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Epstein-Barr virus nuclear antigen 1 (EBNA1) increases the expression levels of MDM2 and MDM4 genes in HeLa cells: a review on MDM2 and MDM4 roles in cancer

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Abstract

Objective Epstein-Barr virus nuclear antigen 1 (EBNA1) is a key viral protein expressed in all latency phases and EBV-associated tumors. It can modulate the expression of various host and viral genes. This study aimed to investigate the impact of EBNA1 on the expression levels of two cellular genes involved in p53 pathway regulation—MDM2 and MDM4—in HeLa cells. This investigation was conducted as part of our broader research on EBV-related oncogenic mechanisms.

Results HeLa cells were transfected with either an EBNA1-expressing plasmid or a control plasmid. Gene expression levels of MDM2 and MDM4 were analyzed using real-time PCR. The results demonstrated a statistically significant increase in MDM4 expression in EBNA1-transfected cells compared to controls ($p=0.028$). Although MDM2 expression was also elevated, the difference was not statistically significant ($p=0.11$). These findings suggest that EBNA1 may play a role in cervical cancer development by upregulating genes that inhibit p53 tumor suppressor activity.

Introduction

Among females, cervical cancer (CC) is a commonly found disease that is connected to substantial levels of morbidity and mortality across the world [1]. There are numerous risk factors linked to the development of CC, and one of the primary causative factors is the infection of the human papillomavirus (HPV), which accounts for approximately 99.7% of all cases [2]. Additionally, various other factors have been identified as contributors to the increased risk of CC, such as smoking, immunosuppression, poor sexual health, and failure to attend regular screenings [3].

New clinical research has confirmed the association between Epstein–Barr virus (EBV) and high risk HPVs (HR-HPVs) in the development of CC [4, 5]. EBV, also identified as human herpesvirus-4, stands as the pioneering oncovirus in humans [6]. This virus is classified under the gamma-herpes viruses and is widely distributed among adults, primarily transmitted through saliva.

A published meta-analysis included 25 publications revealed that the pooled prevalence of EBV in CC was 43.63% [5]. This prevalence was significantly higher when

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compared to healthy controls, who had a prevalence of 19%. Additionally, the analysis found that the expression of EBV increased gradually from 27% in cervical intraepithelial neoplasia I (CIN I) to 35% in CIN2/3. The study also highlighted that EBV coinfection with HPV increased the risk of CC fourfold in EBV-positive women. Furthermore, EBV-positive women were found to have a higher incidence of precancerous cervical lesions compared to EBV-negative cases [5]. These findings suggest that EBV may actively contribute to the development and progression of CC, rather than being a passive participant.

Despite the fact that infected individuals serve as carriers of the EBV for the duration of their lives, it is worth mentioning that only a small proportion of these individuals actually experience the development of EBV-related cancers [7]. One of the most important late proteins of this virus, which is expressed in all viral latency phases, is EBV nuclear antigen-1 (EBNA1) [8]. It is indicated that EBNA1 as a transcription factor has the ability to alter the expression of genes upstream of both viral and cellular promoters [8].

Murine double minute 2 (MDM2) and MDM4 (MDMX) are two of the main regulators of p53 functions [9]. The p53 protein is known to be monoubiquitinated by MDM2, while polyubiquitination of p53 necessitates the development of a heterodimer between MDM2 and MDM4 [9]. MDM2 functions as an oncogene, and it is shown to be overexpressed in a range of human malignancies [10]. By engaging the transactivation domain and directly binding and degrading p21, MDM4 can effectively block p53 transcriptional activity [11]. In this regards, AlQarni et al. declare the connection between EBV infection and increased *MDM2* expression [12]. Moreover, Hashemi and colleagues have recently documented the upregulation of *MDM4* and *MDM2* in MKN-45 cells subsequent to EBNA1 transfection [13].

Therefore, this research aimed to analyze how the EBV-EBNA1 protein affects the expression patterns of the cellular p53 inhibitory genes *MDM2* and *MDM4* in a CC cell line.

Methods

Characterization of EBNA1-containing and control plasmids: transformation, verification, and quality assessment

In this study, we employed the pCEP4 plasmid, an EBV-based plasmid that includes the *EBNA1* gene and a hygromycin B resistance gene, as well as a control plasmid that does not contain the *EBNA1* gene. These plasmids were transformed into *Top10 strain* of *Escherichia coli* and propagated. To confirm the presence of the *EBNA1* gene in the plasmid, we used enzyme digestion and colony PCR techniques. Following this verification, the plasmids were extracted, and their quality and concentrations were assessed using gel electrophoresis and spectrophotometry.

