HPV16 E6 upregulates Aurora A expression

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Received January 14, 2015; Accepted April 13, 2016

DOI: 10.3892/ol.2016.4786

Abstract. Overexpression of Aurora A kinase occurs in certain types of cancer, and therefore results in chromosome instability and phosphorylation-mediated ubiquitylation and degradation of p53 for tumorigenesis. The high-risk subtype human papillomavirus (HPV)16 early oncoprotein E6 is a major contributor inducing host cell immortalization and transformation through interaction with a number of cellular factors. In the present study, co-immunoprecipitation, glutathione S-transferase pull-down and immunostaining were used to show that HPV16 E6 and Aurora A bind to each other in vivo and in vitro. Western blotting and reverse transcription-polymerase chain reaction were used to reveal that HPV16 E6 inhibited cell apoptosis by stabilizing Aurora A expression. The present study may report a new mechanism for the involvement of HPV16 E6 in carcinogenesis, as HPV16 E6 elevates Aurora A expression and the latter may be a common target for oncogenic viruses that result in cell carcinogenesis.

Introduction

Cervical cancer is the second highest cause of female cancer-associated mortality worldwide, accounting for 288,000 mortalities yearly (1). In total, ~510,000 cases of cervical cancer are reported each year, with almost 80% of cases occurring in developing countries. Persistent infection with high-risk human papillomavirus (HPV) is regarded as an etiological origin of cervical carcinogenesis (2,3). HPV16 is the most common type of high-risk HPV, and accounts for >50% of all cervical cancers. HPV16 early protein E6 and E7 are the major oncoproteins that are crucial for host cell immortalization and transformation. In particular, E6 recruits the ubiquitin protein ligase E6-associated protein, and the resulting complex targets the p53 tumor suppressor protein for proteasome-mediated degradation (4,5). HPV16 E6 also interacts with several other cellular proteins, including activating transcription factor 3 (6), E6 binding protein (7), human discs large (8), interferon regulatory factor 3 (9), B-cell lymphoma 2-antagonist/killer 1 (10), E6-targeted protein 1 (11) and human telomerase reverse transcriptase (12). There is also a switch between mouse double minute 2 homolog (Mdm2) and HPV E6-mediated degradation of p53 in cervical cancer cells (13). HPV16 E6 regulates cell differentiation, adhesion, polarity, proliferation, apoptosis, gene transcription and chromosomal stability through these interactions. The interactions are not only important for cell carcinogenesis, but also for the survival of the virus within the host.

Aurora A is a centrosomal serine-threonine kinase that is responsible for proper mitotic progression. This kinase plays an essential role in coordinating mitotic events, including centrosome separation, bipolar spindle assembly, chromosome segregation and cytokinesis (14). The expression and kinase activity of Aurora A are regulated by the cell cycle, peaking when the cells reach M phase. The Aurora A protein is localized in the centrosomes of interphase cells and the spindle of mitotic cells (15). The centrosomes maintain genomic stability through the establishment of bipolar spindles during cell division, ensuring equal segregation of replicated chromosomes to the two daughter cells. The abnormal duplication and distribution of centrosomes in segregation leads to the aneuploidy observed in numerous cancer cell types. The expression of Aurora A is upregulated in several human tumors, including colon, breast, ovarian, gastric and pancreatic cancers, and hematological malignancies (16). Aurora A is also involved in the regulation of drug resistance to several chemotherapeutic agents. The overexpression of Aurora A may inhibit the cell death induced by Taxol (17), and knock-down of Aurora A increases the sensitivity of tumor cells to cisplatin toxicity (18). Aurora A abrogates p53 DNA binding and transactivation activity through the phosphorylation of serine 215 and 315, resulting in the ubiquitylation and proteasomal degradation of p53 via the
Mdm2-mediated pathway (19,20). Overexpression of Aurora A correlates with an advanced clinical stage and shortened survival period (21). Although the deregulated expression and mechanism of the Aurora family involvement in carcinogenesis have been reported (22), the association has yet to be elucidated in virus-mediated tumorigenesis. In the present study, it was demonstrate that HPV16 E6 upregulates the expression of Aurora A.

