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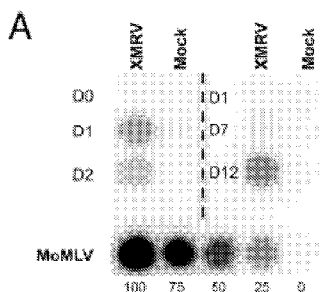


FIG. 1A

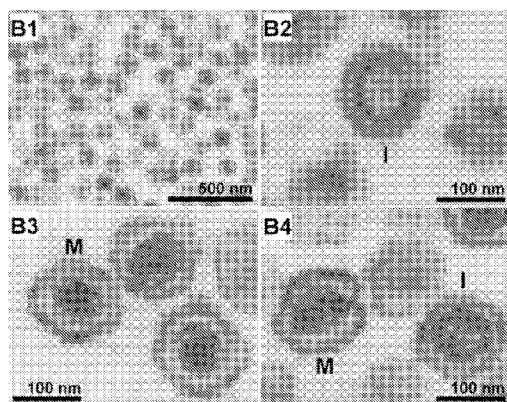


FIG. 1B

(57) Abstract: Disclosed herein are methods and compositions relating to the diagnosis of XMRV infection and XMRV-related diseases, including but not limited to malignancies such as prostate cancer, lymphomas, leukemias, myelodysplastic diseases, thymomas and also non-cancerous conditions such as chronic fatigue syndrome, and other neuroimmune disorders. The methods involve detection of XMRV RNA or DNA, or XMRV proteins, or antibodies to XMRV, and the use of these methods for diagnosis, for monitoring the progression of disease, and in monitoring treatment efficacy of various XMRV-associated diseases. Also disclosed herein are methods and compositions relating to the assessment of the cancer grade and long-term prognosis of a subject with cancer through the detection of anti-XMRV antibodies.



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COMPOSITIONS AND METHODS RELATING TO XMRV-RELATED DISEASES AND CONDITIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 61/216,317, filed May 15, 2009, U.S. Provisional Application No. 61/239,220, filed September 2, 2009, and U.S. Provisional Application No. 61/247,903, filed October 1, 2009, the entire contents of which are hereby incorporated by reference in their entireties.

BACKGROUND

[0002] Cancer is a class of diseases in which a group of cells display uncontrolled growth (division beyond the normal limits), invasion (intrusion on and destruction of adjacent tissues), and sometimes metastasis (spread to other locations in the body via lymph or blood). These three malignant properties of cancers differentiate them from benign tumors, which are self-limited, and do not invade or metastasize. Most cancers form a tumor but some, like leukemia, do not. Cancers are a leading cause of death in animals and humans. The exact cause of cancer is not known, but links between certain activities such as smoking or exposure to carcinogens and the incidence of certain types of cancers and tumors has been shown by a number of researchers. Cancers are caused by abnormalities in the genetic material of the transformed cells. These abnormalities may be due to the effects of carcinogens, such as tobacco smoke, radiation, chemicals, or infectious agents, such as viruses.

SUMMARY

[0003] The present inventors discovered that Xenotropic murine leukemia-related retrovirus (XMRV) has a strong link with human cancer, including prostate cancer and breast cancer. XMRV may also be associated with cervical cancer, hematologic malignancies, including lymphomas and leukemias, and non-cancerous conditions such as chronic fatigue syndrome and other neuroimmune diseases. This disclosure describes a series of methods to detect XMRV infection, and for use of that information in the diagnosis

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of, in monitoring the progression of, in making treatment decisions, and in monitoring treatment efficacy of various XMRV-associated diseases.

[0004] Disclosed herein are methods and compositions for the diagnosing, making prognoses, making treatment decisions, and monitoring the progression of as well as monitoring treatment efficacy of XMRV-associated diseases or conditions such as a cancer. Also disclosed herein are methods and compositions for the diagnosing, making prognoses and monitoring the progression of prostate cancer. Further disclosed are methods and compositions for the diagnosing, making prognoses, and monitoring disease progression as well as monitoring treatment efficacy of non-cancerous XMRV-associated diseases or conditions, such as chronic fatigue and other neuroimmune diseases. Also disclosed herein are methods and compositions for detecting the presence of XMRV in various samples (e.g. screening the blood supply for the presence of XMRV).

[0005] In one aspect, the disclosure provides a method of diagnosing a subject having, or at risk of contracting, a XMRV related disease comprising: screening a sample from the subject for the presence of XMRV; and determining the presence or absence of XMRV in the sample, wherein the presence of XMRV in the sample indicates that the subject has, or is at risk of contracting, an XMRV related disease selected from the group consisting of: prostate cancer, breast cancer, hematological malignancies, other malignancies in tissues that respond to steroid hormones, such as cervix, endometrium, adrenal glands etc. In one embodiment, the XMRV related disease is breast cancer. In another embodiment the XMRV related disease is prostate cancer. In another embodiment, the XMRV related disease is hematological malignancies. In another embodiment, the XMRV related disease is chronic fatigue syndrome and other neuroimmune illnesses.

[0006] In one embodiment, the screening comprises an immunoassay of a body fluid sample for anti-XMRV antibodies. In one embodiment, the screening comprises an immunoassay using an antibody which recognizes an XMRV polypeptide selected from the group consisting of: gag polyprotein (GAG); capsid protein (CA); matrix protein (MA); nucleocapsid protein (NC); envelope protein (ENV), integrase (IN); and combinations of any two or more thereof. In one embodiment, the immunoassay is selected from the group

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consisting of: ELISA, RIA, ELISPOT, western blot, immunofluorescence, and immunohistochemistry.

[0007] In one embodiment, the screening comprises detecting an XMRV nucleic acid sequence using a nucleic acid detection assay. In one embodiment, the nucleic acid detection assay comprises primers which hybridize to the XMRV long-terminal repeat (LTR) or the XMRV integrase (IN) coding region or the XMRV envelope (ENV) coding region. In one embodiment, the nucleic acid detection assay is selected from the group consisting of: PCR, reverse transcriptase PCR, real-time PCR, and quantitative PCR (qPCR).

[0008] In another aspect, the present disclosure provides a method of assessing the prognosis of a subject with prostate cancer comprising: screening a sample from the subject for the presence of XMRV, wherein the screening comprises conducting an immunoassay using an antibody which recognizes an XMRV polypeptide selected from the group consisting of: gag polyprotein (GAG); capsid protein (CA); matrix protein (MA); nucleocapsid protein (NC); envelope protein (ENV), integrase (IN); and combinations of any two or more thereof; and determining the presence or absence of XMRV in the sample, wherein the presence of XMRV is an indication of an aggressive form of the cancer and a poor prognosis for the patient.

[0009] In another aspect, the present disclosure provides a method of assessing the prognosis of a subject with prostate cancer comprising: screening a sample from the subject for the presence of XMRV, wherein the screening comprises of a quantitative PCR that detects XMRV sequences in the LTR, or in regions that code for IN or ENV proteins; and combinations of any two or more thereof; and determining the presence or absence of XMRV in the sample, wherein the presence of XMRV is an indication of an aggressive form of the cancer and a poor prognosis for the patient.

[0010] In another aspect, the present disclosure provides a method for the detection of XMRV in a sample comprising screening the sample for the presence of XMRV, wherein the screening comprises conducting an immunoassay using an antibody which recognizes an XMRV polypeptide selected from the group consisting of: gag polyprotein (GAG); capsid

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protein (CA); matrix protein (MA); nucleocapsid protein (NC); envelope protein (ENV); Integrase (IN); and combinations thereof; and determining the presence or absence of XMRV in the sample.

[0011] In another aspect, the present disclosure provides a method for the detection of XMRV comprising: screening a sample from the subject for the presence of XMRV, wherein the screening comprises of a quantitative PCR that detects XMRV sequences in the LTR, or in regions that code for IN or ENV proteins; and combinations of any two or more thereof; and determining the presence or absence of XMRV in the sample.

[0012] In some embodiments of any of the present methods, the sample is selected from the group consisting of: saliva, semen, peritoneal fluid, synovial fluid, prostatic secretions, cervical secretions, blood, serum, plasma, tissue, and cells. In one embodiment, the sample is tissue selected from the group consisting of prostate tissue, breast tissue lymph nodes, or bone marrow. In one embodiment, the sample is prostate tissue. In one embodiment, the XMRV virus is detected in the epithelial cells or stromal cells of the prostate tissue sample, for example, in malignant epithelial cells and/or benign stromal cells. In one embodiment, the XMRV virus is detected in malignant epithelial cells of the breast tissue sample. In one embodiment, the XMRV virus is detected in immune cells in lymph nodes, bone marrow and peripheral blood from hematological malignancies. In some embodiments, the sample includes Leydig cells from testes. In other embodiments, the sample is blood, plasma or serum from a subject meeting the diagnostic criteria of chronic fatigue syndrome.

[0013] Additional advantages of the disclosed composition(s) and method(s) will be set forth in part in the description which follows, and in part will be understood from the description, or may be learned by practice of the disclosed composition(s) and method(s). The advantages of the disclosed composition(s) and method(s) will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention as claimed.

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BRIEF DESCRIPTION OF THE FIGURES

[0014] Figure 1 shows XMRV molecular clone produces infectious viral particles with similar morphology and composition as Moloney murine leukemia virus (MoMLV). Figure 1A shows reverse transcriptase (RT) activity in tissue culture supernatant of 293T cells transfected with pXMRV1 quantified for 5 months. Increased RT activity was detected peaking one day after transfection (panel on left). No RT detectable RT activity was noted in 293T cells transfected with control plasmid (EGFP). Tissue culture supernatant from transfected 293T cells was used to inoculate naïve LNCap cells, a prostate cancer cell line. As a sign of infection, increased RT activity was detected peaking on day 12 after inoculation (panel on right). RT activity at peak was comparable to that detected in tissue culture supernatant from NIH3T3 cells infected with MoMLV. Inoculation of naïve 293T cells with medium from 293T cells transfected with control plasmid (EGFP) did not result in any detectable RT activity in culture supernatant. Figure 1B shows transmission electron microscopic analysis showed viral particles with a diameter of 100-120 nm and a morphology typical of type C retroviruses (panel B1). Maturation in form of core condensation was observed (panel B2). XMRV particles displayed a size and morphology that is similar to that of related MoMLV (panel B4). Figure 1C shows Western blot analysis of lysed XMRV and MoMLV virions using anti-XMRV whole virus, anti-MoMLV capsid, matrix, nucleocapsid, and envelope antibodies. Molecular weights of XMRV proteins are highly similar to those of MoMLV. The molecular weights for the major structural proteins of XMRV was determined to be 62 kDa for Gag, 15 kDa for MA, 30 kDa for CA, 10 kDa for NC, and 75 kDa for Env. An equivalent to the MoMLV p12 protein can be inferred from sequence comparison studies and bands that likely are intermediates of Gag proteolysis, e.g. p27 (MA-p12), p42 (p12-CA), p38 (CA-NC, panels C and D).

[0015] Figure 2 shows XMRV Prevalence study design. To estimate the prevalence of XMRV in prostate cancer and non-neoplastic prostatic tissues, samples from 233 consecutive cases of prostate cancer and 101 consecutive specimen of benign prostatic hyperplasia were tested. Frozen tissue samples were obtained from a Tissue Bank and formalin-fixed, paraffin-embedded samples were selected from a diagnostic tissue repository. Of the 233 cases of prostate cancer, frozen tissue samples were tested from 138

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cases, fixed tissue was used from 38 cases, and both frozen and fixed samples were tested from 57 cases. All TURP samples were formalin-fixed, paraffin-embedded. DNA was extracted from frozen and fixed prostatic tissue.

[0016] Figure 3 shows detection of XMRV DNA in human prostate tissue by qPCR. Figure 3A shows sequence alignment of a portion of the integrase gene targeted by the XMRV Real-Time PCR with sequences of highly similar murine retroviruses. Primers and probe binding sites were selected to be conserved between the three previously sequenced XMRV isolates and to maximize differences with related murine retroviral sequences. Blast (BLASTN) search of NCBI nucleotide collection using the 122 bp XMRV amplicon (SEQ ID NO:1) as query, all sequences with $\geq 85\%$ identity over the entire amplicon length are shown (11 unique ones, and 3 XMRV sequences); subsequently, a megaBLAST search of the NCBI nucleotide collection using each of the unique sequences as query sequence was performed to identify identical sequences from different sources. MTCR = murine type C retrovirus (X94150.1) and *Mus musculus* chromosome 1, clone RP24-65D16 (AC115959.17) (SEQ ID NO:2). mERV y,1 = *Mus musculus* BAC clone RP24-320A8 from chromosome y (AC182253.3) and *Mus musculus* strain C57BL/6J chromosome 1 clone rp23-116m12 (AC083892.19) (SEQ ID NO:3). mERV y,1' = *Mus musculus* BAC clone RP24-320A8 from chromosome y (AC182253.3) and *Mus musculus* strain C57BL/6J chromosome 1 clone rp23-116m12 (AC083892.19) (SEQ ID NO:4). MelARV = *Mus musculus* isolate MelARV endogenous B-tropic ecotropic murine leukemia virus (DQ366148.1), *Mus musculus* C-type ecotropic endogenous retrovirus (U63133.1) (SEQ ID NO:5). DG-75 = DG-75 Murine leukemia virus (AF221065.1), Murine AIDS virus-related provirus (S80082.1), *Mus musculus* chromosome 9, clone RP23-364M24 (AC103610.9), *Mus musculus* BAC clone RP23-277L21 from chromosome 2 (AC124194.3), Mouse DNA sequence from clone RP23-130L13 on chromosome 9 (CT009721.14), Mouse DNA sequence from clone RP23-259C9 on chromosome 13 (CT030655.7), Mouse DNA sequence from clone RP24-114E18 on chromosome 2 (AL928935.14), Mouse DNA sequence from clone RP23-354H24 on chromosome 4 (AL627314.6), and Mouse DNA sequence from clone RP23-384D6 on chromosome 4 (AL627077.14) (SEQ ID NO:6). Rmcf2 = *Mus musculus castaneus* endogenous virus Rmcf2 (AY999005.1) (SEQ ID NO:7).

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HEMV = Murine leukemia virus serotype HEMV provirus (AY818896.1) (SEQ ID NO:8). NZB-9-1 = Xenotropic murine leukemia virus isolate NZB-9-1 (EU035300.1) (SEQ ID NO:9). mERV 5 = *Mus musculus* chromosome 5, clone RP23-110C17 (AC117614.14) (SEQ ID NO:10). mERV y = *Mus musculus* BAC clone RP24-163J18 from chromosome y (AC175744.2) (SEQ ID NO:11). mERV y' = *Mus musculus* BAC clone CH36-265C6 from chromosome y (AC202413.4), *Mus musculus* BAC clone RP24-302I24 from chromosome Y (AC1 (1844409.3) (SEQ ID NO:12. * = Identical for Xenotropic MuLV-related virus VP35 (DQ241301.1), Xenotropic MuLV-related virus VP42 (DQ241302.1), Xenotropic MuLV-related virus VP62 (EF185282.1).

[0017] Figure 3B shows sensitivity of the real-time PCR assay using plasmid XMRV33 containing a full length XMRV clone. Serial 10-fold dilutions of plasmid pXMRV33 were prepared in human placental DNA (40 ng/ μ l) and used as template in the qPCR assay (200 ng). The qPCR assay was able to consistently detect 50 copies of pXMRV33 per reaction and detected 5 copies of pXMRV33 approximately 50% of the time. The qPCR assay was linear down to a concentration of five copies per reaction. Figure 3C shows sensitivity of the real-time PCR assay using formalin-fixed template DNA. Serial 10-fold dilutions (1:100 to 1:10⁶) of 293T cells chronically infected with XMRV in naïve 293T cells were prepared, formalin-fixed, pelleted, and embedded in paraffin similarly to clinical prostate cancer samples used in this study. Unspiked, naïve 293T cells were used as negative control. One section (10 μ m thick) was added to 9 sections (10 μ m thick) of normal prostate tissue. DNA extracted from a 1:10⁵ dilution resulted in consistent amplification, 1 of 2 duplicate reactions of a 1:10⁶ dilution showed a detectable amplification product. No amplification was seen with naïve 293T cells. Figure 3D shows threshold cycles for XMRV-positive prostate cancer samples (C_T = 36-39) were similar to those obtained with 50-500 copies of pXMRV33 or a 1:10⁵-1:10⁶-fold dilution of XMRV-infected 293T cells.

[0018] Figure 4 shows validation of the Immunohistochemistry Staining to Detect XMRV Protein in Prostate Cancer Tissue Sections. XMRV protein is detected in chronically infected, cultured 293T cells by IHC using anti-XMRV antiserum (panel A). In a mix of XMRV-infected and naïve 293T cells containing only 1% infected cells (panel B), only a small proportion of cells showed the same staining as seen in A. No staining was seen in

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naive 293T cells (panel C) or when using pre-immune control serum with 100% infected 293T cells (panel D). At the cellular level, the staining has a distinct granular appearance that is restricted to the cytoplasm (panel E). Using the same IHC assay with human prostate cancer tissues identified XMRV-infected clusters of neoplastic epithelial (panel G). At the cellular level, these cells showed a staining pattern that was highly similar with that seen in XMRV-infected 293T cells (panel F): darkly staining granules limited to the cytoplasm are clearly appreciated at higher magnification (panel F1, arrowhead). No staining was seen in the same acinus in an adjacent section when using pre-immune control serum from the same rabbit (panel H). N = nucleus, n = nucleolus

[0019] Figure 5 shows XMRV DNA and protein is more prevalent in prostate cancer than in TURP samples and correlates with prostate cancer grade. Figure 5A shows XMRV DNA and/or protein is detected in 26% of 234 prostate cancer specimens and in 6% of control tissues. Presence of detectable XMRV DNA and/or protein is correlated with the prostate cancer grade. Figure 5B shows among 233 unselected cases of prostate cancer, XMRV was detected in approximately 19% of patients with a Gleason score of 6, 27% of those with a Gleason score of 7, 29% of those with a Gleason score of 8, and 44% of those with a Gleason score of 9. Figures 5C and 5D show no association of XMRV with prostate cancer stage (C) or age of the patient (D) was noted.

[0020] Figure 6 shows XMRV protein is detected in neoplastic prostatic epithelial cells by immunohistochemistry. A section from a prostate cancer case shows immunohistochemical staining in multiple acini of neoplastic epithelial cells (brown staining, panel A). The inset (panel A1) shows a higher magnification of a several neoplastic epithelial cells of the large acinus in the center. A schematic representation of the structures contained in this field and the inset are depicted in panel B and B1. In the center of the image, a large, irregular acinus is seen. In the left, top left, and top parts of the image are smaller acini showing similar staining. Counterstaining with hematoxylin highlights nuclei. As a typical feature for prostate adenocarcinoma, many nuclei are large and contain one or more large nucleoli. At higher power, the typical granular cytoplasmic pattern of immunohistochemical staining can be seen (panels A1 and B1). The cytoplasm of contains multiple fine granules. No

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immunohistochemical staining is seen in several acini and the scant stroma present in this field (panels A and B).

[0021] Figure 7 shows XMRV protein is detected in varying proportions of neoplastic prostatic epithelial cells and, in a small number of cases, in rare, scattered stromal cells. Panels A and B represent different fields from the same case depicted in Figure 6 and show the range of staining within the same prostate gland. The field shown in Figure 6 shows strong staining of all epithelial cells of several acini of varying size. Panel A depicts a cluster of small acini and single epithelial cells staining in the XMRV IHC assay. In panel B, fewer cells with somewhat fainter staining are seen. Not all cells of one acinus stain positively in this field. At the cellular level, the staining in all fields is highly similar (Figure 6 A1, panels A1 and B1). Variations of these staining patterns were observed in most IHC-positive cases: Panels C through F show individual or clustered IHC-positive neoplastic epithelial cells in acini of varying size from 4 additional cases. In some cases, epithelial staining was limited to only part of the cytoplasm (Panels H1 and H2). At the cellular level, this staining shows the same granular appearance described earlier. The slide shown in panel H is from the same patient that one of the first XMRV isolates (VP62) was isolated from. IHC-positive cells in the prostatic stroma were seen in 3% of all cases. Scattered rare cells were identified in proximity to neoplastic epithelial cells (panel I) and inflammatory infiltrates (panel J). These cells were usually seen individually in small number (mostly less than 10 per section). Their morphology was consistent with tissue macrophages. The immunohistochemical staining is limited to the cytoplasm. Panels I2 through I4 and J2 through J4 show individual cells in the same section but in a field not contained in panels I and J, respectively.

[0022] Figure 8 shows the presence of XMRV DNA or protein does not correlate with the RNASEL R462Q genotype. The relative frequencies of RNase L R462Q genotypes were similar in individuals with prostate cancer and in controls. There was no significant difference in the RNASEL genotype distribution between men with prostate cancer that tested positive for XMRV by either PCR or IHC.

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[0023] Figure 9 shows identification of 2 patterns of unspecific staining with the immunohistochemistry assay for the detection of XMRV protein: Lipofuscin granules present in epithelial and stromal cells of many prostate glands unspecifically stained with immune (panels A and C) and pre-immune serum (panel B). Their identity was confirmed using a panel of special stains (Luxol fast blue, Congo red, PAS-D, and Giemsa). A second unspecific staining pattern observed was diffuse nuclear staining in many epithelial and stromal cells of a small subset of cases (panel D). Unspecific nuclear staining is a common artifact in immunohistochemical staining [Bussolati G., et al. 2008].

[0024] Figure 10 shows Western blot analysis of human sera that repeatedly tested positive for XMRV, and displayed antibody reactivity to at least two XMRV proteins. MWM = molecular weight markers, V- indicates lanes in which XMRV proteins were electrophoresed. U- indicates uninfected cells processed as for making virus (mock) Top left panel shows viral proteins probed with rabbit anti-XMRV antisera on right and bottom. Each pair of lanes contains XMRV virions run on left (V), and supernatant from non-infected cells treated the same as virions on right (U). Portion of molecular weight markers are seen in each set. Each pair was probed with different human sera (1-13). Viral proteins are shown with arrows (yellow= Env, red = CA and blue = MA proteins respectively). A band around 66kD that reacts positive with some sera even in the supernatant from uninfected cells is most likely bovine serum albumin (BSA, white arrow).

[0025] Figure 11 shows a section from a case of breast cancer processed by IHC using anti-XMRV antisera. Malignant cells are stained brown, indicating that XMRV proteins are expressed in those cells. Adjacent benign epithelial cells show no staining.

[0026] Figure 12 shows the presence of XMRV in the testes of subjects with prostate cancer. XMRV proteins are expressed exclusively in the Leydig cells (central panels). Adjacent sections are stained for calretinin, a marker for Leydig cells. The lower panel shows Leydig cells at higher magnification, illustrating the characteristic granular staining pattern seen in XMRV infected cells.

[0027] Figure 13 shows Western blot detecting presence of antibodies against XMRV polypeptides. A serum sample from a patient with chronic fatigue syndrome shows

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antibodies against SU, CA and p15E polypeptides (A). In (B) is a negative control serum, which does not show reactivity to any of the XMRV polypeptide.

DETAILED DESCRIPTION

[0028] The disclosed methods and compositions may be understood more readily by reference to the following detailed description of particular embodiments and the Example included therein and to the Figures and their previous and following description.

[0029] Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of skill in the art to which the disclosed methods and compositions belong. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present method and compositions, the particularly useful methods, devices, and materials are as described. Publications cited herein and the material for which they are cited are hereby specifically incorporated by reference. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such disclosure by virtue of prior invention. No admission is made that any reference constitutes prior art. The discussion of references states what their authors assert, and applicants reserve the right to challenge the accuracy and pertinency of the cited documents.

[0030] It must be noted that as used herein and in the appended claims, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “an anti-XMRV antibody antibody” includes a plurality of such antibodies, reference to “the anti-XMRV antibody antibody” is a reference to one or more anti-XMRV antibody antibodies and equivalents thereof known to those skilled in the art, and so forth.

[0031] “Optional” or “optionally” means that the subsequently described event, circumstance, or material may or may not occur or be present, and that the description includes instances where the event, circumstance, or material occurs or is present and instances where it does not occur or is not present.

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[0032] Throughout the description and claims of this specification, the word “comprise” and variations of the word, such as “comprising” and “comprises,” means “including but not limited to” and is not intended to exclude, for example, other additives, components, integers or steps.