Establishment of stable EBNA1-expressing HeLa cell lines via transfection and hygromycin B selection

HeLa cells, a cervical adenocarcinoma cell line harboring HPV-18, were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO2 in a 6-well plate. Upon reaching approximately 70% confluence, one group of cells was transfected with an EBNA1-containing plasmid, while another group received a mock plasmid. The transfection was performed using an optimized concentration of DNA-fectamine (BioBasic, Canada). Twenty-four hours post-transfection, the cells were exposed to 350 µg/ml hygromycin B to select for those with stable EBNA1 expression. These selected cells were subsequently cultured for 20 days with hygromycin B, undergoing multiple passages.

Extraction of total RNA, synthesis of cDNA, and confirmation of EBNA1 gene expression

Total RNA was extracted from selected HeLa cells using an RNA Isolation Kit (Dena Zist, Mashhad, Iran). The quality and quantity of the extracted RNA were evaluated through electrophoresis and spectrophotometry, respectively. RNase-free DNase (Sinaclon, Tehran, Iran) was used to remove any plasmid contamination. cDNA synthesis was performed using an EasycDNA Synthesis Kit (AddScript RT-PCR SYBR Master, AddBio, Sweden) with 1000 ng/µl of RNA from each sample. Finally, real-time PCR was utilized to confirm the expression of the *EBNA1* gene.

Primer design and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

expression levels of the cellular genes *MDM2*, and *MDM4* were evaluated using a qRT-PCR assay on an ABI Quant Studio 3TM (Applied Biosystems, Grand Island, NY, USA). The thermal cycling protocol included an initial denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 62 °C. The *beta-actin* gene served as the reference gene for normalization (Table 1) [14].

Normalization of qRT-PCR Ct values and statistical analysis

Normalization of Ct values from the qRT-PCR runs was performed using the CtNorm algorithm, available at http://ctnorm.sums.ac.ir [15]. The means were compared using the Mann-Whitney U test in GraphPad Prism software, with statistical significance defined as a P-value of less than 0.05.

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

Gene Name	Sequence	Product size	Primer position
MDM2 [14]	5′-AACCACCTCACAGATTCCA-3′ 5′-GCACCAACAGACTTTAATA-ACTTC-3′	87 bp	F: 1082–1100 R: 5429 – 5406
MDM4 [14]	5′-GCCTGCCTTGGTGGTT-3′ 5′-CCTAACTGCTCTGATACTGACTC-3′	160 bp	F: 26,457–26,472 R: 28,206 – 28,184
EBNA1 [14]	5′-GGGTGGTTTGGAAAGCATCG-3′ 5′-CTTACTACCTCCATATACGAACACA-3′	156 bp	F: 1257–1276 R: 1413 – 1387

Results

Real-time PCR results of MDM4 gene expression following EBNA1 transfection

MDM4 cellular gene expression level was compared between EBNA1-transfected cells and controls (Fig. 1). Real-time PCR analysis indicated a significant three-fold increase in the expression level of this gene in the presence of EBNA1 ($p = 0.028$).

Real-time PCR results of the MDM2 gene expression following EBNA1 transfection

In Fig. 1, the expression of *MDM2* gene was compared between EBNA1 transfected cells and control cells. Although EBNA1-transfected HeLa cells revealed a two-fold increase in the expression of this gene, this change was not significant ($p = 0.11$).

Discussion

An increased risk of CC has been observed in individuals with EBV/HR-HPV coinfection [5]. Studies indicate a possible collaboration between the EBV and the progression of CC, as evidenced by various research findings [16]. Among the different proteins encoded by EBV, EBNA1 is unique in being present across all viral latency stages. By binding to gene promoters, EBNA1 has the ability to influence the expression of a wide range of genes [8, 17].

