate cancer patients for the RNaseL genotype and for XMRV sequences. Although 76 of our samples (12.9%) displayed the "susceptibility" QQ genotype, consistent with the frequency given in the literature, no XMRV-specific sequences were detected in either the RNA or the DNA from the prostate tumor samples. Given the ratio of approximately 40% positive cases harboring the QQ genotype in the study population of Urisman *et al.* [10], one would have expected at least 30 XMRV positive specimens amongst our 76 RNaseL QQ-allele samples.

At least two other studies have looked for XMRV at the nucleic acid level, albeit with a much smaller sample groups. Fischer and coworkers [11] studied material from 105 German patients with sporadic prostate cancer and found only one individual positive for XMRV by nested RT-PCR, but this individual did not display the QQ RNaseL genotype. Another study carried out in Ireland investigated 139 PCa patients. In 7 QQ patients and two heterozygous RQ samples, no XMRV sequences were detected [12].

It should be mentioned that this study cannot completely rule out the possibility of an infection with another gammaretrovirus in these patients. The design of our PCR approach was done in such a way that one primer pair (In-For/In-Rev) binds to conserved regions, allowing amplification of various MLV types including AKV MLV (J01998), MLV DG-75 (AF221065), MoMuLV (NC_001501), MTCR (NC_001702), MCF 1233 MLV (U13766), and Rauscher MuLV (NC_001819). In this PCR setup a specific signal was obtained with the mouse tail DNA as template, indicating that endogenous MLVs were detected. As additional controls we tested the cell lines 22Rv1 (XMRV positive [17]) and DU145 (XMRV negative [9]). As expected, 22Rv1 was found to be strongly positive for RNA transcripts and for provirus (with In-For/Deletion-Rev primers), while DU145 was negative in both PCR approaches (data not shown).

We also tested 146 sera samples for XMRV antibodies and found none of them to be positive in ELISA or Western blot analyses. The recombinant XMRV proteins that were used reacted positively with sera from immunized mice. As XMRV is closely related to other murine leukemia viruses and therefore immunogenic in mammalian hosts [28], an infection which allows the virus to spread to the stroma cells should induce a humoral immune response. The analysis of sera from prostate cancer patients for antibodies could therefore offer a rapid and valid screening method to investigate the involvement of a virus. Obviously the determination of sensitivity and specificity of these ELISA assays is to a certain degree limited, due to the lack of a human anti-XMRV positive control antibody. Nevertheless, the mouse sera were used to demonstrate the suitability of the recombinant antigens as ELISA antigens, even though the titration cannot be used to determine the amount of antibodies in the human sera samples. The failure to detect XMRV proviruses or transcripts in the 30 cases where DNA, RNA and sera samples were all available, is consistent with the negative ELISA results. It is theoretically possible that the tumor environment itself compromises the immune system and inhibits the antibody response to the tumor-associated viral antigens. This seems unlikely since animal studies have demonstrated that tumor diseases do not dramatically suppress systemic immunity [29]. There was a certain degree of background reactivity to the recombinant Gag proteins, as was also seen in an ELISA using a lysate of ultracentrifuge-concentrated virus as antigen (data not

shown). Difficulties with background signals in testing human sera for reactivity to MLV-derived antigens are well known when using whole virion particles as antigen [30], but this also occurs to a lesser extent when using recombinant proteins [28]. In general, there was a higher background reactivity against Gag in our 146 PCa and healthy control sera tested; and one serum reacted strongly to the pr65 protein. Upon further testing in Western blot and immunfluorescence assay, this serum showed no specificity for XMRV. It might be possible that antibodies directed against the transmembrane protein p15E were missed due to our choice of the gp70 and the pr65 antigen as targets. In other human retrovirus infections, HIV and HTLV antibodies against this region are detectable. Therefore, it should also be mentioned that before the serological assays using XMRV proteins were established all serum samples were screened for cross-reactivity with recombinant gp70, p15E and p27 [31,32] of another gammaretrovirus, the porcine endogenous retrovirus (PERV). All sera were found to be negative for any of these targets despite the obvious sequence homology of XMRV and PERV in the ectodomain of p15E and certain conserved regions in gp70 and p27. Regarding this point, it is also of interest that Furuta *et al.* [33], recently reported the detection by Western blot of antibodies specific for the XMRV Gag protein in blood bank samples from prostate cancer patients and healthy donors, but no Env-specific antibodies.

## Conclusion

In summary, we demonstrate in a large cohort of more than 500 German prostate cancer patients with a median age of 63 years and various stages of disease no evidence for infection by the recently discovered gammaretrovirus XMRV. This result possibly suggests that the rather restricted geographic incidence of XMRV infections, and the epidemiology of XMRV in the United States should therefore be studied closely. In addition, the oncogenic potential of the virus should be thoroughly investigated to exclude (or confirm) this viral infection as a possible trigger for the development of prostate cancer.

## Abbreviations

XMRV: xenotropic murine leukemia virus-related virus; PCa: prostate cancer; MLV: murine leukemia virus; pr: precursorprotein; gp: glycoprotein; SNP: single nucleotide polymorphism; nt: nucleotide;

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

OH carried out the molecular studies and drafted the manuscript. HK carried out the patient's sampling and preparation and drafted together with OH the manuscript. PB, LN and NaB carried out the generation and evaluation of antisera and corrected the manuscript. JD carried out the additional screening against related retroviruses. KM, RK and NB conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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文献4

# XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors

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**Xenotropic murine leukemia virus–related virus (XMRV) was recently discovered in human prostate cancers and is the first gammaretrovirus known to infect humans. While gammaretroviruses have well-characterized oncogenic effects in animals, they have not been shown to cause human cancers. We provide experimental evidence that XMRV is indeed a gammaretrovirus with protein composition and particle ultrastructure highly similar to Moloney murine leukemia virus (MoMLV), another gammaretrovirus. We analyzed 334 consecutive prostate resection specimens, using a quantitative PCR assay and immunohistochemistry (IHC) with an anti-XMRV specific antiserum. We found XMRV DNA in 6% and XMRV protein expression in 23% of prostate cancers. XMRV proteins were expressed primarily in malignant epithelial cells, suggesting that retroviral infection may be directly linked to tumorigenesis. XMRV infection was associated with prostate cancer, especially higher-grade cancers. We found XMRV infection to be independent of a common polymorphism in the *RNASEL* gene, unlike results previously reported. This finding increases the population at risk for XMRV infection from only those homozygous for the *RNASEL* variant to all individuals. Our observations provide evidence for an association of XMRV with malignant cells and with more aggressive tumors.**

Gleason | immunohistochemistry | retrovirus | RNaseL | xenotropic

Prostate cancer is the most common form of nonskin cancer in U.S. men (1). The lifetime risk for developing prostate cancer is ~1 in 6 (2) in the United States, and globally, 3% of men die of prostate cancer (3). Morbidity and mortality from prostate cancer are likely to grow further, given increasing longevity. Epidemiologic studies indicate that infection and inflammation may play a role in the development of prostate cancer (4, 5). A search for viral nucleic acids in prostate cancers led to the identification of xenotropic murine leukemia virus–related virus (XMRV) in ~10% of samples tested (6). Because only malignant tissues were analyzed in the initial report, an association of XMRV with prostate cancer could not be addressed. Our analysis of 233 cases of prostate cancers and 101 benign controls showed an association of XMRV infection with prostate cancer, especially with more aggressive tumors. XMRV proteins were almost exclusively expressed in malignant epithelial cells. Only rarely did we find XMRV proteins in benign stromal cells, in contrast to a previous report (6).

XMRV was originally discovered in patients with a reduced activity variant of the *RNASEL* gene, and a strong correlation between this variant (R462Q) and the presence of XMRV was reported: 89% of XMRV-positive cases and only 16% of XMRV-negative cases were homozygous (QQ) for this variant in a total of 86 cases (6). Our study of 334 cases allowed us to establish the independence of XMRV infection and the R462Q variant. This finding moves the population at risk for XMRV infection from a small, genetically predisposed fraction homozygous for the R462Q *RNASEL* variant to all men. Sequence comparisons have classified XMRV as a gammaretrovirus with a high similarity to murine leukemia viruses. We present experimental evidence that XMRV is indeed a gammaretrovirus. Gammaretroviruses cause leukemias and sarcomas in multiple rodent, feline, and primate species but have not yet been shown to cause cancers in humans. Taken together, our findings provide evidence consistent with a direct oncogenic effect of this recently discovered retrovirus. If established, a direct role for XMRV in prostate cancer tumorigenesis would open up opportunities to develop new diagnostic markers as well as new methods to prevent and treat this cancer with antiretroviral therapies or vaccines.

## Results

**A Molecular Clone of XMRV Infects Human Prostate Cells.** We constructed pXMRV1, a full-length XMRV molecular clone, using 2 overlapping clones from patient isolate VP62 (6) [gift of Don Ganem, University of California, San Francisco (UCSF)]. pXMRV1 was transfected into 293T cells. Reverse transcriptase (RT) activity was detected in the supernatant within 1–2 days of transfection (Fig. 1*A*), indicating the release of viral particles. These were inoculated onto naive 293T cells and LNCaP cells, a human prostate cancer cell line (American Type Culture Collection CRL-1740). Viral release from infected LNCaP cells was first seen on day 7 postinoculation and peaked at day 12. No particles were released from similarly inoculated 293T cells up to day 14. pXMRV1 is therefore an infectious molecular clone, and XMRV replicates efficiently in human prostate cells.

**XMRV Particles Have Type-C Retrovirus Morphology.** Particles released from XMRV-infected cells closely resembled those of a gammaretrovirus, Moloney murine leukemia virus (MoMLV), in size and morphology (Fig. 1 *B–E*). XMRV particles had an average diameter of 137 nm (SD = 9 nm), a spherical to somewhat pleomorphic shape, and characteristic lipid envelopes. The majority of particles contained an electron-dense, polygonal core with an irregular outline (average diameter 83 nm, SD = 8 nm), resembling mature type-C retroviral cores (Fig. 1*C*). Cores defined as "immature," i.e., spherical with an electron-lucent center, were also seen (Fig. 1*D*). A "railroad track," a term used to describe immature MoMLV cores (7), and formed by the radial alignment of the N- and C-terminal halves of the CA protein, was also seen in immature XMRV cores (Fig. 1*D*, arrowhead). These striking ultrastructural similarities between XMRV and MoMLV (Fig. 1*E*) suggest that the 2 viruses are assembled in a very similar manner.

**XMRV Proteins, Except for Env, Closely Resemble Those of MoMLV.** We identified XMRV proteins and defined their molecular weights by comparing Western blots of lysed XMRV and MoMLV virions probed with antisera specific to XMRV or to MoMLV Gag proteins (Fig. 1 *F* and *G*). In accordance with their high (~90%) sequence similarities, the molecular weights of XMRV and MoMLV Gag proteins were found to be very similar. We identified a 75-kDa band as the surface unit (SU) of the envelope (Env) protein, using rabbit antiserum specific to XMRV-Env SU. This antiserum did not react with the MoMLV-SU, consistent with the lower sequence similarity (54%) of the corresponding Env proteins and the general tendency of Env proteins to show greater evolutionary divergence, as compared to Gag or Pol proteins.

Author contributions: H.M.T. and I.R.S. designed research; R.S., D.J.C., and K.R.B. performed research; R.S., H.M.T., and I.R.S. analyzed data; and R.S. and I.R.S. wrote the paper.

The authors declare no conflict of interest.

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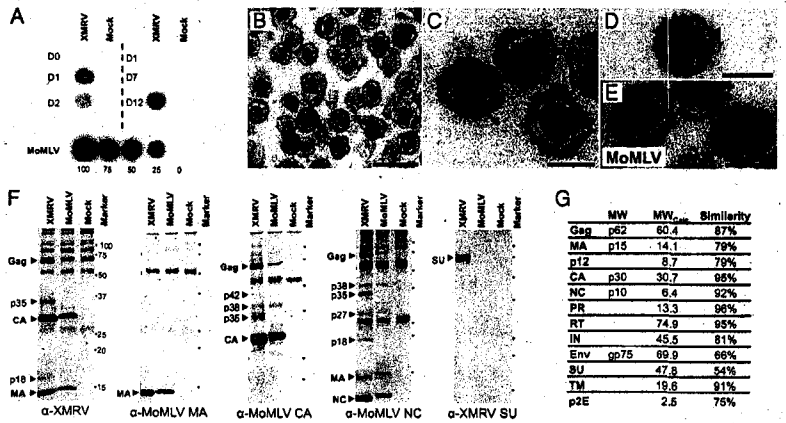


Fig. 1. The XMRV molecular clone produces infectious particles with morphology and composition similar to MoMLV. (*A*) Viral release from cells transfected or inoculated with pXMRV1 or XMRV, respectively. (*Left*) Reverse transcriptase (RT) activity in culture supernatants from cells transfected with pXMRV1 or control EGFP plasmid. (*Right*) RT activity from LNCaP cells inoculated with XMRV. (*Lower*) RT activity from NIH 3T3 cells chronically infected with MoMLV shown for comparison. (*B–E*) Transmission electron microscopy of XMRV particles (*B*), mature XMRV cores (*C*), immature XMRV core, with "railroad track" marked by arrowhead (*D*), and MoMLV particles with mature ("M") and immature ("I") cores (*E*). (*F*) Western blot analysis of lysed XMRV and MoMLV virions, using antisera to XMRV whole virus, MoMLV-CA, MoMLV-MA, MoMLV-NC, and XMRV-Env SU. Comparison of blots allows identification of intermediates of Gag proteolysis, e.g., p27 (MA-p12), p42 (p12-CA), and p38 (CA-NC). (*G*) Molecular weights of XMRV proteins as calculated by Western blot analysis and by sequence prediction and similarity between XMRV and MoMLV proteins. [Scale bars: 250 nm (*B*) and 100 nm (*C–E*).]

**XMRV Proviral DNA Is Detected in 6% of Human Prostate Cancers; Viral Loads of XMRV Are Low.** Our quantitative (q)PCR was designed to efficiently amplify XMRV proviral DNA from formalin-fixed, paraffin-embedded (FFPE) tissues. Primers and probes were chosen in a region of the integrase gene that is 100% conserved between all 3 published XMRV isolates and yet shares at most 80% similarity with the most closely related murine retroviral sequences (Fig. S1*A*). A common forward primer was used with 2 different reverse primers to allow for sequence differences in clinical isolates. Our qPCR was specific for XMRV sequences and did not amplify murine or human endogenous retroviruses; no amplification products were seen when using C57BL/6 mouse genomic DNA or human placental DNA as template. We tested the sensitivity of our qPCR assay in 2 ways. First, in the presence of excess human placental DNA, we could consistently detect 50 copies of the XMRV proviral clone and 5 copies 50% of the time (Fig. S1*B*). Second, because formalin fixation and embedding in paraffin compromise DNA quality, we also used fixed templates to test sensitivity. When DNA from FFPE human prostate tissue sections was spiked with known dilutions of DNA from fixed and embedded XMRV-infected, cultured cells, we consistently detected 1–2 infected cells per qPCR sample (Fig. S1*C*). We developed a second qPCR targeting the single-copy gene–vesicle-associated membrane protein 2 (VAMP2) to test for DNA integrity and amplification inhibitors [details in supporting information (*SI*) *Text*].

To estimate the prevalence of XMRV in men with and without prostate cancer, we analyzed 233 consecutively accessioned prostate cancers and 101 cases of transurethral resection of the prostate (TURP) as benign controls (Fig. S1*D*). We detected XMRV DNA in 14 (6.2%) cases of prostate cancer and in 2 (2.0%) controls. We determined XMRV proviral loads in these tissues. Using XMRV plasmid DNA as a standard, we estimated that qPCR-positive prostate cancers contained 1–10 copies of XMRV DNA per 660 diploid cells (see *Materials and Methods* and Fig. S1*E*). Because the number of tumor cells in any given section varies widely between tumors and even between different areas in the same tumor, it is impossible to estimate how many copies of XMRV DNA are present in each *tumor* cell. Using FFPE XMRV-infected cells as standards, we calculated that each 10-μm section from a prostate cancer contained the same amount of proviral DNA as 6–7 XMRV-infected cultured cells.

**XMRV Protein Is Expressed in 23% of Prostate Cancers and Is Predominantly Seen in Malignant Epithelium.** We developed XMRV-specific antisera and used them for immunohistochemistry (IHC). We first used XMRV-infected and uninfected cells that were mixed at different ratios and fixed in formalin and embedded in paraffin to mimic prostate tissue sections. We saw granular cytoplasmic staining in cells in proportion to the percentage of infected cells in the corresponding mixtures (Fig. 2 *A–C*). No staining was seen in uninfected cells or with preimmune serum (Fig. S2 *A* and *B*), confirming the specificity of our assay. We next performed IHC on prostate samples from XMRV qPCR-positive cases. We saw the same cytoplasmic granular pattern in tissues as in infected cultured cells (Fig. 2 *D* and *E*). Antiserum from a second rabbit resulted in identical staining. No staining was seen with preimmune serum (Fig. 2*F*).

We tested tissue sections from all 334 cases of prostate cancer and

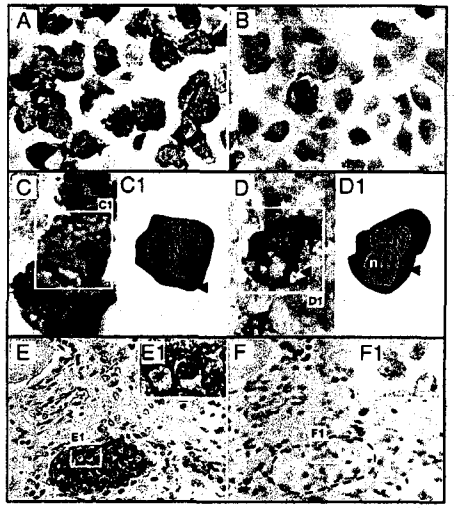


*Fig. 2. XMRV proteins detected in infected cultured cells and in prostate cancer tissue by IHC, using anti-XMRV antiserum. Counterstaining with hematoxylin reveals blue nuclei. (A and B) XMRV-infected cells: 100% infected (A) and 1% infected (B). (C) Cultured infected cells at higher magnification show cytoplasmic granular staining, represented diagrammatically in C1 (arrowhead, granules). (D–F) Human prostate cancers with clusters of malignant epithelial cells (E), with Inset at higher magnification (E1). Granular staining pattern seen at higher magnification. (F and F1) Adjacent section stained with preimmune serum from the same rabbit. N, nucleus; n, nucleolus.*

controls with benign prostatic hyperplasia. We found XMRV protein expression in 54 (23%) cases with prostate cancer and in 4 (4%) controls (Fig. 4*A*). In contrast to a previous report (6) that found XMRV-specific staining only in nonmalignant stromal cells, we observed XMRV-specific staining predominantly in malignant prostatic epithelial cells. XMRV proteins were expressed in epithelial cells in 46 tumors (85%), in both epithelial and stromal cells in 4 tumors (7.5%), and exclusively in stromal cells in another 4 tumors (7.5%). Of the 4 controls, XMRV expression was seen in epithelial cells in 3 and in both epithelial and stromal cells in 1 case. Epithelial cells expressing XMRV protein usually belonged to a single acinus or to a few adjacent acini. The proportion of cells expressing XMRV protein in a given tissue section varied widely (Fig. 3 *A–G*) but positive cells always represented a minority of cells on the slide. The vast majority of IHC-positive epithelial cells showed the same granular staining pattern of the entire cytoplasm that was seen in cultured cells (Fig. 3 *A–F*). However, the staining intensity and the subcellular pattern varied between cases, ranging from intense staining of the entire cytoplasm (Fig. 3*E*) to more discrete granular staining (Fig. 3 *C* and *D*), with some unusual staining patterns (Fig. 3*G*). In summary, XMRV proteins were expressed in 23% of prostate cancers and 4% of controls. Protein expression was seen in clusters of malignant epithelial cells and very rarely in stromal cells (Fig. 3 *H* and *I*).

