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A novel function of HPV16-E6/E7 in epithelial–mesenchymal transition

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ABSTRACT

Human papillomavirus (HPV) 16 is among the most important etiological factors in many human cancers, including head and neck squamous cell carcinomas (HNSCCs) not associated with alcohol or tobacco use. HPV16-E6 and E7 oncoproteins target intracellular signaling networks, altering key molecular and cellular events during tumor progression. The present study investigates the role of HPV16-E6 and E7 oncogenes on the epithelial–mesenchymal transition (EMT), a cellular process thought to be critical for tumor cell invasion and metastasis. Using the epithelial MDCK cell line as an *in vitro* model, we show that the stable expression of HPV16-E6 or E7 induces morphological conversion from cobblestone-shaped epithelium to spindle-shaped mesenchyme-like phenotype. Consistent with these morphological changes, both E6 and E7 induce expression of the EMT-activating transcriptional factors Slug, Twist, ZEB1 and ZEB2, especially ZEBs, accompanied with switch from epithelial to mesenchymal markers. Importantly, E6 and E7 expression results in induction of the migratory and invasive potential, a functional hallmark of EMT. When we examined the association between HPV16 and the EMT signature in HNSCC cell lines derived from head and neck cancer patients, we found a correlation between HPV16 positivity and the expression of EMT transcription factor ZEB1. Taken together, our findings suggest HPV16 induces EMT-like processes via induction of the EMT transcription factors which may contribute to tumor progression and metastasis.

1. Introduction

Human papillomavirus (HPV) is a small circular double-stranded DNA virus with a tropism for squamous epithelium and is known to be the primary cause of cervical cancer [1,2]. In addition, 90% of anal cancers, 40% of vaginal, vulvar, and penile cancers as well as more than 60% of oropharyngeal cancer are related to HPV infection [3]. HPV 16 and 18, among approximately a dozen oncogenic HPV types, are most frequently associated with human cancers such as cervical cancer. HPV 16 in particular is found in approximately 90% of HPV-associated noncervical cancers including head and neck (HN) cancers [3,4]. Squamous cell carcinoma (SCC) is by far the most common histologic type in HN cancers. Despite advances in the management of HNSCC, mortality and morbidity associated with the disease continue to be high. In fact, world-wide incidence and mortality rates from HNSCC are higher than those for cervical cancer [5]. In the United States, despite the overall declining trend in the incidence of head and neck cancer reflecting the decrease in tobacco consumption, incidence of cancer from the oropharyngeal sites, especially the tonsil and the base of the tongue, are rising, most notably in ages 40–55 [6]. Recent studies identified HPV as a causative agent for oropharyngeal SCC particularly in individuals with no history of heavy consumption of alcohol or tobacco [7–9]. However, the oncogenic consequences of oropharyngeal HPV infection and its implications for the best clinical management of patients with HPV-associated HNSCC are just beginning to be understood [10].

It has been well demonstrated that the HPV early gene products E6 and E7 play crucial roles in HPV-mediated carcinogenesis by targeting tumor suppressor p53 and retinoblastoma protein (pRb), respectively. E6 binds to the ubiquitin/protein ligase E6AP and p53, resulting in ubiquitination of p53 and its subsequent proteolytic degradation. The E7 protein, on the other hand, promotes cell cycle progression by destabilizing the pRb-E2F complex [11,12].

HNSCC patients with distant metastasis have extremely poor prognosis with mortality rate of approximately 90% [13]. Although E6 and E7 regulation of cell survival and growth are fairly well studied, their effects on tumor cell invasion and metastasis are not well understood. While normal epithelial cells are stationary, carcinomas often lose their epithelial characteristics and acquire more migratory mesenchymal properties through epithelial–mesenchymal transition (EMT). EMT-regulating genes are often conserved in development of different organs and throughout evolution. Importantly, studies show that carcinoma cells can reactivate the developmental EMT-like process during tumor cell

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invasion and metastasis [14]. Gene expression profiling studies suggested activation of the NF-B pathway, EMT, and deregulation of cell adhesion as prominent genetic alterations for the development and progression of HNSCC [15]. A recent study reported that primary HNSCC tumors with EMT signatures have a twofold increase in the average tumor satellite distance compared to primary HNSCC tumors without an EMT signature [16]. Interestingly, HPV positive HNSCC tends to be poorly differentiated and often diagnosed with regional spread [17]. Although HPV is an important etiological factor in oropharyngeal HNSCC, it is unknown whether HPV oncogenes regulate its invasive phenotype.

In the present study, we determined the effects of HPV16-E6 and E7 on the EMT-like process utilizing the Madin-Darby canine kidney (MDCK) cell line, a well-known *in vitro* model for study of EMT. In addition, we examined the expression levels of EMT-related markers in HNSCC cell lines derived from patients with known HPV status. We report herein that both E6 and E7 induces the expression of EMT transcription factors including SLUG, TWIST, ZEB1 and ZEB2, resulting in the down-regulation of epithelial marker E-cadherin and concomitant up-regulation of the mesenchymal markers N-cadherin, fibronectin, and vimentin. Functionally, both E6 and E7 are capable of inducing fibroblast-like morphological changes of MDCK cells and more importantly promoting cell migration and invasion. In patient-derived HNSCC cell lines, expression levels of EMT genes, in particular ZEB1, were significantly higher in HPV16 positive HNSCC cell lines, validating our findings using MDCK cells.

2. Materials and methods

2.1. Reagents and antibodies

Mitomycin C, anti-vimentin mAb, and peroxidase conjugated antibodies against mouse or rabbit IgG were purchased from Sigma–Aldrich (St. Louis, MO). Anti-E-cadherin mAb was purchased from BD Transduction Laboratories (San Jose, CA). Anti-GAPDH mAb was purchased from Santa-Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture

Canine kidney epithelial MDCK cells were purchased from the American Type Tissue Collection (ATCC) and cultured DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin (Life Technologies Inc., Carlsbad, CA) at 37 °C in a humidified incubator with 5% CO_2. Five human head and neck squamous cell carcinoma cell lines were established from specimens of head and neck squamous cell carcinoma patients at the University of Pittsburg (UP-SCC-090, UP-SCC-154), University of Michigan (UM-SCC-19), and Wayne State University (WSU-HN-12). UP-SCC cells were grown in MEM containing glutamine, nonessential amino acids, gentamicin, and 10% FBS in a humidified atmosphere of 5% CO_2 at 37 °C. UM-SCC cells were cultured in DMEM containing glutamine, nonessential amino acids, and 10% FBS in a humidified atmosphere of 5% CO_2 at 37 °C. WSU-HN cells were maintained in the RPMI1640 containing glutamine and 10% FBS in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Establishment of HPV16-E6 and E7 overexpressing MDCK cells

The following human β-actin promoter expression plasmids from Addgene (Cambridge, MA), a non-profit plasmid repository, were used: p1322 (HPV16-E6), p1324 (HPV16-E7), p1321 (HPV16-E6/E7). Target HPV DNA sequences were obtained by using HindIII and SalI restriction enzymes. The resulting inserts were cloned into HindIII and XhoI sites of pcDNA3.1 vector. The pcDNA3.1-control, pcDNA3.1- E6, pcDNA3.1-E7, and pcDNA3.1-E6/7 vectors were transfected into MDCK cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Subsequently, cells were subjected to 400 μg/ml G418 antibiotic selection for 14 days and pooled for further analysis.

2.4. Immunoblot analysis

Cells were lysed with ice-cold RIPA buffer (0.05 M Tris–HCl, pH 7.4, 0.15 M NaCl, 0.25% deoxycholic acid, 1% NP- 40, 1 mM EDTA) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM sodium metavanadate ($NaVO_3$), 1 mM sodium fluoride (NaF), and protease inhibitor cocktail (Roche). Protein concentration was determined using the BCA procedure (Thermo Fisher Scientific, Pittsburgh, PA). Equal amounts of protein samples in SDS sample buffer [1% SDS, 62.5 mM Tris–HCl (pH 6.8), 10% glycerol, 5% (β-mercaptoethanol, and 0.05% bromophenol blue] were boiled for 5 min and subjected to reducing SDS–PAGE. After electrophoresis, the proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in 100 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.2% Tween-20 (T-TBS) for 1 h at room temperature. The membranes were incubated with T-TBS containing 5% milk and the primary antibodies. After three washes with T-TBS, the blot was incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. The antigen was detected using the Western Blot Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Inc., Boston, MA).

2.5. Semi-quantitative RT-PCR

mRNA was purified from cells using the RNeasy kit (Qiagen, Valencia, CA, USA). cDNA synthesis was performed with Superscript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA), followed by PCR using GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA). Forward and reverse human papillomavirus type 16-specific forward and reverse primers used are as follows: E6: 5′-ACCCACAGGAGCGACCCAGA-3′, 5′-ACCGGTCCACCGACCCCT-TA-3′; E7: 5′-TGAAATAGATGGTCCAGCTGG-3′, 5′- TGCCCATTAA-CAGGTCTTCC-3′.

2.6. Real time RT-PCR

Real time RT-PCR was performed using QPCR SYBR Green Low ROX Mix (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. Relative values of gene expression were normalized to GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = (\Delta Ct_{target\ gene} - \Delta Ct_{GAPDH})_{sample} - (\Delta Ct_{target\ gene} - \Delta Ct_{GAPDH})_{control}$. The fold change in relative expression was then determined by calculating $2^{-\Delta\Delta Ct}$. The sequences of dog and human primers for E-cadherin, N-cadherin, fibronectin, vimentin, Slug, Snail, Twist, ZEB1, and ZEB2 are provided in Supplementary Tables 1 and 2.

2.7. Cell migration and invasion assay

For the scratch wound cell migration assay, cells were grown to 90% confluence in complete medium in a 6-well plate and pre-treated with mitomycin C (25 μg/ml) for 30 min before an injury line was made using a 2-mm-wide plastic pipette tip. After rinsing with PBS, cells were allowed to migrate in serum-free media for 16 h, and photographs were taken (×40) to assess cell motility. The *in vitro* invasive property of cells was assessed using a modified Boyden chamber assay. A total of 1×10^5 cells were placed in the upper compartment of the invasion chamber (BD BioCoat Matrigel

Invasion Chamber, BD Biosciences, Bedford, MA). The chambers were incubated for 16 hr at 37 °C. The filters were then stained with crystal violet. Quantification of the migration or invasion assay was performed by counting the number of cells on the lower surface of the filters.

2.8. Statistical analysis

All results are expressed as mean ± s.d. of triplicates in three independent experiments. Samples were analyzed by one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple range test (parametric). The acceptable level of significance was established at $P < 0.05$.

3. Results

3.1. HPV16-E6. and E7 induce an EMT-like process

To determine the role of HPV16-E6 and E7 oncogenes on the EMT-like process, MDCK cells that stably express E6 (MDCK-E6), E7 (MDCK-E7), both E6 and E7 (MDCK-E6/E7) or control vector (MDCK-neo) were established as described in Section 2. Expression of E6 and/or E7 oncogenes was confirmed by reverse transcription-PCR analysis (Fig. 1B). As shown in Fig. 1A, MDCK-neo cells retained the morphological characteristics of clustered cobblestone-shaped epithelial cells, whereas MDCK-E6, MDCK-E7, and MDCK-E6/E7 cells exhibited a scattered fibroblast-like spindle shape (Fig. 1A). To determine whether these morphological changes are associated with EMT, we examined the expression of epithelial and mesenchymal markers. Consistent with EMT, either E6 or E7 expression or both E6/E7 expression resulted in downregulation of the epithelial marker E-cadherin and upregulation of the mesenchymal marker vimentin (Fig. 1C).

To quantitate the EMT-like process, we performed real-time RT-PCR analysis for EMT marker genes and EMT master transcription factors. As shown in Fig. 2A, E-cadherin mRNA expression was drastically reduced upon E6 or E7 expression. In contrast, the expression levels of mesenchymal markers N-cadherin, fibronectine and vimentin were significantly higher in MDCK-E6, MDCK-E7, and MDCK-E6/E7 cells compared to those in the control MDCK-neo cells. Consistent with the EMT markers, E6 and/or E7 induced the expression of the EMT-activating transcriptional factors SLUG, TWIST, ZEB1, and ZEB2, but not SNAIL expression (Fig. 2B).

