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Human Papillomavirus and Epstein-Barr virus co-infection in Prostate Cancer: Observational Study

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ABSTRACT— Prostate cancer is one of the most common tumors in men worldwide, regardless of ethnic origin or demographics. It is the fifth leading cause of cancer death in men worldwide. In Africa, the disease ranks first in terms of incidence and mortality. What's more, in Morocco, the average age of prostate cancer patients is between 68 and 73. Among the many risk factors for prostate cancer, several studies have shown that specific genetic variants and viral implications are important factors in the initiation or development of prostate tumors. Recent studies have suggested that infection with EBV and HPV may also contribute to the development of this disease. However, the mechanisms by which these viruses may contribute to the development of prostate cancer are not yet fully understood. The aim of this study was to evaluate the potential role of co-infection with human papillomavirus (HPV) and Epstein-Barr virus (EBV) and their correlation with clinical and pathological parameters of prostate cancer. To achieve the objectives of these studies, a total of 100 blood samples were taken from Moroccan men with prostate cancer, after obtaining the necessary authorizations from the scientific research ethics committee. All clinical and pathological data were recorded. Extraction of genetic material according to the required protocol. The DNA samples obtained were stored for the viral applications mentioned below. A series of polymerase chain reactions and statistical analysis procedures were successfully performed. The potential viral influence on prostate cancer, previously proposed in other populations, was explored in Moroccan men. The results presented provide an overview of the main viral functions in prostate cancer in Moroccan men, and highlight the possibility of analyzing their clinical significance.

KEYWORDS: Human papillomavirus, Epstein-Barr virus, Prostate cancer, Viral Coinfection, Prostate-specific antigen, Gleason score.

1. INTRODUCTION

Epstein-Barr virus (EBV) and human papillomavirus (HPV) are two common viruses that have been associated with several types of cancer. In recent years, research has suggested that co-infection with these two viruses may play a role in the development of prostate cancer. HPV is a double-stranded DNA virus

that can immortalize human mammary epithelial cells. More specifically, HPV E5 and E6/E7 Oncoproteins act synergistically in human cancer pathogenesis [1], [2]. Several studies have reported that HPV can co-exist with other human tumour virus strains, such as EBV, and cooperate with this tumour virus to initiate and/or enhance carcinogenesis in different types of human cancers [3]. EBV is a double-stranded DNA gamma herpesvirus that produces persistent infection in memory B lymphocytes, latent EBV infection (EBV core antigen 1) and/or Latent Membrane Protein 2 [4] Latent EBV can reactivate and stimulate viral genes (EBNA, LMP1, and LMP2), thereby inducing epithelial cell growth, proliferation, and angiogenesis while inhibiting apoptosis [5], [6].

Prostate cancer is one of the most common cancers in men, and it is estimated that over 1 million men are diagnosed with the disease each year worldwide [7]. The exact causes of prostate cancer are still not fully understood, but it is believed that genetic, environmental, and lifestyle factors all play a role. Viral infections cause approximately 12% of cancers worldwide, the vast majority (>85%) of which occur in developing countries [8], [9] Recent studies have suggested that infection with EBV and HPV may also contribute to the development of this disease. Several studies have investigated the relationship between EBV and HPV co-infection in prostate cancer. One study found that the prevalence of EBV and HPV co-infection was significantly higher in prostate cancer patients than in healthy controls [10]. Another study reported that the presence of both viruses was associated with more aggressive forms of prostate cancer [11]. However, the mechanisms by which these viruses may contribute to the development of prostate cancer are not yet fully understood.

This study aimed to assess the possible role of co-infection with human papillomavirus (HPV) and Epstein-Barr virus (EBV) and their correlation with clinical and pathological parameters of prostate cancer.

2. Material and methods

2.1 The collection of samples.

The current research is conducted during June 2021 and February 2022. Taking into account the inclusion and exclusion criteria, 100 prostate blood samples were collected during the study at the Urology Department, Military Hospital teaching Mohammed V, Rabat, Morocco (Table I), and 50 normal samples were obtained from the private medical laboratory, Ethical standards were followed prior to and during blood sample preparation, including patient agreements and other verbal agreements signed by patients and investigators. The required ethical approval has been obtained from the Moroccan Biomedical Research Ethics Committee (No. 3/2018/April 30/2018).

The blood samples were directly extracted from the hospital and were accompanied by clinical and pathological parameters. DNA processing and genotyping were performed at the Laboratory of Oncology and Virology, Mohammedia Institute of Technology, Morocco.

Table I. Clinical characteristics of the prostate cancer patients (n=100).

Characteristics	**n (%)**
Total Cases	100 (100)
Age At Diagnosis/Surgery	
≤60 Years	16 (16)

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>60 Years	84 (84)
PSA (ng/ml)	
<2.5	8 (8)
2.5 – 10	23 (23)
>=10	67 (67)
Unknown	2 (2)
Pathological Gleason Score	
<7	15 (15)
7 (3+4)	13 (13)
7 (4+3)	13 (13)
>7	31 (31)
Unknown	28 (28)
Pathological T-Stage	
T1	23 (23)
T2 X	36 (36)
T3 X	7 (7)
T4	9 (9)
Unknown	25 (25)
Alcohol Consumption	
Yes	32 (32)
No	68 (68)
Smoking	
Yes	55 (55)
No	45 (45)

2.2 DNA extraction and genotyping.

Total DNA was extracted from blood samples using the QIAamp DNA Mini Kit according to the kit protocol for PCa blood. DNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc.). Polymerase chain reaction (PCR) was conducted to samples with a DNA concentration of 20-50 ng/µl or higher.

The quality of the extracted DNA was analyzed by amplifying a 268 bp fragment of the β-globin gene using

HotStarTaq DNA polymerase (Qiagen, Düsseldorf, Germany) in the presence of GH20/PCO4 primers. Cycling program consisted of 9 min at 95 °C followed by 35 cycles of an extension cycle of 30 s at 95 °C, 30 s at 55 °C, 1 min at 72 °C and finally 10 min at 72 °C.

2.3 Amplification and detection of HPV and EBV by PCR

All positive β-globin gene PCR products were further confirmed by PCR for polymerase chain reaction.

HPV polyomavirus-specific L1 gene was targeted by polymerase chain reaction using specific primers described elsewhere [12]. PCR in a total volume of 25 µl. PCR reaction with genomic DNA (4 µl), 2x Taq PCR master kit from Qiagen USA mix (25 µl), 4 µl sense primer and 4 µl antisense primer, 12 µl distilled water. PCR amplification was performed using a thermal cycler,35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s. The samples were finally incubated for 10 min at 72°C for a final extension.

Specific EBV gene were targeted by PCR using specific primers described elsewhere [13]. The PCR reaction consisted of a PCR reaction with a total volume of 25 µl, genomic DNA (8 ng), 2x Taq PCR Master Mix kit (Qiagen USA), 2 µmol forward and reverse primers. PCR amplification using Thermal Cycler Amplification procedure for EBV gene amplification Processing was as follows: initial denaturation at 94°C for 3 min, followed by denaturation at 94°C for 1 min, annealing at 43°C for 1 min, extension at 72°C for 1 min, and a final extension of 35 cycles at 72 ˚C for 10 min.

The PCR products for the both virus were resolved using 2 % agarose gel elecrophoresis for 1.5 h at 70 V, Then stained with 1% ethidium bromide and visualized under UV light

2.4 Statistical analysis

Statistical analysis was performed using SPSS software (SPSS version 11.0, Chicago,IL).Data were calculated on 2x2 or more Chi-squared analysis or Fisher's exact test to test for assess the significance of the association between clinic- pathological data (patient's age, Nottingham histological grade and tumour stage) in correlation with the presence/co- presence of HPVs and EBV. Statistical significance was achieved at $p < 0.05$.

3. Results

Our results show that among 100 patients diagnosed with PCa Ten patients (10%) have been reported to have an infection with HPV, and 72% have been determined to be positive for EBV infection. All control cases, however, were free of HPV or EBV infection.

