HPV16 infection regulates RASSF1A transcription mediated by p53

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Abstract. Human papillomavirus (HPV) 16 infection and RASSF1A expression play important roles in tumor development and progression. However, the precise mechanisms underlying their concerted function in the development of reproductive system tumors still remain to be elucidated. In the present study, we showed that HPV16-E6 selectively upregulates RASSF1A expression via degradation of p53, which interacts with the RASSF1A promoter and regulates apoptosis. Overexpression of p53 triggered a decrease in endogenous RASSF1A in SiHa cells, accompanied by apoptosis. Similarly, knockdown of endogenous HPV16-E6 in SiHa cells with RNA interference (RNAi) led to downregulation of RASSF1A mediated by p53 and the subsequent induction of apoptosis. These findings collectively suggest that HPV16 infection regulates p53-mediated RASSF1A expression and suppresses apoptosis. Moreover, RASSF1A may form an element of the negative autoregulatory feedback loops that act on the HPV16 response and are involved in p53-dependent apoptosis. Our results provide novel insights into the cellular mechanism of tumor development, and present a starting point for the development of novel strategies in cancer treatment and effective diagnosis.

Introduction

Human papillomaviruses (HPVs), particularly high-risk types, cause a variety of reproductive system lesions, including cervical neoplasia and cancer. High-risk HPV DNA has been detected in almost all types of cervical cancer, with HPV16 being the most prevalent type in the general population (1). RASSF1A is a tumor suppressor gene with a highly methylated promoter involved in tumorigenesis, development and prognosis (2-6). Previous studies have shown an association between HPV16 infection and RASSF1A expression, although the precise mechanism of action still remains to be elucidated. HPV infection and inactivation of RASSF1A appear to be inversely correlated in several types of cervical tumors and cell lines. The presence of HPV in cervical carcinomas has been shown to alleviate the requirement for RASSF1A inactivation, and no association with RASSF1A methylation has been observed. This suggests that the two events require other interaction mechanisms but engage the same tumorigenic pathway (7,8).

A previous study demonstrated that one of the HPV16 oncoproteins, E5, activates the vascular endothelial growth factor (VEGF) promoter and upregulates its expression via activation of the epidermal growth factor receptor (EGFR) (9). HPV16-E6 and -E7 are known crucial viral oncoproteins which have been shown to be consistently maintained after viral integration into the host cell genome. The probability of neoplasia is increased in HPV16 infections with E6 and E7 oncoprotein expression (10). To date, both p53-dependent and -independent mechanisms of oncogenesis, regulated by HPV proteins, have been described (11). E6 plays a primary role as an anti-apoptotic protein through association with p53 via interactions with E6-associated protein, and mediation of p53 ubiquitination and degradation that prevents eliciting of cellular responses to stress signals, such as DNA damage. However, underlying interaction mechanisms between HPV16 and the host factor RASSF1A still remain to be fully elucidated. Previous experiments conducted by our group showed that p53 binds to the RASSF1A promoter, leading to downregulation of RASSF1A expression (12). Accordingly, it is hypothesized that HPV16 infection, p53 and RASSF1A are closely interrelated.

The present study aimed to investigate whether HPV16 infection regulates RASSF1A expression as well as to determine the underlying mechanisms of action. Our results provide novel insights into the mechanisms of cancer cell development.

Materials and methods

Cells. The human cervical carcinoma cell line, SiHa, was obtained from the Center for Type Culture Collection (Wuhan, Hubei, China).

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Quantitative PCR (qPCR). Total RNAs were prepared with the TRIzol kit (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. All of the RNAs were digested with RNase-free DNase I and purified according to the protocol provided by the manufacturer. In total, ~3 μg RNA was employed as the template for reverse transcription using 0.5 μg oligo(dt) and 200 units of M-MLV reverse transcriptase (Promega, Madison, WI, USA). qPCR was employed for the quantification of gene expression using the multichannel Rotor-Gene 3000 (Corbett Research, Mortlake, Australia) according to the manufacturer's protocol. PCR cycling conditions were as follows: 5 min at 94˚C, followed by 40 cycles of 30 sec at 94˚C, 30 sec at 60˚C and 30 sec at 72˚C in a 25-μl reaction mix containing 1X SYBR-Green I. The primers used were: 5'-TGGAACAACATTAGAACAGC-3' and 5'-CTGCAACAAGACATACATCG-3' for HPV16-E6; 5'-CCTGCTGGATTACATCAAAGCACT-3' and 5'-GTCAAGGGCATATCCTACAACAAA-3' for HPRT. Simultaneous detection of the HPRT gene was performed to normalize HPV16-E6 expression. Similar amplification procedures were employed for HPRT and HPV16-E6. To address robustness issues, each sample was amplified at least in triplicate. Data were analyzed with Rotor-Gene version 6 software and subsequently plotted in Microsoft Excel.

