Supporting Information

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SI Text

Creating an Infectious Clone of XMRV. Overlapping partial clones AM-2–9 and AO-H4 derived from patient isolate VP62 (1) and contained in pCR2.0-TOPO (Invitrogen) were a gift of Don Ganem, UCSF. Site-directed mutagenesis using complementary primers XMRV-M1 and XMRV-M2 (Table S2) was used to introduce a unique MluI restriction site into the overlapping segments of the 2 plasmids (QuikChange II site, Stratagene). The XMRV fragment from one plasmid was joined to the other at this MluI restriction site to obtain the full-length XMRV molecular clone pXMRV33 in pCR2.0-TOPO. The XMRV proviral sequence was then transferred to pcDNA3.1 using HindIII and NotI restriction sites in the pCR2.0-TOPO multiple cloning site (Invitrogen), to generate pXMRV1, a full-length clone of XMRV with a CMV promoter. pXMRV1 sequence was compared with that of the parent XMRV isolate VP62 (EF185282, Table S2). The 2 sequences differed at amino acid residue 411 of RT (M→V) because of substitutions introduced by the MluI restriction site (numbering according to MoMLV sequence NP_955591.1). Three additional differences (C→G at nucleotide 7450, 7694insT, and 7776insG) might represent sequence variations specific to VP62, or sequencing errors in VP62, because at each of these locations, the sequence of pXMRV1 is identical to that of 2 other sequenced clinical isolates of XMRV (2), VP35 (DQ241301.1) and VP42 (DQ241302.1).

Acquisition of Human Prostate Samples: Cancer and Control Tissues. Radical prostatectomy specimens (n = 233) submitted between August 2006 and December 2007 to the Department of Pathology and to the Tissue Bank of the Herbert Irving Comprehensive Cancer Center, Columbia University Medical Center were used to estimate the prevalence of XMRV in human prostate cancer. Prostate tissue specimens removed by transurethral resection for benign prostatic hyperplasia between January 2007 and April 2008 were used as controls. Controls known to contain incidental prostate cancer foci were not included in the study, leaving 101 controls. Of the 233 cancers, 95 were formalin-fixed, paraffin-embedded samples and 195 were frozen samples (with both specimen types available for 57 cases). Hematoxylin and eosin (H&E) stained sections from paraffin-embedded tissue blocks (ranging from 20 to 30 for each case) were examined for the presence of cancer. For each case, the block with the highest amount of cancer was chosen. Because prostate cancer foci cannot always be identified macroscopically at the time of frozen tissue sampling, an H&E-stained section of each case was examined by a pathologist for presence of cancer. Only 20% of our frozen tissue samples contained prostate cancer.

For controls, we collected 101 cases of prostatic tissue removed by transurethral resection of prostate (TURP), most commonly for benign prostatic hyperplasia. It is quite possible that some of these may have had cancer in a part of the prostate not removed by TURP, because prostate cancer usually arises in the periphery of the gland, whereas TURP mostly removes central tissue (Fig. S1*D*). If 1 TURP tissue block was available from a case, the one containing the highest amount of glandular tissue was chosen. The same tissue blocks were used for DNA extraction and immunohistochemical analysis.

DNA Extraction from Human Prostate Tissues. DNA was extracted from 10 sections (10-μm thick) of formalin-fixed, paraffin-embedded tissue, using the QIAamp DNA FFPE Tissue Kit (Qiagen) following manufacturer's directions. Microtome stages were cleaned with 10% bleach and 100% ethanol before each tissue block. New microtome blades were used for each case. The same method was used for extracting DNA from fresh frozen tissue, except the deparaffinization step was omitted. To reduce the risk of sample contamination, tissue processing, DNA extraction, and qPCR were performed in a separate laboratory.

Testing for DNA Integrity in Tissue Samples. We developed a qPCR targeting the single-copy gene–vesicle-associated membrane protein 2 (VAMP2) to test for DNA integrity and amplification inhibitors. Average amounts of amplifiable DNA were similar across sample types: mean quantification cycle (C_q) = 25 for frozen cancers (SD = 1.1), 26.4 for fixed cancers (SD = 3.4), and 25.6 for fixed controls (SD = 1.8). The DNA quality in XMRV PCR-positive cases was similar to that of XMRV PCR-negative cases as seen from equivalent C_q values in both groups. The average template quality was thus similar across sample types. Individual samples were considered unacceptable when the C_q for VAMP2 was 2 SD above the mean; thus, 5 (2.6%) frozen cancers, 7 (7.4%) fixed cancers, and 2 (2%) control samples were excluded from further qPCR analyses.

