Prostate cancer progression correlates with increased humoral immune response to a human endogenous retrovirus GAG protein

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Prediction of prostate cancer progression and mortality has relied on several known biomarkers, including Gleason score, serum PSA and PSMA levels. Here, we explore the expression and immunogenicity of a new endogenous retroviral antigen that correlates with progression of prostate cancer. This antigen was found in primary and metastatic prostate cancer tissues and cell lines, and we described mechanisms driving its specific expression. Moreover, the spontaneous serum antibody response to this antigen in a large number of cancer prostate patients revealed an association with clinical progression of cancer. As a result, we propose that measuring expression and serological responses for this new antigen could have a rapid impact on the clinical course and management of prostate cancer. In a long-term perspective, modulating the immunogenicity to a retroviral endogenous antigen would be a novel immunointervention strategy.
Abstract

**Purpose:** Human endogenous retroviruses (HERV) encode 8% of the human genome. While HERVs may play a role in autoimmune and neoplastic disease, no mechanistic association has yet been established. We studied the expression and immunogenicity of a HERV-K GAG protein encoded on chromosome 22q11.23 in relation to the clinical course of prostate cancer.

**Experimental design:** *In vitro* expression of GAG-HERV-K was analyzed in panels of normal and malignant tissues, microarrays, and cell lines, and effects of demethylation and androgen stimulation were evaluated. Patient sera were analyzed for seroreactivity to GAG-HERV-K and other self-antigens by ELISA and seromics (protein array profiling).

**Results:** GAG-HERV-K expression was most frequent in prostate tissues, and regulated both by demethylation of the promoter region and by androgen stimulation. Serum screening revealed that antibodies to GAG-HERV-K are found in a subset of prostate cancer patients (33/483, 6.8%), but rarely in male healthy donors (1/55, 1.8%). Autoantibodies to GAG-HERV-K occurred more frequently in advanced prostate cancer patients (29/191 in stage III-IV, 21.0%) than in early prostate cancer (4/292 in stage I-II, 1.4%). Presence of GAG-HERV-K serum antibody was correlated with worse survival of prostate cancer patients, with a trend for faster biochemical recurrence in patients with antibodies to GAG-HERV-K.

**Conclusions:** Preferential expression of GAG-HERV-K ch22q11.23 in prostate cancer tissue and increased frequency of autoantibodies observed in advanced prostate cancer patients make this protein one of the first bona fide retroviral cancer antigens in humans, with potential as a biomarker for progression and biochemical recurrence rate of prostate cancer.
INTRODUCTION

Human endogenous retroviruses (HERV) are genome modifiers of exogenous origin that became integrated in the human genome millions of years ago and now represent 8% of total DNA sequences. They are classified into 30-50 families, family K being the most recently identified (1). HERVs contain genes which encode polyproteins flanked by two long terminal repeats (LTRs) (2). Most HERV genes have mutations or deletions in their coding and promoter sequence, which compromise their gene and/or protein expression. Additionally, HERVs expression in normal tissue is usually suppressed by DNA methylation (3-6).

Endogenous retroviruses have been closely linked as the etiological agent to a wide variety of animal cancers. One of the best examples is the MuLV, a mouse endogenous retrovirus associated with leukemia in murine hosts (7, 8). Serological techniques have been critical in establishing the complex relation between these viruses and their hosts (9-12). Our group developed a powerful approach for detecting cancer antigens based on patient humoral immune response (13, 14). Taking advantage of this technique and by using a prostate cancer serum sample, we previously identified a GAG protein derived from human endogenous retrovirus K located on chromosome 22q11.23 (GAG-HERV-K ch22q11.23, NGO-Pr-54) (15). This endogenous retrovirus has been associated with chromosomal rearrangements occurring in prostate cancer that create fusion genes in which the promoter region of GAG-HERV-K ch22q11.23 translocates downstream of ETV1 gene leading to abnormal expression of ETS transcriptional factor (16, 17).

In spite of the fact that most HERVs have mutated or deleted coding sequences, intact ORFs exist and give rise to transcripts that can be detected by real-time PCR (qPCR) (15, 18, 19). Although no expression at the protein level has been clearly identified, immune responses against various HERV components have been widely
demonstrated in different types of cancer, including prostate cancer (20-24). However, a clear correlation between immune response to HERV components and clinical stage has not been intently addressed in prostate cancer.

Prostate cancer is one of the most frequent causes of male cancer-related death (25). Clinical staging of prostate cancer is important in assessing the risk of the disease and therefore for treatment recommendations (26). Still, improved clinical staging biomarkers are required for more reliable prediction of pathological stage in prostate cancer (27-30). The discovery of an endogenous retrovirus antigen highly expressed in prostate cancer and able to elicit humoral immune responses related to disease progression make this an attractive potential new biomarker for clinical staging and a novel target for immunotherapy. In this work, we explore the correlation between humoral immune response to HERV antigen and cancer progression and show mechanisms that could be contributing to HERV expression in prostate cancer.
MATERIAL AND METHODS

Patient samples and cell lines
Sera were collected with informed consent under protocols approved by the Institutional Review Board of Memorial Sloan-Kettering Cancer Center and by the Ethics Review Board from Krankenhaus Nordwest. Serum samples were collected at Memorial Sloan-Kettering Cancer Center, New York, USA, or provided by Dr. Elke Jäger from Krankenhaus Nordwest, Frankfurt, Germany, typically at time of diagnosis. Prostate cancer patient sera were divided according to clinical stage of the disease (I to IV) using the TNM classification system (American Joint Committee on Cancer), as well as according to Gleason score whenever available. Patient characteristics for a subset of patients are shown in Table 1. Cell lines were derived at Memorial Sloan-Kettering Cancer Center, New York, USA or purchased from ATCC. All human cell lines were maintained in RPMI containing 10% FBS, 1% L-glutamine at 37°C in a 5% CO₂ atmosphere.

Probes and monoclonal antibodies
The IgG1 monoclonal antibody to anti-GAG-HERV-K, clone TI-35, was previously generated by our group (15). Antibodies for human androgen receptor (AR-441 - clone ab9474, 1:1,000) and actin (clone ab8227, 1:10,000) were obtained from Abcam (Cambridge, MA - USA). Taqman primers and probes for PSA, BCR, ZDHHC8P1, IGGL1, RGL4 and TFRC genes were obtained from Amersham Bioscience (USA). GAG-HERV-K primers sequences were GAG22q11.23-F (5’-CGC AGG TTA GAC AAG CAC AA-3’) and GAG22q11.23-R (5’-CTC AAG ATC GCC CTG TTT TC-3’).
RNA extraction

RNA extraction was performed using TRizol Reagent (Gibco), according to manufacturer’s instructions with few modifications. Briefly, 5-10 x 10^6 cells, or 100 mg of cancer tissue were homogenized with 1 mL TRizol Reagent and incubated for 5 minutes at room temperature. Two drops of OCT and 100 μL BCP were added to the solution and vortexed. The samples were centrifuged for 15 minutes at 4ºC and the aqueous phase was collected for RNA precipitation by isopropanol. The resuspended RNA was submitted to DNAse treatment according to manufacturer’s instructions (Ambion, USA). The concentration was determined using NanoPhotometer® (IMPLEM, United Kindom). Total RNA derived from normal tissues was obtained from Clontech and Ambion.

Real-time PCR (qPCR)

cDNA was generated from 2 μg of total RNA using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen), according to manufacturer’s instructions. Real-time PCR was performed using Sybr®-green Master Mix (Invitrogen) for GAG-HERV-K chr22q11.23 or Taqman Master Mix (ABI) for AR and PSA on an ABI Prism 7500 Fast real-time PCR system machine (Applied Biosystems, USA) using specific primers and probes. Relative PCR analysis was completed using ABI Prism SDS software. Real-time PCR products were analyzed by electrophoresis and by sequencing. GAPDH (Sybr®-green system) and TFRC (Taqman system) were used as housekeeping genes.

TI-35 mAb characterization

The generation and characterization of mAb TI-35 was previously described by our group (15). In addition, transfection of HeLa cells with GFP-fused GAG-HERV-K
construct or control GFP was performed to assess the subcellular localization of TI-35 staining by confocal microscopy and immunofluorescence. TI-35 specificity was further assessed by IHC with formalin-fixed snap-frozen pellets of Sf9 insect cells infected with GAG-HERV-K recombinant or control baculovirus. Additionally, TI-35 reactivity was assessed by western blot and ELISA against GAG-HERV-K synthetic overlapping long peptides, recombinant GAG-HERV-K proteins produced in E. coli and baculovirus as well as control proteins, and cell extracts from GAG-HERV-K-positive VCaP cells before and after treatment with androgen (see details below).

**Immunohistochemistry**

For the immunohistochemical detection of GAG-HERV-K, monoclonal antibody TI-35 was employed. TI-35 immunostaining was done in snap-frozen specimens as well as in formalin-fixed paraffin-embedded tissue. For paraffin sections, 0.5 μg/ml of TI-35 using EDTA (pH 9.0, 1 mM) as antigen retrieval solution achieved best staining results. For primary detection, the Powervision kit (Leica Bioystems, Richmond, IL) was used. Immunohistochemical staining was done on 5 micron tissue sections applied to slides for immunohistochemistry (Superfrost Plus, Menzel, Braunschweig, Germany). Slides were heated for proper attachment at 60°C for three hours. For immunostaining, slides were deparaffinized in xylene and rehydrated in a series of graded alcohols followed by antigen retrieval. The latter consisted of heating slides in EDTA solution at 97°C for 30 minutes. Primary incubation was done overnight at 4°C followed by the powervision secondary. Diaminobenzidine (liquid-DAB, Biogenex, San Ramon, CA) served as a chromogen and Gill’s #2 hematoxylin was used as a counterstain.

Because mAb TI-35 stained many tissues with different intensity, intensity of staining was scored in addition to the more classic extent of staining. The immunoreactivity of TI-35 was graded based on extent of immunopositive tumor areas as follows: negative (0),
focal, i.e. <5% (1), 5-25% (2), 25-50% (3), 50-75% (4), and >75% (5) of TI-35-positive tumor cells. Intensity of staining was graded as weak (1), moderate (2), and intense (3). Based on our experience with other mAb to cancer antigens, there was more weight given to the extent of staining (on a scale of 0-5) than to intensity of staining (1-3). Cases with moderate (2) and intense (3) staining in more than 50% of the tumor (4 & 5) were considered ‘strong’ expressers; ‘moderate/weak’ expressers were cases with weak (1) staining irrespective of extent and cases with staining in less than 50% of the tumor (1-3) irrespective of intensity.

**Induction of GAG-HERV-K ch22q11.23 expression by 4,5α-Dihydrotestosterone (DHT)**

Cancer cell lines were submitted to DHT (Sigma-Aldrich) stimulation as described before (31). Briefly, 2x10^5 cells were cultured in a 35 mm culture dish for 48 hours in RPMI 10% FCS media at 37°C. The cells were then treated with 0.1 nM, 1 nM or 10 nM of DHT for another 48 hours. Cells were then harvested for RNA extraction and qPCR evaluation of GAG-HERV-K expression, as described previously. The results were expressed as fold increase relative to untreated cells. Cells were also harvested for protein extraction and western blot analysis.

**Luciferase assay**

Cancer cell lines VCaP were plated at 5x10^4 cells per well in a 96 well plate in RPMI 10% FCS medium at 37°C. 24 h later, cells were transfected with pGL3-promoter plasmid (Promega) carrying the 5’LTR promoter region of GAG-HERV-K. Cells were incubated for an extra 8 hours and then treated for 48 h with or without 1 nM DHT. The empty pGL3-promoter plasmid was used as control (MOCK). Cells were harvested and assayed for luminescence count according to manufacturer’s protocol (Promega).
Methylation status of the HERV-Kch22q11.23 promoter region (5’LTR)

Genomic DNA from cancer cell lines was extracted using DNeasy kit (Qiagen) according to manufacturer’s instructions. The methylation status was evaluated by adapting the Methyl-Profiler DNA Methylation PCR Array System protocol (SA Bioscience). Briefly, genomic DNA from cancer cell lines was incubated with methylation-sensitive restriction enzyme (Ms), methylation-dependent restriction enzyme (Md), both (Msd) or any (Mo) and the promoter region was amplified by real-time PCR using specific primers (Methyl-F 5’-ATG TGC CTT GTT AAC AAT GTG TTT A-3’ and Methyl-R 5’-CTC AAC TGC AAG AGG CCT TC-3’). The methylation status was calculated by the following formula: 
PMS=1-FUM, where PMS = promoter methylation status; FUM (unmethylated fraction) =2^-{(Ct Md – Ct Mo)}. The enzyme digestion efficiency (W) was calculated as W = 100 – [100 x (2^-{(Ct Mo – Ct Msd)})]. 70% cutoff digestion efficiency was adopted for experiment validation.

Induction of GAG-HERV-K ch22q11.23 expression by 5-Aza-2’-deoxycytidine treatment

The influence of DNA demethylation in GAG-HERV-K expression was evaluated by 5-Aza-2’-deoxycytidine treatment. Briefly, 2x10^5 cells were cultured in a 35 mm culture dish for 48 hours in RPMI 10% FCS medium at 37°C. The cells were then treated with 0.1 mM or 1 mM of 5-Aza-2’-deoxycytidine for another 48 hours. Cells were then washed with PBS and cultured for another 48 hours at 37°C in RPMI 10% FCS in the presence or absence of DHT. Cells were then harvested for RNA extraction and qPCR evaluation of GAG-HERV-K expression, as described previously. The results were expressed as fold increase relative to untreated cells.
**SDS PAGE and Western blot**

Cell lines were cultured in RPMI 10% FCS and subjected to protein extraction using modified RIPA buffer. The protein extract was submitted to continuous electrophoresis using NuPAGE 4-12% Bis-Tris gel (Invitrogen), under reducing conditions. The separated proteins were transferred to PVDF membrane and incubated with blocking solution (3% BSA, 0.15 M PBS, pH 7.4) for 1 h at room temperature and then incubated with human sera diluted 1:10,000 or TI-35 mouse monoclonal antibody diluted 1:1,000 in dilution buffer (1% BSA, 0.15 M PBS, pH 7.4) for 16 h at 4°C. The membranes were then washed (0.2% Tween 20, 0.15 M PBS, pH 7.4) and incubated with goat anti-human IgG peroxidase-conjugated diluted 1:1,000 (Southern Biotech) or rabbit anti-mouse peroxidase-conjugated (Sigma) diluted 1:20,000 in dilution buffer, for 1 h at room temperature. Membranes were developed with Western Lighting Plus-ECL solution (Perkin Elmer Inc).

**ELISA**

ELISA assays were performed as previously described (32). Briefly, plasma was serially diluted from 1/100 to 1/2,500, added to low-volume 96-well plates (Corning, NY) coated with 0.25 μg/ml full-length recombinant GAG-HERV-K protein or negative control protein DHFR, or with 1 μM GAG-HERV-K overlapping 20mer peptides, and blocked with PBS containing 5% non-fat milk. After incubation, plates were washed by automatic plate washer (Bio-Tek, Winooski, VT) with PBS containing 0.2% Tween and rinsed with PBS. Total IgG bound to antigens was detected with alkaline phosphatase-conjugated anti-human IgG monoclonal antibody (Southern Biotech, Birmingham, AL). Following addition of ATTOPHOS substrate (Fisher Scientific, Waltham, MA), absorbance was measured. A reciprocal titer was extrapolated by determining the intersection of a linear trend.
regression with a cutoff value. The cutoff was defined as 7.5x the average of the OD values from the 3 dilutions of a negative control pool of 4 healthy donor sera. Sera with reciprocal titers >100 to GAG-HERV-K but without reactivity to DHFR were considered significant after confirmation in repeat titration assays.

**GAG-HERV-K_GFP fusion protein expression in HeLa cells, and baculovirus**

GAG-HERV-K gene was amplified using specific primers (pIC113-F 5’-AACTCGAGATGGGGCAAACTGAAATGAAAT-3’ and pIC113-R 5’-AAAAGCTTCTACTCGGGGTGCTGGCTGCTGCTG-3’). The amplified product was digested with Xho-I and Hind-III restriction enzymes and cloned into plasmid pIC113 containing GFP and G418 resistance genes (33). The plasmid was then transfected into HeLa cells using Effectene reagent (Qiagen) and 48 h after the cells were split 1:10 in a 30 cm² plate with RPMI 10% FCS media containing 500 μg/mL G418 selection reagent (Gibco). Two days after selection, positive transfected cells were fixed with 4% formaldehyde and permeabilized with 10% Triton-X. Cells were then stained with TI-35 antibody and analyzed by confocal microscopy (LSM710 Zeiss, Germany). Recombinant baculovirus preparation using GAG-HERV-K plasmid or control was performed following manufacturer’s instructions (Invitrogen) and used to infect Sf9 insect cells (MOI=1).

**Protein Microarray**

Arrays were custom-made from version 4 ProtoArrays® by Invitrogen (California, USA) with the addition of GAG-HERV-K recombinant protein, and used per manufacturer’s instructions. For antibody hybridization, arrays were blocked for 1 h and incubated for 90 min at 4°C with individual sera diluted 1:500 in 4 ml buffer (1 M Hepes, pH 7.5, 5 M NaCl, 0.1% Triton X100 (v/v), 25% glycerol (v/v), 20 mM reduced glutathione, 1 μM DTT
10% Synthetic Block solution (Invitrogen) in H$_2$O) in dishes placed on a horizontal shaker (50 rpm). After 3 washes (0.1% Tween 20 (v/v), 10% Synthetic Block solution (Invitrogen) in PBS), Total bound IgG was detected by incubation with Alexa-Fluor 647 goat anti-human IgG (Invitrogen) diluted 1:2000 in assay buffer for 90 min at 4°C. Arrays were washed again and dried by centrifugation. The slides were scanned at 10 μm resolution using a microarray scanner (Axon 4200AL with GenePix Pro Software, Molecular Devices, Sunnyvale, CA 94089) and fluorescence detected according to the manufacturer's instructions. Images were saved as 16-bit tif files and analysis was performed using GenePix. Local backgrounds were subtracted automatically and the median net intensity in relative fluorescence units (rfu) was reported for each spot. The results were calculated as previously described (34).

**Statistical analyses**

Statistical analyses for comparing multiple columns were done by one-way ANOVA, and with unpaired t-test for two-column comparisons. Frequency of patients with seropositive vs. seronegative responses was compared using two-tailed Fisher’s exact test. Analyses of overall survival and recurrence rates were performed using the Kaplan-Meier method, and analyzed by log-rank or by Gehan-Breslow-Wilcoxon.
RESULTS

Expression of GAG-HERV-K ch22q11.23 in cancer cell line and cancer tissues

We evaluated the expression pattern of GAG-HERV-K ch22q11.23 in normal and cancer tissues as well as in cancer cell lines by qPCR. Since this gene has no introns, RNA was pretreated with DNase to remove genomic DNA prior to reverse transcription. The expression of GAG-HERV-K was highly restricted in normal tissue, with low levels in prostate, breast, and thyroid (Fig. 1A). GAG-HERV-K expression analysis in a large number of tumor cell lines showed absence of detectable expression in most lines from various cancer types. However, significant expression of HERV-K could be detected in some prostate cancer cell lines (VCaP, 22RV-1) and teratocarcinoma cell line (Tera-1) (Fig. 1B). Real-time qPCR analysis of GAG-HERV-K ch22q11.23 expression in prostate cancer tissue showed high expression in 11/11 samples. We found that expression of GAG-HERV-K was mostly restricted to prostate cancer, and generally not present in various other cancer tissue samples such as melanoma, breast cancer, colon cancer, renal cancer, ovarian cancer, esophageal cancer, bladder cancer (except one), lung cancer and hepatocarcinoma (Fig. 1C and Supplementary Fig. S2). We also evaluated neighboring genes located on chromosome 22q11.23 to check for unspecific transcription of GAG-HERV-K in prostate cancer samples due to chromatin remodeling that would favor local gene expression (Supplementary Fig. S1 and Supplementary Fig. S2) (35). Neighboring gene BCR was widely expressed in all cancer samples, while IGLL1 and RGL4 were not significantly altered in any of the cancer samples analyzed. The only gene that displayed a specific increase in prostate cancer was ZDHHC8P1 (Supplementary Fig. S2). This analysis implies that expression of GAG-HERV-K ch22q11.23 is specific to prostate cancer. Although we detected high expression of GAG-HERV-K in all primary prostate cancer tissues analyzed (Fig. 1C), only 2 out of 6 prostate cancer derived cell lines were positive (Fig. 1B). The difference in GAG-HERV-K
K expression observed between prostate cancer cell lines and prostate cancer tissues may be explained by loss of environmental stimuli after *in vitro* culture, such as steroid hormones.

**Stimulation of GAG-HERV-K ch22q11.23 expression by 4,5α-Dihydrotestosterone (DHT) in vitro**

To address the importance of steroid hormones on GAG-HERV-K expression, androgen receptor (AR) positive cell lines (VCaP and LNCaP) and AR negative cell line (PC-3) were stimulated using different concentrations of 4,5α-Dihydrotestosterone (DHT). As a positive control for DHT treatment, the expression of KLK3 (PSA) gene was evaluated before and after DHT stimulation. Expression of GAG-HERV-K was evaluated by qPCR and western blot (Fig. 2A). GAG sequences from different HERV-Ks are highly homologous. To specifically detect GAG-HERV-K from ch22q11.23, we took advantage of a monoclonal antibody TI-35 previously described by our group (15). This antibody is capable of recognizing an epitope of GAG-HERV-K that contains several non-conserved amino acids (Supplementary Fig. S3A and C). Furthermore, TI-35 monoclonal antibody staining colocalized with GFP in HeLa cells expressing GAG-HERV-K_GFP fusion protein (Supplementary Fig. S4A and B) demonstrating its high specificity to the GAG-HERV-K antigen. GAG-HERV-K-specific staining of TI-35 was also confirmed in insect cells expressing baculovirus-encoded GAG-HERV-K or control, using chromogen-based IHC, immunofluorescence, and Western blot (Supplementary Fig. S4C and D, and not shown).

DHT treatment was able to increase the expression of GAG-HERV-K in VCaP cell line, both at RNA and protein levels, but not in any other cell line tested (Fig. 2A). Although LNCaP express AR (Fig. 2B, left), DHT is not sufficient to induce GAG-HERV-
K expression in this cell line (Fig. 2A). PC-3 cells do not express AR and, as expected, did not respond to DHT stimulation. PSA expression on both LNCaP and VCaP was increased after DHT stimulation (Fig. 2B, right). Increased expression of GAG-HERV-K in VCaP cells was confirmed by immunohistochemical analysis of VCaP cells with mAb TI-35. While unstimulated VCaP cells showed only weak and focal immunostaining with mAb TI-35, VCaP cells stimulated with 10 nM or 100 nM of DHT exhibited a more intense immunostaining (Fig. 2C).

To evaluate the direct effect of AR in GAG-HERV-K expression, the promoter region (5'LTR) of HERV-K was cloned into a luciferase reporter plasmid and transfected into VCaP cells. Cells transfected with the promoter construct or the empty plasmid were cultured in the presence or absence of 10 nM DHT. Luciferase signal could be detected only in cells carrying the HERV-K promoter construct, and DHT stimulation increased the signal compared to unstimulated cells (Fig. 2D). These results suggest that DHT plays an essential role in regulating the expression levels of GAG-HERV-K ch22q11.23 in prostate cancer cell lines.

Role of methylation of the HERV-K chr22q11.23 promoter region in prostate cancer cell lines

In addition to the role of androgens on GAG-HERV-K expression, we explored the role of DNA methylation, a known mechanism for suppressing HERV expression (3-5, 36). A recent paper showed that HERV-K ch22q11.23 is preferentially expressed in hypomethylated prostate cancer cell lines and primary prostate tissue samples (37). We confirmed these observations by showing that the HERV-K promoter region (5'LTR) of GAG-HERV-K negative cell lines (LNCaP and PC-3) was hypermethylated when compared to positive cell line VCaP (Fig. 3A), in accordance with GAG-HERV-K expression levels in these lines (Fig. 1B). Moreover, VCaP cells which were
simultaneously treated with 5-Aza-2’dC and stimulated with 1 nM of DHT showed increased expression of GAG-HERV-K compared to cells that were solely DHT-stimulated. This effect was independent of 5-Aza-2’dC concentrations (Fig. 3B, top). LNCaP and PC-3 cell lines did not show increase in HERV-K expression after androgen stimulation (Fig. 3B, middle and bottom). However, 5-aza-2’dC treatment alone was sufficient to induce expression of HERV-K in all tested cell lines (Fig. 3C). Taken together, these results suggest that DNA demethylation together with steroid responsiveness act together to induce GAG-HERV-K ch22q11.23 expression in prostate cancer cell lines.

**Expression of GAG-HERV-K protein in prostate cancer and normal tissues**

After optimization of the immunostaining, a panel of normal tissues was analyzed for GAG-HERV-K expression using mAb TI-35. Normal skin, lung, liver, colon were all negative for staining, with the exception of alveolar macrophages. In kidney tissue, there was granular reactivity in epithelia of the proximal and partially distal tubuli as well as in acini of the pancreas (data not shown). We assessed expression of GAG-HERV-K in prostate using a tissue microarray (TMA) with specimens from primary cancer tissue, benign prostate hyperplasia, and normal prostatic tissue, collected at surgery (Fig. 4A and B). Of the 204 cancer cases of the TMA block, 188 cases were evaluable for IHC. According to our grading system, 93/188 (49.5%) cases showed strong immunoreactivity (Fig. 4A, bottom), while 66/188 (35.1%) displayed moderate to weak staining with mAb TI-35 (Fig. 4A, middle); 29/188 (15.4%) cases remained completely negative (Fig. 4A, top). The association between strong immunoreactivity to GAG-HERV-K and prostate cancer was significant regardless of whether intermediate/weak cases were considered negative or positive. In addition to prostate cancer, the TMA contained 22 samples of normal prostate, 13 of which (59%) were negative and the remaining 9 (41%) displayed
weak GAG-HERV-K expression (Fig. 4D). Finally we analyzed whole block sections of 12 metastatic sites of prostate cancer. All the metastases cases were positive, with 9/12 showing strong and 3/12 weak expression (examples in Fig. 4C).

To assess protein distribution within other tumor types than prostate, we analyzed whole sections of a limited number of other malignant tumors by immunohistochemistry with mAb TI-35. We did not see staining with TI-35 in 2/2 colon cancers; similarly, 7/7 SQCC of the head and neck were completely negative (not shown). However, some tumor types showed staining with TI-35: 1/3 melanomas was positive; 1/4 NSCLC was positive; 2/6 breast carcinomas (invasive ductal type, Fig. 4C, bottom) were positive; and 3/3 ovarian carcinomas were positive. This indicates that GAG-HERV-K protein expression may not be limited to prostate cancer.

**Antibodies against GAG-HERV-K ch22q11.23 in cancer patient sera**

Humoral immune responses against HERV proteins have been identified in different cancer types (15, 23, 24). To evaluate immune responses against GAG-HERV-K ch22q11.23 protein in cancer patients, sera from individual patients with different cancer types (n=1367), as well as healthy donors (n=148), were screened by ELISA for the presence of specific antibodies to recombinant GAG-HERV-K. Reciprocal titers were extrapolated from titration curves and results were considered significant if a serum significantly reacted at dilutions greater than the cutoff of 100. We found that 6.8% (33/483) of prostate cancer patients (all stages combined) had serum antibodies to the GAG-HERV-K protein, with a mean reciprocal titer of 2400 (Fig. 4D, left). Interestingly, despite no detectable GAG-HERV-K expression among tumor lines analyzed in our panel by qPCR, melanoma (15/226 – 6.6%), breast cancer (7/101 – 6.9%), and ovarian cancer (6/98 – 6.1%) patients showed similar frequencies of serum antibodies against GAG-HERV-K, albeit with much lower mean titers (<125) than those observed in the...
prostate cancer group (mean = 2400) (Fig. 4D, left). While commonly observed in prostate cancer, very high titers (>1000) were elicited in only a few patients with melanoma or ovarian cancer, even though these tumor types also show some GAG-HERV-K tissue expression at the protein level by IHC. All other cancer types analyzed showed less than 5% of patients with anti-GAG-HERV-K autoantibodies, with very weak titers. A similar result was observed in the healthy donor group where four individuals (4/148 – 2.7%) had serum antibodies capable of reacting to the GAG-HERV-K protein with titers above 100 (Fig. 4D, left). These results suggested once more the importance of GAG-HERV-K expression in prostate cancer. To further evaluate the specificity of spontaneous serum autoantibodies to GAG-HERV-K, we used patient sera to immunoprecipitate (IP) naturally expressed GAG-HERV-K protein from VCaP cells. High titer serum from a prostate cancer patient seropositive for anti-GAG-HERV-K was successful in recovering the GAG-HERV-K protein from VCaP cells (Supplementary Fig. S3B). We also mapped the recognition of individual epitopes from GAG-HERV-K using overlapping peptides spanning the entire amino acid sequence. We found that most patient sera reacted to the N-terminal area 101-290 of the antigen, or to the C-terminal area 661-690 (Supplementary Fig. S3C). We decided to further investigate the correlation between prostate cancer progression and the GAG-HERV-K ch22q11.23 immune response.

Antibodies to GAG-HERV-K ch22q11.23 as a biomarker for clinical staging of prostate cancer

To evaluate the correlation between antibodies to GAG-HERV-K and prostate cancer progression, sera from prostate cancer patients were divided in 4 groups according to clinical stage (I to IV) and analyzed for the presence of antibody to GAG-
HERV-K protein by ELISA. Sex-matched healthy donor sera were used as control. We found that 21.0% (22/105) of advanced prostate cancer patients with stage IV disease developed antibodies to GAG-HERV-K with titers over 100, while only 1.0% (2/196) and 2.1% (2/96) of early prostate cancer patients with stage I and II disease developed this response, respectively (Fig. 4D, right). Patients with stage III disease showed approximately 8.1% (7/86) of sera positive to GAG-HERV-K protein. Only 1 out of 55 (1.8%) age-matched male healthy donors had antibodies to GAG-HERV-K protein higher than the cutoff (Fig. 4D, right). Together, presence of serum antibodies to GAG-HERV-K was significantly higher in advanced cancer patients compared to early stage patients or healthy donors (p<0.01 with exact Fisher test [stage IV vs. HD], as well as with ANOVA comparison of all stages taking titers into account). We also divided patients according to risk groups, based on Gleason score and eventual metastases (Table 1), and observed a similar trend, in which the highest frequency of antibodies to GAG-HERV-K was observed in patients with the highest risk group (Gleason score ≥7 and eventual metastases) when compared to sex-matched healthy donor sera and other risk groups (Supplementary Fig. S5A). Independently of metastases, when considering Gleason score alone, serum antibodies to GAG-HERV-K were also significantly more frequent in patients with a Gleason score ≥ 8, compared to patients with score ≤ 6, even though not all presenting metastatic cases could be graded (Supplementary Fig. S5B). These results show a clear correlation between eventual prostate cancer progression and detection of antibody to GAG-HERV-K in prostate cancer patients at time of biopsy, highlighting the importance of HERV-K ch22q11.23 as a predictive biomarker of tumor burden and progression.

**Antibodies to self-antigens in prostate cancer GAG-HERV-K Ab+ group**
As the previous results unveiled, only a subset of advanced prostate cancer patients develops antibodies to GAG-HERV-K protein. To evaluate whether patients seropositive for GAG-HERV-K may have overall differences in their immunological response to prostate cancer and/or against self-antigens compared to GAG-HERV-K antibody-negative patients, sera from 28 prostate cancer patients with stage IV disease were divided in 2 groups according to presence of antibodies to GAG-HERV-K (HERV-K+, n=28 and HERV-K−, n=14) and analyzed for serum antibody profiling using custom-made protein microarrays (Protoarrays® with GAG-HERV-K and other cancer antigens added). Sera from sex- and age-matched healthy donors were used as control (n=14).

By applying statistical analyses described previously (32), we were able to identify several antigens that were co-recognized by the HERV-K+ group (Supplementary Table S1). By comparing results from ELISA and seromic profiling, we could validate the microarray results: 89% of samples expected positive for GAG-HERV-K by ELISA were also reactive to GAG-HERV-K on the microarray, missing only 3 patients with low titer samples (data not shown). NY-ESO-1 (18% - 5/28) and CCNB1 (18% - 5/28) were the most frequently recognized antigens by the HERV-K+ group. NY-ESO-1 expression has been associated with advanced prostate cancer (38) and CCNB1 autoantibody has been described in several other cancer types (39, 40). On the other hand, patients in the HERV-K- group preferentially recognized antigens such as TMP4 (29% - 4/14), TLE4 (29% - 4/14), and ARID3A (29% 4/14). TMP4, TLE4, and ARID3A, which have been linked to leukemias or lymphomas (41-43). Moreover ARID3A is regulated by p53 and is a proposed target for cancer immunotherapy (43). This analysis shows heterogeneity of the immune response to cancer antigens among prostate cancer patients with stage IV disease. Understanding the correlation between prostate cancer progression and antigen-specific immune responses could unveil individual etiologies in prostate cancer patients.
Associations with overall survival and biochemical recurrence

To determine whether the anti-HERV-K immune response in prostate cancer can predict progression of disease, patients were divided in 2 groups based on the presence of anti-GAG-HERV-K antibodies and analyzed for overall survival using the Kaplan-Meier method. This analysis could only be performed on a subset of 284 patients undergoing radical prostatectomy with available clinical follow-up data. This cohort unfortunately included only patients with localized disease at the time of surgery when sera were obtained, and therefore did not include any clinical stage IV patients. Patients were stratified and censored according to serological reactivity against GAG-HERV-K at the time of surgery. Patients were retrospectively categorized and assessed for subsequent biochemical recurrence and metastases (Risk group 1: n=102; Risk group 2: n=96; Risk group 3: n=86, see clinical characteristics in Table 1). Although not reaching significance by Log-Rank test (p=0.053, hazard ratio 1.98 [1.23 - 11.85]), there was a correlation with mortality in the GAG-HERV-K antibody-positive group when compared to the GAG-HERV-K antibody-negative group using the Gehan-Breslow-Wilcoxon test (p=0.006, Fig. 5A). We also assessed whether there was a correlation between presence of anti-GAG-HERV-K antibodies and biochemical recurrence of the disease (BCR), based on PSA levels. BCR is associated with a high likelihood of metastatic progression or prostate cancer-specific mortality (44). Though not statistically significant, there was a trend for greater BCR in prostate cancer patients seropositive for GAG-HERV-K (p=0.085, by Log-Rank) compared to the seronegative group (Fig. 5B). The association of GAG-HERV-K serum antibodies with worse survival or greater BCR was explained by the exclusive presence of antibody to GAG-HERV-K in the high-risk groups and none in the lower risk group. However, there was no significant correlation between seropositivity for GAG-HERV-K and metastasis-free survival. Together with the observed
higher frequency of antibody responses to GAG-HERV-K in stage IV patients, these results suggest that the presence of antibodies to GAG-HERV-K may be associated with a worse prognosis for prostate cancer patients and may be indicative of relapse of disease.
DISCUSSION

In the last decade, several studies have shown correlations between human endogenous retrovirus (HERV) expression and human disease such as multiple sclerosis (15, 19, 45). Moreover, immune responses to endogenous retrovirus derived proteins have also been correlated to cancer (20, 21, 23, 46), but no clear clinical significance has yet been suggested. Here we identify an HERV-K GAG protein, located on chromosome 22q11.23, highly expressed in prostate cancer and capable of generating a specific immune response in advanced stages of prostate cancer.

The HERV-K located on ch22q11.23 is a complete retrovirus element, with GAG, POL and ENV genes, flanked by a LTR sequence. The POL and ENV genes have mutations and/or deletions that prevent the proteins from being translated. In contrast, the GAG gene has a complete open reading frame that translates to a protein of 715 amino acids. Restricted expression of GAG-HERV-K ch22q11.23 in prostate cancer tissue suggests this molecule as a suitable biomarker and target for prostate cancer. The expression analysis of the surrounding genes located on chromosome 22q11.23 showed that expression of GAG-HERV-K is not due to unspecific region activation in prostate cancer.

The transcriptional program of androgenic signaling in the prostate consists of thousands of gene targets whose products play a role in almost all cellular functions, including cellular proliferation, survival, lipid metabolism, and differentiation (47). Here we showed that androgen hormone 4,5α-Dihydrotestosterone (DHT) was able to upregulate the expression of GAG-HERV-K in VCaP cell line. Although VCaP and LNCaP are androgen receptor (AR) positive, we were only able to stimulate GAG-HERV-K expression in VCaP cells. One possible explanation is that VCaP cells have higher levels of AR than LNCaP cells, thus rendering the cells more sensitive to DHT. It is well known that prostate cancer cells can overexpress AR and be more sensitive to androgen
hormones (48, 49), which could contribute to the increased expression of GAG-HERV-K observed in prostate cancer samples. Using PROMO, a virtual laboratory for the identification of putative transcription factor binding sites in DNA sequences, we found two potential androgen receptor binding site at positions 328-336 and 496-504 of the promoter of GAG-HERV-K (from its 990 nucleotide upstream sequence). We observed that the DNA methylation status of the HERV-K promoter region (LTR) contributes to GAG-HERV-K expression in cancer cell lines. In accordance with our findings, HERV-K from chromosome 22q11.23 was recently observed to be highly expressed in prostate cancer samples and the expression was dependent of LTR region demethylation (37). Nevertheless, methylation is just one of several mechanisms that impact chromatin structure and gene expression, and it is possible that some of the heterogeneity in GAG-HERV-K expression observed among cancer cell lines may relate to differences in other possible pathways (acetylation, etc.) used to regulate the chromatin structure in the promoter region of GAG-HERV-K. Such mechanisms might reflect different sub-types or stages of prostate cancer, and some of the differences in the induction of GAG-HERV-K observed by androgen treatment may be related to transformation in cell cultures.

Recently, an interesting translocation event in prostate cancer has brought attention to the promoter region of HERV-K. The HERV-K ch22q11.23 5' LTR region was shown to be fused with ETV1 leading to an overexpression of the gene in prostate cancer patients (17). Importantly, ETV1 overexpression in prostate cells confers invasiveness. It is not clear if the HERV-K 5'LTR region alone is sufficient to activate EVT1 expression in these patients. However, our results suggest that the 5'LTR promoter region from HERV-K is not only active but can also increase expression in response to androgen hormone stimulation, in accordance with previous findings (37). Taken together, these findings highlight the importance of HERV-K activity in prostate cancer.
Specific immune response to endogenous retrovirus components is well described in the field (20, 21, 23, 46), but no clear correlation to cancer progression has yet been determined. In this work we showed that advanced prostate cancer patients have a higher incidence of humoral immune response to GAG-HERV-K protein, suggesting an increase in gene expression due to prostate cancer progression. Unfortunately, none of the cohorts that we had access to allowed us to ask whether there is a direct correlation between antigen expression and antibody response, as we could only collect patient serum or tumor, but not both simultaneously. It is known that during prostate cancer progression, hypomethylation of DNA occurs and this event culminates in the expression of endogenous retrovirus (50, 51). Such increase in gene expression could lead to a specific immune response in prostate cancer patients with the progression of the disease. Indeed, our result suggests that progression of prostate cancer is directly correlated to increase of humoral response to GAG-HERV-K protein.

Clinical staging of prostate cancer is important in assessing the risk of the disease and therefore for treatment recommendations (26). Our results indicate that GAG-HERV-K could be a useful tool for diagnostic and/or clinical stage biomarker in prostate cancer. Unfortunately, we were not able to collect serum antibody from patients assessed for protein tumor expression, and the correlation between immunogenicity and expression still needs to be addressed. Although we could not detect significant levels of GAG-HERV-K expression in other cancer type, we detected serum antibody to GAG-HERV-K in a number of melanoma, breast, and ovarian cancer patients, albeit at lower titers compared to prostate cancer patients. Expression of GAG-HERV-K was also seen by IHC in other tumor tissues than prostate, though it may have been missed by qPCR in the small panel of cell lines we used to assess it. Although the presence of autoantibodies to endogenous retrovirus proteins has been correlated to a worse clinical prognostic in melanoma patients (23), we could not determine any correlation between
presence of antibody to GAG-HERV-K ch22q11.23 and clinical staging in melanoma or ovarian cancer (data not shown).

Protein microarray profiling analysis of serum from advanced prostate cancer patients suggests the presence of antibodies to GAG-HERV-K is linked to other antigen-specific immune responses. An increased humoral immune response to cancer testis antigen NY-ESO-1 was observed in the GAG-HERV-K Ab+ group. The presence of antibodies to cancer-testis (CT) antigens has been associated with a worse prognosis in several cancer types, including prostate cancer (32, 38, 52). The evidence of a co-immune response to GAG-HERV-K and NY-ESO-1 in a subset of advanced prostate cancer patients suggests a potential biological association between these two genes during prostate cancer progression and deserves to be further investigated.

We believe that specific expression of GAG-HERV-K in prostate cancer occurs by the combination of epigenetic modification, such as demethylation, and androgen hormone stimulation. Whether the expression of GAG-HERV-K is a causal event or a biological consequence of prostate cancer progression still needs to be addressed. Nevertheless, we show here a clear correlation between the tissue specific GAG-HERV-K protein expression and development of a specific immune response with progression of prostate cancer. Moreover, patients who developed antibodies to GAG-HERV-K also had a lower survival rate and appeared to have higher incidence of disease relapse (BCR). BCR is correlated with an increased mortality and metastatic events in prostate cancer and the early detection of disease relapse is crucial for an efficient treatment (44).

In conclusion, we characterized a GAG protein from a human endogenous retrovirus (HERV-K) located on chromosome 22q11.23 highly expressed in prostate cancer. The expression of GAG-HERV-K is capable of inducing humoral immune response in prostate cancer patients. This immune response was correlated to
advanced stages of the disease possibly due to increase in androgen hormone stimulation and demethylation events triggered by prostate cancer progression. The presence of serum antibodies to GAG-HERV-K was also indicative of worse prognosis and higher BCR among advanced prostate cancer patient. Due to its restricted expression and its ability to elicit a strong humoral response, GAG-HERV-K antigen might be used as an important biomarker for prognostic purposes as well as serving as a target for immunotherapy of advanced prostate cancer. Future directions should address whether GAG-HERV-K serum antibodies change with treatment such as androgen deprivation, or during natural evolution in patients with hormone-refractory disease.
ACKNOWLEDGMENTS

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REFERENCES


Table 1. Patient characteristics within group analyzed by Kaplan-Meier. All values are median (interquartile range)

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<td>22 (23%)</td>
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*Samples obtained at time of surgery or biopsy and assessed retrospectively.
FIGURE LEGENDS

Figure 1: Real-time qPCR expression analysis of GAG-HERV-K in normal tissues, cancer cell lines and cancer tissues. Total RNA from normal tissues, cancer cell lines or cancer tissues was submitted to DNAse treatment and reverse transcriptase reaction for cDNA synthesis. The resulting cDNA was used on qPCR reactions to detect the expression of GAG-HERV-K ch22q11.23. The results are expressed as relative expression to housekeeping gene GAPDH. A-C, Expression of GAG-HERV-K ch22q11.23 in a normal tissue panel (A), cancer cell lines (B) and in cancer tissues samples (C) (Ca, cancer; Prost, prostate; Esoph, esophageal; Hep, Hepatocellular).

Figure 2: Regulation of GAG-HERV-K expression by DHT in prostate cancer cell lines. A, Prostate cancer cell lines were treated with different concentrations of 4,5α-Dihydrotestosterone (DHT) and GAG-HERV-K expression was evaluated by qPCR (left) and western blot (right). In qPCR, HERV-K expression levels were relative to housekeeping GAPDH and plotted as fold increase compared to untreated cells. In the western blot analysis membrane were stained with monoclonal antibody TI-35 (HERV-K), anti-androgen receptor (AR) or anti-actin (actin) antibodies. B, qPCR analysis of AR expression level in prostate cancer cell lines at steady state (left) and PSA expression level before and after treatment with 10 nM of DHT (right). C, Immunohistochemical analysis of GAG-HERV-K expression in paraffin sections of VCaP cell pellets showing low level expression in pellets of native VCaP cells (left) and increased expression after DHT stimulation (right). D, Luciferase assay on VCaP cells transfected with pGL3 luciferase reporter plasmid carrying the HERV-K promoter region (HERV-K) or not (MOCK). Cells were incubated with or without 1 nM DHT for 48 h and harvested for research.
luminescence count. AR and PSA gene expression levels relative to housekeeping gene TRFC using Taqman probes. * p < 0.05 as indicated compared to data with no DHT.

Figure 3: Regulation of GAG-HERV-K expression in prostate cancer cell lines. A, HERV-K promoter methylation status on prostate cancer cell lines was determined by methyl dependent or sensitive enzymatic genomic DNA digestion and the promoter region was amplified by qPCR. 1 = 100% methylated. B, Prostate cancer cell lines were treated with different concentrations of 5-Aza-2 deoxycitidine (5-Aza-2’dC) and then stimulated with DHT. GAG-HERV-K expression was evaluated by qPCR and plotted as fold increase from untreated cells. VCaP cells (top), LNCaP cells (middle) and PC-3 cells (bottom) were evaluated. ● 0 μM of 5-Aza-2’dC; ■ 0.1 μM of 5-Aza-2’dC; ▲ 1 μM of 5-Aza-2’dC. * p < 0.05 compared to other lines (A) or to no 5-Aza-2’dC (B). C, Prostate cancer cell lines were treated with different concentrations of 5-Aza-2 deoxycitidine (5-Aza-2’dC). GAG-HERV-K expression was evaluated by qPCR and plotted as relative expression to housekeeping gene GAPDH. (Top) VCaP cells, (middle) LNCaP cells and (bottom) PC-3 cells were evaluated. * p < 0.05

Figure 4: Tissue expression of GAG-HERV-K protein and ELISA assay to detect serum antibodies against GAG-HERV-K in cancer patients. A, Immunohistochemical analysis of GAG-HERV-K expression in paraffin sections of tissue punches of TMA with normal prostate (top) and prostate carcinoma (middle, bottom) with mAb TI-35, showing a negative staining (top), low staining (middle) and strong staining (bottom) (diaminobenzidine chromogen, brown, original magnification: 10x). B, Summary of frequency of IHC staining for GAG-HERV-K in a set of prostate cancer and normal prostate TMA. C, (Top) Immunohistochemical staining with TI-35 in whole block section
of a metastatic prostate carcinoma site in lung; (Middle) Variable intensity of TI-35 immunostaining with more intense labeling of prostate carcinoma cells next to non-cancerous (asterisks) epithelia (original magnification: 20x); (Bottom), Breast carcinoma with strong homogeneous TI-35 immunostaining of invasive carcinoma cells (original magnification: 10x). D, (Left) Sera from cancer patients and healthy donors (HD) were tested for circulating IgG antibody reactivity against GAG-HERV-K protein by ELISA. Results are plotted as reciprocal antibody titer after confirmation of specificity using control antigens. Each dot represents a representative titer from a patient serum or healthy donor serum. D, (Right) Prostate cancer patients were split in 4 groups according to clinical stage from I to IV, along with male healthy donors (HD). Black lines represent the mean titer of all sera for each group. Dashed lines represent the cutoff above which reactivity is considered specific (100). Percentage of positive sera represented by number of positive sera (titer higher than 1/100) per number of sera in each group. * p < 0.01 by either ANOVA test (titer comparison for III or IV vs. HD or I or II) or Fisher Exact test (frequency positive in IV vs. HD).

**Figure 5: Overall survival curve and BCR free curve of GAG-HERV-K seropositive prostate cancer patients.** A-B, Prostate cancer patients were divided in two groups: GAG-HERV-K Ab+ (antibodies to GAG-HERV-K with titers higher than 1/100) and GAG-HERV-K Ab- (titers lower than 1/100) and survival curve was plotted with the Kaplan-Meier method based on time to death of disease (DOD) (A), or based on time to biochemical recurrence (BCR) of PSA (B). Statistics used are indicated.
**Figure 2**

(A) Graph showing the fold increase of HERV-K expression in VCaP, LNCaP, and PC-3 cells treated with different concentrations of DHT (nM).

(B) Bar graph comparing AR and PSA expression levels in VCaP, LNCaP, and PC-3 cells treated with 0 or 10 nM DHT.

(C) Images of cellular morphology under 0 nM and 10 nM DHT conditions.

(D) Luminescence assay results for MOCK and HERV-K expression levels with varying DHT concentrations.
Figure 4
Figure 5

A  

GAG-HERV-K Ab- (n=275)  
GAG-HERV-K Ab+ (n=9)

Gehan-Breslow-Wilcoxon, p=0.006  
Log-rank (Mantel-Cox), p=0.053  
Hazard Ratio (logrank) = 1.98 (0.9 - 5.9)

B  

GAG-HERV-K Ab- (n=275)  
GAG-HERV-K Ab+ (n=9)

Log-rank (Mantel-Cox) test, p=0.065  
Hazard Ratio (logrank) = 1.85 (0.9 - 5.9)
Clinical Cancer Research

Prostate cancer progression correlates with increased humoral immune response to a human endogenous retrovirus GAG protein

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