[0033] As used herein the terms “diagnose” or “diagnosis” or “diagnosing” refer to distinguishing or identifying a disease, syndrome or condition or identifying a person having a particular disease, syndrome or condition. In illustrative embodiments of the invention, assays are used to diagnose disease, such as cancer, in a subject based on an analysis of a sample.

[0034] By “isolated”, when referring to a nucleic acid (*e.g.*, an oligonucleotide such as RNA, DNA, or a mixed polymer) is meant a nucleic acid that is apart from a substantial portion of the genome in which it naturally occurs and/or is substantially separated from other cellular components which naturally accompany such nucleic acid. For example, any nucleic acid that has been produced synthetically (*e.g.*, by serial base condensation) is considered to be isolated. Likewise, nucleic acids that are recombinantly expressed, cloned, produced by a primer extension reaction (*e.g.*, PCR), or otherwise excised from a genome are also considered to be isolated.

[0035] As used herein, the term “oligonucleotide” refers to a short polymer composed of deoxyribonucleotides, ribonucleotides or any combination thereof. Oligonucleotides are generally between about 10 and about 100 nucleotides in length. Oligonucleotides are typically 15 to 70 nucleotides long, with 20 to 26 nucleotides being the most common. An oligonucleotide may be used as a primer or as a probe. As used herein, a “primer” for amplification is an oligonucleotide that specifically anneals to a target or marker nucleotide sequence. The 3' nucleotide of the primer should be identical to the target or marker sequence at a corresponding nucleotide position for optimal primer extension by a polymerase. As used herein, a “forward primer” is a primer that anneals to the anti-sense strand of double stranded DNA (dsDNA). A “reverse primer” anneals to the sense-strand of dsDNA.

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[0036] An oligonucleotide is “specific” for a nucleic acid if the oligonucleotide has at least 50% sequence identity with a portion of the nucleic acid when the oligonucleotide and the nucleic acid are aligned. An oligonucleotide that is specific for a nucleic acid is one that, under the appropriate hybridization or washing conditions, is capable of hybridizing to the target of interest and not substantially hybridizing to nucleic acids which are not of interest. Higher levels of sequence identity are preferred and include at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity.

[0037] As used herein, the term “sample” or “test sample” refers to any liquid or solid material containing nucleic acids. In suitable embodiments, a test sample is obtained from a biological source (*i.e.*, a “biological sample”), such as cells in culture or a tissue sample from an animal, most preferably, a human. In an exemplary embodiment, the sample is a biopsy sample.

[0038] “Target nucleic acid” as used herein refers to segments of a chromosome, a complete gene with or without intergenic sequence, segments or portions a gene with or without intergenic sequence, or sequence of nucleic acids to which probes or primers are designed. Target nucleic acids may include wild type sequences, nucleic acid sequences containing mutations, deletions or duplications, tandem repeat regions, a gene of interest, a region of a gene of interest or any upstream or downstream region thereof. Target nucleic acids may represent alternative sequences or alleles of a particular gene. Target nucleic acids may be derived from genomic DNA, cDNA, or RNA. As used herein, target nucleic acid may be native DNA or a PCR-amplified product. In one embodiment, the target nucleic acid is a fragment of a XMRV genome.

[0039] As used herein, the term “patient” refers to a subject who receives medical care, attention or treatment. As used herein, the term is meant to encompass a person having or suspected of having a disease including a person who may be symptomatic for a disease but who has not yet been diagnosed.

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Methods

[0040] XMRV is a gamma retrovirus with high sequence similarity to murine leukemia viruses (MLV). Gamma retroviruses are well-characterized oncogenic animal viruses but have not been shown to cause cancers in humans. A role for XMRV in a cancer such as prostate cancer, breast cancer and hematologic malignancies, offers new markers for diagnosis and prognosis, new options for therapy and new options for preventing viral transmission between individuals.

[0041] The methods disclosed herein can be used for the identification and diagnosis of any XMRV associated disease or condition. Such diseases can include cancerous and non-cancerous diseases or conditions, such as chronic fatigue syndrome. Thus, disclosed herein, in one aspect, are methods of identifying a subject having or at risk of having cancer, or chronic fatigue syndrome. Also disclosed herein are methods of identifying a subject with an aggressive form of a cancer, such as prostate cancer. Further disclosed are methods of assessing the aggressiveness of a cancer in a subject. As is noted herein, a search for viral nucleic acids and viral proteins in prostate cancer tissues led to the identification of XMRV in approximately 27% of samples tested. Thus, disclosed herein are methods of identifying a subject having or at risk of having prostate cancer comprising screening a sample from the subject for the presence of XMRV, wherein detection of XMRV is an indication that the subject has or is likely to develop a prostate cancer. It is understood that the detection of XMRV can be accomplished through the detection of the presence of XMRV RNA, DNA or proteins. Thus, in one instance, disclosed herein are methods of identifying a subject having or at risk of having prostate cancer comprising screening a sample from the subject for the presence of XMRV, wherein detection of XMRV is accomplished via the detection of XMRV nucleic acid sequences or XMRV proteins such as, for example, Env, Gag, Pol, In, Matrix, Capsid, or nucleocapsid, and wherein detection of XMRV is an indication that the subject has or is likely to develop a prostate cancer. In some embodiments, the sample is prostate tissue.

[0042] The disclosed methods can be used in conjunction with the diagnosis, prognosis, assessing the aggressiveness of, or monitoring the progression of any disease where

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uncontrolled cellular proliferation occurs such as cancers. A representative but non-limiting list of cancers with which the disclosed methods can be used is the following: myelodysplasia, thymomas, lymphoma, B cell lymphoma, T cell lymphoma, mycosis fungoides, Hodgkin's Disease, myeloid leukemia, bladder cancer, brain cancer, nervous system cancer, head and neck cancer, squamous cell cancers, lung cancers such as small cell lung cancer and non-small cell lung cancer, neuroblastoma/glioblastoma, ovarian cancer, pancreatic cancer, prostate cancer, skin cancer, liver cancer, melanoma, squamous cell carcinomas of the mouth, throat, larynx, and lung, stomach cancer, intestinal cancer, colon cancer, liver cancer, cervical cancer, endometrial cancer, uterine cancer, breast cancer, renal cancer, genitourinary cancer, pulmonary cancer, esophageal cancer, large bowel cancer, hematopoietic cancers; testicular cancer; adrenal cancer, pituitary cancer, colon and rectal cancers, prostatic cancer, soft tissue cancers such as sarcomas, bone cancers, and mesotheliomas.

[0043] The methods may also be used in conjunction with the diagnosis, prognosis, assessing the aggressiveness of, or monitoring the progression of precancer conditions such as cervical and anal dysplasias, other dysplasias, severe dysplasias, hyperplasias, atypical hyperplasias, and neoplasias. Additionally, the methods may also be used for the in the diagnosis, prognosis, assessing the aggressiveness of, or monitoring the progression of non-cancerous conditions associated with XMRV infection such as chronic fatigue syndrome and other neuroimmune diseases.

[0044] It is further understood that the methods disclosed herein may also be practiced via the detection of the presence of antibodies specific for XMRV rather than the direct detection of XMRV itself. Therefore, also disclosed herein are methods of identifying a subject having or at risk of having a cancer, such as a prostate cancer, or having a non-cancerous condition such as chronic fatigue, comprising screening a sample from the subject for the presence of antibodies that specifically bind XMRV, wherein detection of said antibodies is an indication that the subject has or is likely to develop a cancer or non-cancerous XMRV-associated disease or condition. Additionally, the methods may also be used for the detection of XMRV in various samples such as, but not limited to, saliva, semen, peritoneal fluid, synovial fluid, prostatic secretions, cervical secretions, blood,

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serum, plasma, tissue, and cells. In some embodiments, the sample is whole blood, serum plasma or white blood cells. In other embodiments, the sample is tissue from the testes, e.g., a biopsy.

[0045] It is understood and contemplated herein that there are many methods known in the art that can be used to detect the presence of XMRV or anti-XMRV antibodies. For example, the detection can be accomplished through the use of nucleic acid/ protein detection or immunoassays. Thus, disclosed herein are methods of identifying a subject having or at risk of having a cancer, such as prostate cancer, comprising screening a sample from the subject for the presence of XMRV or antibodies that specifically bind XMRV, wherein detection of XMRV or said antibodies is an indication that the subject has a cancer, wherein screening for the XMRV or anti-XMRV antibodies comprises use of an immunoassay. Also disclosed are methods of identifying a subject having or at risk of having a cancer, such as prostate cancer, comprising detecting XMRV DNA in the sample, wherein detection of said XMRV DNA is an indication that the subject has a cancer.

[0046] It is further understood that the disclosed methods can comprise the use of more than one immunoassay. The use of multiple assays can overcome any shortcomings of a single assay and at the same time provide confirmation of results. For example, one assay can be highly selective or highly sensitive to maximize the potential to identify subjects with XMRV infection, or cancers developing from XMRV infection, whereas another assay can be highly specific eliminating the possibility of false positives. Thus, disclosed herein are methods, wherein screening for antibodies comprises use of a first and second immunoassay, wherein the first immunoassay is a highly sensitive immunoassay to identify subjects as potential positives, and wherein the second immunoassay is a highly specific immunoassay to eliminate false positives from the potential positives.

[0047] The disclosed methods can also be used to assess the aggressiveness of a cancer, such as prostate cancer, in a subject or grade an existing cancer. Thus, disclosed herein are methods of assessing the aggressiveness of a cancer, such as prostate cancer, in a subject, comprising screening a sample from the subject for the presence of XMRV or antibodies that specifically bind XMRV and comparing it to a reference standard, wherein an increase

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in XMRV or anti-XMRV antibodies relative to the reference standard is an indication of an increase in the aggressiveness of the cancer. Such methods can include, for example, the detection of XMRV proteins such as Gag, Env, Pol, In, Matrix, and Nucleocapsid. Also disclosed are methods of identifying a subject having a more aggressive cancer, such as, for example, prostate cancer, comprising screening a sample from the subject for the presence of XMRV in the subject, wherein the presence of XMRV in the subject is an indication that the subject has a more aggressive cancer.

[0048] The methods disclosed herein can also be used to monitor the efficacy of a treatment. By monitoring the anti-XMRV antibody levels or monitoring the presence of XMRV, it is possible to assess the effectiveness of a treatment for a cancer, for chronic fatigue syndrome, or other XMRV-associated diseases or conditions. Thus, in one aspect, disclosed herein are methods of assessing the efficacy of a treatment for a cancer such as prostate cancer or a non-cancerous XMRV associated disease such as chronic fatigue comprising screening a sample from the subject for the presence of XMRV or antibodies that specifically bind XMRV and comparing it to a pre-treatment standard, wherein an increase in XMRV or anti-XMRV antibodies relative to the pre-treatment standard is indicates that the treatment is not working and a decrease in XMRV genome sequences or anti-XMRV antibodies relative to a pre-treatment standard indicates a treatment is working.

[0049] It is understood that the sample can comprise any biological substance from a subject such as a nucleic acid, peptide, polypeptide, or tissue sample. Where the sample is a tissue sample, the sample can comprise saliva, cervical tissue and fluids, semen, peritoneal fluid, synovial fluid, cervical secretions, prostatic secretion, blood, serum, or plasma and any tissue samples such as prostatic tissue, breast tissue, lymph nodes or bone marrow. In some embodiments, the sample is whole blood, serum plasma or white blood cells. In other embodiments, the sample is tissue from the testes, e.g., a biopsy. In some embodiments, the tissue sample includes Leydig cells.

Immunoassays

[0050] In some embodiments, anti-XMRV antibody secreting cells (ASC) or anti-XMRV antibodies or XMRV or XMRV proteins can be detected by immunohistochemistry,

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immunofluorescence, ELISPOT, ELISA, or RIA. The steps of various useful immunodetection methods have been described in the scientific literature, such as, e.g., Maggio et al., Enzyme-Immunoassay, (1987) and Nakamura, et al., Enzyme Immunoassays: Heterogeneous and Homogeneous Systems, Handbook of Experimental Immunology, Vol. 1: Immunochemistry, 27.1-27.20 (1986), each of which is incorporated herein by reference in its entirety and specifically for its teaching regarding immunodetection methods. Immunoassays, in their most simple and direct sense, are binding assays involving binding between antibodies and antigen. Many types and formats of immunoassays are known and all are suitable for detecting the disclosed biomarkers. Examples of immunoassays are enzyme linked immunosorbent assays (ELISAs), enzyme linked immunospot assay (ELISPOT), radioimmunoassays (RIA), radioimmune precipitation assays (RIPA), immunobead capture assays, Western blotting, dot blotting, gel-shift assays, Flow cytometry, immunohistochemistry, fluorescence microscopy, protein arrays, multiplexed bead arrays, magnetic capture, *in vivo* imaging, fluorescence resonance energy transfer (FRET), and fluorescence recovery/localization after photobleaching (FRAP/ FLAP).

[0051] In general, immunoassays involve contacting a sample suspected of containing a molecule of interest (such as the disclosed biomarkers) with an antibody to the molecule of interest or contacting an antibody to a molecule of interest (such as antibodies to the disclosed biomarkers) with a molecule that can be bound by the antibody, as the case may be, under conditions effective to allow the formation of immunocomplexes.

[0052] Immunoassays can include methods for detecting or quantifying the amount of a molecule of interest (such as the disclosed biomarkers or their antibodies) in a sample, which methods generally involve the detection or quantitation of any immune complexes formed during the binding process. In general, the detection of immunocomplex formation is well known in the art and can be achieved through the application of numerous approaches. These methods are generally based upon the detection of a label or marker, such as any radioactive, fluorescent, biological or enzymatic tags or any other known label. See, for example, U.S. Patents 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241, each of which is incorporated herein by reference in its entirety and specifically for teachings regarding immunodetection methods and labels.

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[0053] The term “antibodies” is used herein in a broad sense and includes both polyclonal and monoclonal antibodies. In addition to intact immunoglobulin molecules, also included in the term “antibodies” are fragments or polymers of those immunoglobulin molecules, and human or humanized versions of immunoglobulin molecules or fragments thereof, as long as they are chosen for their ability to interact with XMRV. Antibodies that bind the disclosed regions of XMRV are also disclosed. For example, disclosed herein are antibodies that bind to the Env protein, the Gag polyprotein, capsid protein (CA), matrix protein (MA), nucleocapsid proteins (NC), and the SU and TM proteins. The antibodies can be tested for their desired activity using the *in vitro* assays described herein, or by analogous methods, after which their *in vivo* therapeutic and/or prophylactic activities are tested according to known clinical testing methods.

[0054] The term “polyclonal antibody” as used herein refers to an antibody obtained from a substantially heterogeneous population of antibodies, i.e., one or more antibody clones are present in the population. It is understood, that the polyclonal antibodies disclosed herein do not have to be isolated but can be present in an anti-sera. The polyclonal antibodies herein specifically include “chimeric” antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, as long as they exhibit the desired antagonistic activity. Thus, for example, disclosed herein are polyclonal antibodies raised against a whole XMRV such as a heat killed or inactivated XMRV. Also disclosed herein are methods of identifying a subject having or at risk of having a cancer such as prostate cancer or a non-cancerous XMRV-associated condition such as chronic fatigue comprising screening a sample from the subject for the presence of XMRV wherein the presence of XMRV indicates that the subject has or is at risk of having cancer.

[0055] The disclosed polyclonal antibodies can be made using any procedure which produces polyclonal antibodies. For example, disclosed polyclonal antibodies can be prepared by injecting a suitable animal with an antigen, such as XMRV and collecting

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serum from the animal. Optionally, the polyclonal antibodies may then be purified from the collected serum. The particular antigen used can be an antigen such as an inactivated or heat-killed virus, viral lysate, or one or more isolated proteins from XMRV such as, for example, Gag protein, Pol protein, Env protein, Matrix protein, Nucleocapsid, or capsid protein. It is further contemplated herein that the antigen can be synthetic peptides whose sequences are contained in XMRV proteins, or fragments of XMRV proteins. Such peptides or protein fragments can also be conjugated to molecules that enhance antigenicity, such as keyhole limpet hemocyanin and other molecules with similar functions. Animal subjects with the antigen can include but are not limited to rabbits, goats, pigs, or guinea pigs.

[0056] As used herein, the term "epitope" is meant to include any determinant capable of specific interaction with the anti-XMRV antibodies disclosed. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

[0057] By "specifically binds" is meant that an antibody recognizes and physically interacts with its cognate antigen (e.g., a XMRV derived polypeptide) and does not significantly recognize and interact with other antigens; such an antibody may be a polyclonal antibody or a monoclonal antibody, which are generated by techniques that are well known in the art.

[0058] The antibody can be bound to a substrate or labeled with a detectable moiety or both bound and labeled. The detectable moieties contemplated with the present compositions include fluorescent, enzymatic and radioactive markers.

Nucleic Acid Detection

[0059] The disclosed diagnostic methods can also utilize the detection of XMRV nucleic acid or XMRV antibodies as an indicator of the presence of a disease, such as prostate cancer or a non-cancerous XMRV-associated condition, such as chronic fatigue syndrome. For example, nucleic acid molecules and sequences have been amplified and/or detected

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using polymerase chain reaction (PCR), ligase chain reaction (LCR), self-sustained sequence replication (3SR), nucleic acid sequence based amplification (NASBA), strand displacement amplification (SDA), and amplification with Q β replicase. Thus, any form of PCR or real time PCR can be used with the disclosed methods. A variety of PCR forms and protocols are known and can be used. For example, PCR can be mediated by two or more primers, where one or more of the primers comprise a fluorescent label. For example, the primer can comprise a fluorescent change primer. The primers can be designed to hybridize to particular sequences present in the extension products and/or hybridizing nucleic acids. Thus, contemplated herein are methods of identifying a subject having an aggressive form of a cancer, such as prostate cancer, comprising screening a sample from the subject for the presence of XMRV in the subject, wherein the presence of XMRV in the subject is an indication that the subject has an aggressive form of a cancer.

[0060] It is understood herein that the presence of XMRV can be determined through the detection of one or more genes or nucleic acid fragments (including DNA and RNA fragments). Thus, for example, the presence of XMRV can be determined through the PCR detection of Long Terminal Repeats (LTR) or the IN gene of XMRV. Alternatively, the presence of XMRV can be determined through nucleic acid detection comprising two sets of primers wherein a first set of primers is specific for LTR and a second set of primers is specific for IN. It is understood that the detection of two or more different regions of the XMRV genome or genomic DNA increases the sensitivity and specificity of the detection, and these regions can be in other parts of the XMRV genome.

[0061] There are a variety of sequences related to the protein molecules involved in the signaling pathways disclosed herein, for example VP62 of XMRV, or any of the nucleic acids disclosed herein for making VP62, all of which are encoded by nucleic acids or are nucleic acids. The sequences for the human analogs of these genes, as well as other analogs, and alleles of these genes, and splice variants and other types of variants, are available in a variety of protein and gene databases, including Genbank. Those of skill in the art understand how to resolve sequence discrepancies and differences and to adjust the compositions and methods relating to a particular sequence to other related sequences.

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Primers and/or probes can be designed for any given sequence given the information disclosed herein and known in the art.

[0062] Disclosed are compositions including primers and probes, which are capable of interacting with the disclosed nucleic acids as disclosed herein. In certain embodiments the primers are used to support DNA amplification reactions. Typically the primers will be capable of being extended in a sequence specific manner. Extension of a primer in a sequence specific manner includes any methods wherein the sequence and/or composition of the nucleic acid molecule to which the primer is hybridized or otherwise associated directs or influences the composition or sequence of the product produced by the extension of the primer. Extension of the primer in a sequence specific manner therefore includes, but is not limited to, PCR, DNA sequencing, DNA extension, DNA polymerization, RNA transcription, or reverse transcription. Techniques and conditions that amplify the primer in a sequence specific manner are preferred. In certain embodiments the primers are used for the DNA amplification reactions, such as PCR or direct sequencing. It is understood that in certain embodiments the primers can also be extended using non-enzymatic techniques, where for example, the nucleotides or oligonucleotides used to extend the primer are modified such that they will chemically react to extend the primer in a sequence specific manner. Typically the disclosed primers hybridize with the disclosed nucleic acids or region of the nucleic acids or they hybridize with the complement of the nucleic acids or complement of a region of the nucleic acids. The size of the primers or probes for interaction with the nucleic acids in certain embodiments can be any size that supports the desired enzymatic manipulation of the primer, such as DNA amplification or the simple hybridization of the probe or primer.

[0063] The primers for the VP35, VP42, or VP62 XMRV sequences typically will be used to produce an amplified DNA product that contains a region of the VP35, VP42, or VP62 . In general, typically the size of the product will be such that the size can be accurately determined to within 3, or 2 or 1 nucleotides.

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Diagnosis of Disease States

[0064] In certain embodiments, the level of anti-XMRV antibodies or XMRV protein or nucleic acid in a test sample from a patient is used in the diagnosis of a disease, such as cancer. Cancer is a class of diseases characterized by uncontrolled cell division and the ability of these cells to invade other tissues, either by direct growth into adjacent tissue (invasion) or by migration of cells to distant sites (metastasis). Cancer cells may spread throughout the body (*i.e.*, metastasize) by way of the bloodstream or lymphatic system to form tumors in other tissues or organs. Such cancers include, but are not limited to leukemia, lymphoma, breast cancer, lung cancer, esophageal cancer, stomach cancer, colorectal cancer, thyroid cancer, melanoma, bone cancer, prostate cancer, testicular cancer, ovarian cancer, cervical cancer, endometrial cancer, kidney cancer, bladder cancer, and cancer of the central nervous system.

[0065] In some embodiments, the level of anti-XMRV antibodies or XMRV protein or nucleic acid in a test sample is used to monitor therapy. The level of anti-XMRV antibodies or XMRV protein or nucleic acid before treatment can be compared to levels during treatment at regular intervals. A reduction in XMRV proteins or nucleic acids provides an objective assessment of efficacy of therapy, and of patient compliance. A rise in titers following an initial fall would suggest development of viral mutants that are resistant to antiviral therapy, and would signal that a change in therapy is needed.

[0066] In some embodiments, the level of anti-XMRV antibodies or XMRV protein or nucleic acid in a test sample is used to diagnose a disease. The level of anti-XMRV antibodies or XMRV protein or nucleic acid may be compared to a reference value to determine if the levels of XMRV biomarkers are elevated or reduced relative to the reference value. Typically, the reference value is the level XMRV biomarkers measured in a comparable sample from one or more healthy individuals. An increase or decrease in the XMRV biomarkers may be used in conjunction with clinical factors other than XMRV biomarkers to diagnose a disease.

[0067] Association between a pathological state (*e.g.*, cancer) and the aberration of a level of XMRV can be readily determined by comparative analysis in a normal population and an

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abnormal or affected population. Thus, for example, one can study the level of XMRV protein or nucleic acid in both a normal population and a population affected with a particular pathological state. The study results can be compared and analyzed by statistical means. Any detected statistically significant difference in the two populations would indicate an association. For example, if the level of XMRV protein or nucleic acid is statistically significantly higher in the affected population than in the normal population, then it can be reasonably concluded that higher level of XMRV protein or nucleic acid is associated with the pathological state.

[0068] Statistical methods can be used to set thresholds for determining when the level of XMRV in a subject can be considered to be different than or similar to a reference level. In addition, statistics can be used to determine the validity of the difference or similarity observed between a patient's level of XMRV and the reference level. Useful statistical analysis methods are described in L.D. Fisher & G. vanBelle, *Biostatistics: A Methodology for the Health Sciences* (Wiley-Interscience, NY, 1993). For instance, confidence (“p”) values can be calculated using an unpaired 2-tailed t test, with a difference between groups deemed significant if the *p* value is less than or equal to 0.05. As used herein a “confidence interval” or “CI” refers to a measure of the precision of an estimated or calculated value. The interval represents the range of values, consistent with the data that is believed to encompass the “true” value with high probability (usually 95%). The confidence interval is expressed in the same units as the estimate or calculated value. Wider intervals indicate lower precision; narrow intervals indicate greater precision. Preferred confidence intervals of the invention are 90%, 95%, 97.5%, 98%, 99%, 99.5%, 99.9% and 99.99%. A “p-value” as used herein refers to a measure of probability that a difference between groups happened by chance. For example, a difference between two groups having a p-value of 0.01 (or $p=0.01$) means that there is a 1 in 100 chance the result occurred by chance. Preferred p values are 0.1, 0.05, 0.025, 0.02, 0.01, 0.005, 0.001, and 0.0001. Confidence intervals and p-values can be determined by methods well-known in the art. *See, e.g.*, Dowdy and Wearden, *Statistics for Research*, John Wiley & Sons, New York, 1983. Exemplary statistical tests for associating a prognostic indicator with a predisposition to an adverse outcome are described hereinafter.