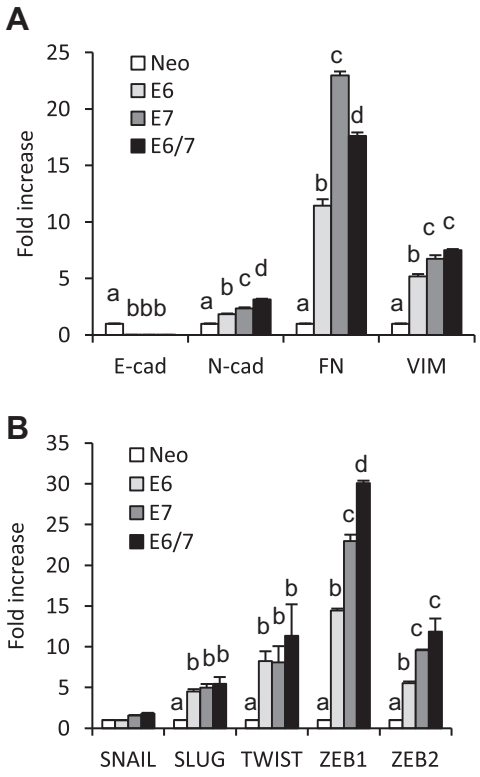

Fig. 2. HPV16-E6 and/or E7 regulate EMT markers and induce EMT-activating transcriptional factors. (A) RNA expression of EMT markers such as E-cadherin, N-cadherin, fibronectin, and vimentin was analyzed using quantitative RT-PCR (B) RNA expression of EMT-activating transcriptional factors Slug, Snail, Twist, ZEB1, and ZEB2 was analyzed using quantitative RT-PCR. Each bar represents the mean ± s.d. Means with different letters (a, b, c, d) are significantly different from one another at P value < 0.05 (ANOVA followed by Newman–Keuls test).

3.2. HPV16-E6 and E7 promote migratory and invasive phenotypes

Epithelial cells undergoing EMT lose their adhesive phenotype and concomitantly acquire a migratory phenotype, a functional

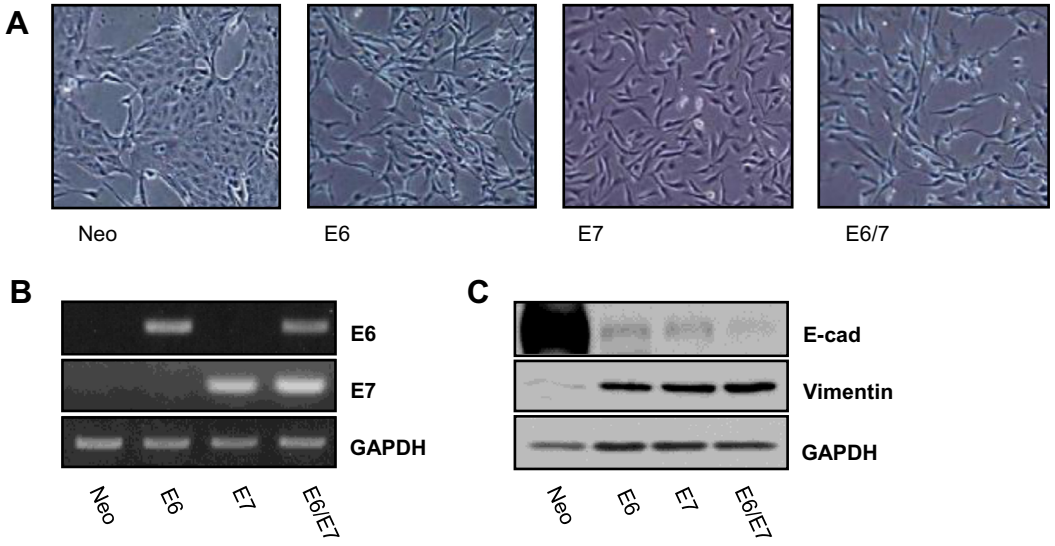

Fig. 1. Stable HPV16-E6 and/or E7 expressing MDCK cells show morphological changes. (A) MDCK cells transfected with vector control (Neo) show typical epithelial cell morphology, while MDCK cells transfected with vector encoding HPV16-E6, E7, and E6/7 show fibroblastic morphology. (B) RT-PCR analysis of E6, E7 expression in the MDCK cells after G418 selection. (C) Immunoblot analysis of E-cadherin and vimentin in MDCK-Neo, -E6, -E7 and –E6/7 cells.