Plasmid constructs

RNA interference (RNAi) clones. siRNA employed for analysis was constructed using the Ambion online siRNA design tool (www.ambion.com/techlib/misc/siRNA_design.html; Ambion, Austin, TX, USA). Hairpin DNA sequences were synthesized as two complementary oligonucleotides, annealed, and ligated between the *Bbs*I and *Xba*I sites to replace the enhanced green fluorescent protein (EGFP) coding sequence of the pmU6pro vector (kindly provided by Dr David Turner, University of Michigan, USA) for generating the interference vectors, HPV16-E6-RNAi and RASSF1A-RNAi. The sequences were the following: HPV16-E6-RNAi sense, 5'-TTTGAATGTGTGTACTGCAAGCATGGCTTGCAGTACACACATTCTTTTT-3' and antisense, 3'-TTACACACATGACGTTCGTACCGAACGTCATGTGTGTAAGAAAAAGATC-5'; RASSF1A-RNAi sense, 5'-TTTGACCTCTGTGGCGACTTCAATGTGAAGTCGCCACAGAGGTCTTTTT-3' and antisense, 3'-TGGAGACACCGCTGAAGTTACACTTCAGCGGTGTCTCCAGAAAAAGATC-5'.

RASSF1A-pcDNA clone. To construct the RASSF1A-pcDNA vector, full-length RASSF1A was amplified from the vector donated by Dr Rongjia Zhou using PCR, and subcloned into the *Bam*HI and *Eco*RI sites of the pcDNA3.0 mammalian expression vector (BD Biosciences Clontech, Palo Alto, CA, USA). PCR cycling conditions were as follows: 5 min at 94˚C, followed by 35 cycles of 1 min at 94˚C, 1 min at 65˚C and 1 min at 72˚C in a 20-μl reaction mix. The sense and antisense primers used for amplification were: 5'-AACGGATCCATGTCGGGGGAGCCTGAGC-3' and 5'-TACGAATTCTCACCCAAGGGGGCAGGCG-3', respectively.

Cell preparation and transfection analysis. The human HPV16-positive cervical cancer cell line SiHa, was obtained from the Center for Type Culture Collection (Wuhan, Hubei, China). The cells were regularly maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS; HyClone, Logan, UT, USA) at 37˚C with 5% CO_2. The cells were passaged every 3 days and seeded onto 24-well plates 12 h prior to transfection. The transfection procedure was performed with Lipofectamine™ 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions.

Assessment of apoptosis with Annexin V/propidium iodide (PI) staining. Apoptotic cell death was measured using the FITC-conjugated Annexin V/PI assay (BioVision, Palo Alto, CA, USA), followed by flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA). Briefly, $1x10^5$ cells were washed with ice-cold phosphate-buffered saline (PBS), resuspended in 0.1 ml binding buffer, and stained with 10 μl FITC-conjugated Annexin V (10 mg/ml) and 10 μl PI (50 mg/ml). After incubation for 15 min at room temperature in the dark, 400 μl binding buffer was added, and the cells were subsequently analyzed with a FACScan flow cytometer (Annexin V excitation at 488 nm and emission at 515 nm; PI excitation at 488 nm and emission at 580 nm).

Western blot analysis. Proteins of freshly obtained SiHa cells were extracted with ice-cold lysis buffer and incubated on ice for 15 min. Following centrifugation for 10 min at 15,000 x g, supernatant fractions were collected, and western blot analysis was performed using routine protocols. Briefly, extracts were analyzed with 12% glycine-SDS-PAGE and transferred onto PVDF membranes with a pore size of 0.2 μm (Hybond-P; Amersham Pharmacia Biotech, Uppsala, Sweden). Nonspecific binding of antibodies was blocked with 5% low-fat milk powder in TBST for 1 h at room temperature. The membranes were incubated with human anti-p53, anti-RASSF1A, anti-HPV16-E6, anti-β-actin (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or anti-caspase 3 (1:500; Epitomics, Inc., Burlingame, CA, USA) antibodies at 4˚C overnight, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:50,000) for 1 h. Proteins were visualized with enhanced chemiluminescence (ECL) regent.

DNA binding assay. Previous experiments conducted by our group demonstrated that p53 binds to the RASSF1A promoter and downregulates its expression (12). Since p53 has been suggested to be the critical mediator linking HPV16 infection with RASSF1A expression, we confirmed the active binding site and used a higher dose of p53 up to 2.5 μg to investigate its regulatory effect. The experimental method used was similar to the method reported previously (12).

Immunohistochemistry. Human testicular chips (no. CC23-01) were purchased from Chao Ying Biotechnology (Xi'an, Shaanxi, China). Each chip contained 23 samples, including normal testis, seminoma, lymphoma and fibroma with three slices in the same area for each sample. The expression levels of RASSF1A and p53 proteins were analyzed with anti-RASSF1A (eBioscience, Inc., San Diego, CA, USA) and anti-p53 (Santa Cruz Biotechnology, Inc.) antibodies, respectively, using streptavidin-biotin complex (SABC) and 3,3'-diaminobenzidine (DAB) visualization

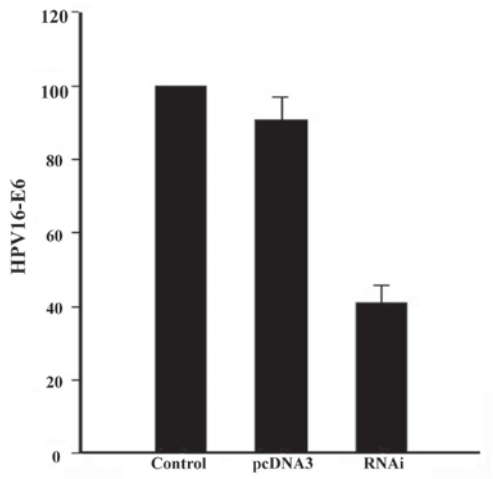

Figure 1. Real-time fluorescent quantitative RT-PCR analysis of HPV-E6 in HPV16 siRNA-transfected SiHa cells. siRNA induced a significant decrease in E6 expression compared with control cells.

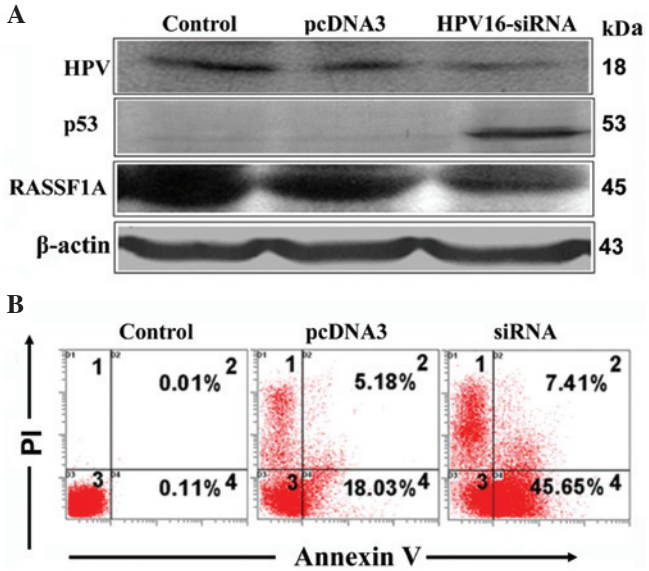

Figure 2. Effects of HPV16-E6 siRNA transfection. (A) Western blot analysis indicated that siRNA induces a significant decrease in E6, resulting in upregulation of p53 and downregulation of RASSF1A. (B) Treatment of cells with E6 siRNA stimulated a marked increase in the number of apoptotic cells.

methods according to the manufacturer's instructions (Boster Biological Technology, Ltd., Wuhan, China).

Results

HPV16-E6 regulates p53 and RASSF1A levels, and suppresses apoptosis. To gain an insight into the association between HPV16-E6 and RASSF1A expression, the HPV16-E6-RNAi vector was cloned and transfected into SiHa cells containing endogenous HPV16, and RASSF1A expression was detected by western blot analysis. HPV16-E6 expression was decreased by 66% (Fig. 1) upon siRNA transfection, subsequently leading to an increase in p53 and decrease in RASSF1A levels (Fig. 2A). Further examination of the biological effect of E6 RNAi revealed a 29.85% increase in apoptosis (Fig. 2B).

