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Epstein-Barr Virus Nuclear Antigen 1 Increases the Expression of Viral Oncogenes and Cellular Genes in the HeLa Cell Line

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ABSTRACT

Epstein-Barr virus (EBV) represents one of the most important viral carcinogens. EBV nuclear antigen-1 (EBNA1) can induce the expression of different cellular and viral genes. In this study, we evaluated the EBNA1 effects on the expression patterns of human papillomavirus type 18 (HPV-18) *E6* and *E7* oncogenes and three cellular genes, including *BIRC5*, *c-MYC*, and *STMN1*, in a cervical adenocarcinoma cell line. HeLa cells were divided into three groups: one transfected with a plasmid containing the *EBNA1* gene, one transfected with a control plasmid, and one without transfection. In all three groups, the expression levels of *E6*, *E7*, *BIRC5*, *c-MYC*, and *STMN1* genes were checked using real-time PCR. Pathological staining was used to examine changes in cell morphology. Real-time PCR results showed that the expression level of HPV-18 *E6* (P=0.02) and *E7* (P=0.02) oncogenes significantly increased in HeLa cells transfected with the EBNA1 plasmid compared to cells transfected with control plasmid. Also, the presence of EBNA1 induced the expression of *BIRC5* and *c-MYC*, which increased tenfold (P=0.03) and threefold (P=0.02), respectively. Regarding the *STMN1* cellular gene, although the expression level in HeLa cells transfected with EBNA1 plasmid showed a twofold increase, this change was insignificant (P=0.11). Also, EBNA1 expression caused the creation of large HeLa cells with abundant cytoplasm and numerous nuclei. The EBV-EBNA1 could increase the expression levels of HPV-18 *E6* and *E7* viral oncogenes as well as *c-MYC* and *BIRC5* cellular genes in the HeLa cell line. These findings indicate that the simultaneous infection of cervical cells with HPV-18 and EBV might accelerate the progression of cervical cancer.

Keywords: Cervical carcinoma, human papillomavirus, Epstein–Barr Virus, EBNA1

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Introduction

Cancer is a global problem, and viruses have long been known as a strong risk factor for different types of cancer. About 15-20% of all cancer cases are related to viruses, including Epstein-Barr virus (EBV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), Human Herpes virus type 8 (HHV8), Human T-cell lymphotropic virus type 1 (HTLV-1), Human Papillomavirus (HPV), and Merkel cell polyomavirus (MCPyV) (1).

Cervical carcinoma (CC) is the fourth most common cancer among women after breast, lung, and colon cancers, which accounts for 1.6% of cancer deaths in women (2). Economic-social and health status, number of births, type of births, race, circumcision factor in men, age of first sexual contact, smoking, use of birth control pills, and age of first childbirth are the risk factors for CC (3). Among these factors, the most important cause of CC is infection with high risk types of HPVs (HR-HPVs) (4). In terms of virology, HPV belongs to the *Papillomaviridae* family, which includes small, non-enveloped viruses, and their double-stranded circular DNA genome is between 5748 - 8607 bp long (5, 6). Most of the HPVs infecting the cervix and anogenital areas are high-risk types, especially two types, 16 and 18, which have a high ability to cause CC (7). Among the eight non-structural proteins of this virus, the role of E6 and E7 in causing CC has been well proven (5). E6 protein can bind to tumor suppressor protein P53 and destroy it by forming a complex with cell ubiquitinating enzyme E6AP, so the proliferation of infected cell increases and the cell moves towards becoming cancerous (5). E7 protein binds to the cell transcription factor E2F and causes its release from retinoblastoma (RB), resulting in transcription of genes related to the proliferative phase of the cell cycle and cell division (5).