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[0069] Once an association is established between a level of XMRV and a pathological state, then the particular physiological state can be diagnosed or detected by determining whether a patient has the particular aberration, *i.e.* elevated or reduced specific XMRV protein or nucleic acid levels. The term “elevated levels” or “higher levels” as used herein refers to levels of a XMRV protein or nucleic acid that are higher than what would normally be observed in a comparable sample from control or normal subjects (*i.e.*, a reference value). In some embodiments, “control levels” (*i.e.*, normal levels) refer to a range of XMRV protein or nucleic acid levels that would normally be expected to be observed in a mammal that does not have a disease. A control level may be used as a reference level for comparative purposes. “Elevated levels” refer to XMRV protein or nucleic acid levels that are above the range of control levels. The ranges accepted as “elevated levels” or “control levels” are dependent on a number of factors. For example, one laboratory may routinely determine the level of XMRV protein or nucleic acid in a sample that are different than the level obtained for the same sample by another laboratory. Also, different assay methods may achieve different value ranges. Value ranges may also differ in various sample types, for example, different body fluids or by different treatments of the sample. One of ordinary skill in the art is capable of considering the relevant factors and establishing appropriate reference ranges for “control values” and “elevated values” of the present invention. For example, a series of samples from control subjects and subjects diagnosed with cancer can be used to establish ranges that are “normal” or “control” levels and ranges that are “elevated” or “higher” than the control range.

[0070] Similarly, “reduced levels” or “lower levels” as used herein refer to levels of XMRV protein or nucleic acid that are lower than what would normally be observed in a comparable sample from control or normal subjects (*i.e.*, a reference value). In some embodiments, “control levels” (*i.e.* normal levels) refer to a range of XMRV protein or nucleic acid levels that would be normally be expected to be observed in a mammal that does not have a disease and “reduced levels” refer to XMRV protein or nucleic acid levels that are below the range of such control levels.

[0071] As used herein, the phrase “difference of the level” refers to differences in the quantity of a particular marker, such as a XMRV protein or nucleic acid, in a sample as

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compared to a control or reference level. For example, the quantity of particular protein or nucleic acid may be present at an elevated amount or at a decreased amount in samples of patients with a disease compared to a reference level. In one embodiment, a “difference of a level” may be a difference between the level of XMRV or XMRV antibodies present in a sample as compared to a control of at least about 1%, at least about 2%, at least about 3%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 50%, at least about 60%, at least about 75%, at least about 80% or more. In one embodiment, a “difference of a level” may be a statistically significant difference between level of XMRV or XMRV antibodies present in a sample as compared to a control. For example, a difference may be statistically significant if the measured level of the f XMRV or XMRV antibodies falls outside of about 1.0 standard deviations, about 1.5 standard deviations, about 2.0 standard deviations, or about 2.5 stand deviations of the mean of any control or reference group.

[0072] The anti-XMRV antibody or XMRV protein or nucleic acid level in a test sample can be used in conjunction with clinical factors other than XMRV to diagnose a disease. Clinical factors of particular relevance in the diagnosis of cancer include, but are not limited to, the patient’s medical history, a physical examination of the patient, complete blood count, cytogenetics, *etc.*

Kits

[0073] The materials described above as well as other materials can be packaged together in any suitable combination as a kit useful for performing, or aiding in the performance of, the disclosed method. It is useful if the kit components in a given kit are designed and adapted for use together in the disclosed method. For example disclosed are kits for diagnosing a cancer, such as prostate cancer or non-cancerous XMRV-associated disease or condition such as chronic fatigue syndrome, the kit comprising XMRV antigens, anti-human antibody (e.g., anti-human IgG), a means of detection, and solid support such as, for example, microtiter plate (e.g., an ELISA or ELISPOT plate). The kits also can contain reagents for visualizing the interaction of the anti-human antibody with an antibody to

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XMRV present in the sample to be used with the kit. The disclosed kits can also include the necessary tools for obtaining a tissue sample from a subject to be diagnosed

EXAMPLES

[0074] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary and are not intended to limit the disclosure. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.), but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C or is at ambient temperature, and pressure is at or near atmospheric.

EXAMPLE 1: PRODUCTION OF AN INFECTIOUS CLONE OF XMRV AND GENERATION OF XMRV SPECIFIC ANTIBODIES

Materials and Methods

[0075] *Infectious clone of XMRV*. Overlapping partial clones AM-2-9 and AO-H4 based on the pCR 2.0-TOPO (Invitrogen) backbone and patient isolate VP62 (Urisman A., et al. 2006) were provided as a gift by Don Ganem (UCSF). Site-directed mutagenesis (QuikChange II site, Stratagene) using complementary primers XMRV-M1 and XMRV-M2 (Table 2) was used to introduce a novel MluI restriction site into the overlapping segments of plasmids AM-2-9 and AO-H4. Briefly, complementary mutagenesis primers were extended using a high-fidelity DNA polymerase. Template plasmid was then digested using DpnI restriction enzyme, the remaining mutated primer extension products were transformed into competent XL10-Gold cells (Stratagene), and the transformed cells were grown at 37°C. Individual colonies were screened for mutated plasmids AM-2-9* and AO-H4* by MluI digestion and direct sequencing using the flanking primers XMRV-S2F and XMRV-S4R (Table 2). Plasmids AM2-9* and AOH4* were then subjected to restriction digest using enzymes HindIII and MluI (AM-2-9*), and MluI and HindIII (AO-H4*). The resulting HindIII-MluI and MluI-HindIII fragments were separated by gel electrophoresis and individually isolated by gel extraction (Gel Extraction Kit, Qiagen). The XMRV genomic fragment isolated from plasmid AO-H4* was then cloned into plasmid AM-2-9*

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using T4 DNA Ligase (New England Biolabs) to obtain the full length XMRV proviral clone pXMRV33. This plasmid was transformed into JM109 cells and verified by direct sequencing using primers XMRV-S1F to -S6F and XMRVS1R to -S9R (Table 2). 293T cells were transfected with pXMRV33 and empty vector pCR 2.0TOPO and supernatants were treated with DNase to remove remaining plasmid DNA and added to naïve 293T cells. Inoculated 293T cells were passaged every 2 to 3 days for greater than 90 days and culture supernatant was periodically monitored for virus production by RT assay, Western blot analysis, and transmission electron microscopy of pelleted material. Low molecular weight Hirt DNA was extracted from infected 293T cells 24 hours after inoculation using the previously described method (Hirt B. 1967). Overlapping viral fragments ~2 kb were amplified and sequenced using primers XMRV-S1R, -S2R, -S1F, -S3R, -S2F, -S4R, -S3F, -S5R, -S6R, -S4F, S7R, -S5F, -S8R, -S6F, -S9R (Table 2) to verify the infectious clone.

[0076] *In vitro production of viral particles.* 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine (2.2 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml). 293T cells were transfected with plasmid pXMRV33 or control plasmid pCMS-eGFP (Clontech) using Lipofectamine PLUS (Invitrogen) following the manufacturer's directions. After the initial transfection, supernatants were harvested at regular intervals and passed through a 0.45µm filter (Whatman). Virions were collected by centrifugation through a cushion of 20% (wt/vol) sucrose, resuspended in TNE buffer (10 mM Tris (pH 7.4), 150 mM sodium chloride, 1 mM EDTA (pH 8)), and quantified by reverse transcriptase (RT) assays. Oligo(dT)/poly(rA) primer-template assays were performed in the presence of (α -³²P) radiolabeled dTTP and Mn²⁺, as previously described (Telesnitsky A., et al. 1995). Aliquots of tissue culture supernatants were stored at -80°C.

[0077] *Transmission electron microscopy.* 293T cells inoculated with XMRV were fixed, dehydrated, sectioned at 60 nm thickness, placed on grids, and stained as described earlier (Auerbach MR., et al. 2007). Samples were examined using a JEOL JEM-1200 EXII electron microscope. Pictures were taken on an ORCA-HR digital camera (Hamamatsu), and size measurements were made using the AMT Image Capture Engine. Diameters of 100 XMRV virions were measured to calculate the mean size of XMRV particles.

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[0078] *Cell-block preparation.* Chronically infected 293T cells, naïve 293T cells, and serial 10-fold dilutions (1:102 to 1:106) of chronically infected 293T cells in naïve 293T cells were fixed with formalin, centrifuged, and the pelleted cells embedded in paraffin. Sections were cut for immunohistochemistry (5 µm thick) or for DNA extraction controls (10 µm thick).

[0079] *Generating XMRV-specific antibodies.* For generation of XMRV whole virus antiserum (anti-XMRV), cell culture supernatant from chronically infected 293T cells was passed through a 0.22 µm filter (Pallcorp) and centrifuged. Details of virus concentration and lysis were as described previously (Telesnitsky A., et al. 1995; Yuan B., et al. 1999). Three rabbits were inoculated with inactivated whole virus, boosted three times after 2, 3 and 7 weeks, and bled 5, 8 and 16 weeks after initial inoculation. All sera were cleared by centrifugation and stored at -80°C in small aliquots.

[0080] *Western blot analysis.* Virions were concentrated and Western blot analysis performed as previously described (Telesnitsky A., et al. 1995; Auerbach MR., et al. 2007; Yuan B., et al. 1999). XMRV proteins were visualized with primary rabbit anti-XMRV (1:5,000), anti-MoMLV CA (NCI 79S-804, 1:10,000), anti-MA (76S-155, 1:10,000), anti-NC (80S008, 1:7,500), anti-SU (1:500) antibodies (Rockland Immunochemicals Inc., Gilbertsville, PA, USA; MoMLV antisera were kind gifts of S. P. Goff, Columbia University). Molecular size standards were used to generate a standard curve of migration distance in Western blot analyses dependent on molecular weight. XMRV protein sizes were determined by interpolation using this standard curve. Data from at least two independent Western blot analyses were used. Empirically determined protein sizes were then correlated with molecular weights calculated from sequence analysis.

Results

[0081] *Generating an infectious clone of XMRV.* pXMRV33, a full-length proviral clone of XMRV, was constructed from two overlapping clones AM-2-9 and AO-H4 obtained from patient isolate VP62 (Urisman A., et al. 2006) (kind gift of Dr. Donald Ganem, UCSF). The introduction of a restriction endonuclease site for MluI allowed for the joining these overlapping clones. The sequence of pXMRV33 was compared to the sequence of

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VP62 (EF185282) Urisman A., et al. 2006). The two sequences differed by the 3811G→C and 3812A→G substitutions introduced by the MluI restriction site, resulting in amino acid substitution: M→V in position 411 of the reverse transcriptase (numbering according to MoMLV sequence NP_955591.1). There were three additional differences between pXMRV33 and the published VP62 sequence (C→G at nucleotide 7450, 7694 insT and 7776 insG). However, these may represent sequencing errors, or sequence variations specific to VP62, because at each of these locations the sequence of pXMRV33 is identical to that of two other sequenced clinical isolates of XMRV, VP35 (DQ241301.1) and VP42 (DQ241302.1) (Jemal A., et al. 2008).

[0082] To test whether viral particles are generated from the full length molecular clone, pXMRV33 was transfected into naïve 293T cells and the culture supernatant monitored for appearance of viral particles by reverse transcriptase (RT) activity and transmission electron microscopy. RT activity was first detected 9 weeks after transfection of pXMRV33 with progressively increasing activity up to 20 weeks after transfection (Figure 1a). The RT activity 20 weeks after transfection was equivalent to the maximal RT activity detected in tissue culture supernatant of NIH3T3 cells transfected with a MoMLV infectious clone (Figure 1a, (Auerbach MR., et al. 2007)). No RT activity was detectable in supernatants from 293T cells transfected with empty control vector (Figure 1a). To test whether released XMRV particles were infectious, supernatants from 293T cells 9 weeks days post-transfection was treated with DNase (to remove any residual DNA from the transfection process), and added to naïve 293T cells. RT activity peaked at day 5 post inoculation. Thus, pXMRV33 is an infectious molecular clone, and the virus replicates efficiently in human cells.

[0083] *Transmission Electron Microscopy of XMRV particles.* Supernatants from 293T cells chronically infected with XMRV were centrifuged to concentrate virions, and the pellets processed for transmission electron microscopy. Particles were abundant (Figure 1B1) and closely resembled those of a type-C murine retrovirus, Moloney murine leukemia virus (MoMLV) in size and morphology (Figure 1B2 and 1B3). XMRV particles had an average diameter of 137 nm (SD = 9 nm), a round to somewhat pleomorphic shape and characteristic lipid bilayer envelopes. The majority of XMRV particles contained an

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electron-dense, polygonal core with an irregular outline, typically defined as mature type-C retroviral core with an average diameter of 83 nm (SD = 8 nm) (Figure 1B2). These central cores had a higher electron density than the space between the core and the envelope. Particles containing well-defined spherical cores with an electron-lucent center (Figure 1B2), typically defined as immature type-C retroviral cores, were also seen. At high magnification, a 'railroad track', a term used to describe MoMLV cores (Yeager M., et al. 1998), was seen on the surface of immature XMRV cores (Figure 1B2). In MoMLV, the N and the C-terminal halves of the CA protein are believed to form the two halves of the railroad track. It appears that the XMRV capsid can be put together in a manner very similar to that of MoMLV.

[0084] *XMRV protein expression.* Antibodies specific to XMRV were generated by injecting rabbits with detergent-lysed XMRV purified from chronically infected 293T cells. A Western blot analysis of released virions using these antisera revealed specific bands that were tentatively identified by comparing lysed XMRV and MoMLV virions in parallel on the same blot (Figure 1C). The blots were probed with antibodies specific to various MoMLV Gag proteins, i.e. anti-MA, anti-CA and anti-NC, to better define various products of XMRV Gag processing. Blots were also probed with antisera generated by immunizing rabbits with the SU portion of XMRV Env protein (gift of Jason Rodriguez and Stephen Goff, Columbia University) to identify the SU portion of Env protein. These allowed for the ability to define the approximate molecular weights of XMRV proteins as follows: Gag-62 kDa, CA-30 kDa, MA-15 kDa, NC-10 kDa and Env-75 kDa. These molecular weights are very similar to those for the corresponding MoMLV proteins (Gag-65, CA-30 kDa, MA-15 kDa, NC-10 kDa Figure 1F, (Goff SP. 2007)). XMRV also appears to have an equivalent of the MoMLV p12 protein, as seen by sequence comparison studies and the presence of bands that appear to be intermediates of Gag proteolysis, e.g. p27, which might correspond to MA-p12; p42, which might correspond to p12-CA; p38, which might correspond to CA-NC (Figure 1C). Two bands of 18 kDa and 35 kDa were seen but they could not be identified with any certainty. The 18 kDa band is likely to be the TM portion of the env protein, based on size and sequence similarity with the TM portion of the MoMLV env. The 35 kDa band did not react with any of the specific antisera used. Most XMRV proteins showed a high

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amino acid sequence similarity with MoMLV (Table 1). The most dissimilar protein is the XMRV envelope protein (66% similarity). Its surface unit (SU) shares only 51% of its amino acid sequence with that of MoMLV. The XMRV structural proteins CA and NC, its enzymes PR and RT, and the transmembrane unit (TM) of the envelope protein all share more than 90% of their amino acid sequence with MoMLV. Taken together, the ability to infect naïve cells using supernatants from cells transfected with pXMRV1 confirms that pXMRV1 is an infectious clone. Its ability to replicate efficiently in human cells, as well as its type-C retrovirus morphology is consistent with being a xenotropic retrovirus. Proteins expressed from this clone correspond well to proteins from MoMLV, a closely related type C murine retrovirus.

Table 1.

	MW	MW (Calc.)	Similarity
Gag	p62	60.4	87%
MA	p15	14.1	79%
p12		8.7	79%
CA	p30	30.7	95%
NC	p10	6.4	92%
PR		13.3	96%
RT		74.9	95%
IN		45.5	81%
Env	gp75	69.9	66%
SU		47.8	54%
TM		19.6	91%
p2E		2.5	75%

EXAMPLE 2. PRESENCE OF XMRV IN HUMAN CANCER

Materials and Methods

[0085] *Human prostatic tissue for immunohistochemical analysis.* Prostatectomy specimens submitted to the Department of Pathology, Columbia University were used to estimate the prevalence of XMRV in human prostate cancer. All prostatectomy specimens are routinely formalin-fixed and an average of 20-30 tissue samples per prostate are paraffin-embedded and stained with hematoxylin and eosin (H&E) for histopathologic examination. Formalin-fixed human tissues are stored in the tissue repository of the Department of Pathology at Columbia University Medical Center. In addition, fresh tissue is

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sampled from prostatectomy specimens and banked at -80°C at the Tissue Bank of the Herbert Irving Comprehensive Cancer Center at Columbia University. Genomic DNA was extracted from 95 formalin-fixed and 195 fixed prostatectomy specimens.

[0086] H&E stained sections from paraffin-embedded tissue blocks (averaging 20-30) of each case were examined for the presence of cancer and the tissue blocks containing the highest amount of cancer were chosen for extraction of genomic DNA. The presence or absence of cancer in frozen tissue samples was determined by a pathologist based on an H&E stained section from each sample. In addition, DNA was extracted from 101 prostatic tissue samples removed by transurethral resection of prostate (TURP) for reasons other than prostate cancer. If more than one tissue block was available from one patient, the one containing the highest amount of glandular tissue was chosen. Sections (5 µm in thickness) were obtained from paraffin-embedded tissue from all cases for immunohistochemical analysis. The same tissue blocks were used for DNA extraction and immunohistochemical analysis in cases where fixed tissue was tested by PCR. For cases where only frozen tissue was tested for the presence of XMRV (pro)viral DNA, one to two paraffin-embedded tissue blocks containing the highest proportion of cancer were chosen. Demographic and medical information consisting of age at time of surgery, ethnicity, pathologic tumor stage (pTNM), and tumor grade (Gleason score) was retained. All protected health information was removed, samples were de-identified, and all experiments were performed in accordance with the Internal Review Board of Columbia University Medical Center (IRBAAAC0089).

[0087] *DNA extraction from human tissue.* Total DNA was extracted from 10 sections (10 µm thick) of formalin-fixed, paraffin-embedded tissue using the QIAamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's recommendations. For DNA extraction from fresh frozen tissue, ten tissue sections (10 µm thick) were processed the same way with the exception that the deparaffinization step was omitted. DNA was quantified (Nanodrop 1000, Thermo Scientific) and stored at -80°C. To reduce the risk of sample contamination, processing of tissue, DNA extraction, and real-time PCR set-up were performed in a laboratory separate from the one where the analysis was performed.

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[0088] *Quantitative PCR amplification of proviral DNA.* Primers and probes were designed to specifically amplify all three full-length XMRV patient isolates sequenced to date but none of the closely related exogenous or endogenous murine retroviruses. BLAST analysis of overlapping 250 bp segments of the XMRV genome (VP 35, GenBank ID DQ241301.1) identified a region of the putative gag-pro-pol gene of XMRV that is 100% conserved between VP35, VP42, and VP62 but shares only 80-85% sequence identity with the most similar murine retroviruses (Figure 2A). A common forward primer (XMRV4552F, Figure 2A and Table 2) and hydrolysis probe (XMRV4572MGB) to be used with two different reverse primers (XMRV4673R, XMRV4653R) were designed in this region using the PrimerExpress software (Applied Biosystems) to result in a 122 bp and 102 bp amplification product, respectively. The reaction mix consisted of 1X TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 900 nM forward and reverse primers, 250 nM hydrolysis probe, and 20-200 ng DNA in a total reaction volume of 20 μ l using MicroAmp Fast Optical 96-Well Reaction Plates. 500 copies of pXMRV33 diluted in 100 ng human placental DNA (SigmaAldrich) was used as positive control. Amplification conditions were 95°C for 20 seconds, followed by 45 cycles of 95°C for 3 seconds and 60°C for 30 seconds. All amplification reactions were carried out in duplicate. Serial 10-fold dilutions of plasmid pXRMV33 in 100 ng human placental DNA was used for assay validation. To control for PCR inhibitors and DNA integrity, a 168 bp segment of the single copy human gene vesicle-associated membrane protein 2 (VAMP2) was amplified in a separate reaction from each sample of extracted DNA. The reaction mix consisted of 1X TaqMan Fast Universal PCR Master Mix, 900 nM primers VAMP2-3043F and VAMP2-3210R, 250 nM hydrolysis probe VAMP2P (Table 2), and 10-100 ng DNA in a total reaction volume of 20 μ l. 100 ng human placental DNA was used as positive control. Amplification conditions were the same as for detection of XMRV. Amplification of the VAMP2 control was carried out in a single reaction. All real-time PCR was performed on a TaqMan 7500Fast instrument (Applied Biosystems). Additionally, amplification of a 63 bp segment of the XMRV R-U5 region in the XMRV LTR region for qPCR uses primers XMRV47F (5'- AATAAAGCCTTTTGCTGTTTGCA-3'; SEQ ID NO: 13), XMRV109R (5'- GAGGAGACCCTCCCAAGGAA-3'; SEQ ID NO: 14), and probe XMR74MGB (5'- 6FAM AAGCGTGGCCTCGC MGB-3'; SEQ ID NO: 15). Amplification conditions using

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a ABI 7900HT instrument are as follows: 2 min at 50C, 10 min at 95C, followed by 45 cycles of 95C for 15 sec and 60C for 1 min. The reaction mix contains 1x TaqMan Universal Master Mix (Applied Biosystems), 900 nM primers, 200 nM probe, 0.01 U/ μ l AmpErase UNG, and 200-1000 ng template DNA.

[0089] *RNase L genotyping.* A commercial TaqMan SNP genotyping assay (assay ID: C___935391_1_, Applied Biosystems) was used with TaqMan SNP Genotyping Mix (Applied Biosystems) for RNase L G1385A (R462Q) genotyping (NCBI SNP reference: rs486907). 9 ng DNA was used in a total reaction volume of 20 μ l. A TaqMan 7500Fast instrument was used for amplification, detection, and allelic discrimination. After amplification, relative normalized fluorescence intensities (Rn) from two allele-specific probes are compared for genotype determination. Ratios of Rn (Q): Rn (R) ranging from 0.6 to 1.1 were interpreted as homozygous wild type cases (RR), from 2.2 to 3.1 as heterozygous individuals (RQ), and from 7.5 to 9.5 as individuals with homozygous R462Q mutation (QQ). DNA from two individuals of each genotype was sequenced to confirm allelic discrimination results. DNA from one individual of each genotype was used as control in all subsequent experiments.

