

Detection of Xenotropic Murine Leukemia Virus–Related Virus in Normal and Tumor Tissue of Patients from the Southern United States with Prostate Cancer Is Dependent on Specific Polymerase Chain Reaction Conditions

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(See the brief report by Henrich et al, on pages XXX–XXX, the brief report by Barnes et al, on pages XXX–XXX, and the editorial commentary by Kearney and Maldarelli, on pages XXX–XXX.)

Background. There are questions regarding the prevalence of xenotropic murine leukemia virus–related virus (XMRV) in patients with prostate cancer and its association with the *RNASEL* R462Q polymorphism. We therefore investigated whether XMRV infection could be found in patients with prostate cancer from the southern United States, and we sought to verify the association with the R462Q.

Methods. Prostate tissue specimens of 144 patients with prostate cancer from the southern United States were genotyped for R462Q by real time polymerase chain reaction allelic discrimination and were screened for XMRV proviral DNA by nested polymerase chain reaction specific for the *env* gene.

Results. The R462Q polymorphism was found at an allelic frequency of 0.33. XMRV was detected in 32 (22%) of the 144 patients. Patients were significantly more likely to test positive for XMRV in both tumor and normal tissue rather than either alone ($\kappa = 0.64$). A positive result for XMRV was not significantly correlated with the R462Q polymorphism ($P = .82$) or clinical pathological parameters of prostate cancer, including Gleason score ($P = .29$).

Conclusions. XMRV is detectable in normal and tumor prostate tissue from patients with prostate cancer, independent of R462Q. The presence of XMRV in normal tissue suggests that infection may precede cancer onset.

Prostate cancer is a leading cancer in men in Western countries, accounting for 25% of incident cancers in American men in 2009 [1, 2]. Despite the high prevalence and gravity of this disease, there are currently few suitable biomarkers to distinguish between cancers

with high and low recurrence potentials and to determine whether patients require immediate therapeutic intervention or only periodic observation [3]. Such biomarkers for classifying prostate cancers into different treatment categories may depend on the underlying etiology of each case. Epidemiological evidence suggests that environmental factors, such as diet and infectious agents, may contribute to chronic inflammation of the prostate and tumorigenesis [2]. An infectious etiology for prostate cancer is supported by the linkage of hereditary prostate cancer to the common R462Q polymorphism in the *RNASEL* gene. The polymorphism, which has been reported to be more prevalent among familial patients with prostate cancer, results in a reduced-activity variant of the innate antiviral factor, ribonuclease L [4, 5]. In 1 study, the R462Q polymorphism was implicated in up to 13% of prostate cancer cases [4]. Correspondingly, xenotropic murine leuke-

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mia virus–related virus (XMRV) was discovered by searching for viruses in patients with prostate cancer homozygous for R462Q with a microarray (Virochip) designed to detect the most conserved regions of all viral families [6]. An expanded screen with patients with prostate cancer harboring wild-type *RNASEL* alleles indicated a strong correlation with the R462Q variant, thus establishing a connection between infection and the disease [6].

The linkage of XMRV to prostate cancer through the *RNASEL* R462Q polymorphism has become the subject of controversy because recent reports indicate that infection occurs independent of R462Q [7, 8]. Additional studies are needed to determine whether *RNASEL* genotype is a reliable indicator of susceptibility to XMRV infection. Furthermore, there is no agreement about the cell types infected in the prostate. XMRV was originally discovered exclusively in the nonmalignant stromal and hematopoietic cells adjacent to the carcinoma [6]. By contrast, another study found XMRV primarily in prostate carcinoma cells [7]. Additional studies are therefore needed to determine whether nonmalignant cells are susceptible to infection by XMRV to address whether infection may precede tumor initiation. Questions regarding the association of XMRV with prostate cancer have also arisen in light of recent studies that detect little to no presence of the virus in patients with prostate cancer [9, 10]. Interestingly, studies that detect XMRV in patients with prostate cancer were conducted in the United States, whereas those that do not detect the virus were conducted in Germany. These conflicting reports emphasize the need to confirm the presence of XMRV in prostate cancer and to define the geographic distribution of the virus.

