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Molecular evaluation of BK and MC human polyomavirus as promising Prostate cancer markers in Moroccan population

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ABSTRACT

Prostate cancer (PCa) is one of the leading causes of cancer deaths in men worldwide. Current evidence of an oncogenic role has emerged for Merkel cell polyomavirus (MCPyV) and BK human polyomavirus (BKPyV). The aim of our study was to describe the molecular evaluation of BKPyV and MCPyV as promising PCa markers and to investigate their correlation with clinical features of the tumor in Moroccan population. Fresh prostate biopsy tissue specimens were from 50 patients with confirmed PCa. The DNA extraction was performed by Invitrogen Genomic DNA Mini Extraction Kit The quality of DNA was checked using Nano drops test. BKPyV and MCPyV genomes were molecularly characterized using polymerase chain reaction (PCR) methods. The PCR products were subjected to the gel electrophoresis. BKPyV and MCPyV were detected with a prevalence of 24% (12/50) and 0% (0/50) prostate tissue specimens, respectively. PCa Gleason scores 7 was the most cancer grades identified with the presence of BKPyV (p<0.05). We suggest that BKPyV and MCPyV confers a risk for PCa, and patients with Gleason scores 7 are at a greatly increased risk in Moroccan population.

1. INTRODUCTION

In 2020, according to the World Health Organisation (WHO), prostate cancer (PCa) is the third most common diagnosed malignancy. PCa is the most commonly diagnosed cancer in over 50% of countries in the world (112 of 185) [1]. PCa incidence rates are 3-fold higher in countries with a high Human

Development Index (HDI) than in countries with a low HDI (37.5 and 11.3 per 100,000, respectively), whereas mortality rates are less variable (8.1 and 5.9 per 100,000, respectively). Its Incidence vary substantially from 6.3 to 83.4 per 100,000 men across the world, with the highest rates found in Northern and Western Europe, the Caribbean, Australia/New Zealand, Northern America, and Southern Africa and the lowest rates in Asia and Northern Africa [1]. PCa pathogenesis involves both heritable and environmental factors. Current evidence highlights the role of viral infections in PCa etiology. In particular, convincing evidence of an oncogenic role has emerged for a specific Human Polyomaviruses (HPyV), the Merkel cell polyomavirus (MCPyV) [2]. Infection with MCPyV is common in the human population, and the virus is detected in several anatomical locations. MCPyV is a small DNA virus with a circular, double-stranded DNA (dsDNA) genome of ~5kb [3]. The viral genome is divided into three major regions: the non-coding control region (NCCR), which contains the viral origin of replication and transcriptional regulatory elements; the early coding region; and the late coding region [3], [4]. The early region encodes large T (LT) antigen, small T (sT) antigen, the 57kT antigen, and a protein called alternative LT Open reading frame. MCPyV LT and sT antigens are the key oncogenes that drive MCPyV-induced tumorigenesis [5].

In 2012, the International Agency for Research on Cancer evaluated the carcinogenicity of the BKPyV, reporting that BKPyV is “possibly carcinogenic to humans”. BKPyV is a ubiquitous human pathogen, with over 80% of adults worldwide persistently infected [6]. The BKPyV genome is a non- enveloped virus with a double-stranded, circular DNA genome. The transcription of early and late coding regions proceeds in a bidirectional way from the origin of replication that is located within the NCCR. The early coding region encodes large tumour antigen (Tag), small tumour antigen (tAg) and truncated TAg (truncTAg) that are produced from different alternatively spliced mRNAs [7]. There is increasing evidence that BKPyV have been implicated in several human cancers. One of BKV’s protein known as agnoprotein may play a role in these pathogenic processes.

To further evaluate the possible role of viral infections in PCa, we carried out this study on the relationship between PCa risk and the presence of MCPyV and BKPyV in PCa samples [7].