**Materials and methods**

*Reagents and antibodies.* The E6 DNA fragment of HPV16 was obtained by polymerase chain reaction (PCR) from SiHa genomic DNA and ligated to a PCDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) as previously described (23). The p3XFLAG-E6 expression vector was generated by PCR cloning of the HPV16 PCDNA3-E6 cDNAs, followed by HindIII and XbaI double digestion and insertion into the HindIII and XbaI sites of the pA3F vector (Sigma-Aldrich, St. Louis, MO, USA). Human Aurora A cDNA (a gift from Dr Bingyi Xiao, University of Pennsylvania, Philadelphia, PA, USA) was subcloned into the pCDNA3.1HA (Invitrogen; Thermo Fisher Scientific, Inc.) and pGEX-4T-2 vectors (GE Healthcare Life Science, Chalfont, UK) to produce hemagglutinin (HA)-tagged and glutathione S-transferase (GST)-tagged plasmids. The antibodies used in the present study were the mouse monoclonal anti-FLAG M2 (catalog no. F1804; Sigma-Aldrich), anti-HA (catalog no. H3663; Sigma-Aldrich), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-Aurora A (catalog no. 610939; BD Biosciences, San Jose, CA, USA) and anti-HPV16 E6 C1P5 (catalog no. sc460; BD Biosciences, San Jose, CA, USA) and anti-HA (catalog no. H3663; Sigma-Aldrich) overnight at 4°C. Immune complexes were captured with 30 µl of a 1:1 mixture of protein A-protein G-conjugated Sepharose beads (1 h at 4°C; Yanhuibio, Shanghai, China). Beads were washed and the supernatant was transferred to a fresh microcentrifuge tube. Lysates were then precleared by end-over-end rotation with normal mouse serum (Sigma-Aldrich) and 30 µl of a 1:1 mixture of protein A-protein G Sepharose beads, pelleted and washed five times with ice-cold RIPA buffer. For western blot assays, input lysates and immunoprecipitated complexes were boiled in Laemmli buffer (Haoran Bio), fractionated by SDS-PAGE and transferred to a 0.45 µm nitrocellulose membrane. The membranes were then probed with appropriate antibodies, followed by incubation with appropriate infrared-tagged secondary antibodies and viewed on an Odyssey imager (LI-COR Biosciences, Lincoln, NE, USA).

*Cell culture and transfection.* The SiHa, CaSkI, C33A and HEK293 cell lines, purchased from the American Type Culture Collection (Manassas, VA, USA) were grown in HyClone Dulbecco’s modified Eagle’s medium (DMEM; GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 µg/ml streptomycin and 2 mM L-glutamine. The cells were transfected by electroporation using a Bio-Rad Gene Pulser II electroporator (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

*Immunohistochemistry.* Slides mounted with sections of paraffin-embedded, archival, cervical tissue specimens were obtained from the Department of Pathology, the First Affiliated Hospital of China Medical University (Shenyang, Liaoning, China) between January 2012 and December 2012. Slides were deparaffinized in xylene and rehydrated using a graded series of alcohol (70, 80, and 100% alcohol; 5 min each). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. Following antigen retrieval in 10 mM sodium citrate buffer (pH 6.0; Haoran Bio, Shanghai, China), samples were blocked with 10% normal rabbit/goat serum (Sangerbio, Shanghai, China) prior to incubation with primary anti-Aurora antibodies overnight at 4°C. The secondary polyclonal biotinylated anti-rabbit/goat IgG antibody (1:200 dilution; S-P kit) and streptavidin-peroxidase conjugate (S-P kit; Dako, Glostrup, Denmark) were added according to the manufacturer’s instructions. The enzymatic reaction was developed in a freshly prepared solution of 3,3’-diaminobenzidine (DAB) using Dako Liquid DAB Color Solution (brown color; Dako). The sections were then counterstained with hemalum, dehydrated, washed with xylene and mounted.