**Presence of XMRV Correlates with Prostate Cancer and Higher Tumor Grade.** We tested for a correlation of XMRV positivity (by qPCR or IHC) with the presence, grade, and stage of prostate cancer. XMRV positivity was 5-fold higher in cancer than in benign controls (odds ratio = 5.7, $P < 0.0001$, Fig. 4*A*). We also tested for a correlation between XMRV positivity and tumor grade as measured by the Gleason score. We saw a correlation between XMRV positivity and higher-grade cancers (Fig. 4*B*). Of the 233 cases with cancer, we found XMRV positivity in 18% of Gleason 6 tumors, 27% of Gleason 7 tumors, 29% of Gleason 8 tumors, and 44% of Gleason 9 tumors ($\chi^2$-test for trend, $\chi^2 = 3.466$, $P = 0.06$, df = 1). Because only 1 case was a Gleason 10, it was not included in the analysis.

Most radical prostatectomy specimens contain relatively low pathological tumor–node–metastasis (TNM) stage cancers, because surgical treatment is not usually performed for higher stages. This is reflected in the distribution of tumor stages (pT) in our series: 75% pT2, 23% pT3, and 2% pT4. XMRV was detected in 25% of stage pT2 tumors and in 32% of pT3 tumors. Of the 5 cases with a pT4 stage, 1 (20%) was XMRV positive (Fig. 4*C*). This moderately increased prevalence of XMRV in advanced stage cancers was not statistically significant. Our sample had very few cases with nodal (N) metastasis and no cases with known distant metastases (M), preventing an investigation of a possible association of XMRV with higher N and M stages. We saw no association between XMRV infection and age at diagnosis (Fig. 4*D*).

**XMRV Infection Is Independent of the R462Q Polymorphism of *RNASEL*.** XMRV was initially discovered in prostate cancers from men homozygous for a common variant of the antiviral enzyme RNase L. This R462Q amino acid substitution results in a 3-fold reduction of enzymatic activity (8). In their study of 86 men with prostate cancer, Urisman et al. reported that 89% of XMRV-positive cases were homozygous for the R462Q variant (QQ) as compared to 16% of XMRV-negative cases (6). We profiled our 334 cases for the RNase L R462Q variant. The distribution was similar between cases with prostate cancer and controls (42.9% RR, 47.2% RQ, and 9.9% QQ in cancers vs. 52.5% RR, 40.6% RQ, and 6.9% QQ in controls, Fig. 4*E*). There was also no difference in allelic distribution between XMRV PCR-positive (50% RR, 43% RQ, and 7% QQ) and PCR-negative cases (42.7% RR, 47.4% RQ, and 10% QQ; Fig. 4*E*). The 2 XMRV-positive controls had RR alleles. When IHC was used to define XMRV-positive and -negative cases, the relative allelic distributions were also similar. We thus found no association between the presence of XMRV and the RNase L R462Q variant.

## Discussion

XMRV is a candidate infectious agent for causing prostate cancer. On the basis of sequence comparison, XMRV was classified as a xenotropic murine gammaretrovirus. We present the first experimental evidence in support of this classification. The morphology of XMRV particles was very similar to MoMLV, a related murine gammaretrovirus. Protein products of the 2 viruses had similar molecular weights, and antisera to most proteins of each virus. The notable exception to this was the SU portion of Env, which determines host specificity and sets xenotropic viruses apart from other related murine viruses. XMRV SU-specific antisera did not cross-react with MoMLV-SU, and the 2 proteins share only a 54% similarity (as opposed to 75–96% similarity for other viral proteins). Our findings thus support the classification of XMRV as a xenotropic murine gammaretrovirus.

We developed 2 sensitive and specific assays for the detection of XMRV in tissues. We used these qPCR and IHC assays to demonstrate the presence of XMRV DNA or proteins in 27% of cases in the largest series of human prostate cancers analyzed thus far. We show that XMRV proteins are expressed almost exclusively in cancerous epithelial cells. Moreover, the presence of XMRV correlated with more aggressive, i.e., higher-grade tumors. These findings provide support for a possible oncogenic effect of XMRV and are crucial for designing studies to investigate mechanisms of transformation.



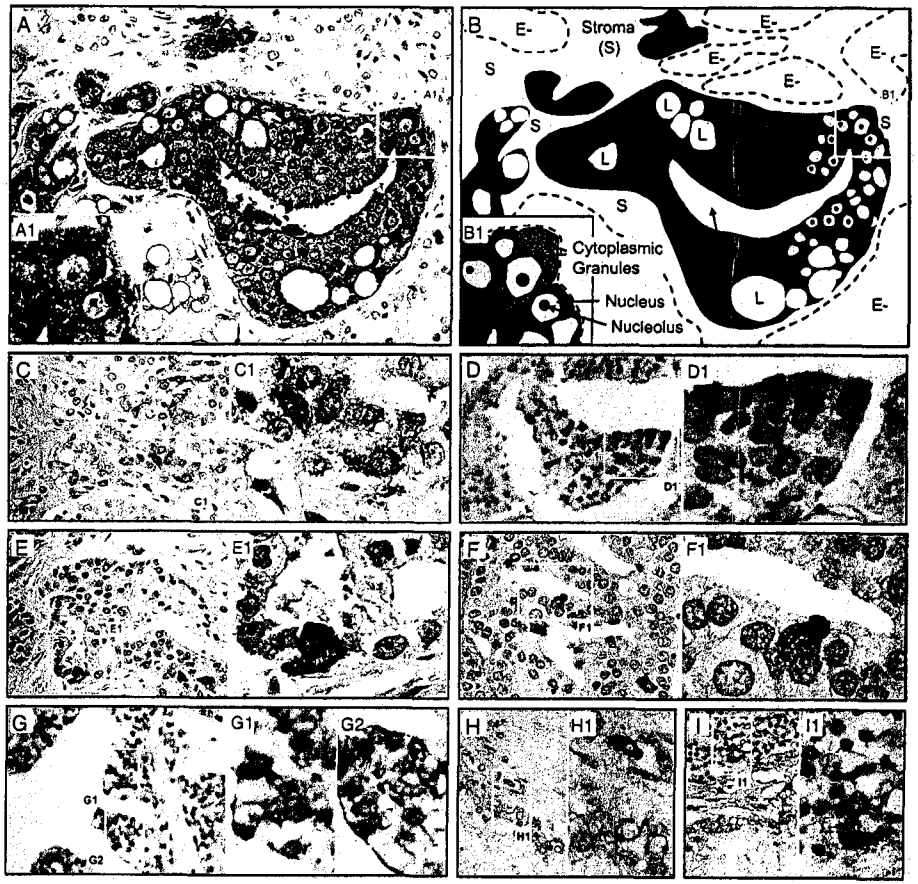


*Fig. 3. XMRV proteins are expressed primarily in malignant epithelial cells and very rarely in stromal cells. (A and B) IHC of a section from a qPCR-positive prostate cancer (A) and its diagrammatic representation (B). Nuclei of malignant cells are large and contain ≥1 large nucleoli (B). Multiple acini of malignant epithelial cells (E+) stain positive. All cells within these acini show intense staining. The stroma (S) and a few other acini (E−) are unstained. Insets (A1 and B1) show corresponding fields at higher magnification, with granular cytoplasmic staining pattern in several malignant epithelial cells. (C) A different field from the same sample as in A shows the range of XMRV protein expression in various acini: fewer cells expressing less protein but the same granular staining pattern. (D–F) Three additional representative samples with different frequencies of malignant epithelial cell clusters and different extents of XMRV protein expression. The intracellular staining pattern remains granular in all. (G) Staining limited to part of the cytoplasm of malignant epithelial cells in a subset of samples, as in this sample from which the XMRV clone VP62 was isolated, courtesy of R. H. Silverman and C. Magi-Galluzzi, Cleveland Clinic (6). (H and I) Scattered rare stromal cells showing cytoplasmic staining were seen close to malignant cells (H) or within inflammatory infiltrates (I).*

The fraction of cases positive for XMRV by qPCR (6%) was lower than by IHC (23%). This variation can be attributed to sampling differences in conjunction with very low viral loads. For the qPCR, detection rates depend on the *proportion* of XMRV-infected cells in the tissue. DNA from infected cells gets diluted in DNA from uninfected cells, thus limiting sensitivity if only a few cells in the sample harbor XMRV. However, qPCR allows a rapid survey of large numbers of tissue samples. In contrast, IHC detects individual XMRV-infected cells, avoiding the dilution effect of PCR. However, the number of cells analyzed is much smaller by IHC (a 5-$\mu$m section vs. a 100-$\mu$m section for DNA extraction) and only actively replicating virus can be detected. Because XMRV produces focal, low-level infections, the 2 assays complement each other and using both is likely to lead to the most accurate estimate of prevalence.

Two of our findings differ significantly from the initial report on XMRV (6). First, we found XMRV proteins in malignant epithelial cells in contrast to initial reports of XMRV proteins in nonmalig-

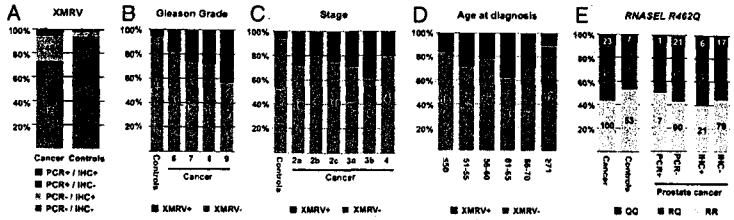


Fig. 4. XMRV DNA and proteins were more prevalent in prostate cancer than in controls, and especially frequent in high-grade cancers, and there was no correlation between presence of XMRV and any particular *RNASEL* genotype. (*A*) Number of prostate cancers or controls that were positive or negative for XMRV, either by qPCR or by IHC. (*B–D*) XMRV-positive cases (by either IHC or qPCR) correlated with Gleason grades (*B*), tumor stage (*C*), or age at diagnosis (*D*). (*E*) Presence of XMRV DNA or protein and the *RNASEL* genotype. Relative frequencies of RR, RQ, and QQ alleles in *RNASEL* at residue 462 were compared in prostate cancer cases and controls (*Left*), in cancers that tested positive or negative for XMRV DNA by qPCR (Center), and in cancers that tested positive or negative for XMRV proteins by IHC (*Right*). Cases are shown as percentages of total on the *y* axis and as number of cases within columns.

nant stromal cells. This can be mostly explained by our use of XMRV-specific antiserum instead of the monoclonal antibody to spleen focus-forming virus Gag protein used in the initial report. We were also able to detect XMRV in malignant epithelium from a case in the initial report (Fig. 3*G*), supporting the notion that antisera specific to XMRV offer a more sensitive means of viral detection. Second, we did not see any association of XMRV with the RNase L R462Q polymorphism as described initially. Methodological differences might account for this discrepancy. We tested prostate cancers for the presence of XMRV DNA and protein, whereas Urisman et al. used a nested RT-PCR to amplify viral RNA. It is conceivable that the reduced-activity variant of RNase L has a more significant effect on the levels of XMRV RNA, rather than on infection per se. Given low viral loads, the chance of detecting XMRV RNA may, therefore, be greater in homozygous individuals. Alternatively, the strength of association may depend on allelic frequencies and prevalence of XMRV. The distribution of RNase L R462Q alleles differed significantly between the 2 studies (23% QQ, 16% RQ, 61% RR in the study by Urisman et al. vs. 10% QQ, 43% RR, and 47% RQ in this study). Consistent with our findings, a survey in Northern European patients identified 2 individuals with XMRV; neither was homozygous for R462Q (9). The independence of XMRV infection from the RNase L R462Q variant indicates that all individuals may be at risk for XMRV infection, not just the ~10% of the population that is homozygous for R462Q. Preventive and antiviral measures will thus benefit a much larger at risk population.

Our finding that XMRV is present in cancerous epithelial cells has important implications for pathogenesis. If XMRV plays a role in prostate cancer development, but infects only nonmalignant stromal cells in the tumor as previously reported, new mechanisms of retroviral oncogenesis would need to be invoked. This finding has discouraged investigation of a causal role of XMRV in prostate cancer thus far. While such a new mechanism is possible, our findings are immediately compatible with classical mechanisms of cell transformation by retroviruses. Retroviruses follow 3 distinct pathways when transforming cells. The first is transduction by an oncogene, where a cell-derived oncogene such as *src* in the viral genome causes rapid transformation. The second is via an essential retrovirus gene transactivating cellular growth-promoting genes, as in the case of the Tax protein of HTLV-I that induces T cell leukemia (10, 11), or the Env protein of Jaagsiekte sheep retrovirus that induces lung cancer in sheep (12). XMRV contains no recognizable oncogene, but we do not understand each XMRV protein enough to rule out any role it might play in transactivation. Finally, there is the insertional activation of a cellular oncogene, a mechanism followed by most leukemia-causing murine gammaretroviruses. Multiple rounds of viral infection are typically needed for the activating insertion to occur. Cells containing the activating insertion are selected over others, leading over time to a distinctly clonal population. While a small number of XMRV integration sites have been sequenced from human prostate cancers (13, 14), no evidence of clonality has emerged yet. Furthermore, the mechanism of insertional activation requires that each cancer cell contains a provirus or, at a minimum, the regulatory sequences from 1 LTR. We estimated that qPCR-positive prostate cancers contained 1–10 copies of XMRV DNA per 660 diploid cells. Because the number of malignant cells in any section varies widely between cases and even between different sections in the same prostate, it is impossible to estimate how many copies of XMRV DNA are present in each *cancer* cell. Our IHC data show that not all malignant cells express XMRV proteins, a finding with 2 possible explanations. It is possible that the malignant cells that lack XMRV protein expression were never infected by XMRV at all—a possibility that is incompatible with any known mechanism of insertional activation by murine gammaretroviruses. Alternatively, it is possible that some XMRV-infected cells lose large portions of their proviral DNA over time, as seen in tumors induced by avian leukosis virus (ALV). In these ALV-induced tumors, an absence of proviral sequences essential for production of viral RNA in most cells, coupled with the absence of viral RNA in tumors, indicates that expression of viral genes is not required for maintenance of the tumor phenotype (15). More studies are required to determine whether XMRV plays any causal role in prostate cancer or whether the presence of the virus in malignant prostatic epithelium is simply a function of its preferential replication in prostate cancer cells.

In line with a slow mechanism for oncogenesis, detection of XMRV in 6% of our controls might indicate that XMRV causes cancer only after a long induction period. Alternatively, these cases may have cancer in an unsampled area of the prostate: TURP removes periurethral tissue whereas cancer usually arises in the periphery of the prostate. It is also possible that XMRV infection does not always lead to cancer. Because our study protocol involves de-identified samples, follow-up of these XMRV-positive controls is not possible.

The finding that XMRV replicates efficiently in a cell line derived from human prostate cancer but not in other human cell lines suggests a viral tropism that warrants further investigation. Is the virus associated with cancers in tissues other than the prostate or in gynecologic malignancies? How is XMRV transmitted? These are all intriguing questions that deserve further exploration. There is growing evidence that current prostate cancer screening algorithms result in early detection of cancers but do not effectively reduce mortality (16, 17). Many cases of prostate cancer are unlikely to manifest themselves during the patient's lifetime. There is a clear need for better markers to detect cancers that pose a significant health threat and to specifically target these for therapy. XMRV, because of its association with more aggressive cancers, might provide such a marker. Furthermore, there are often cases where a screening test is positive, but no tumor is detected on multiple biopsies, leaving the patient and his physician with no clear guidelines. A second XMRV-specific marker might provide further guidance. Large epidemiologic studies are needed to investigate correlation of XMRV with prostate cancer prognosis. The recognition that human papilloma viruses most often initiate cervical carcinomas has focused efforts on viral detection for early diagnosis and on preventive vaccination. Similarly, a determination that a retrovirus can cause prostate cancer would focus efforts on preventing transmission, antiviral therapy, and vaccine development. The pharmacological inhibition of viral replication, as achieved with HIV-1, could dramatically limit the pathological consequences of chronic viral infection.

## Materials and Methods

**Creating an Infectious Clone of XMRV.** Overlapping partial clones AM-2–9 and AO-H4 derived from patient isolate VP62 (6) (gift of Don Ganem, UCSF) were used to generate pXMRV1, a full-length clone of XMRV with a CMV promoter (details of construction and sequencing are in *SI Text*).

**Cell and Virus Production and Assay for Reverse Transcriptase Activity.** 293T cells were maintained in DMEM and LNCaP cells in RPMI, both supplemented with 10% FBS, L-glutamine (2.2 mM), penicillin (100 units/mL), and streptomycin (100 μg/mL). Cells were transfected with plasmid pXMRV1 or control plasmid pEGFP-C1 (Clontech), using Lipofectamine PLUS (Invitrogen) following manufacturer's directions. Supernatants were harvested at regular intervals, passed through a 0.45-μm filter (Whatman), and monitored for virus production by measuring RT activity ((18), details in *SI Text*).

**Transmission Electron Microscopy (TEM).** Virions were centrifuged through 20% (wt/vol) sucrose, resuspended in 2.5% glutaraldehyde in 0.1 M Sorenson's buffer, and processed for TEM as described (19). Samples were analyzed on a JEOL JEM-1200 EXII electron microscope and photographed using an ORCA-HR digital camera (Hamamatsu). Diameters of 100 virions and cores were measured in Adobe Photoshop.

**Anti-XMRV Antisera and Western Blot Analysis.** For generation of XMRV whole virus antiserum (anti-XMRV), supernatant from cultured, infected cells was passed through a 0.22-μm filter (Pallcorp); centrifuged (18, 20); lysed with detergent and inoculated into rabbits (details in *SI Text*). The rabbits were bled before inoculation for preimmune control sera. Western blot analysis of concentrated virions was performed as previously described for MoMLV (18–20). XMRV proteins were visualized with primary rabbit anti-XMRV, anti-MoMLV CA (NCI 795-804), anti-MoMLV MA (765-155), anti-MoMLV NC (805008, 1:7,500), and anti-XMRV-SU (1:500) antisera (MoMLV antisera and XMRV anti-XMRV-SU antisera were gifts of J. Rodriguez and S. P. Goff, Columbia University, New York). Data from at least 2 independent Western blots were used to determine XMRV protein sizes by comparison against molecular weight markers. MoMLV (NC_001501) was used for sequence comparisons.

**Acquisition of Human Prostate Samples: Cancer and Control Tissues.** Radical prostatectomy specimens ($n$ = 233) acquired at the Columbia University Medical Center (CUMC) between August 2006 and December 2007 were used to estimate the prevalence of XMRV in human prostate cancer. Prostate tissues removed by TURP for benign prostatic hyperplasia between January 2007 and April 2008 were used as controls ($n$ = 101). Details of tissue acquisition by banks, specimen selection, and processing are described in *SI Text*. Protected health information was removed and samples were de-identified by the tissue bank. Information about age at time of surgery, ethnicity, tumor stage, and tumor grade was retained (Table S1). Experiments were performed in accordance with the Institutional Review Board of CUMC (IRB-AAAC0089).

**DNA Extraction from Human Prostate Tissues.** DNA was extracted from 10 sections (10-μm thick) of FFPE tissue, quantified (Nanodrop 1000, Thermo Scientific), and stored at −80 °C (details in *SI Text*).

**Quantitative PCR Amplification of Proviral DNA.** BLAST analysis of overlapping 250-bp segments of the XMRV genome (VP35, GenBank ID DQ241301.1) identified a region of the integrase gene of XMRV that is 100% conserved between VP35, VP42, and VP62 but shares only 80–85% sequence identity with the most similar murine retroviruses. A forward primer, a hydrolysis probe, and 2 reverse primers were selected from this region using PrimerExpress (Applied Biosystems) (details in Table S2 and *SI Text*).

**Immunohistochemistry.** FFPE cultured XMRV-infected cells and prostate tissues were sectioned at 5-μm thickness and used for IHC. Details of sectioning, antigen retrieval, antibody treatment, counterstaining, protocol optimization, and controls are in *SI Text*.

**RNase L Genotyping.** The TaqMan SNP genotyping assay (assay ID: C___935391_1_), with the TaqMan SNP Genotyping Mix (both from Applied Biosystems), were used for RNase L G1385A (R462Q) genotyping (NCBI SNP reference: rs486907). Nine nanograms of prostatic DNA was used in a reaction volume of 20 μL. A TaqMan 7500Fast instrument was used for amplification, detection, and allelic discrimination. *RNASEL* genes from 2 individuals of each genotype were sequenced to confirm allelic discrimination results. DNA from 1 individual of each genotype was used as control in each subsequent experiment.

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the pathophysiology of chytridiomycosis appears to be disruption to the osmoregulatory functioning of the skin and consequent osmotic imbalance that leads to cardiac standstill.

To test whether treating electrolyte abnormalities would reduce the clinical signs of disease, we administered an oral electrolyte supplement to *L. caerulea* in the terminal stages of infection, when they lost the righting reflex and could no longer correct their body positions (*26*). Frogs under treatment recovered a normal posture and became more active; one individual recovered sufficiently to climb out of the water onto the container walls, and two individuals were able to jump to avoid capture. These signs of recovery were not observed in any untreated frogs. In addition, treated frogs lived >20 hours longer than untreated frogs [mean time after treatment ± SEM: treated frogs ($N$ = 9), 32 ± 2.8 hours; control frogs ($N$ = 6), 10.7 ± 2.2 hours; Student's $t$ test, $P < 0.001$]. All treated frogs continued to shed skin and ultimately died from the infection, as expected. It is unlikely that electrolyte treatment could prevent death unless the epidermal damage caused by *Bd* is reversed. Although amphibians can generally tolerate greater electrolyte fluctuations than other terrestrial vertebrates (*18*), we suggest that depletion of electrolytes, especially potassium, is important in the pathophysiology of chytridiomycosis. Amphibian plasma potassium concentrations are maintained at constant levels across seasons (*27*), and even moderate hypokalemia is dangerous in humans (*28*).

Our results support the epidermal dysfunction hypothesis, which suggests that *Bd* disrupts cutaneous osmoregulatory function, leading to electrolyte imbalance and death. The ability of *Bd* to compromise the epidermis explains how a superficial skin fungus can be fatal to many species of amphibians; their existence depends on the physiological interactions of the skin with the external environment (*16*–*19*). Disease outbreaks capable of causing population declines require the alignment of multiple variables, including a life-compromising pathophysiology (*1*). Resolving the pathogenesis of chytridiomycosis is a key step in understanding this unparalleled pandemic.

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### Supporting Online Material

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# Detection of an Infectious Retrovirus, XMRV, in Blood Cells of Patients with Chronic Fatigue Syndrome

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Chronic fatigue syndrome (CFS) is a debilitating disease of unknown etiology that is estimated to affect 17 million people worldwide. Studying peripheral blood mononuclear cells (PBMCs) from CFS patients, we identified DNA from a human gammaretrovirus, xenotropic murine leukemia virus–related virus (XMRV), in 68 of 101 patients (67%) as compared to 8 of 218 (3.7%) healthy controls. Cell culture experiments revealed that patient-derived XMRV is infectious and that both cell-associated and cell-free transmission of the virus are possible. Secondary viral infections were established in uninfected primary lymphocytes and indicator cell lines after their exposure to activated PBMCs, B cells, T cells, or plasma derived from CFS patients. These findings raise the possibility that XMRV may be a contributing factor in the pathogenesis of CFS.

Chronic fatigue syndrome (CFS) is a disorder of unknown etiology that affects multiple organ systems in the body. Patients with CFS display abnormalities in immune system function, often including chronic activation of the innate immune system and a deficiency in natural killer cell activity (*1*, *2*). A number of viruses, including ubiquitous herpesviruses and enteroviruses, have been implicated as possible environmental triggers of CFS (*1*). Patients with CFS often have active β herpesvirus infections, suggesting an underlying immune deficiency.

The recent discovery of a gammaretrovirus, xenotropic murine leukemia virus–related virus (XMRV), in the tumor tissue of a subset of prostate cancer patients prompted us to test whether XMRV might be associated with CFS. Both of these disorders, XMRV-positive prostate cancer and CFS, have been linked to alterations in the antiviral enzyme RNase L (*3*–*5*). Using the Whittemore Peterson Institute's (WPI's) national

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tissue repository, which contains samples from well-characterized cohorts of CFS patients, we isolated nucleic acids from PBMCs and assayed the samples for XMRV *gag* sequences by nested polymerase chain reaction (PCR) (*5*, *6*). Of the 101 CFS samples analyzed, 68 (67%) contained XMRV *gag* sequence. Detection of XMRV was confirmed in 7 of 11 WPI CFS samples at the Cleveland Clinic by PCR-amplifying and sequencing segments of XMRV *env* [352 nucleotides (nt)] and *gag* (736 nt) in CFS PBMC DNA (Fig. 1A) (*6*). In contrast, XMRV *gag* sequences were detected in 8 of 218 (3.7%) PBMC DNA specimens from healthy individuals. Of the 11 healthy control DNA samples analyzed by PCR for both *env* and *gag*, only one sample was positive for *gag* and none for *env* (Fig. 1B). In all positive cases, the XMRV *gag* and *env* sequences were more than 99% similar to those previously reported for prostate tumor–associated strains of XMRV (VP62, VP35, and VP42) (fig. S1) (*5*).

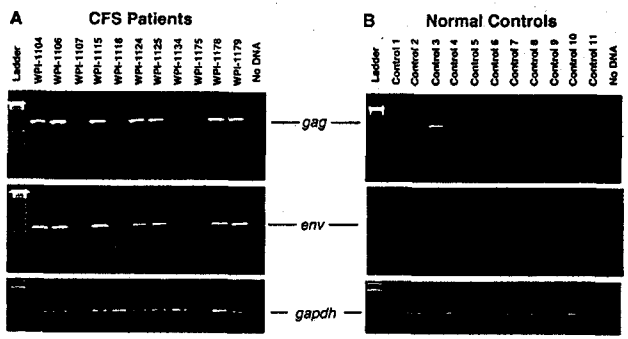


**Fig. 1.** XMRV sequences in PBMC DNA from CFS patients. Single-round PCR results for *gag*, *env*, and *gapdh* sequences in PBMCs of (**A**) CFS patients and (**B**) healthy controls are shown. The positions of the amplicons are indicated and DNA markers (ladder) are shown. These are representative results from one group of 20 patients.

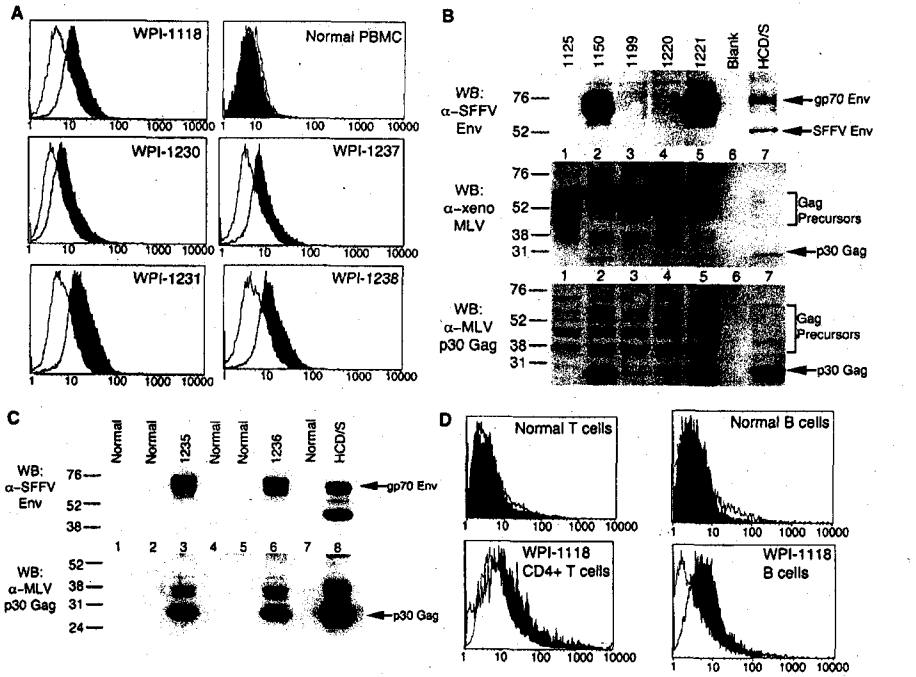


**Fig. 2.** Expression of XMRV proteins in PBMCs from CFS patients. (**A**) PBMCs were activated with phytohemagglutinin and interleukin-2, reacted with a mAb to MLV p30 Gag, and analyzed by IFC. (**B**) Lysates of activated PBMCs from CFS patients (lanes 1 to 5) were analyzed by Western blots with rat mAb to SFFV Env (top panel), goat antiserum to xenotropic MLV (middle panel), or goat antiserum to MLV p30 Gag (bottom panel). Lane 7, lysate from SFFV-infected HCD-57 cells. Molecular weight markers in kilodaltons are at left. (**C**) Lysates of activated PBMCs from healthy donors (lanes 1, 2, 4, 5, and 7) or from CFS patients (lanes 3 and 6) were analyzed by Western blots using rat mAb to SFFV Env (top panel) or goat antiserum to MLV p30 Gag (bottom panel). Lane 8, SFFV-infected HCD-57 cells. Molecular weight (MW) markers in kilodaltons are at left. (**D**) $CD4^+$ T cells (left) or $CD19^+$ B cells (right) were purified, activated, and examined by flow cytometry for XMRV Gag with a mAb to MLV p30 Gag.







Sequences of full-length XMRV genomes from two CFS patients and a partial genome from a third patient were generated (table S1). CFS XMRV strains 1106 and 1178 each differed by 6 nt from the reference prostate cancer strain XMRV VP62 (EF185282), and with the exception of 1 nt, the variant nucleotides mapped to different locations within the XMRV genome, suggesting independent infections. In comparison, prostate cancer–derived XMRV strains VP35 and VP42 differed from VP62 by 13 and 10 nt, respectively. Thus, the complete XMRV genomes in these CFS patients

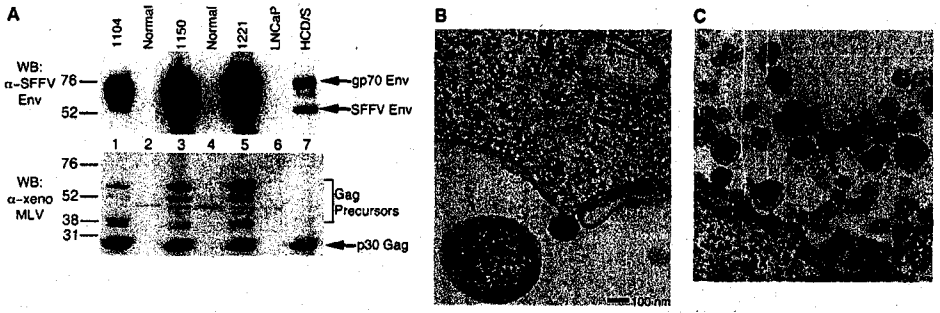


**Fig. 3.** Infectious XMRV in PBMCs from CFS patients. (**A**) Lysates of LNCaP cells cocultured with PBMCs from CFS patients (lanes 1, 3, and 5) or healthy donors (lanes 2 and 4) were analyzed by Western blots with rat mAb to SFFV Env (top panel) or goat antiserum to xenotropic MLV (bottom panel). Lane 6, uninfected LNCaP; lane 7, SFFV-infected HCD-57 cells. MW markers in kilodaltons are at left. (**B**) Transmission electron micrograph of a budding viral particle from LNCaP cells infected by incubation with an activated T cell culture from a CFS patient. (**C**) Transmission electron micrograph of virus particles released by infected LNCaP cells.

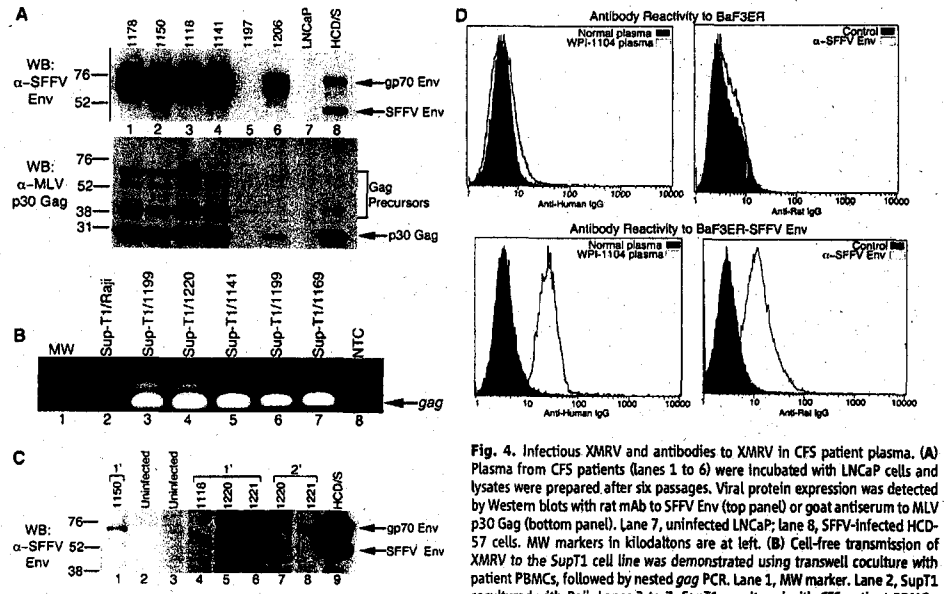


**Fig. 4.** Infectious XMRV and antibodies to XMRV in CFS patient plasma. (**A**) Plasma from CFS patients (lanes 1 to 6) were incubated with LNCaP cells and lysates were prepared after six passages. Viral protein expression was detected by Western blots with rat mAb to SFFV Env (top panel) or goat antiserum to MLV p30 Gag (bottom panel). Lane 7, uninfected LNCaP; lane 8, SFFV-infected HCD-57 cells. MW markers in kilodaltons are at left. (**B**) Cell-free transmission of XMRV to the SupT1 cell line was demonstrated using transwell coculture with patient PBMCs, followed by nested *gag* PCR. Lane 1, MW marker. Lane 2, SupT1 cocultured with Raji. Lanes 3 to 7, SupT1 cocultured with CFS patient PBMCs. Lane 8, no template control (NTC). (**C**) Normal T cells were exposed to cell-free supernatants obtained from T cells (lanes 1, 5, and 6) or B cells (lane 4) from CFS patients. Lanes 7 and 8 are secondary infections of normal activated T cells. Initially, uninfected primary T cells were exposed to supernatants from PBMCs of patients WPI-1220 (lane 7) and WPI-1221 (lane 8). Lanes 2 and 3, uninfected T cells; lane 9, SFFV-infected HCD-57 cells. Viral protein expression was detected by Western blot with a rat mAb to SFFV Env. MW markers in kilodaltons are at left. (**D**) Plasma samples from a CFS patient or from a healthy control as well as SFFV Env mAb or control were reacted with BaF3ER cells (top) or BaF3ER cells expressing recombinant SFFV Env (bottom) and analyzed by flow cytometry. IgG, immunoglobulin G.

were >99% identical in sequence to those detected in patients with prostate cancer. To exclude the possibility that we were detecting a murine leukemia virus (MLV) laboratory contaminant, we determined the phylogenetic relationship among endogenous (non-ecotropic) MLV sequences, XMRV sequences, and sequences from CFS patients 1104, 1106, and 1178 (fig. S2). XMRV sequences from the CFS patients clustered with the XMRV sequences from prostate cancer cases and formed a branch distinct from non-ecotropic MLVs common in inbred mouse strains. Thus, the virus detected in the CFS patients' blood samples is unlikely to be a contaminant.

To determine whether XMRV proteins were expressed in PBMCs from CFS patients, we developed intracellular flow cytometry (IFC) and Western blot assays, using antibodies (Abs) with novel viral specificities. These antibodies included, among others, (i) rat monoclonal antibody (mAb) to the spleen focus-forming virus (SFFV) envelope (Env), which reacts with all polytropic and xenotropic MLVs (*7*); (ii) goat antisera to whole mouse NZB xenotropic MLV; and (iii) a rat mAb to MLV p30 Gag (*8*). All of these Abs detected the human VP62 XMRV strain grown in human Raji, LNCaP, and Sup-T1 cells (fig. S3) (*5*). IFC of activated lymphocytes (*6, 9*) revealed that 19 of 30 PBMC samples from CFS patients reacted with the mAb to MLV p30 Gag (Fig. 2A). The majority of the 19 positive samples also reacted with antisera to other purified MLV proteins (fig. S4A). In contrast, 16 healthy control PBMC cultures tested negative (Fig. 2A and fig. S4A). These results were confirmed by Western blots (Fig. 2, B and C) (*6*) using Abs to SFFV Env, mouse xenotropic MLV, and MLV p30 Gag. Samples from five healthy donors exhibited no expression of XMRV proteins (Fig. 2C). The frequencies of CFS cases versus healthy controls that were positive and negative for XMRV sequences were used to calculate a Pearson $\chi^2$ value of 154 (two-tailed $P$ value of $8.1 \times 10^{-35}$). These data yield an odds ratio of 54.1 (a 95% confidence interval of 23.8 to 122), suggesting a nonrandom association with XMRV and CFS patients.

To determine which types of lymphocytes in blood express XMRV, we isolated B and T cells from one patient's PBMCs (*6*). Using mAb to MLV p30 Gag and IFC, we found that both activated T and B cells were infected with XMRV (Fig. 2D and fig. S4A). Furthermore, using mAb to SFFV Env, we found that >95% of the cells in a B cell line developed from another patient were positive for XMRV Env (fig. S4B). XMRV protein expression in CFS patient–derived activated T and B cells grown for 42 days in culture was confirmed by Western blots (fig. S4C) using Abs to SFFV Env and xenotropic MLV.

We next investigated whether the viral proteins detected in PBMCs from CFS patients represent infectious XMRV. Activated lymphocytes (*6*) were cocultured with LNCaP, a prostate cancer cell line with defects in both the JAK-STAT and RNase L pathways (*10, 11*) that was previously shown to be permissive for XMRV infection (*12*). After coculture with activated PBMCs from CFS patients, LNCaP cells expressed XMRV Env and multiple XMRV Gag proteins when analyzed by Western blot (Fig. 3A) and IFC (fig. S5A). Transmission electron microscopy (EM) of the infected LNCaP cells (Fig. 3B), as well as virus preparations from these cells (Fig. 3C), revealed 90- to 100-nm-diameter budding particles consistent with a gamma (type C) retrovirus (*13*).

We also found that XMRV could be transmitted from CFS patient plasma to LNCaP cells when we applied a virus centrifugation protocol to enhance infectivity (*6, 14, 15*). Both XMRV gp70 Env and p30 Gag were abundantly expressed in LNCaP cells incubated with plasma samples from 10 of 12 CFS patients, whereas no viral protein expression was detected in LNCaP cells incubated with plasma samples from 12 healthy donors (Fig. 4A). Likewise, LNCaP cells incubated with patient plasma tested positive for XMRV p30 Gag in IFC assays (fig. S5B). We also observed cell-free transmission of XMRV from the PBMCs of CFS patients to the T cell line SupT1 (Fig. 4B) and both primary and secondary transmission of cell-free virus from the activated T cells of CFS patients to normal T cell cultures (Fig. 4C). Together, these results suggest that both cell-associated and cell-free transmission of CFS-associated XMRV are possible.

