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%

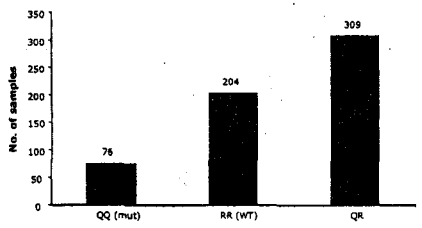

Figure 1
Analysis of sample DNA with allele specific real time PCR for the R462Q genotype. 76 of 589 samples (12.9%) are homozygous for the QQ allele, 204 samples (34.6%) are homozygous for the RR allele and 309 (52.5%) are heterozygous.

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

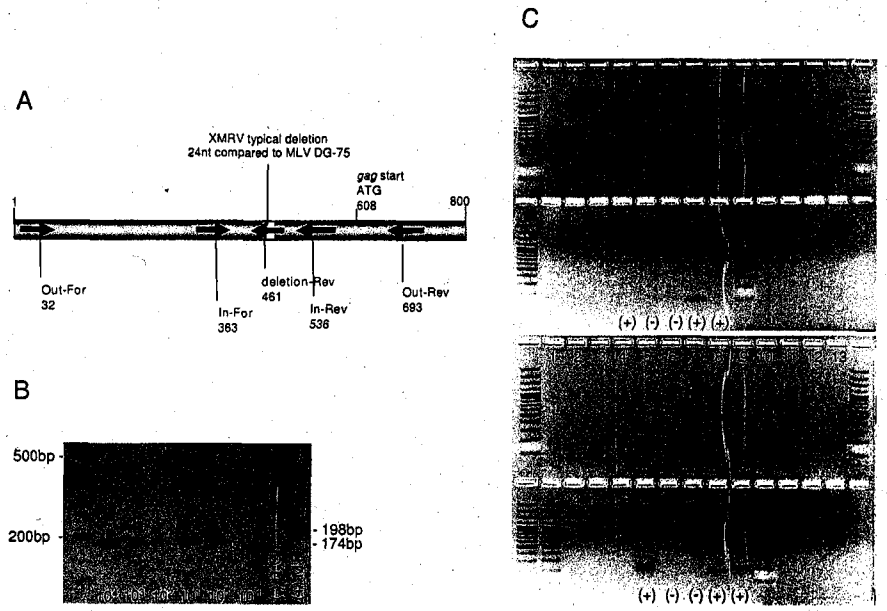

Figure 2
Nested PCR for sensitive screening of patient tumor tissue DNA. (A) A nested PCR primer setup was used as indicated for the screening of 589 PCa patient DNA isolated from prostate tumor and stroma tissue. Primer sites are numbered according to the XMRV VP62 sequence (Genbank EF185282). **(B)** 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-

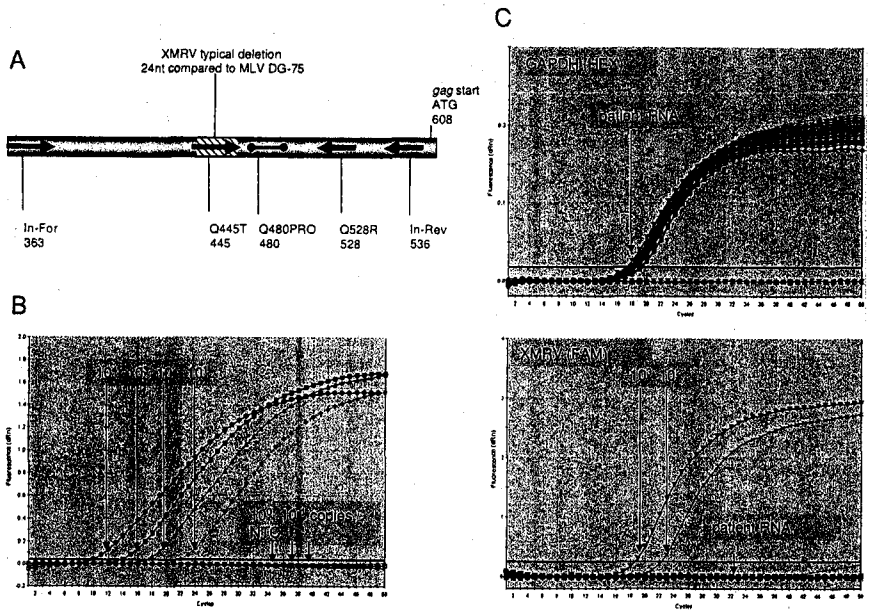

Figure 3
Nested RT-PCR for sensitive and specific screening of patient tumor tissue RNA. (A) RT-PCR for all 589 RNA samples was carried out with In-For and In-Rev primers, followed by a quantitative real-time PCR using primers and probe as indicated. Using the Q445T forward primer spanning the XMRV typical 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

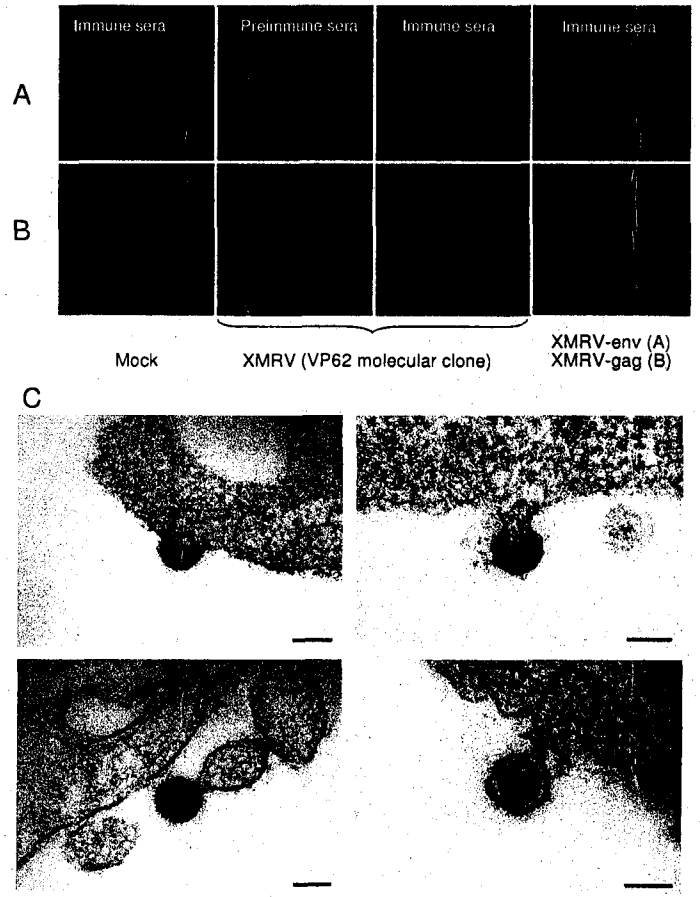

Figure 4
Immunoflourescence microscopy and electron microscopy of transfected 293T cells. Mice were immunized with recombinant gp70 or pr65 protein fragments, and sera were used for immunoflourescence microsocopy of 293T cells transfected two days earlier with the molecular XMRV clone VP62 or with gp70 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.

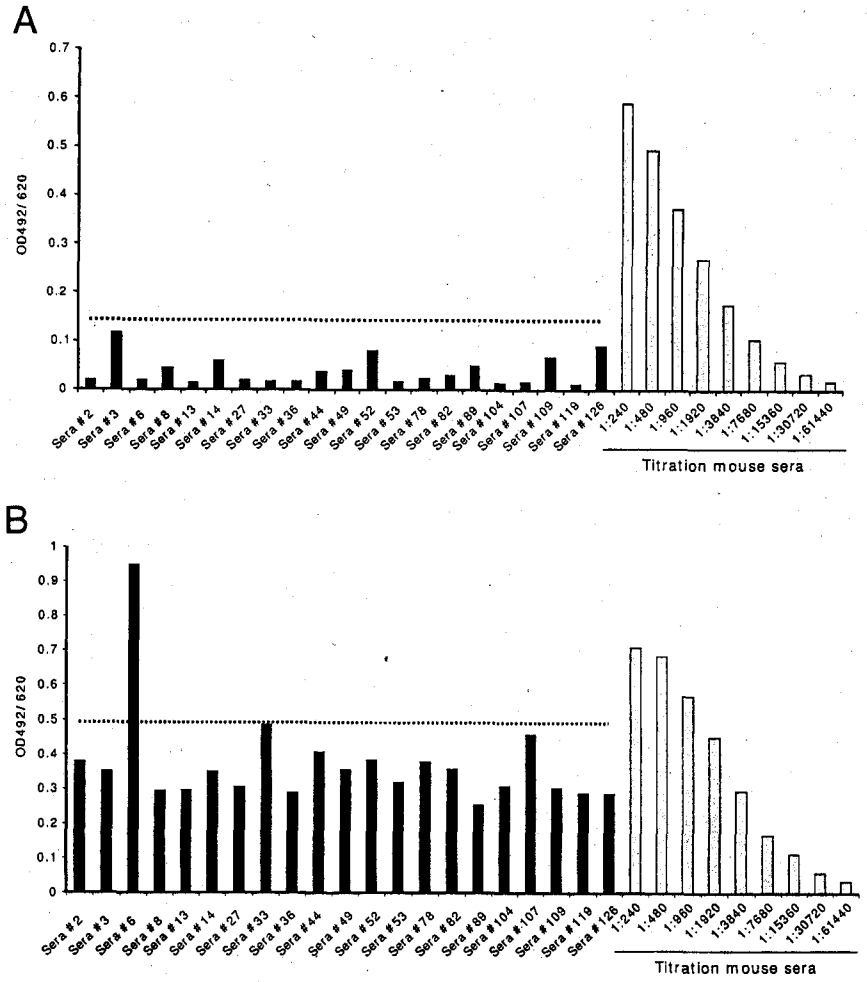

Figure 5
ELISA of PCa patient's sera using recombinant XMRV proteins. Mean ODs with two replicates of each patient sera diluted 1:200 (dark bars) and of serially diluted sera from immunized mice (light bars). Cut-off was calculated as the mean of four (gp70) and five (pr65) sera from healthy controls 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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