Published OnlineFirst on October 21, 2010 as 10.1158/0008-5472.CAN-10-2837

文献１４

XMRV: A New Virus in Prostate Cancer?

Amanda L. Aloia[1,7], Karen S. Sfanos[2,7], William B. Isaacs[3-6], Qizhi Zheng[2,6], Frank Maldarelli[1], Angelo M. De Marzo[2-6]*, and Alan Rein[1]*

HIV Drug Resistance Program[1], National Cancer Institute, Frederick, MD 21702; Department of Pathology[2], Urology[3], and Oncology[4], The Brady Urological Research Institute[5] and the Sidney Kimmel Comprehensive Cancer Center[6] at Johns Hopkins, Baltimore, MD 21231.

Running Title: Lack of detectable XMRV in prostate tissues

Key words: XMRV, prostate, tumors, PCR, immunohistochemistry.

Grant Support: Immunohistochemistry studies and a number of the frozen prostate tissues for PCR assays were supported by NCI-SPORE in prostate cancer P50CA58236. K.S.S is supported by a postdoctoral fellowship award from the Prevent Cancer Foundation. This research was supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

*Address correspondence to: Alan Rein, reina@mail.nih.gov, or Angelo M. De Marzo, ademarz@jhmi.edu.

[7]These authors contributed equally to the work.

Abstract

Several recent papers 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 papers 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, in order to confirm the quality of the sample DNA and its suitability for PCR. Controls demonstrated 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 two rabbit polyclonal antisera, each prepared against a purified 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.

Introduction

In 2006, a new retrovirus was reported to be associated with prostate cancer (PCa) (1). It was recognized as a murine leukemia virus (a member of the gammaretrovirus genus), and was termed “XMRV”, or “xenotropic murine leukemia virus-related virus”. (Murine leukemia viruses [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) (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 transcription”) 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 will thus be 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 transcription-PCR (RT-PCR); fluorescence in situ hybridization (FISH); immunohistochemistry (IHC); screens for anti-viral 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 ½ day meeting held on the NIH campus in September, 2010.

XMRV was initially reported to be more prevalent in prostate cancer 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 prostate cancer patients with the QQ RNASEL 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).

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 BPH tissues from 200 prostate cancer patients using a highly sensitive nested PCR assay; 105 prostate tumors using nested RT-PCR; 589 prostate tumors using nested

PCR and nested RT-PCR as well as 146 serum samples using an ELISA assay; and 130 prostate tumors and control tissue samples using RT-PCR(8-11).

At this point, it would be hard to overstate the discrepancies between 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 two antisera specific for different viral proteins; our results are described below. 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) (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 *E. 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 micro-dissected 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 also performed on 54 of these samples 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 using the primer set described in Urisman et al. (1) and nested PCR was used on 24 cases 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 MLV30 and MLV70 antisera to react with XMRV proteins. As shown in Fig. S1 in Supplementary Information, 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). Additionally, 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 (TMAs), 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 Figs. 2A and 2B. 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 (more than 10,000-fold) of uninfected cell DNA. We also performed IHC with two antisera, each specific for a different MLV protein, under conditions where the sera reproducibly stained XMRV-containing cells but not identically treated control cells. Taken together, the two 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, while 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 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. While 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. Further, unlike PCR primers, the sera we used in our IHC assays are both broadly reactive, since 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, Schlaberg et al. initially reported that positive samples contained 1-10 XMRV copies per 660 cells; 660 diploid cells contain ~ 5 ng of DNA, while we tested

amounts ranging from 25 to 1000 ng (4)). This might explain the negative IHC results with tissue microarrays, but seems unlikely in the >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 Schlaberg 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 Schlaberg 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), so that 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 major histocompatibility complex 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). Based on 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). While we cannot fully explain the discrepancies in staining results, Switzer et al. have also demonstrated 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 positives, 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, ~ 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 anti-polymerase 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) performed PCR and reverse transcription-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 which do not give rise to infectious MLV. While 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 population 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, based on the data presented here, as well as that 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 like 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.

Acknowledgements

We thank Helen Fedor at Johns Hopkins for help with prostate tissues and slides for IHC, Ila Singh for FFPE sections of PCa tissue, and W. Marston Linehan, Kenneth J. Pienta, Peter Pinto, Frank Ruscetti, and Cathy Vocke for prostate tumor DNAs. We are also grateful to Jessica L. Hicks, Jane Mirro, and Demetria Harvin for superb technical assistance and to John M. Coffin, Robert J. Gorelick, Stephen H. Hughes, Michael Piatak, and Saraswati Sukumar for many helpful discussions.