We next investigated whether XMRV stimulates an immune response in CFS patients. For this purpose, we developed a flow cytometry assay that allowed us to detect Abs to XMRV Env by exploiting its close homology to SFFV Env (*16*). Plasma from 9 out of 18 CFS patients infected with XMRV reacted with a mouse B cell line expressing recombinant SFFV Env (BaF3ER-SFFV-Env) but not to SFFV Env negative control cells (BaF3ER), analogous to the binding of the SFFV Env mAb to these cells (Fig. 4D and S6A). In contrast, plasma from seven healthy donors did not react (Fig. 4D and fig. S6A). Furthermore, all nine positive plasma samples from CFS patients but none of the plasma samples from healthy donors blocked the binding of the SFFV Env mAb to SFFV Env on the cell surface (fig. S6B). These results are consistent with the hypothesis that CFS patients mount a specific immune response to XMRV.

Neurological maladies and immune dysfunction with inflammatory cytokine and chemokine up-regulation are some of the most commonly reported features associated with CFS. Several retroviruses, including the MLVs and the primate retroviruses HIV and HTLV-1, are associated with neurological diseases as well as cancer (*17*). Studies of retrovirus-induced neurodegeneration in rodent models have indicated that vascular and inflammatory changes mediated by cytokines and chemokines precede the neurological pathology (*18, 19*). The presence of infectious XMRV in lymphocytes may account for some of these observations of altered immune responsiveness and neurological function in CFS patients.

We have discovered a highly significant association between the XMRV retrovirus and CFS. This observation raises several important questions. Is XMRV infection a causal factor in the pathogenesis of CFS or a passenger virus in the immunosuppressed CFS patient population? What is the relationship between XMRV infection status and the presence or absence of other viruses that are often associated with CFS (e.g., herpesviruses)? Conceivably these viruses could be cofactors in pathogenesis, as is the case for HIV-mediated disease, in which co-infecting pathogens play an important role (*20*). Patients with CFS have an elevated incidence of cancer (*21*). Does XMRV infection alter the risk of cancer development in CFS? As noted above, XMRV has been detected in prostate tumors from patients expressing a specific genetic variant of the *RNASEL* gene (*5*). In contrast, in our study of this CFS cohort, we found that XMRV infection status does not correlate with the *RNASEL* genotype (*6*) (table S2).

Finally, it is worth noting that 3.7% of the healthy donors in our study tested positive for XMRV sequences. This suggests that several million Americans may be infected with a retrovirus of as yet unknown pathogenic potential.

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*Note added in proof:* V.C.L. is operations manager of Viral Immune Pathologies Laboratory, which is in negotiations with the Whittemore Peterson Institute to offer a diagnostic test for XMRV.








**Supporting Online Material**
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# Complete Reconstitution of a Highly Reducing Iterative Polyketide Synthase

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Highly reducing iterative polyketide synthases are large, multifunctional enzymes that make important metabolites in fungi, such as lovastatin, a cholesterol-lowering drug from *Aspergillus terreus*. We report efficient expression of the lovastatin nonaketide synthase (LovB) from an engineered strain of *Saccharomyces cerevisiae*, as well as complete reconstitution of its catalytic function in the presence and absence of cofactors (the reduced form of nicotinamide adenine dinucleotide phosphate and *S*-adenosylmethionine) and its partner enzyme, the enoyl reductase LovC. Our results demonstrate that LovB retains correct intermediates until completion of synthesis of dihydromonacolin L, but off-loads incorrectly processed compounds as pyrones or hydrolytic products. Experiments replacing LovC with analogous MlcG from compactin biosynthesis demonstrate a gate-keeping function for this partner enzyme. This study represents a key step in the understanding of the functions and structures of this family of enzymes.

Nature uses an amazing array of enzymes to make natural products (*1*). Among these metabolites, polyketides represent a class of over 7000 known structures of which more than 20 are commercial drugs (*2*). Among the most interesting but least understood enzymes making these compounds are the highly reducing iterative polyketide synthases (HR-IPKSs) found in filamentous fungi (*3*). In contrast to the well-studied bacterial type I PKSs that operate in an assembly line fashion (*4*), HR-IPKSs are megasynthases that function iteratively by using a set of catalytic domains repeatedly in different combinations to produce structurally diverse fungal metabolites (*5*). One such metabolite is lovastatin, a cholesterol-lowering drug from *Aspergillus terreus* (*6*). This compound is a precursor to simvastatin (Zocor, Merck, Whitehouse Station, NJ), a semi-synthetic drug that had annual sales of more than $4 billion before loss of patent protection in 2006 (*7*).

Biosynthesis of lovastatin proceeds via dihydromonacolin L (acid form **1**, lactone form **2**), a product made by the HR-IPKS lovastatin nonaketide synthase (LovB), with the assistance of a separate enoyl reductase, LovC (*8*) (Fig. 1). LovB is a 335-kD protein that contains single copies of ketosynthase (KS), malonyl–coenzyme A (CoA) acyltransferase (MAT), dehydratase (DH), methyltransferase (MT), ketoreductase (KR), and acyl-carrier protein (ACP) domains, as well as a section that is homologous to the condensation (CON) domain found in nonribosomal peptide synthetases (NRPSs) (*9*). It also contains a domain that resembles an enoyl reductase (ER) but lacks that activity. LovB must catalyze ~35 reactions and use different permutations of tailoring domains after each of the eight chain-extension steps to yield the nonaketide, dihydromonacolin L (**2**). This enzyme also catalyzes a biological Diels-Alder reaction during the assembly process to form the decalin ring system (*10*). In vitro studies of LovB (*11*) have been hampered by an inability to obtain sufficient amounts of the functional purified megasynthase from either *A. terreus* or heterologous *Aspergillus* hosts. As a result, the programming that governs metabolite assembly by LovB or other HR-IPKSs is not understood. Key aspects that remain to be elucidated include (i) the catalytic and structural roles of each domain in the megasynthase, (ii) substrate specificities of the catalytic domains and their tolerance to perturbation in megasynthase functions, and (iii) factors governing the choice of different combinations of domains during each iteration of catalysis. To initiate such studies, we engineered an expression system in yeast to produce large amounts of LovB and examined the influence of cofactors and the ER partner (e.g., LovC) on product formation.

The engineered *Saccharomyces cerevisiae* strain BJ5464-NpgA, which contains a chromosomal copy of the *Aspergillus nidulans* phosphopantetheinyl (ppant) transferase gene *npgA* (*12*), was the expression host. A C-terminal hexahistidine-tagged LovB was placed under the control of the *S. cerevisiae ADH2* promoter (*13, 14*) on an episomal plasmid (YEpLovB-6His). Abundant amounts of the intact LovB could be purified from the soluble fraction to near homogeneity with a final yield of ~4.5 mg/L (fig. S1). We used mass analysis of tryptic digest fragments to verify the identity of the recombinant LovB. The ACP domain of LovB was determined to be nearly completely phosphopantetheinylated by using a ppant ejection assay with high-resolution quadrupole orthogonal acceleration–time-of-flight mass spectrometry (fig. S2). To ascertain activity of the resulting LovB and to examine the necessity for cofactors, malonyl-CoA alone was first added to the purified enzyme in buffer. Whole-cell feeding studies of doubly [$^{13}$C, $^{2}$H]-labeled acetate to cultures of *A. terreus* showed that all three acetate hydrogens were incorporated into the acetate-derived starter units for both the nonaketide and diketide moieties in lovastatin (*15*). The purified LovB can use malonyl-CoA for both chain priming and chain elongation, loading malonate with decarboxylation to make the acetyl starter unit. Although LovB is able to prime with and elongate the chain by two further condensations with malonyl-CoA, in the absence of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), no ketoreduction occurs. The dominant product is lactone **3** (Fig. 2A, trace i), which forms by enolization and cyclization with off-loading of the unreduced triketide. Addition of NADPH to this system enables function of the KR domain. In this and subsequent experiments, the malonyl-CoA could be conveniently synthesized in situ by malonyl-CoA synthase (MatB) from *Rhizobium trifolii* using free malonate and CoA (*16*). With KR enabled, LovB makes penta-, hexa-, and heptaketide pyrones **4** to **6**, as well as ketones **7** and **8** (Fig. 2A, trace ii). The structures were confirmed by chemical synthesis of authentic standards, except for heptaketide **6**, which proved very unstable. However, the mass increase of 26 atomic mass units for **6** and its red shift in the ultraviolet spectrum when compared to **5** are consistent with its proposed heptaketide pyrone structure (table S3). Compounds **7** and **8** result from thioester hydrolysis of penta- and hexaketides stalling on the ACP at the β-keto stage. The resulting β-keto acids spontaneously decarboxylate to afford **7** and **8**. Formation of compounds **4** to **8** illustrates that derailment in the normal programmed steps, namely the lack of methylation due to the absence of *S*-adenosylmethionine

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# Failure to Detect the Novel Retrovirus XMRV in Chronic Fatigue Syndrome

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## Abstract

***Background:*** In October 2009 it was reported that 68 of 101 patients with chronic fatigue syndrome (CFS) in the US were infected with a novel gamma retrovirus, xenotropic murine leukaemia virus-related virus (XMRV), a virus previously linked to prostate cancer. This finding, if confirmed, would have a profound effect on the understanding and treatment of an incapacitating disease affecting millions worldwide. We have investigated CFS sufferers in the UK to determine if they are carriers of XMRV.

***Methodology:*** Patients in our CFS cohort had undergone medical screening to exclude detectable organic illness and met the CDC criteria for CFS. DNA extracted from blood samples of 186 CFS patients were screened for XMRV provirus and for the closely related murine leukaemia virus by nested PCR using specific oligonucleotide primers. To control for the integrity of the DNA, the cellular beta-globin gene was amplified. Negative controls (water) and a positive control (XMRV infectious molecular clone DNA) were included. While the beta-globin gene was amplified in all 186 samples, neither XMRV nor MLV sequences were detected.

***Conclusion:*** XMRV or MLV sequences were not amplified from DNA originating from CFS patients in the UK. Although we found no evidence that XMRV is associated with CFS in the UK, this may be a result of population differences between North America and Europe regarding the general prevalence of XMRV infection, and might also explain the fact that two US groups found XMRV in prostate cancer tissue, while two European studies did not.

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## Introduction

A recent study by Lombardi *et al.* [1] describing a gamma-retrovirus infection in 68 of 101 chronic fatigue syndrome (CFS) patients was notable not only for its claim of a new viral aetiology of a hitherto controversial disease, but also for the fact that proviral DNA could be amplified from the peripheral blood mononuclear cells (PBMC) of 3.75% (8/218) of the healthy controls. This follows an earlier claim that 1.7% (5/300) of healthy Japanese blood donors carried antibodies to the same virus [2]. The virus in question is a recently discovered retrovirus, Xenotropic Murine Leukaemia Virus (MLV)-Related Virus (XMRV).

In the original identification of XMRV in prostate cancer stromal cells, Urisman *et al.* [3] confirmed by sequence analysis that XMRV is not a laboratory contaminant, as is often the case with claims of new retroviral associations with disease. It shares >90% sequence identity in *gag* and *env* (two of the three viral structural genes) with other xenotropic MLVs.

An association between XMRV and prostate cancer was strengthened with the demonstration of XMRV protein expression in malignant epithelial cells [4]. However, these results have not been duplicated in studies conducted in Europe [5–7]. Both prostate cancer and CFS have been linked to an Arg to Gln mutation at codon 462 (R462Q) in the RNaseL gene, an interferon-induced ribonuclease [8]. On activation, RNaseL destroys single stranded cellular and viral RNA, thereby preventing viral replication, blocking protein synthesis, triggering cellular apoptosis and providing an innate anti-viral response. The two US studies are of interest, not only because this would be a further example of a virus association with cancer, but because they represent the first demonstration of a gamma-retrovirus able to infect human cells, over-riding the intrinsic immune mechanisms that were believed to protect humans from MLV infection.

The XMRV sequences derived from prostate cancer tissue are identical to those from CFS patients, but differ from xenotropic MLV sequences, endorsing a genuine cross-species transmission. However, the claim that XMRV is preferentially found in prostate tumours from patients homozygous for the R462Q variant [3] is not borne out by the second prostate cancer study to find XMRV in patients [4], nor was the genetic variant detected in CFS patients carrying XMRV [5].





The finding of Lombardi *et al.* of a 67% XMRV infection rate among CFS patients, if confirmed, would have a serious impact on understanding the pathogenesis of this complex and debilitating disease and its treatment. Therefore, it was important to determine if CFS sufferers in the UK were carriers of XMRV. We have screened DNA extracts from the blood of CFS sufferers by PCRs targeted at an XMRV-specific sequence and at a sequence conserved amongst most murine retroviruses (MRV).

## Methods

### Patients

All patients gave written informed consent for the use of their DNA to test aetiological theories of CFS, and the study was approved by the South London and Maudsley NHS Trust Ethics Committee. The study recruited 186 patients (62% female, age range 19–70, mean 39.6±11.3years) from consecutive referrals to the CFS clinic at King's College Hospital, London. All patients had undergone medical screening to exclude detectable organic illness, including a minimum of physical examination, urinalysis, full blood count, urea and electrolytes, thyroid function tests, liver function tests, 9 a.m. cortisol and ESR. Patients were interviewed using a semi-structured interview for CFS [9] to determine whether they met international consensus criteria for CFS. All subjects met the CDC criteria [10]; patients with the Fukuda-specified exclusionary psychiatric disorders, or somatisation disorder (as per DSM-IV), were not included. The patient set studied is a well-characterised and representative sample of CFS patients who have been described previously: all were routine clinic attendees, referred within the UK National Health Service, who had taken part in prior studies of neuroendocrine functioning [11] and/or of cognitive behaviour therapy [12]. As is typical of the patients seen in this tertiary care centre, they were markedly unwell. Few were working, and 19% were members of patient support groups for CFS/ME [12–14]. The levels of fatigue in this sample were high (mean Chalder Fatigue Scale, 26.3±5.4) [15], as were levels of disability (mean Work and Social Adjustment Scale, total score 28.2±7.2) [16]. The mean GHQ-12 score [17] was 19.7±8.1. Patients had been unwell for a median of 4.0 y (range 1–28 y). Of note was that 45% said their illness definitely related to a viral illness and 45% said it might relate to a viral illness. Overall, we conclude that this sample is typical of CFS patients seen in specialist clinical services in the UK. We also know from collaborative studies that our patients resemble those seen in other specialist CFS services in the United States and Australia [18].

**PCR detection of XMRV and MLV sequences.** DNA was extracted from EDTA whole blood using a standard phenol-based organic deproteinisation procedure [19]. DNA concentrations were determined by absorbance at 260 nm ($A_{260}$). Each sample was amplified in three nested PCRs using primers targeted to an XMRV-specific sequence, to a sequence conserved amongst most MLV and, as a control for sample addition and PCR-inhibition, to a human beta-globin (hBG) sequence (Table 1). Each first-round reaction was performed in a 25 µl volume containing 0.5 units TaqGold (Applied BioSystems, Warrington, UK), 1 x TaqGold reaction buffer (Applied BioSystems), 1.5 mM $Mg^{2+}$, 200 mM each dNTP, 2.5 pmol each primer to which 5 µl DNA extract or control was added. Reaction conditions were one cycle of 94°C, 8 minutes, 35 cycles of 94°C 30 seconds, 55°C 30 seconds, 72°C 30 seconds and one cycle of 72°C, 7 minutes. Second round reaction mixes were identical to the first round and the sample was a 1 µl transfer from the first round reactions. Second round reaction conditions were as for the first round over 30 cycles. PCR amplicons were visualised on a 1% agarose gel stained with ethidium bromide. Each PCR run consisted of test samples, six negative (water) and two positive controls. The positive control was a dilution of a plasmid with a full-length XMRV (isolate VP62) insert, generously gifted by Dr R. Silverman. To validate the sensitivity of the PCR, an end-point dilution of the plasmid was performed. To determine specificity of the PCR, a sample of human DNA from the LNCaP prostate cancer cell line (American Type Culture Collection, code CRL-1740) was amplified with the XMRV and MLV primer sets. To ensure integrity of the DNA extracts, three randomly selected samples were titrated to end-point using the hBG PCR to determine if the PCR copy number equated with the $A_{260}$. To determine if the DNA extracts exhibited low level non-specific inhibition of PCR, 10 samples were subjected to 30 cycles of the first round hBG PCR (reaction mix and conditions as above) followed by 40 cycles of a nested real-time SYBR-green PCR using the SYBR-green Fast PCR kit (Roche, Lewes UK) according to the manufacturer's instructions.

**Table 1.** Oligonucleotide Primers.

| Target | Sequence | Location |
|---|---|---|
| XMRV | Forward outer 5′ CATTCTGTATCAGTTAACCTAC 3′ | 411–432[1] |
| | Reverse outer 5′ ATGATCTCGAGAACACTTAAAG 3′ | 606–588[1] |
| | Forward inner 5′ GACTTTTTGGAGTGGCTTTGT 3′ | 441–461[1] |
| | Reverse inner 5′ ACAGAAGAACAACAAAACAAATC 3′ | 566–544[1] |
| MLV | Forward outer 5′ GGATCAAGCCCCACATACAG 3′ | 2796–2847[2] |
| | Reverse outer 5′ CATCAAACAGGGTGGGACTG 3′ | 3179–3160[1] |
| | Forward inner 5′ AGAAGTCAACAAGCGGGTGG 3′ | 2926–2945[2] |
| | Reverse inner 5′ GGTGGAGTCTCAGGCAGAAA 3′ | 3062–3043[1] |
| hBG | Forward outer 5′ TGGTGGTCTACCCTTGGACC 3′ | 148–162[2] |
| | Reverse outer 5′ GAGGTTGTCCAGGTGAGCCA 3′ | 296–277[2] |
| | Forward inner 5′ GAGGTTCTTTGAGTCCTTTGG 3′ | 170–190[2] |
| | Reverse inner 5′ CATCACTAAAGGCACCGAGCA 3′ | 273–253[2] |

Locations in GenBank accessions [1]EF185282, [2]NM000518.4.
doi:10.1371/journal.pone.0008519.t001

## Results

### Nested PCR Validation

Based on $A_{260}$ of the purified plasmid, both primer sets (XMRV, MLV) were able to amplify a single target copy added to the reaction. Amplification of 600 ng of LNCaP cellular DNA added to XMRV and MLV PCRs yielded no non-specific bands when viewed on an ethidium bromide-stained agarose gel. Quantification of DNA samples from three randomly selected test samples by end-point dilution PCR with the hBG primer set showed concurrence of the PCR-determined copy number with $A_{260}$, thus indicating integrity of the DNA preparations. Nested real-time amplification of 10 samples showed no evidence of non-specific inhibition as determined by the slope of the amplification curves and the height of the signal plateau.

### PCR Analysis of Test Samples

Input DNA ranged from 10 to 600 ng ($1.6\times10^3$ to $1.1\times10^5$ cell equivalents) as determined by $A_{260}$ of which 149 samples had an input of >100 ng and 106 samples >200 ng. None of the 186 test samples analysed yielded a specific PCR product with either the XMRV or MLV primer sets and no non-specific PCR products were observed. A specific hBG product was amplified from all 186 test samples. The positive control was amplified in each run by the

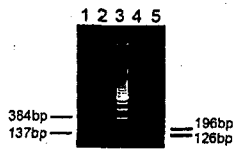


**Figure 1. PCR products of the XMRV VP62 clone.** Primers are generic to MLV (lanes 1 and 2) or specific to XMRV (lanes 4 and 5). The sizes of the respective fragments are shown. Lane 3–200 bp molecular size ladder.
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XMRV and MLV primer sets. A stained gel of the XMRV and MLV PCR products is shown in figure 1 and a representative sample of our results with CFS DNA and MLV primers is shown in figure 2.

## Discussion

Unlike the study of Lombardi *et al.*, we have failed to detect XMRV or closely related MRV proviral DNA sequences in any sample from CFS cases. There have been numerous claims for an infective aetiology to CFS over the years, not least because, as in this sample, many patients report that their symptoms were triggered by an infective episode. Prospective epidemiological studies have confirmed that certain infective agents, for example Epstein Barr virus, are unequivocally associated with subsequent CFS [20], even if the mechanisms are unclear and almost certainly multi factorial. Nearly two decades ago, sequences from another retrovirus, the human T-lymphotropic virus type II, were amplified from the PBMCs of 10/12 (83%) adult and 13/18 paediatric CFS patients, but not from healthy control subjects [21]. However, subsequent studies carried out on small numbers (20–30) of CFS patients, failed to confirm evidence for HTLV (type I or II) [22–25] or other retroviruses, including the closely-related simian T lymphotropic virus type I, the prototype foamy virus, simian retrovirus, bovine and feline leukaemia viruses [26] and HIV-1 [23].

The Lombardi paper is the first to study a significantly larger number of people than that in any previous study and to detect a virus only recently discovered. Our study resembles that of Lombardi *et al.* in certain respects. Both studies use the widely accepted 1994 clinical case definition of CFS[10]. Lombardi *et al.* reported that their cases "presented with severe disability" and we provide quantifiable evidence confirming high levels of disability in our subjects. Our subjects were also typical of those seen in secondary and tertiary care in other centres.

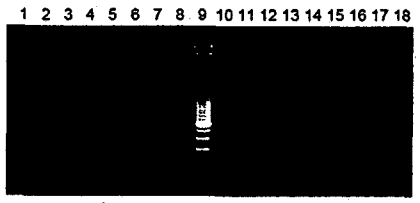


**Figure 2. Nested PCR from the DNA of 8 CFS patients.** Products of generic MLV primers (including XMRV) are shown. Lanes 1–8, CFS patient DNA (2nd round); lanes 9 and 10, XMRV 2nd round and 1st round positive controls; lanes 11 and 12, DNA of uninfected cell line LNCaP; lanes 13–18, water controls.
doi:10.1371/journal.pone.0008519.g002

Our own study also differs from that of Lombardi in other respects. Firstly, the PCR operator was blinded to the provenance of the DNA samples. In fact, with the exception of the PCR controls, all 186 DNA test samples originated from CFS patients. Care was taken to grow the XMRV plasmid in a laboratory in which no MLV had been cultured and no MLV vectors used and the PCR was carried out in a CPA-accredited Molecular Diagnostics Unit which processes only human tissue. Multiple (six) water (negative) controls were included in every run to detect low level contamination and a PCR to amplify a sequence that is conserved in most murine leukaemia viruses was included in order to expose any circulating MLV contamination and to detect any variant of XMRV that might be circulating in the UK CFS population.

Based on our molecular data, we do not share the conviction that XMRV may be a contributory factor in the pathogenesis of CFS, at least in the U.K.

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## Author Contributions

Conceived and designed the experiments: SK MM. Performed the experiments: OWE SK. Analyzed the data: SK MM. Contributed reagents/materials/analysis tools: SK GW DC SW AC. Wrote the paper: SK MM. Facilitated the study by setting up the collaboration: JW. Responsible for providing samples and associated data from a well characterised and valuable cohort of subjects: SW.

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文献7
RETROVIROLOGY

RESEARCH Open Access

# Absence of xenotropic murine leukaemia virus-related virus in UK patients with chronic fatigue syndrome

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**Abstract**

**Background:** Detection of a retrovirus, xenotropic murine leukaemia virus-related virus (XMRV), has recently been reported in 67% of patients with chronic fatigue syndrome. We have studied a total of 170 samples from chronic fatigue syndrome patients from two UK cohorts and 395 controls for evidence of XMRV infection by looking either for the presence of viral nucleic acids using quantitative PCR (limit of detection <16 viral copies) or for the presence of serological responses using a virus neutralisation assay.

**Results:** We have not identified XMRV DNA in any samples by PCR (0/299). Some serum samples showed XMRV neutralising activity (26/565) but only one of these positive sera came from a CFS patient. Most of the positive sera were also able to neutralise MLV particles pseudotyped with envelope proteins from other viruses, including vesicular stomatitis virus, indicating significant cross-reactivity in serological responses. Four positive samples were specific for XMRV.

**Conclusions:** No association between XMRV infection and CFS was observed in the samples tested, either by PCR or serological methodologies. The non-specific neutralisation observed in multiple serum samples suggests that it is unlikely that these responses were elicited by XMRV and highlights the danger of over-estimating XMRV frequency based on serological assays. In spite of this, we believe that the detection of neutralising activity that did not inhibit VSV-G pseudotyped MLV in at least four human serum samples indicates that XMRV infection may occur in the general population, although with currently uncertain outcomes.

## Background

In 2006, pursuing a link between prostate cancer and an inherited mutation in the RNASEL gene, Urisman and colleagues identified a novel gammaretrovirus [1]. Using PCR methodology, this virus was shown to be present in 9/86 (10%) prostate tumours examined. It showed close sequence similarity to xenotropic murine endogenous retrovirus elements and was thus named xenotropic murine leukaemia virus related virus (XMRV). A subsequent study demonstrated receptor usage typical of murine xenotropic virus [2]. Phylogenetic analyses place XMRV firmly within the murine endogenous retroviruses [3] even though no identical element has so far been identified within the mouse genome [4]. More recently, additional groups of samples from patients with prostate cancer have been examined for the presence of XMRV with both positive [5] and negative [6,7] results.

Very recently, a paper reporting the PCR detection of XMRV in PBMC from 68/101 patients with chronic fatigue syndrome (CFS) has been published [8]. Replicating virus could be isolated from stimulated PBMC with sequences almost, but not quite identical to the viruses isolated from prostate cancer patients. Providing apparently compelling evidence against the possibility of laboratory contamination, a number of the patients were shown to have mounted an immune response against XMRV. Interestingly, around 4% of control patients appeared to harbour the virus [8].

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Replication of these results and the possible identification of roles for XMRV in the aetiology of prostate cancer and/or CFS would be of great medical significance. Detection of XMRV might provide potentially useful diagnostic tools and might also suggest therapeutic avenues for treatment. Further, widespread distribution of a potentially pathogenic virus would have important implications concerning its role as a co-factor in other conditions and in the safety of the blood supply. We therefore set out to investigate the distribution of XMRV in UK CFS patients, using PCR to search for the presence of XMRV DNA and neutralisation assays to detect an anti-XMRV immune response. In this study we did not find any association between XMRV infection and CFS.

## Methods

### Sample collection

Samples from the following three centres were tested; St George's University of London (SGUL), Barts and the London Hospital Trust (BLT) and Glasgow Caledonian University (GC).

The SGUL cohort comprised 142 adult CFS patients and 157 healthy blood donors. Both groups were aged between 18 and 65, and the male to female ratios were 45:97 (CFS) and 43:114 (blood donors). At the time of sampling, 2003-2008, blood was collected into three tubes (an EDTA blood tube for DNA preparation; a Paxgene tube for RNA preparation and a plain tube for serum preparation from clotted blood). CFS patients were recruited from clinics in Bristol, Dorset, London, Birmingham, Norfolk and Epsom, and all patients fulfilled diagnostic criteria of Fukuda *et al.* [9]. Blood samples were taken between 1.5 and 4 years following diagnosis. Healthy normal blood donors were enrolled from the National Blood Service (NBS), in Dorset, UK. All subjects provided informed consent, and these studies were approved by Wandsworth Research Ethics Committee, St George's Hospital, Cranmer Terrace, London SW17 0RE.

The BLT cohort comprised 226 anonymised serum samples taken in 2008-2009 (57 from the antenatal clinic; 58 with haematological disorders; 55 liver patients and 56 from the renal clinic). Clotted blood was separated by centrifugation, and the serum supernatant was removed, stored at -20°C and defrosted once. Ethical approval for the use of these samples for assay development was issued by UCLH NHS trust and adopted by chairman's action at BLT.

The GC cohort comprised 28 CFS patients (20 sera and 8 plasma samples) and 12 controls (8 sera and 4 plasma samples) from the West of Scotland catchment area. CFS patients were aged between 28 and 79, with a male to female ratio of 16:12. Samples were collected between 1995 and 2003. Controls were aged between 23 and 63, with a male to female ratio of 7:5. Samples were collected between 2002 and 2004. Some controls were relatives of the patients, and some were hospital staff volunteers. All patients met the Fukuda criteria (9). Ethical permission for blood samples to be analysed for the presence of viruses was granted by Southern General Hospital NHS Trust Local Ethics Committee.

### PCR

Genomic (g)DNA was prepared from PBMC from SGUL patients and controls using the QIAamp DNA mini kit (Qiagen) and amplified using the RepliG Ultrafast Mini Kit (Qiagen), which provides highly uniform amplification of all sequences, with negligible sequence bias. The concentrations after amplification ranged from 108 - 586 ng/μl. Initially, 48 CFS patient gDNA samples were screened by single-round PCR for *gag* and *env* genes, as well as GAPDH, as outlined by Lombardi *et al.* [8] (Table 1). This PCR was performed in a 50 μl reaction volume consisting of 25 μl amplitaq gold PCR mastermix and a final DNA concentration of 2-5 ng/μl. Cycling was modified as appropriate to our mastermix; 95°C for 5 min, (95°C for 30 sec, 57°C for 30 sec, and 72°C for 60 sec) for 45 cycles, hold at 72°C for 7 min, store at 4°C. Products were visualized on 3% agarose gels by ethidium bromide staining. As we did not amplify any products using this PCR, we developed two more sensitive real-time qPCR assays which targeted 2 regions of the *env* gene, beginning at nt 6173 and

**Table 1 Primer sequences used in XMRV-specific PCRs**

| Primer | Sequence | Reference |
|---|---|---|
| 419F gag | ATCAGTTAACCTACCCGAGTCGGAC | Lombardi et al, 2009 |
| 1154R gag | GCCGCCTCTTCTTCATTGTTCT | Lombardi et al, 2009 |
| 5922F env | GCTAATGCTACCTCCCTCCTGG | Lombardi et al, 2009 |
| 6273R env | GGAGCCCACTGAGGAATCAAAACAGG | Lombardi et al, 2009 |
| hGAPDH-66F | GAAGGTGAAGGTCGGAGTC | Lombardi et al, 2009 |
| hGAPDH-291R | GAAGATGGTGATGGGATTTC | Lombardi et al, 2009 |
| Real-time PCR | | |
| 6173 env F | GGCATACTGGAAGCCATCATCATC | |
| 6173 env R | CCTGACCCTTAGGAGTGTTTCC | |
| 6173 env probe | ATGGGACCTAATTTCC | |
| 6682 env F | GTGCTGGCTGTGTCTAGTATCG | |
| 6682 env R | GCAGAGGTATGGTTGGAGTAAGTAC | |
| 6682 env probe | ACGGCCACCCCTTCGT | |

6682, respectively (Table 1). These were used to screen samples of gDNA (prepared from PBMC) or cDNA (prepared from total RNA extracted using the Paxgene system from Preanalytix, UK) from CFS and normal blood donors. In total, 136 CFS gDNA and 140 CFS cDNA samples and 95 control gDNA and 141 control cDNA samples were analysed, such that all 142 CFS patients and 157 blood donors were screened for XMRV using these assays in either genomic DNA, cDNA or both. GAPDH was also amplified as a control using a commercial primer and probe set (Hs_99999905_m1 from Applied Biosystems). Real-time qPCR reactions were performed in 10 μl total volume, consisting of 5 μl PCR mastermix, 0.5 μl (20×) Taqman primers/probe mix, 4.5 μl sample (for gDNA, 1 μl gDNA (100-590 ng) and 3.5 μl DEPC-treated water (Ambion); for cDNA, 4.5 μl cDNA). Cycling times and temperatures were as follows. Initial denaturation occurred for 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 sec and combined primer annealing/extension at 60°C for 1 min. Data were displayed using SDS 1.3.1 software (ABI).

### Plasmids

VP62 XMRV clone was a gift of Robert Silverman [2]. HG1 is a replication-incompetent XMRV clone constructed by site-directed mutagenesis of VP62 (the packaging signal was removed by deleting nucleotides 293-388, as numbered in GenBank EF185282; and nucleotides 7720-8108 were replaced by a BsrG1 site to remove the U3 region). Moloney-MLV Gag-Pol was expressed from KB4, a vector synthesized by cloning the *gag-pol* region from pMD-MLV GagPol [10] into pcDNA3.1. Viral genomic RNA was expressed from an MLV-based retroviral vector encoding β-galactosidase (LTR-LacZ [10]), and envelope proteins were encoded by constructs for either NZB xenotropic envelope, MLV(X) (a gift of Massimo Pizzato), Moloney-MLV env (MOSAF, a gift of Yasu Takeuchi), Friend-MLV env [10], or the G-protein from vesicular stomatitis virus (VSV-G) [11].

### Virus production

Replication defective XMRV virus was prepared for neutralisation assays by co-transfecting 293T cells with HG1 and LTR-LacZ. Pseudotyped MLV was prepared by co-transfecting 293T cells with KB4, LTR-LacZ and an envelope-encoding plasmid (either MLV(X), MOSAF, Friend or VSV-G). After ~18 hours, cells were washed, and fresh media was added for a further ~24 hours, before viral supernatants were harvested, filtered, and the viral titre was measured by ELISA for RT activity (Cavidi tech). Viral stocks were titrated on D17 cells, an established, easily infectable dog cell line, or NIH-3T3 cells for Friend- and Moloney- pseudotyped MLV. After 48 hours, the cells were assayed for β-galactosidase activity using the Galacto-Star system (Applied Biosystems). The amount of virus to be used in the neutralisation assays was determined as the volume of supernatant added to $3.5 \times 10^3$ cells that resulted in ~$4 \times 10^4$ counts per second of chemiluminescence.

### Neutralisation assays

*Neutralisation assays were performed as reported in* [12]. Monoclonal antibodies to MLV Env proteins (shown in Table 2) were gifts from Leonard Evans and have been previously described [13,14]. They were provided and used as untreated hybridoma cell supernatants that were serially diluted two-fold before adding to virus to assess neutralisation activity as for serum, detailed below. Serum samples were heat inactivated at 56°C for 30 min. 5 μl serum were then added to 95 μl media in a 96-well tissue culture plate, and samples were serially diluted two-fold, leaving 50 μl at each dilution. 50 μl virus-containing supernatant were then added to each well, and the plate was incubated at 37°C for 1 hour. Following incubation, 100 μl containing $3.5 \times 10^3$ D17 cells (or NIH-3T3 cells for Friend or Moloney-MLV neutralisation) were added to each well, and the plate was incubated at 37°C. After 48 hours the cells were lysed, and β-galactosidase activity was measured. Infectivity corresponded to counts per second of chemiluminescence.

## Results

### PCR screening

*Lombardi et al. have recently detected XMRV DNA in* 67% of CFS patients by PCR [8]. To confirm an association of XMRV with this disease, we performed PCR for *gag, env* and GAPDH on 48 (of 142) CFS patient gDNA samples from SGUL using the previously published single-round PCR methodology (Table 1 and [8]. However, although all samples were positive for GAPDH, we found no evidence of XMRV DNA in any of the samples (data not shown). In case we were missing low levels of viral DNA, we devised a more sensitive qPCR-based approach. To test the sensitivity of this method, triplicate, serial 1:10 dilutions of VP62 plasmid encoding the full length XMRV genome were added to PBMC DNA from a healthy donor and tested by Taqman PCR with either env 6173 or env 6682 primers (Table 1). All replicates calculated to contain 16 copies of XMRV routinely yielded a product within 37 cycles whereas only one of three replicates of the next dilution scored positive (Figure 1). We concluded that our assay was capable of reliably detecting as little as 16 copies of proviral DNA and was therefore likely to be as sensitive, if not more so, than the assays previously used [8]. We then tested the entire SGUL panel of 142 CFS samples and 157 of the control samples (either gDNA, cDNA or both) with both env 6173 and env 6682 primers. Although positive for GAPDH, all samples were negative for XMRV. To exclude the possibility of specific sample-mediated PCR inhibition, we spiked 3 normal control cDNAs, which had previously tested negative for XMRV nucleic acid, with XMRV VP62 DNA, to a final concentration of $2.3 \times 10^{-6}$ ng/μl and repeated the qPCR using both env 6173 and env 6682 primer sets. We successfully amplified the VP62 in these reactions, proving that the PCR should have amplified XMRV from the patient samples if it was present.

**Table 2 Neutralisation properties of different monoclonal antibodies against XMRV and MLV pseudotyped with three different envelopes.**

| | | | | Neutralisation of | | |
|---|---|---|---|---|---|---|
| Hybridoma[1] | Raised in | Isotype | XMRV | MLV(X) | Friend | Moloney |
| 83A25' | Rat | IgG2A | Y (88) | Y (89) | ND | ND |
| 24-7 | Mouse | IgMK | N | N | ND | ND |
| 48 | Mouse | IgG2A | N | N | Y (95) | Y (83) |
| 538 | Mouse | IgM | N | N | N | Y (63) |
| 603 | Mouse | IgM | N | Y (96) | N | ND |
| 609 | Mouse | IgM | Y (71) | N | ND | ND |
| 610 | Mouse | IgM | N | Y (64) | ND | ND |
| 613 | Mouse | IgM | N | Y (91) | ND | ND |
| 615 | Mouse | IgM | N | N | ND | ND |

Y indicates neutralisation; N indicates no neutralisation; ND is not determined
The number in brackets refers to the percentage neutralisation at the least diluted antibody concentration.
[1] See references [13] and [14] for description of hybridoma cell lines



### Neutralisation assays

In light of the negative data obtained using PCR assays, we set out to search for evidence of XMRV infection using a second method. Viral infection can elicit a neutralising antibody response [12]. Demonstration of such a neutralising activity can be taken as evidence for a viral infection, perhaps in cell types that were not sampled in blood. Defining neutralisation is difficult in the absence of known positive and negative sera. However, a number of neutralising monoclonal antibodies directed against the Env protein of murine retroviruses have been described [13,14]. We therefore obtained several of these (gifts of Leonard Evans) and tested them for neutralisation of XMRV and NZB xenotropic MLV (X) as well as ecotropic Friend and Moloney MLV (Table 2) by assaying for a reduction in virus infectivity following incubation of virus-containing supernatant

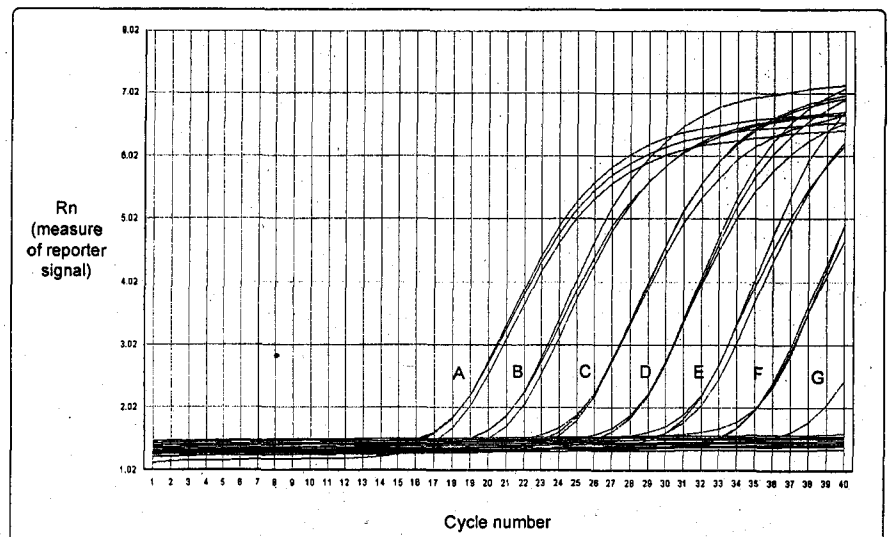


**Figure 1 Sensitivity of PCR screening for XMRV in PBMC DNA.** VP62 plasmid was serially diluted 1:10 into PBMC DNA from a healthy donor and tested by Taqman PCR with env 6173 primers and probe. The final amount of VP62 DNA in the reaction was A, $2.3 \times 10^{-2}$ ng, B, $2.3 \times 10^{-3}$ ng, C, $2.3 \times 10^{-4}$ ng, D, $2.3 \times 10^{-5}$ ng, E, $2.3 \times 10^{-6}$ ng, F, $2.3 \times 10^{-7}$ ng or G, $2.3 \times 10^{-8}$ ng. The limit of sensitivity was $2.3 \times 10^{-7}$ ng (shown by trace F) which equates to 16 molecules of VP62 XMRV clone.

with the monoclonal antibody. As anticipated, some monoclonal antibodies were able to neutralise XMRV (83A25' and 609) whilst others had no effect on XMRV infectivity. Interestingly, we identified three monoclonal antibodies that neutralised MLV(X) but not XMRV (603, 610 and 613) and one that neutralised XMRV but not MLV(X) (609). These reagents may therefore be useful tools with which to distinguish XMRV from other xenotropic MLVs in future investigations. From these experiments we defined two negative (603 and 613) and one positive (83A25') antibody controls for further experiments. To validate the neutralisation assay and examine the possible range of responses to "normal serum", we tested neutralisation using a panel of 226 serum samples from BLT. Previous investigations have detected XMRV DNA in ~1-6% of control samples [5,6,8]. Of our panel only a handful showed possible neutralisation activity, giving curves similar to that shown in Figure 2A, with reductions in viral infectivity similar or greater than that seen with the positive control, monoclonal 83A25'. Over 90% of the samples tested had less than a 2-fold effect on infectivity (Figure 3A). From these data, we have defined a positive as a sample that reduces viral infectivity by at least 70% at a dilution of 1/40 and gives a reduction of 50% at a 1/80 dilution. According to this definition, the BLT sample set contains 3 neutralising sera, identifying 1.3% of samples as positive.

