Human papillomavirus 16/18 E5 promotes cervical cancer cell proliferation, migration and invasion in vitro and accelerates tumor growth in vivo

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Received July 16, 2012; Accepted August 23, 2012

DOI: 10.3892/or.2012.2106

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Abbreviations: HR-HPV, high-risk human papillomavirus; RT, reverse transcriptase; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel; CIN, cervical intraepithelial neoplasm; FACS, fluorescence activated cell sorter; p-paxillin, phosphorylated paxillin

Key words: human papillomavirus, E5, cervical cancer, proliferation, migration, invasion

Abstract. High-risk human papillomaviruses (HR-HPVs) are consistently associated with human cervical cancer. Additionally, the early oncoproteins of HPVs E5, E6 and E7 are known to contribute to tumor progression. The role of E5 is still nebulous. In this study, we aimed to explore the mechanism of E5 action during the human cervical carcinogenesis process. We created four cell models overexpressing HPV16 or HPV18 E5 (HPV16/18 E5) and investigated their ability to proliferate, along with their metastatic characteristics such as migration and invasion. The expression of HPV16/18 E5 protein in various cell lines was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). In addition, we compared the levels of phosphorylated paxillin as well as E-cadherin in cell models and controls by western blot analysis. Finally, we assessed the tumor growth rate of human cervical cancer cells overexpressing HPV16/18 E5 in vitro. We discovered that the expression of HPV16/18 E5 consistently increased the malignant potential of various human cervical cancer cells compared with the primary counterparts. We demonstrated the involvement of HPV16/18 E5 in proliferation, migration, invasion and regulation of the actin cytoskeleton in human cervical cancer cells.

In particular we discovered that HPV16/18 E5 overexpression in human cervical cancer cells correlated with higher levels of paxillin proteins phosphorylated on tyrosine residues and with the downregulation of E-cadherin. Importantly, injection of HPV16/18 E5-overexpressing human cervical cancer cells into mice increased both HPV-and non-HPV-derived tumor growth. Collectively, our data indicate that HPV16/18 E5 influences progression of the human cervical cancer malignant phenotype. This study provides new insights into HPV16/18 E5 as a possible agent that may have an impact on the therapeutic strategies targeting human cervical cancer.

Introduction

Human cervical cancer progression is strongly associated with the infection of high-risk human papillomaviruses (HR-HPVs) (e.g. HPV16 and 18), which are detected in nearly all human cervical cancer (1). HPV is a small, non-enveloped DNA virus expressing three key oncoproteins: E5, E6 and E7, which possess the ability of transforming certain human cells in vitro and are considered to be associated with human cervical carcinogenesis in vivo (2). E6 and E7 are well known for their ability to inhibit the function of tumor suppressor p53 and pRb, respectively (3). However, the role of E5 in cellular transformation is less understood. HPV16 E5 protein is a hydrophobic, 83-amino acid polypeptide that associates with the Golgi apparatus, endoplasmic reticulum and perinuclear membrane (4). E5 is capable of altering growth and differentiation of epithelial cells via a number of pathways, including conferring resistance to apoptosis and affecting several cellular pathways involved in cell adhesion and cell motility signaling (2,5,6). Co-expression of E5 with either E6 or E7 promotes transformation to a greater extent than with either oncoprotein alone (7). Although, multiple studies (8,9) strongly suggest that E5 plays an important role in carcinogenesis at the early stage of HPV-related cancer, the involvement of E5 oncoproteins in multi-step carcinogenesis remains poorly understood and is an intriguing area of active study.

In the present study, we prepared four different cell models to examine the function of the HR-HPV E5 protein. The SiHa and HeLa cell lines were stably transfected with the whole
length of the HPV16 E5 or HPV18 E5 gene, respectively. Meanwhile, we transfected C33A (an HPV-negative human cervical cancer cell line) and HaCaT cells (immortal human keratinocytes used as non-related control) with the whole length HPV16 E5, then screened for stably expressed clones. We demonstrated that E5 protein and mRNA levels increased in the transfected cells compared with the controls. We proved that E5 promotes cell proliferation, migration and invasion in the positive groups. In addition, using an in vivo mouse tumor model, we provided evidence that increased E5 expression promotes both HPV or non-HPV-derived tumor growth. Collectively, our findings indicate that HPV16 E5 plays a critical role in human cervical cancer progression.

