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Transcriptional Suppression of E-Cadherin by HPV-16 E6 and E7 Oncogenes is Independent of Hypermethylation of E-Cadherin Promoter

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Abstract

Background: Cervical cancer is one of the most common cancers observed in women worldwide, and its development is related to E6 and E7 two viral oncoproteins of high-risk human papillomavirus (HPV) types. Aberrant expression of E-cadherin, which is associated with epithelial-to-mesenchymal transition (EMT), is frequently observed in cervical cancer.

Objectives: The mechanisms underlying E-cadherin suppression in cervical cancer are not clear; therefore, this experimental study from Iran was designed to elucidate the relationship of DNA methyltransferase expression and E-cadherin promoter methylation with E-cadherin expression in HPV-16 E6- and E7-expressing cells.

Materials and Methods: Real-time PCR and western blot were used to determine the effects of HPV-16 E6 and E7 on E-cadherin, DNMT1, DNMT3a, and DNMT3b expression in HCT-116 cell line. We also analyzed E-cadherin promoter methylation in cells expressing HPV-16 E6 and E7 oncoproteins by bisulfite sequencing.

Results: HPV-16 E6 and E7 proteins reduced E-cadherin expression 3.7 and 2.2 times when compared with control cells (P = 0.0221 and P = 0.0461, respectively). This reduction was greater in HPV-16 E6-expressing cells than in HPV-16 E7-expressing cells. Although HPV-16 E6 and E7 increased DNA methyltransferase 1 expression 2.6 and 3.4 times, respectively (P = 0.0133 and P = 0.0113) when compared with control cells, they was no E-cadherin promoter methylation.

Conclusions: Unlike other cancer-associated viruses (HBV, HCV, and EBV), reduction in E-cadherin expression in HPV-16 E6- and E7-expressing cells is not due to hypermethylation of the E-cadherin promoter.

Keywords: HPV-16, E-Cadherin, DNA Methyltransferase

1. Background

Cervical cancer is one of the most common cancers observed in women worldwide, with 500,000 cases diagnosed and over 200,000 deaths annually (1). Cervical cancer is caused by the integration of human papillomaviruses (HPVs) in the host genome (2).

HPVs are small, non-enveloped, non-lytic viruses with a double-stranded DNA (dsDNA) genome and are tropic for squamous epidermis. Approximately 40 of the 140 described HPV genotypes are associated with infections of the mucosal epithelia, and are classified into high- and low-risk groups. High-risk HPVs, such as HPV-16 and HPV-18, cause premalignant squamous intraepithelial neoplasias that can progress to cervical carcinomas, whereas low-risk HPVs, such as HPV6 and HPV-11, cause benign genital warts (2).

The carcinogenetic process of cervical cancer is related to E6 and E7 the two viral oncoproteins of high-risk HPVs (2). The high-risk HPV E7 oncoprotein binds and induces the degradation of the retinoblastoma (Rb) tumor suppressor protein, as well as of other pocket proteins, such as p107 and p130. It causes persistent activation of E2F transcription factors, resulting in aberrant S-phase entry (3, 4). High-risk HPV E6 targets the p53 tumor suppressor protein for degradation (5).

Previous studies have suggested that high-risk HPV oncoproteins may contribute to epithelial-to-mesenchymal transition (EMT), which is essential for several physiological and pathological processes, including embryogenesis, inflammation, and cancer progression. One characteristic of EMT is a cadherin switch from E-cadherin expression to N-cadherin expression (6).

E-cadherin, a 120-kDa type I classical cadherin, is primarily expressed on epithelial cells and is found on the surface of keratinocytes and Langerhans cells. It is also an important tumor suppressor protein, and its loss or inactivation is associated with tumor metastasis (7). Decreased or aberrant expression of E-cadherin is frequently found in

cervical carcinomas, as well as other cancers (8, 9). However, the mechanisms underlying E-cadherin suppression in cervical cancer are not clear.

Several proteins downregulate E-cadherin expression, including SNAI1/SNAIL (10), TWIST1 (11), and SNAI2/SLUG (12). Overexpression of these transcription factors appears to be associated with an altered overexpression of transcriptional repressors of E-cadherin in tumor cells (10-12). However, previous studies have suggested that E-cadherin repression in HPV-16-transformed human keratinocytes is independent of SNAIL and SLUG transcription factors (13, 14).

It is evident that tumor suppressor genes may be transcriptionally silenced in association with the aberrant promoter-region CpG island methylation (15). Some studies have shown that loss of E-cadherin expression is also associated with aberrant promoter 5 CpG island methylation in various tumors (16). The specific transfer of methyl groups to form 5 mC is catalyzed by members of the DNA methyltransferase (DNMT) protein family. DNMT1 preferentially methylates DNA containing hemimethylated CpGs and is implicated in the copying and maintenance of methylation patterns from the parental to the daughter strand during DNA replication. In contrast, DNMT3a and DNMT3b induce de novo methylation in unmethylated CpGs (17).