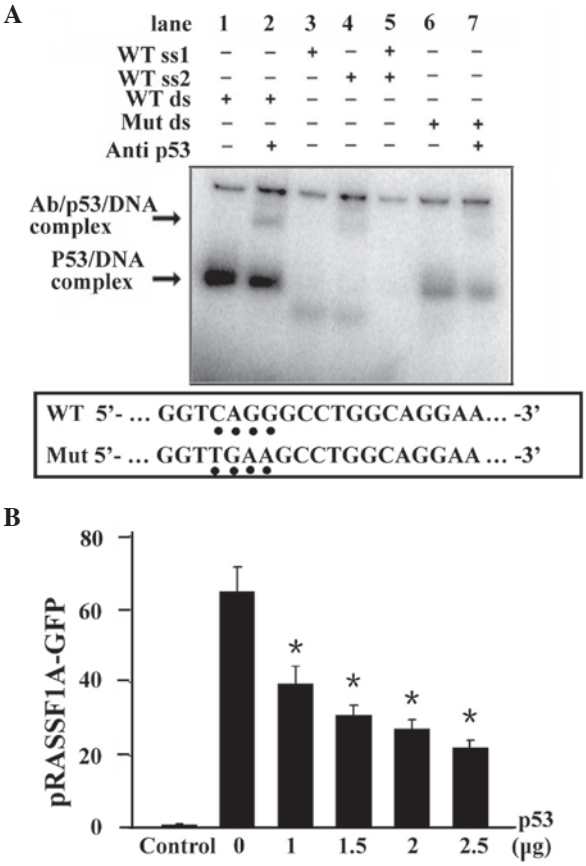

Figure 3. p53 protein binds to RASSF1A promoter and downregulates RASSF1A expression. (A) EMSA was performed to assess the DNA binding capability of the p53 polypeptide with RASSF1A promoter. WT/MT, the wild-type/mutant p53 binding site probe from the human RASSF1A promoter; ds/ss, two complementary oligonucleotide probes/single-stranded probe. The shifted band in lane 1 contained the WT DNA/p53 protein complex and the supershifted band in lane 2 contained the anti-p53/WT DNA/p53 protein complex. Complexes were separated using native gel electrophoresis. The mutant probe sequence is shown in the lower panel. (B) p53 suppresses RASSF1A expression in a dose-dependent manner. Various concentrations of CMV-p53 were co-transfected with pRASSF1A-GFP into COS-7 cells and analyzed by flow cytometry. EMSA, electrophoretic mobility shift assay.

p53 binds to RASSF1A promoter and suppresses RASSF1A expression. According to a previous study conducted by our group, the presence of a p53 binding site in the RASSF1A promoter region was confirmed (12), and a downregulatory effect on RASSF1A was demonstrated upon p53 binding. To validate whether p53 binding is the key factor linking HPV16-E6 and RASSF1A expression, we repeated the experiment with 0-2.5 μg of p53, and examined the binding (Fig. 3A) and regulatory effects (Fig. 3B) of p53. Gel-shift assay showed that His-p53 specifically and efficiently bound to RASSF1A promoter (Fig. 3A, lane 1) which was confirmed by adding the p53 antibody and formed a supershift band (antibody/p53/RASSF1A; Fig. 3A, lane 2). However, single-stranded probes and a mutant RASSF1A probe decreased the formation of the p53/RASSF1A complex (Fig. 3A, lanes 3-7).

p53 inhibits apoptosis induction through RASSF1A regulation. In RASSF1A-expressing SiHa cells, p53 significantly inhibited RASSF1A expression. Consequently, we further investigated the impact of p53 on RASSF1A-induced apoptosis. Flow cytom-