References

1. Urisman A, Molinaro RJ, Fischer N, et al. Identification of a novel Gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant. PLoS Pathog 2006; 2: e25.

2. Lombardi VC, Ruscetti FW, Das Gupta J, et al. Detection of an Infectious Retrovirus, XMRV, in Blood Cells of Patients with Chronic Fatigue Syndrome. Science 2009; 326: 585-9.

3. Silverman RH, Nguyen C, Weight CJ, Klein EA. The human retrovirus XMRV in prostate cancer and chronic fatigue syndrome. Nat Rev Urol; 7: 392-402.

4. Schlaberg R, Choe DJ, Brown KR, Thaker HM, Singh IR. XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors. Proc Natl Acad Sci U S A 2009; 106: 16351-6.

5. Arnold RS, Makarova NV, Osunkoya AO, et al. XMRV infection in patients with prostate cancer: novel serologic assay and correlation with PCR and FISH. Urology 2010; 75: 755-61.

6. Dong B, Kim S, Hong S, et al. An infectious retrovirus susceptible to an IFN antiviral pathway from human prostate tumors. Proc Natl Acad Sci U S A 2007; 104: 1655-60.

7. Kim S, Kim N, Dong B, et al. Integration site preference of xenotropic murine leukemia virus-related virus, a new human retrovirus associated with prostate cancer. J Virol 2008; 82: 9964-77.

8. Fischer N, Hellwinkel O, Schulz C, et al. Prevalence of human gammaretrovirus XMRV in sporadic prostate cancer. J Clin Virol 2008; 43: 277-83.

9. Hohn O, Krause H, Barbarotto P, et al. Lack of evidence for xenotropic murine leukemia virus-related virus (XMRV) in German prostate cancer patients. Retrovirology 2009; 6: 92.

10. Martinez-Fierro ML, Leach RJ, Gomez-Guerra L, et al. Identification of viral infections in the prostate and evaluation of their association with cancer. BMC Cancer 2010; 10: 326.

11. Sfanos KS, Sauvageot J, Fedor HL, Dick JD, De Marzo AM, Isaacs WB. A molecular analysis of prokaryotic and viral DNA sequences in prostate tissue from patients with prostate cancer indicates the presence of multiple and diverse microorganisms. Prostate 2008; 68: 306-20.

12. Knouf EC, Metzger MJ, Mitchell PS, et al. Multiple Integrated Copies and High-Level Production of the Human Retrovirus XMRV from 22Rv1 Prostate Carcinoma Cells. J Virol 2009; 83: 7353-6.

13. Ott DE. Cellular proteins detected in HIV-1. Rev Med Virol 2008; 18: 159-75.

14. Arthur LO, Bess JW, Jr., Urban RG, et al. Macaques immunized with HLA-DR are protected from challenge with simian immunodeficiency virus. J Virol 1995; 69: 3117-24.

15. Cranage MP, Polyanskaya N, McBride B, et al. Studies on the specificity of the vaccine effect elicited by inactivated simian immunodeficiency virus. AIDS Res Hum Retroviruses 1993; 9: 13-22.

16. Bubbers JE, Lilly F. Selective incorporation of H-2 antigenic determinants into Friend virus particles. Nature 1977; 266: 458-9.

17. Switzer WM, Jia H, Hohn O, et al. Absence of evidence of xenotropic murine leukemia virus-related virus infection in persons with chronic fatigue syndrome and healthy controls in the United States. Retrovirology 2010; 7: 57-70.

18. Lo S-C, Pripuzova N, Li B, et al. Detection of MLV-related virus gene sequences in blood of patients with chronic fatigue syndrome and healthy blood donors. Proceedings of the National Academy of Sciences 2010; 107: 15874-9.

19. Voisset C, Weiss RA, Griffiths DJ. Human RNA "rumor" viruses: the search for novel human retroviruses in chronic disease. Microbiol Mol Biol Rev 2008; 72: 157-96.

20. Weiss R. A cautionary tale of virus and disease. BMC Biology; 8: 124.

21. Rosenberg N, Jolicoeur P. Retroviral Pathogenesis. In: Coffin JM, Hughes SH, Varmus HE, editors. Retroviruses. Plainview, N.Y.: Cold Spring Harbor Laboratory Press; 1997. p. 475-585.

22. Hacein-Bey-Abina S, von Kalle C, Schmidt M, et al. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Engl J Med 2003; 348: 255-6.

23. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science 2003; 302: 415-9.