2. Objectives

In the present study, we investigated the effects of HPV-16 E6 and E7 oncoproteins on E-cadherin, DNMT1, DNMT3a, and DNMT3b expression. In addition, we analyzed the E-cadherin promoter methylation state in cells expressing HPV-16 E6 and E7 proteins.

3. Materials and Methods

3.1. Cell Lines

In this experimental study, TC-1, a mouse lung cancer cell line, and HCT-116, a poorly differentiated human colon cancer cell line, were purchased from national cell bank of Iran (NCBI, Tehran, Iran) and were grown in basal medium Dulbecco's modified Eagle's medium (DMEM; life technologies, USA) in the virology department of Tehran University of Medical Sciences (Tehran, Iran). HCT-116 cells are widely used to study E-cadherin regulation, being intact in major E-cadherin repressor pathways such as E-box-mediated repression and having low levels of promoter methylation. Growth media were supplemented with 10% heat-inactivated fetal calf serum (life technologies), 2 mM L-glutamine (Sigma, USA) and 100 μg/mL penicillin/streptomycin (life technologies). The cell lines were maintained at 37°C in a humidified incubator with 5% CO_2.

3.2. Construction of pIRES2-EGFP-E7 and pIRES2-EGFP-E6

The E7 gene was amplified (314 bp) from the extracted DNA of the TC-1 cell line by polymerase chain reaction (PCR). The purified amplification product and plasmid pIRES2-EGFP were digested by restriction enzymes NheI and XhoI (New England Biolabs, Ipswich) at 37°C overnight. These digested PCR products were purified from 0.8% agarose gel using the QIAquick gel extraction kit (Qiagen, Canada). Finally, the purified PCR products were recombined into plasmid pIRES2-EGFP using the T4 DNA ligase kit (Fermentas, Lithuania) and subjected to transformation into competent *Escherichia coli* DH5α cells by a standard heat-shock procedure. Transformants were identified on a selective Luria–Bertani kanamycin agar plate. The 456-bp fragment of the HPV-16 E6 gene was amplified using the extracted DNA of the TC-1 cell line and was similarly cloned into pIRES2-EGFP. The success of the constructions was confirmed by PCR and DNA sequencing.

3.3. Transfection of HCT-116 Cells

HCT-116 cells were transfected transiently using Lipofectamine LTX with Plus reagent (life technologies) according to the manufacturer's instructions. Lipofectamine and plasmid were added to each well, and cells were maintained at 37°C in an incubator with 5% CO_2 for six hours. After this incubation, media were replaced with fresh DMEM with 10% fetal calf serum and 1% penicillin-streptomycin and plates were incubated at 37°C in an incubator with 5% CO_2. For each time point (0, 24, 48, and 72 hours), transfections were conducted three times; that is, three samples of cells were harvested for analyses at each time point.

3.4. Western Blotting Analysis

HCT-116 cells were transfected with 2 μg of plasmids containing HPV-16 E6 and E7, and were collected and lysed in a 200 μL RIPA buffer. Next, 30 μg of protein samples was used for western blot analysis. The following antibodies were purchased from Santa Cruz biotechnology (Santa Cruz, CA): HPV-16 E6 (N-17), HPV-16 E7 (NM2), anti-E-cadherin (G-10), and β-actin (C4).

3.5. RNA Preparation and cDNA Synthesis

HCT-116 cells were cultured in culture plates with confluency over 90% and were transfected with pIRES2-EGFP-E7 and pIRES2-EGFP-E6 plasmids. At 0, 24, 48, and 72 hours after transfection, cells were harvested and suspended in 250 μL volume of sterile phosphate-buffered saline (PBS). Total RNA was extracted from the cells using the TriPure total RNA isolation kit (Roche, USA). cDNA was prepared from total RNA by reverse transcription using M-MuLV, random hexamer primers, and RNase inhibitor (all purchased from

Fermentas, Lithuania). Next, 1 μg of RNA was added to 2 μL of 10 mM DNTPs along with 1 μL of random hexamer primers, 1 μL of M-MuLV, and 0.5 μL of RNase inhibitor. The final volume was made up to 20 μL by adding sterile distilled water and was mixed well. These tubes were incubated at room temperature for 10 minutes and then at 42°C for one hour. The M-MuLV enzyme inactivation step was performed by a step for 10 minutes at 70°C.

