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Human papillomavirus type 16 E7 oncoprotein mediates *CCNA1* promoter methylation

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Key words
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Human papillomavirus (HPV) oncoproteins drive distinctive promoter methylation patterns in cancer. However, the underlying mechanism remains to be elucidated. Cyclin A1 (*CCNA1*) promoter methylation is strongly associated with HPV-associated cancer. *CCNA1* methylation is found in HPV-associated cervical cancers, as well as in head and neck squamous cell cancer. Numerous pieces of evidence suggest that E7 may drive *CCNA1* methylation. First, the *CCNA1* promoter is methylated in HPV-positive epithelial lesions after transformation. Second, the *CCNA1* promoter is methylated at a high level when HPV is integrated into the human genome. Finally, E7 has been shown to interact with DNA methyltransferase 1 (Dnmt1). Here, we sought to determine the mechanism by which E7 increases methylation in cervical cancer by using *CCNA1* as a gene model. We investigated whether E7 induces *CCNA1* promoter methylation, resulting in the loss of expression. Using both *E7* knockdown and overexpression approaches in SiHa and C33a cells, our data showed that *CCNA1* promoter methylation decreases with a corresponding increase in expression in *E7* siRNA-transfected cells. By contrast, *CCNA1* promoter methylation was augmented with a corresponding reduction in expression in *E7*-overexpressing cells. To confirm whether the binding of the E7–Dnmt1 complex to the *CCNA1* promoter induced methylation and loss of expression, ChIP assays were carried out in *E7*-, del CR3-*E7* and vector control-overexpressing C33a cells. The data showed that E7 induced *CCNA1* methylation by forming a complex with Dnmt1 at the *CCNA1* promoter, resulting in the subsequent reduction of expression in cancers. It is interesting to further explore the genome-wide mechanism of E7 oncoprotein-mediated DNA methylation.

Cervical cancer is the third most common cancer in women worldwide.(1) The prevalence of human papillomavirus (HPV)16/18 in cervical cancer cases worldwide is approximately 70%.(2) Broadly speaking, the HPV genome is circular and comprises several genes; of these, the respective products (E6 and E7) of the oncogenic *E6* and *E7* genes have potent transforming capabilities, largely by targeting p53 and pRB, respectively, for degradation.(3) In the integrated form, *E6* and *E7* become overexpressed due to *E2* disruption.(3–5) Hence, integrated HPV represents the major form of infection that is linked to tumorigenesis.(6–9)

In addition to p53 and pRB degradation, data from recent studies have indicated that there may be additional mechanisms by which E6 and E7 contribute to cancer development.(10–14) For instance, E7 may likely be involved in epigenetic alterations by augmenting the activity of DNA methyltransferase 1 (Dnmt1), resulting in a corresponding downregulation of E-cadherin that does not occur through promoter methylation.(12) In another study by Hasan *et al.*, E7 was shown to decrease *tlr9* expression in cervical cancer, but this likely occurred through histone modifications.(13) Recently, genome-wide methylation and expression studies indicated that HPV could increase the methylation and reduce the expression of several genes in head and neck cancer cells.(15,16) A study by Sartor *et al.*, showed the distinctive DNA methylation pattern due to HPV infection in HPV-positive and HPV-negative cell lines, including 84 formalin-fixed paraffin-embedded head and neck tumor samples. They observed that HPV-positive cells showed higher levels of promoter methylation than HPV-negative cells. Seventy-five genes were more highly methylated and expressed at lower levels in HPV-positive head and neck cancer samples compared with HPV-negative samples, including *IRS1*, *GNA11*, *GNAI2*, *EREG*, *CCNA1*, *RGS4*, and *PKIG*.(15) A study by Lechner *et al.* showed increased mRNA expression of both Dnmt3a and Dnmt1 in HPV-positive head and neck cancer cell lines. Moreover, they observed that gene methylation increased in *E6*- and *E6* and *E7*-transfected head and neck cancer cell lines.(16) Nevertheless, the mechanistic basis for this is not well understood. To explore the mechanism by which HPV induces increased methylation in HPV-associated cancers, we used *CCNA1* as a gene model.

We have previously reported that methylation of the *CCNA1* promoter was largely elevated in ~93% of cervical cancer cases, whereas the promoter remains unmethylated in normal

