

XMRV: A New Virus in Prostate Cancer?

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Abstract

Several recent articles have reported the presence of a gammaretrovirus, termed "XMRV" (xenotropic murine leukemia virus-related virus) in prostate cancers (PCa). If confirmed, this could have enormous implications for the detection, prevention, and treatment of PCa. However, other articles report failure to detect XMRV in PCa. We tested nearly 800 PCa samples, using a combination of real-time PCR and immunohistochemistry (IHC). The PCR reactions were simultaneously monitored for amplification of a single-copy human gene, to confirm the quality of the sample DNA and its suitability for PCR. Controls showed that the PCR assay could detect the XMRV in a single infected cell, even in the presence of a 10,000-fold excess of uninfected human cells. The IHC used 2 rabbit polyclonal antisera, each prepared against a purified murine leukemia virus (MLV) protein. Both antisera always stained XMRV-infected or -transfected cells, but never stained control cells. No evidence for XMRV in PCa was obtained in these experiments. We discuss possible explanations for the discrepancies in the results from different laboratories. It is possible that XMRV is not actually circulating in the human population; even if it is, the data do not seem to support a causal role for this virus in PCa. *Cancer Res*; 70(24); 10028–33. ©2010 AACR.

Introduction

In 2006, a new retrovirus was reported to be associated with prostate cancer (PCa; ref. 1). It was recognized as a murine leukemia virus (MLV), a member of the gammaretrovirus genus, and was termed "XMRV", or "xenotropic murine leukemia virus-related virus". (MLVs are found in mice; xenotropic MLVs cannot infect mouse cells, but can generally infect human cells.)

Association of a virus with this important cancer could have enormous implications for detection, prevention, and treatment of PCa, just as the discovery of the role of human papillomavirus in cervical cancer has revolutionized our approach to this disease. Accordingly, many laboratories have begun testing for the presence of XMRV in PCa patients. Remarkably, the same virus was also reported in patients with chronic fatigue syndrome (CFS; ref. 2).

Retroviruses are relatively simple RNA-containing viruses. Their unique properties include the copying of their RNA into double-stranded DNA at the time of infection ("reverse tran-

scription") and the integration of this DNA copy into the chromosomal DNA of the infected cell. Once inserted, this DNA is replicated with the chromosome, and is thus present in the cell and its descendants into the indefinite future.

The methods that have been used to detect XMRV include nucleic acid hybridization; PCR and reverse transcriptase-PCR (RT-PCR); FISH; immunohistochemistry (IHC); screens for antiviral antibodies in patient sera; and virus cultivation. These diverse methods have only given concordant results in a handful of cases. The field was recently reviewed (3), and the state of the science was also covered in a 1.5-day meeting held on the NIH campus in September 2010.

XMRV was initially reported to be more prevalent in PCa tissues from men with homozygous germline RNase L mutations (R462Q) than in men without this QQ genotype (1). In this study, both *in situ* hybridization and IHC found the virus in approximately 1% of stromal cells, but not in tumor cells. A subsequent study reported that 14 of 233 prostate tumors were positive for XMRV by real-time PCR and 54 of 233 were positive by IHC (4). Surprisingly, a number of cases were positive by IHC, but negative by PCR. The IHC in this study localized XMRV proteins primarily in malignant epithelial cells rather than stromal cells and positive staining correlated with high tumor grade. No association with RNase L variants was found in this study. Finally, another study reported that 8 of 20 PCa patients with the QQ RNase L genotype and 3 of 20 with the RQ or RR genotypes were positive in a serum neutralization assay and that 5 of 7 tested tumors were positive by FISH in a subset of stromal cells; FISH and serum positivity correlated with nested PCR results (5). One strong indication that XMRV has infected some human cells in some prostate tumors is the finding of XMRV sequences integrated into human DNA (6, 7).