In this study, we found that EBNA1 upregulated *MDM2* oncogene expression in EBNA1-transfected HeLa cells compared to mock-transfected controls. Hashemi and colleagues demonstrated that EBNA1 has the ability to significantly increase the expression

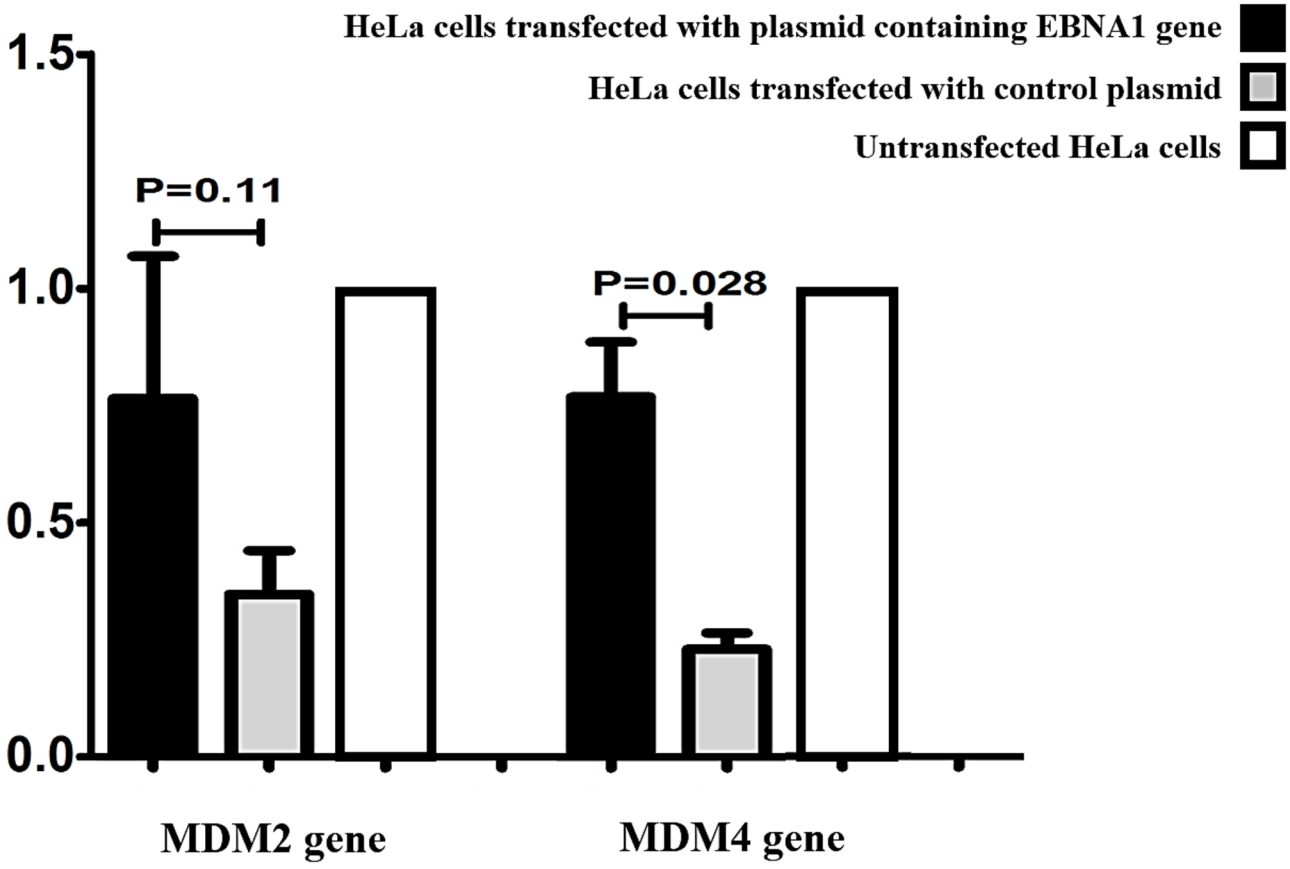

Fig. 1 Real-time PCR analysis of *MDM2* and *MDM4* Gene expression: comparative alterations in EBNA1-Transfected HeLa cells versus mock-plasmid-transfected cells