Table 1. Oligonucleotide sequences and conditions for PCR analyses

Oligonucleotides	Sequence (5′–3′)	Amplicon size, bp	Annealing temperature, °C
Primers			
E7mutF	CCCATCTGTTCTCAGAAACCATATGATGAATTCATCGTGACTG	448	66
E7mutR	CAGTCACGATGAATTCATCATATGGTTTCTGAGAACAGATGGG		
E7del (F)	GATGAATTCTGCAGATATCC	5,655	56
E7del (R)	TAATGGGCTCTGTCCGGTTC		
GAPDHF	GTGGGCAAGGTATCCTG	450	56
GAPDHR	GATTCAGTGTGGTGGGGGAC		
CCNA1metF	TTTCGAGGATTTCGCGTCGT	46	53
CCNA1metR	CTCCTAAAAACCCTAACTCGA		
CCNA1expF	ATTCATTAAGTGAAATTGTGC	170	47
CCNA1expR	CTTCCATTCAGAAACTTATTG		
E7expF	GGGCAATTAAATGACAGCTCAG	142	56
E7expR	GTGTGCTTTGTACGCACAACC		
CCNA1-ChIPF	CAGGAAGCGTAGGTGTGTGAG	205	56
CCNA1-ChIPR	GCTTTGGAAGGGACTGTTTCC		
E-cadherin-ChIPF	CTGATCCCGGTCTTAGTGAGCC	405	58
E-cadherin-ChIPR	GGCTCCAAGGGCCCATGGCTGG		
siRNA			
E7-Sense strand	CAGAACCGGACAGAGCCCAUUACAA		
E7-Antisense strand	UUGUAAUGGGCUCUGUCCGGUUCUG		
Dnmt1-Sense	UUAUGUUGCUCACAAACUUCUUGUC		
Dnmt1-Antisense	GACAAGAAGUUUGUGAGCAACAUAA		

F, forward; R, reverse.

counterparts.(17) In addition, other studies recommended that the *CCNA1* promoter methylation status be used as the optimal molecular marker for distinguishing between normal/low-grade squamous intraepithelial neoplasia and high-grade squamous intraepithelial neoplasia cancer lesions.(18,19) Moreover, we also observed a significant correlation between the integrated form of HPV infection and *CCNA1* promoter methylation.(20) Interestingly, the study by Weiss *et al.* found a significant correlation between HPV-related head and neck cancer and *CCNA1* methylation.(21) Of note, *CCNA1* promoter methylation is now known to be prevalent in other solid tumors, such as head and neck cancers(22) and nasopharyngeal carcinomas,(23) indicating reduced expression and loss of function of cyclin A1 in these tumors.

Recent data suggested that the Zinc finger CR3 region of E7 could interact with Dnmt1. Moreover, E7 can upregulate the DNA methyltransferase activity of Dnmt1.(24) There are several mammalian members of the Dnmt family that display broadly similar functions, but some differences do exist. The function of Dnmt1 is now known to involve DNA methylation maintenance by preferring hemimethylated substrates.(25,26) Dnmt3, comprising Dnmt3a and Dnmt3b, plays a role in de novo methylation.(27) One study revealed that Dnmt3a and Dnmt1 can cofunction in de novo methylation.(28) Taken together, these pieces of evidence prompted us to investigate whether high-risk HPV E7 can decrease the expression of the gene product of *CCNA1* in cervical cancer cell lines through a process that involves promoter methylation and Dnmt1.

Materials and Methods

Cell lines and culture. The human cervical carcinoma cell lines SiHa (HPV type 16-positive)(29) and C33a (HPV-negative)(29) were kindly provided by J. Silvio Gutkind (National Institutes of Health/NIDCR, Bethesda, MD, USA). They were grown and maintained in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Gibco, Carlsbad CA, USA) and 1% antibiotic–antimycotic (Gibco Carlsbad, CA, USA) at 37°C in an atmosphere of 5% CO_2.

5′-Azacytidine treatment. To evaluate methylation and expression changes, 5′-azacytidine (aza) (Sigma-Aldrich, MO, USA) was used to treat the cells. For treatment with aza, SiHa and C33a cells were seeded at 3×10^5 cells/mL in growth media. After overnight incubation, cells were further incubated with fresh media containing aza at the indicated final concentration (0–40 μM) for an additional 5 days, being replaced every 24 h until analysis.

Human papillomavirus type 16 *E7* plasmid construction. To carry out transfection of the *E7* recombinant plasmid in mammalian cells, plasmid 13634: pGEX2T *E7* (Addgene, Cambridge, MA, USA), which expressed only in bacterial cells, was transformed to MAX Efficiency DH5α Competent Cells (Invitrogen, Carlsbad, CA, USA) for propagation. The mutagenesis of this plasmid (c.297A > T) was then carried out to mutate the stop codon TAA to TAT according to the manufacturer's instructions; the E7mutF and E7mutR primers were used as shown in Table 1. The PCR for the mutagenesis was carried out using the QuikChange lighting site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). This mutated vector fragment was then subcloned into the pcDNA 3.1/myc-HIS A (Invitrogen) expression vector in mammalian cells through *Bam*HI (Thermo Fisher Scientific, Waltham, MA, USA) and *Eco*RI (Thermo Fisher Scientific, Waltham, MA, USA) sites. In this vector, the *E7* open reading frame was tagged with MYC and HIS at the C-terminal. The *E7* gene fidelity was then confirmed by sequencing (data not shown).