*Immunoprecipitation and western blotting.* Transfected cells were harvested, washed with ice-cold phosphate-buffered saline (PBS; Solarbio, Beijing, China), and lysed in 0.5 ml ice-cold radioimmunoprecipitation (RIPA) buffer (Novland, Shanghai, China) containing 1% Nonidet P-40 (NP-40), 10 mM Tris (pH 7.5), 2 mM EDTA and 150 mM NaCl, supplemented with protease inhibitors, which consisted of 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml pepstatin and 1 µg/ml leupeptin. Cell debris was removed by centrifugation at 21,000 x g (10 min; 4°C), and the supernatant was transferred to a fresh microcentrifuge tube. Lysates were then precleared by end-over-end rotation with normal mouse serum (Sigma-Aldrich) and 30 µl of a 1:1 mixture of protein A-protein G-conjugated Sepharose beads (1 h at 4°C; Yanhuibio, Shanghai, China). Beads were washed and the supernatant was transferred to a fresh microcentrifuge tube. In total, ~5% of the lysate was saved for input control. The protein of interest was captured by rotating the remaining lysate with 1 µg of anti-HA antibody (catalog no. H3663; Sigma-Aldrich) overnight at 4°C. Immune complexes were captured with 30 µl of a 1:1 mixture of protein A and protein G Sepharose beads, pelleted and washed five times with ice-cold RIPA buffer. For western blot assays, input lysates and immunoprecipitated complexes were boiled in Laemmli buffer (Haoran Bio), fractionated by SDS-PAGE and transferred to a 0.45 µm nitrocellulose membrane. The membranes were then probed with appropriate antibodies, followed by incubation with appropriate infrared-tagged secondary antibodies and viewed on an Odyssey imager (LI-COR Biosciences, Lincoln, NE, USA).

**Purification of GST fusion proteins.** Escherichia coli BL21 (DE3) cells (Biovector, Beijing, China) were transformed with the plasmid constructs to obtain the GST and GST-E6 fusion protein. Single colonies were selected and grown overnight in 3 ml of Luria broth (Seebio, Shanghai, China) supplemented with 100 µg/ml ampicillin (Solarbio). In total, 1 ml of the overnight culture was used to inoculate a 500 ml culture. The larger culture was incubated until the optical density at 600 nm was ~0.6, at which point it was produced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 12 h at 30°C. The bacteria were pelleted, washed once with sodium chloride-Tris-ethylenediaminetetraacetic acid (EDTA) (STE) buffer (Shenxiangbio, Shanghai, China) consisting of 100 mM NaCl, 10 mM Tris and 1 mM EDTA (pH 7.5), resuspended in 3 ml NETN buffer (Helixgen, Guangzhou, China) consisting of 0.5% NP-40, 100 mM NaCl, 20 mM Tris and 1 mM EDTA (pH 8.0), supplemented with protease inhibitors (Haoran Bio), and incubated on ice for 15 min. A volume of 150 µl of 1 M dithiothreitol (DTT; Luanhuabio, Shanghai, China) and 1.8 ml of 10% Sarkosyl solution (Yantuobio, Shanghai, China) in STE buffer was added, and the suspension was sonicated (for 3 min on ice; Misonix Sonicator 4000; QSonica LLC, Newtown, CT, USA) to solubilize the proteins. The lysate was centrifuged (12,000 x g; 10 min; 4°C) to separate the insolubilized fraction. The clear supernatant was transferred to a fresh tube, to which 3 ml of 10% Triton X-100 in STE buffer and 200 µl
of glutathione-Sepharose beads (Weijiaobio, Guangzhou, China) were added. The tube was rotated overnight at 4°C, following which the purified protein bound to glutathione was collected by centrifugation (2 min; 600 x g; 4°C) and washed five times with NETN buffer supplemented with protease inhibitors. The level of purification was determined by SDS-PAGE, and purified proteins were stored at 4°C.