Materials and methods

Construction of recombinant DNA expression vectors. HPV16/18 E5 genes were amplified using PCR from plasmid pBR322-HPV16/18 that included the complete genome of HPV16/18 (kindly provided by Professor H. zur Hausen, Heidelberg University, Germany). PCR was performed using high-fidelity DNA polymerase (Invitrogen Life Technologies, USA) in a 50-µl reaction mixture with primers as shown in Table 1 (10). The reaction conditions were 30 cycles of denaturation at 94˚C for 30 sec, annealing at 56˚C for 30 sec and extension at 72˚C for 45 sec. E5 PCR products were ligated into pEGFP-C1 (Clontech, USA) downstream of their respective CMV promoters. Plasmids were transformed into E. coli DH5α competent cells and selected by kanamycin or ampicillin resistance. Positive colonies were screened by PCR then sequenced to confirm the identity of the DNA inserts.

Preparation of stable cells expressing HPV16/18 E5. Unless specified, all chemicals used in this study are from regular commercial sources. Cells were maintained at 37˚C in a 5% CO2-95% O2 atmosphere. SiHa (human cervical cancer cell line which contains an integrated HPV16 genome from ATCC HTB-35™), HeLa (human cervical cancer cell line which contains HPV18 sequences from ATCC CCL-2™), C33A (human cervical cancer cell line which is negative for HPV DNA and RNA from ATCC HTB-31™, to eliminate potential effects of other HPV parts as the controls) and HaCaT cell lines (immortal human keratinocytes from the Wuhan University Typical Object Preserve Center, China, as the controls) were grown in DMEM containing 10% FBS and supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin and 0.25 µg/ml of amphotericin B. Cells were transfected with E5-containing plasmids (HPV16 E5 was transfected to SiHa, C33A and HaCaT cells, and HPV18 E5 was transfected to HeLa cells) or empty vectors (as mock groups) using Lipofectin™ (Invitrogen Life Technologies) and grown in DMEM/FBS containing 600-1,000 mg/l G418 (a dose cytotoxic for non-transfected cells within 1 week) for 3 weeks. Individual colonies of G418-selected cells were isolated and expanded in DMEM/FBS. Cell lines were examined for E5 mRNA expression using an adaptation reverse transcriptase-polymerase chain reactions (RT-PCR) described by Biswas et al (11). The cells were tested for the presence of the GFP-E5 fusion protein (no commercial E5 antibody) by western blot analyses as described below.

Cell lysis and western blot analyses. Cell lysates were prepared in lysis buffer. Lysates normalized for protein content (Bradford protein assay; Bio-Rad) were prepared in Laemmli buffer, heated, subjected to sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) on 10% acrylamide gels and transferred onto nitrocellulose membranes. For western blot analyses, membranes were blocked in TBS (Tris-buffered saline) and then incubated overnight at 4˚C with indicated primary antibodies [anti-p-paxillin antibody (Tyr118; Cell Signaling Technology, Inc., USA), anti-E-cadherin antibody (BD Biosciences, USA), and anti-β-actin antibody (Sigma-Aldrich, USA) to control equal loading appropriately diluted in the blocking solution. Finally, a signal was detected using ECL. Western blot detection system (GE Healthcare, UK) and exposure to XR film (12).

Proliferation assays. Cell proliferation assays were performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Cells (1x10⁴) were plated in 96-well tissue culture plates and grown to 70% confluence. Seventy-two hours later the cells were stained with 5 mg/ml MTT reagent (Sigma-Aldrich). The absorbance of each well was measured at a wavelength of 570 nm and data were expressed as a raw OD at 570 nm or as a ratio of OD at a specific time point over the initial OD on the first day. Wells without cells but containing medium were used as a blank value that was subtracted from all values. Each assay was performed in triplicate.