Table II: Clinical characteristics of patients Infected with HPV or EBV

Characteristics	Infection with HPV	P-Value	Infection with EBV	P-Value
Total Cases	**10**		**72**	
Age At Diagnosis/Surgery		0,423		0,407
≤60 Years	3		12	
>60 Years	7		60	

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PSA (ng/ml)				
<2.5	0	0,697	6	0,652
2.5 - 10	2		18	
>=10	8		46	
Unknown	0		2	
Pathological Gleason Score				
<7	2	0,733	13	0,253
7 (3+4)	1		10	
7 (4+3)	0		7	
>7	4		20	
Unknown	3		22	
Pathological T-Stage				
T1	2	0,461	19	0,004
T2 X	6		27	
T3 X	0		1	
T4	1		5	
Unknown	1		20	
Alcohol Consumption				
Yes	3	0,597	23	0,581
No	7		49	
Smoking				
Yes	6	0,503	40	0,516
No	4		32	

The patients infected with HPV have an age at diagnosis ranged from 49 to 90 years old (seven are ≤60 years old) (P=0,423). For PSA concentrations 8 patients from the ten infected by HPV have a score over >=10 and the remains 2 have a PSA concentrations between 2.5 - 10 ng/ml with no significant differences (P=0,697). For the pathological Gleason Score 40% of the infected cases with HPV have a score over 7 (P=0,733). While 60 % of them have a pathological T-stage 2 with no significant differences was found (P=0,461).

For those cases that have been confirmed to be positive for EBV, 60 (83,3%) of them have an age at diagnosis over 60 years old (P=0,407). 46 patients have a PSA concentration >=10 ng/ml (P=0,652). For the Gleason Score among 72 EBV positive cases 20 are more than 7 (P=0,253). Whereas, 27 patients have T-stage 2 tumours, and 19 are in T-stage 1 tumours, there was a significant difference between T-stage and EBV infection (P=0,004).

Table III: Association between prostate tumour criteria and HPV, EBV Coinfections vs. non-infected patients.

Characteristics	Coinfection with HPV and EBV cases	Not Infected	P-Value
Total Cases	**9**	**27**	

Age At Diagnosis/Surgery			0,367
≤60 Years	3	4	
>60 Years	6	23	
PSA (ng/ml)			0,761
<2.5	0	2	
2.5 - 10	2	5	
>=10	7	20	
Unknown	0	0	
Pathological Gleason Score			0,671
<7	2	2	
7 (3+4)	1	3	
7 (4+3)	0	6	
>7	4	11	
Unknown	2	5	
Pathological T-Stage			0,634
T1	2	4	
T2 X	5	8	
T3 X	0	6	
T4	1	4	
Unknown	1	5	
Alcohol Consumption			0,596
Yes	3	9	
No	6	18	
Smoking			0,626
Yes	5	14	
No	4	13	

From 100 patients, those who's been determined to be positive for both HPV and EBV represent only 9% of all cases. While, the patients who have been free from HPV and EBV represent 27% (Table III). 66% of the coinfected patients have an age more than 60 years old, while 85% of those who's been free from HPV or EBV infection have an age more than 60 years. For the pathological T-stage, five of nine (55%) coinfected patients are at an early histological T- stage (T2) and 22% are in T-stage 1, however for patients with no infection with HPV and EBV 29% of them have a T-stage 2 and 15% have a T-stage 1, no significant differences was found (P=0,634). Four out of nine have a pathological Gleason Score more than 7 and no patient had a score of 7(4+3), although, 11 patients among the 27 not infected with HPV and EBV have a score more than 7, and 6 out of 27 have a score of 7(4+3) without any significant differences (P=0,671). Regarding the PSA level, 7 out of 9 for the patients with the coinfection, and 20 among the 27 free of HPV and EBV have a PSA more than 10, with no significant differences was found (P=0,761).

4. Discussion

The purpose of the present study was to find out whether EBV-positive samples, HPV-positive samples,

HPV/EBV co-infected samples and control samples differed with respect to EBV and HPV gene expression levels and their association with clinic-pathological parameters.

Viral infections are responsible for around 12% of all cancers worldwide [14], [15]. HPV and EBV infection have been previously reported to represent 38% of all virus-related cancers [16]. In fact, the HPV/EBV coinfection has been reported in some human tumours, such as cervical cancer, breast cancer, nasopharyngeal carcinoma, cervical cancer and in prostate cancer [17]. The present study, HPV and EBV were found in 10% and 72% of patients with prostate cancer respectively, while for the control group it was negative. The HPV/EBV coinfection was found to be at 9%, while those to were free of any infection of HPV or EBV were 27% of the prostate cancer cases.