**GST pull-down assays.** *In vitro* translation of HA-Aurora A was performed using the T7-TNT Quick Coupled Transcription-Translation system (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. For *in vitro* binding experiments, GST fusion proteins were incubated with 35S-labeled *in vitro*-translated Aurora A protein in binding buffer (Yubobio, Shanghai, China) consisting of 1X PBS, 0.1% NP-40, 0.5 mM DTT and 10% glycerol, supplemented with protease inhibitors. The interting proteins were eluted in %X Laemmli loading buffer, boiled and separated on 12% SDS-PAGE. An autoradiography phosphorimager screen (Molecular Dynamics, San Diego, CA, USA) was used and scanning was performed by the Typhoon 9140 imaging system (Molecular Dynamics).

**Reporter assay.** In total, 1.2x10^7 cells were co-transfected with the pGL3-basic or pGL3-Aurora A reporter construct with combinations of various plasmids using a Bio-Rad Gene Pulser II electroporator. At 24 h post-transfection, the cells were harvested, washed in PBS and lysed in cell lysis buffer (BioVision, Milpitas, CA, USA). In total, 50 µl of cell lysate was used for the reporter assay, which was performed using an LMaxII384 luminometer (Molecular Devices, Sunnyvale, CA, USA). In total, 20% of the cell lysate was used for western blotting as aforementioned. The transfected proteins were detected with Odyssey infrared scanning technology (Li-Cor Biosciences, Inc.), using Alexa Fluor 680 and Alexa Fluor 800 (Molecular Probes; Thermo Fisher Scientific, Inc.). All transfections were performed three times, and the results show indicate the means of the data from three independent experiments.

**Lentiviral small hairpin (sh)RNA vector constructs.** For the lentivirus-mediated stable knockdown of HPV16 E6, the E6 shRNA sequence (sequence, 5'-GGACAGGCCCATTCAATAT-3'; LC-Bio, Hangzhou, China) was inserted into the pGIPZ vector according to the manufacturer's protocol (Open Biosystems; GE Dharmacon, Lafayette, CO, USA), resulting in the HPV16 E6 shRNA-expressing vector (sh-E6). In addition, a 21-mer oligonucleotide (sequence, 5'-TCTCGCTTGGCCGAGATAG-3') that had no significant homology to any known human messenger (mRNA) in the databases was cloned in the same vector and used as control shRNA (sh-C).

**Virus production and transduction of CaSki cells.** Lentivirus was produced by transient transfection of the aforementioned plasmids into HEK293T cells. A total of 2x10^6 HEK293T cells were seeded in 10-cm dishes containing DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic and cultured in a 5% CO2 incubator for 24 h prior to transfection. A total of 20 µg plasmid DNA was added to each dish for transfection, including 1.5 µg envelope plasmid pCMV-VSV-G (catalog no. 8454; Addgene, Inc., Cambridge, MA, USA), 3 µg packaging plasmid pRSV-REV (catalog no. 12251 Addgene, Inc.), 5 µg packaging plasmid pMDLg/Pre (catalog no. 12251; Addgene, Inc.) and 10.5 µg lentiviral vector plasmid. The precipitation was formed by adding the plasmids to a final volume of 438 µl H2O and 62 µl 2 M CaCl2, and mixing well. A total of 500 µl of 2X 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES)-buffered saline (Yeasenbio, Shanghai, China) was then added and the solution was incubated at room temperature for 30 min. Chloroquine (Haoran Bio) was added to the 10-ml plated media 5 min prior to transfection at a final concentration of 25 µM. Subsequent to 12 h of chloroquine treatment, the medium was replaced with DMEM supplemented with 10% FBS, 10 mM HEPES and 10 mM sodium butyrate (Sangonbio, Shanghai, China). The medium was replaced again 10 h later by DMEM supplemented with 10% FBS and 10 mM HEPES. The conditioned medium was collected four times at 12 h intervals, filtered through 0.45 µm pore-size cellulose acetate filters, and stored on ice. The virus was concentrated by centrifugation at 70,000 x g for 2.5 h. The concentrated virus was resuspended in RPMI-1640 (Qcbio, Shanghai, China) then used to infect 10^6 cells in the presence of 20 µm/ml Polybrene (Haoran Bio). Following 72 h, puromycin was added to a final concentration of 2 µg/ml for the selection of transfected cells. GFP immunofluorescence was assessed using an Olympus IX71 microscope (Olympus, Tokyo, Japan) filtered with 560 nm excitation and 645 nm emission filters. The cells were grown to 80% confluency in the presence of 2 µg/ml puromycin prior to western blot analysis as aforementioned.