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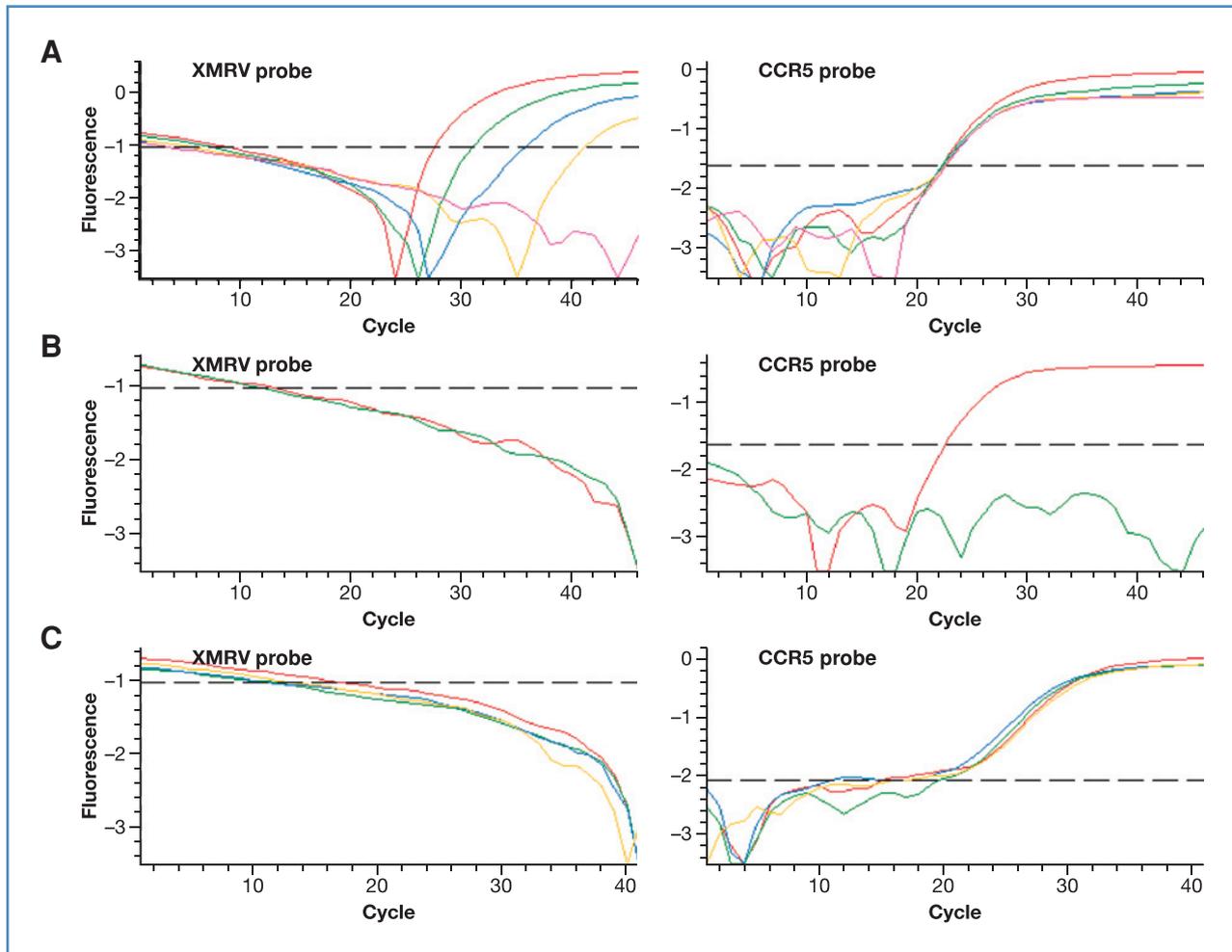


Figure 1. Duplex real-time PCR. A, XMRV-positive control: wells contain 1 ng (red), 0.1 ng (green), 0.01 ng (blue), or 0.001 ng (orange/pink) of 22Rv1 gDNA in 100 ng of HeLa gDNA. B, XMRV and CCR5 negative controls: wells contain 100 ng of HeLa gDNA (red) or 100 ng of *E. coli* gDNA (green). C, typical sample data: wells contain 100 ng of DNA from 4 different prostate tumors.