2. Material and methods

2.1 Specimen collection

Between June 2018 and February 2019, fresh tumor tissue samples from the resection specimens of 50 patients with confirmed PCa were collected at the Urology Department, Military Hospital teaching Mohammed V, Rabat, Morocco. Informed consent was obtained from all participating patients and the study was carried out with the approval of the Ethical Review Committee of the Ethics Committee of Biomedical Research Committee in Morocco (No. 3/2018/April 30/2018).

2.2 Viral DNA extraction

The viral DNA extraction was performed by the Invitrogen Genomic DNA Mini Extraction Kit (Thermo Fisher USA). The purity and concentration of the viral DNA obtained were determined through 260/280 nm absorbance measures using the NanoDrop spectrophotometer 2000 (Thermo Scientific). All steps were performed following the providers' guidelines. Samples with a DNA concentration of 30-60 ng/µl or more are chosen to proceed with the polymerase chain reaction (PCR) and stored at −20°C until further analysis.

2.3 Viral DNA amplification by polymerase chain reaction

- BK human polyomavirus detection

DNA amplified by polymerase chain reaction (PCR). All these DNA samples were tested first of all on the β-globin gene using specific primers GH20/PCO4 [8] (Table I) and the other negative samples were excluded from further testing. And to be sure of the reliability of the assays, a positive control (To be sure of the reliability of the assays, a positive control was performed (DNA BKPyV) was performed during handling.

PCR assays for β-globin screening were performed as follows: initial primary denaturation for 10 min at 94°C, 35 cycles of denaturation at 94°C for 45 s, hybridization at 54°C for 45 s, extension at 72°C for 1 min after a final extension at 72°C for 10 min. All positive β-globin gene PCR products were subjected to further confirmatory PCR of BKV, to proceed with the polymerase chain reaction. The specific gene of BK human polyomavirus were analyzed by polymerase chain reaction using the specific primers described elsewhere [9]. Thus PCR targeting the regulatory regions of BKV was conducted with the BRP-1/2 primers as follows: Polymerase chain reaction consisting of 50 µl total volume. PCR reaction containing genomic DNA (4ul), 2× Taq PCR master Kit from Qiagen USA mix (25ul), 4 µl of primers sense and 4 ul of antisense primers, 12 ul of distilled water. PCR amplification was performed using a Perkin Elmer 2400® thermal cycler, CA, USA.

The BK polyomavirus gene amplification program started with an initial denaturation at 94°C for 3 minutes, followed by 35 cycles of: denaturation at 94°C for one minute, hybridization at 46°C for one minute and extension at 72°C for one minute. And a final extension at 72°C for 10 minutes.

To confirm the size of the PCR products, they were electrophoresed on agarose gel for 1.5 h at 70 V on 1.5% agarose (DNA SUB CELTM, Bio-RAD, Italy), then stained with 1% ethidium bromide, and visualized under UV exposure.

- Mercel cell human poluomavirus detection

Detection of MC human polyomavirus sequences was performed using a PCR amplification using primers (Table I). For PCR, the mixture contained 50 µl total volume. PCR reaction containing genomic DNA (4ul), 2× Taq PCR master Kit from Qiagen USA mix (25ul), 4 µl of primers sense and 4 ul of antisense primers, 12 ul of distilled water. PCR amplification was performed using a Perkin Elmer 2400® thermal cycler, CA, USA.

The mixture was initially denatured at 94°C during 3 minutes followed by 35 cycles of: denaturation at 94°C for one minute, hybridization at 68°C for one minute and extension at 72°C for one minute. And a final extension at 72°C for 10 minutes positive controls, respectively. using specific primers [10]. PCR products were visualized by electrophoresis on 1.5 agarose gels after staining with ethidium bromide (2ul).

Table I: Primers used for BK and MC human polyomavirus detection and typing [8- 10].