Here, we conducted a retrospective study in which we screened a cohort of patients with prostate cancer that is unique from those of previous studies with respect to its location within the United States. Additionally, we selected for patients with a family history of prostate cancer to enrich for carriers of the R462Q polymorphism. The goals of the study were to confirm the presence of XMRV in patients with prostate cancer and to investigate the linkage of XMRV to the R462Q polymorphism. Here, we demonstrate that XMRV is present in 22% of patients with prostate cancer from the southern United States, that infection does not correlate with R462Q, and that reliable detection of viral DNA was dependent on particular conditions of polymerase chain reaction (PCR). Additionally, we show that XMRV is detectable in both normal and cancer tissues in the prostate, suggesting that the virus does not specifically target transformed cells and that infection may therefore precede cancer onset. If XMRV is shown to promote prostate cancer, it may prove to be a valuable biomarker for clinicians when considering treatment for patients.

METHODS

Prostate cancer cohort and tissue preparation. Frozen prostate tissue cores were obtained from a prostate cancer tissue bank at Baylor College of Medicine. Details of the donor patients have been described elsewhere [3]. All prostate tissues used in the study were derived from patients who underwent radical prostatectomy and had provided consent in accordance with the Baylor College of Medicine Institutional Review Board. No patients underwent preoperative treatment for their cancer. To enrich for carriers of the R462Q polymorphism in *RNASEL*, only tissues from patients having at least 1 first- or second-degree relative diagnosed with prostate cancer were selected for XMRV screening and *RNASEL* genotyping. In total, 144 patients were screened for XMRV and the R462Q polymorphism in *RNASEL*. For 57 of the 144 patients, both normal and tumor tissue were available for screening.

All prostate tissues were prepared for DNA extraction in a separate laboratory from the laboratory in which the infectious XMRV clone VP62 (GenBank accession number, NC_007815.1) was handled. DNA was extracted from sections of prostate biopsies with use of the QIAamp DNA Mini kit (Qiagen). All prostate tissue DNA samples were stored at -20°C immediately following extraction in a laboratory free of amplified or cloned DNA.

***RNASEL* genotyping.** All patients were genotyped for *RNASEL* G1385A (R462Q) with use of the Applied Biosystems real-time PCR TaqMan single nucleotide polymorphism assay (Assay ID: C 935391_1_) with TaqMan Universal PCR Master Mix (Applied Biosystems). A 7500 real-time PCR system (Applied Biosystems) was used for amplification and analysis of *RNASEL* genotyping reactions containing 20 ng of prostate tissue DNA. Specimens of predetermined genotypes (homozygous wild-type, heterozygous, and homozygous variant) were used as controls for genotyping reactions. All patients were tested in duplicate.

Provirus screen. A nested PCR assay was developed to screen prostate tissue DNA for XMRV provirus. The first-round primers (5'-ACCAGACTAAGAAGCTTAGAACCTCG-3' and 5'-AGCTGTTCAGTGATCACGGGATTAG-3') amplify an 888-bp region containing the 5' terminus of *envelope* (*env*). The nested, second-round primers (5'-GAACAGCATGGAAAGTCCAGC-GTTC-3' and 5'-CAGTGGATCGATACAGTCTTAGTCC-3') amplify a 653-bp region encompassing the 3 variable regions (VR) of *env*, VRA, VRB, and VRC. First-round reactions contained 650 ng of prostate tissue DNA, 2.5 mmol/L MgCl_2 , 800 $\mu\text{mol/L}$ of dNTPs, 100 ng of each primer, and 1.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems) in a 50- μL total volume. Two microliters of first-round reactions were transferred to 48 μL of a PCR master mix containing 100 ng of each second-round primer and the same concentrations of

Table 1. Summary of *RNASEL* Genotyping and Xenotropic Murine Leukemia Virus–Related Virus Screen