In contrast, several studies have reported the absence, or extremely low prevalence, of XMRV in PCa. These include a study of 338 samples representing tumor, normal, and benign prostatic hyperplasia tissues from 200 PCa patients by using a highly sensitive nested PCR assay; 105 prostate tumors using nested RT-PCR; 589 prostate tumors by using nested PCR and nested RT-PCR as well as 146 serum samples by using an ELISA assay; and 130 prostate tumors and control tissue samples by using RT-PCR (8–11).

At this point, it would be hard to overstate the discrepancies among different laboratories on the basic question of whether XMRV is actually present in the human population, and there is no understanding of its possible role in disease. In an effort to resolve the discrepancies concerning the prevalence of XMRV in PCa, we have tested for the virus using both real-time PCR and IHC, with 2 antisera specific for different viral proteins; our results are described in the following text. Methods are detailed in Supporting Information.

Results

We developed a real-time PCR assay for detection of XMRV sequences in PCa tissues. The quality and concentration of the sample DNAs were confirmed by a duplex PCR procedure, in which the same PCR wells were simultaneously tested for XMRV and for CCR5, a single-copy nuclear gene. For a positive control, we tested the genomic DNA (gDNA) of 22Rv1 cells, an XMRV-infected PCa cell line (12). 22Rv1 gDNA was diluted into 293T or HeLa cell gDNA (typical results shown in Fig. 1). We could routinely detect XMRV sequences in 10 pg of 22Rv1 gDNA (Fig. 1A, blue line), even in the presence of 100 ng or more of background human gDNA. Tests of 1 pg (orange and pink lines) were occasionally positive, but viral sequences were never detected in 0.1 pg of 22Rv1 gDNA (data not shown). Tests with the XMRV plasmid VP62 as standard indicate that there are ~15 copies of XMRV per diploid genome in 22Rv1 gDNA, a number similar to that reported by Knouf et al. (12);

Table 1. Specification of prostate tumor samples tested. The Table shows the number of cases tested by either PCR or IHC

| PCR | | |
|--|-----------|-----------|
| Microdissected prostate tumor | 12 | |
| Prostate tumor metastasis | 10 | |
| Prostate tumor | 139 | |
| IHC | | |
| | MLV30 | MLV70 |
| TMA prostate tumor ^a | 433/1,524 | 433/1,524 |
| TMA prostate benign ^a | 437/1,890 | 437/1,890 |
| TMA prostate tumor metastasis ^a | 52/121 | 52/121 |
| Full sections prostate tumor | 38 | 111 |
| Full sections prostate benign | 5 | 15 |

NOTE: Table shows the number of cases tested by either PCR or IHC.

^aNumbers shown are total number of cases/total number of TMA spots analyzed. Multiple TMA spots (typically at least 4) were analyzed per case.

data not shown). Thus, our assay can always detect ~20 copies of XMRV DNA and can occasionally detect ~2 copies. The CCR5 assays in the same PCR wells were uniformly positive (Fig. 1A). All assays included parallel tests of 293T or HeLa gDNA alone (XMRV negative control, Fig. 1B, red line) and *Escherichia coli* gDNA (CCR5 negative control; Fig. 1B, green line). Using this duplex assay, we screened DNA from 161 prostatic adenocarcinomas including 12 that had been microdissected and 10 that were metastases (Table 1). In all cases, CCR5 was successfully amplified, confirming the quality of the DNA preparation, but there was no amplification from the XMRV primers in any of the cases (Fig. 1C).

Similar real-time PCR assays were performed on 54 of these samples by using a primer-probe set directed at a highly conserved region within the Gag gene. In addition, nested RT-PCR was performed on 41 additional cases by using the primer set described in Urisman et al. (1) and nested PCR was used on 24 cases by using the primers of Hohn et al. (9). In all of these tests, XMRV sequences could be efficiently detected in the RNA or DNA from infected cells, but no positive results were obtained with any of the PCa samples (data not shown).