[0090] *Immunohistochemistry.* Formalin-fixed, paraffin-embedded tissue sections were cut 5 μ m thick and placed on electrostatically charged microscope slides, dried for 30 min at 56°C, deparaffinized for 3 x 3 minutes in xylene (Fisher Scientific), rehydrated in decreasing alcohol concentrations followed by water, and subjected to antigen retrieval. Sections were immersed in High pH Target Retrieval solution (Dako), and heated in a pressure cooker (National SR-206-N), and boiled under pressure for 5 minutes, followed by slow cooling to room temperature. Endogenous peroxidase activity was blocked by treating sections with 3% H₂O₂ for 10 min. Sections were then incubated with 3 ml of primary antibody (anti-XMRV 1:7,500) diluted in antibody diluent with background reducing components (Dako) in individual wells of Antibody Amplifier boxes (Prohisto). Strong staining at primary antibody dilutions of 1:7,500 were observed. Somewhat fainter staining of the same pattern was still present when using the primary antibody at a dilution of 1:15,000. For final analysis a 1:7,500 dilution was used. After washing with 1X TBS, 0.1% Tween 20 (TBS-T) for 3 x 5 minutes, tissue sections were incubated for 30 minutes with

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secondary anti-rabbit HRP-conjugated polymer antibody and washed again for 3 x 5 min. with 1X TBS-T. 3,3'-diaminobenzidinetetrahydrochloride (DAB, Dako) was used as chromogen. Sections were counterstained for 20 seconds with hematoxylin, washed, dehydrated in graded alcohols, and covered with coverslips. Three different approaches were used to assess specificity of staining using XMRV antisera. First, the antisera was pre-adsorbed using sections of prostatic tissues that did not contain prostate cancer. Pre-adsorption had no effect on the intensity of specific staining but did result in a modest reduction of the faint, diffuse background staining seen in the fibrous stroma and in the apical aspect of many epithelial cells. Second, pre-immune serum from the same rabbit was used on sections adjacent to those showing strong staining with the anti-XMRV antiserum. Identical frames were identified based on hematoxylin counterstaining. No immunohistochemical staining was seen in cells within the corresponding structures that showed strong staining in an adjacent section when using XMRV-specific antiserum (Figure 4G and 4H). Third, adjacent sections were stained with an antiserum from a second rabbit inoculated with detergent-inactivated XMRV. This resulted in a staining pattern that was virtually identical to the first antiserum, both at the tissue and the intracellular levels. Tissue sections were used on the test case to further optimize the different antigen retrieval methods, to test varying dilutions and staining parameters of the two anti-XMRV antisera. The protocol resulting in the strongest staining with the least amount of faint, diffuse non-specific staining was used for further analyses of 334 cases. Pre-immune serum from the same rabbit was used as negative control and an IHC-positive case was included as control with each batch. In addition, XMRV infected and naïve cultured 293T cells were fixed, paraffin-embedded, and sectioned at 5- μ m thickness to be used as positive and negative controls, respectively. Pictures were taken with an Olympus IX51 microscope and an Olympus DP71 camera.

Results

[0091] XMRV was initially isolated from cases of human prostate cancer (Urisman A., et al. 2006). Of a total of 86 cases of prostate cancer that were tested in that study, XMRV was identified in 9 by nested RT-PCR. To examine human infection with XMRV in greater depth, use was made of the extensive tissue repositories in the Department of Pathology at

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Columbia University Medical Center, New York. Several novel tools were developed that allowed this analysis: highly-specific anti-XMRV antibodies described above, a very specific quantitative PCR assay (qPCR), and protocols for immunohistochemistry that allowed for sensitive detection of XMRV proteins in cancers.

[0092] To estimate the prevalence of XMRV in men with and without prostate cancer, 233 consecutive cases of prostate cancer were selected. For controls, 101 consecutive cases of transurethral resection of the prostate for urinary obstruction were used (most often due to benign prostatic hyperplasia). Both frozen tissues and formalin-fixed paraffin embedded (FFPE) tissues were used for preparing DNA used in the qPCR analysis and genotyping. Since tissue is typically frozen only in case of tumors, FFPE tissues were used for control tissues. Sections adjacent to the ones used for preparing DNA were used for immunohistochemistry studies.

[0093] *Quantitative PCR detection of XMRV proviral DNA.* The original report on XMRV identification used an RT-PCR assay to detect XMRV RNA in frozen tissues obtained. The nested PCR assay that was used in that study, sequentially amplified 612 and 413 nucleotides from the Gag region of the XMRV genome. It was important that the assay amplified DNA, and that these amplified segments be as short as possible, both desirable features when working with formalin-fixed tissues. The qPCR assay employed a dual-labeled TaqMan probe (5' – fluorescent reporter dye, 3' – minor groove binder/non-fluorescent quencher) flanked by two primers to amplify two regions of the XMRV proviral DNA, 122 bp and 102 bp in length. A common forward primer and a common probe were used in conjunction with two different reverse primers to allow for sequence differences in different clinical isolates. Reverse primer XMRV4673R targets a sequence that is highly divergent between XMRV and the most similar murine retroviral sequences, whereas reverse primer XMRV4653R targets a more conserved region (Figure 2A).

[0094] It was important to ensure that the assay specifically amplified XMRV sequences and not other murine or human endogenous retroviral sequences. In research laboratories, human tissue blocks are often sectioned on the same microtomes used for murine tissues, and contamination with murine samples can result in non-specific amplification of

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exogenous or endogenous murine retroviruses that are present in multiple copies in the mouse genome and have high sequence similarity to XMRV (Figure 2A). Systematic scanning of the XMRV genome identified a region of the putative gag gene that was 100% conserved between all published XMRV clones (total of 3), and yet shared at most 80% similarity with the most closely related 11 murine retroviral sequences (Figure 2A). Primers and probes in this region allowed efficient detection of XMRV without interference from related murine retroviral sequences. To further maximize specificity, primers were selected to allow for greatest mismatch near the 3'-ends, and the probe for greatest mismatch at the 5'-end. To test for amplification of murine ERVs, genomic DNA of a C57BL/6 mouse was used as template. To rule out amplification of human ERVs or other human sequences related to XMRV, commercially available human placental DNA was used as template. Also mouse DNA was mixed with human placental DNA at different ratios. No amplification product was observed in any of the reactions, showing that the qPCR assay was highly specific for XMRV.

[0095] The sensitivity of the qPCR assay was tested in 2 ways. First, serial 10-fold dilutions of plasmid pXMRV33 mixed with 20 ng/μl human placental DNA were used as a template. A consistent signal was obtained when at least 50 copies of XMRV DNA were present in the reaction. When only 5 copies of XMRV DNA were present, a detectable amplification product was seen approximately half the times (Figure 2B). The second method assessed sensitivity of amplification from FFPE samples, using a protocol similar to that used with human prostate tissues. XMRV-infected and naïve 293T cells were mixed together in different ratios: from 1:100 to 1:106 (infected cells: naïve 293T cells). These mixtures of cells were fixed with formalin, embedded in paraffin and sectioned. One such section was mixed with nine sections of normal prostate to closely match this sample to the study material. DNA was extracted and used as template in the qPCR assay. DNA extracted from naïve 293T cells (mixed with normal prostate) did not result in detectable amplification. XMRV proviral DNA was consistently amplified in up to 1 in 105 dilutions of infected cells (further diluted at least ten-fold with normal prostatic tissue). One of two duplicates of a 1 in 106 dilution resulted in detectable amplification (Figure 2C). Using immunohistochemistry to count XMRV-infected cells in an adjacent section, approximately

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1.4 infected cells consistently detected per qPCR sample, making this a highly sensitive qPCR assay.

[0096] The quality of DNA extracted from clinical tissues, whether frozen or fixed, can vary depending on times and conditions of processing and storage. To test for DNA integrity and the absence of inhibitors of DNA amplification, a second qPCR assay was developed amplifying a 168 bp segment of the human single copy gene, vesicle-associated membrane protein 2 (synaptobrevin 2, VAMP2). VAMP2 amplification product was detectable from each of the samples, both frozen and fixed. The mean cycle at which fluorescent signals first exceeded a predetermined background value (quantification cycle or C_q) was similar for each sample type (mean C_q = 25 with SD = 1.1 for frozen tissue, mean CT = 26.4 with SD = 3.4 for fixed cancer tissues, and mean CT = 25.6 with SD = 1.8 for fixed tissue from control cases). This indicated that template DNA quality was similar across all sample types. Samples where the VAMP2 control gene was amplified at a C_q greater than 2 SD above the mean for that sample type were considered to have unacceptable DNA quality and were excluded from further qPCR analyses. This resulted in exclusion of 5 (2.6%) frozen cancers, 7 (7.4%) fixed cancers and 2 (2%) control tissues from the study pool, thus ensuring that any failure to detect XMRV was not a result of poor DNA quality in the sample.

[0097] *Prevalence of XMRV DNA in human prostate tissue.* To estimate the prevalence of XMRV in men, 334 consecutive cases of prostatic disease were selected from men who presented for surgery at Columbia University Medical Center (Figure 3). DNA was analyzed from 189 frozen and 88 FFPE prostate cancers (total of 233 distinct prostate cancer cases) and 101 FFPE control samples. In brief, XMRV DNA was identified in samples from 14 men (6.2%) with prostate cancer and from 2 men (2.0%) of the control group. The C_q values for amplification of the single copy VAMP2 gene were equal or slightly higher for XMRV-positive cases than those for XMRV-negative PCR cases (mean C_q = 25 vs. 24.9 for frozen tissues, 26.4 vs. 25.6 for fixed tissues, and 26.3 vs. 25.3 for fixed control samples respectively). This indicates that the quality of the DNA in the XMRV PCR-positive cases was not superior to that of XMRV PCR-negative cases, and that the different prevalence of XMRV in cancer and non-cancer cases was real.

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[0098] XMRV proviral loads were determined in tissues by comparing the Cq from PCR-positive cases with the Cq of standards with known concentrations of XMRV. Two different external standards were used: plasmid DNA and DNA extracted from cells infected with XMRV that were formalin-fixed and paraffin-embedded. Plasmid DNA standards consisted of 10-fold serial dilutions of pXMRV33 in human placental DNA. These standards revealed that PCR-positive prostate cancers contained between 50 and 500 copies of XMRV DNA per 200 ng of total DNA (equivalent to approximately 40,000 diploid cells). On average, 1% of the total DNA extracted from 10 sections of 10 μ m thickness was used for one qPCR assay. Therefore, the each section of 10 μ m thickness contained between 500 and 5000 copies of XMRV DNA. Since formalin fixation and tissue processing can compromise the quality of extracted DNA, standards were also made with fixed XMRV templates, i.e. from FFPE XMRV-infected cells. Cq of XMRV PCR-positive samples were similar to those obtained from a 1:105-fold dilution of XMRV-infected 293T cells, representing between 6 and 7 XMRV-infected cells per one section of 10 μ m thickness (Figure 2D) assuming comparable number of XMRV DNA targets in prostate tissue and chronically infected 293T cells. This indicates that XMRV-positive prostate tissues contained approximately 500-5000 copies of XMRV DNA (using DNA of optimal quality) or an equivalent of 6 to 7 XMRV-infected cells (using DNA from FFPE tissue) per section of 10 μ m thickness.

[0099] *Detection of XMRV protein by immunohistochemistry.* XMRV proteins are present exclusively in prostatic stromal cells that are near malignant epithelial cells, but never within the cancerous cells themselves. Since all known oncogenic retroviruses transform their host cells directly, new mechanisms need to be invoked if only non-malignant cells harbor the virus. What cell type serves as host for XMRV in human prostate cancer tissues, therefore, has significant implications in understanding possible oncogenic mechanisms. In order to carefully address this question, antisera specific to XMRV was generated (see methods), and then developed an immunohistochemistry (IHC) protocol to determine which cell type expressed XMRV protein in prostate cancer tissues. Cultured 293T cells were used that were chronically infected with XMRV for the development and optimization of an XMRV-specific IHC assay. Chronically XMRV-infected 293T cells were fixed with formalin, embedded in paraffin, and the resulting cellblocks sectioned at the same thickness

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commonly used for IHC analysis of human tissues. Chronically infected 293T cells were serially diluted with naïve 293T cells (mix containing 100% to 0.0001% infected cells). More than 90% of cells showed granular cytoplasmic staining of varying intensity (Figure 4A and 4E). Sections were counterstained with hematoxylin resulting in a blue to purple nuclear staining. In sections containing 1% infected 293T cells and 99% of naïve cells, only a small subset of cells (approximately 1%) stained positive for XMRV (Figure 4B). The pattern of intracellular staining was identical to that observed in sections containing 100% infected cells. No stained cells were identified in sections containing 100% naïve 293T cells (Figure 4C). A pre-immune control serum from the same rabbit was also used on sections containing 100% XMRV-infected 293T cells, and did not see any staining (Figure 4D). It was thus very likely that using the antisera and IHC protocol, XMRV proteins can be visualized in prostatic tissue. In contrast to sections of infected 293T cells, sections of human prostate tissues contain multiple cell types, varying amounts of fibrous stroma and are not typically processed in a uniform manner. All of this may result in increased background staining. Since no well-defined positive control tissues were available, sections from cases that were positive by XMRV qPCR were tested. Conditions were optimized for sections of XMRV-infected 293T cells and identified one XMRV qPCR-positive case that showed particularly strong staining of a subset of malignant epithelial cells (Figure 4G). At the cellular level, the staining had an identical pattern to that seen in XMRV-infected 293T cells (Figure 4F and 4G). This case was used in further analyses to test the observed staining for specificity and further optimize the IHC protocol for use with human prostate tissue sections: by preadsorbing antisera on non-malignant prostatic tissues, using preimmune sera for comparison, and using immune sera against XMRV developed in a second immunized rabbit.

[0100] Anti-XMRV antisera from two different rabbits used at high dilutions recognized the same clusters of malignant epithelial cells with the same intracellular staining pattern. This staining pattern was identical to that seen in XMRV-infected 293T cells. Pre-absorption of anti-XMRV antiserum did not reduce staining intensity, while replacing the anti-XMRV antiserum with pre-immune serum from the same rabbit completely abolished any staining. Given these observations together with the fact that DNA extracted from the

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same case tested positive with the XMRV qPCR it was concluded that the IHC assay was able to specifically detect XMRV proteins in human prostate tissue.

[0101] *Prevalence and distribution of XMRV protein in human prostate tissue.* The human prostate is composed primarily of acinar or ductal epithelial cells, which serve the secretory function of the gland. Almost all cases of human prostate cancer are the result of malignant proliferation of these epithelial cells. In addition to the epithelial cells, the prostate also contains stromal cells, primarily fibroblasts, with a few macrophages, lymphocytes and an occasional granulocyte. To render a pathologic diagnosis of cancer, the entire prostate gland is routinely sampled, resulting in an average of 20-30 tissue blocks that are eventually banked in the tissue repository. Prostate cancer usually follows a focal pattern, with malignant cells seen in only a minority of the sampled tissue blocks. Within each block, the extent of cancer also varies greatly. Sections stained with hematoxylin and eosin dyes were examined that were prepared from each block for routine diagnostic purposes. For each case, one to two tissue blocks were selected that contained the highest proportion of malignant epithelial cells for IHC analysis. For every case that showed any staining, adjacent sections were also tested with control pre-immune serum. With some exceptions (to be detailed below) staining was considered specific if it showed a intracellular pattern similar to that seen in XMRV-infected 293T cells and in the IHC-positive test case shown in Figure 7, without any staining with the pre-immune control serum.

[0102] Of interest was the prevalence of expression of XMRV protein expression in each case series and what the cell type of XMRV expressing cells. The optimized IHC protocol was used to test prostate tissue sections from all 334 men. Overall, expression of XMRV protein was identified in prostatic tissues from 54 men (23%) with prostate cancer and in 4 men (4%) without prostate cancer (Figure 5A). In contrast to previous reports, staining was predominantly observed in epithelial cells that were part of the cancer, i.e. malignant epithelial cells. Of the 54 IHC-positive cases with prostate cancer, expression of XMRV protein was observed in epithelial cells in 46 cases (85%), in both epithelial and stromal cells in 4 cases (7.5%), and exclusively in stromal cells in 4 cases (7.5%). Of the 4 IHC-positive individuals without prostate cancer, XMRV expression was seen in epithelial cells

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in 3 cases and both epithelial and stromal expression in 1 case. Epithelial cells expressing XMRV protein usually belonged to a common acinus or to a few adjacent acini (Figure 6, Figure 7). The proportion of cells expressing XMRV protein in a given section varied widely from case to case (Figure 8A through 8H, low magnification images) with positively staining cells representing a minority of cells. Staining intensity also varied between cases ranging from intense staining of the entire cytoplasm to more discrete staining where the granular pattern could be more readily appreciated. The vast majority of IHC-positive epithelial cells showed the same granular staining pattern of the entire cytoplasm described above. In a small number of cases epithelial staining of only a circumscribed portion of the cytoplasm of epithelial cells was observed. Rare scattered XMRV-expressing stromal cells were seen in proximity to malignant acini (Figure 8I) or in lymphocytic infiltrates adjacent to malignant acini (Figure 8J). Overall, the number of stromal cells expressing XMRV protein was much smaller than the number of IHC-positive epithelial cells. In addition to the 54 cases of prostate cancer and the 4 control cases that showed specific IHC positivity, two main patterns of non-specific staining were also observed. In cases with extensive lipofuscin pigmentation of the prostate, some lipofuscin granules in epithelial cells or stromal macrophages were highlighted with the IHC assay (Figure 9). However, lipofuscin granules were also highlighted with the pre-immune control serum, and upon using protocols that specifically stain lipofuscin. Second, in a small number of cases, homogenous and diffuse nuclear staining was observed in epithelial and stromal cells, a common artifact that can result from suboptimal tissue fixation and antigen retrieval (Bussolati G., et al. 2008). Both types of staining were considered non-specific and not included in subsequent analyses. In summary, expression of XMRV proteins was observed in 23% of prostate cancer cases and in only 4% of control cases. XMRV protein expression was most frequently seen in clusters of epithelial cells that were part of the cancer and rarely in stromal cells adjacent to the cancer.

[0103] *No association of XMRV with the R462Q polymorphism of RNase L.* The association of XMRV with the R462Q variant of RNASEL in the 334 study subjects was also tested. DNA extracted from prostate tissues from all 334 study subjects was genotyped for the 1385 G→A SNP using a commercial TaqMan genotyping assay. Among patients

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with prostate cancer, 42.9% had an RR, 47.2% an RQ, and 9.9% a QQ genotype. Of the 101 control individuals, 52.5% had an RR, 40.6% an RQ, and 6.9% a QQ genotype (Figure 8). Relative genotype distributions vary between ethnic groups with reported frequencies ranging from 32-88% for RR, 12-55% for RQ, and 0-13% for QQ genotypes (HapMap, NCBI SNP database, http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=486907). The relative frequencies of RNASEL genotypes in the study population falls within these reported ranges. When comparing genotype distributions between XMRV-positive and XMRV-negative cases no significant difference was identified (Figure 8): Of the 14 XMRV-positive individuals with prostate cancer, the relative genotype distribution was 50% RR, 43% RQ, and 7% QQ. Of the XMRV-negative cases, the relative genotype distribution was 42.7% RR, 47.4% RQ, and 10% QQ. Both XMRV-positive individuals of the control group had an RR genotype. Thus, no association was found between the presence of XMRV and the RNase L R462Q genotype in the population of over 300 cases. These results do not support an association of XMRV with the common RNase L R462Q variant. The independence of XMRV infection from the RNase L R462Q variant has important implications for prostate cancer development, and indicates that all individuals may be at risk for XMRV infection, not just those homozygous for R462Q.

[0104] *Correlation of XMRV with prostate cancer, tumor grade, and tumor stage.* The correlation of XMRV with the presence, grade, and stage of prostate cancer was analyzed to investigate indirect evidence for a possible oncogenic role of XMRV in the development of prostate cancer.

[0105] First, the prevalence of XMRV in prostate tissues from patients with prostate cancer was compared to that in prostate tissues from patients without prostate cancer. Cases in which XMRV DNA or protein was identified were considered XMRV-positive for this analysis. The odds for XMRV being detected in prostate tissues from men with prostate cancer was more than 5 times higher than those for XMRV being present in tissue samples from men without prostate cancer (OR = 5.7, $p \ll 0.001$). A correlation of XMRV with cancer is also supported by the fact that XMRV protein was identified predominantly in malignant epithelial cells. To test for spatial association of XMRV with prostate cancer, the frequency of XMRV DNA in frozen prostate tissues that did or did not contain prostate

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cancer was compared. Sampling of prostate cancer tissues for the frozen tissue bank is largely blind due to the fact that prostate cancer foci are usually not recognizable macroscopically at the time of sampling. Therefore, an H&E stained section of each frozen tissue sample was examined for the presence of prostate cancer by a pathologist. XMRV DNA was identified in 13.5% of samples containing prostate cancer and in 3.9% of frozen prostate tissue samples that did not contain prostate cancer (but were sampled from a prostate gland that contained cancer elsewhere). The odds for finding XMRV DNA in frozen prostate tissues with prostate cancer were therefore almost 4-times as high as in frozen tissues that did not contain prostate cancer (OR = 3.8, $p < 0.05$). Taken together, a strong correlation of XMRV with prostate cancer was identified when using men with benign prostatic hyperplasia as controls. XMRV protein was identified in malignant prostate epithelial cells and further evidence of XMRV being spatially associated with prostate cancer was obtained by a strong correlation of XMRV DNA with the presence of cancer in the tissue samples used for qPCR analysis.

[0106] Thus, since XMRV infection plays a role in prostate cancer promotion or progression, an association of XMRV infection with tumor grade and stage can also be made. Besides the patient's age, grade and stage have been shown to best correlate with the biological behavior of prostate cancer (Epstein 2006). Tumor grade refers to the level of differentiation of the cancer. The Gleason scoring system, the most common method for prostate cancer grading, defines patterns 1 to 5 of decreasing differentiation, with pattern 5 representing the most undifferentiated architecture. Since many prostate cancers show more than one architectural pattern, the dominant and the subdominant pattern are scored independently and added together for a combined Gleason score; with scores of 2-4 for well-differentiated cancers, 5- 6 for intermediate cancers, 7 for moderate to poorly differentiated cancer, and 8 - 10 for high-grade cancers (Robbins p. 1053). Whether XMRV DNA and protein are more frequently detected in prostate cancer tissues from patients with higher grade or stage of cancer was also determined. (summarized in Table 2). For this analysis, XMRV infection was defined by the presence of XMRV DNA and/or protein. The proportion of XMRV-infected individuals differed significantly across the groups with different Gleason scores with XMRV being more frequently identified in prostate tissues

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from cases with higher-grade cancers. Of the 101 cases without cancer, 6% of individuals were XMRV-positive. Of the 233 cases with cancer, the following fractions were positive for XMRV: 18% of cases with a Gleason score of 6, 27% of cases with a Gleason score of 7, 29% with a Gleason score of 8, and 44% with a Gleason score of 9. Since only one case had a score of 10 (negative by IHC and PCR), it was not included in the correlation analysis. Therefore, there is a significant association of XMRV-infection with a higher-grade of prostate cancer in the cases used for the study.

[0107] Tumor stage is quantified by its extent, most commonly using the pTNM system that describes the extent of the primary tumor in the prostate (pT2 through pT4), the presence of local lymph node metastases (N0 and N1), and distant metastases (M0 or M1). Stages pT2 through pT4 describe prostate cancer with increasing local and regional invasion (Table 3). With the widespread use of serum PSA levels as a diagnostic marker for prostate cancer, most cancer these days is recognized and treated early. This is reflected in the distribution of pT stages in the cases: 75% pT2, 23% pT3, and 2% pT4. XMRV was detected in 25% of stage pT2 cases and in 32% of pT3 cases. Of the 5 cases with a pT4 stage, 1 case (20%) was XMRV-positive (Figure 5C). This moderately increased prevalence of XMRV in advanced stage prostate cancer tissues was not statistically significant. Regional lymph nodes were removed in 46% of cases and lymph node metastases were identified in only 2% of these prostate cancer cases. Distant metastases were not identified in any of the cases in the study. This paucity or lack of metastasis made it impossible to study the association of XMRV with regional lymph node or distant metastases.

[0108] *XMRV present in other tissue and cancer types.* Tissues from approximately 80 autopsies on males aged 18 to 85 years were analyzed for the presence of XMRV using the described methods. Subjects included eight individuals with prostate cancer. Of these eight, two had XMRV present in their prostate cancer, in agreement with earlier results showing that approximately 25% of prostate cancers have detectable XMRV. Tissues from every available organ from the eight subjects with prostate cancer were also analyzed for the presence of XMRV. In these subjects, the only other tissue where XMRV was found to be present was the Leydig cells in the testes, the cells that produce testosterone (Figure 12). Surprisingly, all eight subjects with prostate cancer had detectable XMRV in the testes,

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even subjects for which XMRV was not detected in the prostate cancer tissues. Figure 12 shows sections from the testes, with XMRV proteins being expressed exclusively in Leydig cells (central panels). Adjacent sections were stained for calretinin which is an established marker for Leydig cells. The lower panel shows Leydig cells at higher magnification, illustrating the characteristic granular staining pattern seen in XMRV infected cells. The presence of XMRV in the testes may be a precursor to the development of prostate cancer, and can be used as an indicator to facilitate diagnosis of prostate cancer.

[0109] Additionally, 178 cases of breast cancer were examined for the presence of XMRV using the described methods. Approximately 25% of breast cancers contained either XMRV proviral DNA sequences or XMRV proteins. The XMRV proteins were seen exclusively in the malignant breast epithelium (Figure 11). Adjacent benign epithelium or stroma did not contain XMRV.

