JC Polyoma Virus as a Possible Risk Factor for Prostate Cancer Development - Immunofluorescence and Molecular Based Case Control Study

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Abstract

Background: In vitro studies have produced conflicting results about the significance of the JC Polyoma Virus (JCV) in the human cancers.

Objectives: Our study aims to detect the presence of JCV Large T antigen (LTag) together with viral load quantitation in the prostate tumor samples to assess if JCV harbors risk factor for prostate cancer (PCa).

Method: This was a case control-based study. A total of 110 patients participated in this study, including 55 patients with PCa and another 55 patients with benign prostatic hyperplasia (BPH) as cases and controls, respectively. Tissue, blood and urine samples were collected from each participant. Tissues samples were analyzed for the presence of JCV LTag using a direct immunofluorescence assay (IF). Only positive IF tested samples were subjected to viral quantitation assay. Data were collected and managed using SPSS version 20.

Result: The JCV LTag in the cases group was 23.63% (13/55) which was higher than that of the controls group 5.45% (3/55) with a P. value of .006 and O.R of 5.76. The mean of viral load was significantly higher among cases tissue specimens 20156 ± 5450 copies/ml compared to controls group 6378 ± 2456 copies/ml with P-value of .002. The virus was detected in 11/13 (84.6%) urine samples of cases with a mean viral load of 14068 ± 4590 copies/ml compared to 2/3 (66.6%) of controls viral load 2534 ± 1267 copies/ml.

Conclusion: In conclusion, a higher JCV LTag with more viral load were detected in cases group compared to controls. Our findings support a strong relationship between JCV infection and the probability of developing PCa.

Keywords
JC Polyoma virus, large T antigen, prostate cancer, benign prostate hyperplasia, immunofluorescence assay

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Background

Prostate cancer is the most prevalent and leading cause of cancer death in men, with 241,740 new cases and 28,170 deaths reported in 2012. According to the American Cancer Society, PCa will account for 26% (220,800) of all new cancer cases in males and 9% (27,510) of all male cancer-related fatalities in the United States in 2015. Furthermore, prostate cancer is the most commonly diagnosed cancer in men, with an estimated 161,360 new cases and 29,480 deaths in 2016.

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cancer was the fourth most frequent cancer in the United Kingdom in 2009, accounting for about 13% of all new cases (40,841). Prostate cancer accounts for around 11% of all male cancers in Europe, with 2.6 million new cases diagnosed each year. Prostate cancer incidence and mortality rates in Africa were reported to be 23.2 and 17.0 per 100,000, respectively, in the GLOBOCAN 2012 reports. While this was lesser than in some other parts of the world, it was nevertheless significant. Prostate cancer death rates are generally greater in largely black African populations than in other races, according to the findings. Prostate cancer is the most frequent malignancy in Sudanese men, according to the Radiation and Isotopes Center of Khartoum (RICK) (3.3%). The majority of patients are diagnosed late, often with significant metastatic disease. Prostate cancer affects roughly 600 Sudanese males every year, with a mortality rate of 8.7 per 100,000. The disease was found to be equally widespread throughout tribes, with the majority of cases (85.4%) presenting with stages III and IV. The common age of the sufferers became 72.2 years plus or minus 9.25 years.

Age, race, ethnicity, alcohol intake, hereditary factors, farmers, a high-fat diet, tire factory workers and men who have been exposed to cadmium, and infections with specific viruses such as Human polyomavirus are all risk factors for prostate cancer. The JCV significance in the malignant transformation was first hypothesized in 1971, shortly after its isolation. The function of JCV in human malignancies is debatable, according to in vitro research. The high seroprevalence and high detection rate of JCV in healthy people complicates studies and necessitates a thorough re-evaluation of previously published data. Single case reports of uroepithelial and renal tubular cancers, particularly in the setting of chemotherapy, have provided the most persuasive evidence of an oncogenic impact. In vitro studies indicated the oncogenic impact of the LTag and sTag with the capacity to cooperate with other oncogenic changes, suggesting that human polyomaviruses may have carcinogenic activity in the prostate. This may be owing to their big and tiny T antigen. Previous studies demonstrated JCV LTag gene sequences were also found among 21 of 49 (43%) pediatric malignancy specimens. Another study also determined that the presence of JCV DNA in 58.3% of cancerous and benign prostatic tissue samples, whereas JCV viruria and viremia were found in 62.5% and 8.3% of patients with PC, respectively. On the other hand, in contrast, did not find significant differences in JCV detection between cancerous and benign prostatic tissue specimens screened. Despite the mechanistic evidence, the role of JCV in human cancers is debatable. As a result, this study aimed to detect the presence of JCVLarge T antigen (LTag) together with viral load quantitation in prostate tumor samples to establish or deny the concept that JCV could be a risk factor for prostate cancer and to assess the effectiveness of the confounding factors such as working in tire plan, a alcohol consumption on the infection rate with JCV.

**Material and Method**

**Study Setting and Population**

This was a case-control based study that took place in three hospitals in Khartoum State between September 2017 and November 2018. The study was done according to the STROBE guidelines for Case Control studies. This study includes 110 Formalin-fixed prostate tissue, blood and urine samples from our prior research. Simple random sampling technique was used to select participants of this study. Sudanese patients with PCa are presented the cases group. Sudanese BPH patients with no indications of malignancy throughout histological tissue evaluation form the controls group. All of the subjects had recently been diagnosed and were not chosen from the hospital registry, and they were not receiving any treatment at the time of sample collection. Sample size was calculated by using the following equation:

\[ n = \left( \frac{Z_{\alpha/2}}{\sigma} \right)^2 = \frac{Z_{\alpha/2}^2 \sigma^2}{e^2} \]

Where:

- N: sample size
- \( (Z_{\alpha/2})^2 \): represents the desired level of statistical significance and confidence 95% (typically 1.96).
- \( \sigma \): Standard deviation
- \( e^2 \): Marginal error We wish to be 95% confident of the result i.e.

So, \( Z_{\alpha/2} = 1.96 \) and standard deviation of 1.5 and marginal error of .4 so the sample size will be \( n = \left[ 1.96^2 \times 1.5^2/0.4^2 \right] = 55 \), therefore the estimated sample size was 55 cases and 55 control = 110 study participants.

**Inclusion Criteria**

Only those individuals who were verified to have PCa (in cases) and BPH (in controls) based on prostate tissue biopsy examination and ultrasound imaging, radionuclide scintigraphy, and MRI tests were included in this study. There were no limitations based on age or tribal identity. Our study only included participants who had just received a diagnosis and they didn’t receive any kind of treatment.

**Exclusion Criteria**

Any case or control with history of immunodeficiency or diagnosed with Progressive multifocal leukoencephalopathy or undergo renal transplantation were excluded from this study to avoid any bias that may developed during cases and controls selection and affect on our results.

**Data Collection**

In terms of gender, age, and socioeconomic level, the cases and controls were matched. In this study, all participants were asked about their demographic, socioeconomic, geographical
afflation, cadmium contact, alcohol intake, and clinical data
via an interviewer-administered questionnaire (including
grade and family history of prostate cancer). Data from the
laboratory investigation was also documented.

Ethics Approval and Consent to Participate
The proposal of this study was ethically accepted by Om-
durman Islamic University’s ethics committee (ethics ap-
approval number BC20170922) – Sudan – 22, SEP, 2017. before
the study began. All participants signed a written informed
consent form according to the Declaration of Helsinki.

Immunofluorescence Assay for Detection of JCV-LTag
The primary antibody (PAb 962) Anti-Human Polyomavirus
JCV LTag was used in the immunofluorescence assay. All
stages were carried out according to the formalin-fixed,
paraffin-embedded Santa Cruz Biotechnology direct im-
mune fluorescence assay protocol, which included section
preparation, antigen retrieval, section staining, and inspec-
tion.15 The specimens were then mounted with a mounting
medium and examined under a fluorescence microscope
(Semi-Motorized Fluorescence Microscope BX53) within 30-
60 minutes of staining. The slides were then stored in a dark
box at 4 °C. Positive (JCV-infected urinary epithelial cells) and
Negative controls (normal skin tissue sample) were used to
ensure reagent stability and eliminate false-positive and false-
negative results. Furthermore, to decrease the possibility of
tissue contamination while sectioning and staining, Bancroft
and Gamble standard recommendations of histological
methods were adopted.16

Molecular Analysis and Examination
Only tissues, blood and urine samples of cases and controls
those were demonstrated positive JCV LTag IF reactions
were analyzed for the JCV viral load by using real-time PCR
techniques.

DNA Extraction From Tissue, Urine and Blood Samples
To reduce the effect of the formalin fixation on the extracted
tissue DNA quality, special type of tissue DNA extraction kit
was used in this study. The QIAamp DNA FFPE Tissue Kit is
specially designed for purifying DNA from formalin-fixed,
paraffin-embedded tissue sections. The kit uses special lysis
conditions to release DNA from tissue sections and to
overcome inhibitory effects caused by formalin crosslinking
of nucleic acids. Every tissue sample (25 mg) was processed
with lyses solution (20 μl of 200 mg/mL proteinase K and 5 μl
RNAase A). Genomic DNA was isolated utilizing QIAGEN
Company’s DNeasy® Tissue Kit and stored at 80°C till further
processing. The Nano drops test was performed to evaluate the
DNA's purity. In addition, one mL of urine/blood was
processed with lysis solution (20 μl proteinase K (200 mg/mL)
and 5 μl RNAase A). The DNA was isolated utilizing
QIAGEN Group’s DNeasy® Urine/blood Kit and kept at 80°C
till further investigation.