Deletion analysis. The CR3 Zinc finger deletion of *E7* (del CR3-E7) was done using the Q5 Site-Mutagenesis kit according to the manufacturer's instructions (New England Biolabs, Ipswich, MA, USA). The primers used to introduce this deletion (forward and reverse) are shown in Table 1. The PCR for the mutagenesis was carried out according to the manufacturer's instructions. The deletion of this region was confirmed by sequencing (data not shown).

Transfection. *E7* overexpression was carried out in C33a cells, whereas the *E7* and *Dnmt1* siRNA experiments were carried out in SiHa cells. For *E7* overexpression, C33a cells were seeded into 6-well plates at 3×10^5 cells/mL and incubated overnight. Next, 2 μg *E7* recombinant plasmid and pcDNA 3.1/myc-HIS (PC) empty plasmid (Invitrogen) were transfected using TurboFect (Thermo Fisher Scientific) according to the manufacturer's protocol. For the knockdown experiment, the *E7* siRNA was designed using the BLOCK-iT RNAi designer online tool (accession number NC_001526.2). For Dnmt1 siRNA, the siRNA was designed following Zhang *et al.* (30) and the sequences that were used for E7 and Dnmt1 are shown in Table 1. Prior to transfection, SiHa cells were first seeded at 5×10^4 cells/mL in 12-well plates. Following overnight incubation, 40 pmol *E7* and Dnmt1 siRNA and Stealth RNAi negative control sequences (Invitrogen) were transfected using Lipofectamine 2000 (Invitrogen). Next, 72 h after transfection, SiHa and C33a cells were collected to study *E7* and *CCNA1* mRNA expression, as well as *CCNA1* promoter methylation.

Isolation of DNA. DNA was extracted from SiHa and C33a cultures, digested with lysis buffer II containing SDS (Sigma-Aldrich) and proteinase K (USB, Cleveland, OH, USA) at 50°C overnight. Phenol/chloroform extraction and ethanol precipitation were then carried out as previously described.(31)

Sodium bisulfite treatment and methylation-specific PCR. DNA input of 750 ng for each sample was subjected to bisulfite treatment using the EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA, USA) according to the protocol provided by the manufacturer. The eluted DNA was consequently used to carry out methylation-specific PCR by using methylated and unmethylated specific primers (Table 1).(17) Then, 10 μL PCR products were observed by gel electrophoresis in 8% acrylamide gels and stained with SYBR (Lonza, Walkersville, MD, USA). The methylated and unmethylated band intensities of each sample were visualized and measured using Storm840 and ImageQuant Software (Amersham Biosciences, Little Chalfont, UK).

Preparation of RNA. Total RNA was extracted using the TRIzol reagent (Invitrogen). Then, 5 μg total RNA from each sample was synthesized to cDNA using the RevertAid first-strand cDNA synthesis kit according to the manufacturer's specifications (Thermo Fisher Scientific).

analysis of RNA. Reverse transcription–PCR was carried out to observe the *CCNA1* mRNA expression in SiHa and C33a cells by using CCNA1expF and CCNA1expR. The GAPDHF and GAPDHR primers were used as internal controls (Table 1). *CCNA1* and *GAPDH* products were amplified at 160 and 450 bp, respectively. *CCNA1* and *E7* mRNA expression in aza-treated and transfected cell lines were determined by quantitative real-time PCR. The cDNA was amplified within a 7500-fast real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). Amplification was performed with E7- and CCNA1exp primers (Table 1), including *GAPDH* primers, which was used as a reference gene, and 1× Power SYBR Green PCR master mix (Applied Biosystems). The fold change in expression of *CCNA1* and *E7* between experimental and control cell lines was then determined by using the $^{\triangle\triangle}$CT method.(32)

Table 2. Antibodies for Western blot and ChIP analysis

Antibodies	Ratio	Company
Western blot analysis		
Anti-6x His tag (ab18184)	1:2000	Abcam (Cambridge, UK)
Anti-Dnmt1 (ab13537)	1:200	Abcam
Anti-Dnmt3a (sc-20703)	1:500	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Anti-Dnmt3b (sc-20704)	1:500	Santa Cruz Biotechnology
Goat anti-mouse IgG (HRP) (sc-2062)	1:10 000	Santa Cruz Biotechnology
Goat anti-rabbit IgG (HRP) (sc-2030)	1:10 000	Santa Cruz Biotechnology
Anti-β-actin (HRP) (ab49900)	1:20 000	Abcam
ChIP analysis		
Anti-histone H3-trimethyl K4 (ab8580)		Abcam
Anti-HPV16E7 (sc-6981)		Santa Cruz Biotechnology
Anti-Dnmt1 (ab13537)		Abcam
Anti-Dnmt3a (sc-20703)		Santa Cruz Biotechnology
Anti-Dnmt3b (sc-20704)		Santa Cruz Biotechnology
Mouse IgG isotype control (5415s)		Cell Signaling Technology (Danvers, MA, USA)
Normal rabbit IgG (2729s)		Cell Signaling Technology

Dnmt, DNA methyltransferase.

