Anoikis Resistance and Development in Prostate Cancer: The Possible Role of HPV/EBV Coinfection

Javid Sadri Nahand
Iran university of medical sciences
Khadijeh Khanaliha
Iran university of medical sciences
Hamed Mirzaei
Kashan University of Medical Sciences
Mohsen Moghoofei
Kermanshah University of Medical Sciences
Hossein Bannazadeh Baghi
Tabriz University of Medical Sciences
Maryam Esghaei
Iran university of medical sciences
AliReza Khatami
Iran university of medical sciences
Maryam Fatemipour
Iran university of medical sciences
Farah Bokharaei-Salim (bokharaeifarah@gmail.com)
Iran university of medical sciences

Research Article

Keywords: Human Papillomavirus, Co-infection, Epstein-Barr virus, Prostate cancer, Inflammation, Anoikis

Posted Date: January 4th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-135451/v1

Version of Record: A version of this preprint was published at BMC Cancer on August 17th, 2021. See the published version at https://doi.org/10.1186/s12885-021-08658-y.

Abstract

Background: This study aimed to evaluate the possible role of EBV/HPV co-infection as an etiologic factor in prostate cancer (PCa) development.

Methods: The present case-control research was conducted on 67 cases with prostate cancer and 40 controls. The expression of cellular and viral factors involved in inflammation, tumor progression, and metastasis were quantitated using ELISA and qRT-PCR.

Results: The EBV/HPV co-infection was reported in 14.9% of case group and 7.5% of control group. The high-risk type of HPV, including HPV 16 and 18, were responsible for 50% and 30% of 10 HPV/EBV co-infected PCa samples, respectively. According to the results, a significant relationship was not observed between the PCa and HPV/EBV co-infection (OR=2.9, 95%CI=0.18-45.2, P=0.31). However, the highest percentage of HPV genome integration was found in HPV/EBV coinfected PCa group (8/10, 80%). Moreover, the mean expression levels of inflammatory factors (IL-17, IL-6, TNF-α, NF-κB, VEGF, ROS and RNS), anti-apoptotic mediators (Bcl-2 and Survivin), and anti-anoikis factors (TWIST, N-cad) were higher significantly in the HPV/EBV co-infected PCa cases when comparing with the non-coinfected PCa samples. Nevertheless, the tumor suppressor proteins (p53 and Rb) and the E-cad (inhibiting anoikis resistance) had a significant downregulation in the HPV/EBV co-infected PCa cases than in the non-coinfected PCa samples.

Conclusion: HPV/EBV co-infection probably can act as an etiologic factor in PCa through modulation of cellular behavior.

1. Background

Prostate cancer (PC) has been reported as the second most common male cancer and the fifth main cause of global mortality [1]. There is limited documentation on PC development and the exact etiology of pathogenesis. Various risk factors have been reported for PC in men, including ethnicity, age over 50 years, infections, and acquired or inherited genetic mutations [2]. Chronic inflammation and infection may be associated with the development of cancer in various organs including the thyroid, breast, stomach, liver, cervix and colon. Reportedly, an adult prostate can be prone to inflammation [3–7]. Inflammation of the prostate can occur as a result of a variety of factors, including poor diet, hormonal changes, cell damage, and infection (especially sexually transmitted infections). Tolerance to normal prostate antigens may be lost following epithelial cell damage, which triggers a sustained autoimmune reaction [3, 8]. Of the approximately 1,400 human pathogens, 220 are related to viral agents, a limited number of which are associated with cancer in various ways called oncogenic viruses. A newer concept expresses virus-induced mechanisms altering the biology and aggression of many cancers. Tumor structure alters under the influence of virus-induced mechanisms in cancer cells and stromal cells in the tumor environment, such as inhibited pathways of cancer cell apoptosis, change in tumor metabolism, inhibited anti-tumor immune system, provoked inflammation of the tumor environment, the onset of angiogenesis, the

induction of tumor cell proliferation, higher invasion of tumor, and increased metastasis [9]. Prostate tissue can be impacted by many pathogens, including viruses [2], such as Epstein-Barr virus (EBV), human herpes simplex virus type 2 (HSV2), herpesviruses including cytomegalovirus (CMV) and human papillomavirus (HPV) [10]. However, there is no substantiated evidence to prove a direct correlation between infection frequency and the development of the inflammatory response with prostate carcinogenesis [3, 10].

The EBV and HPV are well-established oncoviruses capable of initiating and progressing various human carcinomas [11–13]. In the high-risk HPV types, E5, E6, and E7 proteins interference with the tumor suppressor proteins in the cell. In addition, the oncoproteins of HPV E6 and E7 can modify the tumor milieu by regulating the certain pro-inflammatory chemokines and cytokines, which can impact the immune reaction efficacy [14]. On the other hand, the oncogenic proteins of LMP1 and EBNA1 in EBV prevent the apoptosis, stimulate the cell motility and proliferation, induce the angiogenesis and cell motility, which all prove the function of EBV in the carcinogenesis [15, 16]. The EBV and HPV co-infection in some carcinomas have been reported by numerous studies [17–22]. Besides, the presence of both HPV and EBV sequences in healthy, malignant, and benign prostate samples [23–26] is of great importance because many experimental findings showed that the HPV and EBV maybe act a role in the development of PCa [27]. In this regard, the function of EBV/HR-HPV co-infection is unclear. The current study aimed to evaluate the association of EBV/HPV co-infection with the PCa development by deregulating the cellular events linked to inflammation (RNS, ROS, TGF-β, VEGF, IL-17, IL-11, IL-8, IL-6, IL-1, TNF-α and NF-kB) and tumor progression (N-cad, Rb, P53, Survivin, Bcl-2, CD44, TWIST, SLUG, PTPN13 and E-cad). Also, we investigate the level of viral gene expression (E7, E6, E2, LMP-1, LMP-2 EBER 1 and EBER2) and the associations among viral genes and cellular factors involved in PCa development.

2. Methods

2.1. The collection of samples

The present multicenter case-control research was conducted during December 2018 to April 2020. First, 67 prostate tumor samples (n = 67) were obtained considering the inclusion and exclusion criteria during the study period in Tehran, Iran. Moreover, healthy tissues (n = 40) dissected from the peripheral area of adenoma removed by surgical procedure were collected as control (in terms of age). At least 24 hours prior to surgery, serum samples were prepared from blood by venipuncture and stored at -80 °C. Additionally, the tumor stage was detected based on the TNM system in accordance with the consultation of a team of experts in cancer including a cancer surgeon, a radiologist and an oncologist. The ethical considerations were in accordance with the Helsinki Declaration, and both verbal and written informed consents were achieved from all research units. Snap-frozen tissue samples in liquid nitrogen were stored at − 80 °C. The clinical profiles of patients included tumor stage, tumor type and age (Table 1).

Table 1
Comparison of the characteristics of research units between prostate cancer and control groups

Characteristics		Prostate Cancer (67)	Control (40)	P	OR (95% CI)
Age (Year)		52.7 ± 12.2	55.6 ± 9.9	0.19	0.95 (0.9–1.02)
HPV positive samples	**Presence**	31.3% (n = 21)	15% (n = 6)	0.060	1.84 (0.08–0.6)
EBV positive samples	**Presence**	49.3% (n = 33)	40% (n = 16)	0.353	1.06 (0.8–1.39)
Mono HPV-infection		16.4% (11)	7.5% (n = 3)	0.289	1.06 (0.26–6.5)
Co-infection (EBV&HPV)		14.9% (n = 10)	7.5% (n = 3)	0.314	2.9 (0.18–45.2)
Mono EBV-infection		34.3% (n = 23)	42.5% (n = 17)	0.48	0.58 (0.17–2.6)
Non-EBV and Non-HPV samples		34.3% (n = 23)	42.5% (n = 17)	0.48	NA
HPV Genotype	6	4.8% (1/21)	0	0.248	1.23 (0.51–4.42)
	11	0	16.7% (1/6)		
	16	47.6% (10/21)	50% (3/6)		
	18	33.3% (7/21)	33.33% (2/6)		
	33	3 (15.8%)	0		
EBV Genotype	1	9.09% (3/33)	0	0.105	0.55 (0.077–3.71)
	2	90.9% (30/33)	100% (20/20)		
Type of Cancer	Acinar adenocarcinoma	40 (59.7%)	-	NA	NA
	Ductal Adenocarcinoma	21 (31.34%)	-	NA	NA
	Squamous cell cancer	6 (8.95%)	-	NA	NA
Stage of Cancer	I	4 (5.9%)	-	NA	NA
	IIA	9 (13.4%)	-	NA	NA

Characteristics		Prostate Cancer (67)	Control (40)	P	OR (95% CI)
	IIB	9 (13.4%)	-	NA	NA
	IIC	5 (7.4%)	-	NA	NA
	IIIA	2 (2.98%)	-	NA	NA
	IIIB	10 (14.9%)	-	NA	NA
	IIIC	6 (8.9%)	-	NA	NA
	IVA	12 (17.9%)	-	NA	NA
	IVB	10 (14.9%)	-	NA	NA
NA; Not applicable					

The study was approved by ethical committee of the Iran University of Medical Sciences (IUMS), Tehran, Iran, under the Ethics code of IR.IUMS.REC.1398.642. As well as, the current study was supported by grant no, 15671 from the research deputy of Iran University of Medical Sciences (IUMS).

2.2. Detection of HPV and EBV by PCR

Total DNA was extracted from the tissue samples by the QIAamp DNA Mini Kit based on the kit protocol from the frozen samples of PCa. The quality of extracted DNA was analyzed using a 268-bp fragment amplification of the b-globin gene with the aid of HotStarTaq DNA polymerase (Qiagen, Dusseldorf, Germany) in the presence of G074 (5'-CAACTTCATCCACGTTCACC- 3') and G073 (5'-GAAGAGCCAAGGACAGGTAC- 3') primers. The cycling program was 95℃ for 9 min, and then 35 cycles at 95C for 30 seconds, 55℃ for 30 seconds, 72℃ for 1 minute, and a final extension at 72℃ for 10 minutes [28].

The DNA extracted from 67 and 40 fresh frozen prostate cancer and control samples, respectively were analyzed by PCR for identification of HPV-L1 gene (MY11: 5'-GCMCAGGGWCATAAYAATGG-3' and MY09: 5'-CGTCCMARRGGAWACTGATC-3') and for detection of HPV E6/E7 gene (pU-H: 5'-GAGCTGTCGCTTAATTGCTC-3' and pU-R: 5'-TGCTAATTCGGTGCTACCTG-3') [29].

For EBV detection nested-PCR method was used by primers that used in Breda et al. study [30], including 5'-GCGGGTG-GAGGGAAAGG-3' and 5'-GTCAGCCAAGGGACGCG-3' for the first PCR round as well as 5'-GCCACCTGGCAGCCCTAAAG-3' and 5'-AGGCTGCCCACCCTGAGGAT-3' for the second PCR round [30]. Ten nM of each primer (Metabion, Germany), 2X Taq Master Mix, 1 µg of template DNA, and water at a final volume of 25 µl. The amplification of the samples was performed by 45 PCR cycles with the thermal program as follows; (1) The first PCR reaction: the initial denaturation for 5minutes at 94 °C; for 30seconds at 94 °C; for 30seconds at 57 °C and for one minute at 72 °C, the final extension at 72 °C for 7minutes, (2) the second PCR reaction: the initial denaturation for 5 minutes at 94 °C, for 30 seconds at

94 °C, for 30 seconds at 50–57 °C and for one minute at 72 °C, and the final extension at 72 °C for 7 minutes.

2.3. EBV and HPV genotyping

In EBV positive samples, the EBV typing was performed by the EBNA2 primers [31]. The size of PCR products expected for EBV strains 1 and 2 is respectively 300 and 250 bp [31].