Real Time PCR for Detection and Quantitation of
JCV Load
Viral quantitation assay was done by using Rotor-Gene Q
(QIAGEN, Germany) machine. Real-time PCR amplifi-
cation was performed following the manufacturing in-
struction (Polyomavirus JCV Real-Time PCR Kit, Shanghai ZJ Bio-Tech Co., Ltd. In a final reaction volume
of 40.5 μl, JCV DNA amplification and quantification have
been conducted in Rotor-Gene Q (QIAGEN, Germany)
machine utilizing 4 μl of 1:10 diluted DNA Template, 34 μl
JCV reaction mix and 2.5 pmol each of forward as well as
reverse primers unique for the JCV Tag gene JCT-1
(Forward: 5 Thermal cycling began with a 10-minute
denaturation stage at 95°C, followed by forty cycles at
95°C for ten seconds and 60°C for 15 seconds, with the
amplified fluorescence measured at 60°C at the completion
of the cycles. The using Rotor-Gene Q (QIAGEN, Ger-
many) Real-Time PCR software was used to examine the
real-time PCR amplification data. Standard curve for JCV
rt-QPCR. JCV plasmid DNA, in serial dilutions ranging
from 10000 to 1000000 copies/ml, has been amplified by
rt-Q PCR. Fluorescence intensity was plotted against cycle
number and calculated as copies/ml. Positive and negative
controls have been included in each run.

Statistical Analysis
The data was analyzed using SPSS version 20 (Statistical
Package for the Social Sciences). A P-value of .05 or less is
considered significant. Various tabular and visual modules
were used to present the results. In addition, a unique statistical
test called chi-square was used to show that JCV can cause
carcinogenic alterations in prostate tissue.

Result
Detection of JCV LTag in the Prostate Tissue Specimens
by IF Assay
The prevalence of JCV LTag among cases was 23.6% (13/
55), which was greater than the prevalence rate among
controls, which was 5.4% (3/55). The Chi-square test was
used to examine the difference in JCV LTag prevalence
between cases and controls, and it was determined to be
statistically significant with a P-value of .006 and an odds
ratio of 5.7 (Figure 1). (Table 1). The highest prevalence of
JCV LTag was found in cases with Gleason score 6 (3 + 3)
and 9(4 + 5) with a percentage of 30.8%, followed by score 7
(3 + 4) and 10 (5 + 5) with a percentage of 15.4%, and the
lowest prevalence was found in cases with Gleason score 8 (4 + 4) with a percentage of 7.6%, but the difference was insignificant with a P-value of .09. (Table 1). The findings revealed that cases with JCV LTag had a mean age of 72.3 ± 7.3 years, which was greater than cases without JCV LTag (62.5 ± 5.6 years), although the difference was not statistically significant (P-value = .520) (Figure 2). The link between various PCa risk variables and the prevalence of JCV LTag was also investigated in the cases group. Alcohol usage, farming, eating a high-fat diet, working in a tire plant, and family history of cancer and cadmium exposure all played a statistically minor influence on the prevalence of JCV LTag among prostate cancer cases, according to the findings (Table 2).

Viral Load Quantitation Result

The mean of viral load was significantly higher among cases tissue specimen 20156 ± 5450 copies/ml compared to control group 6378 ± 2456 copies/ml with P-value of .002. However, the virus was detected in 11/13 (84.6%) urine samples of cases with a mean viral load of 14068 ± 4590 copies/ml compared to 2/3 (66.6%) of controls with viral load mean of 2534 ± 1267 copies/ml and there was significant difference between cases and control in the urinary viral load with P-value of .02. Moreover, JCV was detected in only 2/13 (15.3%) of cases blood samples with viral load mean of 3045 ± 1234 copies/ml compared to zero of the control group, also here the statistical analysis showed significant difference between cases and controls in the blood viral load mean with P-value of .008 (Table 3). Moreover, statistical analysis showed that cases duration of disease, family history and their cancer Gleason scores have no effect on the viral load means with P-value of >.05 for each (Table 3) (Figure 3).