Western blot analysis. To confirm the E7, Dnmt1, Dnm3a, and Dnmt3b protein level in *E7* and empty plasmid-transfected C33a cells, Western blot analysis were carried out as previously described.(33) The total cellular protein concentrations for each sample were measured by the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Then, 40 μg (for detecting E7, Dnmt3a, and Dnmt3b) and 120 μg (for detecting Dnmt1) were loaded for SDS-PAGE. The antibodies used for Western blotting are described in Table 2. All antibodies were prepared in Bløk Noise-Cancelling Reagent (Millipore, Billerica MA, USA). Immunoreactive bands were visualized using the Super Signal West Pico Chemiluminescent Substrate Kit (Thermo Fisher Scientific).

Chromatin immunoprecipitation. Chromatin immunoprecipitation was carried out in *E7* recombinant plasmid, del CR3-*E7*, and empty plasmid-transfected C33a cells, as previously described, with some modifications.(34) To observe the binding of E7 protein (with or without CR3 region) and Dnmt1 at the *CCNA1* promoter, the chromatin fragments were immunoprecipitated with 10 μg each of the antibodies, as described in Table 2. To observe the binding among the Dnmt family at the *CCNA1* promoter, 5 μg each of the antibodies (Table 2) was used. Next, the precipitated DNA was further analyzed by PCR using CCNA1-ChIPF, CCNA1-ChIPR, E-cadherin-ChIPF and E-cadherin-ChIPR primers (Table 1). Then, 10 μL PCR products were observed by gel electrophoresis using 8% acrylamide and 1% agarose gel stained with SYBR (Lonza). The *CCNA1* and *E-cadherin* bands were visualized using Storm840 and ImageQuanNT Software (Amersham Biosciences).

Statistical analysis. To test the *CCNA1* promoter methylation and expression level in aza-treated cells, one-way ANOVA with repeated measures was used to determine statistical significance and level of $P = 0.05$ were considered significant. To test the two groups in each experiment, *E7* siRNA and scrambled siRNA, as well as *E7* and PC overexpression, paired sample *t*-tests were used at confidence intervals of 95%.

Results

***CCNA1* promoter methylation associated with *CCNA1* expression repression in cancer cells.** Two cervical cancer cell lines, SiHa (HPV-positive) and C33a (HPV-negative), confirmed for their HPV status by using L1 primer (MY09/MY11),(20) were subjected to *CCNA1* promoter methylation and expression analysis. As shown in Figure 1a, SiHa cells showed full *CCNA1* methylation, whereas only partial methylation (band intensity of 44.97%) was observed in C33a cells. For *CCNA1* expression, we observed that SiHa cells had lower *CCNA1* expression, giving a band intensity of 26.85%, compared with C33a (70.31%). We also used *GAPDH* as an internal control for normalization (Fig. 1b). The data suggest that the presence of HPV might influence *CCNA1* promoter methylation and expression.

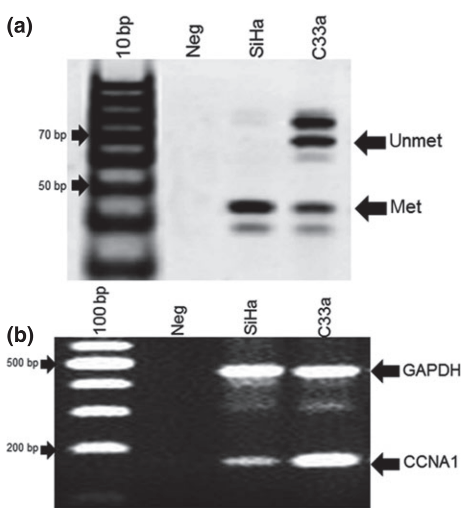

Fig. 1. Profile of *CCNA1* promoter methylation and expression in SiHa (human papillomavirus-positive) and C33a (human papillomavirus-negative) cells. (a) Methylation status of *CCNA1* promoter was detected by methylation-specific PCR using duplex primers for methylated (Met) and unmethylated (Unmet) regions. (b) *CCNA1* expression was observed using duplex primers; expression of *CCNA1* and *GAPDH,* as an internal control to normalize *CCNA1* expression, were measured. Neg, distilled water as a negative control.

Lowering *CCNA1* methylation increases *CCNA1* expression. Next, we subjected C33a and SiHa cells to aza treatment to observe whether *CCNA1* promoter methylation was involved in the control of expression of the product cyclin A1. The results showed that the band density of *CCNA1* methylation in C33a cells gradually decreased with increasing aza concentrations (0–7 μM) while the unmethylated form of *CCNA1* in C33a cells increased with corresponding aza treatment (data not shown). In parallel, the band density of *CCNA1* methylation in SiHa decreased with increasing aza concentrations (0–40 μM). We observed that the methylation status of the *CCNA1* promoter in C33a and SiHa cells decreased significantly with all aza treatment concentrations ($P = 0.0005$ and $P < 0.0001$, respectively) (Fig. 2a,b). *CCNA1* expression was shown by quantitative RT-PCR and $^{\triangle\triangle}$CT(32), with the results revealing that expression continuously increased following aza treatment in C33a and SiHa cells. By one-way ANOVA, both cell lines showed significant increase in *CCNA1* mRNA expression ($P < 0.0001$) (Fig. 2c,d).

Human papillomavirus E7 induces *CCNA1* promoter methylation and decreases *CCNA1* expression. To prove the effects of E7, we evaluated *CCNA1* promoter methylation and mRNA expression changes after *E7* expression reduction. Here, we used *E7* siRNA in HPV-positive SiHa cells. We found that *E7* siRNA-transfected SiHa cells had significantly less *E7* mRNA expression ($P = 0.0002$) (Fig. 3a). *CCNA1* promoter methylation was decreased in *E7* siRNA-transfected SiHa cells compared with scrambled–transfected cells ($P < 0.0001$) (Fig. 3b). The levels of *CCNA1* mRNA expression in E7 siRNA-transfected SiHa cells were increased compared with scrambled siRNA-transfected cells ($P < 0.0001$) (Fig. 3c). This observation revealed that the lower E7 mRNA expression in *E7* siRNA-transfected cells decreased *CCNA1* promoter methylation and increased *CCNA1* mRNA expression.

Moreover, we evaluated whether E7 can de novo methylate the *CCNA1* promoter using an *E7* recombinant expression

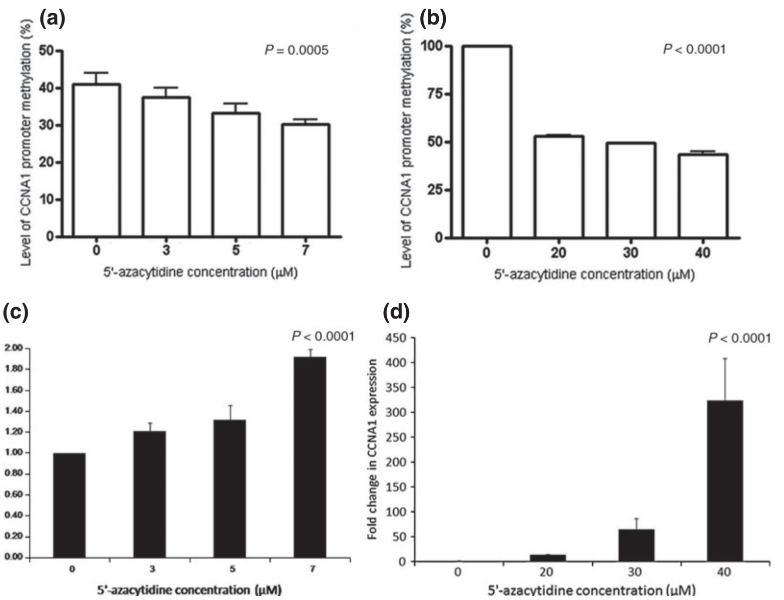

Fig. 2. 5′-Azacytidine at 0, 3, 5, and 7 μM and 0, 20, 30, and 40 μM was used to treat C33a and SiHa cells, respectively, for 5 days, and cells were harvested to observe *CCNA1* promoter methylation and expression. Eight replicates of the experiment were carried out. (a) Diagram and statistical test of the *CCNA1* promoter methylation status in C33a cells. (b) Diagram and statistical test of the *CCNA1* promoter methylation status in SiHa cells. (c) Fold change of *CCNA1* expression in C33a cells. (d) Fold change of *CCNA1* expression in SiHa cells.

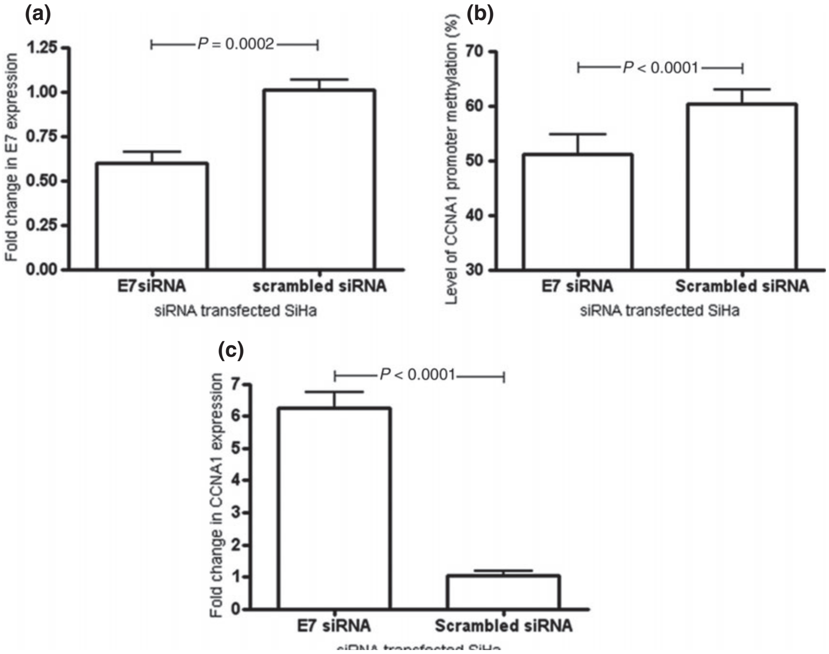

Fig. 3. *E7* and scrambled siRNA were transfected into SiHa cell lines for 72 h. Eight replicates of the experiment were carried out. (a) Fold change of *E7* mRNA expression. (b) *CCNA1* promoter methylation. (c) Fold change of *CCNA1* mRNA expression.

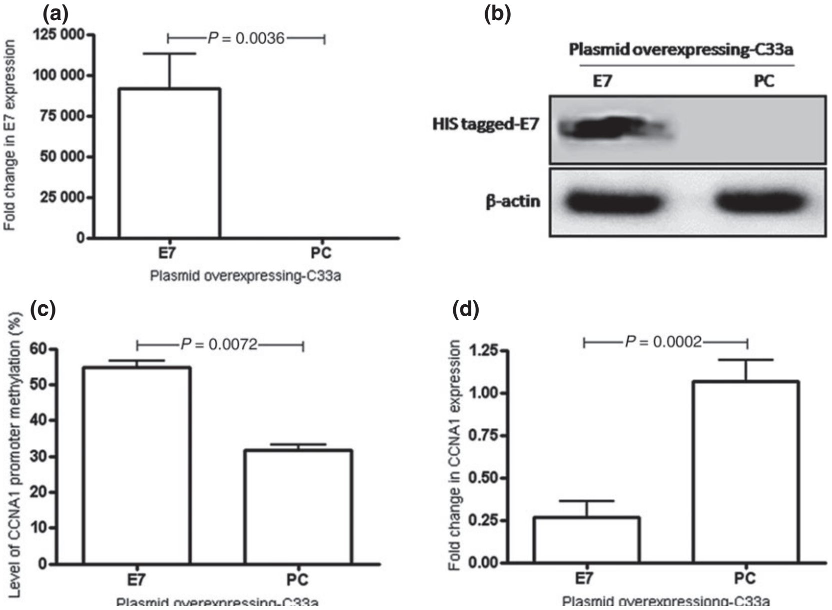

Fig. 4. *E7* recombinant plasmid and empty vector (PC) were overexpressed in C33a cells. (a) Fold change of *E7* expression at the mRNA level. (b) E7 expression at the protein level; β-actin was used as internal control. (c) *CCNA1* promoter methylation status. (d) Fold change of *CCNA1* expression.

plasmid. The *E7* recombinant expression plasmid and empty plasmid control (PC) were transfected into HPV-negative C33a cells. We observed that *E7* gene expression was significantly elevated in *E7* recombinant plasmid-transfected C33a cells compared with the PC control ($P = 0.0036$) (Fig. 4a), and a similar trend was observed for E7 protein expression (Fig. 4b). Furthermore, *CCNA1* promoter methylation was significantly increased in *E7*-overexpressing C33a cells compared with control cells ($P = 0.0072$) (Fig. 4c), broadly suggesting an inverse correlation with *CCNA1* mRNA expression ($P = 0.0002$) (Fig. 4d).

Expression of Dnmt1, Dnmt3a, and Dnmt3b in *E7*-transfected cells. To observe the expression of Dnmt1, Dnmt3a, and Dnmt3b in E7-transfected cells by Western blot analysis, a decreasing level of Dnmt1 protein in *E7*-overexpressing C33a cells was observed compared with control cells. In contrast, there was no difference between the expression of Dnmt3a and Dnmt3b in *E7*-overexpressing C33a and control cells (Fig. 5).

***CCNA1* promoter methylation and expression in *Dnmt1* siRNA-transfected SiHa cells.** After knocking down *Dnmt1*, *Dnmt1* mRNA expression was observed. The results showed its decreased expression (data not shown). Moreover, *CCNA1* promoter methylation was decreased and its expression was increased significantly; $P < 0.0001$ and $P = 0.0420$, respectively (Fig. 6).

***E7*–*Dnmt1* complex targets *CCNA1* promoter.** Because E7 promotes *CCNA1* promoter methylation and is known to form a complex with Dnmt1, we hypothesized that E7 promotes *CCNA1* methylation by forming an E7–Dnmt1–*CCNA1* promoter complex. To demonstrate that our hypothesis was viable, ChIP assays were carried out in C33a cells overexpressing *E7*, del CR3-*E7*, and PC control by precipitation with anti-HPV16 E7 antibody and then carrying out PCR to obtain a 205-bp *CCNA1* product. As shown in Figure 7, the product band of the *CCNA1* promoter was detected in *E7*, and del CR3-*E7*-overexpressing C33a cells, not in the control cells. Notably, by precipitating chromatin with the Dnmt1 antibody, a strong band was identified in the PCR product corresponding to *CCNA1* in *E7*-overexpressing C33a but not in del CR3-*E7* or control cells. There was no E-cadherin PCR band observed after precipitation with E7 and Dnmt1 in all transfected cells, confirming that E7-Dnmt1 binding was specific to the *CCNA1* promoter, but not generally observed with other unmethylated promoters. Next, to investigate whether E7 had a preference for only Dnmt1 to methylate the *CCNA1* promoter, we carried out ChIP–PCR using specific Dnmt1, Dnmt3a, and Dnmt3b antibodies for the immunoprecipitation. The results showed an intense, darker *CCNA1* band following Dnmt1 precipitation in *E7*-overexpressing C33a cells compared with the control cells. There was no difference in the *CCNA1* bands following Dnmt3b precipitation in both cells. However, a darker *CCNA1* band was observed with Dnmt3a precipitation in the control cells compared with the *E7*-overexpressing C33a cells (Fig. 8). These data suggest that the E7–Dnmt1 complex can induce de novo methylation of *CCNA1*.

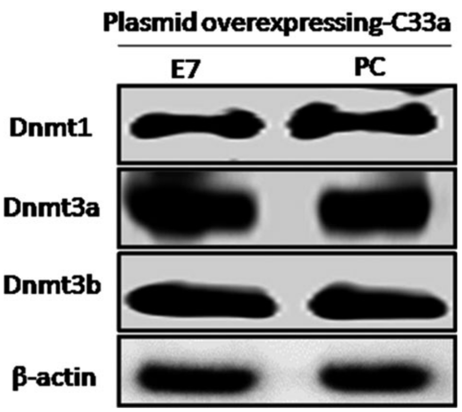

Fig. 5. DNA methyltransferase (Dnmt) family member expression (Dnmt1, Dnmt3a, and Dnmt3b) in E7- and empty vector (PC)-transfected cells. β-actin was used an internal control.

Discussion

We sought to determine the mechanism by which E7 changed the *CCNA1* promoter methylation pattern in cervical cancer. We hypothesized that E7 might interact with Dnmt1 and thereby impact the status of *CCNA1* promoter methylation with a consequent loss of *CCNA1* expression in cervical cancer. To prove our hypothesis, we first investigated the methylation status in two cervical cancer cell lines, SiHa and C33a cells. We found that the *CCNA1* promoter was fully methylated in SiHa cells and only partially methylated in C33a cells. It is possible that the stronger methylation band observed in SiHa cells might have come from E7 induction and, in contrast, the methylation in C33a cells might occur by spontaneous DNA methylation. There are several ways to control gene expression, including promoter methylation.(18,35–38) Here, we provide evidence in our model system that promoter methylation is pivotal in controlling *CCNA1* expression. After treatment of C33a and SiHa cells, decreased methylation was associated with increased *CCNA1* expression. We can conclude that *CCNA1* promoter methylation controls and regulates the expression of its gene product. Ultimately, we were able to show that E7 suppresses *CCNA1* expression through *CCNA1*

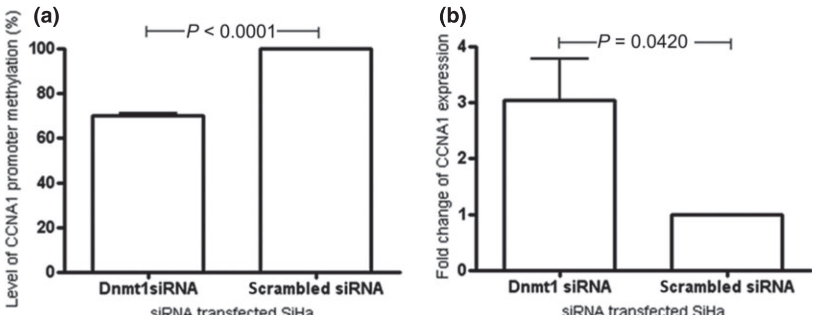

Fig. 6. *Dnmt1* and scrambled siRNA were transfected in SiHa cells for 72 h. Six replicates of the experiment were carried out. (a) Level of *CCNA1* promoter methylation. (b) Fold change of *CCNA1* expression.