Quantitative PCR Amplification of Proviral DNA. The reaction mix for qPCR contained 1× TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 900 nM forward and reverse primers, 250 nM hydrolysis probe, and 20–200 ng DNA in a reaction volume of 20 μL. As a positive control, 500 copies of pXMRV33 diluted in 200 ng human placental DNA (Sigma-Aldrich) were used. Thermocycling conditions were 95 °C for 20 sec, followed by 45 cycles of 95 °C for 3 sec and 60 °C for 30 sec, using MicroAmp Fast Optical 96-Well Reaction Plates and a TaqMan 7500Fast instrument (Applied Biosystems). All reactions were performed in duplicate. Serial 10-fold dilutions of plasmid pXRMV33 in 200 ng human placental DNA were used to validate the assay. To assess quality of DNA extracted from each tissue, a 168-bp segment of the single-copy gene VAMP2 (also known as Synaptobrevin 2) was amplified in a separate reaction. The reaction mix consisted of 1× TaqMan Fast Universal PCR Master Mix, 900 nM primers VAMP2–3043F and VAMP2–3210R, 250 nM hydrolysis probe VAMP2P, and 10–100 ng DNA in a total reaction volume of 20 μL. For positive control, 100 ng human placental DNA was used. Thermocycling conditions were the same as for detection of XMRV. For all primer sequences see Table S2.

Serial dilutions of the XMRV proviral clone were prepared in 200 ng of human placental DNA (50,000–5 copies/reaction). For formalin-fixed template standards, XMRV-infected cultured cells were serially diluted in uninfected cells at ratios of 1:100–1:1,000,000, fixed with formalin, and embedded in paraffin. One section of each dilution was added to 9 sections of normal prostate and DNA was extracted using the same method described above. To calculate the number of XMRV-infected cells contained in formalin-fixed qPCR standards, XMRV IHC-positive cells were counted in a section of infected cells diluted at a ratio of 1:100,000. For the remaining dilutions, the number of infected cells per section was extrapolated.

Immunohistochemistry. Formalin-fixed, paraffin-embedded prostate tissue sections were cut 5-μm thick, placed on electrostatically charged microscope slides, dried at 56 °C, deparaffinized in xylene, and rehydrated in decreasing alcohol concentrations.

Initial studies were performed with prostate sections from XMRV qPCR-positive cases. A strongly staining case was chosen for further optimization of the protocol. For antigen retrieval, sections were immersed in High pH Target Retrieval solution (Dako), pressure cooked (National SR-206-N) for 5 min, and slowly cooled to room temperature. Endogenous peroxidase activity was blocked with 3% H_2O_2. Sections were incubated with anti-XMRV antisera, diluted 1:7,500 in antibody diluent with background reducing components (Dako) in individual wells of Antibody Amplifier boxes (Prohisto). Tissue sections were incubated for 30 min with secondary anti-rabbit HRP-conjugated polymer antibody. Washes were with 50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 8.0, and staining was detected with 3,3′-diaminobenzidinetrahydrochloride (Dako). Sections were counterstained with hematoxylin, dehydrated with graded alcohols, and covered with coverslips.

1. Urisman A, et al. (2006) Identification of a novel Gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant. *PLoS Pathog* 2(3):e25.
2. Jemal A, et al. (2008) Cancer statistics, 2008. *CA Cancer J Clin* 58(2):71–96.