Target	Primers	Sequenses 5'- 3'	Annealing temperature	Product size, bp
β-globin	PCO3	5'-CAACTTCATCCACGTTCACC-3'	54 °C	256 pb
	GH20	5'-GAAGAGCCAAGGACAGGTAC-3'		
MCPyV	PEP-1	5' -GCAAAAAAACTGTCTGACGTGG-3'	68 °C	351 bp
	PEP-2	5'-CCACCAGTCAAAACTTTCCCA-3'		

BKPyV	BRP-1	5'-TTGAGAGAAAGGGTGGAGGC-3'	46 °C	265 pb
	BRP-2	5'-GCCAAGATTCCTAGGCTCGC-3'		

3. Statistical analysis

The results of all statistical calculations were computed using SPSS software. Frequency variations were evaluated by the Chi-Square Calculator for 5 x 5 (or less) Contingency Table test, which was used to calculate P-values for prostate adenocarcinoma (positive cases) and prostate adenocarcinoma (negative cases) in relation to clinical characteristics. Almost half of all p-values of <0.05 were considered statistically significant.

4. Results

Among the 50 total fresh biopsy samples tested using classical PCR reference technique 24% (12/50) were positive for BK polyomavirus and 76% (38/50) were negative, On the other hand all 50 samples were found to be negative for MC polyomavirus (Figure 1,2).

The clinical parameters of 50 patients are shown in (Table II); Most of the patients are generally people 60 years or older (86%), 14% of the patients, about 60 years precisely 57-60 years. In addition to this, 17 (34%) patients were considered to be T2 stage tumor, which means that the tumor occupies half of one side of the prostate. or in the 2 sides of the prostate. Although 14 (28%) were classified as T1 stage which means the tumor is in 5% or more of the prostate tissue removed at surgery. 3 men (6%) at stage T3 indicates that the tumor has spread both within the prostate and to one or both sides of it. prostate or the tumor has enlarged into the seminal vessels. 15 patients (30%) have a T4 tumor stage meaning that the cancer has spread to nearby structures other than the seminal vesicles. The pathological stage of the tumor, 5 (41.66%) of the BK polyomavirus carriers were in T3 stage, while 12 (31.57%) of the non-carriers were in the same T3 stage, and 4(33.33%) were in T4 same stages, while 10 (26.36%) of the non-carriers were in the same T4 stage (P-value = 0.6602).

For each tumor parameter, the number and percentages of patients who are BK polyomavirus carriers and non-carriers. 11 patients (22%) have a pathological Gleason score>7, which translates into cancers that are likely to develop very quickly or high grade. high grade. And the same proportion in those with a score < 7, indicating that the cancer is probably growing slowly. While (16%) of all patients have (4+3) a score of 7, which means the tumor is more likely to grow and spread. And 40% of patients have (3+4) of 7, which shows a less severe cancer. Among the BK polyomavirus carriers, 8 (66.66%) patients have a Gleason score of seven or more, while 14 (36.84%) of the Bk polyomavirus non-carriers have a Gleason score of seven or more. (With no significant difference between carriers and non-carriers at $p < 0.05$. P value=.303784).

Eight (66.66%) patients with BK polyomavirus have a PSA concentration of 10 ng/ml or more, while 20 (52.63%) non-carriers have a PSA concentration of 10 ng/ml or more. For patients with PSA concentrations between 2.5-10ng/ml there are 3(25%) patients who have BK polyomavirus, while 10 (26.36%) non-carriers of BK polyomavirus Concerning patients who have PSA concentration <2. 5; 1(8.33%) patients who have BK polyomavirus (positive cases), While 8(2.05%) patients negative for BK polyomavirus (With no significant difference between carriers and non-carriers at $p < 0.05$. P value=.563673). Ten (83.33%) of those diagnosed at 60 years or older, were virus carriers, while 18 (47.36%) of non- carriers were 60 years or older at the time of diagnosis. While 2 (16.66%)of those diagnosed at less than 60 years, were virus carriers, while 20 (52.63%)of non-carriers were less than 60 years old at the time of diagnosis at $p < 0.05$ (P-value=0.028666).