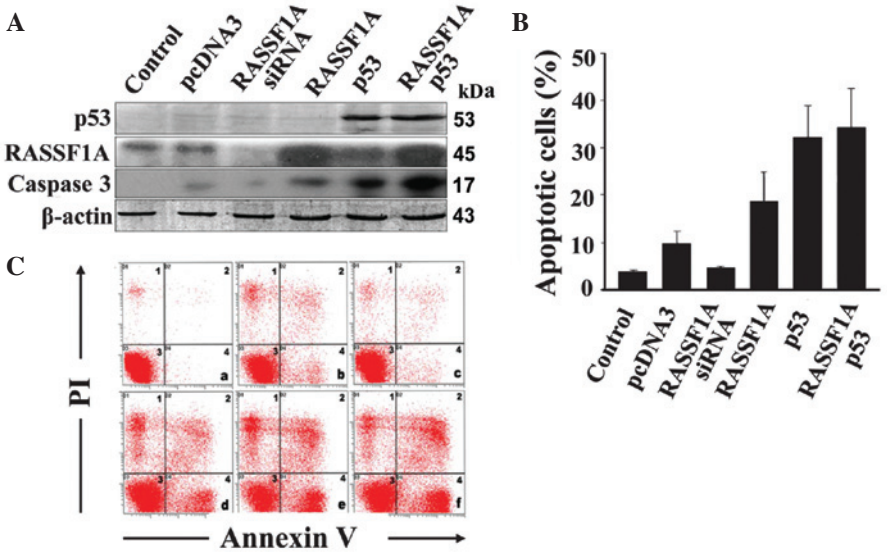

Figure 4. Effects of the overexpression of p53 or/and RASSF1A on the apoptosis of SiHa cells. (A) Overexpression of p53 suppressed RASSF1A protein levels and induced caspase 3 shear, as observed following western blot analysis using anti-RASSF1A, anti-p53 and anti-caspase 3 antibodies. Elevated caspase 3 shear (17 kDa) implied the correlation between p53 and RASSF1A in apoptosis. β-actin protein was used as an internal control. Protein molecular weights are shown on the right. (B) Apoptosis of SiHa cells transfected with p53 or/and RASSF1A vector. Both p53 and RASSF1A induced apoptosis, although cotransfection led to lower levels of apoptosis relative to the expected additive effects of the individual proteins. (C) Annexin V-PI analysis of apoptosis by flow cytometry. (a-f) Control to RASSF1A + p53 cells as indicated in the figure. PI, propidium iodide.

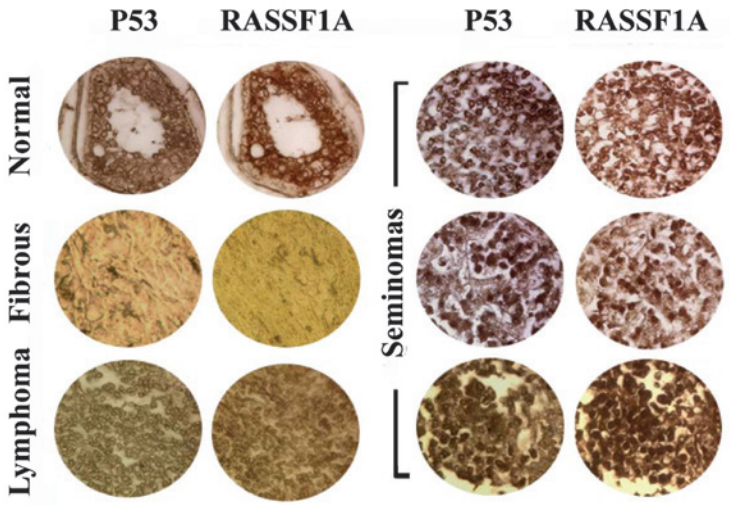

Figure 5. Co-localization of RASSF1A and p53 proteins observed by immunohistochemical analysis using antibodies against RASSF1A and p53 in human testicular chips. Upper left panel, normal testicular samples; left middle panel, fibrous tissue samples; lower left panel, lymphoma samples; right three panels, seminoma samples. RASSF1A and p53 signals were observed in normal, spermatocytic seminoma and lymphoma samples, in contrast to fibrous tissues. In spermatocytic seminoma and lymphoma samples, signals were observed around the nuclear membrane, and also in the nuclei in spermatocytic seminomas, in contrast to the predominant cytosolic signals in normal testis. Furthermore, RASSF1A and p53 proteins were co-localized in each sample.

etry combined with Annexin V/PI staining (Fig. 4B and C) revealed that treatment of RASSF1A-expressing SiHa cells with RASSF1A siRNA inhibits apoptosis by 54%, compared to pcDNA. Upon overexpression of RASSF1A, the proportion of apoptotic cells increased from 10.1 to 19.4%, compared with pcDNA, while in the presence of p53, the proportion of apoptotic cells increased to 33.53%. Although both RASSF1A and p53 are apoptosis inducers, overexpression of the two proteins induced a significantly lower increase in the apoptotic cell percentage (35.8%) compared with the expected additive effect (52.93%; 19.4 + 33.53%), indicating that apoptosis induction by RASSF1A is at least partially inhibited by p53. Furthermore, caspase 3 appears to be involved in the apoptotic pathways of p53 and RASSF1A (Fig. 4A).

