

## Forming Complex of HPV16 E7 to Transcription Factor AP2 Alpha and DNMT1 in Cervical Cancer Cells

### การสร้างคอมเพลกซ์ระหว่าง HPV16 E7 กับทรานสคริปชันแฟกเตอร์ AP2 อัลฟา และ DNMT1 ใน เซลล์มะเร็งปากมดลูก

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#### ABSTRACT

Cause of the cervical cancer development is recognized by viral oncoproteins E6 and E7 of high risk human papillomavirus (HR-HPV) that bind to tumor suppressor proteins, p53 and pRb, respectively. However, viral regulation of host epigenetics by HR-HPVs remains elusive, particularly, the association of HPVE7 and host promoter hypermethylation of proto-oncogene. Our previous studies suggested that presence of HPV E7 contributes to hypermethylation of *CCNA1* promoter, which might regulate a production of CCNA1 tumor suppressor protein. We postulated that HPVE7 forms complex with DNMT1 and putative transcription factor regulating *CCNA1* gene. We performed pull-down assay using Ni-NTA purification and using HPVE7 as bait protein. Co-purification proteins as putative E7 interacting proteins were detected by western blot analysis using antibodies against targeted proteins, which are DNMT1 and TFAP2A. The pull-down assay confirmed that HPVE7 potentially binds to DNMT1 and TFAP2A, which supported our hypothesis.

#### บทคัดย่อ

สาเหตุของการเกิดมะเร็งปากมดลูกเกิดจากโปรตีน E6 และ E7 ของเชื้อไวรัสฮิวแมนแพปพิโลมาชนิดที่มีความเสี่ยงสูง จับกับ โปรตีน tumor suppressor p53 และ pRb ของโฮสต์ตามลำดับ แต่อย่างไรก็ตามกระบวนการเปลี่ยนแปลงในระดับเหนือพันธุกรรมยังไม่เป็นที่เข้าใจ ซึ่งการศึกษาก่อนหน้านี้พบว่าการแสดงออกของโปรตีน E7 ของไวรัสฮิวแมนแพปพิโลมา ส่งผลต่อการกระตุ้นการเติมหมู่เมทิลที่ตำแหน่งโปรโมเตอร์ของยีน *CCNA1* จึงได้ตั้งสมมติฐานว่า โปรตีน E7 HPV E7 สร้างคอมเพลกซ์กับ DNMT1 และทรานสคริปชันแฟกเตอร์ AP2A ซึ่งเป็นหนึ่งในโปรตีนทรานสคริปชันแฟกเตอร์ของยีน *CCNA1* การเตรียมโปรตีนบริสุทธิ์ด้วย Ni-NTA ถูกใช้เพื่อทดสอบการจับกันของโปรตีน E7 (pull-down assay) โดยการใช้โปรตีน E7 เป็นตัวล่อ (bait) จากนั้นตรวจสอบโปรตีนที่ถูกตกมาด้วยกันโดยใช้แอนติบอดีต่อ DNMT1 และ TFAP2A ด้วยเทคนิค western blot และจากผลการทดลองพบว่าตรวจพบ DNMT1 และ TFAP2A ในตะกอนโปรตีน E7 ซึ่งผลที่ได้จากการทดลองนี้สนับสนุนตามสมมติฐานที่ตั้งไว้

**Keywords:** Human papillomavirus, Cervical cancer, Pull-down assay

**คำสำคัญ:** ไวรัสฮิวแมนแพปพิโลมา มะเร็งปากมดลูก การตกตะกอน โปรตีน

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## Introduction

Cervical cancer is the most common gynecologic malignancy in women around the world (Lopez et al., 2017). More than 90% of this cancer are caused by infection of high risk HPV type 16 and 18 through sexual intercourse (Munoz et al., 2003). Nowadays, viral oncoproteins, E6 and E7, are commonly known to interact with host regulatory proteins resulting in oncogenesis. Interaction between HPV E6 and p53 leads to the preventing apoptosis and enabling transformed cells to replicate (Tommasino et al., 2003). Moreover, the interaction between HPV E7 and retinoblastoma protein results in release and activation of E2F transcription factors that drive the expression of genes which precipitate in cell-cycle entry and promotes DNA synthesis (Crosbie et al., 2013). Additionally, HPV E7 plays important role to suppress expression of tumor suppressor genes by DNA methylation (Jin et al., 2011).

Previously, our studies have been demonstrated that 93% of methylation of *CCNA1* gene promoter was found in cervical cancer patients by comparing to normal cervix (Kitkumthorn et al., 2006). *CCNA1* or cyclin A1 is a gene which contributes to G1 to S cell cycle progression in somatic cells and also correlates with the repair of DNA from destruction (Ji et al., 2005). Furthermore, HPV16E7 is able to induce hypermethylation at the promoter of *CCNA1* gene by forming a complex with DNMT1 (DNA (cytosine-5-)-methyltransferase1) leading to reduction of gene expression (Chalertpet et al., 2015). DNMT1 is a major epigenetic gene silencer in the human genome. It plays an important role in maintenance methylation in somatic cells. In cancer cells, DNMT1 is responsible for the aberrant hypermethylation of CpG islands at promoter regions of tumor suppressor genes leading to genes silencing (Pradhan et al., 2008). A previous study demonstrated that leading to a decrease of *E-cadherin* expression was due to the reduction of the expression of transcriptional factor (Laurson et al., 2010). This finding suggested that decreasing of *E-cadherin* expression is not caused by DNA methylation but by chromatin remodeling. The reports from many studies showed that HPV16E7 protein was able to bind to many transcription factors such as SP1 (Gloss and Bernard, 1990), AP1 (Thierry et al., 1992), GRE, NF1, YY1, Oct-1 and C/EBP (Hoppe-Seyler and Butz, 1994) etc.

TFAP2A is a member of the AP2 transcription factor family that binds to the consensus sequence 5'-GCCNNNGGC-3' and regulates genes for controlling cell division and apoptosis (Su et al., 2014).

## Objective of the study

This study aimed to demonstrate that complex formation of host DNMT1 and transcription factors with HPV16E7.

## Methodology

### Bioinformatics analysis for prediction of *CCNA1* transcription factor and binding region

Bioinformatics were used for prediction transcription factors based on nucleotide sequences of *CCNA1* promoter and selection of candidate transcription factor. The position of *CCNA1* promoter's sequences was referred from the work of Chalertpet et al., in 2015. The sequence was found by [http://epd.vital-it.ch/search\\_EPDnew.php](http://epd.vital-it.ch/search_EPDnew.php).

After that, transcription factors were predicted by <http://jaspar.genereg.net> and <http://algggen.lsi.upc.es>. Finally, the protein that had the highest score of binding to promoter of *CCNA1*, derived from the Cytoscape program was chosen for investigation.

#### **Cell line and culture**

The Human embryonic kidney cell line (HEK293) was kindly provided by P. Chienwichat (Faculty of Medicine and Public Health, HRH Princess Chulabhorn College of Medicine Science, Chulabhorn Royal Academy). It was grown and maintained in MEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Gibco, Carlsbad CA, USA) at 37°C in an atmosphere of 5% CO<sub>2</sub>.

#### **Human papillomavirus type 16 E7 plasmid construction**

It was designed by carrying out the transfection of the HPV16E7 recombinant plasmid in mammalian cells, plasmid 13634: pGEX2T E7 (Addgene, Cambridge, MA, USA), which expressed only in bacterial cells, was transformed to MAX Efficiency DH5a 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 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 BamHI (Thermo Fisher Scientific, Waltham, MA, USA) and EcoRI (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 already confirmed by DNA sequencing. This plasmid construction was kindly provided by K. Chalertpet (Center of Excellence in Molecular Genetics of Cancer and Human Diseases, Department of Anatomy, Faculty of Medicine, Chulalongkorn University).

#### **Transfection**

HEK293 cell was seeded into 100 mm dish at  $2 \times 10^6$  (JET BIOFIL, Guangzhou, China) in 10 mL of MEM medium 24 hours before transfection. After 24 hours of incubation, 20 µg of each HPV16E7 plasmid and empty vector were diluted in 1 mL serum growth medium and 20 µL of Turbofect (Pierce Biotechnology, Thermo Fisher Scientific, Rockford, USA). Next, 24 hours after transfection, cells were collected for study of the protein interaction.

#### **Pulldown assay**

After cell collection, protein lysate was extracted by RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% TritonX-100) with protease inhibitor. Lysates were cleared by centrifugation at 14000g for 15 minutes. BCA assay (Pierce Biotechnology, Thermo Fisher Scientific, Rockford, USA) was performed for measurement the concentration of protein. Pulling down the specific protein (HPV16E7 protein) was used HisPur<sup>TM</sup> Ni - NTA Resin (Pierce Biotechnology, