RESEARCH ARTICLE

No Detection of Xenotropic Murine Leukemia Virus-Related Viruses in Prostate Cancer in Sanandaj, West of Iran

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Abstract

<u>Background</u>: Multiple etiologies have been hypothesized for prostate cancer, including genetic defects and infectious agents. A recently reported gamaretrovirus, xenotropic murine leukemia virus-related virus (XMRV) has been reported to be detected in prostate cancer. However, this virus has not been detected in similar groups of patients in other studies. Herein, we sought to detect XMRV in prostate cancers and benign controls in Sanandaj, west of Iran. <u>Materials and Methods</u>: In a case-control study, genomic DNA was extracted from formalin fixed and paraffin embedded prostate tissues from a total of 163 Iranian patients. We developed a conventional and a nested PCR assay using primers targeting to an env specific sequence of XMRV. PCR assays were carried out on 63 prostate cancers and 100 benign prostate hyperplasias. <u>Results</u>: Beta-actin sequences were successfully detected in the DNA extracts from all prostate tissues, confirming DNA extraction integrity. We did not detect XMRV in samples either from prostate cancers or benign prostate hyperplasias using XMRV specific primers. <u>Conclusions</u>: We conclude that in our population XMRV does not play a role in genesis of prostate cancer.

Keywords: XMRV - MLV - prostate cancer - Iran

Introduction

Prostate cancer is the most common malignancy in men worldwide (Groom et al., 2012). The annual incidence rates of prostate cancer among Iranian population are dramatically higher than in other countries of Asia (Akbari et al., 2008). Multiple etiologies have been hypothesized for the cause of prostate cancers, including genetic defects, infectious agents and associated inflammation (Silverman et al., 2010). Identification of a potential virus as the cause of prostate cancer is very important because it could facilitate management of this disease such as treatment and prevention. A recently described retrovirus, the xenotropic murine leukemia virus-related virus (XMRV) was detected in about 40% of familial prostate cancer using a DNA microarray (Virochip) containing oligonucleotides comprising conserved sequences from known viral genomes and was strongly associated with homozygosity for the R462Q reduced activity, a variant of the antiviral enzyme, RNase L (Urisman et al., 2006).

Murine leukemia viruses (MLVs) belong to gammaretroviruses in the retroviridae family, which viral RNA is reverse-transcribed to DNA during replication. They are endogenous viruses that have integrated their provirus DNA into the mouse genome and can cause leukemias, lymphomas, neurologic diseases, and immunodeficiency disorders in their hosts (Rezaei et al., 2013). Xenotropic MLV (XMLV) replicates only in non-mouse hosts, and ecotropic murine leukemia virus (EMLV) replicates only in mice, but polytropic murine leukemia virus (PMLV) has wider host range. The *envelope (env)* sequences of XMRV were being so related to endogenous XMLV, hence the naming of the new virus as XMRV. There is about 96% nucleotide sequence homology between XMRV and MLV in the genome (Silverman et al., 2010; Zhou et al., 2012; Rezaei et al., 2013). Also, conformational and cross-reactive epitopes are shared between mouse gammaretroviruses and XMRV.

Since original report, some researchers have detected XMRV in human prostate cancers, while others did not (Schlaberg et al., 2009; Arnold et al., 2010; Danielson et al., 2010; Verhaegh et al., 2011). Schlaberg et al. (2009) in USA showed higher prevalence of XMRV DNA by PCR (6%) and viral proteins by immunohistochemistry (IHC) (23%) in tissues from prostate cancers in comparison to noncancerous controls (Schlaberg et al., 2009). A similar study reported, a 22% XMRV prevalence in prostate cancers from Texas but the virus was found in both tumor and non-tumor tissues (Danielson et al., 2010). Moreover neutralizing antibodies for XMRV were identified in 11 of 40 patients with prostate cancer and XMRV sequences were confirmed in five of 11 by nested

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PCR and fluorescence in situ hybridization (Arnold et al., 2010; Switzer et al., 2011). Recently a study reported that, XMRV was detectable in some patients with prostate cancer in Kerman, southeast of Iran (Reza et al., 2012). In contrast, in other studies have been found very low or no XMRV prevalence in prostate cancers in Europe (Germany, and the Netherlands) and USA using only RT-PCR (Fischer et al., 2008; Hohn et al., 2009; Aloia et al., 2010; Silverman et al., 2010; Sakuma et al., 2011; Sfanos, 2011; Stieler et al., 2011). Moreover other findings imply that XMRV does not present in humans, and is only seen in the laboratory contamination (Sfanos et al., 2012). Thus, the role of XMRV in human pathogenesis is controversial (Switzer et al., 2011). XMRV was also reported to be presence in patients with chronic fatigue syndrome (CFS) (Lombardi et al., 2009). However, the link between XMRV and CFS is unclear, because in other studies it has failed to detect XMRV infection in CFS patients (Erlwein et al., 2010; Groom et al., 2010; Switzer et al., 2010; van Kuppeveld et al., 2010; Knox et al., 2011; Satterfield et al., 2011; Shin et al., 2011). However, gammaretroviruses are known to induce cancer in animals, understanding XMRV or related MLV infections in human prostate cancer tissues will shed light on their potential contribution to human disease.

Proposed reasons to explain the conflicting data are unknown but may be: technical differences, lack of standardized XMRV PCR assays, assay sensitivity, contamination by and cross-reactivity of XMRV PCR assays with closely related endogenous MLVs such as trace quantities of mouse genomic DNA found in reagents and samples (Hue et al., 2010; Oakes et al., 2010; Robinson et al., 2010; Sato et al., 2010; Knox, Carrigan et al., 2011; Tuke et al., 2011), differences in the geographical distribution of XMRV, sequence differences among XMRV genomes (Silverman et al., 2010; Singh et al., 2010; Knox et al., 2011) and factors related to the population genetic factors (Switzer et al., 2011).

Due to public health and medical consequences of potential XMRV or related MLVs infection in humans, we considered it is important to confirm or reject their association with prostate cancer in Iranian context. Herein, we developed a conventional and nested PCR assays specific for env region of XMRV and MLVs, seeking evidence of infection with XMRV or MLV in DNA content of formalin fixed and paraffin embedded pathologic specimens from prostate cancers and benign prostate hyperplasia (BPH) as controls to analysis the association of XMRV or related MLVs with prostate cancer in Iran.

Materials and Methods

Study population and specimens

63 prostate adenocarcinoma cancers and 100 BPH patients were used in this case-control study. Samples were taken from paraffin embedded blocks archived in pathology laboratory, Tohid hospital, Sanandaj, capital of Kurdistan province, in the west of Iran, during 2010-2012. To reduce the exposure of patient samples to potential sources of mouse, XMRV and MLV DNA, standard laboratory procedures for sterile DNA extraction were

practiced when handling and processing of specimens.

Tissue deparaffinization

Prostate tissue sections with 10 μm thicknesses were deparaffinized using Genetbio kit (Korea), according to manufacturer's instruction. Briefly, prostate tissue sections were thrown into 1ml xylene at 50-60ºC for 15 minutes. Then supernatant was separated by centrifugation at 13000 rpm and repeated 3-4 times and washed in 100%, 90%, 70%, 50% ethanol for 5 minutes.

DNA extraction

DNAs were extracted using PrimePrep™ genomic DNA isolation kit (GenetBio, Korea) from formalin fixed and deparaffinated prostate tissue sections. Genomic DNA of YAC-1 cells containing MLV provirus, was used as positive control purchased from national cell bank, Iran, Pasteur institute (NCBI Code: C552). Nucleic acid concentrations were determined by spectrophotometry.

Molecular detection of XMRV

Prostate tissue specimens were screened for XMRV and MLV provirus by nested PCR using primers targeting to an XMRV specific *envelope (env)* sequence, a sequence conserved amongst MLVs. Briefly, we used a first set of primers that encompasses an 1070bp, and a second set of primers to amplify 300bp of env region of the XMRV. The first round (external) primer sequences were as; **F1**: 5´-AAC AGC ATG GAA AGT CCA GC-3´; **R1**: 5´-CGT GAT TCC ACT TCT TCT GG-3´; The second round (internal) primer sequences were as: **F2**: 5´-GGA CGA TGA CAG ACA CTT TC-3´; **R2**: 5´-GAG TGT TTC CTC GCT TAA GG-3´. In addition, a conventional PCR assay using only second set (internal) primers to amplify 300 bp sequences was done to exclude any detection loss due to possible fragmentation of extracted long DNA molecules from pathologic blocks.

PCR master mix (CinnaGen, Tehran, Iran) was a premixed 2× concentrated solution of 0.04U/μl Taq DNA Polymrease (recombinant), reaction buffer, 3mM MgCl$_2$, 0.4mM of each dNTPs. Primers were added as 10pM **F1**, 10pM **R1** in 1.5μl each in first reaction, and 10pM **F2**, 10pM **R2** 1.5μl each in second PCR reaction. 2μl volumes of DNA were used as a template and the final volume of PCR reactions was 20μl. PCR reactions were heated to 94°C for 10 minutes and then cycled 35 times through 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds. Final extension 72°C for 10 minutes and was hold in 4ºC. Nested PCR products for XMRV and MLV env region would be about 1070 bp and 300 bp DNA bands. Negative controls consisted of reactions with H$_2$O in place of DNA templates. All PCR products were visualized on Ethidium Bromide-stained 1.5% agarose gels. PCR products from the first round and second-round PCR of the correct size were photographed. All specimen preparation, DNA extraction, and PCR testing was done in physically isolated rooms for contamination prevention.

Beta-actin PCR

To verify the integrity and quality of genomic DNA purified from prostate tissues and to rule out the presence

of PCR inhibitors, ß-actin sequence was amplified. ß-actin primers were designed as; ß-actin F: 5´-GCG TTA CAC CCT TTC TTG AC-3´, ß-actin R: 5´-TTG TGA ACT TTG GGG GAT GC-3´; ß-actin PCR program was as: initial denaturation at 94°C for 10 min; 94°C for 3 min, 58°C for 30 seconds and 72°C for 45 seconds (35 cycles). Final extension 72°C for 10 minutes and was hold at 4ºC. It was done for all of the prostate tissue DNA extracts before main XMRV PCRs.

Results

The average age of the prostate patients was 70.77 years, ranged from 33-88 years old. Using the Gleason system, 39.7% of the study population had grade <5 cancer, 25.4% had grade 5-6 cancer, 14.26% had grade 7, and 20.64% had high grade carcinoma. Of total 163 samples, 69 were from rural and 94 from urban; and of cancer cases 28 were from rural and 35 from urban regions.

Beta-actin sequences were successfully detected in the DNA extracts from all prostate tissues, confirming DNA extraction integrity. Designed XMRV and MLV specific primers were able to detect MLV provirus in YAC-1 cell. But testing of DNA extracted from all prostate tissue sections of total 163 specimens including 63 prostate cancers and 100 BPH was negative for XMRV and MLV
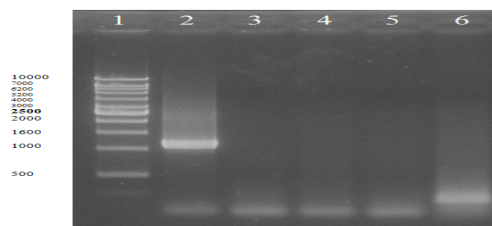

Figure 1. 1.5% Gel Agarose Electrophoresis of DNA Molecules to detect XMRV Provirus DNA in Prostate Tissues. Lane 1: molecular weight DNA markers (vivantis); lane 2: positive control (YAC-1 cell genomic DNA) using XMRV external primers, a 1070 bp PCR product is seen; lane 3: prostate cancer tissue DNA, using XMRV external primers; lane 4: prostate benign tissue DNA; using XMRV external primers; lane 5: negative control; lane 6: a sample of prostate tissue β-actin amplification (180 bp PCR product) to show DNA integrity, it was achieved for all of the prostate tissue DNA extracts before XMRV PCRs
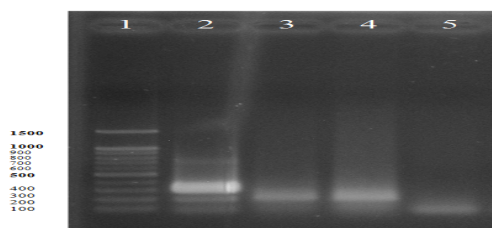

Figure 2. 1.5% Gel Agarose Electrophoresis of DNA Molecules to Detect XMRV Provirus in Prostate Tissues. lane 1: molecular weight DNA markers (Fermentas); lane 2: positive control (YAC-1 cells genomic DNA) using XMRV internal primers, and β-actin primers, (300 bp and 180 bp PCR products are seen, respectively); lane 3: prostate cancer tissue DNA, using XMRV internal primers and β-actin primers; lane 4: prostate benign tissue DNA, using XMRV and β-actin primers; lane 5: negative control

sequences in nested PCR using external and internal primers targeting to an XMRV and MLV specific envelope (env) sequence, a sequence conserved amongst XMRV and MLVs. Consistently, all of the samples were negative using XMRV and MLV specific internal primers. Thus, the overall prevalence for XMRV and MLV in our samples was 0%. The size of the beta-actin amplimer (180bp) was designed to be similar to the expected amplimer size for PCR detection of XMRV and MLV-related viruses using internal primers. A representative stained gels following doing of PCR assays are shown in Figures 1 and 2.

Discussion

Our finding is consistent with studies demonstrating no association between XMRV and related gammaretroviruses in prostate cancers. Only MLV env sequences were detected in DNA extraction of YAC-1 mouse cell lines. Beta-actin sequences were also detected by PCR in the DNA extractions from all prostate tissues, confirming their DNA integrity and sensitivity of DNA amplification. The reasons of failure to detect XMRV sequences in our specimens are unlikely due to the methods used, such as DNA extraction and PCR amplification, because both beta-actin in all prostate specimens and the env region of MLV in YAC-1 cells were successfully amplified.

Because of the high degree of homology between XMRV and related MLV provirus sequences present in mouse genomic DNA, it has been shown that XMRV specific PCR assays can cross-react with MLV provirus sequences (Urisman et al., 2006; Silverman et al., 2010; Zhou et al., 2012; Rezaei et al., 2013). PCR assays described herein detected MLV provirus env sequences present in mouse genomic DNA (YAC-1 cells) agrees with result of other studies (Danielson et al., 2010; Tang et al., 2012; Rezaei et al., 2013).

A high prevalence of XMRV has been reported in patients with prostate cancer and chronic fatigue syndrome (CFS) in the USA (Urisman et al., 2006; Schlaberg et al., 2009; Arnold et al., 2010). But, in Europe (Fischer et al., 2008; Hohn et al., 2009; Verhaegh et al., 2011), the USA, and recent study in Australia detection of XMRV in prostate (Aloia et al., 2010; Switzer et al., 2010; Rezaei et al., 2013), and breast cancer tissues (Khan et al., 2012) have been failed. Herein, we did not detected XMRV and related MLVs DNA in 163 samples of prostate cancer and BPH, which is in consistence with other reports from the USA, Europe, central Africa, China, and Australia (Simmons et al., 2011; Rezaei et al., 2013; Williams et al., 2013). The reasons for this variation in results might be factors such as, geographic distribution, genetic variations of the virus (Hohn et al., 2009; Luczkowiak et al., 2012) and absence of XMRV and related MLVs in human infections. It is also possible that the quality of the specimens were low due to preservation, handling, and the duration of storage prior to DNA isolation.

Several laboratory methods were applied to detect XMRV in prostate cancers; the results of them do not support any association between XMRV and related gamaretroviruses in prostate cancer (Mendoza et al., 2012; Rezaei et al., 2013). As well, the absence of XMRV or

related MLV may not be due to decreased sensitivity of PCR assay. Since we detected MLV provirus in YAC-1 cells using same primers.

Mouse DNA contamination in laboratory reagents was also reported to be the source of XMRV in studies of prostate cancer tissues and CSF (Switzer et al., 2011; Tang and Hewlett, 2011; Tang et al., 2012) and resulted to retraction of some papers. These results are important science laboratory contamination by endogenous MLVs would be false-positive XMRV detection due to the high homology in DNA sequences between these viruses (Tang and Hewlett, 2011). In addition, primers originally used for XMRV detection were also able to detect homolog MLV sequences from mouse DNA. In a study the absence of XMRV DNA or neutralizing anti-XMRV antibodies suggests no or very low prevalence of XMRV in their cohorts. They conclude that real-time PCR and IHC positive samples were laboratory contamination and false-positive immune reactions (Sakuma et al., 2011). Our PCR result in specimens was negative for XMRV and related MLV, but only YAC-1 cells was positive for MLV; thus we did not have any mouse cross contamination.

Potential limitations of our study are that, due to the limited amount of samples we were unable to test more prostate cancer patients. For viremia examination, unfortunately, it was not achievable to extract RNA from plasma specimens of our patients to analyze by RT-PCR. As well, we were not able to do serologic tests for antibodies that are important features of retroviral infections for additional evidence for non tissue-specific infections. In addition, studies failing to detect XMRV or related MLVs in prostate cancer and CFS patients outnumber the studies confirming the original findings (Korn et al., 2012; Mi et al., 2012; Paolucci et al., 2012; Rezaei, Hearps et al., 2013). In a study the presence of XMRV was detected among some patients with prostate cancer in the Iran, but did not find any correlation between XMRV infection and various clinical pathological parameters of prostate cancer (Reza, Fahimeh et al., 2012).

In conclusions, several studies have used primers that simultaneously detect XMRV and related MLVs in prostate cancers (Hohn et al., 2009; Aloia et al., 2010; Robinson et al., 2011; Switzer et al., 2011; Das Gupta et al., 2012; Rezaei et al., 2013). But, XMRV or related gamaretroviruses (MLVs) were not detected in our samples. We conclude that in our population XMRV or related MLVs does not play a role in tumorigenisis of prostate cancer and adds additional evidence to this area.

Acknowledgements

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