

The Use of ELISA and PCR in Identifying Correlations between Viral Infection and Benign Prostatic Hypertrophy

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Benign prostatic hyperplasia (BPH) is induced by a persistent local inflammatory process that leads to cell proliferation. Viral infections associated with immune deficiencies can trigger the chronic inflammation of the prostate. Therefore, we have investigated several viral expressions in BPH patients and tried to establish a link with the diagnosed hyperplasia. 50 patients with BPH without urinary tract infection were tested for the presence of the following viruses: human papilloma virus (HPV), cytomegalovirus (CMV) and Epstein-Barr virus (EBV). These viruses are the most common cause of asymptomatic viral infections. HPV-specific DNA detection by polymerase chain reaction (PCR) was used for freshly surgical removed tissue sample. Both anti-CMV (IgG, IgM) and anti-EBV (IgG, IgM) antibodies were detected in the patients' serum with standard enzyme-Linked Immunosorbent Assay technique (ELISA). Specific HPV-DNA in prostate tissue was found only in 4% of patients, while 98% and 100% patients were positive for serum anti-CMV IgG or anti-EBV, proving intense earlier contact with the virus. IgM anti-CMV evaluation was found in around 10% of the cases which were also negative for EBV, sustaining that this was a non-acute infection. The findings showed that BPH may be associated with a chronic inflammation due to the post-viral infection with CMV or EBV, or secondary to the presence of these viruses in the prostate, while the involvement of HPV infection in BPH development is comparably lower. Our data suggests that viral investigation in BPH should be considered in the screening protocol of BPH as an indicator of possible inflammatory-mediated tumorigenesis of urinary tract.

Keywords: benign prostatic hypertrophy, human papilloma virus, cytomegalovirus, Epstein-Barr virus

Benign prostatic hyperplasia (BPH) is the most common urological disease encountered in men, being age dependent, thus in the 51-60 years group about one in two men will develop BPH, and over 80 years of age, and more than eight of ten will be diagnosed with this disease [1]. Although it is a public health problem, up to now it has not yet been found a direct relationship between BPH and a certain causative agent, often the etiology of hyperplastic process being multi-factorial [1]. The main assumption is that cell proliferation is generated through steroid hormones action and due to the inflammatory response to a local infection [1-4]. Hence, persistent inflammation of the prostate can be sustained by a viral infections doubled by an inefficient immune anti-viral response. Therefore, a weak antiviral immune response developed by cytotoxic T-lymphocytes (T-CD8+) or insufficient/un-efficient antiviral antibodies secreted by B lymphocytes can sustain the infection in the prostate tissue [5]. Given the frequency of unapparent/asymptomatic viral infections with human papilloma viruses (HPV), cytomegalovirus (CMV) and Epstein-Barr (EBV) and their relation to prostate pathology, it is important to detect in the benign pathology of the prostate the presence of these viruses as triggers to possible neoplastic transformation of the BPH [6-8]. This may

improve patient's surgical treatment, taking into account that large transurethral resection (TUR-P) may lead to serious complications such as renal deficiency and dialysis [9,10]. Therefore, early diagnosis and therapy may reduce the incidence of complications. HPV infection has been incriminated in the clinical history of both sexes [9,10], thus in 75% of men has been associated with malignant prostatic diseases, namely in the genesis of prostatic adenocarcinoma [8,11-13]. It is important to identify all factors that may influence the oncogenesis process in order to prevent tumor formation. Regarding the morbidity associated with bladder, uterus or prostatic tumors, it is well known that obstructive renal failure followed by dialysis and complications such as vascular calcifications, peritonitis, anemia, bone demineralization, involves a great cost for the medical insurance companies associated with low life quality and life expectancy for the patient [14-19].

The actual identification of HPV in BPH patients has not been reported, most of the information coming from the control groups of patients diagnosed with prostatic adenocarcinoma [20-24]. A large number of studies have linked HPV with the tumorigenesis process, e.g. in the neoplasia of the esophagus, bladder, lung, breast and so on [25-29]. CMV infection is another wide spread infection,

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being detected in 50-85% of young adults [6,30], but in contrast to HPV, the involvement of CMV in the benign or malignant prostate disease is controversial since several reports acknowledge that prostate CMV infection could be a contributing factor to prostate cancer [31,32], but infection of benign prostate with CMV is very rare [33-35]. Regarding EBV, only few studies have researched its presence in the prostate gland [33,36,37]. Acknowledging the already reported data and the possible involvement of persistent viral infections in benign prostatic proliferation as triggers for future tumorigenesis, we have investigated the presence of HPV viruses, the CMV and EBV specific serum antibodies in a group of patients diagnosed with BPH.