The samples positive for HPV were genotyped using INNO-LiPA HPV Genotyping v2 test (Innogenetics, Ghent, Belgium) based on the kit protocol.

2.4. Quantitative real-time PCR

The expression of cellular gene (SLUG), HPV genes (E7, E6, and E2) and EBV genes (EBER 1, EBER-2, LMP-1 and LMP-2) was quantified by real-time PCR in the HPV-EBV-positive and HPV-positive PCa samples, respectively. To this end, the extraction of total RNA (1 μg) was performed by QuantiNova Reverse Transcription Kit (QIAGEN, Germany), followed by constructing the cDNA in a thermal cycler as 27 °C for 10 minutes, 38 °C for 15 minutes, 44 °C for 40 minutes, and 72 °C for 15 minutes. (The reaction condition was set as followed: 45 °C for 2 minutes in the DNA elimination reaction, 25 °C for 3 minutes in the annealing phase, 45 °C for 10 minutes in the reverse transcription phase and 5 minutes 85 °C in the inactivation of reaction).

2.4.1. EBV genes

LMP-1. The expression level of LMP-1 in EBV-infected PCa tissue was measured by qRT-PCR technique by the probes and primers according to Kubota et al. [32].

LMP-2A. Quantitative real-time PCR was carried out in EBV-positive tissues for LMP-2A by QuantiNova SYBR Green PCR kit (Qiagen, Hamburg, Germany) which has been described in Busson et al [33].

EBER. The EBER1 and EBER2 levels were measured in EBV-positive PCa tissue using qRT-PCR according to Shannon-Lowe et al. [34].

2.4.2. HPV genes

QuantiNova Reverse Transcription® Kit, one step RT-PCR® kits (QIAGEN, Hilden, Germany) and Quantitative SYBR green TaqMan Universal PCR Master Mix® (QIAGEN, Germany) were respectively applied to recognize the viral E7, E6 and E2 genes. The serial dilutions of E7, E6 and E2 genes cloned in PUC57 vector (GenScript, Jiangsu, China) were utilized for viral genes, which contained equivalent volumes of these genes from 72 to 865 million copies/reaction, as control.

The viral genes of E2 (forward primer: 5'-CTACGAATTCATGGAGACTCTTTGCCAACG-3' and reverse primer: 5'-GATAGAATTCTCATATAGACATAAATCCAG-3'), E6 (forward primer: 5'-GCAATGTTTCAGGACCCACA-3' and reverse primer: 5'-ACAGCATATGGATTCCCATCTC-3') and E7 (forward primer: 5'-AAGTGTGACTCTACGCTTCGGTT-3', reverse primer: 5'-GCCCATTAACAGGTCTTCCAAA-3' and Probe of FAM-TGCGTACAAAGCACACACGTAGACATTCGTA-BHQ), were respectively detcted using one step RT-

PCR® kits (QIAGEN, Hilden, Germany), QuantiNova Reverse Transcription® Kit and Quantitative SYBR green TaqMan Universal PCR Master Mix® (QIAGEN, Germany) [35]. The serial dilutions of E7, E6 and E2 genes cloned in PUC57 vector (GenScript, Jiangsu, China) were utilized for viral genes, which contained equivalent volumes of these genes from 72 to 865 million copies/reaction, as control.

2.4.3 Cellular gene (SLUG)

The SLUG gene was amplified by the qRT-PCR in the presence of specific primers. The real time PCR device (Rotor-Gene® Q; Qiagen, Hilden, Germany) was utilized exploiting the Power SYBR Green PCR Master Mix (TaKaRa Bio; Kusatsu; Japan). The normalization of the relative expression level for the gene was carried out by a GAPDH as housekeeping gene. The sequences of F and R primers to amplify the SLUG gene were as follows: the forward primer sequence was as: 5'-GCCTCCAAAAAGCCAAACTACA-3', the reverse primer sequence was as: 5'-GAGGATCTCTGGTTGTGGTATGACA-3' [36].

In addition, the expression level was measured by the equation of $2^{(-\Delta\Delta Ct)}$ exploiting the online data analysis tool of QIAGEN (Gene Globe; http://www.qiagen.com/us/ shop/genes-and-pathways/data-analysis-center-overview-page/). All reactions were repeated three times, and the internal control was considered to be GAPDH for the normalization of gene expression level (E7, E6, and E2) for various specimens.

2.5. Physical status of HPV DNA

The qPCR technique was used to compute the E2/E6 ratio for the characterization of the HPV-DNA physical status (episomal or integral status) in the specimens infected with the HPV. The E2/E6 = 0 was defined as a fully integrated status and the E2/E6 ≥ 1 as an episomal status, and the E2/E6 = 0–1 as a mixed status [37].

2.6. Enzyme linked immunosorbent assay (ELISA)

2.6.1. Estimation of serum cytokines and survivin levels

The taken whole blood was poured into the SST™ serum separation tubes. Each sample had five complete inversions. The blood was left at room temperature for 15–30 minutes in order to clot. No longer than two hours after collecting, the separator gel tubes had centrifugation, followed by discarding the clot for 15 minutes at 1,500 × g. The serum samples were distributed aseptically at 50-μl volumes and kept − 80 °C for subsequent testing.

The serum levels of IL-17, IL-11, IL-8, IL-6 and IL-1 were measured by the related ELISA kit (Abcam, Cambridge, MA, USA) based on the suggested protocol. Moreover, the serum levels of VEGF, TGF-β and TNF-α were measured by Quantikine Assay Kit (R&D Systems), Human TGF-β Quantikine ELISA® Kit (Minneapolis) and Human TNF-α PicoKine ELISA Kit (Boster), respectively, based on the kit protocols.

The survivin (anti-apoptotic mediator) expression level was measured by Survivin Human SimpleStep ELISA® Kit (Abcam) based on the manufacturers' protocol.

2.6.2. Measurement of Bcl-2 and anoikis-related factors in tissue samples

In brief, all prostae tissue samples were grounded individually in the liquid nitrogen, and then lysed by BioPlex lysis buffer (Bio-Rad, Hercules, CA) inside the microcentrifuge tubes. The tissue lysate was homogenized in a Dounce homogenizer, followed by centrifugation with 13,000 rpm for 10 minutes at a temperature of 4 °C to obtain a clear supernatant containing the lysate of prostate tissue.

The Bcl-2 level was quantified in all tissue lysate samples by the Human Bcl-2 ELISA Kit (Abcam) based on the related kit protocol.

The expressin level of anoikis-related proteins including PTPN13, TWIST, N-cadherin and E-cadherin in prostate tissue lysates were measured using Human Tyrosine-Protein Phosphatase Non-Receptor Type 13 (PTPN13) ELISA Kit (MyBioSource, USA), TWIST ELISA Kit (Aviva Systems Biology, CA, USA) and Human E-Cadherin, N-Cadherin ELISA Kit (Abcam, Cambridge, MA, USA) respectively, based on the related kit protocols.

2.6.3. Quantification of Reactive Oxygen/Nitrogen Species, p53, Retinoblastoma and NF-kB in prostate tissue lysates

The level of ROS and RNS were determined in tissue lysate samples by the OxiSelect™ Intracellular ROS/RNS Assay Kit (Cell Biolabs, Inc., San Diego, CA), based on the kit protocol.

Quantification of NF-kB, Rb and p53 was performed in the tissue lysates by NF-kB p65 Transcription Factor Assay® Kit, Human Retinoblastoma ELISA® Kit (Sigma-Aldrich, Saint Louis, USA) and Abcam's p53 Simple Step ELISA® Kit (Cambridge, USA), respectively, based on the kit protocols.

2.7. Statistical methods

Statistical analysis was performed by GraphPad Prism 6 and STATA versions 11.2 software. Kolmogorov–Smirnov test was used to assesse the normality of data distribution. Data were reported as mean ± standard deviation (SD) of parametric variables, and the median and the interquartile range for nonparametric data. In the comparison of central tendency parameters, including the mean for the normal variables and the median for the non-normal variables, two-independent samples t-test or Mann-Whitney non-parametric tests were applied between two groups, as well as one-way ANOVA or kruskal-wallis tests were applied between more than two groups. Fisher’s exact test or Chi-square was utilized to evaluate the associations between the categorical data. Spearman’s rank correlation was measured for obtaining the extent of coexpression between the cellular factors and the viral genes. The statistically sinificance level was considered to be $P < 0.05$, adjusted by Bonferroni’s correction test for ANCOVA analysis of data. Benjamini-Hochberg method was applied to correct the false discovery rate for multiple comparisons. Heat maps were plotted by one matrix CIMminer using Ward cluster algorithm and Euclidean distance approachs.

3. Result

3.1. The profiles of research units

The demographic profiles of the study participants (n = 107) are shown in Table 1. Totally, 64 PCa cases and 40 control were collected between August 2018 and March 2020. The cases and controls were homogeneous for the age (P = 0.19). The observed types of PCa tissues included acinar adenocarcinoma (40/64, 59.7%), ductal adenocarcinoma (21/64, 31.3%) and squamous cell cancer, SCC (6/64, 8.9%). In the current study, the lowest and highest cancer stages were respectively IIIA (2.98%) and IVA (17.9%). EBV-DNA was found among 49.3% of PCa samples, HPV DNA in 31.3%, while in control group in 40% and 15%, respectively. Among 67 PCa tissue specimens, HPV mono-infection was detected in 11 (16.4%), EBV mono-infection was detected in 23 (34.3%), and HPV/EBV co-infection was detected in 10 (14.9%). As well as, among 40 control tissue specimens, HPV mono-infection was detected in 3 (7.5%), EBV mono-infection was detected in 17 (42.5%), and HPV/EBV co-infection was detected in 3 (7.5%). No significant association was found between the EBV, HPV and HPV/EBV co-infection and the PCa (OR = 1.06, 95%CI = 0.8–1.39, P = 0.35, OR = 1.84, 95%CI = 0.08–0.6, P = 0.06, and OR = 2.9, 95%CI = 0.18–45.2, P = 0.34, respectively). HPV 16 and EBV 2 were the highest genotypes isolated in both case group (47.6% and 100%, respectively) and control group (50% and100%, respectively).The most frequent HPV genotypes in HPV/EBV co-infected positive groups were HPV-16 (6/11, 54.54%), followed by HPV-18 (3/11, 27.24%), HPV-33 (1/11, 9.09%), and HPV-6 (1/11, 9.09%). Also, there was no significant association between all HPV and EBV genotypes and the PCa development (P = 0.24 and 0.1, respecitvely). No statistically association was reportd between EBV/HPV co-infection and histopathological types of tumor (P = 0.268, and 0.353). No significant difference was seen in the frequency distributions of PCa stages between the EBV-positive PCa and the EBV-negative PCa, as well as between the HPV-positive PCa and the HPV-negative PCa (P = 0.17 and 0.54).

3.2. Physical status of the HPV genome

The HR-HPV genome integration in the host chromosome importantly deregulates the expression of E6 and E7 oncogenes, thereby resulting in cell transformation [38]. It should be noted that the association of integration with contributing to prostate malignancy has not been the reported. A fully integrated type was seen in 42.8% of PCa group and 16.6% of control group. In 9.5% of PCa and 16.6% control groups, HPV genome was present in a purely episomal form. Also, mixed forms (episomal and integrated genomes) were found in 47.6% and 66.66% of the PCa and control specimens, respectively (Table 2). Furthermore, the frequency of the fully HPV genome integrated type was significantly higher in the PCa group co-infected with the HPV/EBV when comparing with the mono-HPV infected PCa group (*P*: 0.0009 and 0.002, respectively). It is noteworthy that all integrated form of HPV DNA was found in the HPV/EBV co-infection samples. The HPV 16 DNA integrated type was observed in 5 of 21 (23.8%) HPV-positive PCa, in 5 of 10 (50%) HPV/EBV-co-infected positive PCa and in 1 of 1(100%) HPV/EBV co-infected positive control groups. The HPV-18 DNA was integrated in 3 of 21 (14.2%) HPV-positive PCa, and in 3 of 10 (30%) HPV/EBV-co-infected positive PCa groups (Table 2).

Table 2
The HPV genome physical state in the studied groups

Status	PCa group	Controls group	Mono HPV infected PCa group	Co-infected PCa group	Mono HPV infected (%) Control group	Co-infected Control group
Fully integrated state (E2/E6 = 0)	9/21 (42.8%)	1/6 (16.6%)	1/11 (9.09%)	8/10 (80%)	0	1/3 (33.33%)
	P: 0.81		P: 0.0009		P: NA	
Episomal state (E2/E6 = 1)	2/21 (9.5%)	1/6 (16.6%)	2/11 (18.18%)	0	0	1/3 (33.33%)
	P: 0.53		P: NA		P: NA	
Mixed state (E2/E6 = > 0 to < 1)	10/21 (47.6%)	4/6 (66.66%)	8/11 (72.72%)	2/10 (20%)	3/3 (100%)	1/3 (33.33%)
	P:0.6		P: 0.003		P: 0.002	
NA; Not applicable						

3.3. The comparison of expression pattern of viral genes between study groups

Table 3 shows the expression level calculated for the EBV genes (EBER-1, EBER-2, LMP-1 and LMP-2) and HPV genes (E2, E6 and E7) in both stages and types of PCa. The maximum expression level of EBV genes examined was EBER 2 in stage IIA samples (mean ± SD:22.4 ± 9.7) as well as the lowest expression level of EBV genes was that of LMP 2 in stage IIIC samples (mean ± SD: 7.1 ± 8.3).The lowest and highest expression of LMP-1 gene were observed in stage IIIB (mean ± SD: 7.5 ± 10.2) and IVB (mean ± SD: 15 ± 11.9) samples, respectively. The expression of both LMP-2 and EBER-1 had the maximum mean levels in stage I specimens, and EBER 2, has the lowest expression in stage IIIB samples. Stratification of the specimens in terms of the types of cancer demonstrats the EBER 1 level in SCC (mean ± SD: 18.6 ± 7.5) and EBER 2 in ductal adenocarcinoma (mean ± SD: 8.7 ± 9.5) samples were respectively the maximum and the minimum. The LMP-1 and LMP-2 gens had the highest expression level in acinar adenocarcinoma and SCC samples, respectively (Table 3). Figure 1B shows that a significant difference was in the EBV gene expression between mono EBV-positive PCa samples, whereas, except for EBER-2 gene, no significant difference was found between the mono EBV-positive PCa and HPV/EBV-coinfection positive PCa samples.

Table 3
Comparison of the expression level of HPV and EBV genes between types and stages of Prostate Cancer

Cancer characteristic		E2	E6	E7	LMP-1	LMP-2	EBER 1	EBER 2
Types of Cancer	Acinar adenocarcinoma	2.5 ± 2.5	13.1 ± 6.2	12.89 ± 6.3	14.2 ± 7.4	12.3 ± 11.3	17.8 ± 15.3	16.2 ± 10.2
	Ductal adenocarcinoma	2.4 ± 2.8	12.91 ± 5.9	13.87 ± 4.63	13.07 ± 11.4	9.7 ± 10.8	8.6 ± 10.1	8.7 ± 9.5
	Squamous cell cancer	0	17.33 ± 4.7	19.17 ± 4.3	11.8 ± 2.7	13.5 ± 11.1	18.6 ± 7.5	17.2 ± 8.1
	P	Ns	ns	ns	Ns	ns	ns	ns
Stages of Cancer	I	0	0	0	14.5 ± 9.2	17.5 ± 12.1	20.1 ± 11.5	19.8 ± 10.8
	IIA	0	13 ± 2.8	14 ± 4.2	15 ± 9.50	17.4 ± 9.6	20.3 ± 8.2	22.4 ± 9.7
	IIB	3 ± 1.85	6.6 ± 2.7	7.3 ± 1.5	10.8 ± 5.33	7.6 ± 12.6	9.8 ± 16.3	8.21 ± 4.04
	IIC	0	0	0	12.8 ± 6.9	12.8 ± 11.9	17.3 ± 7.2	15 ± 5.11
	IIIA	0	0	0	8 ± 6.31	11 ± 3.2	9.8 ± 11.2	12.3 ± 10.4
	IIIB	4.5 ± 1.99	13.8 ± 4.9	11.6 ± 4.8	7.5 ± 10.2	7.9 ± 7.3	16.8 ± 5.9	11.3 ± 4.8
	IIIC	3 ± 0.54	14.5 ± 7.5	17 ± 1.8	9.8 ± 9.01	7.1 ± 8.3	8.9 ± 9.02	10.8 ± 3.3
	IVA	5.4 ± 2.8	12.7 ± 4.3	13.4 ± 3.9	12 ± 11.7	15.1 ± 11.8	11.5 ± 7.2	12.6 ± 4.52
	IVB	0	15.5 ± 2.7	17.2 ± 2.5	15 ± 11.9	16.5 ± 11.2	17.2 ± 7.7	13.7 ± 6.2
	P	Ns	ns	ns	ns	ns	ns	ns

Geometric Mean ± Standard Deviation, NA: Not available, ns: not significant.

The lowest and highest levels were found for both E6 and E7 genes in stage IIB (mean ± SD: 6.6 ± 2.7 and 7.3 ± 1.5, respectively) and IVB (mean ± SD: 15.5 ± 2.7 and 17.2 ± 2.5, respectively) samples, respectively (Table 3). The minimum HPV gene expression level was related to E2 that was observed in the stage IIIC (mean ± SD: 3 ± 0.54) samples. Concerning the cancer type, the E7 gene expression level were the highest in the SCC (mean ± SD: 19.17 ± 4.3), as well as, the highest E6 gene level was observed in the SCC. According to Fig. 1, the expression level of HPV genes between the mono HPV-positive PCa cases and the

mono HPV-positive controls as well as between the mono HPV positive PCa cases and the HPV/EBV coinfected PCa cases was not significantly different. Nonetheless, the expression level of E7 gene was significantly higher in the HPV/EBV coinfected PCa group when comparing with the HPV/EBV coinfected controls.

3.4. Comparison of expression pattern of inflammatory-related factors among study groups

We examined the expression levels of different inflammatory mediators between the PCa samples with the control samples and between EBV/HPV-positive samples with EBV/HPV-negative samples. The result is shown in Tables 4, 5, and 6. According to the results obtained, the level of inflammation-related factors including NF-κB, TNF-α, IL-17, IL-11, IL-8, IL-7, IL-6, IL-1, VEGF, TGF-β, ROS, and RNS in the PCa group higher than in the control group. For detection of whether HPV and/or EBV infection or/and HPV/EBV-coinfection are implicating in regulating inflammation in PCa, we analyzed the expression level of these factors among EBV infected, HPV infected, and HPV/EBV coinfected samples. All inflammatory factors mentioned above was higher significantly in the groups positive for HPV compared to the groups negative for HPV (Table 5). Furthermore, as shown in the Table 5, the maximum levels of expression were seen for the ROS ($P < 0.0001$, fold change: 7.5), RNS ($P < 0.0001$, fold change: 2.6) and IL-6 ($P < 0.0001$, fold change: 2.51) in the PCa group positive for HPV compared with the PCa group negative for HPV. The correlation results in Table 7 showed significantly positive correlation between the expression level of the viral proteins (E2, E6 and E7) and the inflammatory agents ($P < 0.001$). The strongest positive correlation was between ROS and E6/E7 ($r = 0.763$, $P = 0.0001$), and then between Survivin and E6/E7 ($r = 0.75$, $r = 0.76$, respectively, p value = 0.0001 for both).

Table 4
Comparison of cellular factors levels between the EBV positive and negative samples

Cellular Factors	PCa group (a) *vs* control group (b)	EBV-positive samples (c) *vs* EBV-negative samples (d)	EBV-positive PCa (e) *vs* EBV-negative PCa (f)	EBV-positive control (g) *vs* EBV-negative control (h)	P*	P+	P$	P^
NF-κB	a: 19.4 ± 7.4 b: 9.5 ± 7	c: 18 ± 7.8 d: 12.7 ± 9	e: 22.4 ± 4.3 f: 15.4 ± 8.8	g: 10.9 ± 7 h: 7.1 ± 6.6	< 0.0001	0.006	0.004 1.45	ns
TNF-α	a: 17 ± 7.4 b: 11.7 ± 6.7	c: 18 ± 5.5 d: 11.03 ± 8.1	e: 19.7 ± 5.7 f: 13.4 ± 7.9	g: 15.2 ± 4.1 h: 6 ± 6.4	0.002	< 0.0001	0.001 1.47	0.001
IL-1	a: 13 ± 5.7 b: 7.8 ± 5.3	c: 13.1 ± 5.3 d: 8.4 ± 6.1	e: 15 ± 4.6 f: 10.4 ± 6	g: 10 ± 4.8 h: 4.2 ± 4	0.0002	0.001	0.01 1.44	0.05
IL-6	a: 14.4 ± 6 b: 8.9 ± 6.6	c: 14.8 ± 5.8 d: 9 ± 6.6	e: 16.4 ± 5.5 f: 11.8 ± 5.9	g: 12.3 ± 5.5 h: 3.4 ± 3.9	0.0004	0.0003	0.02 1.3	0.004
IL-8	a: 12.62 ± 6.2 b: 5.68 ± 5.1	c: 10.4 ± 6.6 d: 9.7 ± 6.8	e: 12.7 ± 6.3 f: 12.4 ± 6.01	g: 6.7 ± 5.5 h: 4.1 ± 3.7	< 0.0001	ns	ns	ns
IL-11	a: 15.6 ± 8.3 b: 7.1 ± 6.2	c: 13.4 ± 8.6 d: 11.3 ± 8.5	e: 16.5 ± 8.3 f: 14.3 ± 8.2	g: 8.3 ± 6.4 h: 5.5 ± 5.2	< 0.0001	ns	ns	0.049
IL-17	a: 9.8 ± 3.9 b: 4.3 ± 3.8	c: 8.2 ± 4.8 d: 7.4 ± 4.6	e: 10.2 ± 4.1 f: 9.4 ± 3.7	g: 4.9 ± 4.01 h: 3.3 ± 3.6	< 0.0001	ns	ns	ns