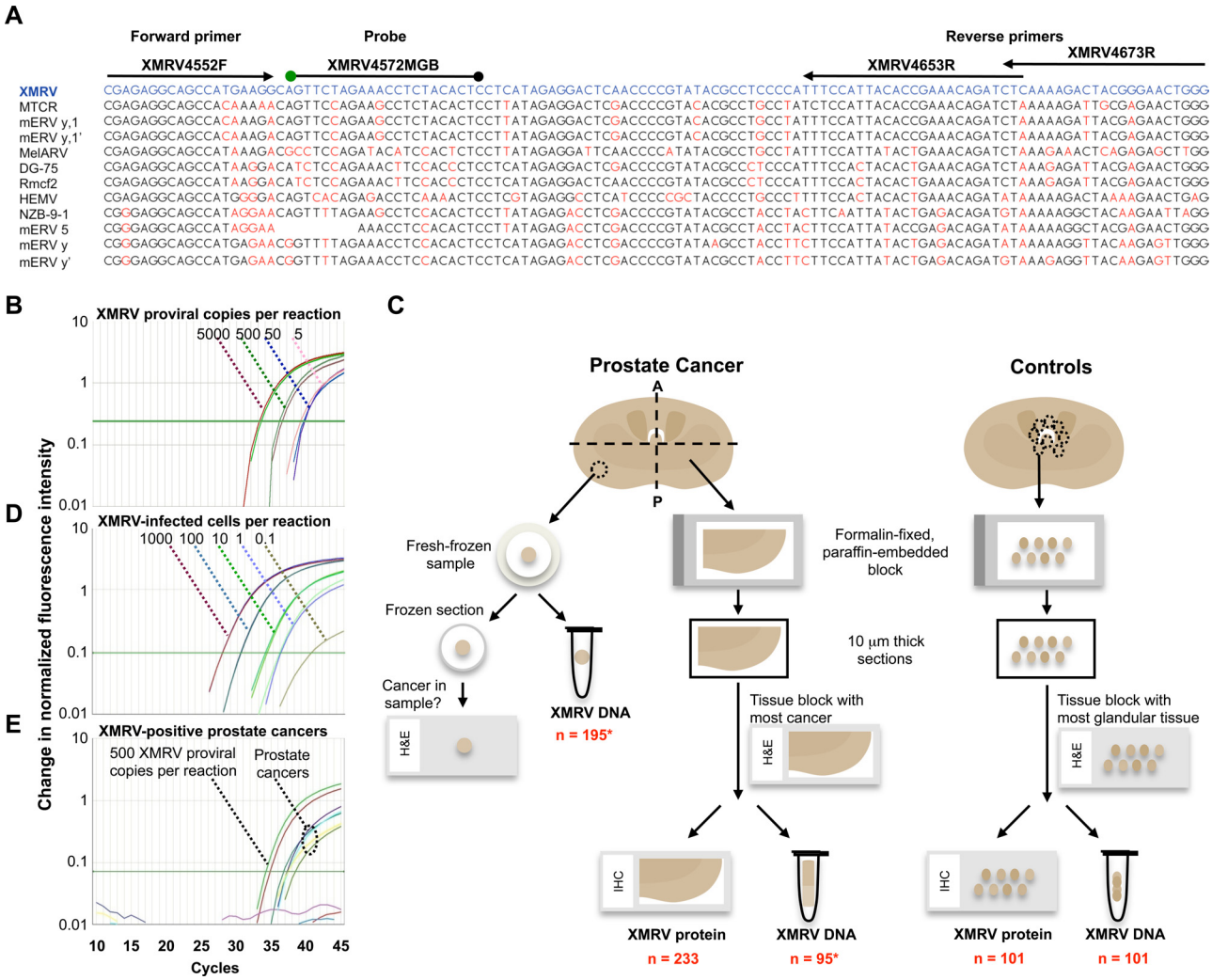

Fig. S1. Detection of XMRV DNA in prostate tissues by a qPCR and scheme for processing tissues for DNA extraction and immunohistochemistry. (*A*) Primer and probe hybridization sites in the XMRV integrase gene compared with equivalent regions of the most closely related murine retroviral sequences. For abbreviations and accession numbers, see Table S2. (*B* and *C*) Sensitivity of the qPCR assay: Detection of 5-5,000 copies of XMRV molecular clone in 200 ng human placental DNA (*B*). Serial 10-fold dilutions containing DNA from FFPE cultured XMRV-infected cells. No amplification was seen with uninfected cells (*C*). With both methods, the lowest detectable template concentrations resulted in quantification cycles (C_q) between 36 and 40. (*D*) Analysis of 233 consecutive cases of prostate cancer resections and 101 controls. Cancers consisted of frozen (n = 138), FFPE (n = 38), or frozen and fixed (n = 57) tissues. All controls were FFPE tissues. A, anterior; P, posterior; H&E, hematoxylin & eosin staining; *, for 57 cases, both frozen and fixed tissues were tested. (*E*) C_q for XMRV-positive prostate cancer samples compared to that for a standard reaction containing 500 copies of XMRV proviral DNA diluted in normal prostatic DNA.