Variable	Screen result
Total no. of patients	144
Positive PCR result, no. (%) of patients ^a	32 (22.2)
R462Q allelic frequency	
Total patients, frequency of R462Q alleles	0.33
Patients with positive PCR result, frequency of R462Q alleles ^b	0.297
Genotypic distribution	
Wild-type RR, no. (%) of patients	66 (45.8)
Heterozygous RQ, no. (%) of patients	61 (42.4)
Homozygous variant QQ, no. (%) of patients	17 (11.8)

NOTE. PCR, polymerase chain reaction.

^a Patients who had positive results by PCR for xenotropic murine leukemia virus–related virus regardless of tissue type.

^b R462Q allelic frequency for the 32 patients with positive results for xenotropic murine leukemia virus–related virus DNA.

each of the components of the first-round reactions. Thermocycling conditions were as follows: 94°C for 5 min; 35 cycles of 94°C for 30 s, 56°C for 1.5 min, and 72°C for 1 min; and ending with 72°C for 10 min. The master mixes for each set of PCR reactions were tested for sensitivity and nucleic acid contamination by incorporation of positive and negative controls, respectively. The positive control consisted of 3 separate reactions, each with 100 ng of DNA isolated from XMRV-infected LNCaP cells diluted 1 to 1×10^3 in uninfected LNCaP cells. The master mix was considered to be adequately sensitive only if all 3 positive control reactions had positive results. Negative controls consisted of 3 separate reactions with H₂O in place of DNA template and 3 separate reactions of 650 ng of uninfected LNCaP DNA. After thermocycling, second-round reactions were electrophoresed on agarose gels containing ethidium bromide and were visualized under ultraviolet light. All tissues were screened in triplicate, and patients/tissues were considered to be positive if ≥ 1 PCR reactions had positive results.

Cell culture and generation of PCR sensitivity controls.

The LNCaP human prostate carcinoma cell line was used to test the sensitivity of the PCR assay and to generate XMRV stocks. XMRV has been shown to infect and replicate within this cell line [11]. LNCaP cells were cultured in RPMI 1640 (Invitrogen), 10% heat-inactivated fetal bovine serum (Sigma), glutamine, and penicillin and streptomycin (Invitrogen) and were incubated at 37°C with 5% CO₂.

To generate PCR sensitivity controls, LNCaP cells were transfected with an infectious XMRV clone (VP62), a generous gift from Robert Silverman (Cleveland Clinic) [11]. One day after transfection, the cells were washed with phosphate-buffered saline and were supplied with fresh media. Two days posttransfection, the conditioned media was passed through a 0.45- μ m

syringe filter and was used to infect a new stock of LNCaP cells. The infected cells were cultured for 40 days, splitting them 1:10 every 5–7 days. The infected cells and a separate stock of uninfected LNCaP cells were washed with phosphate-buffered saline, trypsinized, and mixed together at ratios of 1:100, $1:1 \times 10^3$, $1:1 \times 10^4$, and $1:1 \times 10^5$ (infected cells:uninfected cells). Without further culturing, total cellular DNA was extracted from the cell mixtures with use of the QIAamp DNA Mini kit (Qiagen). Extracted DNA was used as template to test the sensitivity of the XMRV *env* nested PCR assay.

To test for VP62 plasmid contamination in prostate specimens, a set of 4 primers were designed for nested PCR. The 2 forward primers (5'-TCTGGCTAACTAGAGAACCCACTG-3' and 5'-AATACGACTCACTATAGGGAGACC-3') were specific to the multiple cloning site of pCDA3.1(-) (Invitrogen). The 2 reverse primers (5'-AAGGTAACCCAGCGCCTCTTC-3' and 5'-GTTACGGTCTGTCCCATGATCTC-3') were specific to the 5' terminus of VP62 *gag*. The VP62 nested PCR assay was found to be capable of detecting 10 plasmids diluted in 600 ng of uninfected LNCaP DNA in 3 of 3 samples and 1 plasmid in 600 ng of uninfected LNCaP DNA in 1 of 3 samples (data not shown).