Cellular Factors	PCa group (a) *vs* control group (b)	EBV-positive samples (c) *vs* EBV-negative samples (d)	EBV-positive PCa (e) *vs* EBV-negative PCa (f)	EBV-positive control (g) *vs* EBV-negative control (h)	P*	P+	P$	P^
VEGF	a: 38.64 ± 10.8 b: 13.7 ± 6.9	c: 33.5 ± 14 d: 27.9 ± 15.07	e: 40.8 ± 8.5 f: 32.8 ± 14.3	g: 23 ± 12.4 h: 14.05 ± 6.08	< 0.0001	0.03	0.01 1.2	0.02
TGF-β	a: 15.2 ± 7.6 b: 7.5 ± 5.1	c: 13.5 ± 7.9 d: 10.9 ± 7.3	e: 16.4 ± 7.8 f: 13.6 ± 7.1	g: 8.7 ± 5.3 h: 5.4 ± 4.2	< 0.0001	ns	ns	0.039
Rb	a: 9.1 ± 5.7 b: 14.06 ± 6.4	c: 10.7 ± 6.2 d: 11.1 ± 6.8	e: 9.4 ± 6.1 f: 8.8 ± 5.4	g: 13 ± 5.8 h: 15.8 ± 7.3	0.0006	ns	ns	ns
P53	a: 10.4 ± 6.7 b: 13.9 ± 6.6	c: 10.8 ± 6.3 d: 13 ± 7.4	e: 10.1 ± 6.01 f: 10.9 ± 6.8	g: 11.9 ± 5.6 h: 17.3 ± 6.9	0.04	ns	ns	0.02
ROS	a: 10.2 ± 9.7 b: 6.09 ± 5.3	c: 8.9 ± 8.09 d: 9.07 ± 8.1	e: 9.8 ± 10.44 f: 9.6 ± 10.11	g: 5.2 ± 4.7 h: 7.5 ± 6.1	0.03	ns	ns	ns
RNS	a: 11.8 ± 10.3 b: 5.2 ± 5.3	c: 9.6 ± 9.6 d: 9.2 ± 9.08	e: 12.52 ± 10.57 f: 10.9 ± 10.1	g: 5 ± 5.6 h: 5.3 ± 5.06	0.0002	ns	ns	ns
Survivin	a: 17.8 ± 4.8 b: 9 ± 6.2	c: 16.3 ± 6.5 d: 12.3 ± 6.5	e: 19.9 ± 3.3 f: 15.1 ± 5.1	g: 10.4 ± 6.3 h: 6.5 ± 5.2	< 0.0001	0.031	0.02	ns
Bcl-2	a: 12.1 ± 4.2 b: 6.4 ± 4.5	c: 11.4 ± 5.4 d: 8.2 ± 4	e: 14.1 ± 3.9 f: 9.5 ± 3.5	g: 17.6 ± 0.5 h: 5.7 ± 3.9	0/0001	0.033	0.008	0.0005

Cellular Factors	PCa group (a) *vs* control group (b)	EBV-positive samples (c) *vs* EBV-negative samples (d)	EBV-positive PCa (e) *vs* EBV-negative PCa (f)	EBV-positive control (g) *vs* EBV-negative control (h)	P*	P+	P$	P^
CD44	a: 7.9 ± 5.1 b: 4.03 ± 2.4	c: 8.6 ± 4.7 d: 3.5 ± 2.6	e: 10.7 ± 4.7 f: 3.5 ± 1.6	g: 5.3 ± 2.2 h: 3.5 ± 3.5	0.0003	< 0.0001	< 0.0001	0.004
TWIST	a: 10.2 ± 12.2 b: 6.4 ± 7.2	c: 8.6 ± 10.5 d: 9.2 ± 11.3	e: 9.9 ± 11.9 f: 10.7 ± 12.8	g: 6.5 ± 7.4 h: 6.2 ± 7.01	ns	ns	ns	ns
E-cad	a: 12.9 ± 8.4 b: 13.5 ± 5.6	c: 13.6 ± 7.7 d: 12.5 ± 7.3	e: 13.3 ± 8.6 f: 12.4 ± 8.3	g: 14.1 ± 6.2 h: 12.6 ± 4.7	ns	ns	ns	ns
N-cad	a: 10.7 ± 11.6 b: 5.8 ± 8.4	c: 8.6 ± 10.5 d: 9.5 ± 11.4	e: 10.2 ± 11.1 f: 11.4 ± 12.1	g: 6 ± 8.8 h: 5.6 ± 8.1	0.009	ns	ns	ns
PTPN13	a: 9.7 ± 5.9 b: 12.9 ± 6.6	c: 10.8 ± 6.4 d: 11.03 ± 6.3	e: 10.09 ± 6.22 f: 9.3 ± 5.7	g: 12 ± 6.7 h: 14.5 ± 6.3	0.03	ns	ns	ns
SLUG	a: 2.1 ± 1.8 b: 1.1 ± 2.7	c: 1.8 ± 2.04 d: 1.9 ± 2.3	e: 2.05 ± 1.7 f: 2.6 ± 1.7	g: 1.6 ± 2.4 h: 0.2 ± 3.02	ns	ns	ns	ns

Geometric Mean ± Standard Deviation, *: comparison between group a versus group b, +: comparison between group c versus group d, $: comparison between group e versus group f, ^: $: comparison between group g versus group h, FDR correction for multiple comparisons by Benjamini-Hochberg method.

Table 5
Comparison of cellular factors levels between the groups positive and negative for HPV

Cellular Factors	PCa group (a) *vs* control (b)	HPV-positive samples (c) *vs* HPV-negative samples (d)	HPV-positive PCa (e) *vs* HPV-negative PCa (f)	HPV-positive control (g) *vs* HPV-negative control (h)	P*	P+	P$	P^
NF-κB	a:19.4 ± 7.4 b:9.5 ± 7	c: 23.5 ± 5.8 d: 13.2 ± 7.9	e: 24.2 ± 6 f: 17 ± 7	g: 20 ± 3.9 h: 8 ± 6.05	< 0.0001	< 0.0001	0.0009 1.42	0.004
TNF-α	a:17 ± 7.4 b:11.7 ± 6.7	c: 21.1 ± 6 d: 12.8 ± 6.6	e: 22.4 ± 6.4 f: 14.3 ± 6.4	g: 19.5 ± 3.1 h: 10.6 ± 6.3	0.002	< 0.0001	< 0.0001 1.56	0.03
IL-1	a:13 ± 5.7 b:7.8 ± 5.3	c: 17.2 ± 4.1 d: 9.1 ± 5.2	e: 17.3 ± 4.1 f: 10.9 ± 5.1	g: 16.7 ± 4.2 h: 6.5 ± 4.1	0.0002	< 0.0001	0.0003 1.57	0.002
IL-6	a:14.4 ± 6 b:8.9 ± 6.6	c: 18.9 ± 5 d: 10.2 ± 5.8	e: 18.9 ± 5.2 f: 12.2 ± 5.2	g: 19 ± 3.9 h: 7.5 ± 5.6	0.0004	< 0.0001	< 0.0001 2.51	0.0004
IL-8	a: 12.62 ± 6.2 b: 5.68 ± 5.1	c: 17.3 ± 5.1 d:7.6 ± 5.3	e: 17.7 ± 5.4 f: 10.1 ± 5.09	g: 15.5 ± 3.1 h: 4.2 ± 3.5	< 0.0001	< 0.0001	0.004 1.77	0.006
IL-11	a: 15.6 ± 8.3 b: 7.1 ± 6.2	c: 20.52 ± 6.8 d: 9.8 ± 7.4	e: 21.2 ± 7.2 f: 12.8 ± 7.4	g: 17 ± 5.08 h: 5.7 ± 4.1	< 0.0001	< 0.0001	0.02 1.6	ns
VEGF	a: 38.64 ± 10.8 b: 13.7 ± 6.9	c: 48.5 ± 9.5 d: 25.8 ± 12.5	e: 52.58 ± 3.5 f: 35.8 ± 4.2	g: 29.5 ± 1.2 h: 11.9 ± 3.7	< 0.0001	< 0.0001	0.0009 1.46	0.005

Cellular Factors	PCa group (a) *vs* control (b)	HPV-positive samples (c) *vs* HPV-negative samples (d)	HPV-positive PCa (e) *vs* HPV-negative PCa (f)	HPV-positive control (g) *vs* HPV-negative control (h)	P*	P+	P$	P^
TGF-β	a: 15.2 ± 7.6 b: 7.5 ± 5.1	c: 19.2 ± 5.7 d: 10.1 ± 7	e: 19.9 ± 6.1 f: 12.9 ± 7.2	g: 16 ± 1.6 h: 6.2 ± 4.2	< 0.0001	< 0.0001	0.02 1.54	ns
Rb	a: 9.1 ± 5.7 b: 14.06 ± 6.4	c: 2.4 ± 1.08 d: 13.8 ± 4.6	e: 2.6 ± 1.01 f: 12.3 ± 4.1	g: 1.5 ± 1 h: 15.8 ± 4.6	0.0006	< 0.0001	< 0.0001	0.0003
P53	a: 10.4 ± 6.7 b: 13.9 ± 6.6	c: 3 ± 1.3 d:14.7 ± 5.2	e: 3.2 ± 1.35 f: 14.03 ± 5.2	g: 2 ± 0.8 h: 15.68 ± 5.09	0.04	< 0.0001	< 0.0001	0.002
ROS	a: 10.2 ± 9.7 b: 6.09 ± 5.3	c: 22.3 ± 5.7 d: 3.7 ± 2.7	e: 23.4 ± 5.5 f: 3.1 ± 2.04	g: 17 ± 3.6 h: 4.5 ± 3.3	0.03	< 0.0001	< 0.0001 7.5	ns
RNS	a: 11.8 ± 10.3 b: 5.2 ± 5.3	c: 19.7 ± 8.9 d: 5.9 ± 6.5	e: 20.2 ± 9.7 f: 7.7 ± 7.8	g: 17.2 ± 2.6 h: 3.5 ± 2.8	0.0002	< 0.0001	0.0004 2.6	0.003
Survivin	**a**: 17.8 ± 4.8 **b**: 9 ± 6.2	c: 22 ± 3.2 d: 12.1 ± 5.8	e: 21.9 ± 3.2 f: 15.8 ± 4.1	g: 22.7 ± 3.5 h: 7.03 ± 3.2	< 0.0001	< 0.0001	0.0006	< 0.0001
Bcl-2	**a**: 12.1 ± 4.2 **b**: 6.4 ± 4.5	c: 15.9 ± 3.4 d: 8.1 ± 3.9	e: 15.6 ± 3.7 f: 10.4 ± 3.4	g: 17.3 ± 0.8 h: 4.9 ± 1.9	0/0001	< 0.0001	0.002	< 0.0001

Cellular Factors	PCa group (a) *vs* control (b)	HPV-positive samples (c) *vs* HPV-negative samples (d)	HPV-positive PCa (e) *vs* HPV-negative PCa (f)	HPV-positive control (g) *vs* HPV-negative control (h)	P*	P+	P$	P^
CD44	a: 7.9 ± 5.1 b: 4.03 ± 2.4	c: 7.8 ± 5.1 d: 6.1 ± 4.5	e:7.9 ± 5.4 f: 8 ± 5.01	g: 7.5 ± 3.8 h: 3.5 ± 1.8	0.0003	ns	ns	ns
TWIST	a: 10.2 ± 12.2 b: 6.4 ± 7.2	c: 25.7 ± 7.5 d: 3.1 ± 2.6	e: 26.2 ± 8.1 f: 2.4 ± 1.3	g: 23 ± 1.6 h: 4.03 ± 3.5	ns	< 0.0001	< 0.0001	ns
E-cad	a: 12.9 ± 8.4 b: 13.5 ± 5.6	c: 3.4 ± 2.08 d: 16.5 ± 5.6	e: 3.5 ± 2.2 f:17.5 ± 6.2	g: 3 ± 0.8 h: 15.07 ± 4.2	ns	< 0.0001	< 0.0001	< 0.0001
N-cad	a: 10.7 ± 11.6 b: 5.8 ± 8.4	c: 25.8 ± 7.6 d:3.2 ± 2.4	e: 25.7 ± 8.09 f: 3.4 ± 2.1	g: 26.2 ± 6.2 h: 2.9 ± 2.7	0.009	< 0.0001	< 0.0001	0.004
PTPN13	a: 9.7 ± 5.9 b: 12.9 ± 6.6	c: 4.4 ± 5.6 d: 13.1 ± 5	e: 5 ± 6.01 f:12.08 ± 4.4	g: 2 ± 0.8 h: 14.5 ± 5.5	0.03	< 0.0001	0.0007	0.004
SLUG	a: 2.1 ± 1.8 b: 1.1 ± 2.7	c: 3.8 ± 1.4 d: 1.01 ± 1.9	e: 3.9 ± 1.4 f: 1.2 ± 1.3	g: 3.4 ± 1.2 h: 0.6 ± 2.6	ns	< 0.0001	< 0.0001	Ns

Geometric Mean ± Standard Deviation, *: comparison between group a versus group b, +: comparison between group c versus group d, $: comparison between group e versus group f, ^: $: comparison between group g versus group h, FDR correction for multiple comparisons by Benjamini-Hochberg method.