Mislocalization of p53 and RASSF1A proteins in human testicular tumors. To investigate the potential association between localization of the p53 and RASSF1A proteins and tumorigenesis, we analyzed their expression patterns in human testicular tissue chips. Immunohistochemical analysis using specific antibodies showed p53 and RASSF1A signals in samples of normal testis, spermatocytic seminoma and lymphoma, while not in fibrous tissues (Fig. 5). RASSF1A was weakly expressed in a number of spermatocytic seminoma samples.

In spermatocytic seminoma and lymphoma samples, signals were observed around the nuclear membrane, and additionally in nuclei in spermatocytic seminoma cases, compared with mainly cytosolic signals in normal testis. Furthermore, localization of p53 and RASSF1A was coincident in each sample. Our results indicate co-localization of the two proteins, with altered localization patterns in human testicular tumors.

Discussion

Both oncogenes and tumor suppressor genes contribute to the genesis of cancer, which involves multiple genes, including those functioning in DNA repair, signal transduction, apoptosis and cell cycle regulation. For instance, HPV16-E6 and RASSF1A are known oncogenic and tumor suppressor genes that are critical in apoptosis regulation (13-16).

The results of the present study indicate a novel function of RASSF1A in the HPV16 pathway. Treatment of cells with HPV16-E6 siRNA led to upregulation of p53 and downregulation of RASSF1A, indicating that RASSF1A acts as an element of the negative autoregulatory feedback loops activated in response to p53. Decreased expression of RASSF1A is known to be sufficient for maintaining a dynamic equilibrium of cell growth and apoptosis, and the high RASSF1A level induced by HPV16 infection could partly counteract tumorigenesis. RASSF1A may play a pivotal role in tumorigenesis, distinct from its earlier reported function as a tumor suppressor. The oncoprotein E6 promotes p53 degradation whose carcinogenic effect is suppressed by RASSF1A. In response to p53, transcriptional networks of p53-responsive genes interact with a number of transduction pathways and positive and negative autoregulatory feedback loops. In the present study, RASSF1A was identified as a novel member of the negative autoregulatory feedback loops. While RASSF1A is a known conventional tumor suppressor, the precise mechanisms by which it interacts with other oncogenes and tumor suppressors remain to be elucidated.

High-risk HPV types, including types 16 and 18, have been identified in ~2/3 of all cervical cancer patients worldwide (17,18). HPV16-E6 binds to and degrades the p53 tumor suppressor protein, leading to malfunction of its DNA repair mechanism (19,20). Previous studies have shown that RASSF1A inactivation and HPV infection are mutually exclusive, and highlight a possible correlation between HPV infection and RASSF1A expression, which may reflect functional interactions between RASSF1A and viral E6 (7,21). One hypothesis is that methylation underlies this correlation. However, in the present study, a novel mechanism is reported where HPV16-E6 regulates RASSF1A transcription mediated via p53 protein. Treatment of cells with HPV16-E6-siRNA led to upregulation of p53 protein, and subsequently, to a decrease in RASSF1A transcription. RASSF1A induces apoptosis and cell cycle alterations via its capability to bind and stabilize the microtubule, control mitosis and regulate genome stability. Specific effector factors include cyclin D1, p120E4F, Cdc20, PMCA4b, Bax, CNK1 and Raf1-MST2 (14,22-26). Our experiments demonstrated that p53 and RASSF1A induce apoptosis through caspase 3 activation, maintaining their reported identities as tumor suppressors. However, overexpression of both proteins resulted in significantly lower apoptosis compared to the expected additive effect, indicating an additional potential role of RASSF1A in a feedback regulatory loop to balance cell survival and death.

In summary, our findings provide novel insights into the cellular mechanism of tumor development that might facilitate cancer therapy and diagnosis. Further knowledge of the molecular mechanisms downstream of RASSF1A is required to provide a reference for tumor gene therapy.

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