of p53-inhibiting genes such as *MDM2* and *MDM4* in a Burkitt's Lymphoma Cell Line [18]. MDM2 is a crucial negative regulator of the p53 tumor suppressor protein. It acts as an E3 ubiquitin ligase, targeting the N-terminal trans-activation domain (TAD) of p53 for ubiquitination, and also serves as an inhibitor of p53's transcriptional activation [19, 20]. Based on a study by Gnanasundram et al., EBV can modulate the MDM2-P53 pathways via EBNA1-induced mRNA translation stress [21]. A 2018 study further demonstrated that MDM2 is overexpressed in EμEBNA1 tumor cells, with its levels being associated with EBNA1 expression [22]. In this context, Alipour et al. observed that EBNA1 can increase the expression of HPV-18 *E6* and *E7* oncogenes, along with several cellular genes, in the HeLa cell line [23]. Our studies have shown that EBNA1 can boost *MDM2* gene expression in cervical cells, reduce p53 activity, prevent cell death in infected cells, and contribute to the pathogenesis of EBV in CC.

We also found that HeLa cells transfected with the EBNA1 plasmid exhibited significantly higher levels of *MDM4* transcripts compared to cells transfected with a mock plasmid. This gene encodes a nuclear protein that features a p53 binding domain at the N-terminus and a RING finger domain at the C-terminus, displaying structural similarities to the p53-binding protein MDM2. Both proteins interact with the p53 tumor suppressor protein to inhibit its activity and have been found to be overexpressed in various human cancers [24]. Unlike MDM2, which targets p53 for degradation, this protein inhibits p53 by binding to its transcriptional activation domain. Additionally, it interacts with MDM2 through its RING finger domain, preventing the degradation of MDM2. So MDM4 can reverse MDM2-targeted degradation of p53 while maintaining suppression of p53 transactivation and apoptotic functions [25, 26]. Consistent with our findings, Hashemi et al. showed that the presence of EBV-EBNA1 protein in MKN-45 cells led to a significant increase in the expression of both *MDM2* and *MDM4* genes [13]. Consequently, the overexpression of *MDM2* and *MDM4* mediated by EBNA1 may be associated with p53 suppression in CC cells.

Although this study focused on HeLa cells, which are HPV-18 positive, we acknowledge that the use of a single cell line may limit the generalizability of our findings. To determine whether EBNA1-mediated upregulation of MDM2 and MDM4 is cell-line-specific or influenced by HPV subtype, future studies should examine additional cervical cancer cell lines. These include HPV-16–positive lines such as CaSki or HCB-514, and other HPV-18–positive lines like TMCC-1. Such comparative analyses could clarify whether the observed regulatory effects are broadly applicable across HPV-driven cervical cancers or specific to certain genetic backgrounds.

On the basis of these results, we hypothesized that EBNA1 would be related to the degree of *MDM2* and *MDM4* expression as well as the likelihood of developing CC.

E6 from high-risk HPV types is an oncoprotein that promotes host cell transformation by targeting p53 for degradation [27]. Additionally, studies have indicated that the E6 protein from HPV-16 hinders p53 function, which aids in tumor progression [28]. Our findings also reveal that EBNA1 expression significantly elevates the levels of two p53-inhibiting genes, MDM4 and MDM2, suggesting that EBNA1, along with the E6 oncoprotein, may further suppress p53. Supporting this, our previous research demonstrated that the EBV-EBNA1 protein can enhance the expression of HPV-18 E6 and E7 oncogenes [29].

In this study, we confirmed the elevated expression of two important cellular p53 inhibitory genes at the mRNA level by real-time PCR technique. Previous studies have shown that genetic variants in the MDM2 gene may influence p53 activity and cancer susceptibility [30–33]. Researchers have suggested that this genetic polymorphism may contribute to an increased risk of cancer [34]. For instance, Hong et al. demonstrated that the 309GG genotype of MDM2 is linked to a higher risk of esophageal squamous cell carcinoma [35]. Additionally, the 309G allele has been reported to be associated with the early diagnosis of estrogen receptor-positive breast cancer [36]. Furthermore, the presence of the 285 C allele of rs117039649 has been linked to a reduced risk of breast, ovarian, and endometrial cancer (EC) in patients with the 309G allele, indicating that it might counteract the effect of SNP309 in MDM2 [36].