The HPV infection in prostate cancer has been reported in several studies. Previous studies conducted in our laboratory on prostate cancer patients identified HPV in the prostate tissue and blood with 16% and 10% respectively of the cases analysed [12], [18]. The results of this study on HPV also report that 10 out of 100 patients were positive, the association between HPV infection and tumour clinic-pathological parameters revealed that there is no significance association. Nevertheless, 4 out of 10 of the infected men have a pathological Gleason Score over 7, with 3 out of the 10 have a Gleason Score unknown, and from a pathological outstanding, tumours with a score greater than 7 have a highly undifferentiated and aggressive cancer cells. Moreover, from the Pathological T-Stage viewpoint, the majority of infected patients (80%) are in T2 with 60% and 20% were in T1 stage. This indicates that the prostate tumours were at an early stage of development. This indicates that the HPV viral infection occurred at an early stage, just as the tumours were developing. We therefore concluded that HPV could contribute to the early development of prostate tumours. However, this result should be viewed with caution, as non of the tumour criteria in particular PSA concentration, histological tumour stage and the Gleason Score, were not significantly different between the infected and non-infected men with HPV.

The EBV infection in prostate cancer, was also reported in several studies, a previous study in Sweden report that EBV was detected in 8.8% (31 out of 352) of malignant and benign prostate cancer [19], and in 8% of prostate Cancer patients (16 of 200) in the USA [20]. A separate study reported EBV infection in almost 37% of prostate cancer patients [21]. However, in our study, we have detected EBV in 72% of prostate Cancer patients. The association between EBV infection and tumour criteria indicated a significant difference in only the pathological T-stage (P=0,004). These findings led us to conclude that EBV viral infections were present at an early stage, when tumours were developing, however, no other significant difference was found especially for the PSA and the Gleason score. Yet the majority of the infected men (46 out of 72 EBV infected men) were at a PSA level more or equal than 10 ng/ml, furthermore for the pathological Gleason Score 20 out of the 72 EBV infected men have a score over 7, considering that 22 of them have an unknown score. These results could be interpreted as the possible major role of the EBV infection in the early stages of the development of the prostate tumours.

High level of EBV and HPV gene sequences were detected in previous studies, [22] have found in 2013 that these sequences were in normal and benign prostate cancer samples. EBV/HPV coinfection was significantly more frequent in patients with prostate cancer (55%) than in those with benign prostate cancer (15%) and normal prostate (30%). In our study, we detect EBV/HPV coinfection in 9% of all cases of prostate cancer, while the control group was free of booth EBV and HPV. Although there is no significant difference between clinical parameters and viral coinfection, the highest number of infected men (4 out of 10) have a Gleason score over 7, which indicate that they a highly undifferentiated and aggressive cancer cells. Additionally, over 77% of them were in the early stages (T2 55%, T1 22%), revealing that the

prostate tumours were at an initial stage of development. Added to that 7 out of the coinfected 9 patients have a PSA level more than 10 ng/ml.

Our results are in accordance with those of [23]. They found that the HPV/ EBV co-infection was detected in 14.9% of prostate cancer cases. Although there was no statistically significant association between HPV/EBV coinfection and prostate cancer creteria, the expression profile of certain cellular factors involved in inflammation, tumour progression and metastasis could be different between the HPV/EBV co-infected prostate cancer group with mono-EBV infection and mono-HPV infection, these differences indicate that the co-presence of these viruses may alters the expression patterns of cellular factors, compared with mono-infections, suggesting that HPV/EBV coinfection is a contributing factor in the development of prostate cancer, and indicating the role of EBV in HPV genome integration.

Although, the links between viral infections and prostate tumours have yet to be fully explored. Our finding suggests that there could be a role of viral infections in the development of prostate tumours. With this in mind, further studies are needed to explain the role of viral infections and prostate cancer development.

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Availability of data and materials
The data generated in this study is available from the corresponding author.

Author contribution
All authors contributed to the study conception, design, material preparation, data collection and analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Agreement of the Ethics Committee of Biomedical Research in Morocco code: (n°3/2018/April 30/2018-Morocco).

Competition interests
The authors declare that they have no competing interests.

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