One (8.33%) underwent treatment by radical prostatectomy, while 20 (52.63%) of non-carriers treated by radical prostatectomy at $p < 0.05$ (P-value=0.006719).18 men (36%) were self-reported smokers and 10 (20%) alcoholics. these were alcoholics.

Pathology has become major factors in determining subjects with newly diagnosed prostate cancer. Descriptive statistics showed that there was a significant correlation between serum PSA level and clinical stage.

Table II: Clinical characteristics of patients with prostate cancer.

	Number (%)	***Positive cases (%)***	***Negative cases (%)***	***P-value***
Age at diagnosis / surgery.				
<60 years	7 **(14%)**	2 **(16.66%)**	20 **(52.63%)**	0.028666**
>60 years	43 **(86%)**	10 **(83.33%)**	18 **(47.36%)**	
Tumor stage.				
Local tumor	18 **(36%)**	9 **(75%)**	16 **(42.10%)**	0 .046945**
Metastasis	32 **(64%)**	3 **(25%)**	22 **(57.89%)**	
Smoking.				
Yes	18 **(36%)**	10 **(83.33%)**	18 **(47.36%)**	0.028666**
No	32 **(64%)**	2 **(16.66%)**	20 **(52.63%)**	
Alcohol consumption.				
Yes	10 **(20%)**	10 **(83.33%)**	17 **(44.73%)**	0.019352**
No	40 **(80%)**	2 **(16.66%)**	21 **(55.26%)**	
Preoperative PSA ng/ml.				
<2.5	7 **(14%)**	1 **(8.33%)**	8 **(21.05%)**	0 .563673*
2.5 – 10	10 **(20%)**	3 **(25%)**	10 **(26.36%)**	
>10	33 **(66%)**	8 **(66.66%)**	20 **(52.63%)**	
Pathological Gleason score.				
<7	11 **(22%)**	2 **(16.66%)**	8 **(21.05%)**	0.303784*
7(3+4)	20 **(40%)**	1 **(8.33%)**	6 **(15.78%)**	
7(4+3)	8 **(16%)**	1 **(8.33%)**	10 **(26.36%)**	
>7	11 **(22%)**	8 **(66.66%)**	14 **(36.84%)**	
Pathological T-stage.				
pT1	14 **(28%)**	2 **(16.66%)**	6 **(15.78%)**	0.619677*
pT2 x	17 **(34%)**	1 **(8.33%)**	10 **(26.36%)**	
pT3 x	3 **(6%)**	5 **(41.66%)**	12 **(31.57%)**	
pT4	15 **(30%)**	4 **(33.33%)**	10 **(26.36%)**	
Radical prostatectomy.				
Yes	29 **(58%)**	1 **(8.33%)**	20 **(52.63%)**	0.006719**
No	21 **(42%)**	11 **(91.66%)**	18 **(47.36%)**	

This study evaluated the association of an BK polyomavirus sequence with prostate cancer in Moroccan men.

Detection of BK human polyomavirus sequences is shown in Figure 1, 2 by targeting the BK polyomavirus

sequence embedded in the human genome, a 256 base pair fragment.

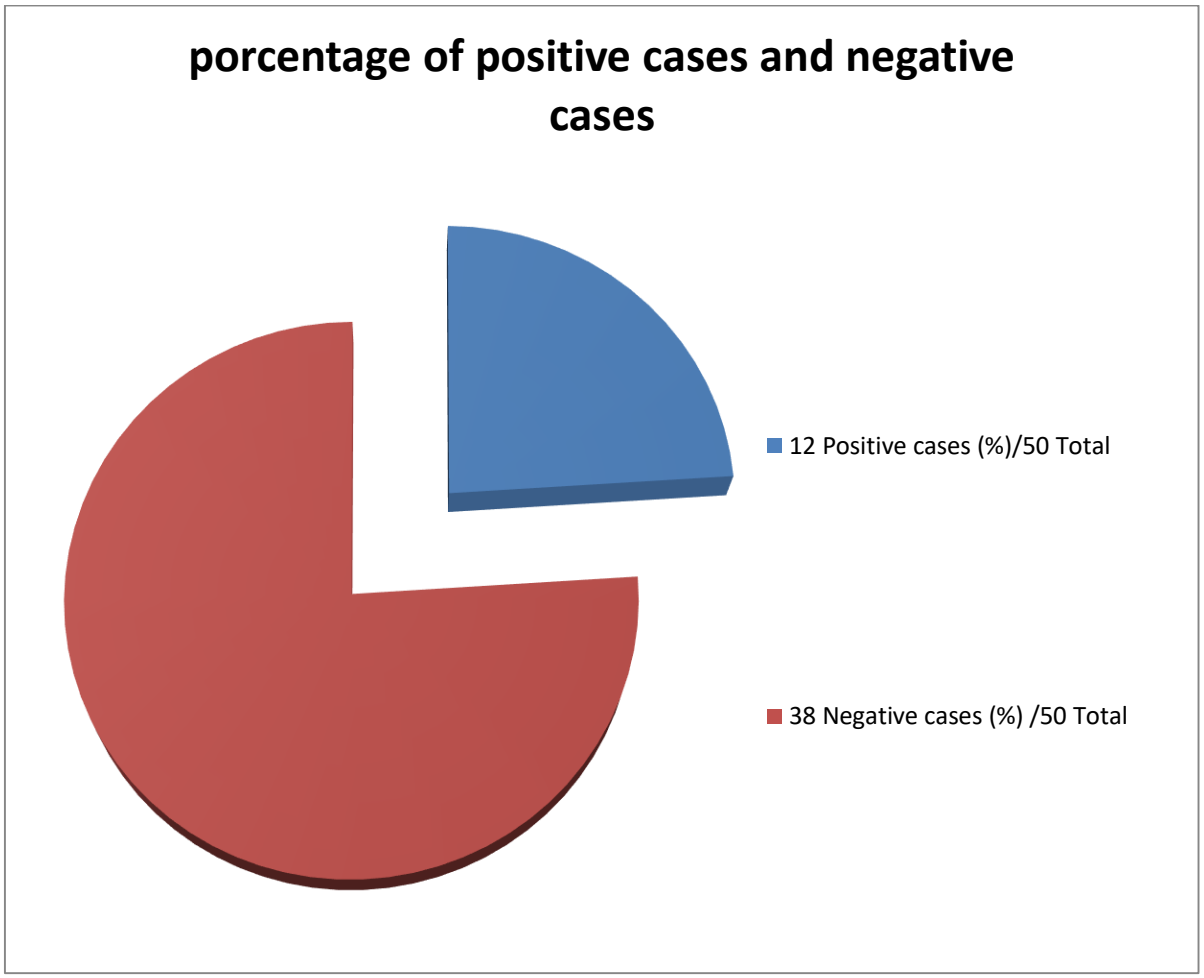

Figure 1: Detection of BK human polyomavirus: pourcentage of positive cases and negative cases.