Several studies have also indicated that these two significant polymorphisms, rs2279744 and rs117039649, may be linked to an increased susceptibility to gynecological cancers (GCs) [37, 38]. In a meta-analysis, Zhang et al. demonstrated that both rs2279744 (SNP309) and rs117039649 (SNP285) are associated with the risk of GCs. Additionally, researchers in another meta-analysis found that polymorphisms at the rs2279744 loci of the *MDM2* gene are linked to an increased risk of EC [39]. The del1518 variant has been reported to be associated with a reduced risk of EC among individuals with the SNP309TT genotype, in both dominant and recessive models [40]. In a hospital-based case-control study, Miedl et al. reported that the SNP285 and SNP309 genetic variants of the *MDM2* gene are associated with an increased risk of breast cancer (BC) in Central European women. Moreover, these polymorphisms have also been linked to an earlier onset of BC and its prognosis [31].

The expression of MDM2 and MDM4 proteins is being investigated across various cancer cell lines and tissues. In this line, it has been confirmed that the MDM4

protein is overexpressed in several human tumor cell lines [41, 42]. Additionally, tissues from soft-tissue sarcomas exhibit significantly higher levels of MDM4 protein compared to healthy controls [43]. Moreover, Danovi et al. revealed overexpression of the MDM2 protein in BC tissues [44]. Analysis of MDM2 and MDM4 proteins also showed higher steady state levels in retinoblastoma than control group [31].

Taken together, it is now evident that in many human cancers, p53 activity is inhibited by high levels of MDM proteins. Therefore, inactivating MDM proteins with therapeutic molecules might be a valuable strategy for tumor treatment [32]. Several studies suggest that small-molecule inhibitors targeting MDM2 and MDM4 could restore p53 activity and offer therapeutic benefits [11, 45].

Other inhibitors of MDM4 and MDM2, such as 5-fluorouracil, have also demonstrated anti-tumor activity in colon and gastric cancer cells [46]. Researchers found that malignant rhabdoid tumor (MRT) cells were particularly sensitive to MDM2 inhibition by idasanutlin (specific to MDM2) and to dual inhibition of MDM2/4 by ATSP-7041, compared to other p53 wild-type cancer cell lines. These compounds led to a significant upregulation of the p53 pathway in MRT cells, and this sensitivity was eliminated by CRISPR-Cas9-mediated inactivation of TP53 [32]. Furthermore, park et al., found that simultaneous inhibition of both MDM2 and MDM4 in virus-positive Merkel cell carcinoma led to an increased p53 response [47]. It has also been reported that trametinib, which inhibits MDM4/MDM2, enhanced G1 cell cycle arrest and induced apoptosis in colon and gastric cancer cells [26]. Recently, Langenbach et al. demonstrated that MDM2 inhibitors induce the production of MHC-II and IL-15, suggesting a novel approach to disrupting the immunosuppressive tumor microenvironment [48].

Limitations

- The study was conducted using only a single cervical cancer cell line (HeLa), which may not fully represent the biological diversity of HPV-positive or HPV-negative cervical cancers.
- Normal cervical epithelial cells were not included as a control group, limiting our ability to compare gene expression levels against a physiological baseline.
- Gene expression analysis was restricted to the mRNA level using real-time PCR. No protein-level validation (e.g., Western blotting or immunohistochemistry) was performed to confirm whether changes in transcription translated into functional protein changes.
- Functional assays were not included in this study. Therefore, the biological consequences of EBNA1-induced gene expression changes—such as their effects on cell proliferation, apoptosis, or migration—remain to be determined.
- Only a limited number of target genes were evaluated. A broader transcriptomic or proteomic analysis would be valuable to identify other potential pathways regulated by EBNA1.
- As this was an in vitro study, the results may not fully reflect in vivo conditions. Further animal model or tissue-based studies are needed to validate these findings in the context of cervical cancer progression.

Conclusion

In conclusion, our results suggest that the EBV-EBNA1 protein can elevate the expression levels of the *MDM2* and *MDM4* genes in HeLa cells. This leads to significant suppression of the p53 protein and enhanced cancer cell survival, potentially accelerating the onset of CC. Additionally, findings from other studies highlight the critical role of MDM2 and MDM4 in cancer development, indicating that inhibiting their function with various molecules could be considered a therapeutic strategy for treating different types of cancer. Further research is recommended to clarify these findings.

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SMA.H. and AH.A. contributed equally to conducting the research and writing the manuscript, S.E. assisted in data collection and statistical analysis, A.F. contributed to laboratory experiments and result validation, J.S. supervised the study, provided conceptual guidance, and finalized the manuscript. All authors read and approved the final manuscript.

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Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

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Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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