Discussion

[0110] Infectious agents are known to cause approximately 20% of all human cancers and it has been proposed that chronic infections might also play a role in prostate cancer development and progression. XMRV is a novel human gammaretrovirus that has first been identified in prostate cancer tissues and is the most recent candidate for an infectious agent playing a role in prostate cancer. Evidence is also presented showing the presence of XMRV in human prostate cancer tissues and, for the first time, the presence of XMRV proteins in neoplastic epithelial cells in human prostate cancer. XMRV DNA was detected in 6% and XMRV proteins were detected in 23% of prostate cancer tissues tested from patients treated surgically for prostate cancer. Intriguingly, XMRV protein expression was detected almost exclusively in neoplastic epithelial cells, in contrast to a previous report in which XMRV protein was detected exclusively in non-epithelial cells of the prostatic stroma using a monoclonal antibody against the Spleen Focus Forming Virus (SFFV) p30 gag capsid protein. In contrast, this study used antiserum raised against inactivated XMRV particles, and validated for sensitivity and specificity using both chronically infected, cultured human cells and human prostate cancer tissues. When testing tissue sections from all 233 patients with prostate cancer and 101 controls, 23% of prostate cancer tissues and 4% of control tissues showed specific staining. This is the first report of a large survey of

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prostate cancer and control tissues using IHC. By both methods, XMRV was more frequently detected in tissues from men with prostate cancer than in control tissues. An increased prevalence of XMRV was also observed in higher-grade prostate cancers. Together, these observations provide the first evidence for an association of XMRV with the malignant cells in prostate cancer, and possibly with its development.

[0111] The localization of XMRV to neoplastic epithelial cells has important implications. Several different mechanisms have been characterized for the transforming potential of oncogenic retroviruses. All of these share the feature that retroviral infection leads to transformation of infected cells. The pattern of staining observed in neoplastic epithelial cells in prostate cancer tissue closely resembled that seen in chronically infected, cultured 293T cells. IHC-positive cells were clustered in the same acini, and these acini were often in close proximity to each other with the majority of adjacent acini not showing any staining. Further support for a spatial association of XMRV with neoplastic epithelial cells was obtained from qPCR analysis of frozen tissues: within a prostate gland containing cancer, XMRV was more frequently found in close proximity of the cancer than in samples collected away from the cancer.

[0112] There are several possible reasons for the finding of XMRV proteins in malignant epithelial cells. An XMRV-specific antiserum, raised in rabbits against whole XMRV, was used, making the IHC assay more sensitive and specific for the detection of XMRV proteins in human prostate cancer tissues. This notion is further supported by the fact that IHC-positive epithelial cells were identified in a prostate cancer case (Figure 7G).

[0113] The prevalence of XMRV as determined by qPCR and IHC assays most likely represents an underestimate of the true prevalence of XMRV in prostate cancer tissue. This is particularly true for detection of XMRV DNA by qPCR but also to be expected for detection of XMRV protein by IHC. XMRV proteins was found to be expressed in a variable, but small, subset of cells in any given tissue section, rendering its detection sensitive to sampling error. When detecting XMRV DNA by qPCR, DNA from infected cells gets highly diluted in DNA contributed from noninfected cells. The overall detection rate is therefore determined by the proportion of XMRV-infected cells in the sampled tissue

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and by the sensitivity of the PCR assay. The advantage of detecting viral nucleic acids is that a larger portion of the tissue can be surveyed (i.e. DNA from all cells in the sample are pooled and tested) with the disadvantage being reduced overall sensitivity. The choice to test for XMRV DNA rather than RNA is another factor contributing to the sensitivity of XMRV detection by the qPCR approach. Since most frozen tissue was not available for the control cases, and since RNA is often degraded in formalin-fixed tissues, the presence of XMRV DNA was tested. Detection of XMRV protein by IHC allows (sub)cellular localization and detection of individual XMRV-infected cells in a given tissue section, removing the dilution effect that becomes a factor in interpreting the qPCR results. The limits of this approach however are that only actively replicating virus can be detected and that the sample size analyzed is much smaller. DNA was extracted from a total thickness of 100 μm for qPCR analysis, whereas sections of only 5 μm thickness were used for IHC. The main limitations for estimating the prevalence of XMRV via detection of XMRV protein by IHC therefore are sampling differences and the ability to test only a small fraction of each sample.

[0114] The findings by immunohistochemical analyzes that XMRV infects only a small proportion of cells are also supported by quantitative PCR analyses. Threshold cycles of PCR-positive prostate cancer cases were similar to those obtained from a 1:10E+5 to 1:10E+6-fold dilution of XMRV-infected 293T cells (Figure 3). This indicates that a similar amount of template DNA was present in the XMRV-positive prostate cancer cases as in highly dilute, chronically infected 293T cells. Since integrated provirus and reverse transcribed but not integrated virion DNA can serve as template, both the proportion of infected cells (number of proviruses) and concentration pre-integrated viral DNA determines the amount of template DNA. Therefore, the prostate tissue samples either contained a very small proportion of XMRV-infected cells with similar concentration of viral DNA or a larger proportion of infected cells with XMRV replicating at a lower level. Comparing threshold cycles of XMRV-positive prostate cancer cases to 10-fold serial dilutions of pXMRV33 in human placental DNA indicates an equivalent proviral load as approximately 50-500 copies of pXMRV33 per 200 ng of genomic DNA. These findings also help explain discrepancies of results obtained by detection of XMRV nucleic acids and

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identification of XMRV in tissue sections. Results obtained by different tests did not always concur for a given case: Of the 14 prostate cancer cases with detectable XMRV DNA, XMRV protein was identified in 6. None of the 2 TURP samples from men without prostate cancer that tested positive for XMRV DNA had detectable XMRV protein (Figure 5A). Besides the absence of a gold standard for assay validation, the low level, focal infection pattern of XMRV most likely explains variable results.

[0115] In several cases only one of the two duplicate real-time PCR reactions resulted in detectable amplification products, further supporting low level, focal infection. Polymorphisms in the primer binding sites are possible alternative explanations for these findings. Given high threshold cycles even for cases, where both duplicates with both primer combinations resulted in detectable amplification, it is most likely that the viral load for most cases was close to the detection limit of the qPCR assay. All PCR products were sequenced from XMRV DNA positive cases. No sequence variation was identified in this region of the XMRV genome that is 100% conserved between the 3 fully sequenced isolates reported thus far. Detection rates also varied by the type of tissue tested: The rate of XMRV-positivity was higher in frozen tissue samples than in formalin-fixed samples. Of the 14 PCR-positive prostate cancer cases, XMRV was detected in 11 frozen and in 3 fixed tissue samples. Overall, XMRV was detected in 3 out of 88 individuals (3.4%) with prostate cancer when FFPE tissue was tested and in 11 out of 189 individuals (5.8%) when frozen tissue was tested.

[0116] However, no association of XMRV with the RNASEL R462Q polymorphisms was observed. The initial identification of XMRV was based on the observation that individuals homozygous for the common RNASEL R462Q polymorphism have a higher risk for developing prostate cancer. It had been hypothesized that a homozygous carriers of this polymorphism may be more susceptible to persistent viral infections, which in turn may increase their risk for prostate cancer. Based on this observation, prostate cancer tissues from individuals homozygous for the RNASEL R462Q polymorphism were screened for the presence of viral nucleic acids (Urisman A., et al. 2006). A strong association of detectable XMRV RNA and the RNASEL R462Q genotype were reported. All individuals in this study were genotyped and observed no association of the presence of XMRV DNA or

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protein with the RNASEL R462Q genotype. There are several differences between the initial and the study that might account for these differences: First, prostate cancer tissues were tested for the presence of XMRV DNA and protein while Urisman et al. tested for viral RNA. It is conceivable, that the reduced-activity variant of RNase L has a more significant effect on the levels of XMRV RNA transcripts and genomes than on proviral DNA and pre-integrated cDNA in infected tissues. In the initial study, a nested RT-PCR was used for the detection of XMRV RNA while a qPCR assay was used to detect XMRV DNA. The results of both studies indicate that the viral load of XMRV in the tested tissues is low. The chance of detecting XMRV RNA may therefore simply be greater in individuals that are homozygous for the reduced-activity variant of RNase L. If levels of viral RNA transcripts and genomes are more affected by the RNASEL R462Q polymorphism than levels of XMRV DNA or protein, the mode of XMRV detection may determine whether an association is identified or not. Secondly, a different study population was used. The strength of the association of XMRV with the RNASEL genotype may vary from population to population dependent on the prevalence of XMRV and the relative genotype distribution in the study population, or another gene near the RNASEL locus, and not RNASEL itself, may be linked to prostate cancer.

[0117] Correlation of XMRV with prostate cancer. Availability of suitable control tissue from men without prostate cancer is a limiting factor for the study of a possible association of XMRV with prostate cancer. In this retrospective study, prostate tissue removed by transurethral resection of the prostate was used for reasons other than prostate cancer as control tissue. There are several limitations to the comparability of this tissue to prostate tissues removed by radical prostatectomy for treatment of prostate cancer. During the transurethral procedure, tissue is resected with an electric knife causing cautery artifact in the collected tissue chips. In addition, tissue is predominantly removed from the transition zone. However, prostate cancer most commonly arises in the peripheral zone. To reduce the effect of the first limitation, only those tissue samples were chosen that contained large tissue chips limiting the cautery artifact to a thin external layer. In addition, tissues were selected to contain the maximum amount of glandular tissue. In order to conduct a preliminary association analysis, the prevalence of XMRV DNA and protein was compared

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in prostate cancer and control tissue samples. XMRV DNA was found in approximately twice as many cases of prostate cancer as in control cases. Even when limiting the analysis to fixed prostate cancer tissues, the prevalence of XMRV DNA was 50% higher in tissues from men with prostate cancer than in control tissue. As discussed above, detection of XMRV protein by IHC provides more reliable prevalence estimates. XMRV protein was detected in 23% of prostate cancer cases and in 4% of control tissues. These initial findings indicate that XMRV is associated with prostate cancer when using tissue removed by transurethral resection as control.

[0118] The findings have several important implications for the study of a possible oncogenic effect of XMRV. XMRV was identified in up to 23% of prostate cancer tissues and localized XMRV protein almost exclusively to neoplastic epithelial cells. This latter finding indicates that XMRV plays a role in cancer development or progression through one of the several direct mechanisms that have been well characterized for known oncogenic retroviruses.

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TABLE 2

Primer	Start Position*	Sequence	SEQ ID NO:	Application
XMRV-M1	3800	5'-CCT TGC CTA CGc gTG GTA GCA GCC-3'	16	Mutagenesis
XMRV-M2	3823	5'-GGC TGC TAC CAc gCG TAG GCA AGG-3'	17	Mutagenesis
XMRV-S1R	602	5'-CGA GAA CAC TTA AAG ACA GAA GAA-	18	Sequencing
XMRV-S2R	944	5'-CGG GAG CTG TCG GTA A-3'	19	Sequencing
XMRV-S1F	1339	5'-TGA AGA TCC AGG TAA ATT GAC G-3'	20	Sequencing
XMRV-S3R	1575	5'-TCT GTA GTG GTG TAA TCC CAA TC-3'	21	Sequencing
XMRV-S2F	3733	5'-GGG ACC TTG GCG TCG GCC TGT GGC-3'	22	Sequencing
XMRV-S4R	3853	5'-CTT GCC TGC ATC CTT TGT CA-3'	23	Sequencing
XMRV-S3F	5522	5'-GCC GCT GCT TAT CAG GAC CAG-3'	24	Sequencing
XMRV-S5R	5702	5'-GTA TCC ACG CAG AGA TGC C-3'	25	Sequencing
XMRV-S6R	5731	5'-AGT TGT CGC CGC CTT TAC GTG-3'	26	Sequencing
XMRV-S4F	6598	5'-GGG ACG GGA GAC AGG CT-3'	27	Sequencing
XMRV-S7R	6761	5'-GGG GCA GAG GTA TGG TTG G-3'	28	Sequencing
XMRV-S5F	7380	5'-TGG CGT AGT AAG AGA TAG CAT-3'	29	Sequencing
XMRV-S8R	7567	5'-GAA TAC AGG GTC CGA AGA G-3'	30	Sequencing
XMRV-S6F	7727	5'-ACC CCA CCA TAA GGC TTA GCA C-3'	31	Sequencing
XMRV-S9R	7942	5'-TTA GTT TCG CTT TAT CTG AGG ACC A-3'	32	Sequencing
XMRV4552	4552	5'-CGA GAG GCA GCC ATG AAG G-3'	33	Detection
XMRV4572	4572	5'-6FAM AGT TCT AGA AAC CTC TAC ACT C-3'	34	Detection
XMRV4653	4653	5'-GAG ATC TGT TTC GGT GTA ATG GAA A-3'	35	Detection
XMRV4673	4673	5'-CCC AGT TCC CGT AGT CTT TTG AG-3'	36	Detection
VAMP2	3043	5'-TCT GCC ACT TCG GGT TTC TC-3'	37	Control
VAMP2	3210	5'-GGT AGC CAC CCC TCT CAC AA-3'	38	Control
VAMP2P	3067	5'-HEX CAT TCC TGC TCC CCA GTT TTC -3'	39	Control

TABLE 3

Prostate Cancer					TURP
Sample Type	All	Frozen	Fixed	Both	Fixed
Number	233	138	38	57	101
Age, median (range)	60	60	60	61	72
	(41-76)	(41-74)	(43-76)	(50-73)	(44-85)
Race/ethnicity (%)					
African American	17	19	16	14	27

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Caucasian	53	55	53	49	38
Hispanic	12	13	8	14	29
Other/not provided	17	13	24	23	6
Tumor grade (%)					n/a
6	16.3	18.1	10.5	15.8	
7	70.4	71.0	73.7	66.7	
8	6.0	4.3	10.5	7.0	
9	6.9	5.8	5.3	10.5	
10	0.4	0.7	0	0	
Tumor stage (%)					
Primary Tumor					n/a
pT2a	9	13	5	4	
pT2b	2	2	3	2	
pT2c	63	57	79	67	
pT3a	18	19	11	19	
pT3b	6	7	3	4	
pT4	2	1	0	5	
Regional LN					n/a
pNX	54	52	58	54	
pN0	44	46	40	42	
pN1	2	1	3	4	

EXAMPLE 3: DETECTION OF XMRV IN BODY FLUIDS

Materials and Methods

[0119] qPCR and Immunohistochemical methods can also be used to detect the presence of XMRV in body fluids, including saliva, semen, peritoneal fluid, synovial fluid, prostatic or cervical secretions, blood, serum, or plasma, as follows.

[0120] *Total nucleic acid extraction for qPCR detection of XMRV.* If a sample of body fluids contains cell suspensions, cells are pelleted by centrifugation at 1500 rpm for 10 minutes, followed by removal of all but 500 μ l supernatant. Cells are resuspended by vortexing and then processed for nucleic acid extraction. Body fluids that are cell free are mixed by vortexing and used directly for nucleic acid extraction. Total nucleic acids are extracted using the QIAamp Virus BioRobot 9604 Kit following the manufacturer's instructions.

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[0121] *Detection of XMRV by qPCR.* The reaction mix contains 1 x TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 900 nM forward and reverse primers, 250 nM hydrolysis probe, and 20-200 ng DNA in a reaction volume of 20 μ l. As a positive control, 500 copies of pXMRV33 diluted in 200 ng human placental DNA (Sigma-Aldrich) are used. Thermocycling conditions are 95°C for 20 seconds, followed by 45 cycles of 95°C for 3 seconds and 60°C for 30 seconds, using MicroAmp Fast Optical 96-Well Reaction Plates and a TaqMan 7500Fast instrument (Applied Biosystems).

[0122] To assess quality of DNA extracted from each sample, a 168 bp segment of the single copy gene vesicle-associated membrane protein 2 (VAMP2, also known as Synaptobrevin 2) is amplified in a separate reaction. The reaction mix consists of 1X TaqMan Fast Universal PCR Master Mix, 900 nM primers VAMP2-3043F and VAMP2-3210R, 250 nM hydrolysis probe VAMP2P, and 10-100 ng DNA in a total reaction volume of 20 μ l. For positive control, 100 ng human placental DNA are used. Thermocycling conditions are the same as for detection of XMRV as previously described. Primer sequences are listed in Table 4a-f.

Table 4a. Sequences of LTR primers used for qPCR and qRT-PCR

LTR (R-U5, 63 bp)			
Name	Sequence	Start	SEQ ID
XMRV47F	5'-AATAAAGCCTTTTGCTGTTTGCA-3'	47	40
XMRV109R	5'-GAGGAGACCCCTCCCAAGGAA-3'	109	41
XMRV74MGB	5'-6FAM-AAGCGTGGCCTCGC-MGB-3'	74	42

Table 4b. Sequences of IN short primers used for qPCR and qRT-PCR

IN short (65 bp)			
Name	Sequence	Start	SEQ ID
XMRV4552F	5'-CGAGAGGCAGCCATGAAGG-3'	4552	43
XMRV4616R	5'-GCGTATACGGGGTTGAGTCC-3'	4597	44
XMRV4572MGB	5'-6FAM-AGTTCTAGAAACCTCTACACTC-MGB-3'	4572	45

Table 4c. Sequences of IN long primers used for qPCR and qRT-PCR

IN long (122 bp)			
Name	Sequence	Start	SEQ ID
XMRV4552F	5'-CGAGAGGCAGCCATGAAGG-3'	4552	46
XMRV4673R	5'-CCCAGTCCCGTAGTCTTTTGAG-3'	4673	47
XMRV4572MGB	5'-6FAM-AGTTCTAGAAACCTCTACACTC-MGB-3'	4572	48
XMRV4653R	5'-GAGATCTGTTTCGGTGTAATGGAAA-3'	4653	49

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Table 4d. Sequences of Env primers used for qPCR and qRT-PCR

Env (86 bp)			
Name	Sequence	Start	SEQ ID
XMRV6356F	5'-GGATGCCCCAAAACATG-3'	157	50
XMRV6441R	5'-GGACCTGGCGGGTCAGA-3'	242	51
XMRV6393MGB	5'-TCCACTGGGGCCGAC-3'	194	52

Table 4e. Sequences of VAMP2 primers used for qPCR and qRT-PCR

VAMP2 (78 bp)			
Name	Sequence	Start	SEQ ID
VAMP2-3613F	5'-CCCCACACTTCTGGTTTTCTG-3'	3613	53
VAMP2-3690R	5'-CAGCATCTCTCTACCCTTTCAC-3'	3690	54
VAMP2-3645 MGB	5'-AGCAGGGATATCTAAGC-3'	3645	55

Table 4f. Sequences of VAMP2 primers used for qPCR and qRT-PCR

VAMP2 (168 bp)			
Name	Sequence	Start	SEQ ID
VAMP2-3043F	5'-TCTGCCACTTCGGGTTTTCTC-3'	3043	56
VAMP2-3210R	5'-GGTAGCCACCCCTCTCACAA-3'	3210	57
VAMP2-3067MGB	5'-[HEX]CATTCCTGCTCCCCAGTTTTTCATGTGG[Tamra]-3'	3067	58

MGBNFQ – minor groove binder/non-fluorescent quencher
Nucleotide positions are based on VP62 (EF185282.1)

[0123] For detection of XMRV RNA, a reverse transcription reaction is carried out prior to amplification as follows: 1 µl random hexamers and 1 µl 10mM dNTPs are mixed per sample/control, and 2 µl of the mix is aliquoted into a RNase free 0.2 ml tube for each sample/control. 8 µl of sample RNA is added (up to 5 µg) per tube. Tubes are incubated at 65°C for 5 minutes in a thermocycler, then placed on ice for 1 minute.

[0124] The following cDNA synthesis mix is prepared: 2 µl, 10X RT buffer; 4 µl, 25mM MgCl₂; 2 µl, 0.1M DTT; 1 µl, RNaseOUT (40U/µl); 1 µl, SuperScript III RT (200U/µl). 10 µl of cDNA synthesis mix is added to each tube, mixed, and spun down briefly. Reactions are incubated for 10 min at 25°C, then incubated for 50 min at 55°C in thermocycler. Reactions are terminated by incubation at 85°C for 5 min in thermocycler, then chilled on ice and spun down briefly. 1 µl of DNase-free RNase H is added per tube, and incubated at 37°C for 20 min. cDNA is stored at -20°C.

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[0125] *Detection of XMRV by immunohistochemistry (IHC) in cervical secretions.* Cell block preparation from cervical brush samples in ThinPrep Transport Media is performed as follows: Specimens are transferred to 15 ml Falcon tubes, and centrifuged at 1500 rpm for 10 minutes. Following aspiration of supernatant with a serological pipette, 10 ml of freshly made alcohol/formalin solution (made by mixing 1 ml of 10% formalin with 9 ml 95% ethanol) is added to pellet and vortexed. Samples are then centrifuged sample at 1500 rpm for 10 minutes, followed by aspiration of all but about 0.5 ml of the supernatant. Pellets are transferred onto Bio-Wrap paper, which is folded and put into labeled cassette, which is placed in a jar containing 10% formalin. Cassettes are then subjected to tissue processing, embedding, and sectioning onto glass slides for immunohistochemical staining for the detection of XMRV proteins, as is previously described.

[0126] *Detection of XMRV sequences in cervical fluid* It is still unknown by which route XMRV infects people. Related retroviruses spread through transfer of blood or body fluids from an infected person to a healthy person. The ability to detect XMRV in cervical fluids as described here has allows for determining a possible route of spread of virus, including spread from mother to infant during childbirth, and has important implications for public health. Furthermore, detection of XMRV in cervical fluids offers a non-invasive method to detect XMRV. Cervical fluid is centrifuged at 1500 rpm for 10 min and all but 500 μ l supernatant is removed. Pelleted cells are resuspended in the remaining fluid by vortexing and used for nucleic acid extraction using the QIAamp Virus BioRobot 9604 Kit following the manufacturers instructions. XMRV proviral DNA sequences are then detected by qPCR as detailed above. XMRV sequences by PCR in approximately 5-10% of samples from women on whom a test to detect other viruses (such as human papilloma virus, or herpes simplex virus) had been ordered.

[0127] *Detection of XMRV in seminal fluid and spermatic fractions obtained from human semen.* The ability to detect XMRV in semen is also important to determine possible routes of XMRV transmission. Semen for 300 de-identified donors was separated into the spermatozoon fraction and the seminal plasma fraction by centrifugation (Carrell, D. T., et al., 1998). DNA was isolated from 220 microliters of the spermatozoon fraction and qPCR for XMRV sequences was used to detect XMRV proviral DNA. RNA was isolated from

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220 microliters of the seminal plasma, and qRT-PCR was used to detect XMRV in this fraction. All nucleic acids are extracted using the QIAamp Virus BioRobot 9604 Kit following the manufacturer's instructions. XMRV DNA or RNA was found in approximately 7% of all samples tested, indicating that XMRV is present in semen obtained from otherwise healthy men who are not known to have prostate cancer or chronic fatigue syndrome, or other XMRV-associated conditions. This finding has important implications for viral spread, and tests for detection of XMRV in semen have an application in testing donor semen samples used in fertility clinics.

EXAMPLE 4: DETECTION OF XMRV ANTIBODIES IN HUMAN SERA

Materials and Methods

[0128] *ELISA Protocol.* Materials used in the ELISA assay were Maxisorp Strip Plates (Nunc), Binding buffer (1X PBS), 10X Wash buffer (0.030M Potassium Phosphate, 0.080M Sodium Phosphate *7H₂O, 2.90M NaCl, 4.0 % (v/v) Tween 20, pH 7.2), 10X Sample Dilution buffer (0.030M Potassium Phosphate, 0.080M Sodium Phosphate *7H₂O, 1.45M NaCl, 1.0%(v/v) Tween 20, 1.0% BSA, pH 7.2), Blocking is StabilCoat (Surmodics, Eden Prairie, MN), TMB substrate, Stop solution (1 N Sulphuric Acid), Human Secondary: Peroxidase-conjugated AffiniPure F(ab')₂ Fragment Goat Anti-Human IgG + IgM (H+L) (code 109-036-127, Jackson ImmunoResearch, West Grove, PA), Human Secondary: Peroxidase-conjugated AffiniPure F(ab')₂ Fragment Goat Anti-Human IgG (H+L) (code 109-036-088, Jackson ImmunoResearch, West Grove, PA), Rabbit Secondary: Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) (code 111-035-003, Jackson ImmunoResearch, West Grove, PA). . The following concentrations were used: viral lysate – 0.5 µg/mL; MLV-CA protein – 0.5-5 ug/mL; rabbit serum – 1:16,000; rabbit conjugate – 1:20,000 (with reference to the 50% glycerol stock); and human conjugate – 1:20,000 (with reference to the 50% glycerol stock). The conjugates and serum were diluted in 1X Sample Dilution Buffer.