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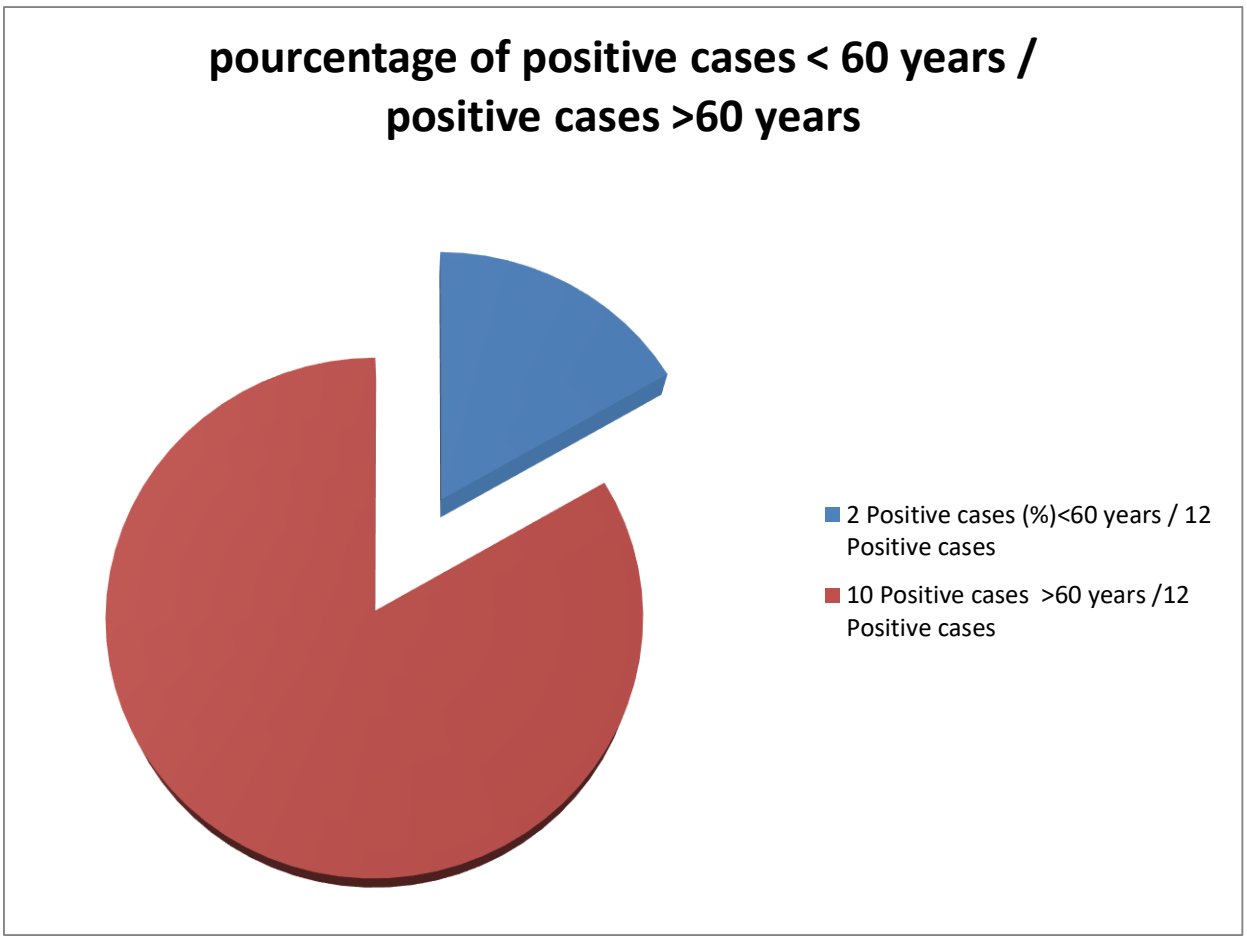

Figure 2: Detection of BK human polyomavirus: pourcentage of positive cases < 60 years / positive cases >60 years.

In conclusion, the results are in agreement with numerous reports that have confirmed the presence of BKPyV DNA and its early protein TAg in many human neoplasms. These parameters are therefore likely to be used as markers, specific for the diagnosis of prostate cancer.

5. Discussion

In comparison, several studies conducted on relatively small samples and using several PCR techniques have demonstrated a possible association of BKPyV with human prostate adenocarcinoma. [11] found the presence of BKPyV Tag sequences in four of seven prostate adenocarcinomas and then confirmed these results in a subsequent report [12]. Confirmed these results in a subsequent report [12]. BKPyV with human prostate adenocarcinoma. Similarly, [13] looked for BKPyV DNA by nested PCR in two of seven prostate adenocarcinoma samples. And more recently, among 16 prostate cancer samples analyzed by PCR, [14] demonstrated early and regulatory BK DNA sequences.