Cloning and sequencing of patient-derived PCR products.

Positive PCR reactions were electrophoresed on agarose gels and were extracted using the Qiaex II Gel Extraction Kit (Qiagen), according to the manufacturer's instructions. Extracted

This figure is available in its entirety in the online version of the *Journal of Infectious Diseases*.

Figure 1. Xenotropic murine leukemia virus–related virus *env* polymerase chain reaction fragment nucleotide sequence alignment.

A

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VP62  MESPAFSKPLKDKINPWGPLIIMGILVRAGASVQRDSPHQVFNVTWKITNLMTGQTANATSLLGTMDDTFPK
PCA1  .....A.....
PCA2  .....
PCA3  .....
PCA4  .....
PCA5  .....
PCA6  .....
PCA7  .....
PCA8  .....
PCA9  .....
PCA10 .....
PCA11 .....
PCA12 .....
PCA13 .....
PCA14 .....
PCA15 .....
PCA16 .....
PCA17 .....

                VRA                VRC
VP62  LYFDLCLVGDNDWDDPEPDIGDGRSPGGRKRRLYDFYVCPGHTVLTGCGGPREGYCGKWGCETTGQAYWK
PCA1  .....G.....
PCA2  .....
PCA3  .....
PCA4  .....
PCA5  .....*
PCA6  .....
PCA7  .....D.....
PCA8  .....
PCA9  .....
PCA10 .....
PCA11 .....
PCA12 .....
PCA13 .....
PCA14 .....
PCA15 .....
PCA16 .....
PCA17 .....

                VRB
VP62  PSSSWDLISLKRGNTPKQGQPCFDSSVSGSIIQGATPGGRCNPLVLEFTDAGKRASWDAPKTWGLRLYRST
PCA1  .....G.....
PCA2  .....
PCA3  .....
PCA4  .....
PCA5  .....
PCA6  .....
PCA7  .....
PCA8  .....
PCA9  .....
PCA10 .....
PCA11 .....
PCA12 .....
PCA13 .....
PCA14 .....
PCA15 .....
PCA16 .....
PCA17 .....

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B

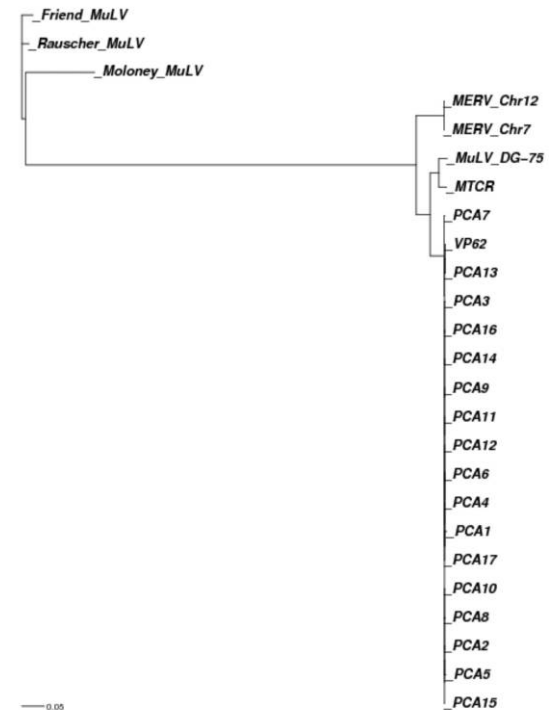


Figure 2. Sequence analysis of patient-derived polymerase chain reaction (PCR) products. *A*, Comparison of the predicted Env protein sequences from the PCR products of 17 patients with the Env sequence of the xenotropic murine leukemia virus–related virus (XMRV) clone VP62; variable regions (VR) A, B, and C are indicated; *dots* indicate identical residues; and a stop codon is indicated by an *asterisk*. *B*, Phylogenetic tree of the XMRV patient clones, compared with other murine retroviruses.