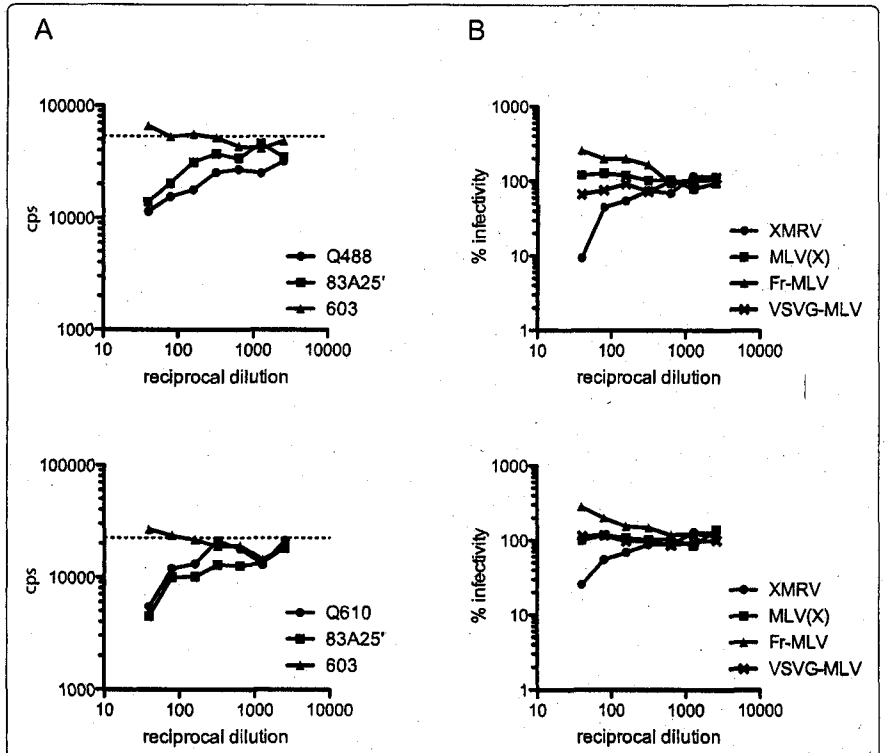


**Figure 2 Examples of BLT positive serum neutralisation activity.** A, Infectivity of XMRV (measured as counts per second of chemiluminescence produced from β-galactosidase activity) after incubation with patient serum or hybridoma cell supernatant. Infectivity is plotted against the reciprocal dilution of the BLT serum (black circles, top panel, sample Q488, bottom panel, sample Q610; triangles, negative control, monoclonal 603; squares, positive control, monoclonal 83A25'). The dashed line indicates viral infectivity in the absence of sera. B, Infectivity data for viruses with four different envelopes (circles, XMRV; squares, MLV(X); triangles, Friend-MLV; crosses, VSV-G) after incubation with patient serum. Data were normalised by setting the infectivity for each virus in the absence of patient serum at 100%, and plotted against the reciprocal of serum dilution for two positive sera, top panel sample Q488 and bottom panel sample Q610.



To confirm that the neutralisation activity demonstrated was specific for XMRV, we tested a subset of sera for neutralisation of XMRV alongside MLV particles pseudotyped with different envelope proteins from MLV (X), Friend-MLV or VSV. As shown in Figure 2B, of these four virus preparations, only XMRV infectivity was inhibited by any of the sera tested. Even the infectivity of particles expressing the closely related MLV(X) envelope that is 94% identical to XMRV was unaffected by sera that inhibited XMRV (Figure 2B, squares). Thus, it seems that the neutralising activity is specific for XMRV.

We therefore felt this assay was sensitive and specific enough to examine the neutralising ability of the SGUL cohort of blinded patient serum samples. After unblinding the samples, it emerged that of the 142 CFS patient sera tested none was positive as defined by the criteria above (Figure 3B). These results suggested that there was no link between XMRV and CFS. By contrast, the control group of 157 blood donors contained 22 positives, a frequency of 14%, considerably higher than that seen in the BLT group (Figure 3C). It was also noticeable that the neutralising activity of all but one of the SGUL positive samples was much stronger than the BLT positive samples (compare Figure 2A with Figure 4A). In fact, most of the SGUL positive sera reduced XMRV infectivity by 100 fold at both 1/40 and 1/80 dilutions. Intriguingly, many of these serum samples were collected from a single blood donation session. Some samples from this session, however, were negative. Surprisingly, PCR analyses of DNA samples corresponding to the positive sera from the SGUL controls were uniformly negative. We therefore investigated the specificity of this response by testing 21 of the positive sera for neutralisation of MLV pseudotyped with the envelope proteins from MLV(X), Friend-MLV or VSV. In every case, the serum was able to neutralise additional viruses to XMRV, including particles pseudotyped with the non-retroviral envelope from VSV (Figure 4B and Table 3). This implied that the strong positive neutralising activity demonstrated by the SGUL blood donor controls was not specific to XMRV, and in all likeliness was not elicited by this virus.

To test whether the SGUL cohort of CFS patients was unique, we also tested 40 samples (including some

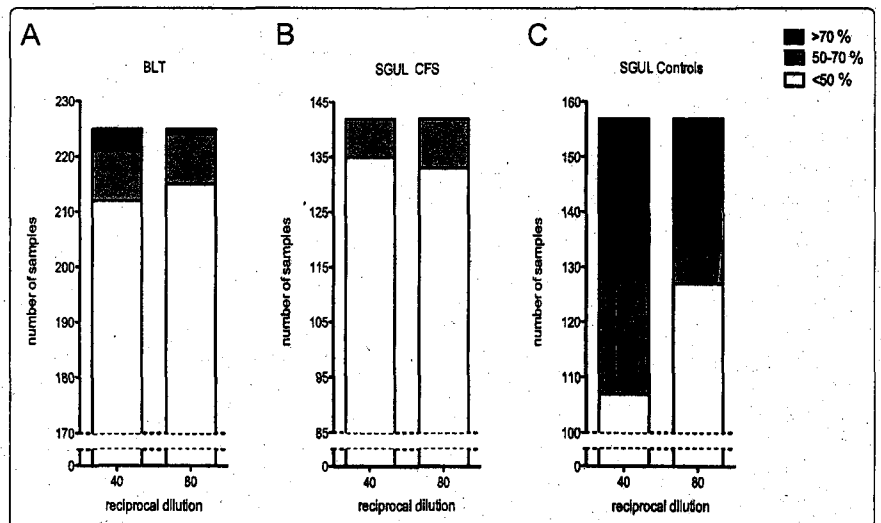


**Figure 3 Distribution of neutralisation activity in three samples sets.** Numbers of patients showing different degrees (>70% , 50-70% and <50%) of neutralisation of XMRV infectivity are shown for the 1/40 and 1/80 serum dilutions. A, Total BLT cohort (n = 226); B, SGUL CFS cohort (n = 142); C, SGUL control blood donor cohort (n = 157).

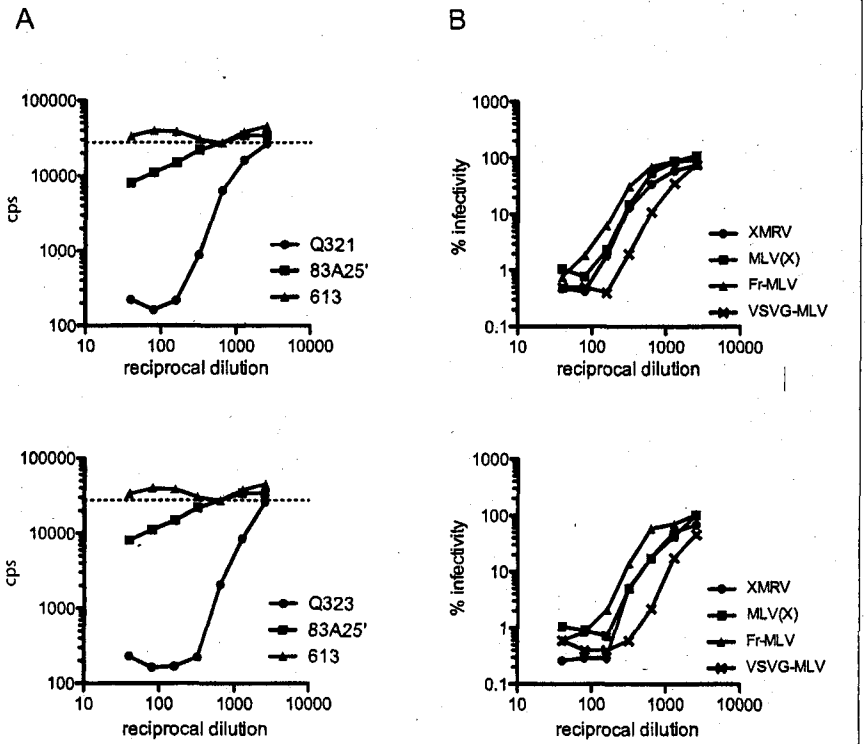


**Figure 4 Examples of SGUL positive serum neutralisation activity.** A, Infectivity of XMRV (measured as counts per second of chemiluminescence produced from β-galactosidase activity) after incubation with patient serum or hybridoma cell supernatant. Infectivity is plotted against the reciprocal dilution of the SGUL serum (black circles, top panel, sample Q321, bottom panel, sample Q323; triangles, negative control, monoclonal 613; squares, positive control, monoclonal 83A25'). The dashed line indicates viral infectivity in the absence of sera. B, Infectivity data for viruses with four different envelopes (circles, XMRV; squares, MLV(X); triangles, Friend-MLV; crosses, VSV-G) after incubation with patient serum. Data were normalised by setting the infectivity for each virus in the absence of patient serum at 100%, and plotted against the reciprocal of serum dilution for two positive sera, top panel sample Q321 and bottom panel sample Q323.

plasma samples as well as sera) from a separate CFS cohort in our neutralisation assay. This GC cohort revealed a solitary positive out of 28 CFS samples (3.6%), and no positives out of 12 control samples. The positive CFS patient serum was also able to neutralise MLV pseudotyped with either MLV(X) or Friend envelopes, although interestingly, it was not able to neutralise VSV-G pseudotyped MLV (Table 3). Neutralisation data from the different cohorts are summarized in Table 4. Thus, in summary, we found no association of XMRV with either CFS cohort.

## Discussion

We set out with the intention of confirming the results of Lombardi *et al.* [8] concerning the association of XMRV with CFS. In total, we tested 142 CFS samples for both the presence of XMRV DNA in PBMCs by PCR and for the presence of neutralising antibodies against XMRV in our viral neutralisation assay, and a further 28 CFS samples for neutralising antibodies only. However, in contrast to Lombardi *et al.*, we found no evidence of XMRV DNA in any patient samples tested, and only a single neutralisation-positive patient serum. Our findings



**Table 3 Neutralisation properties of different human sera against XMRV and MLV pseudotyped with three different envelopes.**

| | Neutralisation of | | | | |
|---|---|---|---|---|---|
| Sample ID | XMRV | MLV(X) | Friend | VSV | XMRV detected by PCR |
| Barts and the London | | | | | |
| Q488 | + | - | - | - | ND |
| Q610 | + | - | - | - | ND |
| Q663 | + | ND | ND | ND | ND |
| St George's University of London | | | | | |
| Q302 | ++ | ++ | ++ | ++ | no |
| Q304 | ++ | ++ | ++ | ++ | no |
| Q305 | ++ | ++ | ++ | ++ | no |
| Q306 | ++ | ++ | ++ | ++ | no |
| Q307 | ++ | + | + | - | no |
| Q308 | ++ | ++ | ++ | ++ | no |
| Q309 | ++ | ++ | ++ | ++ | no |
| Q310 | ++ | ++ | ++ | ++ | no |
| Q311 | ++ | + | + | + | no |
| Q312 | ++ | ++ | ++ | ++ | no |
| Q313 | ++ | ++ | ++ | ++ | no |
| Q314 | ++ | ND | ND | ++ | no |
| Q315 | ++ | ++ | ++ | ++ | no |
| Q316 | ++ | ++ | ++ | ++ | no |
| Q317 | ++ | ++ | ++ | ++ | no |
| Q319 | ++ | ND | ND | ++ | no |
| Q320 | ++ | ++ | ++ | ++ | no |
| Q321 | ++ | ++ | ++ | ++ | no |
| Q323 | ++ | ++ | ++ | ++ | no |
| Q324 | ++ | ++ | ++ | ++ | no |
| Q326 | ++ | ND | ND | ND | no |
| Q372 | + | - | - | + | no |
| Glasgow Calendonian University | | | | | |
| Q125 | + | ++ | ++ | - | ND |

+ indicates neutralising activity; ++ indicates strong neutralising activity; - indicates no neutralising activity; ND is no determined.

therefore appear inconsistent with the previous report that isolated XMRV from PBMCs of CFS patients. We are confident that, although we are unable to replicate the PCR detection of XMRV in PBMC DNA from CFS patients, our PCR assay is more sensitive than the published single round PCR method and should have possessed the necessary sensitivity to detect XMRV if it was indeed present (Figure 1). Furthermore, we were able to detect neutralising activity in one patient and in several control serum samples (Table 4 and Figure 3), implying that our neutralisation assay also has the required sensitivity. The lack of neutralising activity in CFS samples compared to controls could reflect an inability to mount an immune response in these patients. However, in that case, the virus would be expected to replicate to higher levels in CFS patients making it easier to detect by PCR. As we could not detect any evidence of XMRV infection by our PCR assays, we think this is an unlikely explanation. Thus, in our cohorts, we found no association of XMRV with CFS. This is in stark contrast to the result of Lombardi *et al.* [8]. However, it is thought likely that the term CFS defines multiple diseases [15-17], and it remains formally possible that a fraction of these are associated with XMRV. During the submission of this manuscript another report was published by Erlwein *et al.* that also failed to detect XMRV in CFS patients by PCR [18]. The publication of these results has promoted much discussion and controversy amongst CFS researchers and patients alike, and has highlighted the need for additional investigations in this area. Following the findings reported here, it would seem a prudent next step for subsequent studies to compare samples and protocols between different laboratories around the world.

There have also been conflicting reports describing the association of XMRV with prostate cancer. Two studies from the USA [1,5] have found an increased

**Table 4 Summary of number of positive sera with XMRV neutralisation properties**

| Sample cohort | Positive | Total number |
|---|---|---|
| Barts and the London | | |
| Control | 3 | 226 |
| St Georges University of London | | |
| CFS | 0 | 142 |
| Control | 22 | 157 |
| Glasgow Caledonian University | | |
| CFS | 1 | 28 |
| Control | 0 | 12 |

prevalence of the virus in prostate cancer patients, although they differed as to whether this was dependent on the RNASEL genotype of the patient. Conversely, two German studies failed to establish a link between the virus and disease [6,7]. Nevertheless, XMRV has been detected in the control groups in multiple investigations [5,6,8], with the incidence varying between 1 and 6%. In our serological studies we have also identified neutralising activity against XMRV in around 4% of all the samples examined. Remarkably many (but not all) of the seropositive samples were identified in a relatively small group of blood donors within the SGUL cohort, possibly suggesting a local outbreak of infection. There is no evidence that this group are related or that they have a particularly high risk of acquiring a retroviral infection. Therefore, an outbreak of this kind seems unlikely. Moreover, all but one of the positive samples from the SGUL set we tested were also able to neutralise MLV pseudotyped with the envelope protein from VSV (Table 3). The one serum that failed to neutralise VSV-G pseudotyped MLV was, however, able to neutralise MLV particles pseudotyped with other retroviral envelopes. We therefore consider these positives from healthy blood donors to be non-specific cross reacting responses. The remaining four positive samples from the BLT and GC cohorts had much weaker neutralisation activities and did not neutralise VSV-G pseudotyped MLV, although, again, the positive serum from GC did neutralise particles expressing other retroviral envelopes (Table 3). Although we cannot rule out the possibility that the activity of these samples against XMRV is also non-specific, one possible explanation for these serological findings remains that XMRV infection has occurred in around one percent of the population. This figure is consistent with the general prevalence in control samples previously reported. Given the common oncogenic properties of gammaretroviruses [19] and the reported link between XMRV and prostate cancer [1,5], such an observation might be of considerable significance, particularly for the blood transfusion services. It should, however, be noted that we have so far been unable to reliably detect bacterially expressed XMRV Gag proteins by using these sera in immunoblotting experiments. It is therefore conceivable that these neutralising activities were not elicited by XMRV. Further investigations are required to determine the nature of these antiviral activities.

## Conclusions

In summary, we have studied 299 DNA samples and 565 serum samples for evidence of XMRV infection. We have not identified XMRV DNA in any samples by PCR, however, some serum samples were able to neutralise XMRV infectivity in our assay. Only one of these positive sera came from a CFS patient, implying that there is no association between XMRV infection and CFS. Furthermore, most of the positive sera were also able to neutralise MLV particles pseudotyped with other envelope proteins, indicating there may be cross reactivity with other retroviruses and even other enveloped viruses. It therefore seems unlikely that these responses were elicited by XMRV. However, the detection of neutralising activity that did not neutralise VSV-G pseudotyped MLV in at least four human sera may indicate that XMRV infection does occur at in the general population, although the outcome of such infections is currently uncertain.

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**Authors' contributions**

JK, JS and KB conceived and designed the investigation. HG and VB carried out the viral neutralisation assays and analysed the data. KM, ER, SB and JK performed the PCR analyses. SH, JG, FM, JB and JK provided patient samples. JS and KB analysed the data and drafted the manuscript. All authors read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.