The MLV proteins p30^{CA} and gp70^{SU} are cleavage products of the viral Gag and Env polyproteins, respectively. We tested the ability of the anti-p30 ("MLV30") and anti-gp70 ("MLV70") antisera to react with XMRV proteins. As shown in Supplementary Figure S1, both antisera reacted with the expected viral proteins in virus particles (the MLV30 blot shows that some uncleaved Gag polyprotein, Pr65^{Gag}, as well as the normal cleavage product p30^{CA}, is present in the virus particles). Thus, these antisera cross-react with the corresponding polypeptides from XMRV. Western blots with MLV30 and MLV70 using lysates from 22Rv1 cells were similarly positive (data not shown), but not using lysates of the human PCa cell lines DU145 and PC3, which were negative for XMRV by PCR (data not shown).

By IHC, both sera showed clear and reproducible staining of 293T cells transfected with the VP62 clone of XMRV (Fig. 2B), but never stained 293T cells that had been transfected with an

empty plasmid (Fig. 2A). In addition, both antisera stained 22Rv1 cells (Fig. 2C), indicating that the staining did not require the overexpression typically associated with transient transfection. A total of 596 prostatic adenocarcinomas and 452 benign prostate tissue specimens, prepared either as full tissue sections or as tissue microarrays (TMA), were analyzed with MLV30 and/or MLV70 (Table 1). Many of the prostatic tissues evaluated from these cancer patients included areas of acute and chronic inflammation, atrophy, benign prostatic hyperplasia, and high-grade prostatic intraepithelial neoplasia (Table 1). Each experiment included positive and negative controls, which always gave results as in Figure 2A and B. However, no staining of prostate tissue samples was ever observed with either antiserum (Fig. 2D).

Discussion

We used a real-time PCR assay capable of detecting XMRV sequences in DNA from a very small number of infected cells, even in the presence of a vast excess (>10,000-fold) of uninfected cell DNA. We also performed IHC with 2 antisera, each specific for a different MLV protein, under conditions in which the sera reproducibly stained XMRV-containing cells but not identically treated control cells. Taken together, the 2 assays surveyed nearly 800 prostate tumors, including microdissected tumor specimens; metastatic tumor tissue; and intermediate- and high-grade primary tumors. No signs of XMRV infection were found in any of these tests. The results suggest that the prevalence of XMRV in prostate tumors may be far lower than has been reported previously.

How can our negative results be reconciled with the positive reports from other laboratories? It has been suggested that XMRV might be present in North American, but not European, prostate tumors (9). However, our samples, like those of Schlaberg et al. (4), were from North American men. Also, although we did not select RNase L R462Q homozygotes for analysis, the number of cases we examined was high enough to include a substantial number of these individuals. Another