PCR products were cloned into pCR2.1-TOPO with use of the TOPO TA Cloning Kit (Invitrogen), according to the manufacturer’s protocol. The cloned PCR sequences were propagated in NEB 10-beta (New England BioLabs) *Escherichia coli* and were isolated with the QIAprep Spin Miniprep Kit (Qiagen), and the sequences of the DNA inserts were determined.

Phylogenetic analysis. *Env* sequences were aligned using Clustal and were trimmed to the same length with gaps. The maximum likelihood tree of *env* sequences was generated using PhyML [12].

Statistical analysis. Statistical analyses were performed us-

ing Stata, version 10 (StataCorp). Correlation between XMRV positivity and tissue type was analyzed by measuring the simple kappa coefficient. Correlations between XMRV positivity and Gleason score or seminal vesicle invasion were analyzed using Fisher’s exact test. Correlation between XMRV positivity and extracapsular extension or surgical margin invasion was assessed using the χ^2 test.

Accession numbers. Sequences of cloned XMRV *env* genes were deposited in GenBank under accession numbers GU812341–GU812357. Accession numbers from GenBank for other viral sequences are as follows: DG-75, af221065;

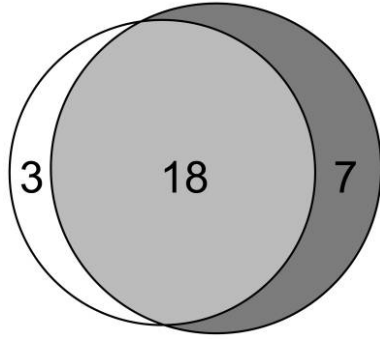


Figure 3. Distribution of xenotropic murine leukemia virus–related virus (XMRV) between normal and cancer tissue of 57 patients with prostate cancer. The *white circle* represents patients from whom XMRV DNA was detected by polymerase chain reaction in normal tissue, the *dark gray circle* represents patients from whom XMRV DNA was detected by polymerase chain reaction in tumor tissue, and the *light gray overlap* represents patients for whom both tissue types had positive results for XMRV DNA. Twenty-nine of the 57 patients had negative results for XMRV DNA. Patients were found to be more likely to have positive results for both tissue types (simple κ coefficient, 0.64).

Raucher MuLV, NC_001819; Friend MuLV, M93134; Moloney MuLV, NC_001501.1; MERV Chr12, ac153658; MTCR, NC_001702; and MERV Chr7, ac127565.

RESULTS

Distribution and frequency of RNASEL R462Q. To investigate the linkage of XMRV infection to *RNASEL* R462Q, we obtained prostate tissue specimens from patients with prostate cancer to screen for the virus and the R462Q polymorphism in *RNASEL*. Because XMRV was originally found to be strongly associated with R462Q, we screened patients with prostate cancer with a family history of prostate cancer, which are reported to have an increased R462Q allelic frequency [4–6]. In total, 144 prostate cancer cases were screened by a real-time PCR-based allelic discrimination assay for the R462Q polymorphism in *RNASEL*. We found there to be 66 (45.8%) wild-type (RR) individuals, 61 (42.4%) heterozygotes (RQ), and 17 (11.8%) individuals homozygous for the Q variant (Table 1). The allelic frequency for R462Q was determined to be 0.33, which is intermediate in comparison to other described prostate cancer cohorts of unselected or sporadic cases (allelic frequency of R462Q, 0.25 and 0.38) [4, 7, 10].