24. Ruscetti SK. Deregulation of erythropoiesis by the Friend spleen focus-forming virus. Int J Biochem Cell Biol 1999; 31: 1089-109.

Table 1. Tabulation of Tissues Assayed

PCR		
Microdissected prostate tumor	12	
Prostate tumor metastasis	10	
Prostate tumor	139	
IHC	**MLV30**	**MLV70**
TMA prostate tumor*	433/1524	433/1524
TMA prostate benign*	437/1890	437/1890
TMA prostate tumor metastasis*	52/121	52/121
Full sections prostate tumor	38	111
Full sections prostate benign	5	15

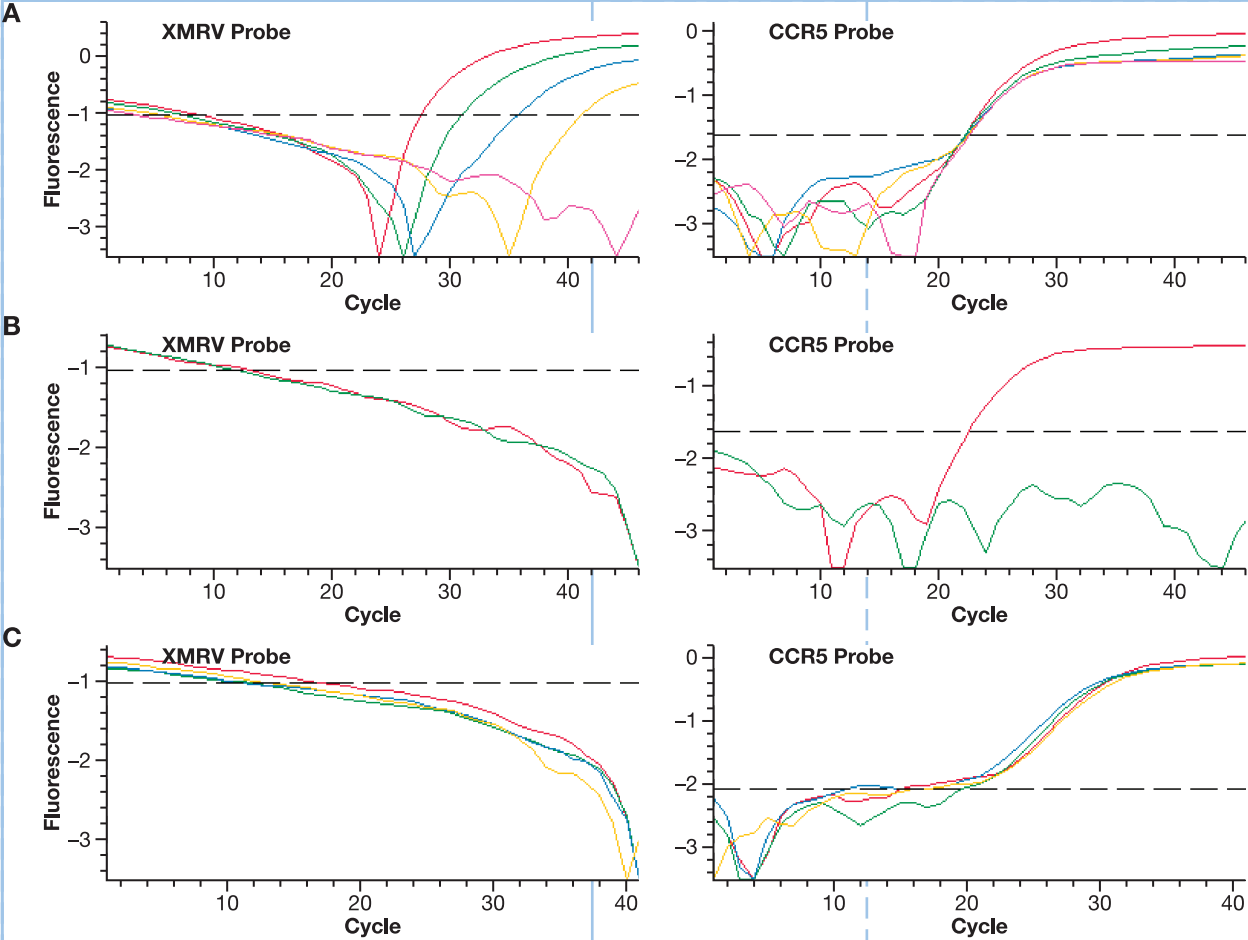
* Numbers shown are total number of cases / total number of TMA spots analyzed. Multiple TMA spots (typically at least 4) were analyzed per case.