Table 1. Shows the prevalence of JCV LTag in cases and controls (samples were examined using an immunofluorescence assay) and comparison of cases Gleason score grades in the PREVELANCE JCV LTag (P-value of .05 or less is considered significant). JCV, JC Polyoma Virus; LTag, Large T antigen.

| Study variable                  | Positive | Negative | Total | P-value |
|--------------------------------|----------|----------|-------|---------|
| Type of study participant      |          |          |       |         |
| Cases                          | 13 (23.6%) | 42 (76.4%) | 55    | .006    |
| Controls                       | 3 (5.4%)  | 52 (94.6%) | 55    |         |
| Prostate cancer Gleason scores|          |          |       |         |
| 3 + 3                          | 4 (30.8%) | 9 (21.5%)  | 13    | .090    |
| 3 + 4                          | 2 (15.4%) | 14 (33.3%) | 16    |         |
| 4 + 4                          | 1 (7.6%)  | 7 (16.6%)  | 8     |         |
| 4 + 5                          | 4 (30.8%) | 6 (14.3%)  | 10    |         |
| 5 + 5                          | 2 (15.4%) | 6 (14.3%)  | 8     |         |
| Total                          | 13        | 42       | 55    |         |

Figure 1. *JC Polyoma Virus* Large T antigen expression was detected using a direct immunofluorescence assay (Semi-Motorized Fluorescence Microscope BX53). A. Prostate cancer tissue section shows a negative IF reaction 10X magnification. B, C, D, and E are prostate cancer sections that showed a positive IF reaction by 10X magnification.
Discussion

Using the IF assay and Real-time-PCR, we assessed the rate of JCV infection in patients and controls in this study. The prevalence of JCV in 50% of 12 PCA patients in prostatic tissues or urine specimens. On the other hand, in the current study after investigation of JCV LTag + ve cases and control by using RT-PCR, the JCV viral load mean was higher significantly among cases group compared to controls in the three types of samples tissue, blood and urine. However, two previous independent studies found no significant changes in JCV occurrence between malignant and benign prostatic tissue specimens similarly; another study in Mexico found no JCV sequences in any of the 55 prostate cancer tissues examined. Technical differences in viral detection methodologies and sample size could explain discrepancies in the outcomes of numerous research, including ours.

We employed a combine of IF and molecular approaches to evaluate the tissue, whereas most of the earlier investigations used a molecular technique to detect viral DNA. The IF approach was used to illustrate the Large T antigen of JCV for prostate cells as well as to detect JCV infection. Only one study employed immunohistochemical staining, and they discovered that 38.2% (29/76) of the cases in their study were positive for JCV. In vitro studies indicated the monogenic impact of the LTag and sTag with the capacity to cooperate with other oncogenic changes, suggesting that human polyomaviruses may have carcinogenic activity in the prostate.
And therefore, the presence of Large T antigen in the cases prostate tissue may give strong evidence that JCV may contribute to the ontogenesis process. However, studies showed that there are about 75 to 80% homology in the Large T antigen amino acids sequences between BKV and JCV, therefore to confirm the presence of virus we did the RT-PCR for detection and quantification of JCV in the IF positive samples and we get confirmation in most of the tissue samples leading us to find an additional strong evidence that JCV could be a possible risk factor of PCa development. On the other hand, viruses are less to be detected in cases urine sample and rare to be identified in the cases blood samples this may suggest that JCV may inhibit and colonize the urinary tract and then start to invade the prostate cell and breaking the apoptosis mechanism through binding of tumor suppressor antigen such as P53 by Large T antigen. Despite the small sample size in this study, our findings show the presence of JCV in PCa patients’ tissue, urine, and blood samples, confirming the findings of an earlier study in which JCV was defined as a widespread inhabitant of the prostate. Despite the fact that the identification of viral nucleic acids and antigens within tumor tissue is critical, but not adequate, in illustrating any possible

Figure 3. Scatter graph of Q- Real time PCR. A & B showed Standard curve for JCV rt-QPCR. JCV plasmid DNA, in serial dilutions ranging from 10000 to 10000000 copies/ml, has been amplified by rt-Q PCR. Fluorescence intensity was plotted against cycle number. C & D describe the distribution of JCV loads expressed as DNA copies per milliliter. Short lines indicate medians and ranges of the viral load in the samples. The highest viral load was 27421 while the lowest viral load of 92 copies/ml. E & F combined scatter diagram between the samples curve and the standard curve. The Linear range of the assay obtained by use of known serial dilutions of the JCV plasmid as a template. The JCV copies number per samples were calculated from the standard curve. JCV, JC Polyoma Virus.
significance of viral infection in human cancer, the discovery of a high frequency of JCV DNA in prostate tissue opens the door to further research into its potential role in PCa. Further studies are needed to determine how a primarily neurotropic virus like JCV might play role in PCa development and progression, particularly through regulatory region rearrangements.

We attempted to clarify the relationship between PCa and JCV in this work, which was recognized as one in a line of investigations attempting to show a connection between the presence of oncogenic viruses and the emergence of PCa. The prevalence of HPV in prostate cancer has been found to range from 0% to 100%.26,27 It is helpful to consider a recent meta-analysis by Lawson et al. They evaluated 14 serology-based studies that included 5149 prostate cancer patients and 7794 benign prostate controls and found that 20% of both groups had HPV antibodies. They concluded that there is no difference in the prevalence of HPV antibodies between men with and without PCa when assessed serologically. However, in another part including only PCR-based studies conducted after the year 2000 (including 1071 prostate cancer patients and 1103 benign prostate controls), the HPV prevalence was found to be 21.6% in prostate cancer patients and 6.7% in controls \( (P = 0.001) \).23 A recent study in men from Tobago, a region with one of the highest rates of PCa incidence and mortality, found that HHV-8 causes a latent infection in the prostate that is associated with macrophage infiltration and inflammation.24 However, a meta-analysis to investigate the relationship between infections brought on by several sexually transmitted diseases, such as HSV-1, HSV-2, HHV-8, and CMV, found no conclusive evidence of an elevated risk of PCa.25 Due to all of these conflicting findings regarding the connection between infectious agents and PCa, funding organizations should pay closer attention to this issue and work to support the research centers financially so they can conduct an extensive, multinational study that will result in results that are both conclusive and accurate and will affected by the variation in techniques methods or sample size.

In this study, we divided the cases into five Gleason scores. With a percentage of 30.8%, cases with Gleason score 6 \((3 + 3)\) and 9(4 + 5) had the highest prevalence of JCV LTag. However, because the infection rate was statistically negligible, our findings show that JCV infection does not affect prostate cancer progression. Also, PCa Gleason score did not show any effect on the viral load among cases group. Previous research had found that individuals with high-grade PC (Gleason score \( \geq 7 \)) had high levels of mRNA expression of particular JCV genes.26-28 The limited number of cases in this study may result in such finding, therefore, the correlation between JCV infection and prostate cancer staging and grading should also be determined in the future studies with inclusion of a greater number of cases for better finding. The impact of PCa risk variables such as alcohol consumption, farming, a high-fat diet, working in the tire industry, and a family history of cancer on the JCV infection rate among cases was investigated by both IF and molecular assay. All of the PCa risk factors studied did not affect the rate of JCV infection in the cases group. The last finding requires further examination and larger sample size.

Corticosteroids are frequently prescribed for the treatment of advanced prostate cancer and have the potential to have agonist growth stimulatory effects on the tumor in certain circumstances. They have also been used to relieve pain from osseous metastases, reduce cancer-associated weight loss, fatigue, and chemotherapy-related adverse events.29 High-dose or long-term, low-dose corticosteroid use, on the other hand, can cause immune suppression and increased susceptibility to infections.30 To rule out the possibility that the infection was caused by a reduction in immunity caused by long-term steroid therapy, we asked our cases and controls about their history of steroid medication at the time of the study, and we received responses from a total of 93/110 participants, 42 cases and 51 controls. Only 5 of them had a history of steroid medication at the time the study began, one of the cases vs four controls. This will not provide statistical value; therefore, additional research is needed to better understand the relationship between JCV infection and PCa in the presence or absence of long-term medication.

**Conclusion**

In conclusion, a higher JCV-LTag was detected in cases group compared to controls. Tissue, blood and urine specimens from PCa patients had higher JCCopies compared to BPH patient’s specimens. As a result, our findings to gather with previous finding support a strong relationship between JCV infection and the probability of developing PCa.

**Limitation**

Our research has several limitations; regrettably, we were not given any financing. As a result, we only include a small number of cases and controls, and not all of the samples from the cases and controls (110) were evaluated using both IF and qRT-PCR simultaneously. We start with the IF assay and then perform a PCR analysis. Future research should incorporate additional cases and controls, and all samples should be examined using the two aforementioned methods.

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Authors Contribution

B.M.T.G. was responsible for Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Resources; Software; Supervision; Roles/Writing – original draft; Writing – review & editing. B.M.T.G. read and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Approval

The proposal of this study was ethically accepted by Omdurman Islamic University’s ethics committee (ethics approval number BC20170922) – Sudan. before the study began.

Consent to Participate

All participants signed a written informed consent form according to the Declaration of Helsinki.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available upon reasonable request from the corresponding author.

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