

Detection of Xenotropic Murine Leukemia Virus-Related Virus in Prostate Biopsy Samples

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ABSTRACT

Objective: To determine the association of Xenotropic murine leukemia virus related virus (XMRV) infection with prostate cancer and compare it with benign prostate hyperplasia.

Study Design: Case control study.

Place and Duration of Study: Department of Histopathology and Molecular Pathology, Dow University of Health Sciences, Karachi, from January 2009 to December 2012.

Methodology: XMRV was screened in 50 prostate cancer and 50 benign prostatic hyperplasia biopsies using conventional end-point PCR. Other studied variables were family history of prostate cancer, patients age and Gleason score.

Results: XMRV was detected in 4 (8%) of the 50 prostate cancer biopsy specimens compared to none in biopsies with benign prostatic hyperplasia. However, there was no significant statistical association of XMRV infection with the other variables.

Conclusion: A low frequency of XMRV infection was found in this case-control study. Men, who harbor XMRV infection, may be at increased risk of prostate cancer but this needs to be investigated further at a larger scale.

Key Words: *Xenotropic murine leukemia virus related virus (XMRV). Pakistan. Prostate cancer. Gleason score.*

INTRODUCTION

Prostate cancer is the second most common malignancy among men globally.¹ Scientists have reported several risk factors, linked to the development of prostate cancer. An important risk factor recently identified in samples of prostate cancer is xenotropic murine leukemia virus (MLV)-related virus (XMRV). Phylogenetic analysis has demonstrated that XMRV is a gammaretrovirus bearing close resemblance with endogenous murine leukemia virus. It is the first gamma retrovirus known to infect humans.²

XMRV was initially identified in non-malignant stromal cells of prostate cancer tissues homozygous with R462Q polymorphism in the RNaseL gene, suggesting viral involvement in tumorigenesis via paracrine mechanisms.² However, a subsequent study suggested direct viral involvement in oncogenic mechanism by demonstrating viral antigens in malignant prostatic epithelial cells especially in higher-grade tumors, independent of the RNaseL polymorphism.³ Later, demonstration of specific viral integration sites near cancer breakpoints, micro-RNA genes and XMRV

neutralizing antibodies in serum of prostate cancer patients, further strengthened the hypothesis of viral oncogenesis.⁴ Contrary to these findings, several recent studies have failed to report XMRV in variety of prostate cancer samples, making its link with prostate cancer elusive.⁵⁻⁸

The aim of this study was to detect XMRV in biopsies with adenocarcinoma of prostate and compare it with non-tumor prostate biopsies. The association of XMRV with family history of prostate cancer, patient's age and Gleason score was also determined.

METHODOLOGY

Ethical approval was sought from Institutional Review Board (IRB) of Dow University of Health Sciences (DUHS) to collect 100 formalin-fixed paraffin-embedded (FFPE) samples of prostate biopsies, which included newly diagnosed 50 cases of prostate adenocarcinoma and 50 age matched benign prostatic hyperplasia as controls. The samples were collected from Dow Diagnostic Research and Reference Laboratory (DDRRL), from January 2009 to December 2012. The samples were from patients aged 40 - 85 years, diagnosed with adenocarcinoma of prostate and benign prostatic hyperplasia. Mean age was 67.1 years. These patients underwent TURP surgery at various tertiary care hospitals of the country and represented wide social and ethnic backgrounds.

All prostate cancer biopsies were reviewed by a panel of expert histopathologist at DDRRL, to report final

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diagnosis and tumor score by consensus. Family history of prostate cancer, age and informed consent was obtained from patients. Family history of the disease was determined as described by Carter *et al.*⁹

All FFPE prostate tissues were prepared for DNA extraction in a separate laboratory free from all mice contamination. DNA was extracted from 10 µm sections of prostate biopsies by using Invitrogen PureLink™ Genomic DNA extraction Kit according to manufactures instruction. All prostate tissue DNA samples were stored at -20°C immediately following extraction in a laboratory free of amplified or cloned DNA. To authenticate a successful extraction β-Globin gene was amplified by conventional PCR.