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA from cells was extracted using TRIzol reagent and cDNA was generated using a Superscript II Reverse Transcription kit (Invitrogen; Thermo Fisher Scientific, Inc.). The primers for RT-qPCR were as follows: Aurora A sense, 5'-GGAGAGCTTAAAAATTGCAGATTTTG-3' and antisense, 5'-GGC AAACACATACACAGACCT-3'; HPV E6 sense, 5'-GACCCAGAAAATTTACACAG-3' and antisense, 5'-CACAACGTTGTTGGATTTG-3' and GAPDH sense, 5'-CTCTCCTGACTTCAACACCGG-3' and antisense, 5'-GCCAAAATTCGTTGTCATACAG-3'. cDNA was amplified using 10 µl Master Mix from the DyNAmo SYBR green RT-qPCR kit (MJ Research; Bio-Rad Laboratories, Inc.), 1 mM of each primer, and 2 µl of the cDNA product in a 20 µl total volume. Thirty cycles of 1 min at 94°C, 30 sec at 55°C, and 40 sec at 72°C were followed by 10 min at 72°C in an MJ Research Opticon II thermocycler (Bio-Rad Laboratories, Inc.). A melting curve analysis was performed to verify the specificity of the amplified products. The values for the relative levels of change were calculated using the 2^ΔΔCt method (24), and each sample was tested in triplicates.

**Results**

**HPV16 E6 combines with Aurora A.** It has been shown that Aurora A accumulates and functions as an inhibitor of p53 in the majority of cancer cells. In order to determine the association between HPV16 E6 and Aurora A, these two molecules were first confirmed to form a complex by co-immunoprecipitation assays. HEK293 cells were co-transfected
with expression constructs for Flag-tagged HPV16 E6 and HA-tagged Aurora A, with empty vectors acting as negative controls. Whole-cell extracts of the transfected HEK293 cells were precipitated with anti-HA antibody, and the precipitates were analyzed with anti-Flag antibody. The transfected Flag-HPV16 E6 and HA-Aurora A were found to associate with each other, but not empty vectors, indicating a specific interaction between ectopically expressed HPV16 E6 and Aurora A in cervical cancer tissue.
whether HPV16 E6 combines with Aurora A in cervical carcinoma cell lines was then investigated. Endogenously expressed HPV16 E6 was immunoprecipitated from HPV16 positive cervical carcinoma CaSki and SiHa cells. The co-immunoprecipitation of Aurora A was monitored by the polyclonal antibody reactive to Aurora A. The HPV-negative cervical carcinoma C33A cell line was used as a negative control. The results revealed that HPV16 E6 formed a stable complex with Aurora A (Fig. 1B). An in vitro binding assay was also performed to determine whether HPV16 E6 directly interacts with Aurora A. The GST-E6 and GST expression constructs were bacterially expressed and incubated with in vitro translated 35S-labeled Aurora A, and the GST pull-down assay result showed that GST-E6 beads, but not GST alone, precipitated a significant amount of Aurora A protein with radioactivity. The result showed there is a direct association between HPV16 E6 and Aurora A (Fig. 1C). This interaction between HPV16 E6 and Aurora A was also shown by an immunostaining assay. Endogenous HPV16 E6 accumulated in the nucleus of CaSki cells co-localized with Aurora A (Fig. 1D).