3.6. Quantitative Real-Time PCR

The expression of E-cadherin, DNMT1, DNMT3a, and DNMT3b mRNAs was analyzed by quantitative real-time PCR using the SYBR Premix Ex Taq II kit (TAKARA BIO INC, Japan). The following primer sequences were used: E-cadherin, forward: 5'-AGGGGTTAAGCACAACAGCA-3' and reverse: 5'-GGTATTGGGGGCATCAGCAT-3'; DNMT1, forward: 5'-CCTTGGAGAACGGTGCTCAT-3' and reverse: 5'-CTTAGCCTCTCCATCGGACT-3′; DNMT3a, forward: 5'-CTTTTGCGTGGAGTGTGTGG-3' and reverse: 5'-CGGATGGGCTTCCTCTTCTC-3'; DNMT3b, forward: 5'-AGGAGTGTGAAGCAAGGAGC-3' and reverse: 5'-CCGAGCTTTGCAGTTTTCCC-3'; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward: 5'-ATGTTCGTCATGGGTGTGAA-3' and reverse: 5'-GGTGCTAAGCAGTTGGTGGT-3'. The temperature profile was 95°C for 1 minute; 40 times, 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds; the melting curve program was set at 60°C to 95°C. All samples were run in triplicate simultaneously with negative controls.

3.7. Bisulfite Sequencing

For gene-specific methylation analysis, bisulfite sequencing was used. Analysis of DNA methylation is based on bisulfate treatment of genomic DNA, which converts unmethylated cytosine to uracil but methylated cytosines remain unaltered in this process. In brief, 500 ng of DNA isolated from the transfected cell line, using the QIAamp DNA mini kit (Qiagen, CA), were bisulfite converted using the EZ DNA methylation-gold kit (Zymo Research, Orange, CA). This DNA, which was used for the nested PCR (14) and PCR products, was ligated into the TA cloning vector using the RBC TA cloning vector kit (RBC Bioscience, Taiwan). Ten individual clones were sequenced to determine the methylation state of CpGs within the E-cadherin promoter.

3.8. Statistical Analysis

An unpaired, two-tailed student t-test was used to compare and analyze statistical differences using graph-pad prism software. Variables are expressed as mean $\pm$ SD of at least three separate experiments. $P < 0.05$ was considered statistically significant for the differences and is indicated by an asterisk in the corresponding illustration.

4. Results

4.1. HPV-16 E6 Decreased E-Cadherin Transcription and Protein Levels in HCT-116 Cell Line

HCT-116 cells were transfected with pIRES2-EGFP-E6 (Figure 1A) and RNA extracted from these cells was reverse transcribed. The cDNA of E-cadherin and GAPDH was measured by quantitative real-time PCR. The results showed that E-cadherin mRNA levels were reduced in HCT-116 cells expressing the HPV-16 E6 protein ($P = 0.0221$; Figure 2A). Western blot detection of HPV-16 E6 and E-cadherin proteins was achieved using total cell extracts from HPV-16 E6-treated cells. The E-cadherin protein level was found to have decreased by the expression of HPV-16 E6 in HCT-116 cells (Figure 3A, 3C).

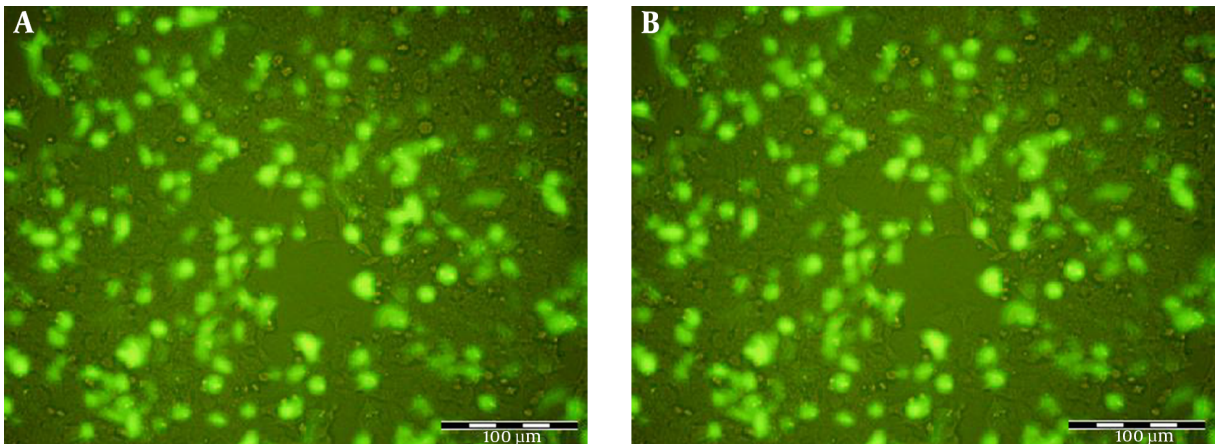
4.2. E-Cadherin mRNA and Protein Levels were Reduced in HPV-16 E7-Expressing Cells

HCT-116 cells were transfected with pIRES2-EGFP containing the HPV-16 E7 gene (Figure 1B). HCT-116 cells expressing E7 were harvested 24, 48, and 72 hours after transfection, and RNA was analyzed using real-time PCR. E-cadherin mRNA levels were reduced in HCT-116 cells expressing the HPV-16 E7 oncoprotein when compared with control cells ($P = 0.0461$; Figure 2B). Lysates of HCT-116 cells expressing E7 were analyzed by western blotting by using antibodies against the HPV-16 E7 protein (Figure 3B). Western blot analyses showed that the E-cadherin protein level was reduced in HCT-116 cells harboring the HPV-16 E7 oncoprotein (Figure 3C). The reduction in E-cadherin expression in HCT-116 cells expressing the HPV-16 E7 oncoprotein was smaller than that in HCT-116 cells harboring the HPV-16 E6 protein.

4.3. Co-Expression of HPV-16 E6 and E7 Oncoproteins Further Suppressed E-Cadherin Expression

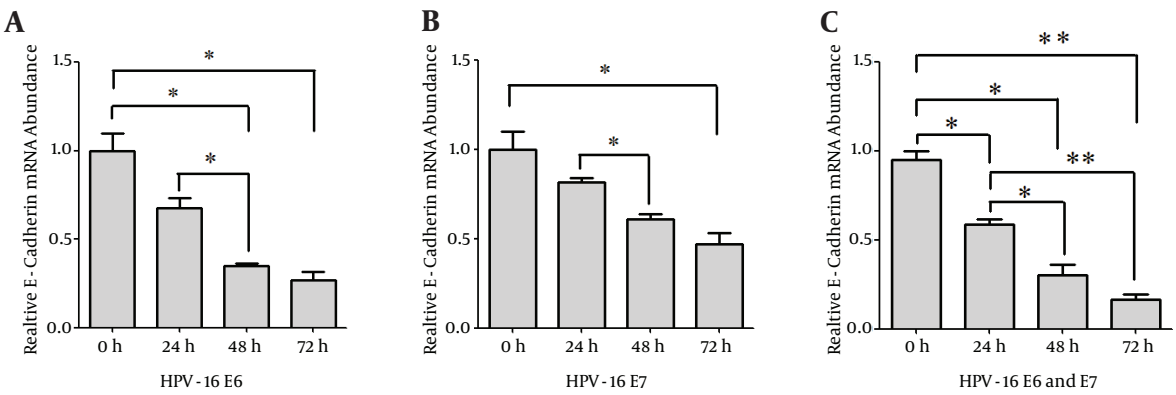
It was shown that E-cadherin expression was remarkably reduced in HCT-116 cells expressing the E6 and E7 oncoproteins of HPV-16 when compared with control cells. The reduction in E-cadherin expression in these cells was further compared with HCT-116 cells transfected with pIRES2-EGFP containing the HPV-16 E6 or E7 genes ($P = 0.0049$; Figure 2C). Western blot analysis showed that the E-cadherin protein was significantly reduced in HCT-116 cells expressing the E6 and E7 oncoproteins of HPV-16 when compared with control cells and HCT-116 cells harboring the HPV-16 E6 or E7 oncoproteins (Figure 3C). Statistical analysis results of E-cadherin expression, including mean, SD, and P values, are represented in Table 1.

Figure 1. Green Fluorescent Protein (GFP) Visualization of HCT-116 Cells Transfected With pIRES2-EGFP-E6 and pIRES2-EGFP-E7



A, HCT-116 cells were transfected with pIRES2-EGFP-E6; B, HCT-116 cells were transfected with pIRES2-EGFP containing the HPV-16 E7 gene. At 48 hours after transduction, GFP-expressing cells were imaged with magnification 100 × by fluorescence microscopy.

Figure 2. E-Cadherin mRNA Levels



A, E-cadherin mRNA levels in HCT-116 cells expressing HPV-16 E6; B, E-cadherin mRNA levels in HCT-116 cells expressing HPV-16 E7; C, E-cadherin mRNA levels in HCT-116 cells expressing HPV-16 E6-E7. Error bars represent 6 SEM. * $P < 0.05$, ** $P < 0.01$.