Recently, many clinical studies have acknowledged the role of EBV/HPV co-infection in the development of cancers related to HR-HPVs, especially CC (8, 9). EBV can easily infect cervical epithelial origin cells that are even infected with HPVs due to the abundance of EBV/C3d receptors on the surface of uterine cells (10-12). It is important to note that as the severity of an HPV-infected epithelial cell lesion increases due to further integration of the HPV genome in the host's chromosome, the conditions for EBV entry into such a cell improve (13, 14). Also, EBV infection in an HPV-derived cells can have a wide range of effects on cell signaling pathways, hastening the development of CC (11, 12).

EBV is classified as an oncogenic double-stranded DNA virus (length of roughly 170 kb) (12). It belongs to the family of *herpesviridae* and is found in more than 90% of adult humans, the majority of whom do not exhibit any symptoms (15). Saliva and genital secretions are the primary means of transmission for EBV (12, 16). EBV nuclear antigen-1 (EBNA1) is the sole viral protein present in all EBV latency types (17). EBNA1 has a regulatory role in the transcription of both viral and cellular promoters (17). The promoters of the genes whose transcription is controlled by EBNA1 are among the DNA sequences in the cellular genome that this protein can bind to as a viral transcription factor (18, 19).

The first cellular gene investigated in this study was *BIRC5*. The product of this gene inhibits cell apoptosis by interfering with the function of caspases. So the increase in the expression of this gene has been reported in many malignancies (20, 21). MYC, as a transcription factor, has a significant impact on a number of biological functions, including cell growth, proliferation, apoptosis, and cellular metabolism (22). Stathmin-1 (also known as oncoprotein 18) is a 17-kDa protein that plays an important role in cytoskeleton

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regulation (23). Numerous cellular processes, such as cytoplasmic organization, cell division, and cell motility, depend on the cytoskeleton, which serves as a scaffold (24).

Our understanding of cancer and its molecular underpinnings can be improved by identifying host-virus interactions, which can also help us in the discovery of prognostic indicators, novel antiviral therapies, and therapeutic interventions. In this study, we aimed to evaluate the EBV-EBNA1 effects on the expression patterns of HPV-18 *E6* and *E7* oncogenes as well as three cellular genes, including *BIRC5*, *c-MYC*, and *STMN1* in a cervical adenocarcinoma cell line.

Materials and methods

Plasmids, bacterial transformation, and plasmid extraction

In this study, the Invitrogen pCEP4 plasmid (an EBV-based plasmid containing the *EBNA1* gene and the hygromycin B resistance gene) as well as pcDNA3.1/hygro+ as control plasmid that contains the hygromycin B resistance gene but lacked the *EBNA1* gene were used. The plasmids were transformed into *Escherichia coli* (the *Top10* strain) and multiplied. To confirm the presence of the *EBNA1* gene in the plasmid, enzyme digestion and colony PCR methods were used. Then, these plasmids were extracted, and their quality and concentrations were determined using gel electrophoresis and spectrophotometry.

Cell culture, transfection, and clonal selection by hygromycin B

HeLa cells (cervical adenocarcinoma cell line containing HPV-18) were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS) at 37°C and 5% CO2 in a six-well plate. After reaching the cell confluency of about 70%, one group of cells was transfected with the pCEP4 plasmid (containing the *EBNA1* gene) and the other group with the control plasmid (lacking the *EBNA1* gene) using an optimized concentration of DNA fectamine (BioBasic, Canada). All experiments were performed in duplicate, i.e. we have two well for each group (two well for EBNA1 transfected group, two well for control plasmid transfected group). After 24 hours of transfection, both EBNA1- and control-plasmid transfected cell groups were treated with 350 µg/mL hygromycin B to select EBNA1 transfected cells (cells with stable EBNA1 expression) and cells containing control plasmid. These cell groups were cultured for 16 days in the presence of hygromycin B during several passages to achieve 100% selection.

RNA isolation and cDNA synthesis

An RNA Isolation Kit (Dena Zist, Iran) was used to extract the total RNAs. To assess the quality and quantity of the extracted RNAs, gel electrophoresis and spectrophotometry (Nanodrop™ Spectrophotometer, Thermo Scientific, USA) were used. Following the manufacturer's instructions, isolated RNA (1000 ng/µL from each sample) was reverse transcribed into cDNA using an EasycDNA Synthesis Kit (AddScript RT-PCR SYBR Master, AddBio, Sweden).