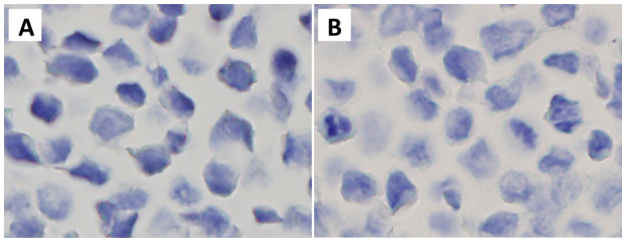

Fig. S2. Specificity of detection of XMRV proteins by IHC. (*A*) Using anti-XMRV antiserum on mock-infected cells results in no staining. (*B*) Similarly, no staining is observed when pre-immune serum from the same rabbit is used to stain XMRV-infected cells. Counterstaining with hematoxylin reveals blue nuclei.

Table S1. Samples acquisition with demographic information, tumor grade, and stage

	Prostate cancer				
Sample type	All	Frozen	Fixed	Both	Controls: fixed
Number	233	138	38	57	101
Age, median (range)	60 (41–76)	60 (41–74)	60 (43–76)	61 (50–73)	72 (44–85)
Race/ethnicity, %					
African–American	17	19	16	14	27
Caucasian	53	55	53	49	38
Hispanic	12	13	8	14	29
Other/not provided	17	13	24	23	6
Tumor grade, %					NA
6	16.3	18.1	10.5	15.8	
7	70.4	71.0	73.7	66.7	
8	6.0	4.3	10.5	7.0	
9	6.9	5.8	5.3	10.5	
10	0.4	0.7	0	0	
Tumor stage, %					
Primary tumor					NA
pT2a	9	13	5	4	
pT2b	2	2	3	2	
pT2c	63	57	79	67	
pT3a	18	19	11	19	
pT3b	6	7	3	4	
pT4	2	1	0	5	
Regional LN					NA
pNX	54	52	58	54	
pN0	44	46	40	42	
pN1	2	1	3	4	

Age is given in years and tumor grade is given as the Gleason score. Tumor stage is according to the American Joint Committee on Cancer (1): pT2a, unilateral, involving one-half of 1 lobe or less; pT2b, unilateral, involving more than one-half of 1 lobe but not both lobes; pT2c, bilateral disease; pT3a, extraprostatic extension; pT3b, seminal vesicle invasion; pT4, invasion of bladder, rectum; pNX, regional lymph nodes were not assessed; pN0, no regional lymph node metastasis; pN1, metastasis in regional lymph node(s). Distant metastasis was not assessed in any of the patients with prostate cancer. Abbreviations: LN, Lymph node; NA, not applicable.