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RESEARCH

# 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

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## ABSTRACT

**Objective** The presence of the retrovirus xenotropic murine leukaemia virus-related virus (XMRV) has been reported in peripheral blood mononuclear cells of patients with chronic fatigue syndrome. Considering the potentially great medical and social relevance of such a discovery, we investigated whether this finding could be confirmed in an independent European cohort of patients with chronic fatigue syndrome.

**Design** Analysis of a well defined cohort of patients and matched neighbourhood controls by polymerase chain reaction.

**Setting** Certified (ISO 15189) laboratory of clinical virology in a university hospital in the Netherlands.

**Population** Between December 1991 and April 1992, peripheral blood mononuclear cells were isolated from 76 patients and 69 matched neighbourhood controls. In this study we tested cells from 32 patients and 43 controls from whom original cryopreserved phials were still available.

**Main outcome measures** Detection of XMRV in peripheral blood mononuclear cells by real time polymerase chain reaction assay targeting the XMRV *integrase* gene and/or a nested polymerase chain reaction assay targeting the XMRV *gag* gene.

**Results** We detected no XMRV sequences in any of the patients or controls in either of the assays, in which relevant positive and negative isolation controls and polymerase chain reaction controls were included. Spiking experiments showed that we were able to detect at least 10 copies of XMRV sequences per $10^5$ peripheral blood mononuclear cells by real time as well as by nested polymerase chain reaction, demonstrating high sensitivity of both assays.

**Conclusions** This study failed to show the presence of XMRV in peripheral blood mononuclear cells of patients with chronic fatigue syndrome from a Dutch cohort. These data cast doubt on the claim that XMRV is associated with chronic fatigue syndrome in the majority of patients.

## INTRODUCTION

Chronic fatigue syndrome, also named myalgic encephalitis, is characterised by disabling physical and mental fatigue, lasting for at least six months, without an apparent physical cause.[1-3] The hallmark of the illness is debilitating fatigue, but symptoms like myalgia, disrupted sleep, difficulty with concentration, sore throat, and lymphadenopathy may also be present, albeit more variably. More than two thirds of patients are women. Although the cause is unknown and the illness may cover more than one entity, many have suggested that infectious agents have a role.[4] Indeed, the onset of chronic fatigue syndrome is often preceded by an acute flu-like illness or infectious mononucleosis with seemingly impaired recovery.[5] A role of chronic infection and changed immunity has been postulated. Most cases of the illness are sporadic, but some clustered cases have been described, particularly suggesting an infectious cause. However, despite extensive studies, no causative infectious agent has been conclusively identified, neither has an immune defect been established to explain the symptoms.[2,6]

In a recent publication in *Science*, Lombardi et al[7] reported the detection of xenotropic murine leukaemia virus-related virus (XMRV)—a human gamma retrovirus that was first identified in tumour tissue of patients with prostate cancer[8]—in peripheral blood mononuclear cells of patients with chronic fatigue syndrome. In that study, XMRV was detected by polymerase chain reaction in 67% of patients (68 of 101 samples) and in 4% of healthy individuals (eight of 213 samples). Furthermore, antibodies to XMRV were identified in the blood of patients but not in controls. Lombardi et al showed that XMRV was infectious and transmittable from clinical material of patients to T cell cultures and a





permissive cell line. The genetic sequence of XMRV in patients was nearly identical to that in patients with prostate cancer, indicating that the identified retrovirus is a genuine human virus rather than a mouse leukaemia virus contamination.

This report was considered a major scientific breakthrough and attracted a lot of attention. However, the paper fell short in the description of the patients: what was the nature of the cohort, what was the age and sex distribution, how well were the controls matched? Investigation of an independent cohort is therefore necessary before a causal association between XMRV infection and the development of chronic fatigue syndrome can be ascertained. We investigated the presence of XMRV in a well established Dutch cohort of patients with chronic fatigue syndrome using previously described real time and nested polymerase chain reaction assays on two different target genes.[7-9]

## METHODS

### Patient cohort

All patients and controls examined in this study were part of a Dutch cohort of 298 patients, which has been described in detail.[10,11] All patients of this cohort fulfilled the Oxford criteria and reported severe, unexplained, debilitating fatigue of at least one year in duration.[12] The median duration of their symptoms was seven years (range 2-45 years). The enrolled patients came to our outpatient clinic twice in a three month period. On the second visit, each patient was accompanied by a neighbourhood control (who was selected by the patient) of the same sex and within two years of the same age. Patients and controls visited our clinic between December 1991 and April 1992. All patients underwent a physical examination and an extensive laboratory work-up and completed a set of questionnaires.[10] Blood samples were obtained from 76 patients (randomly chosen using a table of random numbers[10] from the 298 patients described above) and 69 matched neighbourhood controls. Blood samples were sent to the central laboratory of the blood transfusion service in Amsterdam, where peripheral blood mononuclear cells were isolated for a study of lymphocyte subsets and apoptosis.[11] After isolation, a fraction of the peripheral blood mononuclear cells was directly cryopreserved according to a standard protocol in a computerised device. Cells were aliquoted in phials and stored with 10% dimethyl sulfoxide at −196°C (liquid nitrogen) in a density of about $10^7$ cells per ml. The quality of the storage conditions at the central laboratory of the blood transfusion service has been amply demonstrated by Jansen et al, who showed that peripheral blood mononuclear cells stored for 12 years remained fully viable and immunologically competent.[13]

In this study, we examined peripheral blood mononuclear cells of all patients (n=32) and controls (n=43) from whom original cryopreserved vials were still available. This group included 25 patients and their matched controls, as well as seven patients and 18 controls that were not matched to each other. The male to female ratio of the patient group that was tested in this study was 1:2. Average age of the male patients was 40.7 years (range 25-61) and of female patients was 40.5 years (25-67).

### Nucleic acid isolation and copy DNA synthesis

Nucleic acid was isolated from 100 µl of peripheral blood mononuclear cells (about 0.5 to $2\times10^6$ cells) using the MagNA-Pure LC and the MagNA-Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Almere, Netherlands) according to the instructions of the manufacturer and eluted in 50 µl of elution buffer. A fixed amount of phocine distemper virus, a paramyxovirus that was used as internal control, was added to the samples before nucleic acid isolation so that we could monitor RNA quality and possible inhibition of amplification of the samples.[14] RNA in the total nucleic acid isolates was reverse transcribed to copy DNA using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Nieuwerkerk aan den IJssel, Netherlands) in a 50 µl reaction mix containing 20 µl of nucleic acid isolate (concentration 25-150 ng per µl) and random hexamers as primers, according to the manufacturer's instructions.

### Real time polymerase chain reaction assay

A duplex real time polymerase chain reaction assay was developed, adapted from the XMRV *integrase* real time polymerase chain reaction assay described by Schlaberg et al,[9] to detect XMRV and phocine distemper virus simultaneously. The reaction mixture contained 12.5 µl of 2X LightCycler 480 Probes Master (Roche Diagnostics), 1 µM of each primer and 400 nM of each probe, and 5 µl of copy DNA in a reaction volume of 25 µl. The XMRV and phocine distemper virus primers were as described.[9,14] The XMRV probe was used as a 5′-(6-carboxyfluorescein)-labelled, locked nucleic acid hydrolysis probe and the phocine distemper virus probe was used as a 5′-yakima yellow-labelled, locked nucleic acid hydrolysis probe. All primers and probes used in this study are shown in the table. Cycling conditions were 95°C for five minutes, followed by 50 cycles of 95°C for 15 seconds and 60°C for 45 seconds using the LightCycler 480 instrument (Roche Diagnostics). The result of the sample was considered a valid result only if the crossing point value for the spiked phocine distemper virus was within two cycles of the average of uninhibited samples.

Positive and negative controls for isolation, reverse transcription, and polymerase chain reaction were included in each run. β-globin real time polymerase chain reaction was performed using primers and hybridisation probes as described.[15] Mean crossing point value of β-globin real time polymerase chain reaction assay was 23.84, standard deviation 0.95. As a positive control for the polymerase chain reaction assay, we used nucleic acid isolated from 22Rv1, a prostate carcinoma cell line (American Type Culture Collection number CRL-2505) that was recently shown to contain multiple integrated copies of XMRV and to produce

high levels of infectious virus.[16] Total nucleic acid isolation and sample preparation from this cell line was as described above for peripheral blood mononuclear cells.

To determine the sensitivity of the XMRV real time polymerase chain reaction assay, we generated a 192 base pairs XMRV *integrase* polymerase chain reaction product using primers XMRV-F2 (which is located upstream of XMRV-F1) and XMRV-R3 (which is located downstream of XMRV-R2), and 22Rv1 copy DNA as a template. The polymerase chain reaction product was purified using the Wizard PCR preps DNA purification system (Promega Benelux, Leiden, Netherlands). The concentration was determined using a NanoDrop 1000 (Thermo Scientific/Isogen, De Meern, Netherlands) and the number of copies per µl was calculated. A dilution series was made in which $10^1$ to $10^7$ copies of the calibrator were added to $10^6$ peripheral blood mononuclear cells before nucleic acid isolation. This corresponds to 1 to $10^6$ copies per reaction, since a tenth of the isolated nucleic acid was used as input for the polymerase chain reaction, which was performed as described above.

### Nested polymerase chain reaction assay

The XMRV *gag* nested polymerase chain reaction assay was adapted from Urisman et al.[8] The reaction mixtures contained 25 µl of 2X PCR Master (Roche Diagnostics), and 200 nM of each primer in a reaction volume of 50 µl. In the first reaction, 5 µl of copy DNA was used. Subsequently, 5 µl of the first reaction was used as input for the nested reaction. Primers were as described, except for the reverse primer of the nested reaction (GAG-I-R), which was replaced by GAG-I-R2 to yield a 92 base pairs reaction product (we used primer GAG-I-R2 because it produced less background in the nested reverse transcription polymerase chain reaction). The target sequence of GAG-I-R2 is 100% conserved among all XMRV isolates published to date (data not shown). Cycling conditions were as previously described.[8] Polymerase chain reaction products (20 µl) were analysed on a 2.5% agarose gel.

To determine the sensitivity of our XMRV nested polymerase chain reaction assay, a 708 base pairs XMRV *gag* polymerase chain reaction product was generated using primers GAG-UNIQ-F described by Dong et al[17] (which is located upstream of GAG-O-F) and GAG-O-R, and 22Rv1 copy DNA as a template. Purification and determination of the amount of the polymerase chain reaction product were performed as described above for the real time polymerase chain reaction calibrator. A dilution series was made and $10^1$ to $10^7$ copies of the calibrator were added to $10^6$ peripheral blood mononuclear cells prior to nucleic acid isolation. This corresponds to 1 to $10^6$ per reaction since a tenth of the isolated nucleic acid was used as input for the nested polymerase chain reaction, which was performed as described above. In the same way, we tested the sensitivity of the nested polymerase chain reaction assay described by Urisman et al[8] using primer GAG-I-R instead of GAG-I-R2.

**Sequences of primers and probes used in this study**

| | Sequence | Reference |
|---|---|---|
| **XMRV integrase gene** | | |
| XMRV-F1 | 5'-CGAGAGGCAGCCATGAAGG-3' | 9 |
| XMRV-F2 | 5'-AACCTGATGGCAGATCAAGC-3' | This study |
| XMRV-R1 | 5'-GAGATCTGTTTCGGTGTAATGGAAA-3' | 9 |
| XMRV-R2 | 5'-CCCAGTTCCCGTAGTCTTTTGAG-3' | 9 |
| XMRV-R3 | 5'-TTTGCCTTGTAGGACCCAAT-3' | This study |
| XMRV-Probe | 5'-AGTTCTAGAAACCTCTACACTC- 3' | 9 |
| **XMRV gag gene** | | |
| GAG-O-F | 5'-CGCGTCTGATTTGTTTTGTT-3' | 8 |
| GAG-O-R | 5'-CCGCCTCTTCTTCATTGTTC-3' | 8 |
| GAG-I-F | 5'-TCTCGAGATCATGGGACAGA-3' | 8 |
| GAG-I-R | 5'-AGAGGGTAAGGGCAGGGTAA-3' | 8 |
| GAG-I-R2 | 5'-CAGACTGGTTGGATGCAATG-3' | This study |
| GAG-UNIQ-F | 5'-GACTTTTTGGAGTGGCTTTGT-3' | 17 |
| **PDV haemagglutinin gene** | | |
| PDV-F | 5'-GGTGGGTGCCTTTTACAAGAAC-3' | 14 |
| PDV-R | 5'-ATCTTCTTTCCTCAACCTCGTCC-3' | 14 |
| PDV-probe | 5'-ATGCAAGGGCCAATT-3' | 14 |
| **Human β-globin gene** | | |
| bGLO-for | 5'-GAGCCATCTATTGCTTACATTTGC-3' | 15 |
| bGLO-rev | 5'-TTGGTCTCCTTAAACCTGTCTTGT-3' | 15 |
| bGLO-FL | 5'-CCAGGGCCTCACCACCAACTTC-3' | 15 |
| bGLO-LC640 | 5'-CCACGTTCACCTTGCCCCACAG-3' | 15 |

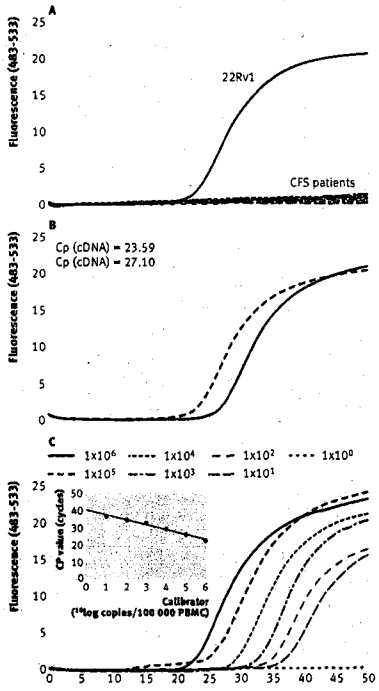


**Fig 1| Results of XMRV *integrase* real time polymerase chain reaction assay. (A) All 32 patients with chronic fatigue syndrome (CFS) compared with positive 22Rv1 control, which yielded a crossing point value of about 23. Results for neighbourhood controls not shown. (B) 22Rv1 total nucleic acid (DNA, solid), reverse transcribed total nucleic acid (cDNA, dashed). The additional reverse transcription step increased the sensitivity of the polymerase chain reaction, decreasing the crossing point (Cp) value by 3.5. One of three independent experiments is shown. (C) Sensitivity of the assay. The inlay shows linear relation between number of spiked molecules and crossing point value from $10^1$ to $10^6$ copies per reaction**





## RESULTS

Total nucleic acid was isolated from peripheral blood mononuclear cells of 32 patients and 43 healthy controls. Nucleic acid was subjected to copy DNA synthesis to increase the sensitivity of our polymerase chain reaction assays. This was done because we observed that the real time polymerase chain reaction assay on nucleic acid isolated from a XMRV positive prostate cancer cell line, 22Rv1, and subjected to copy DNA synthesis—allowing detection of both proviral DNA and viral RNA—was about 10 times more sensitive than without copy DNA synthesis (fig 1B). Nevertheless, all samples from patients with chronic fatigue syndrome and from controls tested negative for both the XMRV *integrase* gene (fig 1) and the XMRV *gag* gene (fig 2).

Our negative XMRV polymerase chain reaction results were unlikely to be due to low amounts of nucleic acid tested or low sensitivity of the assays used. We used 50-300 ng of total nucleic acid from peripheral blood mononuclear cells per polymerase chain reaction, which is similar to the amount used by Lombardi et al.[7] Moreover, by adding 10-fold serial dilutions of a defined amount of template DNA to peripheral blood mononuclear cells before nucleic acid isolation, we demonstrated that both the real time polymerase chain reaction assay (fig 1C) and the nested polymerase chain reaction assay (fig 2B) could detect at least 10 copies of XMRV per $10^5$ peripheral blood mononuclear cells, indicating a high sensitivity. A similar sensitivity of the nested polymerase chain reaction assay was observed when we used the same primers as described by Urisman et al (inner reverse primer GAG-I-R instead of the inner reverse primer GAG-I-R2 used in our assay) (data not shown).[8]

Our negative XMRV polymerase chain reaction results are also unlikely to be due to problems with nucleic acid isolation, loss of RNA or DNA integrity, synthesis of copy DNA, or the polymerase chain reaction procedure, since both the phocine distemper virus RNA (an internal control of which a fixed amount was added to each of the samples before nucleic acid isolation) and the β-globin gene were efficiently amplified in all samples tested (data not shown).

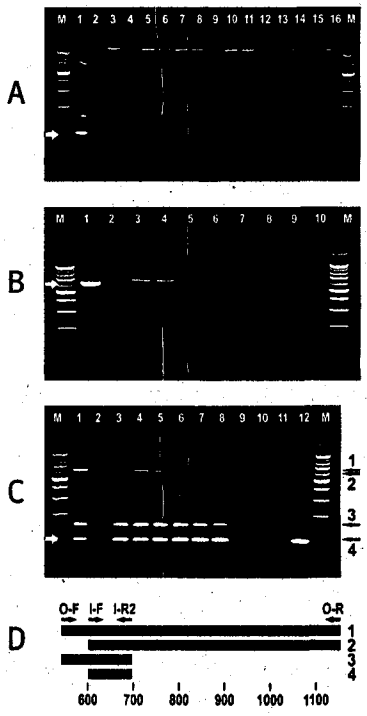


**Fig 2| Results of XMRV *gag* nested polymerase chain reaction assay. (A) Results for 11 patients with chronic fatigue syndrome and negative controls. Results for neighbourhood controls not shown. (1) The positive 22Rv1 control yielded a product of the expected size of 92 base pairs (arrow); (2) negative polymerase chain reaction control; (3) phocine distemper virus (internal control); (4) negative reverse transcription control; (5)-(11) and (13)-(16) patient samples; (12) negative isolation control; (M) 100 base pairs size marker. (B) Sensitivity of the XMRV *gag* first polymerase chain reaction. (C) Sensitivity of the nested reaction. White arrows indicate the 613 base pairs (B) and 92 base pairs (C) reaction products. (1) 22Rv1; (2) negative polymerase chain reaction control; (3-9), dilution series of $10^6$ to $10^0$ copies of calibrator per reaction; (10) negative isolation control; (11) negative nested polymerase chain reaction control; (12) positive nested polymerase chain reaction control (22Rv1); (M) 100 base pairs size marker. Black arrows 1-4 in (C) indicate polymerase chain reaction products that are formed in the nested reaction (see D). (D) Positions of the *gag* primers and the *gag* polymerase chain reaction products formed in the nested reaction. In addition to the primers of the nested reaction (GAG-I-F, I-F and GAG-I-R2, I-R2), primers from the first reaction (GAG-O-F, O-F and GAG-O-R, O-R) are also present in the nested reaction, yielding reaction products 1-4 that correspond to the black arrows in (C). Numbers represent the positions on the XMRV genome (VP42, accession DQ241302)**

## WHAT IS ALREADY KNOWN ON THIS TOPIC

Chronic fatigue syndrome is a debilitating disease of unknown cause that affects millions of people worldwide

A study from the United States reported the detection of the retrovirus xenotropic murine leukaemia virus-related virus (XMRV) in peripheral blood mononuclear cells in a cohort of patients with chronic fatigue syndrome, suggesting a possible causal relation and a satisfactory explanation for their problems

## WHAT THIS PAPER ADDS

We found no evidence for the occurrence of XMRV in peripheral blood mononuclear cells of patients with chronic fatigue syndrome from a well defined Dutch cohort

These data cast doubt on the claim that XMRV is associated with chronic fatigue syndrome in the majority of patients

## DISCUSSION

### Principal findings

We assessed the presence of XMRV in peripheral blood mononuclear cells isolated from patients with chronic fatigue syndrome from a well characterised Dutch cohort. We found no evidence for the presence of XMRV in any of these sporadic cases of chronic fatigue syndrome or in controls.