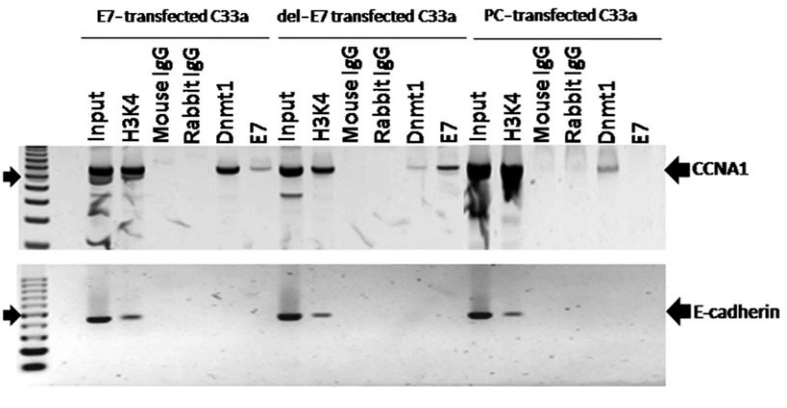

Fig. 7. Chromatin immunoprecipitation was used to confirm the binding of the E7–DNA methyltransferase 1 (Dnmt1) complex at the *CCNA1* and *E-cadherin* promoter in E7-transfected cells using *CCNA1* and E-cadherin primers (distilled water was used as a negative control for PCR). Input DNA was used as a positive control for PCR, and H3K4me3 was used as a positive control antibody for the ChIP assay. E7 and Dnmt1 antibodies were used to observe the binding of the E7–Dnmt1 complex at the *CCNA1* and *E-cadherin* promoter. Mouse IgG and rabbit IgG served as negative control antibodies for ChIP. PC, empty vector.

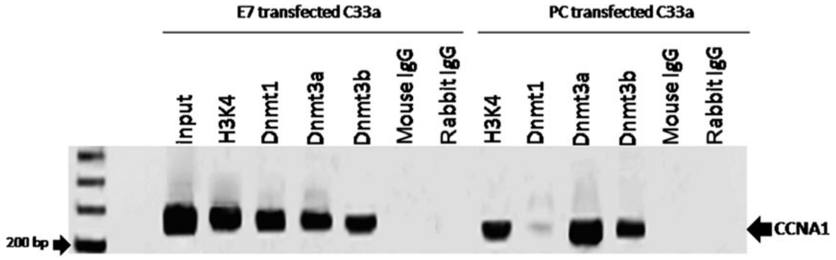

Fig. 8. Chromatin immunoprecipitation was carried out to confirm the binding of DNA methyltransferase (Dnmt) family members (Dnmt1, Dnmt3a, and Dnmt3b) to the *CCNA1* promoter. Input DNA was used as a positive control for PCR, and H3K4me3 was used as a positive control antibody for the ChIP assay. PC, empty vector.

promoter methylation. Using ChIP assays, we detected the PCR product of *CCNA1* after pulling down E7 and Dnmt1 antibodies in *E7*-overexpressing C33a cells compared with control cells. This implies that E7 and Dnmt1 formed a complex and bound to the *CCNA1* promoter. This complex might be the cause of de novo methylation of the *CCNA1* promoter in HPV-infected cells. Moreover, in support of our observations, recent data has shown that Dnmt1 is involved in *CCNA1* promoter methylation and expression in SiHa cells.(39) Interestingly, we observed dark and intense PCR bands after precipitating Dnmt3a and Dnmt3b in both *E7*- and PC control-transfected cells. These results also relate to our prior observations, which showed that the *CCNA1* promoter was methylated in untransfected C33a cells. Taken together, these results confirm the role of the E7–Dnmt1 complex in inducing *CCNA1* promoter methylation.

The studies by Sartor *et al.* and Lechner *et al.*(15,16) confirm the impact of HPV-mediated promoter methylation of genes in HPV-associated cancers. However, the mechanism of virus-induced methylation was unknown. Here, we discovered this new mechanism using *CCNA1* as a gene model. In addition to the induction of *CCNA1* promoter methylation, there are several other methylated genes also induced by HPV. In addition, expression observation by mass spectrometry revealed evidence that E7 could upregulate and downregulate several genes in C33a cells transfected with HPV E7.(14) Moreover, E7 can bind to several transcription factors such as E2F, p300, and TBP.(40–42) Therefore, the proposed mechanism by which HPV induces methylation and suppresses expression of the genes is that the E7–Dnmt1 complex interacts with other transcription factors at gene promoters containing cis elements that respond to these transcription factors. This proposed mechanism requires further confirmation as to whether E7 can promote genome-wide methylation targeted genes.

There are many types of virus-associated cancers in addition to HPV-related cancers, including Epstein–Barr virus, and hepatitis B- and hepatitis C-associated cancers. There is evidence showing a correlation between the promoter methylation of genes and viral infection in these cancers.(43–45) A study by Kaneda *et al.* suggested that the overexpression of Dnmts by latent proteins and epigenomic changes, such as 3-D conformational changes and histone modifications, might be involved in the extensive induction of methylation in Epstein–Barr virus-associated cancer.(46) Nevertheless, there is no evidence revealing the mechanism by which methylation pattern changes occur due to viral carcinogenesis. Our study is the first to discover the mechanism of the increased methylation of genes due to viral infection.

In conclusion, this study revealed that E7 of HPV is able to induce *CCNA1* promoter methylation by forming a complex with Dnmt1, resulting in decreasing expression of cyclin A1 mRNA. This finding provides a mechanistic model by which E7 of HPV mediates the methylation of several gene promoters in HPV-associated cancers. This present observation could be very useful for drug discovery in treating cancers associated with HPV infection, as well as many other diseases in the future, using an epigenetic treatment model.

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Disclosure Statement

The authors have no conflict of interest.

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