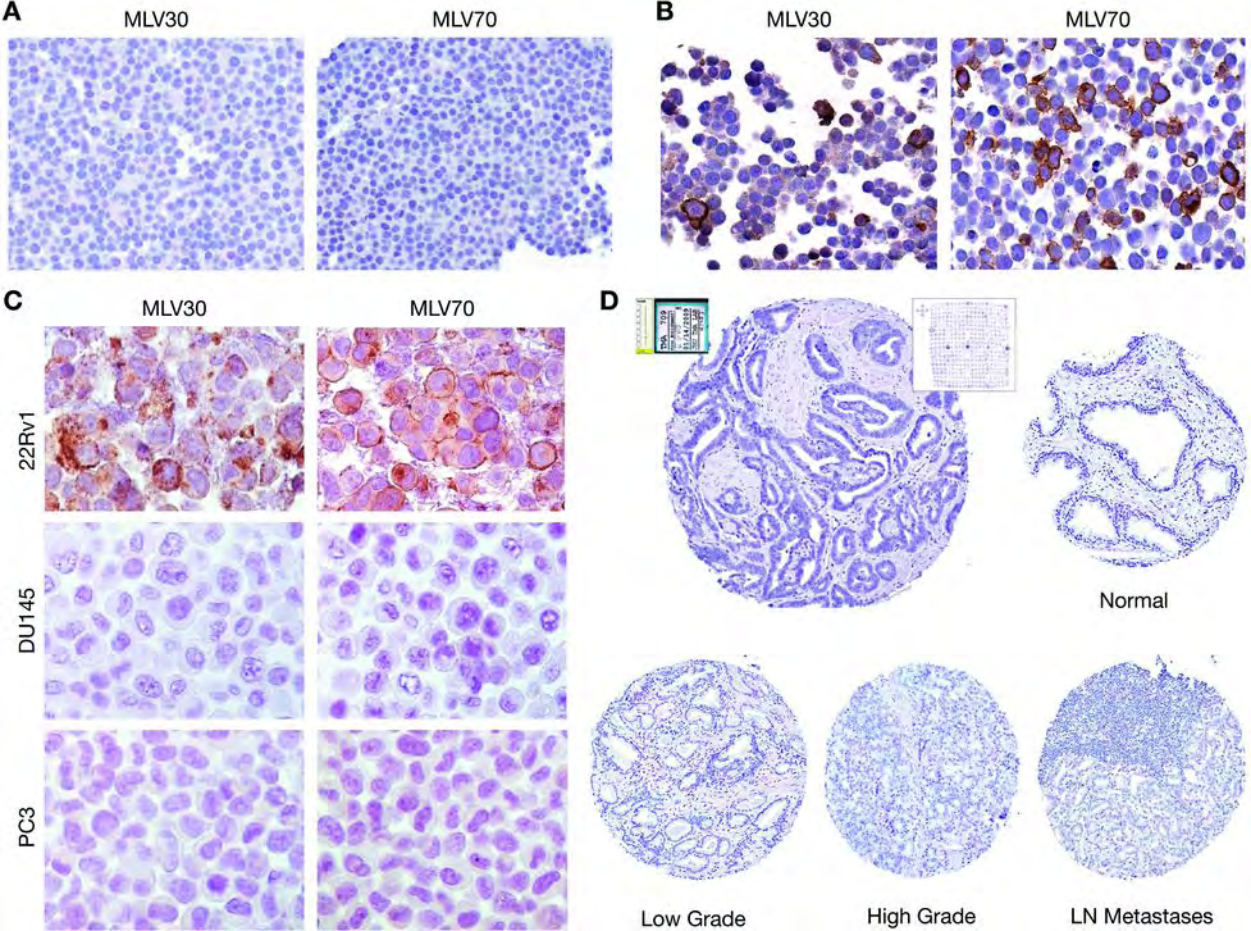
Table 1. Specification of prostate tumor samples tested. The Table shows the number of cases tested by either PCR or IHC.

Figure Legends

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

Figure 2. Immunohistochemistry 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 do not stain 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 metastases. Examples shown were stained with MLV30 antisera.


A
XMRV Probe
CCR5 Probe
Fluorescence
Cycle
B
XMRV Probe
CCR5 Probe
Fluorescence
Cycle
C
XMRV Probe
CCR5 Probe
Fluorescence
Cycle


A
MLV30
MLV70
B
MLV30
MLV70
C
MLV30
MLV70
22Rv1
DU145
PC3
D
Normal
Low Grade
High Grade
LN Metastases