Primer design and real-time PCR

The NCBI gene database and AlleleID software (version 7) were used to design primers. Table 1 displays the sequences of designed primers. SYBR green-based real-time PCR was used to assess the expression of the *E6* and *E7* viral oncogenes and cellular genes, including *BIRC5*, *c-MYC*, and *STMN1*. The qRT-PCR ABI QuantStudio™3 instrument (Applied Biosystems, Grand Island, NY, USA) was employed for this purpose. The *beta-actin* gene was used as the reference gene. 2x Master Mix Green (Ampliqon Inc.,

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Denmark), cDNA, primers (at a concentration of 10 pmol), and water made up the final volume of each reaction, which was 15 µL. For the PCR program, a denaturation phase of 95 °C lasting 15 minutes was followed by 40 cycles of 95 °C for 15 seconds and annealing/extension at 62 °C for 1 minute.

Table 1. Primers used for evaluation of gene expression by relative quantitative real-time PCR.

Gene Name	Sequence	Product size	Primer position
E6	5'-TTAATAAGGTGCCTGCGGTG-3' 5'-GCGTCGTTGGAGTCGTTC-3'	156 bp	F: 304-323 R: 459-442
E7	5'-TCACGAGCAATTAAGCGACT-3' 5'-CACGGACACACAAAGGACAG-3'	219 bp	F: 81-100 R: 299-280
c-MYC	5'-TCACACCCTTCTCCCTT-3' 5'-CGCTCCACATACAGTCC-3'	180 bp	F: 1432-1448 R: 1611-1595
BIRC5	5'-AGTTGGAGTGGAGTCTGG-3' 5'-CTTGCTGGTCTCTTCTGG-3'	144 bp	F: 2346-2363 R: 2489-2472
STMN1	5'-GCTTGTCTTCTATTCACCAT-3' 5'-TTGCGTCTTTCTTCTGC-3'	203 bp	F: 239-258 R: 441-425
EBNA1	5'-GGGTGGTTTGGAAAGCATCG-3' 5'-CTTACTACCTCCATATACGAACACA-3'	156 bp	F: 1257-1276 R: 1413-1387
Beta-actin	5'-GCCTTTGCCGATCCGC-3' 5'-GCCGTAGCCGTTGTCG-3'	90 bp	F: 135-150 R: 240-224

Verification of *EBNA1* gene expression

To remove plasmid contamination, total RNA extracted from transfected cells was treated with DNase (Sinaclon, Tehran, Iran) according to the manufacturer's instructions. The expression of EBNA1 was verified using real-time PCR. Total RNA that had been subjected to DNase was used as the negative control.

Pathology staining

At the end of the 16th day, cells with stable EBNA1 expression and cells containing control plasmid were harvested. To examine morphological changes, hematoxylin and eosin (H&E) staining was used.

Data analysis

All experiments were performed in duplicate. The final results were calculated based on the average of corresponding experiments for each group. The CtNorm algorithm, which can be accessed online at http://ctnorm.sums.ac.ir, was used to equalize the Ct values of the qRT-PCR runs (25). The data were computed in Microsoft Excel after Ct normalization. Mann –Whitney U test in GraphPad Prism software was applied to compare means. Statistical significance was defined as a *P*-value less than 0.05.

Results

Real-time PCR results analysis of the expression of HPV-18 *E6,* and *E7* oncogenes following EBNA1 transfection

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The expression levels of two HPV-18 oncogenes, *E6* and *E7*, were compared between EBNA1 transfected cells and mock plasmid transfected cells (Figure 1A). Real-time PCR results showed that the expression of *E6* (P=0.02) and *E7* (P=0.02) genes was considerably increased in HeLa cells containing *EBNA1* gene compared to mock plasmid transfected controls (*E6* gene expression increased twofold, and *E7* gene expression increased thrice).

Real-time PCR results analysis of *BIRC5, c-MYC,* and *STMN1* gene expression following EBNA1 transfection

The expression levels of *BIRC5*, *c-MYC,* and *STMN1* cellular genes were compared between cells transfected with the EBNA1 plasmid and controls (Figure 1B). According to real-time PCR results, in the presence of EBNA1, the expression levels of *BIRC5* and *c-MYC* genes was increased tenfold (P = 0.03) and threefold (P=0.02), respectively. Regarding the *STMN1* cellular gene, although the expression of this gene in EBNA1 transfected HeLa cells showed a twofold increase, this change was not significant (P = 0.11).

Pathological staining for morphological examination

We observed alterations in the morphology of EBNA1 transfected cells after selecting hygromycin B-resistant clones. Pathological staining revealed, as depicted in Fig. 2, that EBNA1 expression changed the shape of HeLa cells in comparison to the control (mock plasmid-transfected) cells. HeLa cells transfected with the EBNA1 plasmid became abnormally large cells with numerous nuclei and abundant cytoplasm

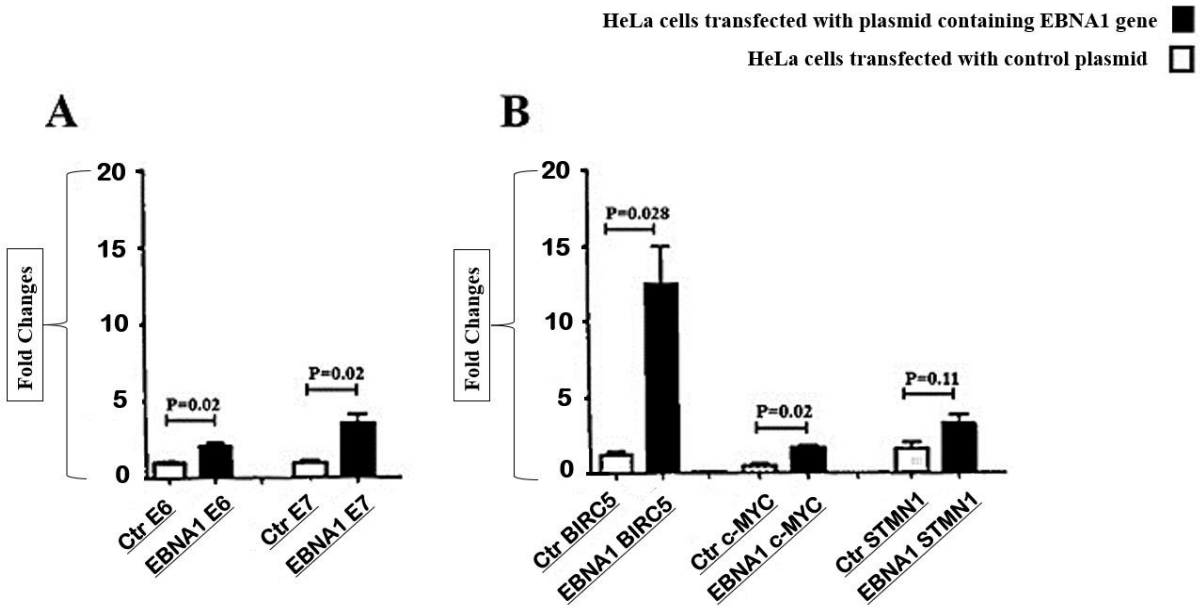

Fig.1. Real-time PCR results analysis of the expression of *E6, E7, BIRC5, STMN1*, and *c-MYC* genes: **A)** Comparison of expression changes of *E6*, and *E7* viral oncogenes between EBNA1 transfected HeLa cells and control cells **B)** Comparison of expression changes of *BIRC5*, *STMN1*, and *c-MYC* genes between EBNA1 transfected HeLa cells and control cells