XMRV is detected in patients with prostate cancer. A highly-sensitive, nested PCR assay for XMRV *env* was developed to screen patients for XMRV infection. The PCR assay was found to be capable of detecting a single copy of VP62 plasmid (data not shown). We also tested the sensitivity of the PCR assay in the context of integrated provirus. We found that XMRV provirus could be detected at a dilution of 1 infected human prostate carcinoma cell per 1×10^4 uninfected cells in

3 of 3 samples with use of 600-ng of DNA ($\sim 1 \times 10^5$ cells). With the same quantity of DNA, the nested PCR assay was found to be capable of detecting XMRV provirus in 1 infected cell per 1×10^5 uninfected cells in 1 of 3 samples (data not shown). Importantly, assuming XMRV provirus is present at a frequency of 0.15%–1.5%, which has been estimated in other reports, our nested PCR assay is greater than 15–150 times more sensitive than that which is minimally required to detect the virus [6, 7]. Thus, the nested PCR assay is a highly sensitive method to detect XMRV provirus. Each patient specimen was screened in triplicate with use of 650 ng of prostate tissue DNA. In total, 32 (22%) of 144 patients were found to have positive results for XMRV by PCR (Table 1). The majority of tissue specimens that were positive for XMRV had positive results in only 1 or 2 of 3 replicates (data not shown).

To confirm that XMRV was detected, the 653-bp *env* PCR products were sequenced from 17 patients with positive results (nucleotide sequence alignment is presented in Figure 1). In comparison to reference strain VP62, 3 of the 17 sequences encoded nonsynonymous nucleotide differences, representing a total of 5 amino acid differences (Figure 2A). With respect to all 17 predicted Env peptide sequences, differences from VP62 were observed at a rate of 0.14%, ranging from 0% to 1.4% (patient PCA1). The high degree of sequence identity with VP62 suggests that positive PCR results for the tissue specimens were attributable to the presence of XMRV DNA. This was confirmed by phylogenetic analysis of the sequences (Figure 2B). Additionally, we tested for the presence of contaminating VP62 plasmids in the DNA isolated from the patient tissue specimens with use of a nested PCR assay targeting the pCDNA3.1(-) multiple cloning site–XMRV genome junction. We found no evidence for contamination in specimens with positive results by PCR for XMRV *env* (data not shown).

XMRV is present in cancer and normal tissues. For 57 of the 144 patients, both normal and tumor prostate tissues were available for screening, whereas only tumor tissue was available for the remaining 87 patients. In this subset of patients, XMRV was detected in 21 (36.8%) of 57 normal tissue samples and in 25 (43.9%) of 57 tumor tissue samples. The virus was de-

Table 2. Xenotropic Murine Leukemia Virus–Related Virus Screening by Nested Polymerase Chain Reaction (PCR) for *env*

PCR result	RNASEL genotype			Total
	RR	RQ	QQ	
Positive	16	13	3	32
Negative	50	48	14	112
All ^a	66 (24.2)	61 (21.3)	17 (17.6)	144 (22.2)

NOTE.Data are no. or no. (%) of specimens. RR, homozygous wild-type; RQ, heterozygous; QQ, homozygous R462Q variant.

^a The numbers in parentheses are percentages that indicate the proportion of xenotropic murine leukemia virus–related virus PCR-positive specimens.

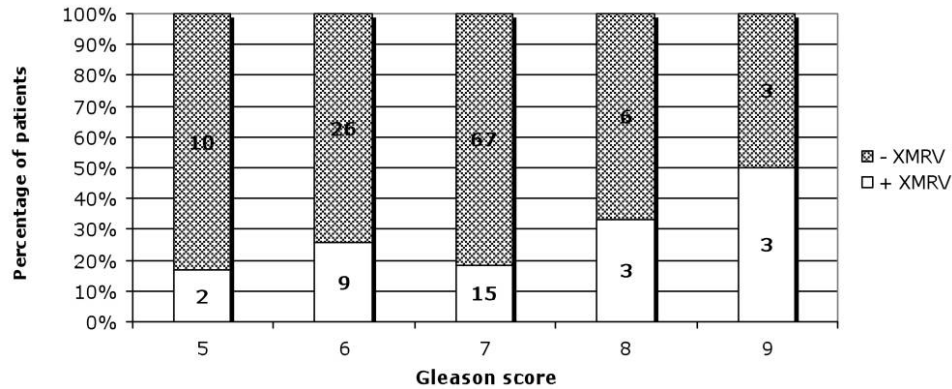


Figure 4. Xenotropic murine leukemia virus–related virus (XMRV) infection is not significantly correlated with Gleason score. Numbers of infected patients (*light gray*) and uninfected patients (*dark gray*) are graphed according to Gleason score. No association between detection of provirus and Gleason score was found ($P = .29$, by Fisher’s exact test; $P = .30$, by 2-sample t test).