Wound healing assays. Cell migration was assessed in classical wound healing assays (12). Confluent monolayer cells in a 6-well plate were wounded using a plastic pipette tip (P200) and rinsed with PBS before being replaced in the culture medium. The bottoms of the wells were marked to indicate where the initial images of the wounded area were captured. After 24-h incubation at 37˚C, images (x10) of the same areas were recorded using an Axiovert 200M microscope (Zeiss) equipped with a CoolSNAP™ ES camera (Photometric®, Roper Scientific) and closure of the wound was evaluated using Metamorph® (V6.3; Molecular Devices Corp.).

Cell invasion assays using Transwells. In vitro invasion was determined in a Matrigel-based Transwell assay essentially as previously described by Pelletier et al (13). The upper chambers of 24-well Transwell plates (Corning Costar, Cambridge, MA, USA) with a pore size of 8 µm were coated with Matrigel (0.7 mg/ml; Sigma-Aldrich) and the lower compartments were filled with serum-free conditioned DMEM of NIH3T3. HPV16 or HPV18 E5 (HPV16/18 E5)-positive cells (1x10⁵) re-suspended in a 10% serum-containing medium were added to the top chamber of the Transwell (8 µm, BD Biosciences, Franklin Lakes, NJ, USA) pre-coated with Matrigel™ diluted in ice-cold PBS (175 µg/ml) at a total of 35 µg/well and allowed to migrate for 24 h. Then, the total number of invasive cells were estimated using Calcein AM (BD Biosciences) as recommended by the manufacturer.

Immunofluorescence. Cells were stably transfected with pEGFP-C1 or GFP-E5 and fixed as usual, followed by nuclear staining with phospholine iodide (PI). To observe the cytoskeleton, cells were blocked with 2.5% BSA plus 1% goat serum
and doubled stained for F-actin using tetramethylrhodamine B isothiocyanate-phalloidin (Sigma-Aldrich). Cells were examined for fluorescence using laser scanning microscopy (Olympus FluoView FV1000).

**Tumor growth in vivo.** HPV16/18 E5-positive cells were grown in DMEM/FBS to 80% confluency. Cells (5x10^5) resuspended in 200 µl of physiological saline were injected subcutaneously in the right flank of 6-week-old nude mice (Chinese Medical Science College, Experiment Animal Research Center, China) (n=5 for each group). All studies involving mice were approved by the Huazhong University of Science and Technology Animal Care and Use Committee. Tumor development was followed for 10 weeks. Mice were monitored twice a week for tumor growth.

**Table I.** HPV16/18 E5 PCR primers.

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growth. Tumor size was measured every week with calipers to assess tumor volume ($\text{length} \times \text{width}^2/2$). The incidence of the tumors and the survival of mice were recorded.

Statistical analysis. Statistical analyses and graphical presentations were conducted by SPSS 13.0 and SigmaPlot 10.0. Results are presented as the means ± standard error of the mean (SEM). Statistical analysis of significance was based on analysis of variance or $\chi^2$ analysis; $P$-values <0.05 were considered to indicate a statistically significant difference.

Results

Analysis of the expression level of E5 before and after transfected in different cell models. To explore the role of HPV16/18 E5, we prepared four cell lines stably expressing HPV16/18 E5 as described in Materials and methods. As a result, HPV16/18 E5 was expressed as a GFP fusion protein.

To determine the expression of HPV16/18 E5 before and after transfection, we compared both instances by RT-PCR. As shown in Fig. 1A, we observed a high level of HPV16/18 E5 in all the transfected cells. HPV18 E5 also appeared at low levels in the mock (transfected with empty plasmids, as the blank control) and untransfected HeLa cells. To further substantiate these results the protein extracts were blotted with the GFP antibody. We discovered bands with similar molecular weights (GFP+E5) as shown in Fig. 1B. Further analyses of the subcellular localization of the GFP+E5 fusion protein in HPV18 E5 stably transfected HeLa cells revealed that most of the GFP+E5 fluorescence accumulated at the perinuclear region of the cells (Fig. 1C).

HPV16/18 E5 promotes cell proliferation. To demonstrate the role of HPV16/18 E5 in cell functions, we first examined whether E5 regulates cell proliferation in different cell lines. We found that overexpression of HPV16/18 E5 significantly enhanced cell proliferation and showed higher proliferative abilities compared to its paired groups (Fig. 2). As expected, we found no significant difference in HPV16 E5 and HPV18 E5 (Fig. 2A).