Cloned XMRV RNA fragments (Norgen Biotek Corp.) were used to generate XMRV cDNA stock for PCR controls. cDNA synthesis was performed by M-MLV Reverse Transcriptase Kit (Bioneer) according to manufacturers protocol. The retrieved cDNA was stored at -20°C in a laboratory free of amplified or cloned DNA for further use.

The XMRV proviral DNA screening was performed by conventional end-point PCR using commercially available XMRV specific primers (Norgen Biotek Corp.).¹⁰ β-Globin gene was amplified using PG04/GH20 primers.¹¹ Primer details are given in Table I. The PCR conditions for these primers were as follows:

For XMRV primers, the total 20 µL PCR reaction mixture contained 5 µL sample, 10 µL 2x PCR master mix (Merck), 2 µL primers set mix (Norgen Biotek Corp.), and 3 µL nuclease free water. The PCR thermal profile was: 95°C for 3 minutes, 40 cycles of 94°C for 15 seconds, 60°C for 30 seconds, 72°C for 45 seconds and final extension of 5 minutes at 72°C. For β-Globin primers, the total 20 µL PCR reaction mixture contained 5 µL sample, 10 µL 2x PCR master mix (Merck), 1 µL of each primer and 3 µL nuclease free water. The PCR thermal profile was 94°C for 5 minutes, and 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and 10 minutes final extension at 72°C (Figure 1).

Amplified PCR product was run on 2% Agarose gel stained with ethidium bromide. The PCR products were identified based on their predicted fragment size. To validate the PCR data, XMRV cDNA was subjected to every PCR run as positive control, whereas to access template contamination negative controls without DNA template were included in each PCR session. Finally, it is very important to highlight that we took all necessary measures to ensure that entire bench work is free from all sorts of contamination.

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS), version 21. Association of XMRV infection with tissue type, patient's age, family history of prostate cancer and Gleason score

were analyzed by Fisher's exact test whereas odds ratio was estimated to determine risk of disease in infected patients.

RESULTS

Four out of fifty prostate adenocarcinoma biopsies were positive for XMRV DNA; however, none of the benign control showed positivity (Figure 2). An insignificant statistical value ($p = 0.059$) and risk (OR=0.479; CI= 0.38 - 0.59) was determined, among case versus control group. However, statistical parameters of control (benign prostatic hyperplasia) could not be calculated, as the virus was not found in the control group.

The association of XMRV infection with family history, patient's age and Gleason score of prostate cancer cases was also investigated. However, none of these research parameters showed significant statistical link with XMRV infection. The statistical estimates are presented in Table II.

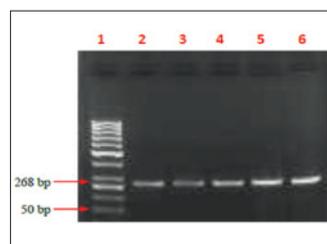


Figure 1: Representative gel of isolation controls. Line 1 shows 50 bp molecular weight ladder. Lanes 2-6 displays 268 bp β-Globin gene amplicon.

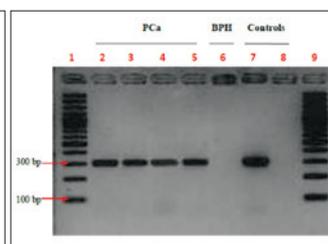


Figure 2: Representative gel of XMRV PCR. Lanes 1, 9 shows the 100 bp molecular weight ladder. Lanes 2 - 5 are results from XMRV positive prostate adenocarcinoma (PCa) specimens. Lane 6 represents a XMRV negative benign prostatic hyperplasia (BPH) specimen. Lanes 7 - 8 are positive and negative controls respectively for XMRV PCR.

Table I: Primers used for XMRV screening.

Primers	Sequence / description	Amplimer length
β-Globin		268bp
PG04	CAACTTCATCCACGTTCCACC	
GH20	GAAGAGCCAAGGACAGGTAC	
XMRV	Catalog # 34710; ISO Certified (Norgen Biotek Corp.)	300bp

References for each primer are cited in the Materials and methods section.

Table II: Association of XMRV with age, family history and Gleason's score of prostate cancer.