1. Jemal A, et al. (2008) Cancer statistics, 2008. *CA Cancer J Clin* 58(2):71–96.

Table S2. Sequences of primers used for mutagenesis, sequencing, and qPCR

Primer	Start position	Sequence	Application
XMRV-M1	3,800	5′-CCT TGC CTA CGc gTG GTA GCA GCC-3′	Mutagenesis
XMRV-M2	3,823	5′-GGC TGC TAC CAc gCG TAG GCA AGG-3′	Mutagenesis
XMRV-S1R	602	5′-CGA GAA CAC TTA AAG ACA GAA GAA-3′	Sequencing
XMRV-S2R	944	5′-CGG GAG CTG TCG GTA A-3′	Sequencing
XMRV-S1F	1,339	5′-TGA AGA TCC AGG TAA ATT GAC G-3′	Sequencing
XMRV-S3R	1,575	5′-TCT GTA GTG GTG TAA TCC CAA TC-3′	Sequencing
XMRV-S2F	3,733	5′-GGG ACC TTG GCG TCG GCC TGT GGC-3′	Sequencing
XMRV-S4R	3,853	5′-CTT GCC TGC ATC CTT TGT CA-3′	Sequencing
XMRV-S3F	5,522	5′-GCC GCT GCT TAT CAG GAC CAG-3′	Sequencing
XMRV-S5R	5,702	5′-GTA TCC ACG CAG AGA TGC C-3′	Sequencing
XMRV-S6R	5,731	5′-AGT TGT CGC CGC CTT TAC GTG-3′	Sequencing
XMRV-S4F	6,598	5′-GGG ACG GGA GAC AGG CT-3′	Sequencing
XMRV-S7R	6,761	5′-GGG GCA GAG GTA TGG TTG G-3′	Sequencing
XMRV-S5F	7,380	5′-TGG CGT AGT AAG AGA TAG CAT-3′	Sequencing
XMRV-S8R	7,567	5′-GAA TAC AGG GTC CGA AGA G-3′	Sequencing
XMRV-S6F	7,727	5′-ACC CCA CCA TAA GGC TTA GCA C-3′	Sequencing
XMRV-S9R	7,942	5′-TTA GTT TCG CTT TAT CTG AGG ACC A-3′	Sequencing
XMRV4552F	4,552	5′-CGA GAG GCA GCC ATG AAG G-3′	Detection
XMRV4572MGB	4,572	5′-6FAM AGT TCT AGA AAC CTC TAC ACT C MGBNFQ-3′	Detection
XMRV4653R	4,653	5′-GAG ATC TGT TTC GGT GTA ATG GAA A-3′	Detection
XMRV4673R	4,673	5′-CCC AGT TCC CGT AGT CTT TTG AG-3′	Detection
VAMP2–3043F	3,043	5′-TCT GCC ACT TCG GGT TTC TC-3′	Control
VAMP2–3210R	3,210	5′-GGT AGC CAC CCC TCT CAC AA-3′	Control
VAMP2P	3,067	5′-HEX CAT TCC TGC TCC CCA GTT TTC ATG TGG Tamra-3′	Control

MGBNFQ, minor grove binder/nonfluorescent quencher. Nucleotide positions are based on VP62 (EF185282.1).

Table S3. Sequences used for alignment and design of specific primers

Abbreviation	Definition (GenBank accession no.)
XMRV	XMRV VP35 (DQ241301.1)
	XMRV VP42 (DQ241302.1)
	XMRV VP62 (EF185282.1)
MTCR	Retroviridae complete genome, murine type C retrovirus (X94150.1)
	Mus musculus chromosome 1, clone RP24–65D16 (AC115959.17)
mERV y,1	*M. musculus* BAC clone RP24–320A8 from chromosome y (AC182253.3)
	M. musculus strain C57BL/6J chromosome 1 clone rp23–116m12 (AC083892.19)
mERV y,1′	*M. musculus* BAC clone RP24–320A8 from chromosome y (AC182253.3)
	M. musculus strain C57BL/6J chromosome 1 clone rp23–116m12 (AC083892.19)
MelARV	*M. musculus* isolate MelARV endogenous B-tropic ecotropic murine leukemia virus (DQ366148.1)
	M. musculus C-type ecotropic endogenous retrovirus (U63133.1)
DG-75	DG-75 Murine leukemia virus (AF221065.1)
	Murine AIDS virus-related provirus (S80082.1)
	M. musculus chromosome 9, clone RP23–364M24 (AC103610.9)
	M. musculus BAC clone RP23–277L21 from chromosome 2 (AC124194.3)
	Mouse DNA sequence from clone RP23–130L13 on chromosome 9 (CT009721.14)
	Mouse DNA sequence from clone RP23–259C9 on chromosome 13 (CT030655.7)
	Mouse DNA sequence from clone RP24–114E18 on chromosome 2 (AL928935.14);
	Mouse DNA sequence from clone RP23–354H24 on chromosome 4(AL627314.6)
	Mouse DNA sequence from clone RP23–384D6 on chromosome 4 (AL627077.14)
Rmcf2	*M. musculus castaneus* endogenous virus Rmcf2 (AY999005.1)
HEMV	Murine leukemia virus serotype HEMV provirus (AY818896.1)
NZB-9–1	Xenotropic murine leukemia virus isolate NZB-9–1 (EU035300.1)
mERV 5	*M. musculus* chromosome 5, clone RP23–110C17 (AC117614.14)
mERV y	*M. musculus* BAC clone RP24–163J18 from chromosome y (AC175744.2)
mERV y′	*M. musculus* BAC clone CH36–265C6 from chromosome y (AC202413.4)
	M. musculus BAC clone RP24–302I24 from chromosome Y (AC1 (1844409.3)

mERV, murine endogenous retrovirus.