hallmark of EMT [14]. When we examined the effects of E6 and/or E7 on the migratory and invasive phenotype, MDCK-E6, MDCK-E7, and MDCK-E6/E7 cells displayed a more motile phenotype, as assessed by a scratch wound migration assay, compared to MDCK-Neo cells (Fig. 3A). Similarly, the Matrigel transwell invasion assay showed a more invasive phenotype in MDCK-E6, MDCK-E7 and MDCK-E6/E7 cells compared to the control MDCK-neo cells (Fig. 3B). These results suggest that HPV-mediated carcinogenesis involves E6 and E7-induced EMT-like process, resulting in a more motile and invasive phenotype, a functional consequence of EMT critical for tumor cell invasion and metastasis.

3.3. HPV positivity is associated with the EMT transcription factor ZEB1 in HNSCC cells derived from head and neck cancer patients

To evaluate the clinical relevance of the above results, we examined the relationship between the HPV 16 positivity and the EMT signatures in 5 established HNSCC cell lines derived from head and neck cancer patients. Characteristics of these cell lines are summarized in Table 1. Expression of E6 and E7 oncogenes in HPV-positive cell lines UPCI-SCC090 and UPCI-SCC154 was confirmed by RT-PCR analysis (Fig. 4A). Although not all the EMT transcription factors are elevated in HPV-positive cell lines, ZEB1 and ZEB2 expression, especially ZEB1, was significantly higher in HPV-positive cell lines UP-SCC-090 and UP-SCC-154, compared to HPV-negative cell lines UPCI-SCC003, UM-SCC19, and WSU-HN12 (Fig. 4B). It should be noted that no correlation was observed between HPV 16 positivity and EMT marker gene expression such as E-cadherin, N-cadherin, fibronectin and vimentin (Fig. 4C).

4. Discussion

In the present study, we demonstrated a novel function of HPV16-E6/E7 oncoproteins in inducing the expression of EMT activating transcriptional factors leading to activation of the EMT-like process in MDCK cells. The ZEB family (ZEB1, ZEB2), the Snail superfamily of zinc finger transcription factors (Slug, Snail), and the Twist family of bHLH factors are the typical developmental

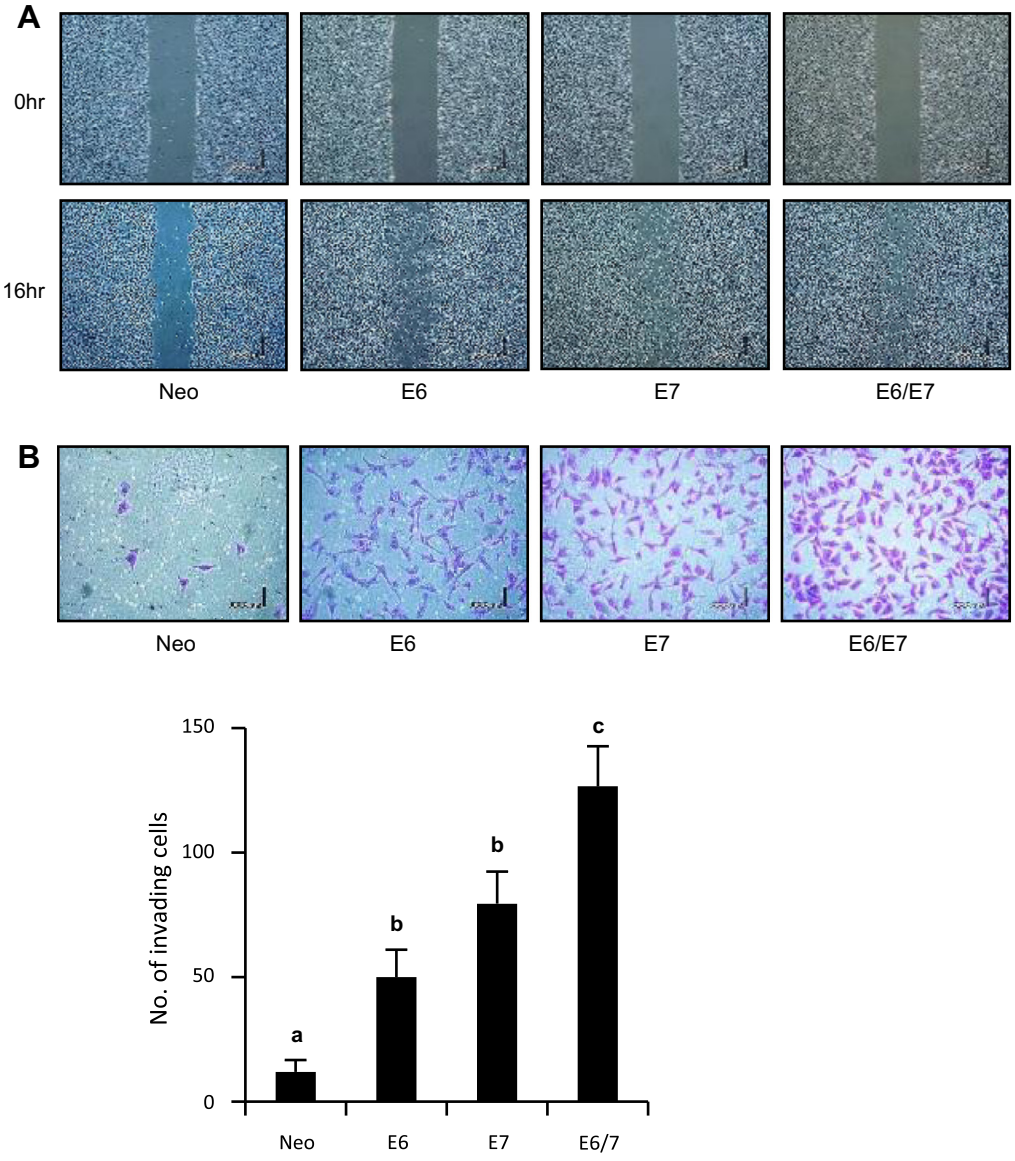

Fig. 3. HPV16-E6 and/or E7 induce motility and invasion of MDCK cells. (A) A scratch migration assay was performed in MDCK-Neo, -E6, -E7, -E6/7 cells for 18 h. (B) Cell invasion was assessed using a BioCoat Matrigel invasion chamber for 16 h. Invading cells were counted and plotted as mean ± s.d. Means with different letters (a, b, c) are significantly different from one another at P value < 0.05 (ANOVA followed by Newman–Keuls test).

Table 1
Features of HNSCC cell lines derived from patients.

Name	HPV16	p53 status	Site	Primary	Stage	Differentiation	Gender
UPCI-SCC003	–	Wild type	Tonsil	New	T1N0	Well	Female
UM-SCC19	–	Deletion	Base of tongue	New	T2N1	Moderate-Poor	Male
WSU-HN12	–	Truncated	Base of tongue	New	T4N1	No data	No data
UPCI-SCC090	+	Wild type	Base of tongue	Recurrence	T2N0	Poor	Male
UPCI-SCC154	+	Wild type	Tongue	New	T4N2	Poor	Male

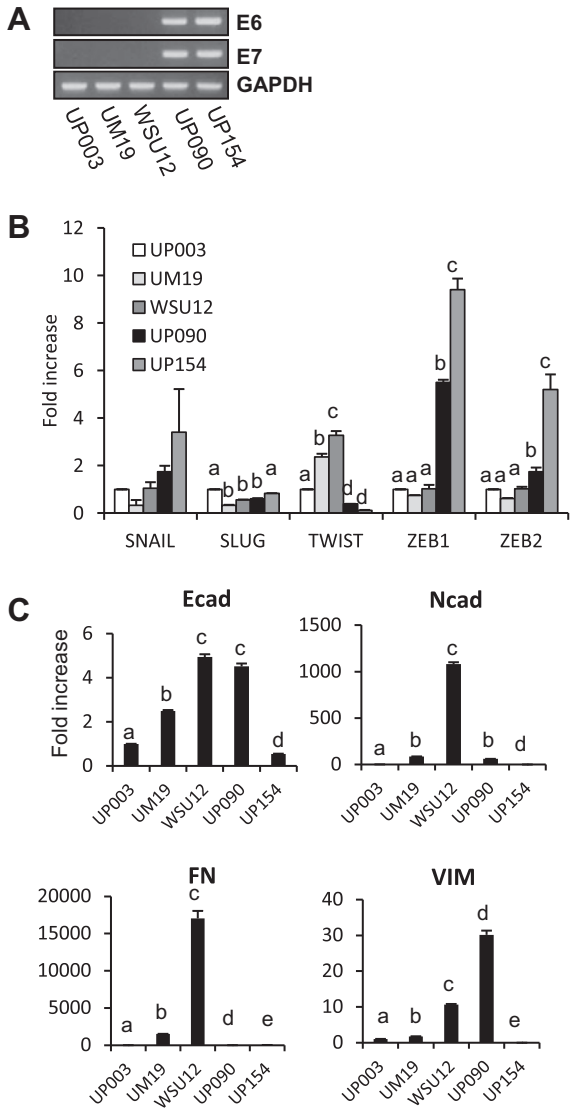

Fig. 4. Differential expression of EMT markers and related transcriptional factors between HPV16 negative- and positive HNSCC cell lines. (A) RT-PCR analysis of E6, E7 expression in the HNSCC cell lines established from patients. (B) RNA expression of EMT-activating transcriptional factors Slug, Snail, Twist, ZEB1, and ZEB2 was analyzed using quantitative RT-PCR. (C) RNA expression of EMT markers such as E-cadherin, N-cadherin, fibronectin, and vimentin was analyzed using quantitative RT-PCR. Each bar represents the mean ± s.d. Means with different letters (a, b, c, d, e) are significantly different from one another at P value < 0.05 (ANOVA followed by Newman–Keuls test).

EMT master transcription factors that bind to the promoters of epithelial and mesenchymal specific genes for the regulation of promoter activities [18–20]. Among several EMT master transcription factors tested in MDCK cells, ZEB1 is the most significantly upregulated by E6 or E7 expression. Consistently, we found that ZEB1 is greatly increased in HPV16-positive HNSCC cell lines derived from head and neck cancer patients. It is thought that many of the molecular and cellular changes such as gene expression, cell adhesion and migratory phenotype, associated with normal developmental EMT are recapitulated during human cancer progression. These changes are often transient and regulated by somewhat redundant, but distinct members of the EMT master transcription factors depending on the genetic background of the cells and extracellular factors. Recent evidence indicates that the ZEB factors are downstream of the Snail and Twist families for the regulation of EMT [21]. The hierarchical Snail-Twist/ZEB relationship supports an earlier report of the strongest correlation between the ZEB factors, especially ZEB1, and E-cadherin loss and EMT across cancer cell types [22]. Although the oncogenic activities of HPV16 in human cancers are well-recognized, only a few studies have investigated the EMT processes, mostly focusing on HPV16-E6 and -E7 regulation of E-cadherin. E-cadherin expression in the epidermis was shown to be reduced or lost during HPV16 infection, which is associated with depletion of Langerhans cells at the site of infection [23,24]. *In vitro* studies also showed that cell surface E-cadherin expression is reduced upon E6 or E7 expression, implicating a role for E6 and E7 for the suppression of E-cadherin expression [23,25]. Recent studies suggested that E-cadherin expression is silenced by DNA hypermethylation via HPV16-mediated DNA methyltransferase 1 (DNMT1) activity [26,27]. Here, we found that the EMT-activating transcriptional factors, especially ZEB1, are increased by E6 and E7. These EMT transcription factors are well known to bind to the E-box motif of E-cadherin promoter and suppresses its activity [20]. Although these EMT transcription factors are shown to regulate expression of the EMT-related markers at the level of transcription, mounting studies emphasized the existence of multifactorial regulation of the EMT markers at the levels of DNA, RNA, and protein, which may partially explain inconsistent correlation between the EMT transcription factor and EMT markers such as E-cadherin, N-cadherin, fibronectin, and vimentin in human tumor tissues in our study [20].

HNSCC includes numerous sub-sites, such as nasopharyngeal, oropharynx, oral, and larynx which represent distinct microenvironments for tumor progression. These tumor-microenvironmental interactions, in addition to the genetic background of the cells, are likely to influence HPV oncogene interactions with the signaling networks and cellular events such as EMT. Thus, further research is warranted to better understand the interactions between the HPV16 oncogenes and EMT process. The subsequent functional significance may provide molecular insights into the invasive and metastatic tumor cell program between HPV-negative and -positive HNSCC.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.04.060.

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