To assess whether other HPV parts affect cell proliferation, we used C33A-HPV16 E5 as HPV negative controls and HaCaT-HPV16 E5 as the non-related controls to eliminate any influence from other HPV parts. As demonstrated in Fig. 2B-D we demonstrated that the cell proliferation ability had no significant difference in the HPV-positive or -negative groups. Together these results indicate that HPV16/18 E5 contributes to the control of cell proliferation.

HPV16/18 E5 modulates migration and invasion of human cervical cancer cells. Cancer progression involves the transformation of cells to acquire motility and invasive abilities (12). Therefore, we next explored whether HPV16/18 E5 is critical
to human cervical cancer cell migration and invasion. We compared the migration of SiHa-HPV16 E5 with SiHa-GFP cells and the untreated SiHa cells in wound healing assays. As shown in Fig. 3A and B, increasing levels of HPV16 E5 in human cervical cancer cells promoted migration and this was significant in SiHa-HPV16 E5 groups compared with the
controls. We then compared the migration of HeLa-HPV18 E5 cells with HeLa-GFP cells or the untreated HeLa cells and obtained similar results.

To determine whether the expression of HPV16/18 E5 promotes invasion in a tumor-like context, we evaluated invasion of human cervical cancer cells through Matrigel™ matrix in Transwells assays with a collagen type I matrix (Fig. 3C). As expected, human cervical cancer cells expressing HPV16/18 E5, including SiHa-HPV16 E5 and HeLa-HPV18 E5 cells, grew significantly outward after 48 h in culture. In contrast, in empty plasmid-transfected cells (SiHa-GFP and HeLa-GFP) or untreated SiHa and HeLa cells the number of invasive cells were less.

To explore the mechanism involved we studied whether HPV16/18 E5 affected the expression of E-cadherin. E-cadherin expression in cancer cells is associated with cancer progression, invasion, metastasis and cytoskeleton rearrangement (14). E-cadherin expression was significantly downregulated in SiHa-HPV16 E5/HeLa-HPV18 E5 cells compared to the control cells (Fig. 3D). Together these results suggest that HPV16/18 E5 promotes cell migration and invasion in human cervical cancer cells through the E-cadherin pathway.

HPV16/18 E5 affects the cytoskeleton of human cervical cancer cells. The actin cytoskeleton is critical for maintaining cell morphology and is required for cell motility. HPV16/18 E5 played an important role in regulating cell proliferation, migration and invasion as demonstrated above. Furthermore, we compared actin staining in SiHa cells expressing either GFP+E5 (Fig. 4A) or GFP (Fig. 4B). We discovered that in SiHa-HPV16 E5 cells stably transformed by E5, the phalloidin staining revealed multiple heavy actin cables brightly stained (Fig. 4A) whereas vector-transfected SiHa-GFP cells contained only a few thin actin fibers (Fig. 4B). We compared the actin cytoskeleton of HeLa-HPV18 E5 with HeLa-GFP cells, and obtained exactly the same results.

Tyrosine phosphorylation of paxillin (p-paxillin) plays a key role in the regulation of actin cytoskeleton organization (15). As expected, we demonstrated the increased expression of p-paxillin in SiHa-HPV16 E5/HeLa-HPV18 E5 cells as compared to the control cells (Fig. 4C). Collectively, these results revealed that the increased expression of HPV16/18 E5 in human cervical cancer cells significantly enhance cell motility.

HPV16/18 E5 promotes tumor growth in vivo. To establish the biological relevance of our findings (12), we examined whether the overexpression of HPV16/18 E5 in human cervical cancer cells confers tumorigenic advantage in subcutaneous tumors using a mouse model. To examine this, SiHa-HPV16 E5 and HeLa-HPV18 E5 cells, parental SiHa or HeLa cells, SiHa-GFP or HeLa-GFP were injected subcutaneously into nude mice. Tumors derived from SiHa-HPV16 E5 and HeLa-HPV18 E5 cells (Fig. 5A and B) grew more rapidly and reached the maximal volume allowed during 8 weeks post-injection compared with parental SiHa and HeLa cells or SiHa-GFP and HeLa-GFP treated groups (Fig. 5D and E). Together, these observations suggest that the increased expression of HPV16/18 E5 in human cervical cancer cells not only increases the tumor volume but also accelerates...
the tumor growth rate both in HPV- and non-HPV-derived tumors in vivo (Fig. 5C and 5F). Due to the appearance of a spontaneous tumor necrosis core, mice were required to be euthanized 8 weeks post-injection. Nevertheless, in line with our previous in vitro studies, these results strongly support the important role of HPV16/18 E5 in tumor growth in vivo.