XMRV	Age		Family history of prostate cancer		Gleason score	
	< 55	≥ 55	Familial	Non-familial	Low ^a	High ^b
Present	1	3	2	2	1	3
Absent	2	44	8	38	8	38
Total	3	47	10	40	9	41
P-value	0.22		0.17		0.56	
Odds ratio	0.13		4.75		1.58	
C.I	0.00 - 1.96		0.58 - 38.9		0.14 - 17.2	

^a Gleason score 1-6; ^b Gleason score 7-10.

DISCUSSION

The present study, to the best of our knowledge is first of its kind to report XMRV in Pakistani patients with prostate adenocarcinoma. Presence of XMRV proviral DNA was documented in four prostate cancer biopsy specimens. However, we were unable to confirm the correlation between XMRV infection with prostate cancer, age, family history and Gleason score. These rates are in agreement to those reported by PCR in an earlier case-control study but much lower than demonstrated by immunohistochemistry (IHC) in the same investigation.³ Although it has been recently suggested that IHC antibody used in that investigation, might have detected non-viral proteins.¹²

In sharp contrast, previous cohort investigations reported a high prevalence of XMRV in variety of samples from prostate cancer patients whereas; recent studies have failed to detect the virus in multiple sets of specimens.^{2,8,12-14} Such discrepancies in results might have been contributed either by differences in detection methods used or technical skills.³ Additionally, cross-contamination by mice DNA leading to false positive result and in particular recent reports of contaminated human cell lines employed in experiments, has suggested re-evaluation of previous results.¹⁵⁻¹⁸ Alternatively, geographical distribution of the virus as noted in case of other retroviruses or total absence of XMRV in clinical samples might have accounted for such variation in results.¹⁹

In the present work, we have employed a conventional end-point PCR assay to screen proviral DNA, whereas viral RNA was amplified in most of the previous studies by RT-PCR assay.^{2,5,20,21} Although, proviral DNA screening was also demonstrated in several investigations by real-time and nested assays using PCR primers, manufactured to author's specification.^{3,12,14,22} However, contrary to those, a qualitative PCR was performed in this study, using XMRV specific commercially available PCR primer set of renowned brand and followed standardized PCR protocol. Furthermore, to the best of our knowledge, use of commercially available PCR primer has not been documented in any published XMRV research to-date.

To ensure contamination free experiment, all research work was carried out in a separate mice free laboratory along with inclusion of negative control in every PCR run. Moreover, entire work was carried out on clinical biopsy specimens and no human cell line was introduced in research. Samples were processed in BSC II - A safety cabinets and considered positive if three consecutive extractions followed by PCR replicates amplified a DNA fragment of the expected size.

An important aspect of the role of XMRV in prostate cancers that needs further investigation is the potential synergism between the virus and familial prostate

cancer with R462Q variant.² Contrary to initial report, the virus has been identified in non-familial tumors independent of genetic polymorphism in significant numbers.^{3,14} Consistent with those findings, we have determined an equal presence of XMRV in familial and non-familial cases (2:2), although R462Q status was not investigated. It is, therefore, suggested that virus can be detected independent of familial tumors; however, the present results were unable to comment on RNaseL (R462Q) polymorphism.

Along with genetics, age is another major risk factor of prostate cancer.²³ We have observed that the majority of XMRV bearing cases were aged above 55 years, suggesting high frequency of viral detection with advancing age. Similar findings were reported in an earlier case-control analysis.³ Significant statistical power and risk could not be documented, likely due to low viral prevalence and limited sample size. For precise estimation we recommend research with large number of sample representing both younger and older patients.

The association of XMRV infection with higher Gleason score has been reported previously.³ Although, the present results did not find a statistical correlation between XMRV infection and higher Gleason score, we have observed a trend slightly in favor of high Gleason score. Interestingly, immunosuppression produced by cancer immunoediting is commonly observed with tumor progression.²⁴ Additionally, cancer patients are also recipients of chemotherapy, which further suppress immune system.²⁵ It is, therefore, suggested that acquisition of XMRV infection might be due to deteriorating state of cellular immunity. Additional studies with a larger sample size will be required to precisely evaluate the link between XMRV infection and Gleason score.

CONCLUSION

A low prevalence of XMRV was indicated in samples with prostate adenocarcinoma. In the light of the present findings, we recommend further investigation of prostate carcinogenesis in men who fails to clear XMRV, to get a full insight of this virus and its importance in the disease.

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