[0129] Antigen binding (XMRV viral lysate or purified MLV-CA protein) to the plate was performed using 100µL of the binding buffer and incubation of the samples overnight at 4°C. Wells were washed 5 times with 300µL of wash buffer (120 sec soak). 100µL of

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blocking buffer was added and samples were incubated for 1-2 hours at room temperature, followed by aspiration of the blocking buffer. 100 μ L of rabbit or human serum (diluted if necessary in Sample Dilution Buffer) was added to the plates, and then incubated for 2 hours at room temperature. Wells were washed 5 times with 300 μ L of wash buffer (120 sec soak). 100 μ L of secondary antibody conjugates were added to each well and incubated for 30 minutes at room temperature, then wells were washed 5 times with 300 μ L of wash buffer (120 sec soak), followed by addition of 100 μ L of TMB and incubation for 30 minutes at room temperature in the dark. 100 μ L of stop solution was added to each well, and measurements were taken at 450nm & 650nm. Results are expressed as OD450-OD650 to correct for irregularities in the plate).

[0130] *Protocol for Western Blots.* The following viral protein preparations were prepared: XMRV lysed in 1% Triton X-100 and purified His-tagged MoMLV CA protein. Viral protein preparations were resolved on 12% SDS-PAGE gels. Protein(s) were transferred from the gel onto a PVDF membrane after prewetting membrane in methanol. Following protein transfer, the membranes were stained with Ponceau S to visualize protein bands. Using the Ponceau S stained proteins as a guide, the membrane is cut into strips. Each strip is blocked for 1 hour at room temperature in 5% milk in 1x Tris buffered saline (TBS), without any detergent, and then incubated with human serum at dilutions ranging from 1:8 to 1:100 in 5% milk in TBS with Tween 20 (TBST). Incubation was carried out for 2h at room temperature or overnight in cold room, with gentle agitation on a rocker plate. Membranes were rinsed once briefly in TBST, then washed three times, 5 minutes per wash, with TBST, on a rocker plate. The secondary antibody conjugated to a IR-dye was diluted 1:10,000 (800nm) or 1:20,000 (680nm) in 5% milk in TBST + 0.01% SDS and incubated with the strips for 1 hour at room temperature on the rocker. Membranes were rinsed once, washed three times, 5 minutes per wash, with TBST. Blots were scanned one at a time on the Odyssey scanner, using default intensity 5.

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Results

[0131] Experiments using a combination of ELISA and Western blot-based assays were conducted in order to obtain evidence of XMRV infection in the form of anti-XMRV antibodies in sera.

[0132] *An ELISA to screen for XMRV infection.* The current ELISA protocol consists of detergent-lysed XMRV attached to the bottom of wells in a 96-well plate. Human sera, at various dilutions (1:2, 1:4, 1:8, 1:8, 1:16, 1:32, 1:100), are bound to wells, the wells washed, treated with peroxidase-conjugated secondary antibodies and the reaction developed and read at 450/650 nm. The protocol is applied to human sera that have a high likelihood of containing anti-XMRV antibodies, for example, sera from patients with a past history of prostate cancer who were being monitored for cancer recurrence. Since XMRV is found in over one fourth of prostate cancers examined, a similar proportion of sera from prostate cancer patients can contain anti-XMRV antibodies. Anti-XMRV antibodies may also be found in sera of patients with chronic fatigue syndrome and other XMRV-associated conditions. These positive sera, if confirmed to be positive by a Western blot (see below) provide good positive controls for screening large numbers of unselected samples. In this selected population of patients with past history of prostate cancer, approximately 20-25% of samples that can be considered positive were found from the qPCR studies on prostate cancer samples.

[0133] *Western blots as a confirmatory test for XMRV infection.* To confirm that the antibodies detected by ELISA were indeed against XMRV, a Western blot analysis of lysed XMRV virions was performed using serum samples. Several of the samples reacted against the CA (p30) and MA proteins of XMRV, and a few reacted against the Env protein of XMRV, or its proteolytic products, viz. SU and p15E (Figure 10 and Figure 13). The only other band that appeared was around 65-66 kD, this is most likely to be BSA, since it is present in lanes that do not contain virus, but contain supernatants from uninfected cells. Approximately 30% of serum samples, taken from men with a history of prostate cancer, reacted against the XMRV proteins on a Western blot. This proportion (30%) is similar to the 27% of prostate cancers that score positive for XMRV by qPCR and IHC (Figure 10).

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[0134] Similarly, to detect if patients with Chronic Fatigue Syndrome have evidence of XMRV infection, we collected sera from 105 patients diagnosed as having chronic fatigue syndrome and fulfilling the Fukuda criteria for diagnosis. For comparison, we also collected sera from 200 healthy volunteers. Each of these sera was used to probe a PVDF membrane onto which XMRV proteins had been transferred from a gel. A positive sample usually contained antibodies reactive to at least two of the following three XMRV polypeptides: SU, CA and p15E (see Fig. 13A). Non-reactive samples (most healthy volunteers) did not contain antibodies to SU or p15E (Fig. 13B). Antibodies to CA alone, however, were quite prevalent in the healthy volunteer population, and are not to be interpreted as evidence of XMRV infection.

[0135] A potential testing algorithm for determining the presence of XMRV antibodies includes a highly sensitive screening test to identify all potential positives (such as the ELISA test), followed by a highly specific test (such as the Western blot) to eliminate the false positives.

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* * * *

[0136] While certain embodiments have been illustrated and described, it should be understood that changes and modifications can be made therein in accordance with ordinary skill in the art without departing from the technology in its broader aspects as defined in the following claims.

[0137] The present disclosure is not to be limited in terms of the particular embodiments described in this application. Many modifications and variations can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. Functionally equivalent methods and compositions within the scope of the disclosure, in addition to those enumerated herein, will be apparent to those skilled in the art from the foregoing descriptions. Such modifications and variations are intended to fall within the scope of the appended claims. The present disclosure is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled. It is to be understood that this disclosure is not limited to particular methods, reagents, compounds compositions or biological systems, which can of course vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

[0138] In addition, where features or aspects of the disclosure are described in terms of Markush groups, those skilled in the art will recognize that the disclosure is also thereby described in terms of any individual member or subgroup of members of the Markush group.

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[0139] As will be understood by one skilled in the art, for any and all purposes, particularly in terms of providing a written description, all ranges disclosed herein also encompass any and all possible subranges and combinations of subranges thereof. Any listed range can be easily recognized as sufficiently describing and enabling the same range being broken down into at least equal halves, thirds, quarters, fifths, tenths, *etc.* As a non-limiting example, each range discussed herein can be readily broken down into a lower third, middle third and upper third, *etc.* As will also be understood by one skilled in the art all language such as “up to,” “at least,” “greater than,” “less than,” and the like, include the number recited and refer to ranges which can be subsequently broken down into subranges as discussed above. Finally, as will be understood by one skilled in the art, a range includes each individual member. Thus, for example, a group having 1-3 cells refers to groups having 1, 2, or 3 cells. Similarly, a group having 1-5 cells refers to groups having 1, 2, 3, 4, or 5 cells, and so forth.

[0140] All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

[0141] Other embodiments are set forth within the following claims.

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CLAIMS

What is claimed is:

1. A method of diagnosing a subject having, or at risk of contracting, a xenotropic murine leukemia virus-related virus (XMRV) related disease comprising:
 - screening a sample from the subject for the presence of XMRV, wherein the screening comprises an immunoassay using an antibody which recognizes an XMRV polypeptide selected from the group consisting of: gag polyprotein (GAG); capsid protein (CA); matrix protein (MA); nucleocapsid protein (NC); envelope protein (ENV), integrase (IN); and combinations of any two or more thereof; and
 - determining the presence or absence of XMRV in the sample, wherein the presence of XMRV in the sample indicates that the subject has, or is at risk of contracting, an XMRV related disease selected from the group consisting of: prostate cancer, breast cancer, cervical cancer and chronic fatigue syndrome.
2. A method of diagnosing a subject having, or at risk of contracting, a xenotropic murine leukemia virus-related virus (XMRV) related disease comprising:
 - screening a sample from the subject for the presence of XMRV; and
 - determining the presence or absence of XMRV in the sample, wherein the presence of XMRV in the sample indicates that the subject has, or is at risk of contracting, an XMRV related disease selected from the group consisting of: breast cancer and cervical cancer.
3. The method of claim 2, wherein the screening comprises an immunoassay of a body fluid sample for anti-XMRV antibodies.
4. The method of claim 1 or claim 3, wherein the antibody is polyclonal or monoclonal.
5. The method of claim 1 or claim 3, wherein the immunoassay is selected from the group consisting of: ELISA, RIA, ELISPOT, western blot, immunofluorescence, and immunohistochemistry.

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6. The method of claim 1 or claim 2, wherein the screening comprises detecting an XMRV nucleic acid sequence using a nucleic acid detection assay.
7. The method of claim 6, wherein the nucleic acid detection assay comprises primers which hybridize to the XMRV long-terminal repeat (LTR), the XMRV integrase (IN) coding region, or the XMRV envelope (ENV) coding region.
8. The method of claim 6, wherein the nucleic acid detection assay is selected from the group consisting of: PCR, reverse transcriptase PCR, real-time PCR, and quantitative PCR (qPCR).
9. The method of claim 1 or claim 2, wherein the sample is selected from the group consisting of: saliva, semen, peritoneal fluid, synovial fluid, prostatic secretions, cervical secretions, blood, serum, plasma, tissue, and cells.
10. The method of claim 9, wherein the sample is tissue selected from the group consisting of prostate tissue, breast tissue or cervical tissue.
11. The method of claim 10, wherein the sample is prostate tissue.
12. The method of claim 11, wherein the XMRV virus is detected in the epithelial cells or stromal cells of the prostate tissue sample.
13. A method of assessing the prognosis of a subject with prostate cancer comprising:
screening a sample from the subject for the presence of xenotropic murine leukemia virus-related virus (XMRV), wherein the screening comprises conducting an immunoassay using an antibody which recognizes an XMRV polypeptide selected from the group consisting of: gag polyprotein (GAG); capsid protein (CA); matrix protein (MA); nucleocapsid protein (NC); envelope protein (ENV), integrase (IN); and combinations of any two or more thereof; and
determining the presence or absence of XMRV in the sample, wherein the presence of XMRV is an indication of an aggressive form of the cancer and a poor prognosis for the patient.

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14. The method of claim 13, wherein the immunoassay comprises a plurality of antibodies which recognize at least the following XMRV polypeptides: gag polyprotein (GAG); capsid protein (CA); matrix protein (MA); nucleocapsid protein (NC); and envelope protein (ENV).
15. The method of claim 13, wherein the antibody is polyclonal or monoclonal.
16. The method of claim 13, wherein the immunoassay is selected from the group consisting of: ELISA, RIA, ELISPOT, western blot, immunofluorescence, and immunohistochemistry.
17. The method of claim 13, wherein the screening further comprises detecting an XMRV nucleic acid sequence using a nucleic acid detection assay.
18. The method of claim 17, wherein the nucleic acid detection assay comprises primers which hybridize to the XMRV long-terminal repeat (LTR), the XMRV integrase (IN) coding region, or the XMRV envelope (ENV) coding region.
19. The method of claim 17, wherein the nucleic acid detection assay is selected from the group consisting of: PCR, reverse transcriptase PCR, real-time PCR, and quantitative PCR (qPCR).
20. The method of claim 13, wherein the sample is selected from the group consisting of: prostatic secretions, prostate tissue; and prostate cells.
21. The method of claim 20, wherein the sample is prostate tissue.
22. The method of claim 21, wherein the XMRV virus is detected in the epithelial cells or stromal cells of the prostate tissue sample.
23. A method for the detection of xenotropic murine leukemia virus-related virus (XMRV) in a sample comprising screening the sample for the presence of XMRV, wherein the screening comprises conducting an immunoassay using an antibody which recognizes an XMRV polypeptide selected from the group consisting of: gag polyprotein (GAG); capsid protein (CA); matrix protein (MA); nucleocapsid protein

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- (NC); envelope protein (ENV); Integrase (IN); and combinations thereof; and determining the presence or absence of XMRV in the sample.
24. The method of claim 23, wherein the immunoassay comprises antibodies which recognize at least the following XMRV polypeptides: gag polyprotein (GAG); capsid protein (CA); matrix protein (MA); nucleocapsid protein (NC); and envelope protein (ENV).
 25. The method of claim 23, wherein the antibody is polyclonal or monoclonal.
 26. The method of claim 23, wherein the immunoassay is selected from the group consisting of: ELISA, RIA, ELISPOT, western blot, immunofluorescence, and immunohistochemistry.
 27. The method of claim 23, wherein the screening further comprises the use of a nucleic acid detection assay.
 28. The method of claim 27, wherein the nucleic acid detection assay comprises primers which hybridize to the XMRV long-terminal repeat (LTR), the XMRV integrase (IN) coding region or the XMRV envelope (ENV) coding region.
 29. The method of claim 27, wherein the nucleic acid detection assay is selected from the group consisting of: PCR, reverse transcriptase PCR, real-time PCR, and quantitative PCR (qPCR).
 30. The method of any one of claims 23-29, wherein the sample is selected from the group consisting of saliva, semen, peritoneal fluid, synovial fluid, prostatic secretions, cervical secretions, blood, serum, plasma, tissue; and cells.
 31. The method of claim 30, wherein the sample comprises Leydig cells from testes.