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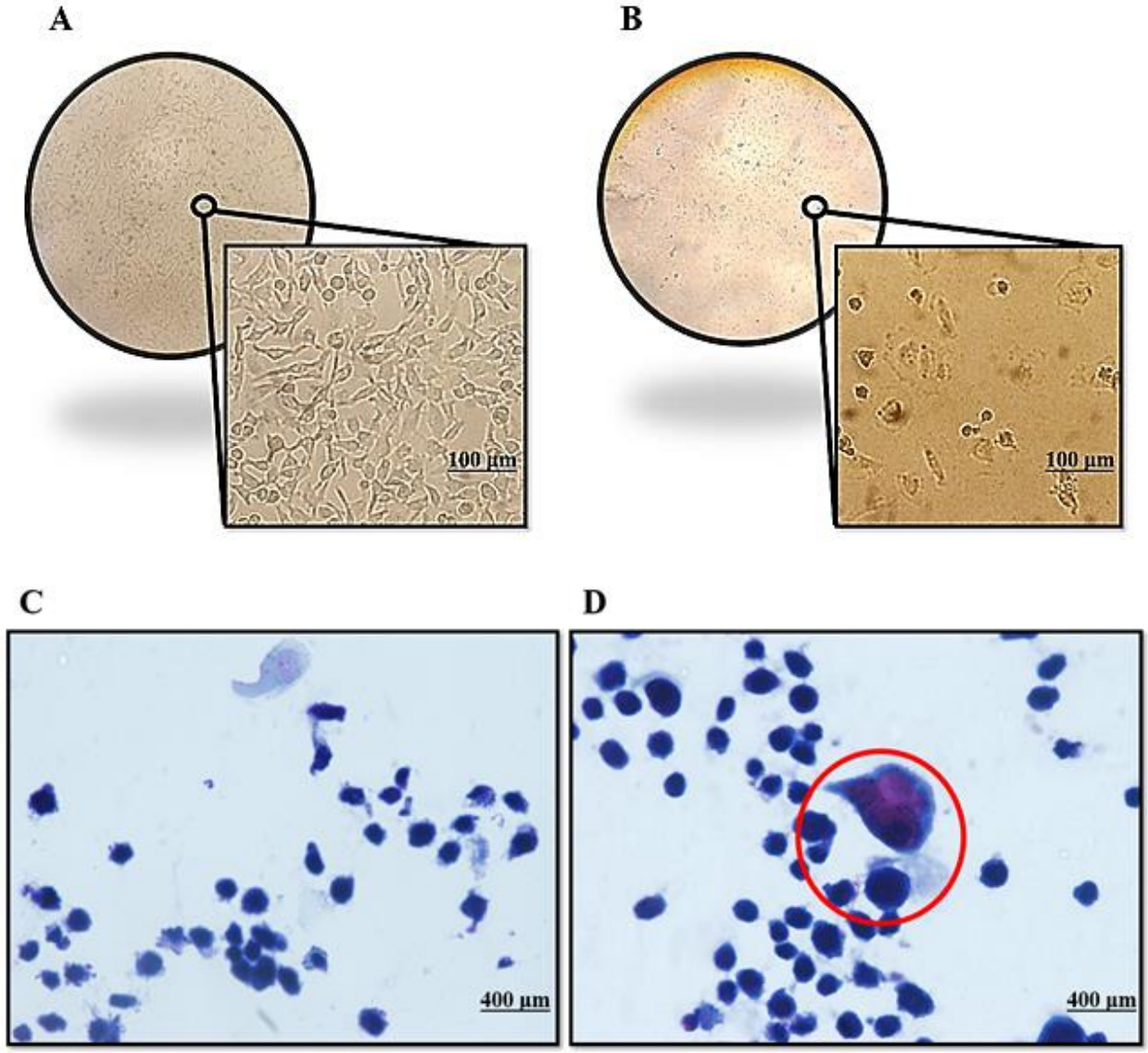

Fig.2. Morphological changes of HeLa cell line transfected with EBNA1 and control plasmid: **A)** HeLa cells transfected with the control plasmid; **B)** abnormal morphology in the culture of the HeLa cells transfected with EBNA1; **C)** pathological staining of control plasmid transfected cell; **D)** pathological staining of EBNA1 plasmid transfected cell.

Discussion

Today, the study of the connection between viruses and cancer is of interest to many researchers. Numerous cancers and EBV have been proven to be related thus far. However, the effect of this virus on some cancers, including CC, remains unknown. Recently some studies have reported the frequency of EBV in epithelial tissues isolated from CC patients (9, 26, 27). Based on a study conducted in 2019 by Guidry *et al*., it is stated that when EBV enters the cell infected with HR-HPV, the prevailing conditions of the cell cause EBV not to replicate, and enter to its latent phase (28). The only viral protein presents in all EBV

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latency types is EBNA1 (17). EBNA1 can affect the cellular and viral genes expression by binding to their promoters (17, 29).

In this study, we found that HPV-18 *E6* oncogene, was upregulated at the mRNA level in HeLa cells that were transfected with a plasmid containing *EBNA1* gene compared to control plasmid transfected cells. E6 protein, by forming a complex with the cellular ubiquitination enzyme E6AP, can bind to the tumor suppressor protein P53 and cause its destruction through the *proteasomal degradation* pathway (5). Following the reduction of P53 protein in the cell, E6 can induce the *BIRC5* gene promoter. Therefore, this drop in P53 level and increase in the *BIRC5* gene product, both can inhibit apoptosis of the infected cell (5, 30). Also, the E6/E6AP complex, by binding to another cellular protein called NFX1-91, which plays a role in inhibiting the expression of the human telomerase reverse transcriptase (hTERT) enzyme, causes its destruction (31). Therefore, the NFX1-91 inhibitory effect on the expression of hTERT is removed (31). On the other hand, E6 protein of HPV-18 by forming a complex with c-MYC cellular protein (E6/c-MYC), directly affects the hTERT gene promoter and increases its transcription. So this enzyme is produced in a high amount, which results in the immortalization of the host cell (31). The E6/E6AP complex also interacts with other cellular proteins, including the pro-apoptotic protein BAK1, the Fas-associated protein with death domain (FADD), and procaspase 8, to inhibit apoptosis in infected cells (32). In 2018, Al-Thawadi *et al*. revealed that the co-expression of the *E6* gene of HR-HPVs and the EBV-*LMP1* gene is associated with a phenotype of poorly differentiated squamous cell carcinoma (26). So, the expression of the DNA-binding protein inhibitor 1 (Id-1) increases greatly and, in combination with nuclear NF−κB p65, can be associated with aggressive CC behavior and poorer clinical outcomes (33, 34). As a result, increased *E6* expression by EBNA1 may hasten the progression of cervical lesions to cancerous status in HPV/EBV coinfection compared to HPV mono-infection.

Also we found that the expression level of HPV-18 *E7* gene was significantly increased in *EBNA1* transfected HeLa cells. The product of this viral oncogene binds to the cell transcription factor E2F, causing it to be released from RB. In this way, E2F enters the cell nucleus and transcribes genes related to the proliferative phase of the cell cycle, so the infected cell goes from G1 to the S-phase and continues to multiply (5). E7 protein also interacts with cellular histone deacetylase 1 and 2 (HDAC 1, 2) enzymes and thus leads to activation of transcription (35). Also, E7 can bind to the TMEM173/STING protein complex in the infected cell and prevent the identification of the viral genome in the cytoplasm. So, the innate immune responses of interferon alpha and beta are inhibited (36). It has also been reported that in the case of HPV/EBV co-infection, the HPV-E7 protein prevents the replication of EBV and drives this virus to its latent phase, which might contribute to the progression of the cell to become cancerous (28). Hence, the expression of EBNA1 in HPV/EBV co-infection could increase the potency of viral carcinogenesis more than in HPV mono-infection. To the best of our knowledge, for the first time, we showed that EBV-EBNA1 could increase the expression level of HPV-18 *E6* and *E7* oncogenes in HeLa cell line.