Table 1. Findings From Statistical Analysis of E-Cadherin Expression Using Unpaired, Two-Tailed Student t-test[a]

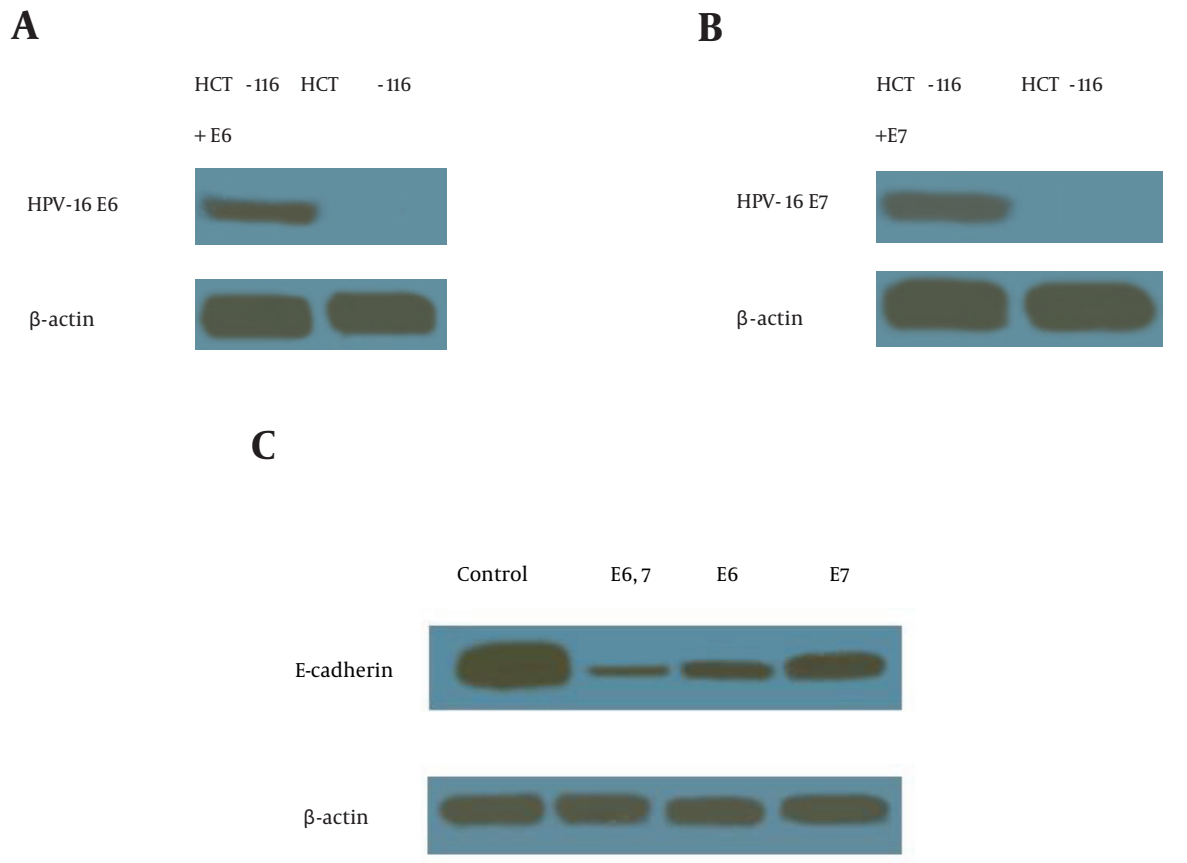
E-Cadherin Expression, HPV-16 protein	24 Hours After Transfection	P Value	48 Hours After Transfection	P Value	72 Hours After Transfection	P Value
HPV-16 E6	0.6761 (0.07918)	0.1057	0.3473 (0.02415)	0.0233	0.2677 (0.06750)	0.0221
HPV-16 E7	0.8155 (0.03596)	0.2156	0.6086 (0.04054)	0.0639	0.4690 (0.08888)	0.461
HPV-16 E6, E7	0.5867 (0.03965)	0.0240	0.2632 (0.07570)	0.0112	0.1584 (0.03514)	0.0049

[a]Values are expressed as mean ± SD.

4.4. HPV-16 E6 Gene Increased DNMT1 mRNA Levels in HCT-116 Cells

Hypermethylation of gene promoter regions could lead to transcriptional repression of genes mediated by DNMTs. For example, HBV X protein increases the level and activity of DNMT1, which reduces E-cadherin expression (18). Therefore, we studied the effects of HPV-16 E6 on-

Figure 3. Western Blots



Western blot detection of HPV-16 E6 and E7 proteins in total cell extracts from A, HCT-116 cells harboring pIRES2-EGFP-E6 and; B, pIRES2-EGFP-E7; C, lysates of HCT-116 cell line (control) and of HCT-116 cells expressing E6, E7, and E6, E7 were analyzed by western blotting using antibodies against E-cadherin and β-actin.

coprotein on DNMT1, DNMT3a, and DNMT3b mRNA levels. DNMT1 expression was increased in HCT-116 cells expressing E6 when compared with control cells (P = 0.0133; Figure 4A). There was no significant change in DNMT3a and DNMT3b mRNA levels (data not shown).