Discussion

Recent findings implicate HPV16 E5 as an important mediator of oncogenic transformation (16). In this study, we provide evidence that HPV16/18 E5 plays a role in promoting the proliferation, migration and invasion of human cervical cancer cells in vitro and accelerated the growth of human cervical cancer-derived tumors in vivo. Our investigation revealed that E5 overexpression induces cell proliferation in both HPV-positive and -negative groups pointing towards E5 sufficiency to promote cell growth (Fig. 2). Venuti et al (17) suggested that the expression of E5 is increased upon differentiation to promote proliferation of differentiated cells and productive viral replication. The localization of E5 was observed in internal cell membranes (Fig. 1), as previously reported (4). We suggest that the localization of HPV E5 to the endoplasmic reticulum indicated that its activity may be related to the trafficking of cytoplasmic membrane proteins through this cellular compartment.

To explore the possible mechanism involved, we performed a thorough investigation. In this study, we observed that HPV16/18 E5 overexpression affects cell migration, invasion and significantly downregulated E-cadherin protein expression (Fig. 3). E-cadherin, found at adherens junctions, is the principal effector of cell-cell adhesion (18). E-cadherin impairment represents the hallmark of malignancy and is strongly associated with poor prognosis of a number of tumors (19). Loss of E-cadherin expression in cancer cells weakens cell-cell adhesion and is associated with cancer progression, invasion, metastasis and cytoskeleton rearrangement (14). Our results suggest that the enhancement of migration and invasion in HPV16/18 E5-overexpressing human cervical cancer cells may be, at least partly, due to the E-cadherin downregulation. Further investigation is required to elucidate the molecular events responsible for the association of E-cadherin and E5, and this is an ongoing focus of our research.

Transformed cells commonly exhibit altered morphology and reduced cell adherence due to the disruption of cytoskeletal structures (15) and cell migration is strictly regulated by the re-organization of the actin cytoskeleton (20). It may be possible that E5 was directly or indirectly involved in these processes. Our investigation (rhodamine-phalloidin was used to label F-actin) (Fig. 4) demonstrated that the cell shape of the clones overexpressing HPV16/18 E5, revealed polygonal cell shapes, which was brightly stained and longitudinal actin bundles were formed. In contrast to the control cells, we observed shrunken cell shapes and stress fibers were almost invisible. Previous studies revealed that tumor cells with different motile activities differ in terms of morphology and that these differences may be attributable to the reorganization of the actin cytoskeleton (21). Tyrosine phosphorylation of paxillin (p-paxillin) was found to be involved in the regulation of actin cytoskeleton organization (12). In this study, we discovered that the overexpression of HPV16/18 E5 upregulated p-paxillin and may in this manner, contribute to cancer progression by altering signaling pathways regulating the actin cytoskeleton network supporting cell migration. We hypothesize that the downregulation of E-cadherin and the upregulation of p-paxillin by HPV16/18 E5 may endow human cervical cancer cells with altered spatial relationships that favor uncontrolled proliferation, migration and invasion.

The role of HPV16/18 E5 in carcinogenesis seems to be limited to the early stages of cervical carcinogenesis since the E5 gene is frequently deleted when the HPV genome is integrated during malignant progression (22). Therefore, targeting E5 which is frequently expressed in earlier stages of malignant transformation may be a rational approach for preventing premalignant lesions from progressing into invasive human cervical cancer and may be advantageous particularly in the early stage of HPV infections and precancerous lesions.

Acknowledgements

This study was partially supported by the National Science Foundation of China (30901586, 30973205, 81172464), The ‘973’ Program of China (2009CB521800), the Huibei Province Science Fund (2011CDB191) and the Central University Basic Science Research Fund (2012QN188).

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