According to our study, the expression level of *BIRC5* gene was about tenfold higher in EBNA1 transfected HeLa cells (compared to control cells). In agreement with our study, Lu *et al*. stated that in Burkitt's lymphoma, EBNA1 increases the expression of the *BIRC5* gene by forming a complex and binding to the promoter of this gene (37). BIRC5 (also named survivin) is a member of the inhibitors of apoptosis

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proteins (IAPs) family, which by interfering in the functions of caspases 3, 7 and also inhibiting Bax and Fas-induced apoptotic pathways, causes abnormal cells to survive (38). So, increased expression of *BIRC5* by EBNA1 might contribute to the pathogenesis of CC in HPV/EBV co-infected cervical cells.

Our study indicated that the expression level of the *c-MYC* gene increased threefold in HeLa cells transfected with EBNA1 plasmid compared to control plasmid transfected cells. Based on the study by Ferber *et al*., it was found that in CC, the HPV-18 genome is integrated in a region near the *c-MYC* gene and can cause the activation of this proto-oncogene (39). Also, it is stated that the product of this gene in the presence of HR-HPVs-E6 protein, can induce the cell hTERT enzyme and ultimately lead to the immortality of the infected cell (31, 39). According to studies conducted in Burkitt lymphoma cases, it is clear that EBV is involved in 90% of *c-MYC* gene translocations, which can cause the activation of this proto-oncogene, and subsequently, be involved in the development and/or progression of cancers (40). Also, c-MYC protein is able to bind to the promoter region of vascular endothelial growth factor A (VEGFA) to stimulate more production of this growth factor and then cause angiogenesis (41). Therefore, as a multi-functional proto-oncogene, increased expression of *c-MYC* by EBNA1 in cervical cells co-infected with HR-HPVs and EBV might push cervical lesions to CC more than in HPV infection alone.

The results of our study indicated that *STMN1* gene transcript level was doubled in HeLa cells transfected with EBNA1 plasmid, although this change in *STMN1* gene expression was not significant. According to Miceli *et al*., *STMN1* gene expression is increased in breast cancer and is directly related to tumor size and degree of damage (42). Also, in agreement with our results, a study performed in 2012 on nasopharyngeal carcinoma (NPC) determined that the expression level of the *STMN1* gene increased threefold in the presence of EBV-EBNA1 (43). Also, in another study, it is stated that in cancers related to HPVs, such as oropharyngeal cancer, the expression level of this cellular gene increases significantly (44).

Based on the results of our study, pathological staining revealed that the expression of EBNA1 had an impact on the morphology of HeLa cells. Similar results were obtained by Wang *et al*., who found that EBNA1 affected NPC cell shape as well as the expression of markers for the epithelial-mesenchymal transition (EMT) (45). They also showed that the EBNA1 was highly expressed in NPC tissue samples, tying this expression to NPC lymph node metastasis (45). To the best of our knowledge, for the first time, we showed that the expression of EBNA1 can lead to significant morphological changes in HeLa cells. Other studies did not report such morphological changes in epithelial cells, which could be attributed to their method of transiently transfecting the cells, whereas we looked at stabilized long-term EBNA1 effects. All of these findings point to a link between EBNA1 expression and the expression of viral and cellular genes in the HeLa cell line, and we believe that such a relationship, in the case of simultaneous infection with HR-HPVs and EBV, causes the development and progression of CC more than HPV infection alone.

In conclusion, our results indicated that the EBV-EBNA1 could increase the expression levels of HPV-18 *E6* and *E7* oncogenes as well as cellular genes, including *BIRC5*, and *c-MYC* in the HeLa cell line. Therefore, simultaneous EBV and HPV-18 infection of cervical cells might accelerate the progression of CC to a higher degree not only by HPV carcinogenesis mechanisms but also by inducing and over-expression of HPV-18 *E6* and *E7* oncogenes, as well as *BIRC5*, and *c-MYC* cellular genes through EBV-EBNA1. Further studies are recommended to clarify these results.

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