tected exclusively in the normal tissue of 3 patients and was detected exclusively in tumor tissue of 7 patients, whereas 18 patients had provirus detected in both tissue types (Figure 3). Statistical analysis of these results indicate that patients were more likely to harbor provirus in both normal and tumor tissue rather than one or the other (κ coefficient of agreement, 0.64), suggesting that XMRV does not specifically target tumor tissue in the prostate.

XMRV infection does not correlate with R462Q, Gleason score, or other pathological parameters of prostate cancer.

We investigated whether XMRV infection is enriched among carriers of the R462Q polymorphism of *RNASEL* in this cohort. XMRV was detected in 24.2%, 21.3%, and 17.6% of wild-type (RR), heterozygous (RQ), and homozygous variant (QQ) patients, respectively (Table 2). However, infection was not found to be significantly associated with the R462Q polymorphism of *RNASEL* ($P = .82$, by χ^2 test).

XMRV infection is reportedly associated with higher Gleason score prostate cancers [7]. We therefore examined whether XMRV infection correlates with tumor grade. The patients in our study consisted of 12, 35, 82, 9, and 6 patients with Gleason scores of 5, 6, 7, 8, and 9, respectively. Although there appears to be a trend between XMRV infection and increasing Gleason

score in Figure 4, no statistically significant association was found ($P = .29$, by Fisher’s exact test). Furthermore, we examined whether XMRV infection correlates with seminal vesicle invasion, extracapsular extension, and surgical margin invasion, which are indicators of spreading prostate cancer (Table 3). However, we found no significant correlation between XMRV infection and seminal vesicle invasion ($P = .33$, by Fisher’s exact test), extracapsular extension ($P = .59$, by χ^2 test), or surgical margin invasion ($P = .89$, by χ^2 test).

DISCUSSION

Our screen of patients with prostate cancer confirms the presence of XMRV among patients with prostate cancer in the United States. We detected XMRV DNA in normal and tumor tissue, indicating that nonmalignant cells may be susceptible to infection. In agreement with recent studies, we find no correlation between the presence of XMRV infection and the R462Q polymorphism of *RNASEL*, confirming that the population at risk of infection is not confined to homozygous carriers of the Q variant [7, 8].

Interestingly, 3 independent studies, including 2 surveys of German prostate tissue specimens and a screen of English chronic

Table 3. Statistical Analysis of Xenotropic Murine Leukemia Virus–Related Virus Positivity Versus Clinical Pathological Parameters of Spreading Prostate Cancer

Parameter	Positive clinical pathology		Negative clinical pathology		Test	P
	PCR positive, no. (%) of patients	PCR negative, no. of patients	PCR positive, no. (%) of patients	PCR negative, no. of patients		
ECE	11 (25.6)	32	21 (21.4)	77	χ^2	.59
SVI	5 (33.3)	10	27 (21.4)	99	Fisher’s exact	.33
SMI	8 (23.5)	26	24 (22.4)	83	χ^2	.89

NOTE. Patients either scored positive or negative for clinical pathological parameters of prostate cancer and were either positive or negative by PCR for xenotropic murine leukemia virus–related virus. ECE, extracapsular extension; SMI, surgical margin invasion; SVI, seminal vesicle invasion.