Table 6
Comparison of cellular factors levels between the co-infection and mono-infection groups

Cellular Factors	Co-infected PCa (a) vs not co-infected PCa (b)	Co-infected Positive control (c) vs not co-infected control (d)	Co-infected PCa (a) vs HPV-mono-infected PCa (e)	Co-infected PCa (a) vs EBV-mono-infected PCa (f)	Co-infected PCa (a) and non-HPV/non-EBV PCa (g)
NF-κB	a: 26.9 ± 3.1	c: 21.6 ± 2.5	a: 26.9 ± 3.1	a: 26.9 ± 3.1	a: 26.9 ± 3.1
	b: 17.8 ± 7.1	d: 8.2 ± 6	e: 20.3 ± 7.2	f: 18.4 ± 3.2	g: 12 ± 8
	P^{*} (0.0004)	P^{+} (0.004)	$P^{\$}$ (ns)	P^ (ns)	$P^{\#}$ (0.0001)
TNF-α	a: 25.5 ± 2.7	c: 19.5 ± 3.1	a: 25.5 ± 2.7	a: 25.5 ± 2.7	a: 25.5 ± 2.7
	b: 15.2 ± 6.8	d: 10.6 ± 6.3	e: 19 ± 7.6	f: 16.2 ± 4.8	g: 10.2 ± 6.3
	P^{*} (0.001)	P^{+} (ns)	$P^{\$}$ (ns)	P^ (0.04)	$P^{\#}$ (0.0001)
IL-1	a: 18.4 ± 3.6	c: 18.3 ± 3.5	a: 18.4 ± 3.6	a: 18.4 ± 3.6	a: 18.4 ± 3.6
	b: 14.2 ± 4.4	d: 6.7 ± 4.2	e: 16.1 ± 4.6	f: 12.5 ± 4.2	g: 7.1 ± 4
	P^{*} (ns)	P^{+} (0.004)	$P^{\$}$ (ns)	P^ (ns)	$P^{\#}$ (0.0001)
IL-6	a: 21.3 ± 2.1	c: 19 ± 3.9	a: 21.3 ± 2.1	a: 21.3 ± 2.1	a: 21.3 ± 2.1
	b:12.2 ± 5.2	d: 7.5 ± 5.6	e: 16.3 ± 6.5	f: 13.3 ± 5.2	g: 8.2 ± 1.6
	P^{*} (0.004)	P^{+} (0.003)	$P^{\$}$ (ns)	P^ (ns)	$P^{\#}$ (0.001)
IL-8	a: 17.3 ± 4.9	c: 16.3 ± 3.2	a: 17.3 ± 4.9	a: 17.3 ± 4.9	a: 17.3 ± 4.9
	b: 11.6 ± 6.1	d: 4.5 ± 3.8	e: 18.2 ± 6.1	f: 4.8 ± 3.9	g: 9.1 ± 3.6
	P^{*} (ns)	P^{+} (0.009)	$P^{\$}$ (ns)	P^ (0.01)	$P^{\#}$ (ns)
IL-11	a: 21.8 ± 6.4	c: 17 ± 5	a: 21.8 ± 6.4	a: 21.8 ± 6.4	a: 21.8 ± 6.4
	b: 14.3 ± 8.1	d: 6.1 ± 5.4	e: 20.6 ± 8.2	f: 14.3 ± 8.1	g: 10.7 ± 5.9
	P^{*} (ns)	P^{+} (ns)	$P^{\$}$ (ns)	P^ (ns)	$P^{\#}$ (0.04)
IL-17	a: 15.2 ± 1.8	c: 13 ± 2	a: 15.2 ± 1.8	a: 15.2 ± 1.8	a: 15.2 ± 1.8
	b: 8.7 ± 3.3	d: 3.4 ± 2.7	e: 13.2 ± 2.4	f: 8 ± 2.7	g: 7.3 ± 2.4
	P^{*} (0.008)	P^{+} (0.002)	$P^{\$}$ (ns)	P^ (0.006)	$P^{\#}$ (0.003)
VEGF	a: 53.6 ± 3.8	c: 29.6 ± 1.5	a: 53.6 ± 3.8	a: 53.6 ± 3.8	a: 53.6 ± 3.8
	b: 38.7 ± 7.3	d: 12.5 ± 4.8	e: 51.44 ± 3	f: 35.8 ± 4.7	g: 35.8 ± 3.6
	P^{*} (0.04)	P^{+} (ns)	$P^{\$}$ (ns)	P^ (0.02)	$P^{\#}$ (0.02)

Cellular Factors	Co-infected PCa (a) vs not co-infected PCa (b)	Co-infected Positive control (c) vs not co-infected control (d)	Co-infected PCa (a) vs HPV-mono-infected PCa (e)	Co-infected PCa (a) vs EBV-mono-infected PCa (f)	Co-infected PCa (a) and non-HPV/non-EBV PCa (g)
TGF-β	a: 21.5 ± 4.5	c: 16 ± 2	a: 21.5 ± 4.5	a: 21.5 ± 4.5	a: 21.5 ± 4.5
	b: 13.9 ± 7.5	d: 6.6 ± 4.5	e: 18.2 ± 7.3	f: 13.3 ± 3.5	g: 11 ± 5.6
	P^{*} (ns)	P^{+} (ns)	$P^{\$}$ (ns)	P^ (0.03)	$P^{\#}$ (0.01)
Rb	a: 2.3 ± 1	c: 1.6 ± 1.1	a: 2.3 ± 1	a: 2.3 ± 1	a: 2.3 ± 1
	b: 10.5 ± 5.3	d: 15.3 ± 5.2	e: 2.7 ± 0.9	f: 12.4 ± 4.7	g: 12.3 ± 3.3
	P^{*} (< 0.0001)	P^{+} (< 0.0001)	$P^{\$}$ (ns)	P^ (< 0.0001)	$P^{\#}$ (< 0.0001)
P53	a: 2.6 ± 0.9	c: 2.3 ± 0.5	a: 2.6 ± 0.9	a: 2.6 ± 0.9	a: 2.6 ± 0.9
	b: 12.1 ± 6.2	d: 15.1 ± 5.7	e: 3.7 ± 1.4	f: 13.3 ± 5.4	g: 14.9 ± 5
	P^{*} (< 0.0001)	P^{+} (0.0006)	$P^{\$}$ (ns)	P^ (< 0.0001)	$P^{\#}$ (< 0.0001)
ROS	a: 24.5 ± 5.8	c: 15.6 ± 3	a: 24.5 ± 5.8	a: 24.5 ± 5.8	a: 24.5 ± 5.8
	b: 6.6 ± 8	d: 5.1 ± 4.4	e: 22.2 ± 5.3	f: 3.4 ± 1.9	g: 2.6 ± 2.1
	P^{*} (< 0.0001)	P^{+} (ns)	$P^{\$}$ (ns)	P^ (< 0.0001)	$P^{\#}$ (< 0.0001)
RNS	a: 20 ± 9.4	c: 17.3 ± 3.2	a: 20 ± 9.4	a: 20 ± 9.4	a: 20 ± 9.4
	b: 10.1 ± 9.7	d: 4 ± 3.7	e: 20.4 ± 10.6	f: 9.2 ± 9.3	g: 5.6 ± 4.3
	P^{*} (0.02)	P^{+} (0.01)	$P^{\$}$ (ns)	P^ (0.02)	$P^{\#}$ (0.004)
Survivin	a: 22.5 ± 1	c: 21.3 ± 0.5	a: 22.5 ± 1	a: 22.5 ± 1	a: 22.5 ± 1
	b: 13.7 ± 4.9	d: 7.4 ± 4	e: 19.5 ± 1.8	f: 17.2 ± 4.3	g: 12.3 ± 4.7
	P^{*} (< 0.0001)	P^{+} (< 0.0001)	$P^{\$}$ (ns)	P^ (ns)	$P^{\#}$ (< 0.0001)
Bcl-2	a: 14.1 ± 1.2	c: 15.4 ± 1.3	a: 14.1 ± 1.2	a: 14.1 ± 1.2	a: 14.1 ± 1.2
	b: 8.8 ± 3	d: 5 ± 2.6	e: 13.5 ± 1	f: 8 ± 2.1	g: 7.2 ± 1.9
	P^{*} (0.005)	P^{+} (0.0004)	$P^{\$}$ (ns)	P^ (0.005)	$P^{\#}$ (0.001)
CD44	a: 10.6 ± 5.4	c: 9.3 ± 1.5	a: 10.6 ± 5.4	a: 10.6 ± 5.4	a: 10.6 ± 5.4
	b: 7.2 ± 4.8	d: 3.5 ± 1.7	e: 3.7 ± 1.4	f: 10.8 ± 4.4	g: 3.9 ± 2
	P^{*} (ns)	P^{+} (ns)	$P^{\$}$ (0.03)	P^ (ns)	$P^{\#}$ (0.01)

Cellular Factors	Co-infected PCa (a) vs not co-infected PCa (b)	Co-infected Positive control (c) vs not co-infected control (d)	Co-infected PCa (a) vs HPV-mono-infected PCa (e)	Co-infected PCa (a) vs EBV-mono-infected PCa (f)	Co-infected PCa (a) and non-HPV/non-EBV PCa (g)
TWIST	a: 26.8 ± 6.7	c: 23 ± 2	a: 26.8 ± 6.7	a: 26.8 ± 6.7	a: 26.8 ± 6.7
	b: 6.8 ± 10.1	d: 4.6 ± 4.9	e: 25.6 ± 9.9	f: 2.5 ± 1.3	g: 2.3 ± 1.4
	P^{*} (< 0.0001)	P^{+} (0.009)	$P^{\$}$ (ns)	P^ (< 0.0001)	$P^{\#}$ (< 0.0001)
E-cad	a: 2.7 ± 0.6	c: 2.6 ± 0.5	a: 2.7 ± 0.6	a: 2.7 ± 0.6	a: 2.7 ± 0.6
	b: 15.1 ± 7.7	d: 14.6 ± 4.6	e: 4.5 ± 3	f: 18 ± 5.8	g: 16.8 ± 6.9
	P^{*} (< 0.0001)	P^{+} (0.007)	$P^{\$}$ (ns)	P^ (< 0.0001)	$P^{\#}$ (< 0.0001)
N-cad	a: 25.9 ± 6.8	c: 25.3 ± 7.3	a: 25.9 ± 6.8	a: 25.9 ± 6.8	a: 25.9 ± 6.8
	b: 7.6 ± 9.8	d: 3.8 ± 5.5	e: 25.6 ± 9.7	f: 3.4 ± 1.8	g: 3.4 ± 2.5
	P^{*} (0.001)	P^{+} (0.02)	$P^{\$}$ (ns)	P^ (0.0004)	$P^{\#}$ (0.0003)
PTPN13	a: 6.2 ± 7.6	c:2 ± 1	a: 6.2 ± 7.6	a: 6.2 ± 7.6	a: 6.2 ± 7.6
	b: 10.5 ± 5.3	d: 14.1 ± 5.8	e: 3.6 ± 3.6	f: 12.7 ± 4.7	g: 12.5 ± 4
	P^{*} (ns)	P^{+} (0.002)	$P^{\$}$ (ns)	P^ (0.03)	$P^{\#}$ (0.03)
SLUG	a: 3.5 ± 1.6	c: 3.2 ± 1.4	a: 3.5 ± 1.6	a: 3.5 ± 1.6	a: 3.5 ± 1.6
	b: 1.8 ± 1.8	d: 0.96 ± 2.8	e: 4.3 ± 1.1	f: 0.9 ± 1.4	g: 1 ± 1.1
	P^{*} (ns)	P^{+} (ns)	$P^{\$}$ (ns)	P^ (0.004)	$P^{\#}$ (0.01)

Geometric Mean ± Standard Deviation, *: comparison between group a versus group b, +: comparison between group c versus group d, $: comparison between group e versus group f, ^: $: comparison between group g versus group h, FDR correction for multiple comparisons by Benjamini-Hochberg method.

Table 7
Spearman's correlation coefficient between the cellular factors with the HPV and EBV genes

	LMP-1	LMP-2	EBER 1	EBER 2	E2	E6	E7
NF-κB	0.6****	0.43***	0.33**	0.31**	0.37**	0.64****	0.64****
TNF-α	0.49**	0.18 ns	0.19 ns	0.28*	0.271**	0.50***	0.504***
IL-1	0.501**	0.42**	0.34**	0.22*	0.40***	0.533***	0.529***
IL-6	0.66***	0.41**	0.11 ns	0.28*	0.309**	0.444***	0.435***
IL-8	0.54***	0.11 ns	0.07ns	0.08 ns	0.393**	0.603***	0.596***
IL-11	0.55***	0.3*	0.34*	0.136 ns	0.341***	0.504***	0.509***
IL-17	0.56***	0.41**	0.104 ns	0.09 ns	0.420***	0.726***	0.724***
VEGF	0.57****	0.39*	0.302*	0.07 ns	0.323**	0.595***	0.604***
TGF-β	0.51***	0.25*	0.103 ns	-0.08 ns	0.31**	0.55***	0.59****
Rb	-0.47***	0.39**	0.28**	0.22*	-0.553***	-0.751***	-0.751***
P53	-0.51***	-0.401*	-0.201*	-0.05 ns	-0.456***	-0.712***	-0.715***
Survivin	0.76****	0.39**	0.308**	0.17 ns	0.528***	0.757***	0.761***
Bcl-2	0.49***	0.39**	0.102 ns	-0.03 ns	0.556***	0.743***	0.743***
ROS	0.46**	0.29*	0.09 ns	0.105 ns	0.525***	0.763***	0.763***
RNS	0.301*	0.08 ns	0.02 ns	0.106 ns	0.460***	0.654***	0.652***
CD44	0.8****	0.68****	0.48***	0.58****	0.21*	0.34**	0.29**
TWIST	0.53****	0.44***	0.44***	0.045 ns	0/53**	0/83****	0/86****
E-cad	-0.5***	-0.2*	-0.11*	−0.096 ns	-0/47**	-0/71***	-0/71***
N-cad	0.51****	0.41**	0.42**	0.206 *	0/51**	0/85****	0/87****
PTPN13	-0.409***	-0.15 ns	-0.15 ns	−0.01 ns	-0/48**	-0/41*	-0/38*
SLUG	0.35*	0.05 ns	0.05 ns	0.10 ns	0/35*	0/46**	0/46**
ns: not significant, * p < 0.05; ** p < 0.01; *** p < 0.001, ****<0.0001							

Compared with the EBV-negative group, EBV-negative PCa group and EBV-negative control group, the expression level of VEGF, IL-6, IL-1, NF-κB and TNF-α was higher in EBV-positive control, EBV-positive PCa and EBV-positive groups, respectively (Table 4). As shown in Table 7, there was a strongest correlation between LMP-1 and IL-6 (R = 0.66, P = 0.001). Table 7 presents more details. Since some of the samples studied were both infected with the EBV and infected with the HPV (HPV/EBV coinfection), the question may now be whether the increased expression of inflammatory factors was the result of the effect of EBV-infection or the presence of HPV-infection and/or effect of HPV/EBV-co-infection? Therefore, as can be seen in Table 6, the level of inflammatory factors was compared between the co-infected and mono-infected samples. Some inflammatory factors (VEGF, IL-17, IL-6, TNF-α and NF-κB) had statistically higher expression level in the HPV/EBV co-infected PCa group when comparing with not co-infected PCa group. It is worth mentioning, not-coinfected PCa group including mono HPV-infected PCa samples, mono EBV-infected PCa samples, and non HPV and non EBV PCa samples. The results showed that all inflammatory factors had an increase in the mean expression level in the HPV/EBV co-infected PCa group when comparing with the mono HPV infected PCa group, but this increase was not significant. Although increase in mean expression was not statistically significant, can it be concluded that cause is due to a presence of EBV infection? In comparing inflammetory factors between HPV/EBV co-infection PCa group with mono EBV infected PCa group, it was observed that there was a significant higher mean expression level of TNF-α, IL-17, IL-8, RNS, ROS, TGF-β and VEGF in coinfected group than in the mono EBV infected group, but the two groups showed no significant difference in the mean expression level of IL-11, IL-6, IL-1 and NF-κB ($P > 0.05$). Therefore, it can be concluded that an elevated expression level of RNS, ROS, TGF-β, VEGF, TNF-α, IL-17 and IL-8 probably more due to the presence of HPV infection than to EBV infection and also an elevated expression level of IL-11, IL-6, IL-1 and NF-κB factors may be due to the simultaneous presence of HPV and EBV infections. In addition, the level of all inflammatory factors except IL-8 in HPV/EBV co-inffected PCa group was significantly higher than in non-HPV and non-EBV PCa group (Table 6).

3.5. Comparison of the expression pattern of Apoptosis-related, tumor suppressor, Anoikis-related factors and CD44 among the study groups

Following the identification of differences in the expression levels of inflammatory-related mediators between studies groups, we examined the association between the concentrations of antiapoptotic factors (survivin and Bcl-2), tumor suppressor factors (p53 and Rb), anoikis-related mediators (SLUG, PTPN13, N-cadherin, E-cadherin and TWIST) and CD44 (as a cell adhesion glycoprotein) in the samples infected with HPV, the samples infected with EBV, the samples coinfected with HPV/EBV, and control groups. In comparison with the control group, the p53 and Rb expression levels were significantly lower and also Bcl-2, Survivin and CD44 levels were higher significantly in the PCa group. Furthermore, among anoikis-related factors, only N-cad and PTPN13 levels significantly were higher and lower, respectively, in the PCa group when comparing with the control group (Table 4). Analysis of the results also showed no significant difference in the expression level of tumor suppressor proteins and anoikis-related factors between EBV-positive samples and EBV-negative samples. However, the concentrations of CD44, Survivin and Bcl-2 in the EBV-positive samples were significantly higher compared to the EBV-negative samples

(Table 4). As well, a significant positive correlation was observed between the EBV-LMP1 gene expression levels and the expression levels of CD44 ($r = 0.8$, $P < 0.0001$) and Survivin ($r = 0.76$, $P < 0.0001$) (Table 7). As seen in Table 5, there was a significant reduction and elevation in the concentration of tumor suppressor proteins (p53 and Rb) and the anti-apoptotic proteins (survivin and Bcl-2), respectively, in the HPV-positive groups in comparison with the HPV-negative groups. Also, the concentration of E-cad and PTPN13 proteins was significantly lower and the concentration of N-cad, SLUG, TWIST was significantly higher in the HPV-positive groups when comparing with the HPV-negative groups. In addition, no statistically significant difference was found in the expression pattern of CD44 between HPV-positive groups when comparing with the HPV-negative groups ($P > 0.05$). As shown in Table 7, there was a significantly positive correlation between the E7 and E6 level and Survivin (r:0.75, and 0.76, respectively, $P < 0.001$), Bcl-2 (r:0.74, and 0.74, respectively, $P < 0.001$), TWIST (r:0.83, and 0.86, respectively, $P < 0.0001$) and N-cad (r:0.85, and 0.87, respectively, $P < 0.0001$). Beside, there was a significantly negative correlation between E7 and E6 level and Rb (r:-0.75, and − 0.75, respectively, $P < 0.0001$), p53 (r:-0.71, and − 0.71, respectively, $P < 0.0001$), E-cad (r:-0.71, and − 0.71, respectively, $P < 0.0001$).

Based on correlation results in Table 6, significantly lower and higher expression levels were reported for the tumor suppressor proteins (p53 and Rb) and Bcl-2 in the HPV/EBV co-infection PCa group, respectively in compared to the mono EBV positive PCa group, and the non-HPV/non-EBV PCa group.As well, the mean expression level of these factors between HPV/EBV co-infected PCa and mono HPV-infected PCa groups are different which it probably due to the presence of the EBV infection and it can contribute to the progression of prostate cancer by modulation of cellular factors.In comparing Survivin between HPV/EBV co-infection PCa group with mono HPV- and mono-EBV-infected PCa group, it was observed that the mean expression level of survivin had no significant difference between the these groups ($P > 0.05$). Therefore, it can be concluded which high concentration of Survivin in coinfected PCa group maybe can due to the simultaneous presence of HPV and EBV infections.

Expression level of CD44 in PCa and EBV-positive groups were significantly higher than control and EBV-negative groups, respectively (Table 4). As shown in Table 6, the mean CD44 expression level was significantly different only between the two groups (HPV/EBV coinfection PCa group versus mono HPV-positive PCa group), but not between coinfection PCa group and mono EBV-positive PCa group. Thus, the increase in mean expression level of CD44 in the coinfection group is most likely due to the presence of EBV virus.

According to the results of Table 4, among anoikis-related factors, the expression level of N-cad and PTPN13 in the PCa group is significantly different from the control. Also, no significant difference was seen in the mean expression level of anoikis-related factors between EBV-positive and EBV-negative samples (Table 4). Conversely, a significant difference was reported between the mean expression levels of all of these factors in HPV-positive compared to HPV-negative samples (Table 5). The mean expression level of TWIST, N-cad and SLUG in HPV/EBV co-infected PCa group was higher compared to EBV/HPV negative PCa, mono EBV-infected PCa, non HPV/non EBV PCa groups. As shown in Table 7, a significant negative correlation was seen between the expression level of E-cad with E7 and E6 (r=-0/71,

P < 0.0001), and also between E7 and E6 levels with the TWIST (r = 0/83, r = 0/86, respectively, P < 0/0001) and with the N-cad (r = 0/85, r = 0/87, respectively, P < 0/0001). More information is given in Table 7.

In compared HPV/EBV coinfected PCa group with coinfection negative PCa, mono EBV-positive PCa and non-HPV/non-EBV PCa groups, the E-cad level and the TWIST and N-cad levels had significantly decreased and increased expression, respectively (Table 6). However, no significant difference was found in the mean expression level of Anoikis-related factors between the mono HPV positive PCa group and the co-infection positive PCa group. In conclusion, the HPV infection is probably the cause of these changes and HPV plays important role in contribute to anokis resistance than the EBV virus.

4. Discussion

The present research tried to find the differences among EBV-positive samples, HPV-positive samples, HPV/EBV coinfected samples, and controls concerning the expression levels of EBV and HPV genes, the physical status of HPV integration, and the expression levels of some inflammatory, tumor suppressor, anti-apoptotic and anoikis related mediators including IL-17, IL-11, IL-6, IL-1, IL-8, TNF-α, NF-κB, VEGF, TGF-β, ROS, and RNS, Rb, p53, Survivin, Bcl-2, CD44, TWIST, E-cad, N-cad, PTPN13, and SLUG.