HPV16 E6 enhances Aurora A expression. Aurora A has been shown to aberrantly accumulate in numerous types of cancer cells (25,26). In order to investigate Aurora A expression and its association with HPV in cervical cancer, the present study assessed the protein level of Aurora A in HPV-positive cervical cancer tissue and HPV-negative normal cervical tissue by immunohistochemistry assays. The result revealed that Aurora A is highly expressed in cervical cancer tissue, but barely expressed in normal cervical tissue (Fig. 2A). To determine that the elevated Aurora A level is due to HPV16 E6, stable CaSki cells carrying HPV16 E6 knockdown (Sh-E6) or control (Sh-Cr) were created by transduction of the cells with shRNA-containing lentivirus followed by selection of lentivirus-expressing cells using puromycin (23). The Aurora A level was determined by western blot analysis. The protein level of Aurora A in E6-knockdown cells was evidently decreased compared with the control cells (Fig. 2B). In addition, the present study confirmed the effect of HPV16 E6 on Aurora A expression by transient transfection. In total, 1.5x10⁷ HEK293 cells were transiently transfected either with the empty vector or increasing amounts of Flag-tagged E6. At 36 h post-transfection, the cells were harvested and subjected to western blot assay. The results showed that the mRNA level of Aurora A in HPV16 E6-transfected cells was decreased compared with the empty vector (Fig. 3A). To confirm this result, the luciferase reporter gene driven by the Aurora A promoter was generated and assessed using a reporter
assay. HEK293 cells were cotransfected with pGL3-basic or pGL3-Aurora A and either Flag-E6 or the empty vector, and the cells were harvested at 24 h post-transfection. The cell lysates were subjected to a luciferase reporter assay. The results were presented as the relative luciferase unit (RLU) compared with pGL3-basic and vector alone-cotransfected cells. Data is expressed as the means ± standard deviation of three independent experiments. The immunoblotting results of Flag-tagged E6 and GAPDH are shown in Fig. 3B. A reporter assay was performed of pGL3-Aurora A promoter cotransfection with an increasing amount (0, 5, 10, 15, 20 mg) of Flag-E6 (Fig. 3C). At 24 h post-transfection, the cells were harvested and subjected to a luciferase reporter assay. The results were presented as the RLU fold compared with the pGL3-Aurora A and empty vector alone-cotransfected cells. HPV16 E6 raised the RLU of pGL3-Aurora A in a dose-dependent manner.

Discussion

In the present study, it was demonstrated that HPV16 E6 combines with Aurora A and enhances its expression. The results are consistent with other studies that have shown that Aurora A is frequently overexpressed in a variety of human tumors and cancer-derived cell lines (27-29).

Aurora A, B and C are highly conserved serine/threonine kinases that play vital and distinct roles in the process of chromosomal segregation, such as chromosome condensation, alignment, control of spindle checkpoints, chromosome segregation and cytokinesis, and these kinases have been identified as oncogenes (14,30). Specifically, Aurora A localizes to centrosomes and spindle microtubules proximal to centrosomes during mitosis, as this kinase is required for the assembly of the mitotic spindle, where Aurora A accumulates on centrosomes at the spindle poles during prophase until the cell reaches metaphase. The expression and activity of Aurora A kinase varies as the cell cycle progresses. The levels are low in the G1/S phase, upregulated during the G2/M phase and rapidly reduced subsequent to mitosis (31). Abrupt expression of Aurora A induces oncogenic transformation (32) and inhibition of Aurora kinase activity leads to the failure of multiple events in mitosis, such as incorrect separation of centriole pairs, misalignment of chromosomes on the metaphase plate and incomplete cytokinesis (33). Thus, it is reasonable to conclude that the Aurora kinases may be good molecular therapeutic targets for cancer.

In the present study, a novel mechanism of HPV oncoprotein E6 functions to upregulate aurora kinase A in cervical cancer cells.

Acknowledgements

The present study was supported by grants from the National Natural Science Foundation of China (grant nos., 81171649 and 81572054).

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