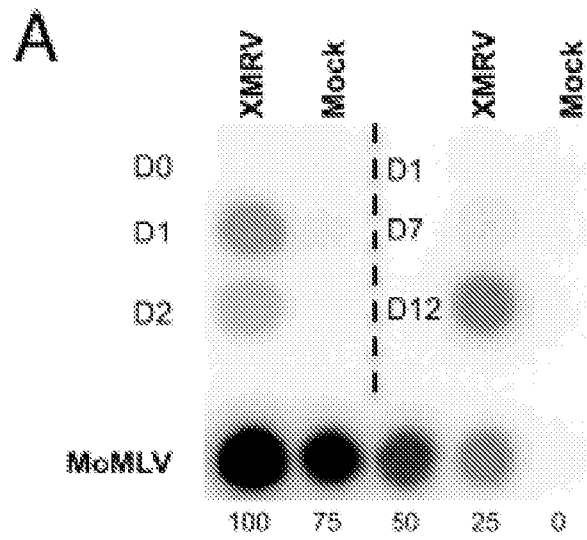


FIG. 1A

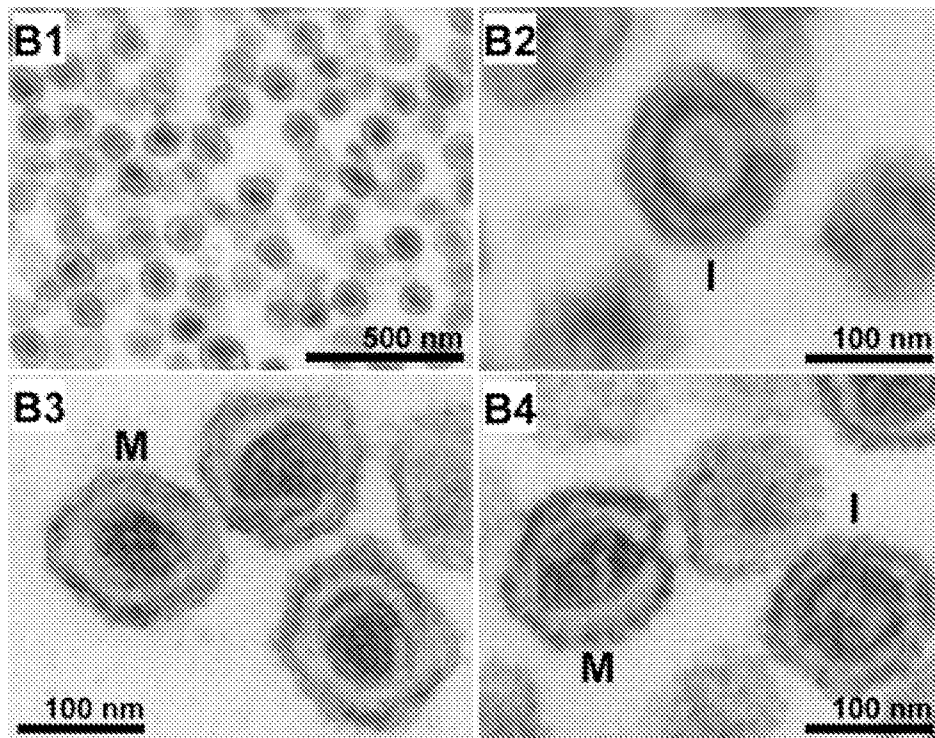


FIG. 1B

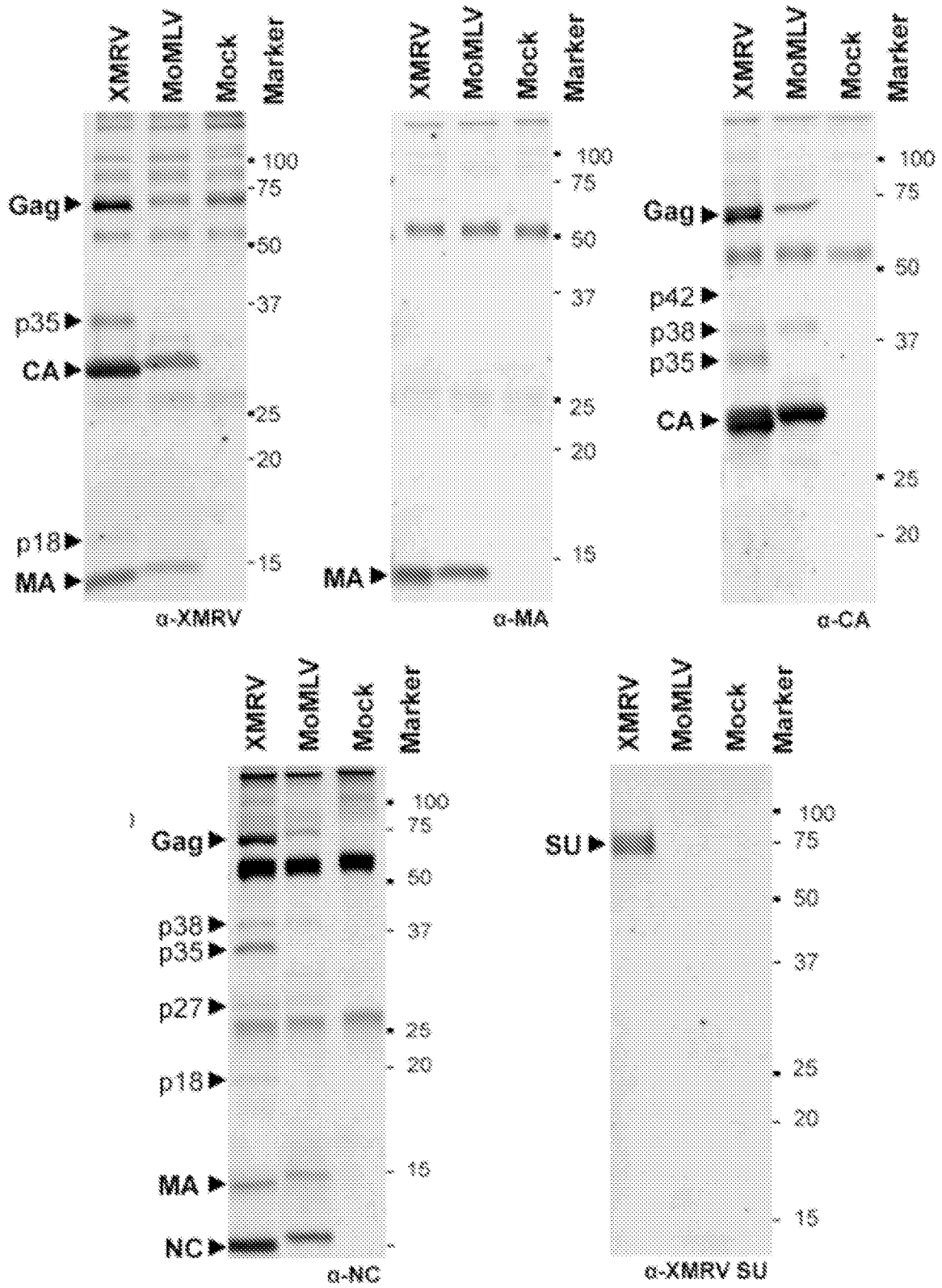


FIG. 1C

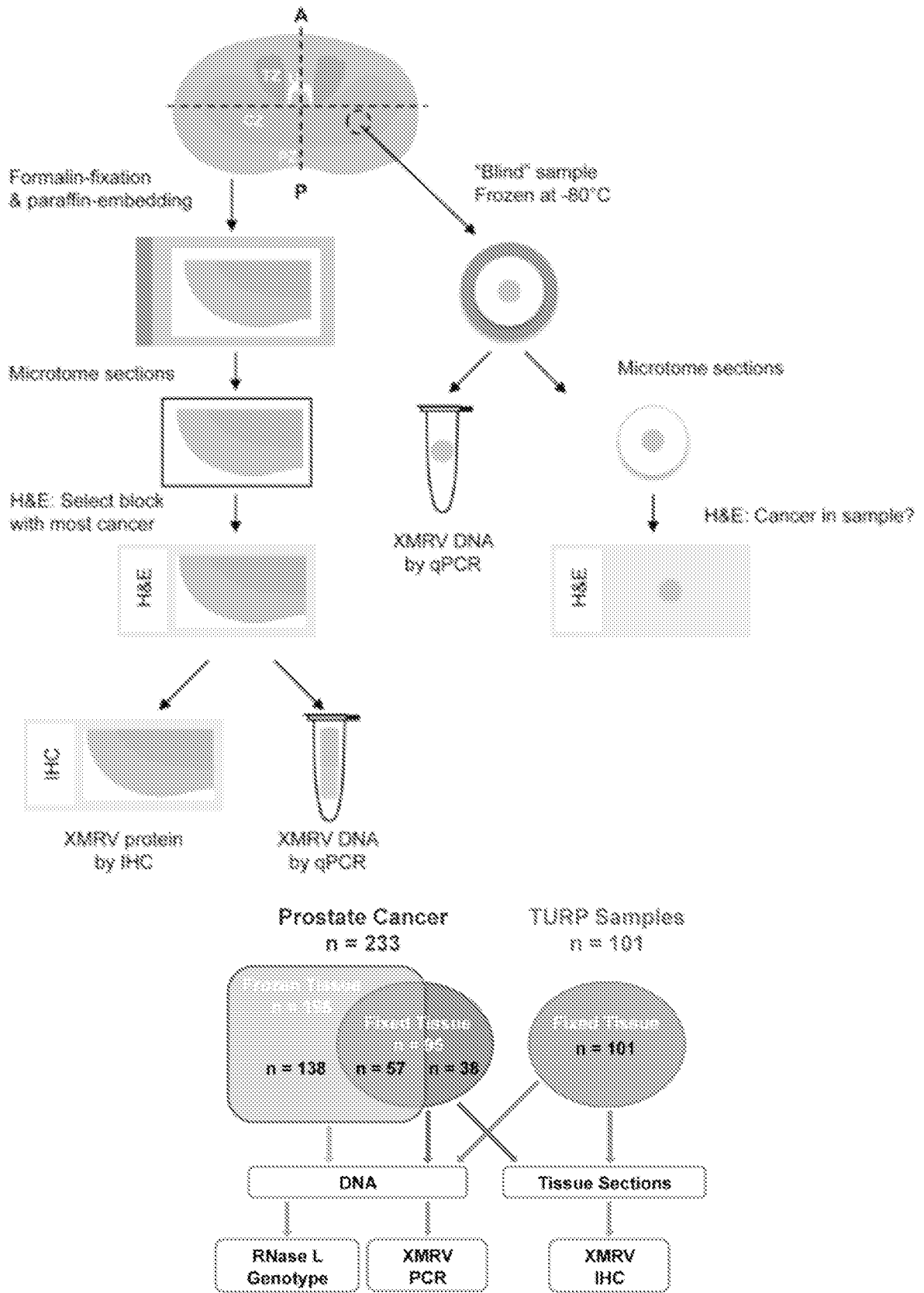


FIG. 2

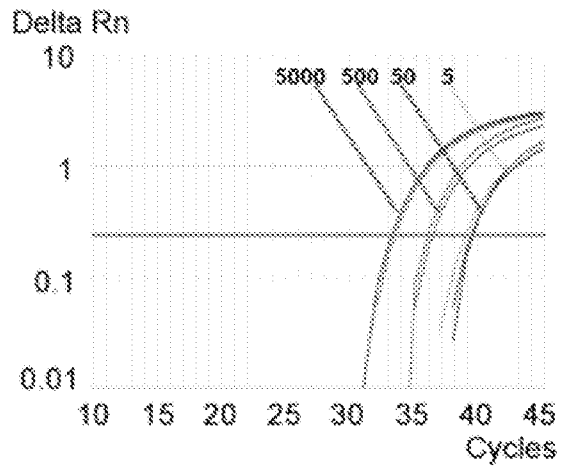


FIG. 3B

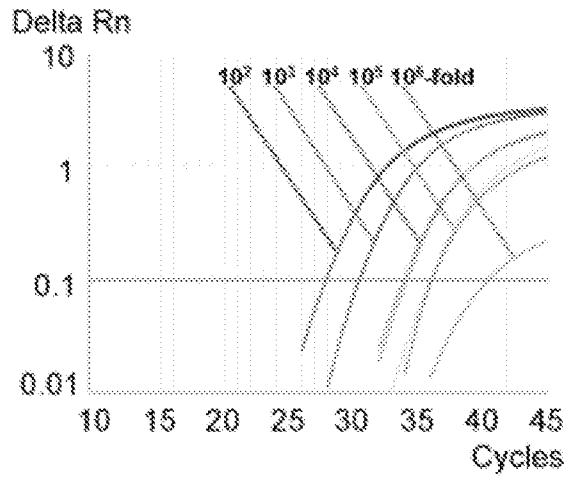


FIG. 3C

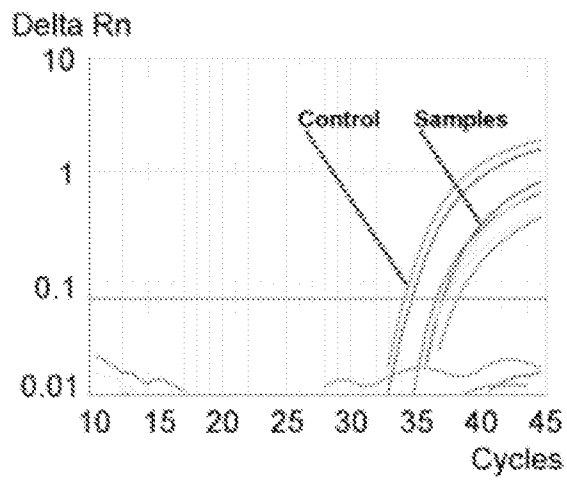


FIG. 3D

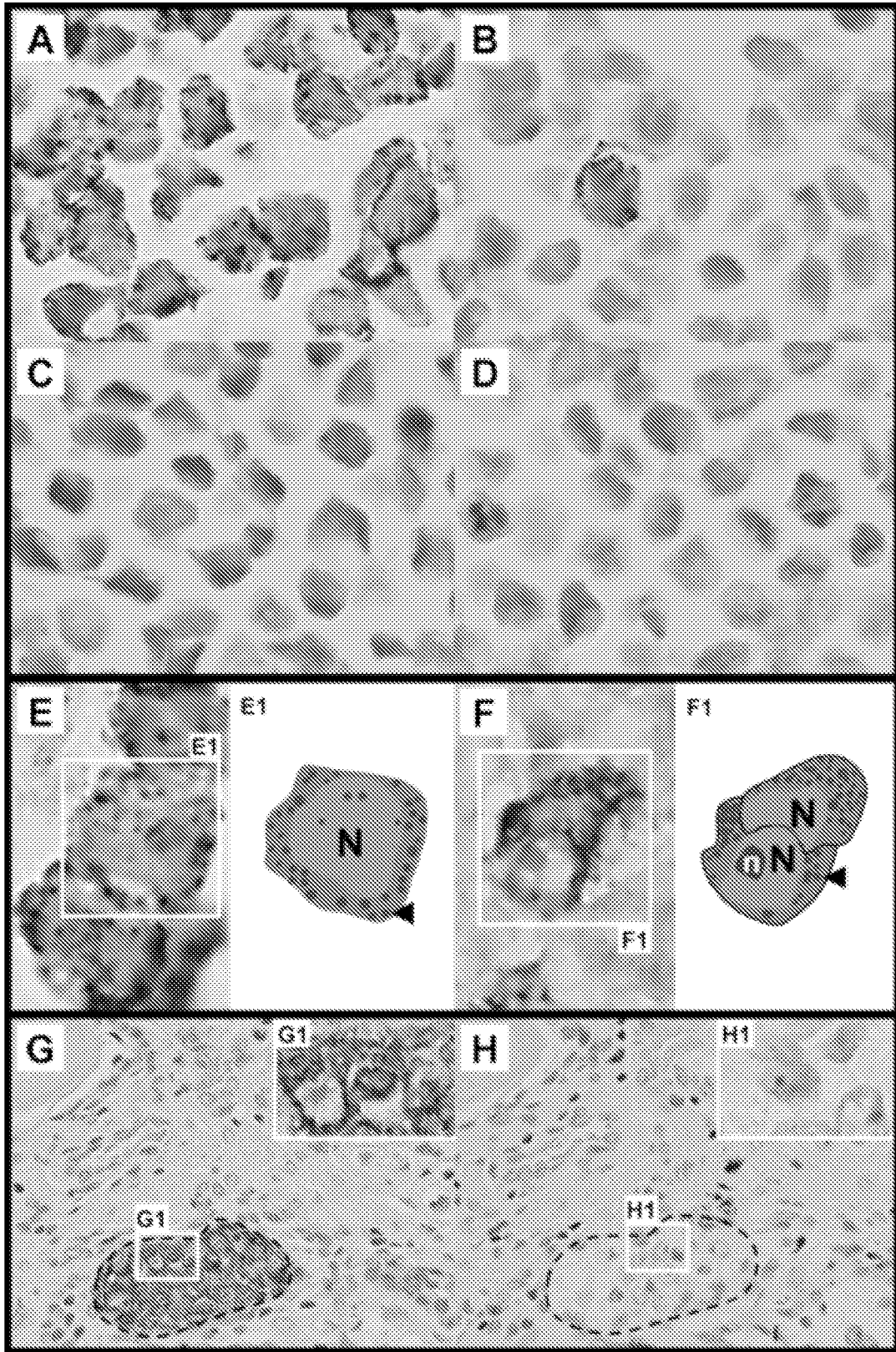


FIG. 4

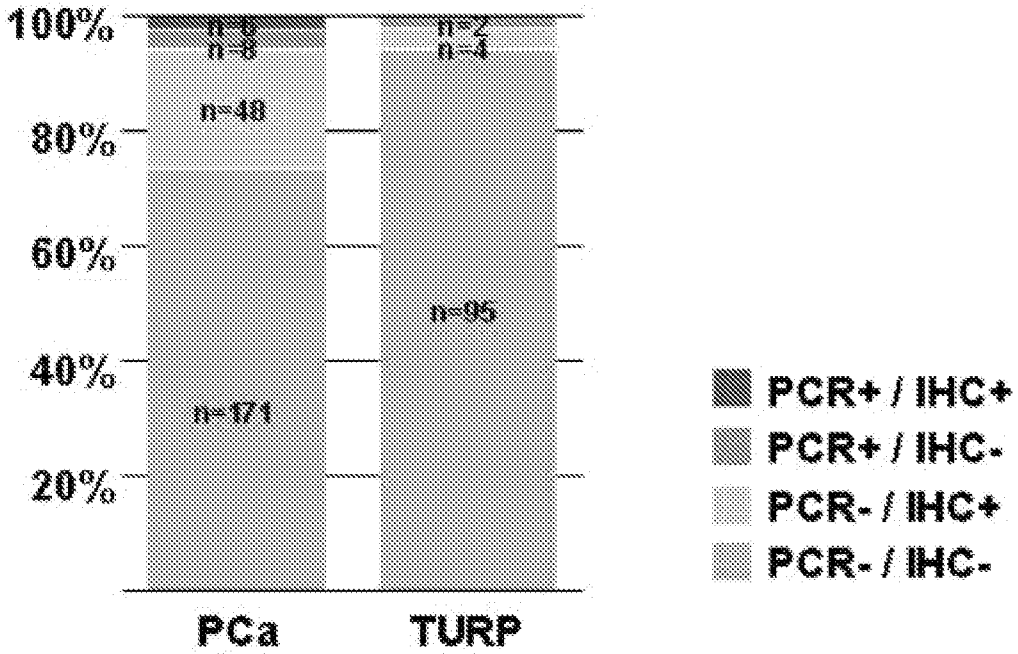


FIG. 5A

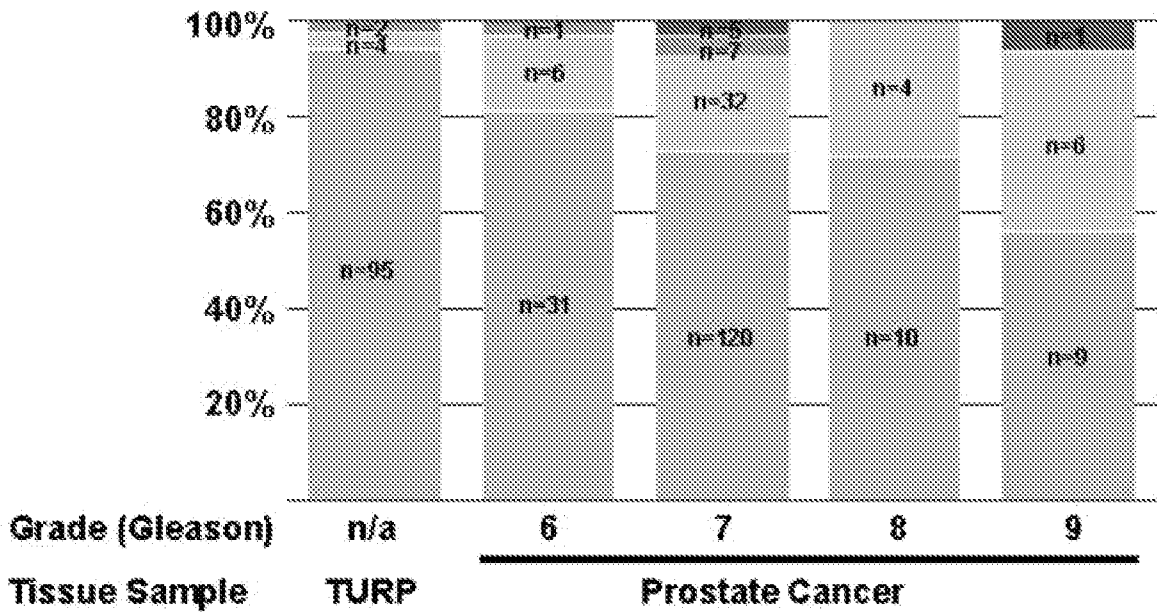


FIG. 5B

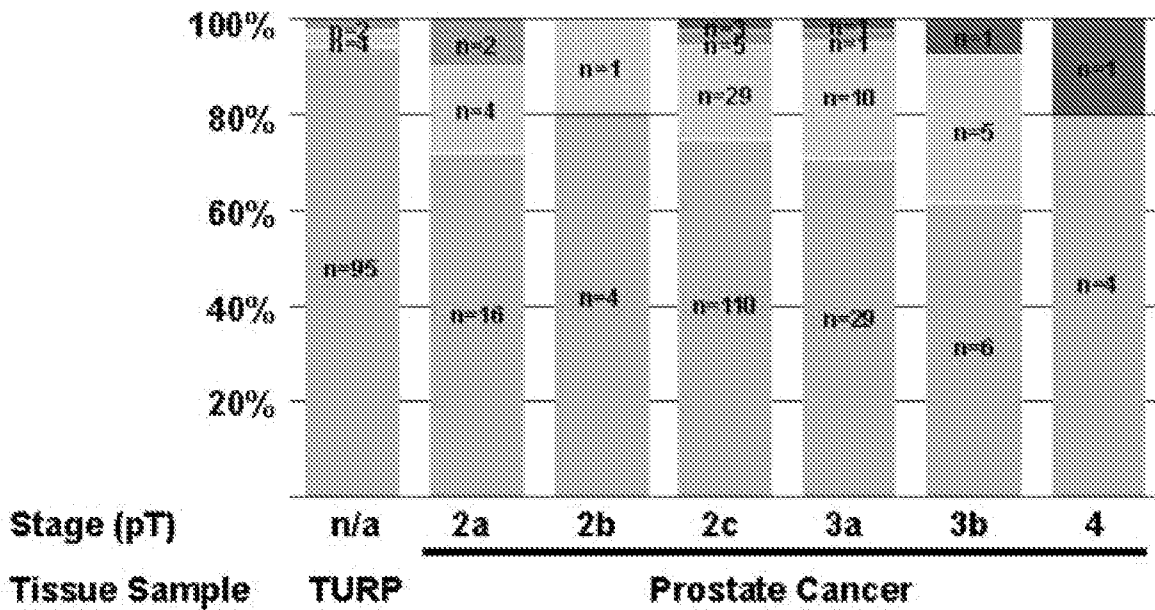


FIG. 5C

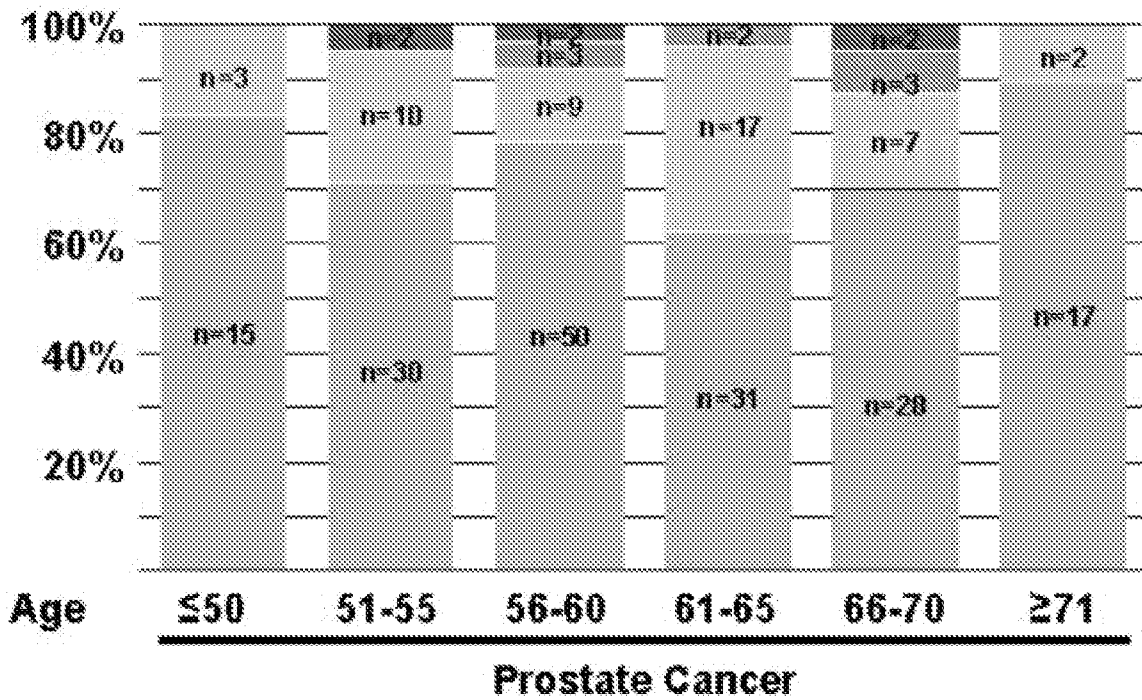


FIG. 5D

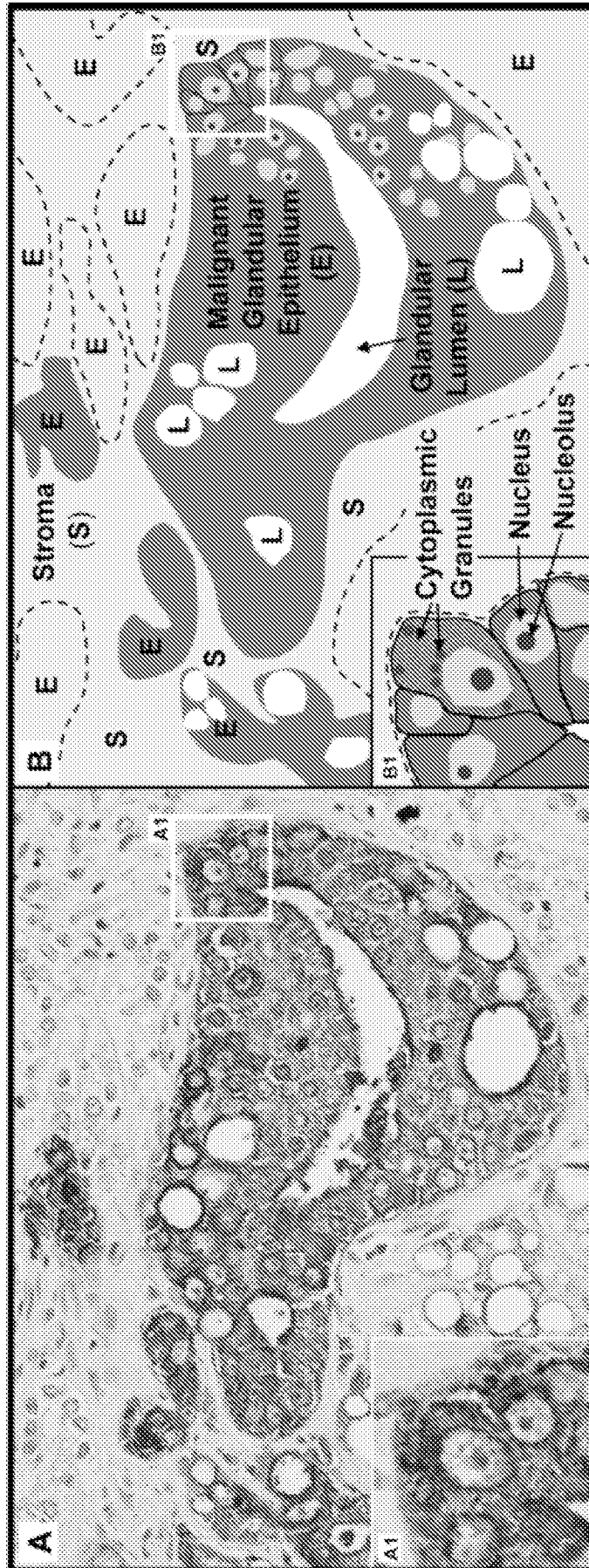


FIG. 6

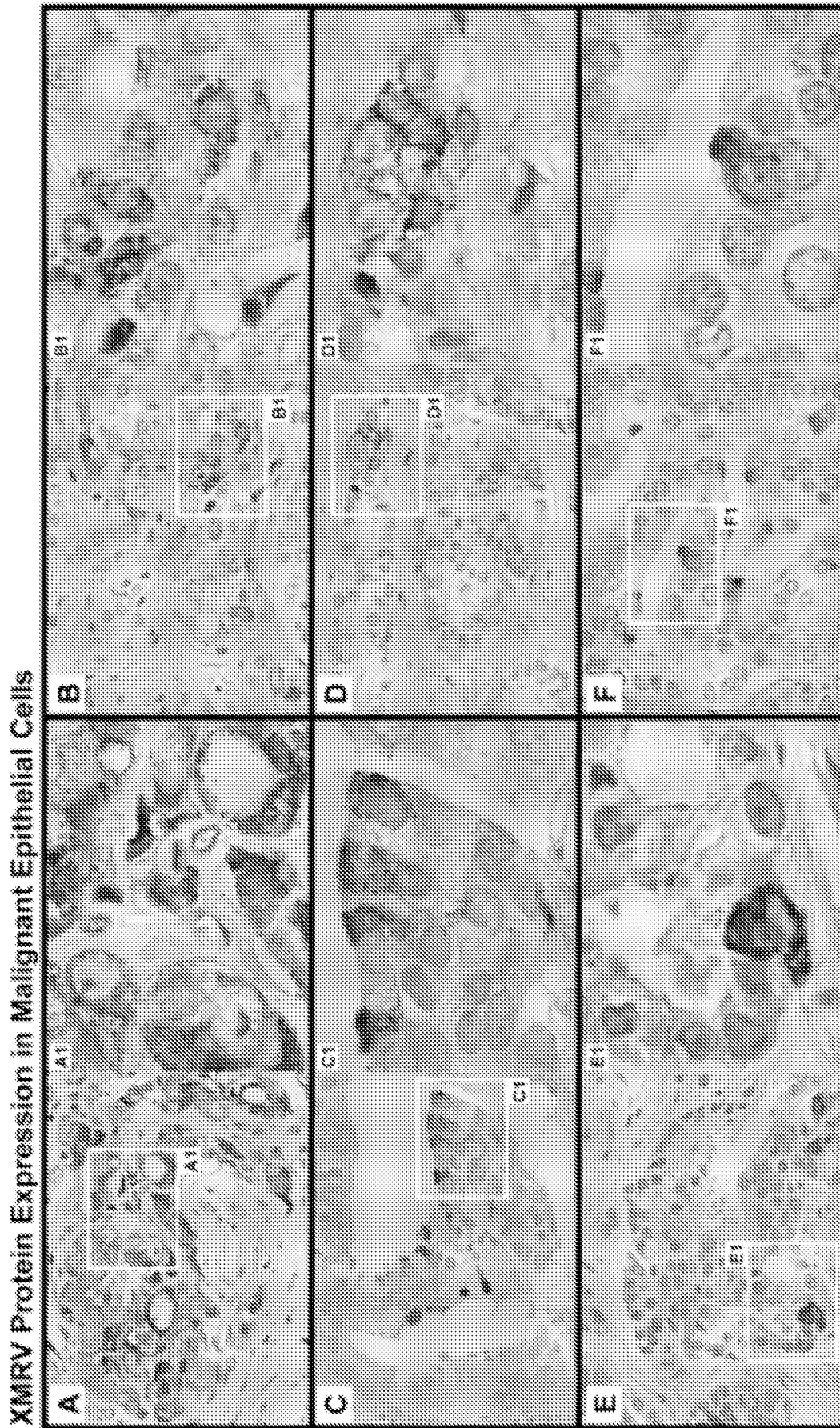


FIG. 7A-F

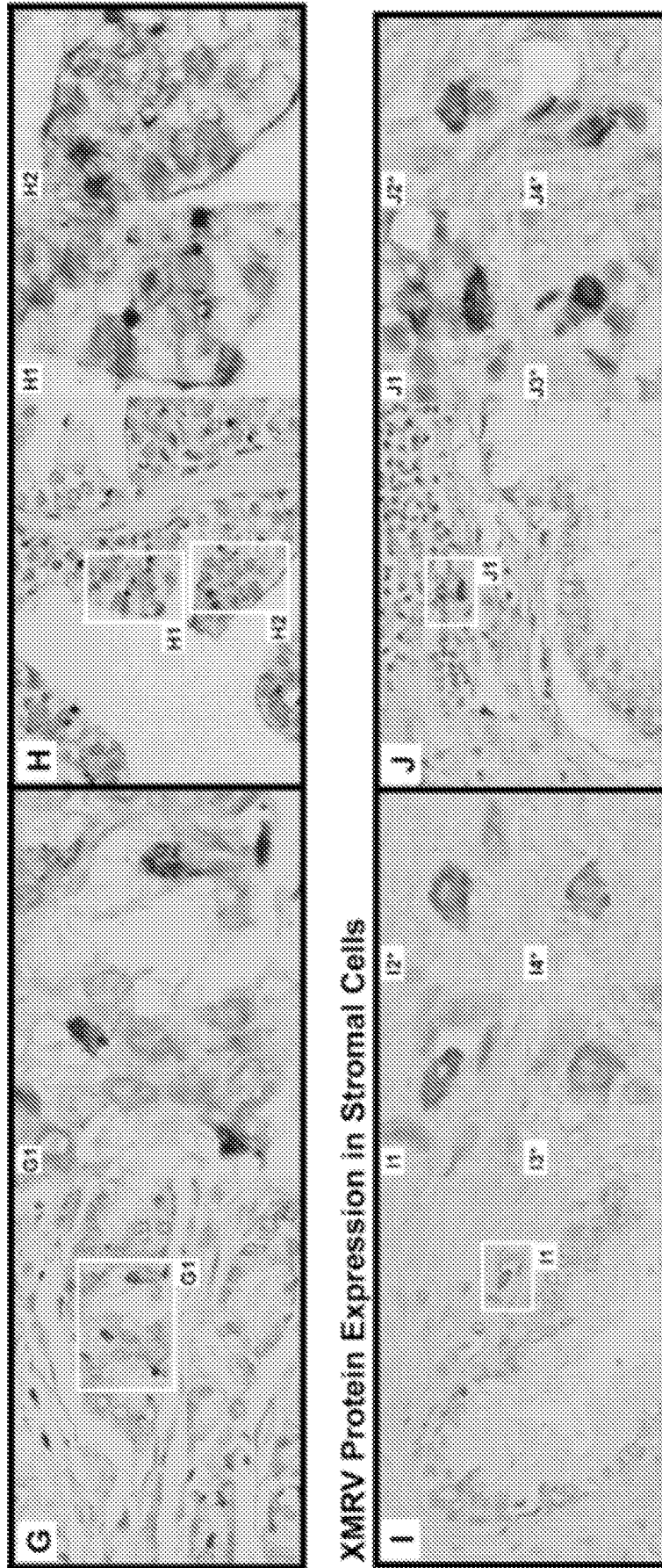


FIG. 7G-J

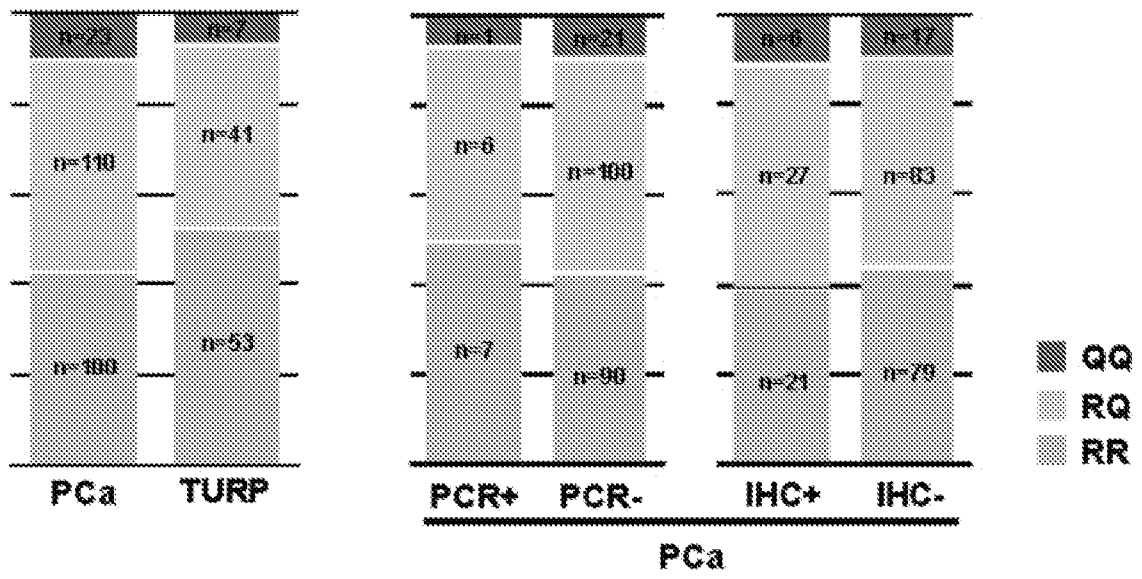


FIG. 8

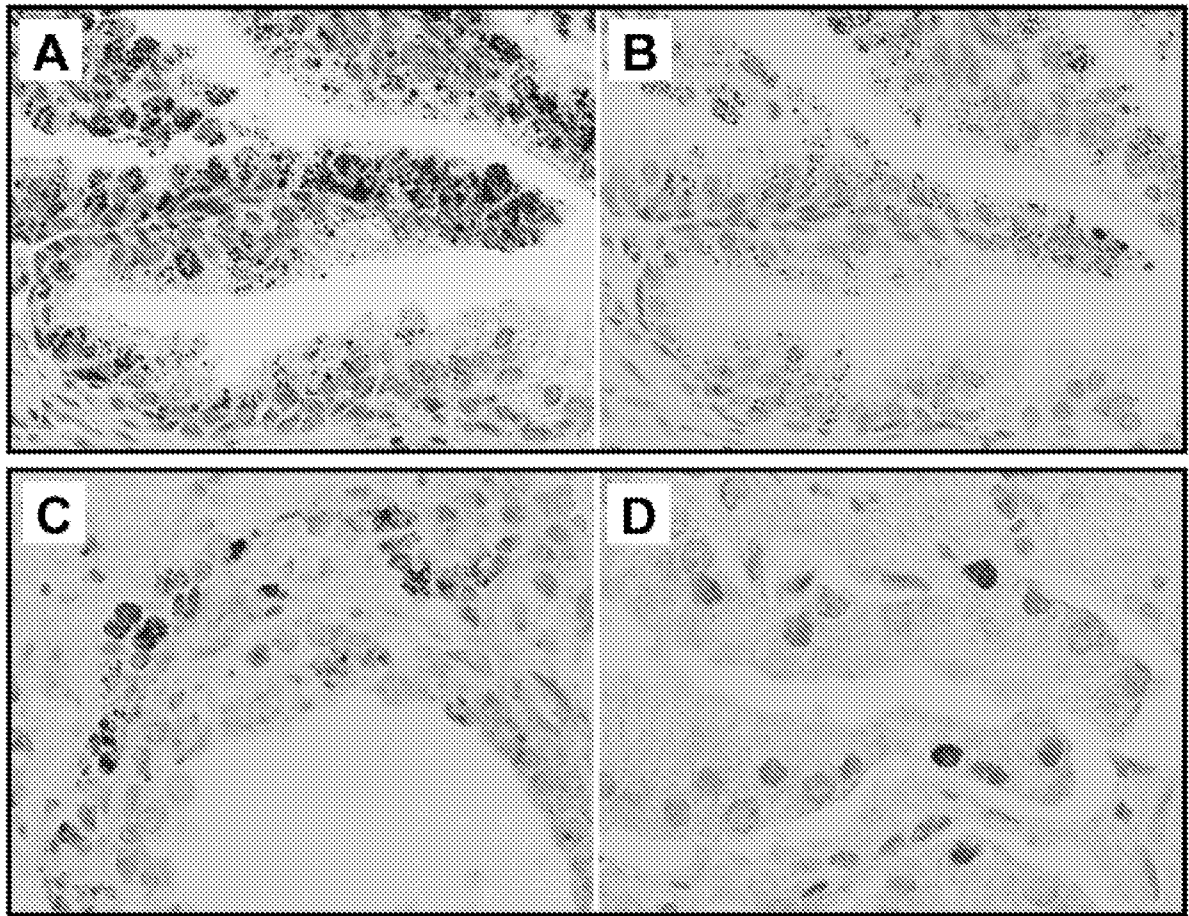


FIG. 9

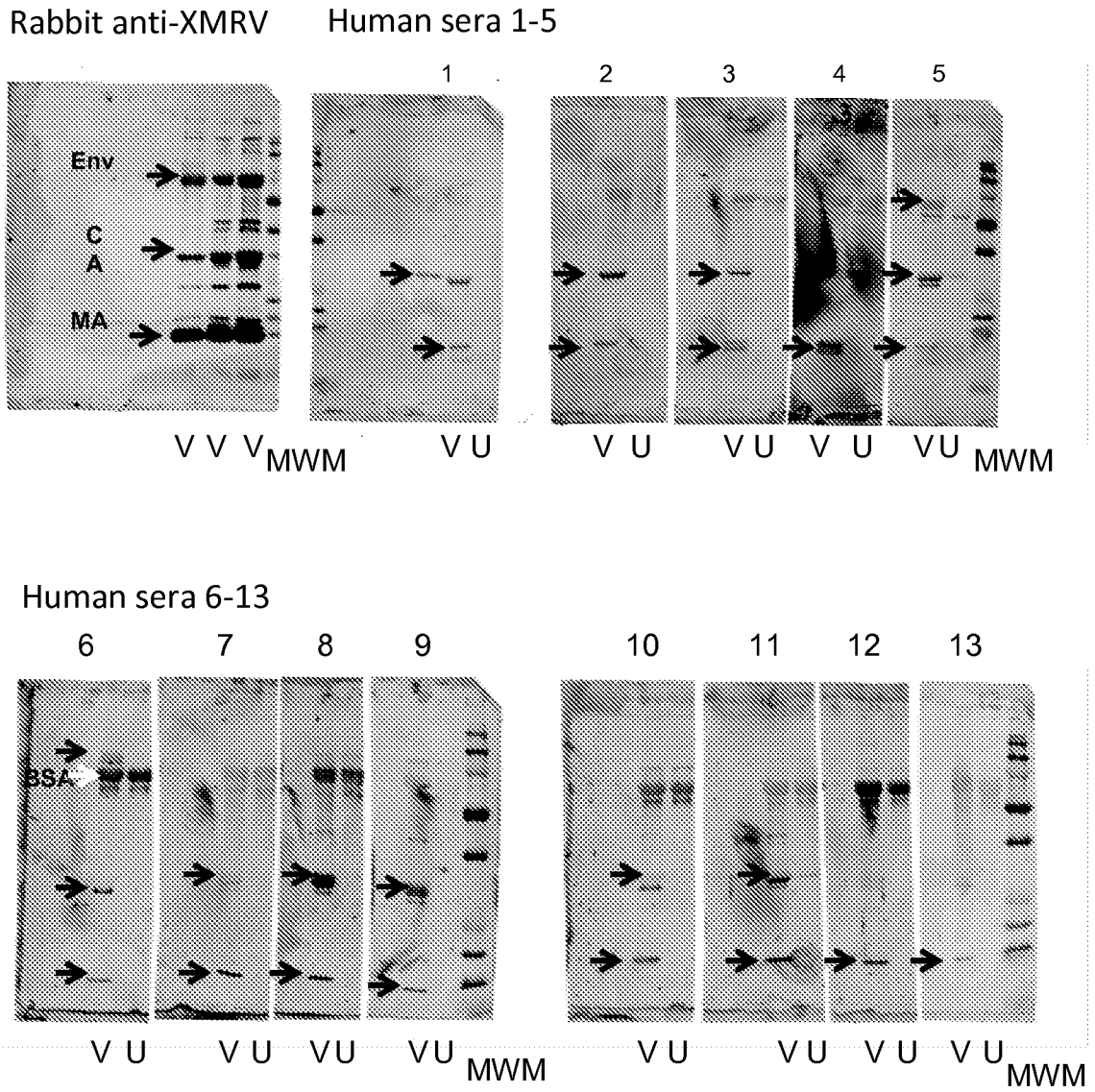


FIG. 10

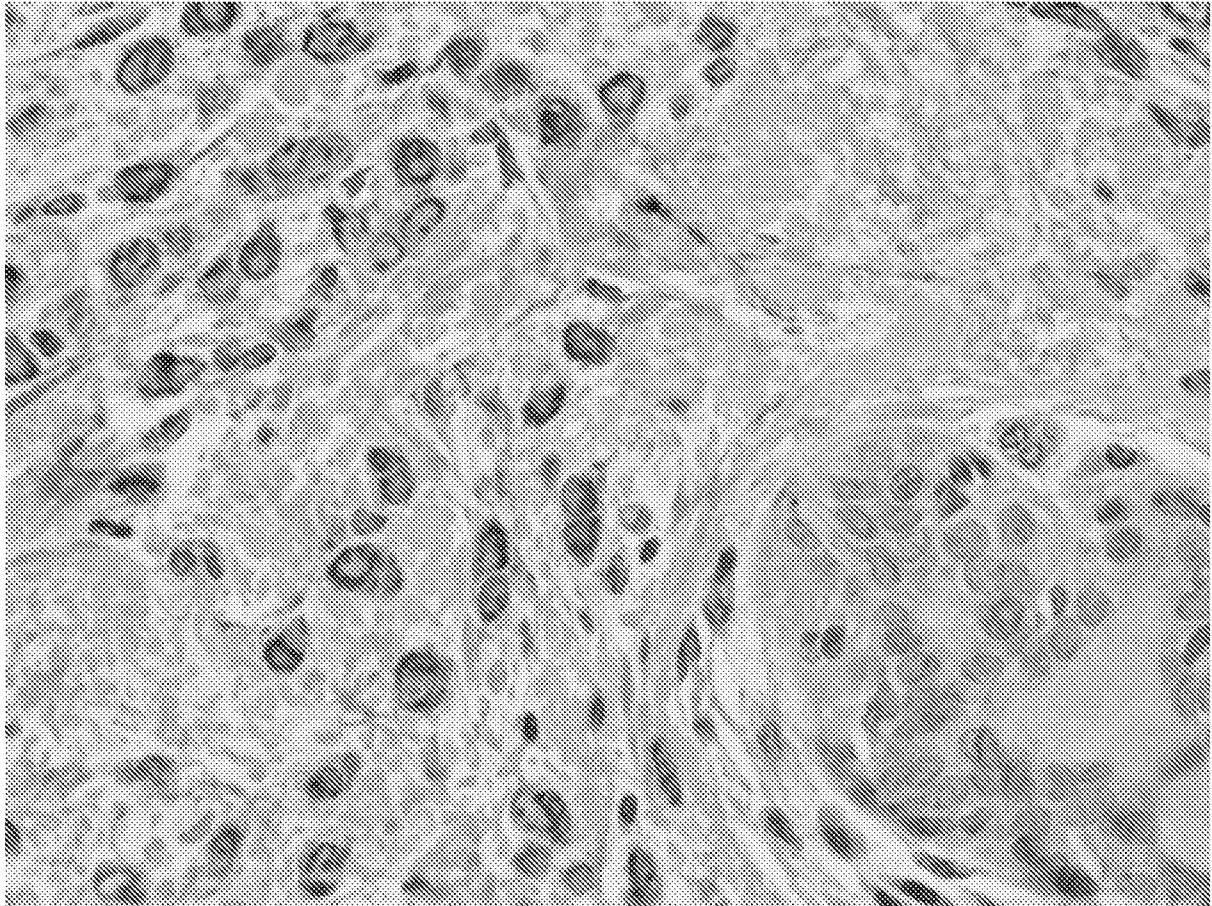


FIG. 11

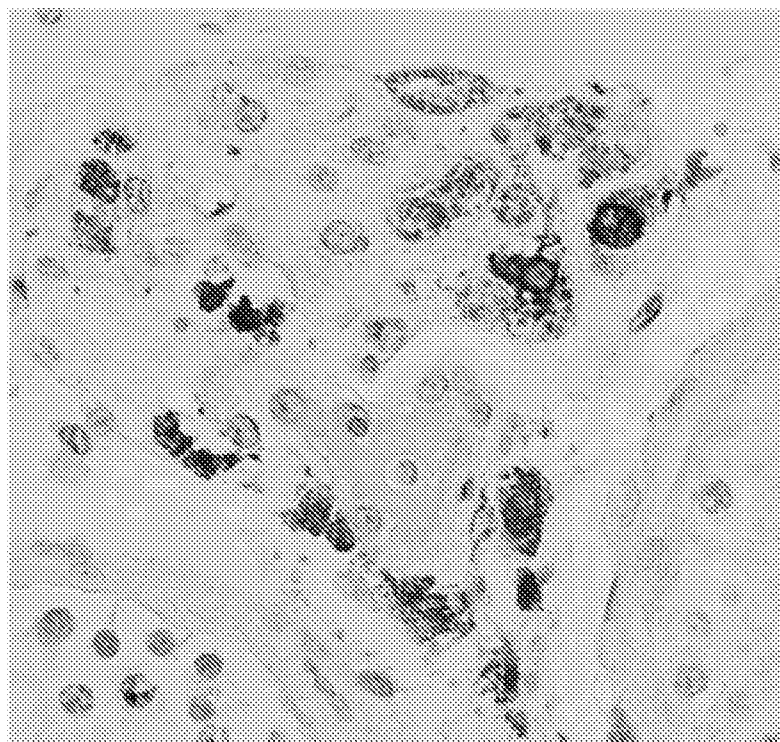
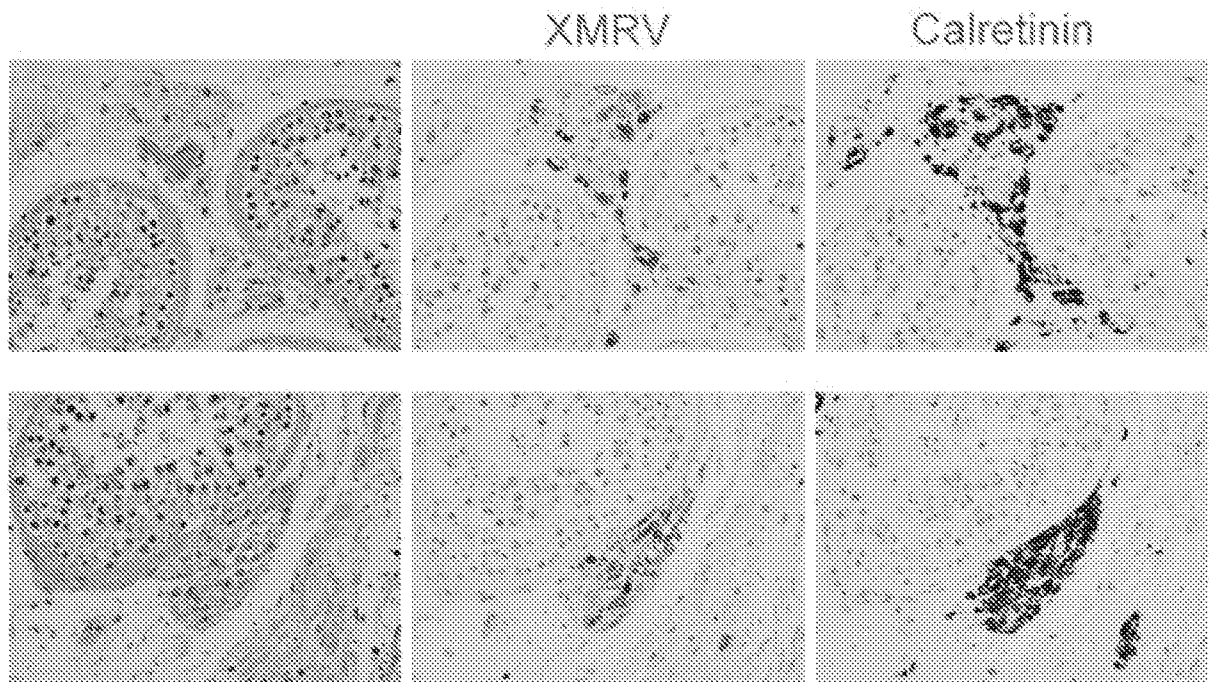


FIG. 12

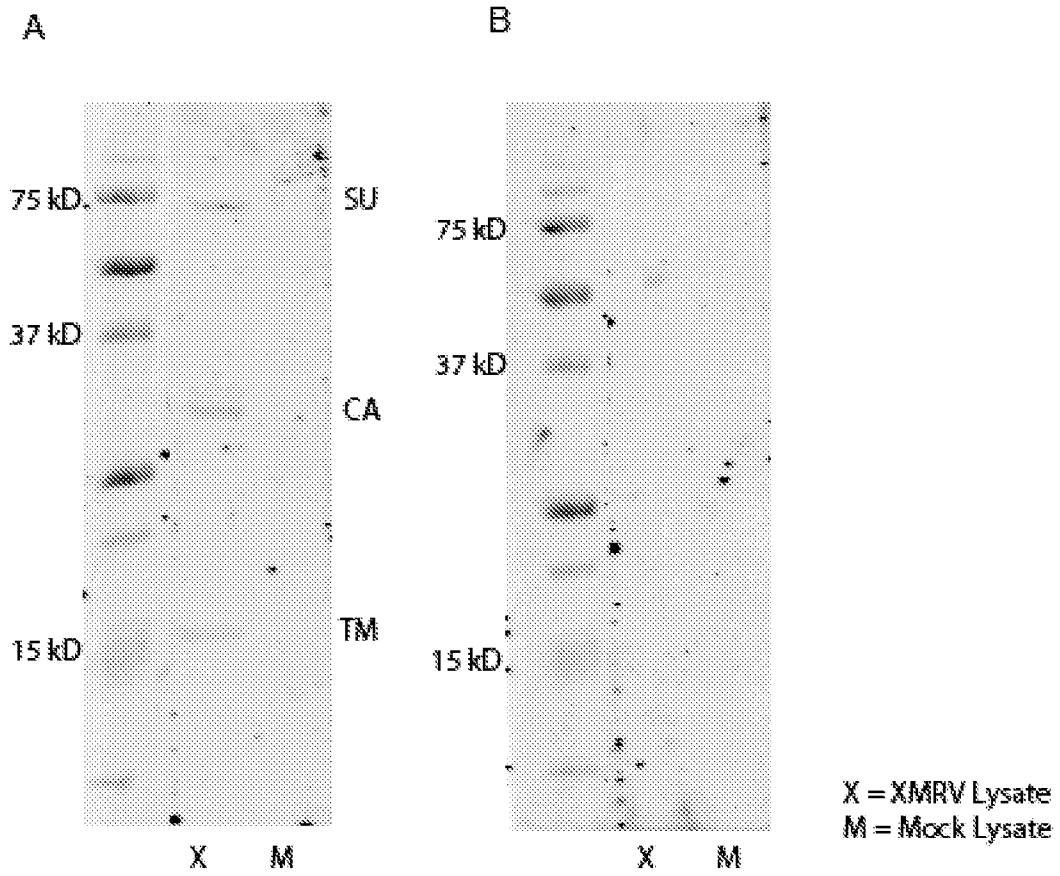


FIG. 13

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2010/035144

A. CLASSIFICATION OF SUBJECT MATTER Int. Cl. <i>C12Q 1/04</i> (2006.01) <i>C12N 7/00</i> (2006.01) <i>C07K 16/08</i> (2006.01) <i>G01N 33/53</i> (2006.01)		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) MEDLINE, BIOSIS, CAPLUS, WPIDS, EPODOC (XMVR, Xenotropic murine leukemia virus related)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/110589 A2 (THE CLEVELAND CLINIC FOUNDATION) 19 October 2006 (Abstract, page 2 line 32 to page 3 line 19, page 44 line 18- page 46 line 8, page 46 line 9-page 47 line 12, page 62 lines 20-33, page 67 line 1-14, page 81 line 19-28, page 79 lines 16-22, page 82 line 6, page 98 line 25, Fig 12, Example 1.2, Claims 16-20)	1, 4-12 and 23-30
X	URISMAN et al. 'Identification of a novel Gammaretrovirus in prostate tumors of patients homozygous for R462Q <i>RNASEL</i> variant', PLoS Pathogens. 2006, Vol 2, No 3, pages 0211-0255. (Whole document)	1, 4-12 and 23-30
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "E" earlier application or patent but published on or after the international filing date "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "O" document referring to an oral disclosure, use, exhibition or other means "&" document member of the same patent family "P" document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search 29 June 2010	Date of mailing of the international search report <p style="text-align: center; font-weight: bold;">30 JUL 2010</p>	
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. +61 2 6283 7999	Authorized officer ANITA PREMKUMAR AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 2 6283 2572	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2010/035144

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FISCHER et al, 'Prevalence of human gammaretrovirus XMRV in sporadic prostate cancer', Journal of Clinical Virology, 2008, Vol 43, pages 277-283. (Abstract, page 280, 2.1, 2.2 and 3.1)	6 and 8-11

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2010/035144

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	2006110589	AU	2006235266	CA	2603863	EP	1882032
		MX	2007012361				

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX