RESEARCH ARTICLE

Evaluation of Xenotropic Murine Leukemia Virus and its R426Q Polymorphism in Patients with Prostate Cancer in Kerman, Southeast of Iran

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Abstract

A role for the xenotropic murine leukemia virus (XMRV) in prostate cancer development has been postulated. To answer questions regarding the prevalence of XMRV in Iranian patients with prostate cancer and its association with the *RNASEL* R462Q polymorphism, we here investigated a series of cases in Kerman, in the Southeast of Iran, and sought to verify the association with the R462Q using Real Time PCR Method. Prostate tissue specimens of 200 patients with prostate cancer were genotyped for R462Q by real time polymerase chain reaction allelic discrimination and were screened for XMRV proviral DNA by real time polymerase chain reaction specific for the envelope gene. Of 200 patients in this study 8 (4%) cases were positive for XMRV, the QQ allele being the most frequenct regarding the R426Q polymorphism while in negative patients it was the RQ allele. There was significant correlation between high pathological scores and XMRV positive samples. No significant relationship was found between age groups and XMRV results. XMRV was only found in patients with QQ and RQ alleles, not RR. XMRV is detectable in tumor prostate tissue from some patients with prostate cancer, independent of R462Q.

Keywords: Prostate cancer -XMRV - R426Q - real time PCR - Iran

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Introduction

Prostate cancer is one of the most common malignant cancers in men and after lung cancer it is the second leading cause of mortal cancers in males. The risk of prostate cancer for men in their lifetime is 1 in 6 cases, which is statistically significant and the high incidence of this cancer in middle-aged men is remarkable compared to other cancers (Alvarez et al., 2012). According to recent studies, 30-50 percent of men over age 50 are infected with prostate cancer. Differences in prostate cancer incidence and its mortality around the world may be related to differences in genetic and environmental factors (Ferrís et al., 2011). Recently, the genetic factors involved in prostate cancer have been reported and the cause of variant of R462Q (rs486907) of the *RNASEL* gene in this cancer has been indicateded. Proteins encoded by *RNASEL* (*RNaseL*) gene have the effective role in the antiviral response induced by interferon Class 1 and 2 for human immunity (Casey et al., 2002).

The relationship between variants of *RNASEL* (which is a gene with viral activity) with prostate cancer, makes this theory that there is likely a viral infection in prostate cancer (Chen et al., 2003). To review this issue a Panviral Microarray test has been done on patient's prostate tissues who were homozygous individuals for R462Q variant. From total 11 samples, 7 patients had a Gama retrovirus (Wainberg et al., 2011). These results indicated that there is a significant relationship between *RNASEL* gene malfunction and retroviral infection in the prostate. Subsequent reviews to the recognition of this virus showed that this is a similar virus with Murine leukemia virus (MLV) and therefore it was named xenotropic murine leukemia virus - related virus (XMRV) (Urisman et al., 2006; Robinson et al., 2011).

After identification of this virus in prostate cancer tissue, different studies on the incidence of this viral infection in patients with prostate cancer were done and also association of R462Q variant and *RNASEL* gene with the risk of this infection was observed (Arnold et al., 2010). The results were different, but in most studies the high prevalence of XMRV infection in prostate cancer patients than controls has been reported. For the first time, (10%) incidence rate of XMRV infection in prostate cancer as well as its relationship with mutant R462Q of *RNASEL* gene was reported in 2006 by Urisman and colleagues (Urisman et al., 2006). In 2009 a study was conducted by Schlaberg and colleagues and the XMRV incidence in patients with prostate cancer was reported 27% but no association between XMRV infection and mutant

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R462Q of *RNASEL* gene was found (Garson et al., 2011). In 2010 Arnold and colleagues reported high incidence (27.5%) of XMRV infection in prostate cancer patients and in similar review study which was done by Urisman the mutant R462Q of *RNASEL* gene was associated with XMRV infection (D'Arcy et al., 2008). In 2009, XMRV infection was detected in 22RV1 cell line which is derived from prostate epithelial cancer cells and also Furuta and colleagues reported the 6% incidence of XMRV in prostate cancer patients in PBMC samples (Maric et al., 2010). Unlike those studies which their results were favor of XMRV involvement in prostate cancer, other studies has been investigated in which no XMRV infection in prostate tissue and prostate cancer patients has been reported.

Fortunately, this new virus is sensitive to antiviral drugs such as Zidovudine, Tenofovir and Reltegravir and with definitive proof about the role of this virus as an etiologic factor for prostate cancer; we can reduce the risk of this cancer by anti-viral therapy (Sakuma et al., 2010). According to the conflicting results about the relationship between XMRV and prostate cancer, further studies to clarify this issue are required. So the aim of this study is the evaluation of XMRV infection incidence, the existence of R462Q mutant of *RNASEL* gene in patients with prostate cancer and the association of R462Q mutant of *RNASEL* gene with the risk of XMRV infection which was done for the first time in Iran.

Materials and Methods

Prostate cancer and tissue preparation

Paraffin embedded blocks or biopsy samples from patients with prostate cancer, referred to the Department of Pathology, University Hospital, Kerman Central Pathobiology Lab, Kerman province, Iran, and during 2010-2012 were identified. A total of 460 specimens found, but 200 formalin-fixed paraffin-embedded tumor tissues of patients collected and entered to our study and others patients were lost due to inadequate, absence of histological material. No patient had received chemo- or radiotherapy before surgery. The present study is based on a retrospective examination of prostate carcinoma diagnostic biopsy or surgery samples from clinical cases, all original hematoxylin and eosin (H&E) slides and/or H&E recut from tissue blocks were reviewed. In total, 200 samples were screened for XMRV and the R462Q polymorphism in *RNASEL*. This project was approved by the human research ethics committee of the Kerman University of Medical Sciences and participating Centers, and written informed consent was obtained from all participants.

Deparaffination samples

Paraffinated blocks from the 200 tumor samples were cut in 5-μm sections and 8 sections/patient were collected in the same microcentrifuge tube. Samples were de-waxed in 500 μl xylene, All microcentrifuge tube located for 10 min in a 60 °C heated block and centrifuged at 8,000 rpm, supernatant was removed. This step was then repeated 3 times. Add 500 μl absolute ethanol, centrifuge at 10,000 rpm for 1 min, the samples were then dried in a 60°C heated block with open lids for 10-20 min for remove residual ethanol.

Tissue digestion

According to samples (biopsy or surgery), 200-400 μl of Tissue Lysis Buffer was added to each tube [4 M Urea, 200 mM Tris, 20 mM NaCl, 200 mM EDTA; PH=7.4 (25°C)]. To all tubes added 20-40 μl proteinase K, Samples were gently vortexes and located for 10 min in a 60°C heated block, and all samples were subsequently incubated at 37°C overnight.

Provirus DNA Extraction

The next day, 200 μl of Binding Buffer [6 M Guanidine-Hcl, 10mM Urea, 10mM Tris-Hcl, 20% Tritonx-100(v/v); PH=4.4(25°C)] was added to each tube with gently vortex. DNA was isolated using a QIAamp DNA Mini kit (Qiagen, Germany) according to the manufacturer's instructions. Extracted DNA pellets were resuspended in 100μl of prewarmed Elution buffer and stored at -20°C until use.

Quality Control

The quality and concentration of DNA was measured either on an ethidium bromide-stained 1% agarose gel or by standard spectrophotometric methods (data not shown). Also, presence of DNA and accuracy of DNA extraction was confirmed by PCR with human β-globin primer as an internal control. The positive control consisted of 2 separate reactions, each with DNA isolated from XMRV-infected LNCaP cells. The master mix was considered to be adequately sensitive only if all 2 positive control reactions had positive results. Negative controls consisted of 2 separate reactions with H_2O in place of DNA template and 2 separate reactions of uninfected LNCaP DNA. After thermo cycling, second-round reactions were electrophoreses on agarose gels containing ethidium bromide and were visualized under ultraviolet light.

Provirus detection

A Real Time PCR assay was developed to screen prostate tissue DNA for XMRV provirus. The primers amplify a 102bp region containing the 3´ variable regions of envelope. Primers and probes designed by H.R.Mollaie after alignment of complete genomes XMRV consist in Nucleotide pubmed database. reactions contained 8μl of prostate tissue DNA, 2.5 mmol/L $MgCl_2$, 800 mmol/L of dNTPs, 100 ng of each primer, 30 ng of probe and 1.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems) in a 50 mL total volume.

Thermo cycling conditions were as follows: 95°C for 10 min; 40 cycles of 95°C for 15s, 60°C for 40 sec,

Table 1. Sequence of Primers and Probes and their Position with Melting Temperature

Name	Sequence Primer and Probes	Tm	Position
Forward primer	GGAAGATGTGGAAAATGA	58.3	266
Reverse primer	GGGACTCACCTATTAAGA	58.3	405
R probe	ATGAATTTGCCCAAAATGTCCTGTC	67.7	289
Q probe	ATGAATTTGCCCGAAATGTCCTGTC	69.2	289
Forward primer	CCTGTCTATCTACTACTGTG	59.8	725
Reverse primer	GGCCATAAACATAATTAGGG	59.9	826
XMRV probe	CCAGAGTTCAACCAGGACACAGTA	69	786

Table 2. Basic Characteristic of Patients with Prostatic Cancer

Age Group	No.	Biopsy	Embedded Paraffin Block	Extra capsular Extension(ECE)	Seminal Vesicle Invasion (SVI)	Surgical Margin Invasion (SMI)	XMRV Result
<40	4	4	0	4 (2%)	0 (0%)	0 (0%)	0 (0%)
40-50	12	10	2	11 (5.5%)	1 (0.5%)	0 (0%)	1 (0.5%)
50-60	44	36	8	28 (14%)	8 (4%)	8 (4%)	1 (0.5%)
>60	140	140	0	52 (26%)	22 (11%)	46 (23%)	6 (3%)

Quantitative determination of the amplified products was done with the Rotor Gene 6000 (Corbett Research, Australia). To synthesis of our ideal genes, the test should be done by two pairs of forward and reveres primers and three different probes to detect allele R, Q and XMRV agent. Our specific probes were designed by different fluorescent labels for tracking our targets separately. In Table 1 the sequences of primers and probes and their position with melting temperature are shown.

RNaseL genotyping

A TaqMan Real-time PCR setup designed by H.R.Mollaie, was used for *RNaseL* genotyping of tumor samples which detects the single nucleotide polymorphism G1385A (rs486907) responsible for the R462Q mutation (Allele change CGA to CAA, R=Arg change to Q=Gln). PCR was carried out following a two-step protocol with 95°C for 10min, 95°C for 15sec and 60°C for 40sec. Data acquiring in green channel for R and yellow channel for Q polymorphism.

Statistical analysis

Chi-square test or Fisher's exact test was conducted using SPSS version 17 for the association between the presence of provirus genome and other characterizes (values P=0.05 were considered statistically significant).

Results

Two hundred patients with prostatic cancer were selected during two years (2010-2012). Paraffinated blocks or biopsy samples from these patients were selected for detection of XMRV and evaluation frequency of *RNaseL* polymorphism using Real Time PCR. Different age groups are categorized by less than 40 year old, 40-50, 50-60 and upper than 60 years. From total 200 samples, 140 were upper than 60 year which is reported by biopsy specimens. According to this individual information, most of samples from extra capsular extension (ECE), seminal vesicle invasion (SVI) and surgical margin invasion (SMI), were found in patient who were more than 60 years. After analysis of data by statistical software (SPSS V-14), Pearson correlation of age and PSA was 0.633 and the p value=0.000 (see Table 2).

Table 3. Frequency of XMRV Results and R426Q Allele Polymorphism in Different Gleason Scores

Gleason	5	6	7	8	9
Negative	19 (9.5%)	26 (13%)	50 (25%)	33 (16.5%)	64 (32%)
Positive	0 (0%)	0 (0%)	2 (1%)	4 (2%)	2 (1%)
R426 Q allele					
QQ	1 (0.5%)	0 (0%)	17 (8.5%)	11 (5.5%)	16 (8%)
RQ	4 (2%)	14 (7%)	18 (9%)	18 (9%)	23 (11.5%)
RR	14 (7%)	12 (6%)	17 (8.5%)	8 (4%)	27 (13.5%)

In total patients, frequency of R462Q alleles was following QQ=45(22.5%), RQ=77(38.5%), RR=78(39%), 192(96%) of 200 patients was negative results for XMRV and 8 samples (4%) was positive for XMRV. In this study in patients with positive results, QQ allele 5 patients with mean=97.4±70.4 and in RQ allele 3 patients with mean=111.7±42.3 are reported and interestingly no XMRV gene was detected in RR allele. In patients with negative results, QQ allele 40 patients with mean=121.3±54.4 and

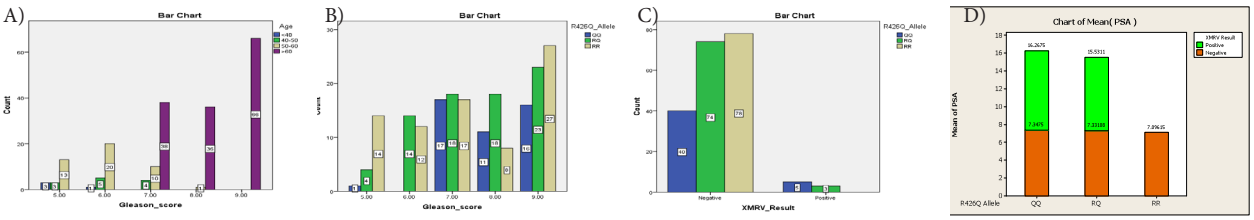

Figure 1. Frequency. A) Frequency of Gleason Score Age Group, B) Frequency of Gleason Score R426Q Allele. D) Frequency of XMRV results for R426Q allele. E) Frequency results of PSA with the combination of XMRV and R426Q (Mean ± SD).

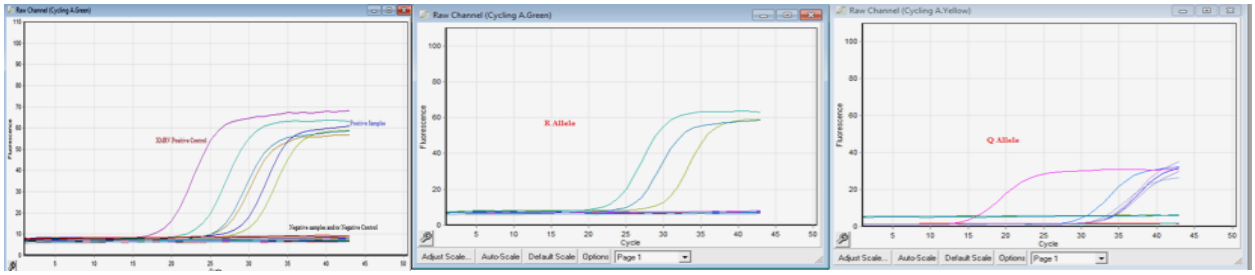

Figure 2. Positive Control Samples for XMRV are Positive and Other Positive Samples are Shown but Negative Control and Negative Samples are Represented Linearly. About XMRV positive control, because of high titer, our detection was done sooner than other positive samples. The specific peak is detected in green channel which identified R allele. On the other hand the specific peak is detected in yellow channel which identified presence of Q allele in our samples.

in RQ allele 74 patients with mean=104.9±63.4 and also in RR allele, 78 patients with mean=85.4±50.5 are reported and in all XMRV gene is negative. From total samples, the mean of Gleason score in QQ, RQ and RR alleles are 13.6, 14.2 and 5.8 respectively and no XMRV was found in RR allele groups (see Figure 1 and Table 3). Mean of Gleason scores in the combination of XMRV results and frequency of R426Q alleles are 5.6(QQ), 11.9(RQ) and 17.7(RR) alleles respectively in cases that are negative for XMRV gene. QQ and RQ with the mean=8 and 16 are shown that are carrying XMRV gene.

In Figure 1D three columns are shown which results are about Prostatic specific Antigen (PSA) Mean±SD with the combination of XMRV and R426Q frequency. In QQ and RR homozygous alleles and RQ heterozygote allele, XMRV results are shown. From total samples, the mean of PSA in QQ, RQ and RR alleles are 16.2, 15.5 and 7.0 respectively and interestingly the XMRV results are positive (green color) in QQ and RQ columns and no XMRV was found in RR allele groups (see Figure 2). After analysis of data, Pearson correlation of Gleason score and PSA was 0.206 and the p value=0.003 were reported.

Discussion

In 2000, in Italy and Spain, prostate cancer incidence was ranked third (approximately 10%), while in France it was the most common male cancer (approximately 19%) (Quaglia et al., 2003).Some previous studies have reported that opposite of Europe and America, prostate carcinoma is not common in Iran. It has been estimated that the incidence of prostate cancer has increased in Iranian immigrants to Western countries (Hosseini et al., 2010). Although current data on the epidemiology of prostate cancer in Iran are vague and incomplete, this cancer is the eighth leading cause of death from cancer in Iran and the incidence of this cancer has been rising in the last 10 years in Iran. It is thought that 42 percent of prostate cancer cases due to genetic factors and 58 percent are because of environmental factors but various causes of prostate cancer is still not well known even in some cases different results have been seen in this field. Prostate specific antigen (PSA) testing for prostate cancer screening can signal the presence of impalpable and thus previously undetectable cancer. Consequently, intensified effort in early detection of prostate cancer through PSA screening has amplified the incidence of prostate cancer by allowing the detection of localized latent cancer lesions, whereas in our country, data on incidence of prostate cancer only reflect the clinically obvious diseases (Sadjadi et al., 2007). In 2002, Carpten et al. proposed *RNASEL* as an effective gene for prostate cancer. To test this hypothesis that *RNASEL* sequence variants are associated with prostate cancer, we studied SNPs variants R462Q and 200 prostate cancer patients. Overall, our study provided support for the hypothesis that *RNASEL* is a prostate cancer susceptibility gene and suggestive evidence of relation between mutations (RR, RQ, and QQ) and prostate cancer was observed and we observed identical frequencies of R462Q mutation carriers among sporadic prostate cancer cases (Summers et al., 2008). The common missense mutation R462Q has been studied in prostate cancer risk, using 423 prostate cancer cases and 454 sibling controls, Casey et al. showed that heterozygous carriers of this variant have 50% greater risk of prostate cancer than no carriers, and homozygous carriers have more than double the risk (Casey et al., 2002). They also reported a decrease in enzymatic activity of the variant to one-third of normal activity. Rökman et al. reported moderate evidence for an increased risk of prostate cancer for homozygous carriers of the R462Q variant in 66 index patients from HPC families (Rökman et al., 2001). In addition, Nakazato et al. reported an association between this variant and prostate cancer risk among 473 affected men from 181 families; however, they found an opposite trend with odds ratios of 0.8 for heterozygotes and 0.5 for homozygotes (Nakazato et al., 2003). This missense variant was significantly associated with an increased prostate cancer risk in a study from Japan based on 101 familial prostate cancer cases and 105 controls, yet other studies have failed to find an association between this variant and prostate cancer risk (Nakazato et al., 2003).

In conclusion, we confirm the involvement of *RNASEL* in the prostate cancer and we provide some evidence for an association of the *RNASEL* gene with prostate cancer in Iranian men. The prostate cancer risk is varied between racial/ethnic groups, indicating that race/ethnicity plays a role in the development of prostate cancer. This is because each case brings genetic material that sets each race and ethnicity apart and also the combination between environmental factors and genetic background can play a role in this cancer(Huihua et al., 2006). Our study of patients with prostate cancer confirms the presence of XMRV among patients with prostate cancer in the Iran. The results presented here identify XMRV infection in prostate tissue from patients with prostate cancer as judged by Real time-PCR with XMRV-specific primers. It clearly demonstrated that human XMRV infection is strongly linked to RNase L activity. This result supports the view that the R462Q RNase L variant leads to a subtle defect in innate (IFN-dependent) antiviral immunity.

Interestingly, some studies, including 2 surveys of German prostate tissue specimens and a screen of English chronic fatigue syndrome patients, found no evidence of XMRV infection (Hong et al., 2012). However, in agreement with studies performed in the United States, they found the presence of XMRV in prostate cancer tissues (Bjartell, 2006). It is possible that XMRV is mostly present in the Iranian population. If so, it would be interesting to know the reason for this geographic distribution. Alternatively, the inability to detect XMRV in the world may possibly reflect genetic differences between different strains. However, this seems unlikely considering the high degree of sequence conservation among XMRV isolates and the variety of primer target sequences used for detection among the studies in Europe (Klein and Silverman, 2010). It is possible that the quality of the specimens were low because of preservation, handling, and the duration of storage prior to DNA isolation. However, we were able to detect the patients for R462Q with use DNA without difficulty. We did not find a correlation between XMRV infection and various clinical

pathological parameters of prostate cancer, including seminal vesicle invasion, extra capsular extension, and surgical margin invasion. From total samples, the mean of Gleason score in QQ, RQ and RR alleles are 13.6, 14.2 and 5.8 respectively and no XMRV was found in RR allele groups. Similar to another report, which found a correlation with higher Gleason scores, we found a slight trend in favor of increasing Gleason score (Trottier and Fleshner, 2010) . In conclusion, our study supports a hypothesis that XMRV is endemic to Middle East and Iran. However, more investigation into the relation of XMRV with prostate cancer and other human diseases is needed.

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