### Strengths and limitations of the study

A limitation of our study is that the numbers of patients and controls in our study were relatively small. Based on these low numbers, the upper limit of the 95% confidence interval is a prevalence of 9% for the patient group and 7% for the control group, as calculated according to Eypasch et al (by the formula p=3/n).[18] Although we cannot formally rule out a role of XMRV, our data cast doubt on the claim that this virus is associated with chronic fatigue syndrome in the majority of patients.

### Comparison with findings of previous studies

The results of the present study are in contrast with the findings of Lombardi et al, who detected XMRV in 67% of the patients with chronic fatigue syndrome analysed.[7] Technical aspects are unlikely to explain the difference in XMRV positivity rate between our data and their data. The possibility that the relative long duration of chronic fatigue syndrome in our cohort may have led to our negative results seems unlikely, because retroviruses integrate into the genome of the host. Given the high sensitivity of our real time and nested polymerase chain reaction assays, a positive signal should have been obtained in the presence of the virus. The fact that our samples were cryopreserved for many years is also unlikely to account for the negative results. Peripheral blood mononuclear cells cryopreserved for 12 years under these conditions have remained viable and immunocompetent.[13] Moreover, we found no difference in efficiency of β-globin gene amplification from stored samples compared with samples that were used directly after isolation (data not shown), indicating good quantity and quality of the nucleic acid isolated from cryopreserved samples.

As technical aspects do not seem to provide an explanation, the difference might be explained by the two cohorts studied. Our patients met the Oxford criteria for chronic fatigue syndrome, whereas the patients studied by Lombardi et al were reported to fulfil the Centers for Disease Control criteria,[7] but this is unlikely to explain the absence of XMRV in our patients' samples. Unfortunately, the paper of Lombardi and colleagues lacked a clear description of their patient cohort. Recently, at the Tri-Society Annual Conference 2009 in Lisbon, a presentation reported that the peripheral blood mononuclear cells were derived from patients from the outbreak of chronic fatigue syndrome at Incline village at the northern border of Lake Tahoe, United States (1984-5).[10] This outbreak has long been thought to have been caused by a viral infection and has been associated with a number of viruses, most notably Epstein-Barr virus[20] and human herpes virus 6,[21] but firm evidence for a role of viruses in this particular outbreak has never been provided. It is possible that the study of Lombardi et al has unravelled the viral cause of the chronic fatigue syndrome outbreak, but it seems unlikely that their study demonstrates a viral association for sporadic chronic fatigue syndrome cases, such as those we tested, or represents the majority of patients. Studies of XMRV in sporadic chronic fatigue syndrome cases from the United States would be of great interest.

XMRV was initially identified in tumour tissue of about 10% of patients with prostate cancer in the United States.[8] This association was recently confirmed in another independent study from the United States, in which XMRV was detected in 23% of patients.[9] Remarkably, in three independent European cohorts of patients with prostate cancer, no XMRV was detected.[22-24] Whether this discrepancy is due to differences in the geographic distribution of the virus remains to be established.

Recently, a team from the United Kingdom reported the failure to detect XMRV in all 186 tested peripheral blood mononuclear cell samples from a well characterised cohort of British patients with chronic fatigue syndrome.[25] This team, however, did not use the same primer sets as used by Lombardi et al, leaving open a possible explanation for the difference in results. In our study, we used the same primer sets as used by Lombardi et al. Although our patient group was relatively small and more research is required, our findings—together with those of Erlwein et al[25]—cast doubt on the claim that XMRV is associated with chronic fatigue syndrome in the majority of patients.

Lombardi et al also detected XMRV in about 4% of healthy controls.[7] We failed to detect XMRV in peripheral blood mononuclear cells of healthy controls in our study, but the number of controls tested (n=43) is too low to exclude the occurrence of XMRV in blood in a part of the population. Clearly, more research is needed to establish the distribution of XMRV in healthy controls and, of course, in blood supply products in Europe and in the United States.





### Implications

In conclusion, we found no evidence for a role of XMRV in the cause of chronic fatigue syndrome in Dutch patients. Over the past decades we have seen a series of papers prematurely claiming the discovery of the microbial cause of chronic fatigue syndrome. Regrettably, thus far none of these claims has been substantiated.

**Contributors:** FJMvK, MGN, JMDG, and JWMvdM designed the study and wrote the paper. ASdJ, KHL, GWV, and WJGM performed experiments and analysed the data. CMAS, GB, JMDG, and JWMvdM established the chronic fatigue syndrome patient cohort. All authors had full access to all of the data (including statistical reports and tables) in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. FJMvK and JWMvdM are guarantors of the paper and accept full responsibility for the work and/or the conduct of the study, had access to the data, and controlled the decision to publish.
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**Competing interests:** All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare that none of them (1) has support from companies for the submitted work; (2) has relationships with companies that might have an interest in the submitted work in the previous 3 years; (3) has spouses, partners, or children that have financial relationships that may be relevant to the submitted work, and (4) has non-financial interests that may be relevant to the submitted work.
**Ethical approval:** Ethical aspects of this study were approved by the Commissie Mensgebonden Onderzoek from Radboud University Medical Centre (CMO-1991).
**Data sharing:** No additional data available.

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平成２２年５月１８日開催
薬事・食品衛生審議会血液事業部会
運営委員会提出資料

**資料５**

日本赤十字社血液事業本部

## 日本赤十字社血液事業本部組織の変更について（平成 22 年 4 月）

今後予定している新規製剤の製造販売承認取得に向けた体制の整備、また、採血業に係る体制の明確化を図るなど、事業運営体制を強化すること等を目的に以下のとおり組織の変更を行った。

### １．臨床開発課

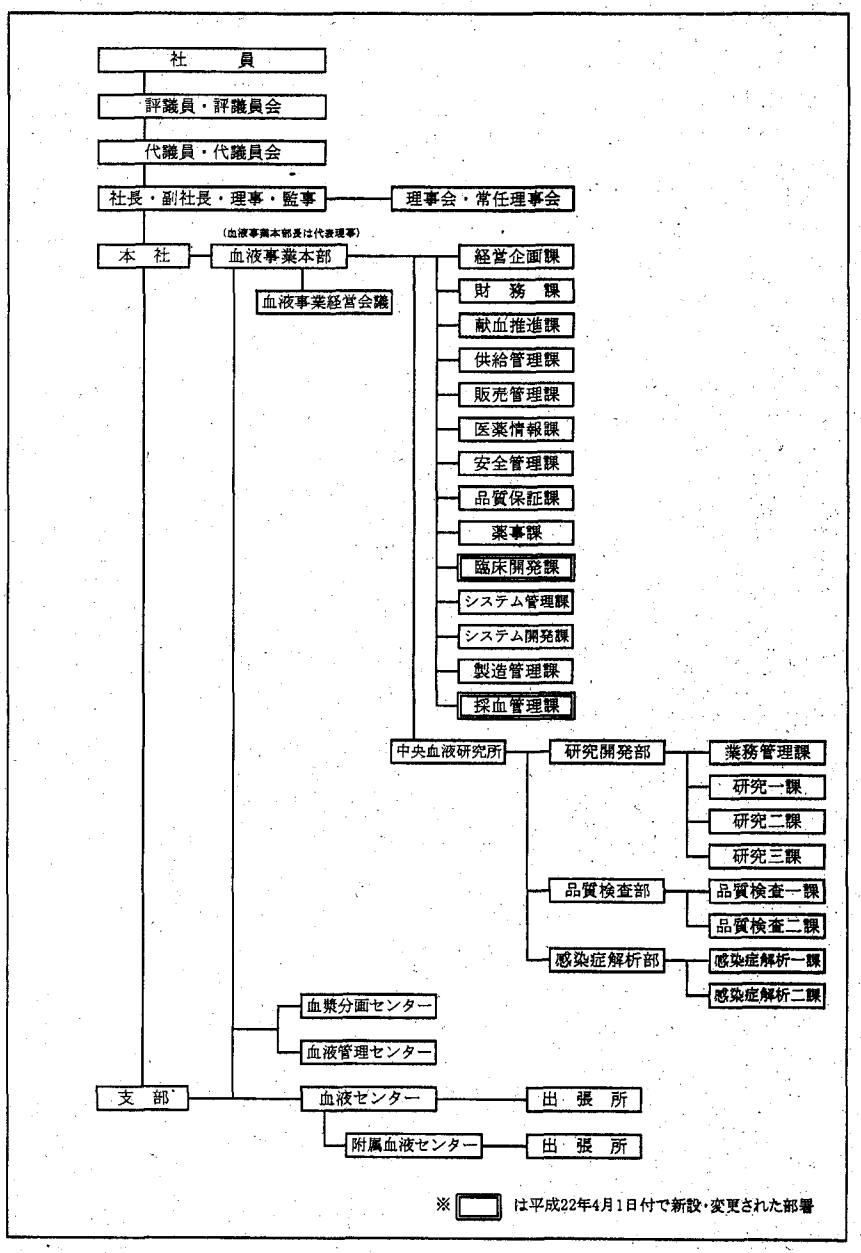
新規製剤の製造販売承認並びに不活化技術の導入検討については、昨年「開発・申請担当」参事･主査及び「不活化担当」参事・主査を配置し、準備を進めてきましたが、今後、臨床試験を含めて申請・承認に至る手続きを円滑に進める必要があることから、従来の参事・主査の体制から移行し、新たに臨床開発課を設置することにより執行体制の強化を図った。

### ２．採血管理課

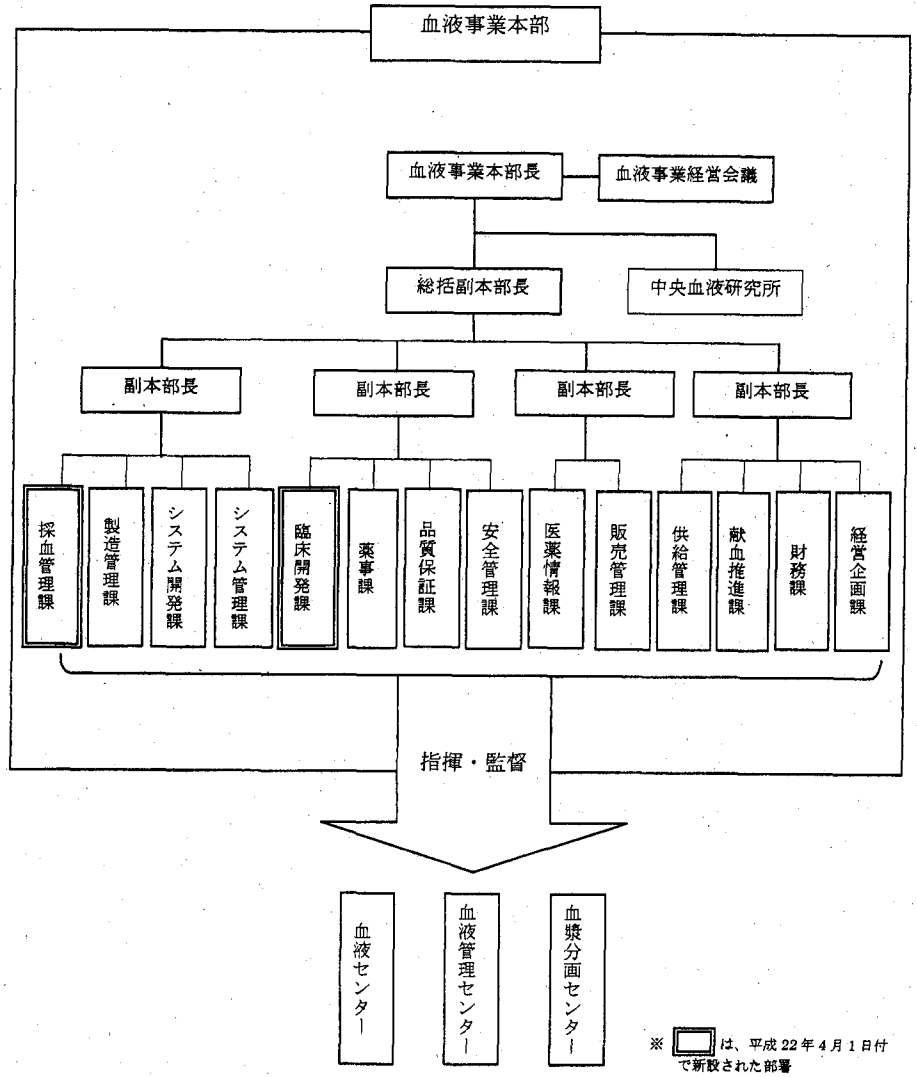
採血業に係る体制については従来、「採血担当」参事・主査を置き業務を遂行してきたが、新たに採血管理課を設置することにより体制の明確化及び強化を図った。

〔別 紙〕

## 平成22年4月現在における血液事業の執行体制について（日本赤十字社）



## 日本赤十字社血液事業本部の事業執行体制について




ひと、くらし、みらいのために
厚生労働省
Ministry of Health, Labour and Welfare

資料6－1

**Press Release**


平成22年5月14日
医薬食品局血液対策課
(担当・内線) 課長　亀井 (2900)
企画官 光岡 (2901)
(代表電話) 03(5253)1111
(直通電話) 03(3595)2395
(F A X) 03(3507)9064


報道関係者 各位

# フィブリノゲン製剤納入先医療機関の追加調査について

平成１６年１２月９日に公表したフィブリノゲン製剤納入先医療機関を対象として、平成１９年１１月７日付で実施した追加調査の結果について、平成２２年４月３０日までに回収した医療機関からの回答を取りまとめた状況をお知らせいたします。

1　回答状況

（1）追加調査実施期間　　平成１９年１１月７日～１２月５日（※1）
（ただし、現在も回収中）

（※1）（1）の調査以降、平成２０年８月２５日及び平成２１年１月１６日にも元患者の方へのお知らせ状況等について再度調査を行っており、（3）回答施設数以降はそれらの結果を反映したものである。

（2）追加調査対象施設数　　医療機関　６，６１０施設
（平成１６年公表施設のうち、所在地等が不明であった施設を除いた医療機関）

（3）回答施設数
- 平成１６年公表時に存続していた５，３９７施設のうち、５，２８９施設（98%）から回答があった。
- なお、このほか平成１６年公表時に廃院等していた１，２１３施設のうち、５００施設から回答があった。

## 2　主な調査結果

（1）投与の年月について回答があった医療機関数と元患者数

医療機関数　　８９７施設
元患者数　　１２，９２３人　（投与年別は別表）

（2）上記以外に、過去に投与の事実をお知らせしたという記録が残されているが、現在では投与の年月は特定できないとする回答があった医療機関数と元患者数

医療機関数　　８８施設
元患者数　　２７５人

（3）（1）と（2）の合計

医療機関数　　９６７施設（※2）
元患者数　　１３，１９８人

（※2）厚生労働省ホームページ「C型肝炎ウイルス検査受診の呼びかけ（フィブリノゲン製剤納入先医療機関名の再公表について）」の公表医療機関等リスト上の該当医療機関の「備考」欄に、「フィブリノゲン製剤を投与されたことが判明した元患者の方がいるとの報告あり。」と記載した。

（4）元患者の方への投与の事実のお知らせの状況

| | | 元患者数 |
|---|---|---|
| お知らせした | | ７，５３７人　(57%)　(※3) |
| お知らせしていない | | ５，６６１人　(43%) |
| 理由 | 投与後に原疾患等により死亡 | １，９６６人　(15%) |
| | 連絡先が不明又は連絡がつかない | ２，５４９人　(19%) |
| | 肝炎ウイルス検査の結果が陰性 | ４１７人　( 3%) |
| | 今後お知らせする予定である | ２０８人　( 2%) |
| | その他（未記入含む） | ５２１人　( 4%) |
| 合　計 | | １３，１９８人 |

（※3）元患者の方に一人でも投与の事実をお知らせした医療機関は８００施設であった。

（5）診療録等の保管状況

平成６年以前の診療録等が次のいずれかにより保管されている施設数
（括弧内は調査対象施設数に対する割合）

２，０６０施設（31%）（※4）

（内訳）（※5）

| | |
|---|---|
| 診療録（カルテ） | １，５３３施設（23%） |
| 手術記録あるいは分娩記録 | １，６０２施設（24%） |
| 製剤使用簿 | １３８施設（ 2%） |
| 処方箋 | １４０施設（ 2%） |
| 輸液箋あるいは注射指示箋 | ２７３施設（ 4%） |
| レセプトの写し | ８０施設（ 1%） |
| 入院サマリーあるいは退院サマリー | ２９３施設（ 4%） |
| その他の書類 | ２９２施設（ 4%） |

（※4）平成１６年の調査では「昭和６３年６月３０日以前にフィブリノゲン製剤を投与した記録（診療録、使用簿など）が保管されていますか。」との設問であったのに対し、今回の調査では、「平成６年以前のカルテ等の各種書類が保管されていますか。」との設問であったため、保管していると回答した施設の割合が異なったものと思われる。

（※5）厚生労働省ホームページ「C型肝炎ウイルス検査受診の呼びかけ（フィブリノゲン製剤納入先医療機関名の再公表について）」の公表医療機関等リスト上の「カルテ等の有無」欄に、平成６年以前のカルテ等の記録が一部でも保管されている場合、△印を付していたが、さらに保管されている記録の保管期間、保管状況等を記載した。

（別表）

投与の年月について回答があった元患者数の投与年別の内訳

| 投与年 | 人数 |
|---|---|
| 昭和３９年 | ０人 |
| ４０年 | ７人 |
| ４１年 | ８人 |
| ４２年 | １２人 |
| ４３年 | １５人 |
| ４４年 | １８人 |
| ４５年 | １９人 |
| ４６年 | ２２人 |
| ４７年 | ２５人 |
| ４８年 | ３５人 |
| ４９年 | ４８人 |
| ５０年 | ４９人 |
| ５１年 | ６４人 |
| ５２年 | ８６人 |
| ５３年 | １２４人 |
| ５４年 | １９９人 |
| ５５年 | ２９３人 |
| ５６年 | ３４９人 |
| ５７年 | ４７４人 |
| ５８年 | ８３２人 |
| ５９年 | １，３３４人 |
| ６０年 | １，６６４人 |
| ６１年 | ２，３３２人 |
| ６２年 | ２，８１２人 |
| ６３年 | １，６５３人 |
| 平成　元年 | １８３人 |
| ２年 | １１１人 |
| ３年 | ８２人 |
| ４年 | ３７人 |
| ５年 | ２５人 |
| ６年 | １１人 |
| 計 | １２，９２３人 |

ひと、くらし、みらいのために
厚生労働省
Ministry of Health, Labour and Welfare

資料6－2

**Press Release**

平成２２年４月２６日（月）
医薬食品局総務課医薬品副作用被害対策室
室長補佐：信沢　（内線）２７１７
管理係長：内沼　（内線）２７１８
（直通）０３－３５９５－２４００

# C型肝炎訴訟の和解について

本日、大阪地方裁判所において、下記のとおり和解が成立しましたので、お知らせします。

平成２０年１月以降、同地裁に係属している原告（患者数６人）についての和解。製剤はフィブリノゲン製剤。

上記６人の症状は、慢性肝炎４人、無症候性キャリア２人である。

（参考）

○和解等成立人数※1　１４６０人

○新規提訴等人数※2　１６５９人　（４月２６日現在）

※１「和解等成立人数」は、今回の和解成立者は含まず、これまでに和解が成立した人数（患者数）である。また、調停が成立した４人を含む。
※２「新規提訴等人数」は、救済法施行後に提訴等し、訴状等が国に送達された人数（患者数）である。このうち、１２５２人は既に和解等が成立している。