fatigue syndrome patients, found little to no evidence of XMRV infection [9, 10, 13]. However, in agreement with studies performed in the United States, we found the presence of XMRV in prostate cancer tissues [6, 7, 14]. It is possible that XMRV is mostly absent from the European population. If so, it would be interesting to uncover the reason for this geographic distribution. Alternatively, the inability to detect XMRV in Europe may possibly reflect genetic differences between American and European strains. However, this seems unlikely considering the high degree of sequence conservation among XMRV isolates and the variety of primer target sequences used for detection among the studies in Europe [6, 8–10, 13]. Additionally, the failure to detect XMRV may be attributable to differences in the detection techniques employed. We have found that detection of XMRV required rather specific conditions. For instance, at least 600 ng of prostate tissue DNA was necessary for reliable detection with our PCR assay. XMRV was detected in 3.2% of the patients when we initially used 100–140 ng of prostate tissue DNA, compared with 22.2% of the patients when we used 650 ng. Additionally, we found that detection of XMRV from patient specimens, but not from LNCaP cells infected *in vitro*, depended on the gene targeted in the PCR assay. We were unable to detect XMRV in the patient tissue samples by nested PCR with primers specific for the *gag* and *pol* genes, regardless of whether 100 or 650 ng of DNA was used as template. We found the *gag* primers to be at least 10-fold less sensitive than the *env* primers, and the *pol* primers tended to amplify a competing region from the human genome (data not shown). It is unclear whether these deficiencies account for the inability to detect XMRV in patient samples or whether XMRV is mainly present as an incomplete provirus in the cells of these patients. Nonetheless, the difficulty associated with detecting XMRV in patient samples may perhaps explain studies that do not detect the virus among large cohorts.

We found our nested PCR assay for XMRV *env* to be capable of detecting 1 infected cell per 1×10^5 uninfected LNCaP cells in 1 of 3 samples with use of 600 ng of DNA. The fact that the PCR-positive tissue specimens tested positive in only 1 or 2 of 3 replicates may indicate that XMRV provirus is present at a very low copy number. This interpretation would be consistent with another report [7]. Alternatively, it is possible that the quality of the tissue specimens was low because of preservation, handling, and the duration of storage prior to DNA isolation. However, we were able to genotype the patients for R462Q with use of 20 ng of DNA without difficulty.

Our finding that XMRV can be detected in the normal tissue of patients with prostate cancer suggests that nonmalignant cells may also be susceptible to XMRV infection. If this is correct, XMRV infection may precede and possibly participate in the process of tumorigenesis. There is currently little evidence to suggest that XMRV employs any traditional mechanisms for transforming cells. The virus harbors no known oncogenes,

and a clonal integration pattern indicative of insertional mutagenesis has not been observed in prostate cancer specimens. In accord with other studies, we predict a proviral copy number of far less than 1 per cell, arguing against insertional mutagenesis as a mechanism of transformation [6, 7].

A limitation of our PCR-based screen is that it does not identify the infected cell types. It is possible that the XMRV we detected was exclusively from nonmalignant cells, because tumor tissue consists of both malignant and nonmalignant cell types. It is important to note that XMRV may promote tumorigenesis through paracrine and cell-cell interactions. Prostate cancer has been shown to depend on the biology of the surrounding stromal microenvironment, and a reactive stromal phenotype has been shown to promote cancer progression [3, 15–18]. It would be interesting to determine whether XMRV elicits the conversion of prostate stromal cells to a reactive phenotype, regardless of the cell type infected.

We did not find a correlation between XMRV infection and various clinical pathological parameters of prostate cancer, including seminal vesicle invasion, extracapsular extension, and surgical margin invasion. Similar to another report, which found a correlation with higher Gleason scores, we observed a slight trend in favor of increasing Gleason score [7]. However, our results were not statistically significant. Additional studies with a greater number of patients will be required to evaluate a correlation between XMRV infection and Gleason score.

In conclusion, our data support a hypothesis that XMRV is endemic to North America. However, further investigation into the association of XMRV with prostate cancer and other human diseases is needed. If established as an agent of human disease, XMRV may prove to be an important biomarker for selecting a suitable course of treatment.

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