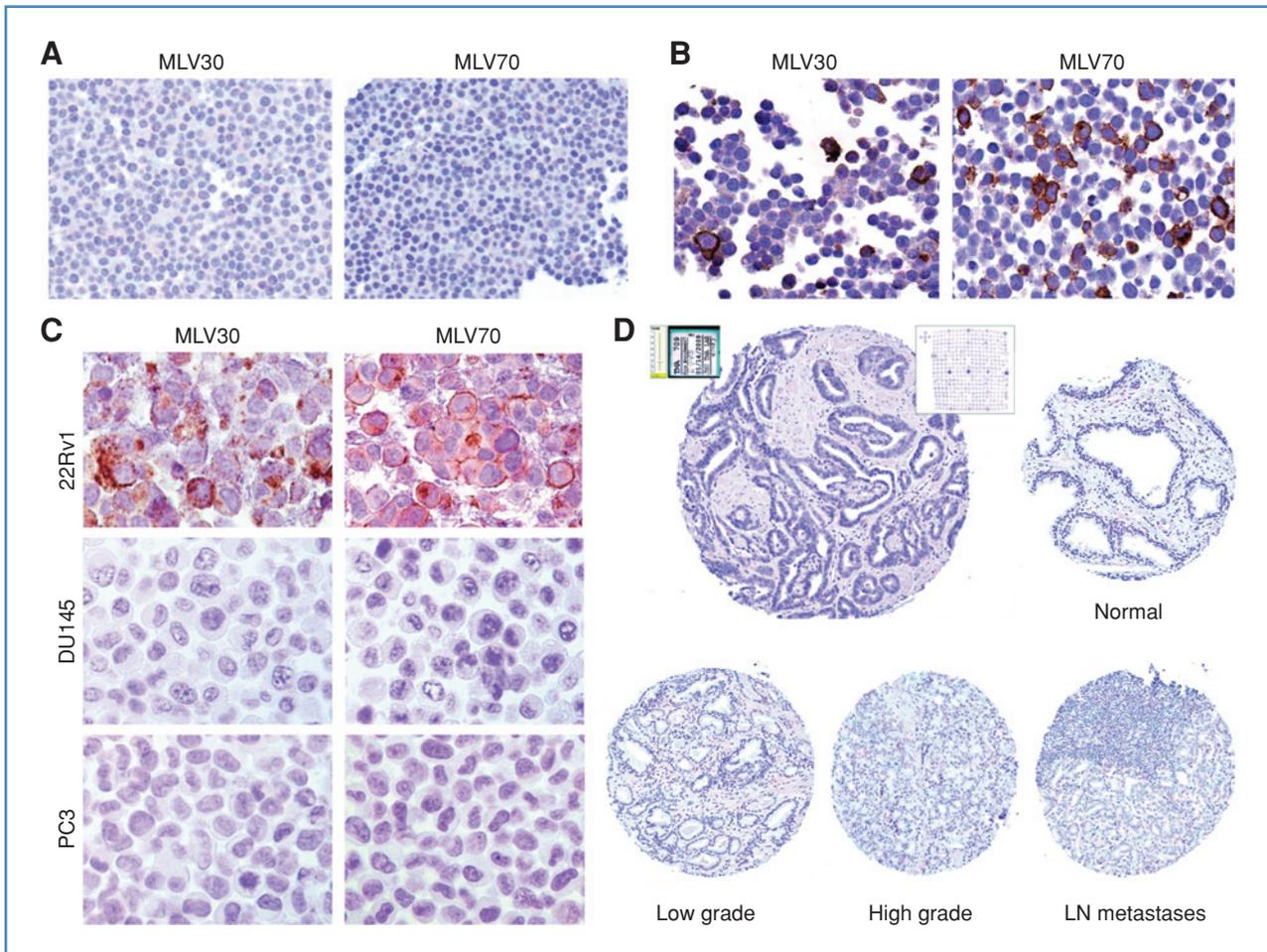


Figure 2. IHC with MLV30 and MLV70 antisera. A, antisera do not stain 293T cells transfected with pcDNA3.1. B, antisera stain 293T cells transfected with VP62 XMRV. C, antisera stain 22Rv1 cells, but not DU145 and PC3 cells. D, typical sample data. Top left, example of TMA and typical TMA spot. No antisera staining was observed for normal prostate, low-grade PCa, high-grade PCa, or lymph node (LN) metastases. Examples shown were stained with MLV30 antisera.

possibility is that XMRV was present in our samples, but we failed to detect it because the viral sequences were somewhat different from the published XMRV sequences. Although little variation in XMRV sequences has been observed to date (the reported sequences are ~97% identical), this could potentially explain our negative PCR results. However, we used several primer sets, some against highly conserved MLV sequences, and still saw no MLV signals. Furthermore, unlike PCR primers, the sera we used in our IHC assays are both broadly reactive, as they were generated using Mo-MLV proteins but reacted with the XMRV proteins in our positive controls (Mo-MLV and XMRV are 82% identical at the amino acid level). Thus, it seems extremely improbable that sequence polymorphisms can explain our failure to detect XMRV by IHC.

It could also be proposed that infected cells are present at such a low level in virus-positive tumors that the samples we tested were too small to contain infected cells. [Contrary to this, Schlager et al. (4) initially reported that positive samples contained 1–10 XMRV copies per 660 cells; 660 diploid cells

contain ~5 ng of DNA, whereas we tested amounts ranging from 25 to 1,000 ng]. This might explain the negative IHC results with TMAs but seems unlikely in the more than 100 tumors for which we analyzed standard slides, which generally contain more than 10^5 cells.