Experimental part

Material and methods

Patients

50 patients with clinically and laboratory proven BPH, admitted in the Department of Urology of Dr. Carol Davila Central Military Emergency University Hospital between 2013 and 2014 were investigated. All patients underwent surgery: transurethral resection (TUR-P)-37 patients, transvesical adenomectomy - 13 patients. Patients with urinary tract infection and those who had previously received treatment with 5-alpha reductase inhibitor were excluded from the study to minimize the impact on prostate inflammation.

All patients signed an informed consent before study entry. In order to conduct this study the consent of the local ethics committee of the hospital was obtained.

Detection of HPV

Fresh prostatic tissue fragments were collected from the patients for each prostatic lobe. The fragments were frozen at -20° C until use. Isolation and purification of genomic DNA (gDNA) of the prostate tissue samples was performed with the kit QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The method involved proteolysis followed by adsorption on the micro-formed silicate membrane in micro-spin format. Contaminants were removed by washing and centrifugation, and elution of gDNA from the membrane was carried out using a saline buffer to finally obtain 200µL gDNA. The analysis of samples resulting from the extraction procedure was performed with the spectrophotometers *Biospec-nano* (Shimadzu Biotech, Kyoto, Japan) and *Epoch-multivolume* (Biotek, Winooski, VT, USA), to quantify DNA amount of (ng/µL) and quality (ratio OD260 / OD280). The ratio OD260 / OD280A having at least 2 indicated a high purity extracted DNA.

Determination of the presence of the HPV consisted in HPV-specific DNA (HPV-DNA) detection by polymerase chain reaction (PCR). The kit HPV *High Risk Screen Real-TM Quant* (Sacace Biotechnologies, Como, Italy) was used. The RT-PCR detects both high risk HPV (types 16, 18, 31, 45) and low-risk HPV (type 33, 35, 39, 51, 52, 56, 58, 59) strains. The kit identifies the most common oncogenic HPV genotypes. Each set of samples contained 10 µL gDNA, 3 standards with known concentrations of HPV-DNA and a negative control (NTC - No Template Control). Mix reaction tubes were processed in the device *RotorGene 6000* (Corbett Life Sciences, Qiagen, Hilden, Germany) according to the manufacturer's program amplification kit (table 1).

Results were automatically calculated using the *High Risk HPV Screen 4x Quant*, supplied by the manufacturer along with the kit. Test results are considered invalid in the

Table 1

THE POLYMERIZATION REACTION AMPLIFICATION PROGRAM

Stage	Temperature (°C)	Time	No. of cycles
1	95	15 min	1
2	95	5 sec	5
	60	20 sec	
	72	15 sec	
3	95	5 sec	40

absence of a fluorescent signal, or negative if fluorescent signal is present only in FAM channel. The results are considered positive for HPV types 16, 31, 33, 35, 52, 58 if there is a positive signal (Ct ≤ 33) in the JOE channel; HPV type 18, 39, 45, 59 if there is a positive signal in the ROX channel; HPV type 51, 56 if there is a positive signal in the Cy5 channel.

HPV-DNA load is calculated using the following formula:

$$\log\left(\frac{\text{HPV DNA copies per reaction}}{\text{genomic DNA copies per reaction}} \times 200000\right) = \log(\text{HPV DNA in } 100\,000 \text{ cells})$$

Detection of CMV

Anti-CMV IgG and IgM antibody types were detected in the patient's serum using Enzyme-Linked Immunosorbent Assay technique (Novalis Cytomegalvirus IgG / IgM ELISA immunodiagnostic Novatec GMBH, Dietzembach, Germany) according to the manufacturer's instructions. Briefly, serum samples were added to CMV antigen coated ELISA plates. Each kit contains 3 controls (positive, negative, cut-off). After washing the wells, peroxidase-coupled human anti-IgG / IgM antibodies were added. To the formed complex tetra-methyl-benzidine (TMB) was added, generating a color reaction proportionally with the serum antibody level. Absorbance at 450/620 nm was read with the plate reader *Sunrise* (Tecan, Männedorf, Switzerland). Cutoff value was determined by mediating the two absorbances obtained (cut off control was assayed in duplicate). According to the manufacturer, samples were considered positive when their absorbance exceeded the cut-off by more than 10%, while negative when less with more than 10%. Samples were considered in the *grey area* if the value resided in less than 10% above or below cut-off.

The results were expressed in Novatec units (NTU) according to the manufacturer formula.