The viral infections globally accounts for about 12% of cancers, predominantly (> 85%) in developing countries [11, 12]. The HPV and EBV are reportedly associated with 38% of all cancers linked with viruses [39]. Evidence has proved an associstion between the HPV infection and the cervical cancer prognosis and carcinogenesis. There is also reportedly a strong association between the HPV infection and other cancers as an etiologic factor [40]; as well, studies reported the presence of EBV in the cervical cancer [41] and the prostate cancer [26]. In addition, the EBV/HPV co-infection has also been detected in the breast cancer [42, 43]. In our study, the HPV and EBV were isolated from in 31.3% and 49.3% of the PCa group, as well as in 15% and 40% of the control group, respectively. The EBV/HPV co-infection was present in 14.9% of PCa patients, 7.5% of control samples. Moreover, the high-risk strains (HPV 16 and 18) were responsible for 50% and 30% of HPV/EBV co-infected PCa samples, respectively. Also, the most common EBV genotype in the study was genotype 2. The EBV infection has been reported in the prostate tissues samples. The presence of the EBV was reported in 8.8% of malignant and benign prostate tissue samples (31 of 352) in Sweden [24], and in 8% of malignant, benign and normal prostate tissues (16 of 200) in the United States [25]. A study reported the EBV infection in about 37% of prostate cancer patients (n = 19) [23]. HPV-18 and EBV (EBNA1) gene sequences were detected in high proportions and almost equal to normal, benign and prostate cancer samples [26]. The EBV/HPV co-infection was significantly more prevalent in prostate cancer (55%) when comparing with benign samples (15%) and normal prostate samples (30%) [26]. Moreover, the EBV and HPV act simultaneously to enhance the cultured cervical cell proliferation, indicating that the same may happens to prostate epithelial cells [26, 44], in line with the present experiments about the high-risk HPVs and about the EBV in PCa.

The HPV genome integration is the key genetic phenomenon in the cervical carcinogenesis. Some studies revealed that the EBV infection accelerates the HPV genome integration into cervical cell genome in the cervix, which increases their genomic instability [18, 45] and develops lymphoepithelioma-like cervical carcinoma in some cases [41]. In line with this hypothesis, our results showed the maximum HPV genome integration rate was detected in the HPV/EBV co-infected PCa group (8/10, 80%). Also, the purely integrated HPV (47.4%) was significantly higher than the purely episomal HPV (5.2%) in the tumor tissues positive for the HPV. The purely integrated HPV was significantly higher in HPV/EBV co-infected PCa samples compared to the mono HPV-infected PCa samples (P: 0.0009).

Since no studies have been performed on the effect of the HPV/EBV coinfection on PCa development, we compared inevitably our findings with the results obtained from other cancers. The EBV acts probably as a co-factor for the HPV to induce the pathology of uterine cervix, verified by Szkaradkiewicz et al. [46] who reported a possible sexually EBV transmission route. Also, It has been found that sexually transmissible infections have been associated with increased risks of PCa [47]. However, there is no convincing evidence to support the hypothesis that EBV or HPV infection plays a direct role in the pathogenesis of prostate cancer. Additionally, a recent meta-analysis by de Lima et al. shows that EBV infection posed a two-fold increased risk of precancerous cervical lesions and four-fold increased risk of cervical cancer in HPV positive women [45]. However, in the current study, there were no significant associations between the presence of EBV, HPV, and HPV/EBV co-infection with PCa ($P = 0.35$, $P = 0.06$, and $P = 0.34$, respectively).

There are limited empirical techniques to analyze the molecular pathways regarding the EBV/HPV coinfection. However, EBV LMP1 mixed with HPV16 E6 proteins in the transformed mouse embryonic fibroblasts (MEFs) caused a decrease in the residues of DNA Damage Response (DDR), including p27, pRb and p53, as well as an elevation in the levels of checkpoint kinase 1 (CHK1), Akt and MAPK signaling and NF-κB signaling [48]. Similarly, as mentioned in result 3.5 section, mean expression level of p53 and Rb in co-infecction group were lower than mono HPV- and mono EBV-infected groups but was not significantly different between co-infected group with mono HPV-infected group, while, there was a significant between coinfected group with mono EBV-infected group. Besides, there was no significant difference between EBV-positive samples with EBV-negative specimens. Additionally, a significant reverse correlation was found between the HPV E7 and E6 mRNA expression levels with those of p53 and Rb, respectively (Table 7). Therefore, it can be concluded which lower concentration of Rb and p53 in coinfected PCa group probably more due to the simultaneous presence of HPV and EBV infections but maybe HPV infection effect was more than EBV infection.

One of the important defense strategies of innate immunity against pathogens is inflammation that helps to heal damaged tissue [49]. However, inflammation during pathogenesis can play a dual role. Prolonged chronic inflammation promotes tissue damage and autoimmune disorders, in addition to can lead to the accumulation of damage to DNA and give rise to cancers [50, 51]. Chronic or recurrent inflammation can be developed following the persistent viral infection. The pivotal pathogenic mechanism in the development of cancer caused by EBV and HPV is to induce the cytokine impact and the chronic

inflammation [14] but the function of HPV/EBV co-infection in PCa is unknown. In addition, the NF-κB has a key role in coordinating the expression of genes related to the chronic inflammatory diseases [52]. A wide variety of factors such as viral infection, oxidative stresses, cytokines (TNF-α, IL-1β), and tumor promoters can activate NF-κB. Also, NF-κB modulates expression of the various genes including cytokines (e.g. IL-1, 6, 8, TNF) and cell cycle regulatory molecules [49, 52]. A crosstalk between inflammation and cancer mediates by NF-kB thoroughly accepted [49]. It has been observed that an elevated EBER1 level in cervical cells may play a role in the transition from the inflammation to the oncogenesis of cervical cancer caused by HPV through the modulation of innate immunity [53]. The results of the current study showed that all inflammatory factors had significantly higher expression level in HPV-positive specimens when comparing with the HPV-negative ones (Table 5, result 3.4 section), while the concentration of VEGF, IL-11, IL-6, IL-1 and NF-kB was significantly elevated in the EBV-positive specimens in comparison with the EBV-negative ones (Table 6). There was also a significant association among the expression levels of LMP-1, E7 and E6 with NF-kB as well as between IL-6 and LMP-1 (Table 7). These results indicate that a possible association between inflammation with HPV and EBV in PCa. The level of NF-κB, TNF-α, IL-6, IL-17, VEGF, ROS and RNS in HPV/EBV co-infected PCa samples was higher than non-coinfected PCa samples. Besides, the mean expression level of TNF-α, IL-8, RNS, ROS, TGF-β, VEGF and IL-17 in co-infection positive PCa samples was significantly higher than mono EBV positive samples. Also, the inflammatory factors had greater mean expression level in the HPV/EBV co-infection group when comparing with mono HPV and mono EBV-infected groups but no significant difference was reported between coinfection and mono HPV groups. Therefore, by comparing the mean expression level of inflammatory factors between co-infection and mono-infection (Table 6, result 3.4 section) it can be concluded that HPV/EBV coinfection can increase the mean expression of inflammatory factors compared to mono HPV or mono EBV infection.

In a study by Grace et al., a significantly positive correlation was observed between the HPV-induced SCC and the expression levels of Bcl2/p53 proteins [54], confirming that the HPV could develop the cervical lesions by deregulating the induction of apoptosis [54]. In fact, it can be said that the high-risk HPV-E6 oncoprotein is able to enhance the Bcl-2 protein expression in the cervical cancer through the elimination of inhibitory activity of p53 over the Bcl-2 [55]. Survivin and Bcl-2 (as anti-apoptotic proteins), which widely expressed in most tumor tissues, are critical factors to regulate the progression of cell cycle and to impede the apoptosis [56]. In a study by Guo et al., the p53-induced survivin upregulation is promoted by LMP1 through an elevation activity of survivin promoter and activity of p53-survivin DNA binding, which means that the complexity of p53 regulation in Survivin occurs via the viral oncoprotein of LMP1 in NPC. Their model of p53-induced G1/S cell cycle progression could upregulate the LMP1-mediated expression of survivin in the pathogenesis of nasopharyngeal carcinoma [57]. Moreover, an LMP1-induced upregulation of Bcl-2 has been reported in B cells [58], while LMP1 has no effect on Bcl-2 levels in the leukemic T cell line Jurkat [59]. In a study of Muzio et al., the evaluation of survivin expression, oral premalignant lesions and oral carcinoma in the presence of HPV infection showed significantly higher expression level of survivin in the samples positive for HPV when comparing with those negative for HPV. The evidence suggested that the expression level of survivin may be influenced directly or indirectly by

the HPV [60]. In this study, it was observed that the Survivin and Bcl-2 expression level in the HPV-infected, EBV-infected and HPV/EBV-coinfected samples were significantly higher than in HPV-negative, EBV-negative and non-HPV/EBV-coinfected samples, respectively. Additionally, in the current study, there was a direct association between the E6 and E7 expression level and the Survivin and Bcl-2 expression level as well as between the expression levels of LMP-1 and Survivin. The EBV/HPV co-infection may initiate a neoplastic transformation of carcinogenesis [22]. Moreover, an in vivo interaction occurs between HPV and EBV as well as an interaction is there between EBV and HPV oncoproteins [44]. According to result of this study, the Survivin and Bcl-2 expression level significantly was higher in HPV/EBV coinfected PCa group than in non coinfected PCa group as well as there was not significantly different between mean expression level of Survivin in HPV/EBV co-infected PC with mono HPV- and mono EBV-infected PCa samples. In general, these results suggest that the co-presence of HPV and EBV infection may lead to the resistance of cancer prostate cells to apoptosis, although probably the effect of the HPV infection is greater than that of EBV.

Metastasis refers to the removal of tumor cells from their original location and then their dissemination to and proliferation in distant locations [61]. To this end, epithelial cancer cells must achieve a flexible migratory phenotype. In this regard, the epithelial-mesenchymal transition acts as a developmental pathway for tumor cells to reach this phenotype [62]. One of the physiological barriers for the tumor metastasis is anoikis, but most tumor cells become resistant to the anoikis [63].

According to the findings, the Slug and Twist (EMT- inducing transcription factors) are responsible for the anoikis resistance, in contrast, E-cadherin significantly inhibits anoikis resistance [63]. The CD44 as a cell membrane receptor with multifunctional potential is highly expressed in many cancers such as PCa and accounts for cell-cell adhesion and tumor metastasis and invasion [64]. The tumor aggression may be inhibited by the PTPN13 phosphatase. The invasion of different cancer cells is suppressed by the PTPN13 [65]. In a study by Castilla et al., the PTPN13 gene silencing elevated the expression level of invasion-related genes in the PCa cells [66]. The HPV-E6, by losing the PTPN13, triggers the anchorage-independent growth in the human epithelial cells [67, 68]. Accordingly, the present study aimed to analyze the expression levels of SLUG, Twist, N-cadherin, E-cadherin and PTPN13 proteins were in prostate tissue lysates. As mentioned in result 3.5, the mean expression level of N-cad, SLUG and TWIST were significantly higher in the cases positive for HPV compared to that in the groups negative for HPV, while the PTPN13 and E-cad level in the HPV-positive PCa group was significantly downregulated compared with HPV-negative PCa group (Table 5). Based on the findings, the E6 and E7 expression levels had a significant negative and positive correlation with E-cadherin and N-cadherin/TWIST/SLUG, respectively (Table 7). It should be noted that no significant difference in the concentrations of PTPN13, TWIST, SLUG, N-cad and E-cad was found between EBV-positive and EBV-negative groups (Table 4). However, the mean CD44 expression level was significantly greater in EBV-positive samples than in EBV-negative ones and there was a positive correlation between the LMP-1 and LPM-2 expression level and the CD44 expression level (Tables 4 and 7) but no significant difference was seen between the HPV-positive and HPV-negative groups. It has been reported that the LMP1 down-regulates the E-cadherin expression and up-regulates TWIST and other transcription factors linked to the cell motility [69, 70]. The EBV-protein, LMP-1, leads to

anoikis resistance by inducing the expression of anti-apoptosis proteins survivin, CD44, the inhibitor of DNA binding 1 (Id1), Bim and ROS [63, 71]. Moreover, the co-expression of E6 and LMP1 triggers some processes, including tumor-formation ability, anchorage-independent growth, resistance to apoptosis and cell proliferation, in nude mice when comparing with the expression of E6 or EBNA1 alone [72]. The LMP1 and HR-HPV E6 co-expression is associated with more aggressive malignant tumors, including cervical SCC and breast adenocarcinoma [73, 74]. In current study, the level of TWIST, and N-cad in HPV/EBV-coinfected PCa group compared to non-coinfected PCa group were increased, while the E-cad had significantly decreased expression level. However, no significant difference was reported in the mean expression level of anoikis-related factors between the PCa group positive for co-infection and the PCa group positive for mono HPV. As well as, the mean level of CD44 expression was not significant differences between HPV/EBV coinfection PCa group vs mono EBV-positive PCa group. Thus, the increase in mean expression level of CD44 in the coinfection group is most likely due to the presence of EBV virus. In conclusion, the existence of the HPV and EBV is probably the cause of these changes and viral infection plays important role in contribute to anoikis resistance and PCa development.

One of the limitations of this study was we had to sample a peripheral area of surgically dissected benign prostatic hyperplasia because of lacking access to normal or healthy prostate samples.

5. Conclusion

According to the results from the present study, the HPV/EBV coinfection was present in 14.9% of PCa samples and that the high-risk strains (HPV 16 and 18) were responsible for 50% and 30% of 10 PCa samples co-infected with HPV/EBV, respectively. The maximum percentage of HPV genome integration was found in HPV/EBV coinfected PCa group (8/10, 80%). In current study, although there were not significant association between the HPV/EBV co-infection with PCa, the expression pattern of some cellular factors involved in inflammation, tumor progression and metastasis were different between PCa infected with virus and between HPV/EBV co-infected PCa samples with mono EBV or mono HPV infection. These differences may mean that the simultaneous presence of these viruses has altered the pattern of expression of cellular factors compared to mono infection (Fig. 2), suggesting the HPV/EBV co-infection as a contributing factor in the development of PCa, in addition to the fact that the EBV has a role in the HPV genome integration.

List Of Abbreviations

PCa: Prostate Cancer

HPV: Human papilloma virus

EBV: Epstein-Barr Virus

EBER-1: Epstein-Barr Virus-Encoded RNA-1

EBER-2: Epstein-Barr Virus-Encoded RNA-2

LMP-1: Latent Membrane Proteins-1

LMP-2: Latent Membrane Proteins-2

OR: Odds ratio

qRT-PCR: quantitative RT-PCR

RT-PCR: Real-time PCR

Bcl-2: B-cell lymphoma 2

ECM: Extracellular matrix

IL: Interleukin

ELISA: Enzyme-linked immunosorbent assay

NF-κB: Nuclear factor-kB

ROS: reactive oxygen species

TNF-α: Tumor necrosis factor α

TGF-β: Transforming growth factor β

VEGF: Vascular endothelial growth factor

RB: Retinoblastoma

PTPN13: protein tyrosine phosphatase non-receptor type 13

Declarations

Ethics approval and consent to participate

The study was approved by ethical committee of the Iran University of Medical Sciences (IUMS), Tehran, Iran, under the Ethics code of IR.IUMS.REC.1398.642.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study could become available through the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

The current study was supported by grant no, 15671 from the research deputy of Iran University of Medical Sciences (IUMS).

Authors' contributions

J.N. wrote the main manuscript text and Investigation. Kh.Kh. Investigation and Methodology. H.M. Conceptualization and Software. M.M. Conceptualization and Software H.B. Conceptualization, Writing-Reviewing, and Editing, M.E. Conceptualization, Writing manuscript draft preparation. A.KH. Methodology, and Data curation, M.F. Methodology, and Data curation. F.S. Conceptualization, Investigation, Writing-Reviewing, and Editing. All authors approved the final version for submission.

Acknowledgements

Not applicable

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Figures

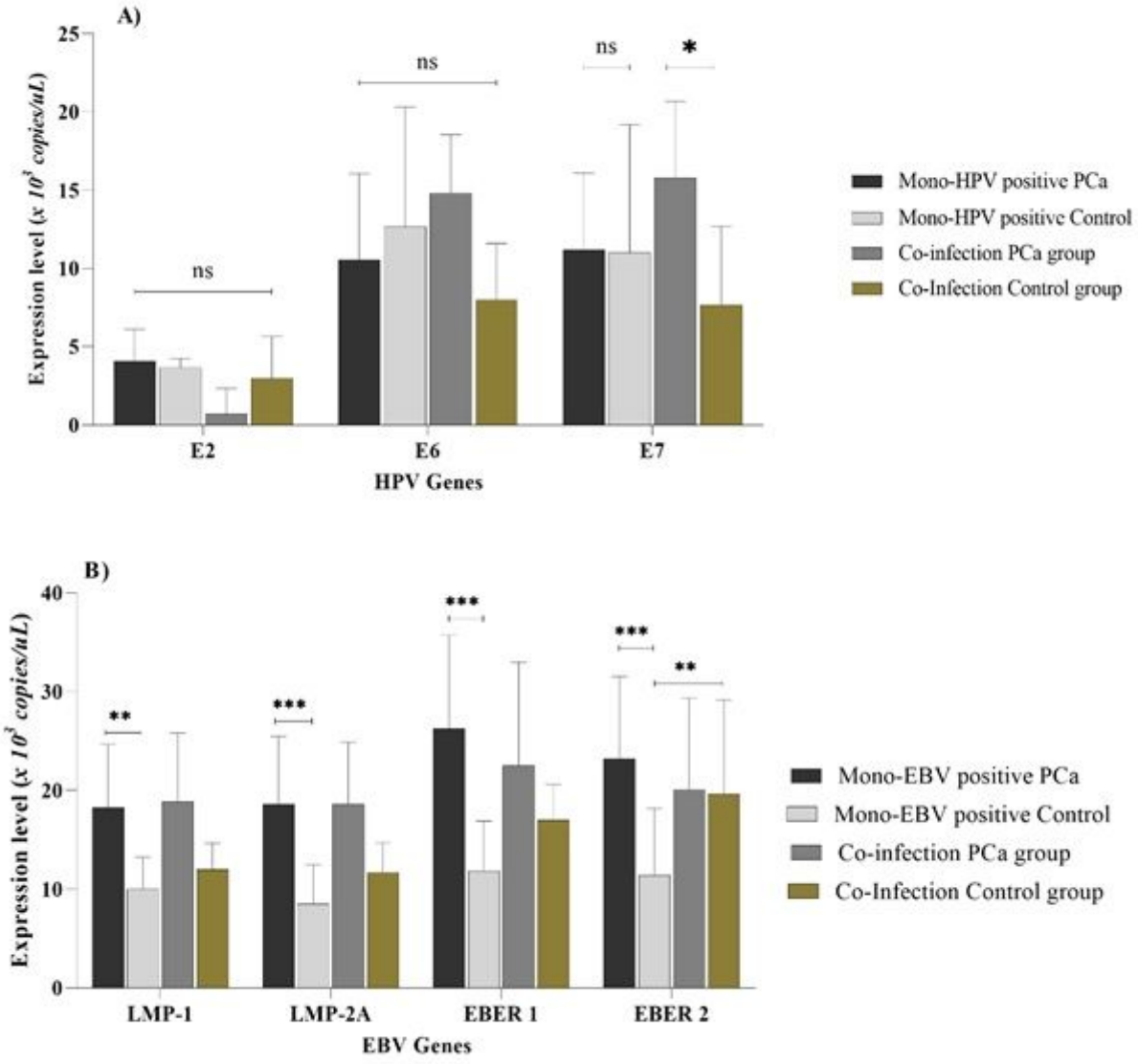

Figure 1

Differences in the expression level of viral genes in the two mono-infection and co-infection groups. NS: not significant at the level of 0.05. (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

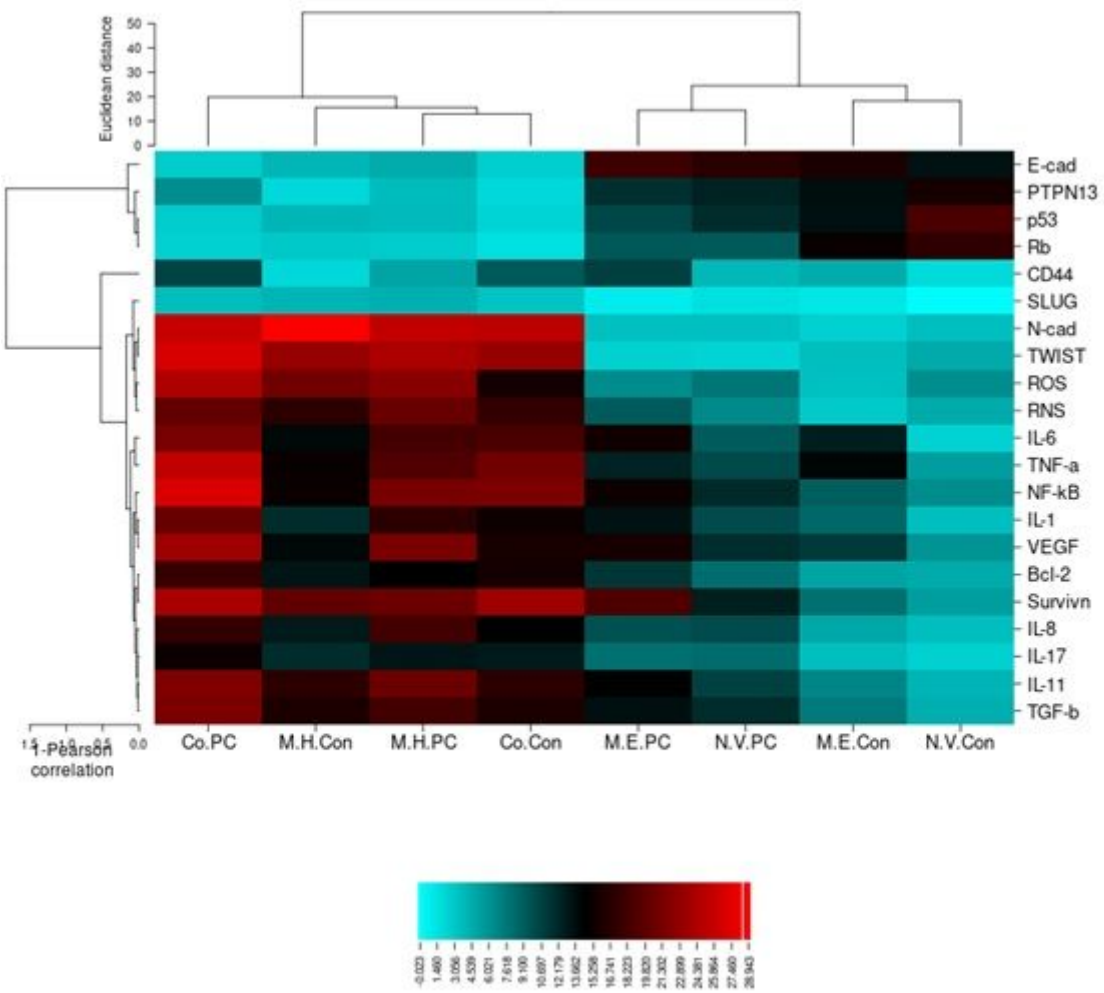

Figure 2

Hierarchical clustering of the differentially expressed Proteins and mRNAs between the studied groups. (M.H.Con: mono HPV-infected control samples, Co.PC: HPV/EBV-Coinfected PCa, M.H.PC: mono HPV-infected PCa samples, Co.Con: HPV/EBV coinfected control samples, M.E.PCa: mono EBV-infected PCa samples, N.V.Con: non-HPV/non-EBV control samples, N.V.PC: non-HPV/non-EBV PCa samples, and M.E.Con: mono EBV-infected control samples).The mean expression level of cellular factors in different groups, indicates that the expression pattern of cellular factors in Co-infected groups and mono-HPV samples is similar.