Finally, another conceivable explanation for the staining seen by Schlager et al. (4) is that the anti-XMRV serum used in their experiments contains antibodies directed against cellular proteins, in addition to the antibodies against XMRV proteins. The XMRV used as immunogen by Schlager et al. was apparently produced in human cells. It is thus difficult to exclude the possibility that human proteins were present in the virus preparation used as immunogen. HIV-1 virus particles are known to incorporate a wide variety of proteins from the virus producing cells (13) and so these proteins are impossible to remove from the virus; indeed, early vaccine trials with simian immunodeficiency virus were confounded by this phenomenon (14, 15). Incorporation of MHC proteins into MLV particles has also been reported (16). We received

PCa tissue sections (kindly provided by Dr. Ila Singh, University of Utah) from a number of cases from specimens used by Schlaberg et al. (4). On the basis of their results with the anti-XMRV antiserum, these samples were predicted to be IHC-positive. However, the sections did not stain with our MLV30 or MLV70 antisera (data not shown). Although we cannot fully explain the discrepancies in staining results, Switzer et al. have also showed that under immunoblotting conditions, the anti-XMRV antiserum (4) reacts with proteins in uninfected HeLa cells (17).

Many laboratories have used PCR to detect XMRV in clinical samples. However, the extraordinary sensitivity of this technique magnifies the risk of finding false-positive results, as well as the ability to find authentic positives. The risk is compounded by the widespread use of mice in biomedical research. Every mouse cell contains, in its DNA, about 100 MLV genomes, termed "endogenous viruses". These genomes reflect past infections of germ cells and the resulting integration of the viral sequences into the mouse germline. As PCR is capable of amplifying and detecting a single molecule of viral DNA, this means that, for example (depending, of course, on the specificity of the primers), a millionth of a microliter of mouse blood is a potential source of a positive signal in a PCR assay for MLV. Indeed, there are anecdotal reports of false-positive MLV signals ultimately traced to the use of the same microtome blade for cutting mouse and PCa sections and to the tiny amounts of mouse DNA contaminating the mouse antipolymerase monoclonal antibody used in commercial "hot start" PCR kits.

The existence of endogenous MLVs may be pertinent to another recent set of observations. In an attempt to reproduce the detection of XMRV in cases of CFS, Lo et al. (18) conducted PCR and RT-PCR on blood samples from CFS patients and healthy blood donors. They obtained positive signals from a high proportion of the CFS cases (and a much lower proportion of the healthy donors). However, when the PCR products were sequenced, they were found to differ from XMRV; thus, these results are completely distinct from the reports of XMRV detection. In fact, the sequences match endogenous MLV sequences almost exactly. It should be emphasized that (unlike the studies reporting isolation of XMRV) this report does not include direct evidence for the presence of an infectious virus; the data consisted exclusively of amplification and detection of MLV-like sequences. Notably, the endogenous MLVs that they resemble most closely are defective MLV genomes that do not give rise to infectious MLV. Although the authors provided strong experimental evidence arguing against contamination of their clinical samples with mouse DNA, this remains a possible explanation for their results.

In conclusion, the fundamental question of whether XMRV is really an infectious agent circulating in the human popula-

tion is still unresolved. This question will not be settled until reproducible assays for the virus are established and validated; in turn this will require exchange of samples and testing of well-characterized standards, followed by cross-comparison of results obtained in different laboratories. Efforts in this direction are now underway at the U.S. National Institutes of Health. However, on the basis of the data presented here, as well as those from other investigators (8–11), we are doubtful that XMRV is commonly found in PCa. Over the years, many claims associating viruses with diseases have turned out to be mistaken (19, 20), and it is still possible that XMRV will fall into this category.

Finally, it is crucial to distinguish the question of the existence and prevalence of XMRV in the human population from the question of its causal role in PCa. In general, gammaretroviruses such as XMRV induce malignant transformation by insertional mutagenesis, so that tumors induced by a gammaretrovirus are clones in which all the cells are infected (21). This mechanism of carcinogenesis has been observed not only in laboratory animals, but also in children exposed to gammaretrovirus-derived vectors in gene therapy trials (22, 23). Although some exceptions to this insertional mutagenesis mechanism have been described (24), the viral genome is present in the transformed cells in all known cases. Thus, infection of an extremely minute fraction of the cells in some prostate tumors, even if confirmed, would seem to be incompatible with the possibility that XMRV plays a causal role in prostate tumorigenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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