Detection of EBV

Anti-EBV IgG and IgM antibodies were detected in the patient's serum with the Enzyme-Linked Immunosorbent Assay technique (Novalis Epstein-Barr virus IgG / IgM ELISA immunodiagnostic Novatec GMBH, Dietzembach, Germany) according to the manufacturer's instructions. Briefly, serum samples were added to EBV antigen coated ELISA plates. After adding peroxidase-coupled human anti-IgG / IgM antibodies the formed complex was revealed with tetra-methyl-benzidine (TMB), generating a color reaction proportionally with the antibody level. The absorbance was read with the plate reader *Sunrise* (Tecan, Männedorf, Switzerland).

Cutoff value was determined by mediating the two absorbances obtained (cut off control was assayed in duplicate). According to the manufacturer, samples were

considered positive when their absorbance exceeded the cut-off by more than 10%, while negative when less with more than 10%. Samples were considered in the *grey area* if the value resided in less than 10% above or below cut-off.

Results were processed using SPSS version 16.0 for Windows. Same software was used for tables and graphics. The continuous numerical data was represented as median and minimum-maximum range. Fisher exact tests were used to determine if there are differences between ordinal variables. Differences between continuous variables were performed using the Mann Whitney U test.

Results and discussions

HPV presence in benign tissues

Evaluating all tissue samples, we have obtained 4% HPV positivity (fig. 1). Table 2 presents the distribution of fluorescent signals (number of signals) on the 4 pursued channels. NTC has not issued a positive fluorescent signal on the 4 channels, and the 3 standards have shown positive signals depending on the concentration on all channels. Just one sample has issued a positive fluorescent signal (Ct ≤ 33) on Cy5 channel, which corresponds to the presence in the prostatic tissue of HPV type 51 or 56. Another sample (case 48) which showed a weak positive result (Ct = 38.21), is also included in statistics.

Table 2
DISTRIBUTION OF FLUORESCENT SIGNALS ON 4 CHANNELS

Channel	Fluorescent signals		
	NTC	Standards	Samples
FAM	0	3	0
JOE	0	3	0
Rox	0	3	0
Cy5	0	3	2

Given that this type of kit also evaluates the risk of cervical cancer induced by HPV, the result has been inserted by the program in the non-significant risk group. Overall, the certain presence of HPV in prostate occurred in 4% of the studied cases.

Determination of anti-viral antibodies

Out of all investigated patients, none of them revealed the presence of anti-CMV IgM antibodies, while 49 (98%) patients showed positive serum anti-CMV IgG antibodies (table 3, fig. 1). A total of 5 cases showed an uncertain outcome (gray area) for anti-CMV IgM, which may suggest a possible acute infection (table 3).

Table 3
CMV DETECTION RESULTS FROM 50 PATIENTS WITH BPH

Determination	No. of positive results	No. of uncertain results
IgM anti-CMV	0	5
IgG anti-CMV	49	1

Out of all patients, none revealed the presence of anti-EBV IgM antibodies, whereas all patients showed positive for anti-EBV IgG antibodies (table 4, fig. 1). No patient had an uncertain outcome (gray area) for anti-EBV IgM or IgG (table 4).

Table 4
EBV DETECTION RESULTS FROM 50 PATIENTS DIAGNOSED WITH BPH

Determination	No. of positive results	No. of uncertain results
IgM anti-EBV	0	0
IgG anti-EBV	50	0

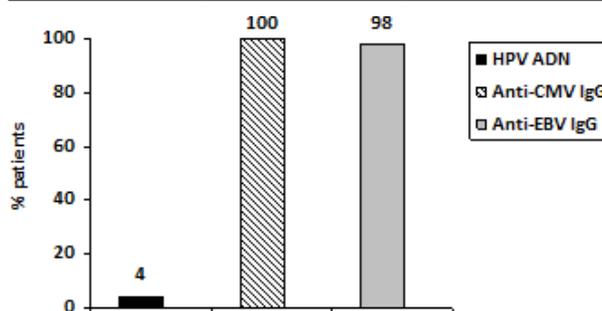


Fig. 1. Frequency of viral infection evidence in tested cases

Our results emphasize that BPH patients have a low rate of tissue HPV infection, while a chronic CMV and EBV infections evidence.