4.5. DNMT1 Expression Increases in HPV-16 E7-Expressing Cells

No significant difference was observed in DNMT3a and DNMT3b expression in HCT-116 cells expressing E7, and DNMT1 mRNA levels were increased when compared with control cells (P = 0.0113; Figure 4B). In HCT-116 cells expressing the E6 and E7 oncoproteins, DNMT1 expression was remarkably increased when compared with HCT-116 cells that were transfected with the HPV-16 E6 or E7 genes (P = 0.0482; Figure 4C). Mean, SD, and P values of DNMT1 expression are presented in Table 2.

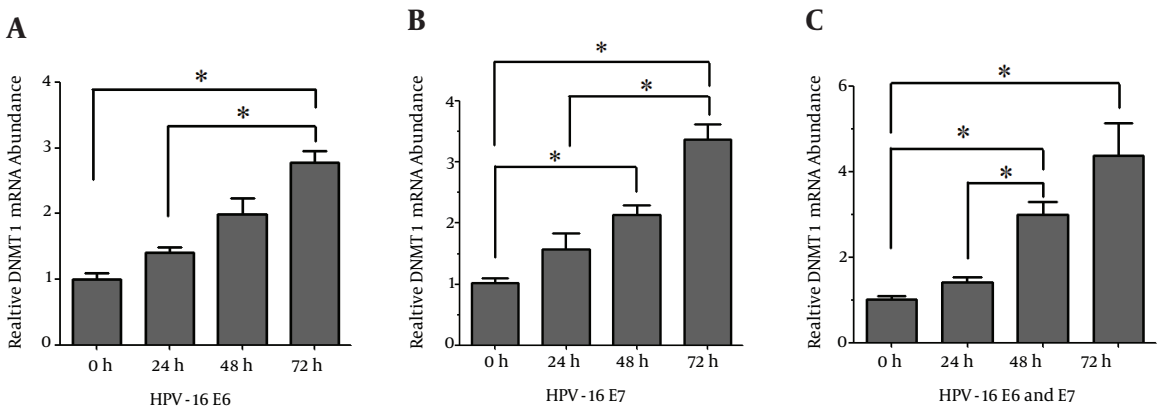
4.6. HPV-16 E6 and E7 Oncoproteins do not Change E-Cadherin Promoter Methylation

It is possible that overexpression of DNMT1 in HCT-116 cells expressing E6 and E7 results in hypermethylation of the E-cadherin promoter and reduces E-cadherin expression. To test this hypothesis, we extracted DNA from HCT-116 cells that were transfected with E6 and E7 plasmids and conducted bisulfite reaction followed by PCR cloning and bidirectional sequencing. No significant differences were observed in the 29 CpGs islands in the E-cadherin promoter in HCT-116 cells expressing E6 and E7 when compared with control cells (Figure 5A, 5B).

5. Discussion

EMT is characterized by the loss of cell-cell and cell-extracellular matrix interactions and the gain of mes-

Figure 4. DNMT1 mRNA Levels in HCT-116 Cells



A, DNMT1 mRNA levels in HCT-116 cells expressing HPV-16 E6; B, DNMT1 mRNA levels in HCT-116 cells expressing HPV-16 E7; C, DNMT1 mRNA levels in HCT-116 cells expressing HPV-16 E6, E7. Error bars represent 6 SEM. *P < 0.05.

Table 2. Statistical Analysis of DNMT1 Expression Using Unpaired, Two-Tailed Student t-test

DNMT1 Expression, HPV-16 Protein	24 Hours After Transfection	P Value	48 Hours After Transfection	P Value	72 Hours After Transfection	P Value
HPV-16 E6	1.403 (0.1310)	0.0979	1.989 (0.3563)	0.0676	2.780 (0.2573)	0.0133
HPV-16 E7	1.575 (0.3745)	0.1836	2.143 (0.2096)	0.0214	3.374 (0.3403)	0.0113
HPV-16 E6, E7	1.425 (0.1624)	0.1001	2.993 (0.4268)	0.0241	4.374 (1.074)	0.0482

enchymal properties in epithelial cells. This process is currently classified into three subtypes: Type 1 EMT is an essential process that leads to cell lineage identity during embryogenesis. Type 2 EMT is associated with inflammation and injury. The type 3 EMT developmental program enables epithelial cells to acquire invasive mesenchymal phenotype characteristics in carcinoma progression and metastasis (19). In tumor metastasis, many molecular and morphological properties of this EMT program are similar to those of the developmental EMT program. A critical molecular feature of EMT is the down regulation of E-cadherin that is expressed on the plasma membrane of most normal epithelial cells (7).