The basic molecular mechanisms that control the course and progression of prostate cancer are poorly understood [15], [16]. However, research has shown that a complex interplay between several genes and environmental factors occurs [17]. Recent findings from several studies have shown that infectious agents, such as viruses, may play an important role in the pathogenesis and/or proliferation of prostate cancer [14]. BKPyV is an infectious agent that may be involved in the genesis of prostate cancer. it infects humans and is typically acquired early in life with an antibody seroprevalence rate of nearly 90% in adults. Because BKV is so prevalent in the human population, it appears to have oncogenic potential [18]. Urinary tract

cancers are the primary target for etiologic correlation with BKPyV. There are also two other studies on the presence of BKV DNA in prostate cancers [19]. Revealed the presence of BKPyV DNA in 3/12 prostate samples by performing PCR (94), and Lau et al. revealed BKPyV DNA in tumor cells of 2/30 prostate samples by following the ISH method (In situ hybridization) [13]. BKPyV infects normal epithelial cells and causes a switch from normal cells to IPA by increasing TAg expression; in turn, the switch to IPA causes TAg expression. This results in triggering of proliferation and binding of p53 in the cytoplasm. As cells multiply, they accumulate mutations at a higher-than-normal rate because of this lack of p53 activity. Eventually, a cell accumulates a large number of mutations to completely lose control of its growth and multiply clonally to form a tumor.

The disappearance of BKPyV in tumor cells may be due to selection against TAg by the immune system [20], dilution of viral episomes due to lack of replication, or TAg-induced proapoptotic effects that are not considered consistent with other production regulatory mutations in tumor cells [21], leading to selection against TAg expression. Loss of TAg expression has been found in studies of TRAMP (transgenic mouse adenocarcinoma of the prostate) mice, which develop tumors due to the presence of the early SV40 region [22]. When tumor cells are extracted from these animals and cultured, their TAg expression disappears [23]. A similar loss of oncoprotein expression is noted in bovine alimentary canal cancers caused by bovine papillomavirus type 4: the virus is required for papilloma induction, but its presence is not essential for growth or maintenance of the transformed state [24]. Further studies are needed to establish whether there is a causal link between BKV and prostate cancer. If so, the unique viral characteristics of BKPyV can be studied for prophylactic or therapeutic vaccination or for treatment by designing drugs that target TAg.

6. Conclusion

Our results show a considerable prevalence of BKPyV infections in prostate cancer patients, whereas there is no correlation between MCPyV infection and prostate cancer patients, however, there was a significant association between infection and age of prostate cancer patients. Interestingly, infections have a positive feedback effect on cancer. Thus, data on personal history of infection can be used to improve cancer treatment and prevention strategies. The observation of a high prevalence of BKV infections in the present study indicates that an approach combining traditional cancer therapies with antivirals and antimicrobial agents would improve the prospects for therapeutic success.

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List of abriviation

- CaP : Prostate Cancer
- DNA : Deoxyribonucleic acid
- WHO : World Health Organisation
- PCR : Polymerase Chaine Reaction.
- PSA : prostate specific antigen.
- BKPyV : BK polyomavirus
- MCPyV : MerkelCellpolyomavirus
- TAg : Large tumour antigen
- tAg : small tumour antigen

- LT : large T
- sT : small T
- ISH : In situ hybridization
- HDI : Human Development Index

Contribution of the authors

- I.T: conceived and designed the experiments, optimized the experimental approach, realized the experiments, wrote the manuscript, all authors approved the final version.
- B.A: analyzed all the patients' data, performed the statistical analyses.
- I.S: manuscript Reviewing.
- Y.E: manuscript Reviewing.
- S.A: analyzed all the patients' data, performed the statistical analyses.
- A.L: managed the collection of samples and the processing and storage of all samples, proofreading and technical support.
- I.A.M: analyzed all the patients' data, performed the statistical analyses, manuscript Reviewing.
- M.M. E: conceived and designed the experiments, optimized the experimental approach, realized the experiments, wrote the manuscript, all authors approved the final version.

Conflict of interest statement

The authors declare no conflict of interest.

Ethic Approval consent to participate

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