This paper aims to investigate the presence of viral infection in BPH as a possible cause for persistent inflammation and hence prostate hypertrophy. HPV usually causes unapparent/asymptomatic infections, but the possibility of particular strains to cause tissue dysfunctions must be taken into consideration. A number of inflammatory syndromes can be the result of infections with these viruses [3]. The existence of female genital cancer risk after unapparent HPV infection is a proof of this, bringing arguments for an investigation in male patients. Since the unapparent HPV infection is frequent in males (men being the virus reservoir that generates carcinogenic infections in women), intra-prostatic search for the presence of HPV becomes an element of interest. There is a high risk of urethral compression associated with BPH or malignant tumors of the pelvic region which may lead to recurrent infections of the urinary tract, lithiasis or renal failure that may require dialysis or very expensive treatments [38-40]. Therefore, preventing or early diagnosis of this pathology may be of great interest in urology or nephrology. During the conducted investigation, the presence of HPV-DNA was detected in two of the 50 investigated cases (4%). Because the investigation kit is intended to establish the degree of risk of cervical cancer in women, the results obtained in men cannot be assigned to a certain degree of risk. Considering that the use of this kit for analyzing the presence of HPV-DNA was performed for the first time in our country for men and targeted a particular tissue, the results should be interpreted only as intra-prostatic viral presence, without being able to determine whether such presence gives or not a certain degree of risk of developing prostate cancer. The literature provides conflicting information on a definite link between HPV infection and cancer, especially prostatic adenocarcinoma [41-44].

The fact that it was possible to identify cases of BPH with the presence of HPV, can be a starting point for introducing the investigation for the detection of HPV in the male genital secretions or excised tissue after surgery of the male genitals. Both identified cases were positive for 51, 56 strains, recently employed as a high-risk category. So far, we have not found in literature any association between these virus strains and prostate adenoma.

We believe it is too early to establish a link between the presence of HPV and the development of BPH, but we can do a number of correlations regarding the immune response of patients. The HPV-positive patients showed a decreased number of cytotoxic T-lymphocytes (T-CD8+), which was the general tendency in BPH patients we investigated [5]. This suggests a possible risk of infection in these patients, knowing the involvement of CD8+ T-lymphocytes in cellular antiviral immune response. Contact with the HPV does not necessarily mean that the virus entered into the prostate, the viral infection being able to be maintained in genital secretions only. The existence of the virus in prostate is proof that a possible HPV infection is within the body and can be also found in this organ.

The investigations for the presence of CMV and EBV revealed a similar immune pattern. Therefore, the presence of anti-CMV IgG in 98% of cases and anti-EBV IgG in 100% of cases is accompanied by the uncertain presence of anti-CMV IgM in 10% of cases and the absence of anti-EBV IgM. This emphasizes the rarity of acute viral infection cases or with a virus that replicates in the body, although there is an anamnestic contact with the virus. Our results also suggest that even if the virus could theoretically be stationed in the sleeping form in an organ, in most cases the conditions for viral replication are not met. The presence of antiviral IgG in an overwhelming majority of cases is somewhat surprising, and could conclude that most likely the infections were unapparent. We have observed that most patients had decreases in B lymphocytes, which, associated with the presence in their medical history of infection with EBV in 100% of cases, can generate the assumption that these patients suffered effects of the lymphotropic action of EBV on B lymphocytes [5]. However, there are cases of rare lymphomas with B-cells involving the prostatic tissue, for which this viruses may be responsible for the increasing number of abnormal B lymphocytes, but there is not enough data in the literature to conclude [45,46]. In patients with BPH, the evolution towards adenoma can be the result of a chronic inflammation post-viral infection or an effect of the presence of the virus, even if just transiently, in the prostate. Under the circumstances of unapparent acute infections, the prostatic cells reaction to the presence of the virus cannot be established or predicted, given the fact that the infection even cannot be diagnosed in the acute phase. From an anamnesis point of view, the existence of such an infection can be evidenced by the presence of specific IgG antibodies. Patients with high levels of anti-CMV IgG or anti-EBV IgG prove the existence of previous contact with the virus, but we cannot determine precisely whether or not the virus had affected the prostate. The presence of these antibodies in almost all patients with BPH may question whether CMV and EBV are involved in chronic inflammatory processes that generate the cellular reaction inducing prostatic hypertrophy.

Conclusions

Acknowledging the already reported data and the possible involvement of persistent viral infections in benign prostatic proliferation as triggers for future tumorigenesis, we have investigated the presence of HPV viruses and the

CMV and EBV specific serum antibodies in a group of patients diagnosed with BPH using ELISA and PCR. Development of BPH is a result of a succession of events that start from an impaired immune response associated with a viral infection that can sometimes be asymptomatic.

HPV oncogenic activity in female patients has been well established. In addition to the other two viruses, CMV and EBV, it has been proven to alter the DNA sequences in many cell populations. We have been able to prove the presence of HPV in male patients. The role of these viruses in the development of BPH or even carcinoma of the prostate is still under debate. We can only make assumptions based on the role of these viruses in other affections. The analysis of the casework and the conducted investigations do not provide sufficient data to support viral infection influence on a possible malignant transformation of the prostate. However, based on our results, we support the viral investigation as a possibility of enlarging tumor screening in inflammatory diseases of urinary tract and this screening can have therapeutic implications.

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