EMT has been implicated in the metastasis of primary tumors, and provides molecular mechanisms for cervical cancer metastasis (20). Previous studies have shown that carcinomas of the cervix frequently have decreased or aberrant expression of E-cadherin (8, 9).

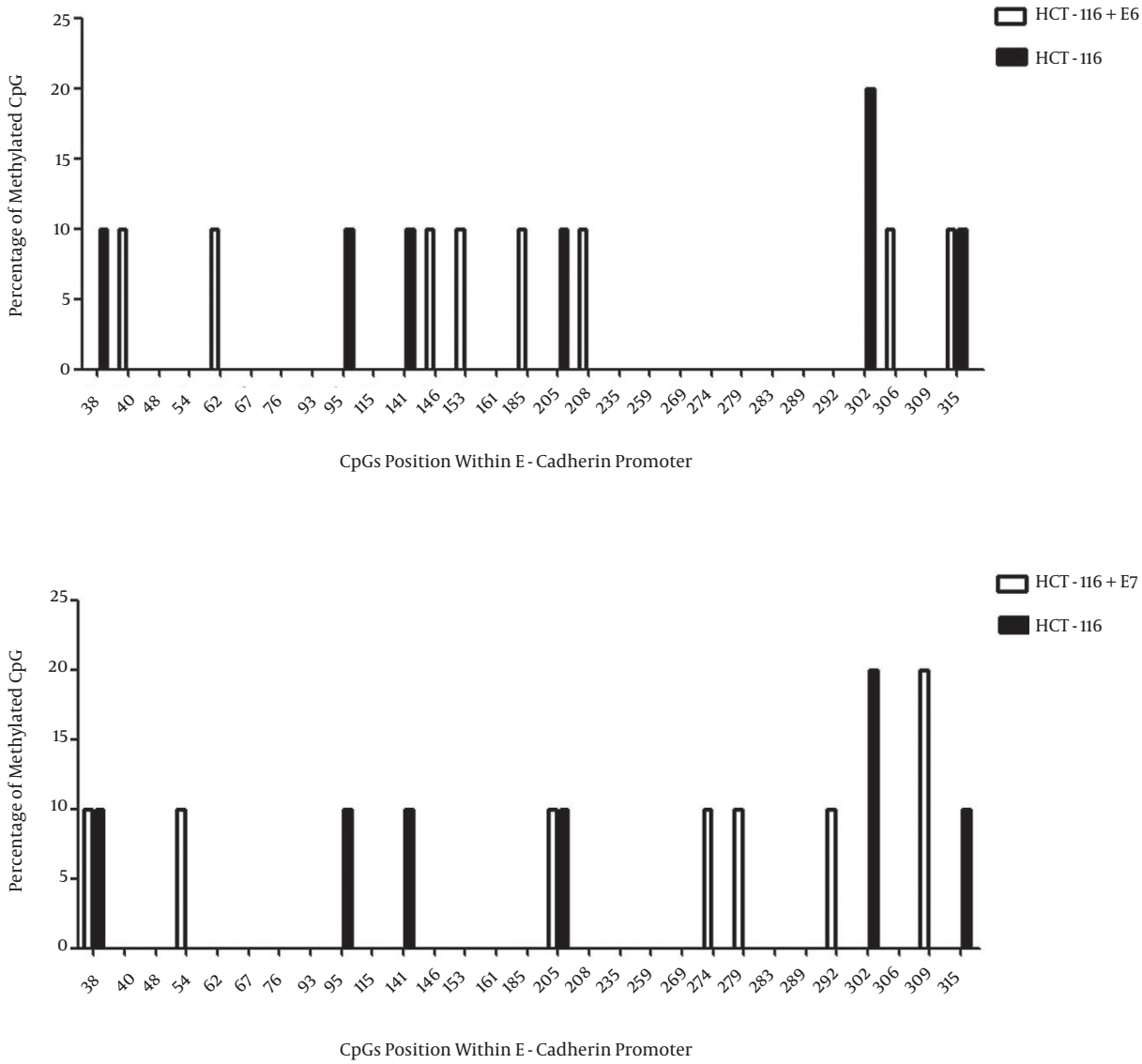
Cervical cancer develops because of the integration of high-risk HPVs in the host genome (2). A significant correlation exists between the presence of HPV DNA and cervical cancer development (21). EMT induction may directly contribute to HPV E6 and E7 viral proteins (22), and the HPV-18 E6 oncoprotein has been expressed in keratinocytes, leading to a morphological conversion to a fibroblast-like morphology (23).

According experimental studies, we repeated our experiments three times to achieve valid results, and performed real-time PCR and western blot analysis for each experiment using three samples.

The following were the inclusion criteria: cell viability over 90%, high transfection efficiency, mycoplasma-free cells, cells intact in the major E-cadherin repressor pathways such as E-box-mediated repression, presence of low levels of promoter methylation, and extraction of high quality of extracted RNA. Exclusion criteria included Mycoplasma-positive cell line, low transfection efficiency, and poor quality of extracted RNA.

Our results showed that E-cadherin expression was decreased in HCT-116 cells expressing the HPV-16 E7 oncoprotein. This finding is consistent with that of a previous study showing that HPV-16 E7 expression in MDCK cells reduced E-cadherin expression and induced EMT (24). Caberg et al. used siRNA for the E7 oncoprotein in HPV-16-transformed human keratinocytes, and showed that the suppression of HPV-16 E7 resulted in restoration of E-cadherin expression

Figure 5. The Methylation State of CpGs Within the E-Cadherin Promoter (Position -319 to +16 Relative to the Transcription Start Site)



A, The methylation state of each CpG within the E-cadherin promoter in HCT-116 cells encoding HPV-16 E6; B, the methylation state of each CpG within the E-cadherin promoter in HCT-116 cells encoding HPV-16 E7.

in these cells, and that this restoration was independent of E-cadherin suppressor transcription factors, such as SLUG and SNAIL (13). Furthermore, depletion of pRB by siRNA reduced E-cadherin expression in MCF7 breast cancer cells (25). pRB is targeted for degradation by HPV-16 E7, so it is possible that the capacity of HPV-16 E7 to reduce E-cadherin expression is linked to its ability to destabilize pRB.

HPV-16 E6 oncoprotein expression reduced E-cadherin mRNA and protein levels in HCT-116 cells. This finding is similar to that of a previous study showing that the HPV-16 E6 protein induces E-cadherin repression and that the E-cadherin promoter is repressed in cells expressing E6, causing reduced E-cadherin transcription (26).

In the present study, co-expression of HPV-16 E6 and E7 proteins in HCT-116 cells further reduced E-cadherin expression. Moreover, HPV-16 E6-expressing cells show a greater reduction in E-cadherin expression than HPV-16 E7-expressing cells, and our data suggest that HPV-16 E6 is es-

sential for reducing E-cadherin expression and inducing EMT (26).

The mechanisms by which HPV-16 E6 and E7 oncoproteins reduce E-cadherin protein are not clear. E-cadherin expression is reduced in infection with other cancer-associated viruses (HBV, HCV, and EBV), which act via transcription suppression related to the E-cadherin promoter. The X protein of HBV increases DNMT1 expression and reduces E-cadherin expression (18). Likewise, the HCV core protein activates DNMT1 and DNMT3b, and reduces E-cadherin expression (27). The LMP1 protein of EBV increases DNMT1 expression and represses E-cadherin expression (28).

We studied DNMT expression in HCT-116 cells expressing HPV-16 E6 and E7 oncoproteins, and showed that E6 and E7 increased DNMT1 expression. There was no significant change in DNMT3a and DNMT3b mRNA levels.

The augmentation of DNMT expression influences DNA methylation; therefore, we studied E-cadherin promoter methylation states in HCT-116 cells expressing E6 and E7. The bisulfite sequencing showed that these cells were not significantly different from control cells in terms of E-cadherin promoter methylation; that is, the E-cadherin promoters in HCT-116 cells expressing HPV-16 E6 and E7 oncoproteins were almost fully unmethylated, similar to control cells.

It was found that although HPV-16 E6 and E7 oncoproteins increased DNMT1 expression, their actions did not result in E-cadherin promoter methylation. Therefore, the reduction of E-cadherin expression cannot be attributed to hypermethylation of this promoter; that is, the mechanism by which HPV-16 reduces E-cadherin expression appears to be different from those of HBV, HCV, and EBV.

In conclusion, our results showed that, although HPV-16 E6 and E7 increase DNMT1 expression, they do not result in E-cadherin promoter methylation. Therefore, the reduction of E-cadherin expression is not due to E-cadherin promoter hypermethylation. Further studies should aim to investigate activities of HPV-16 E6 and E7 and uncover pathways that regulate E-cadherin expression. Knowledge about these pathways may help discover better treatment options for cervical cancer metastasis.

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Footnotes

Authors' Contribution: Ebrahim Faghihloo designed and coordinated the study and participated in examinations, writing, and final editing of the manuscript. Talat Mokhtari-Azad assisted in the study design and manuscript editing. Majid Sadeghizadeh and Shohreh Shahmahmoodi participated in the design and planning of the study. All authors have read and approve the content of the manuscript.

Conflict of Interest: All authors declare that there are no conflicts of interest.

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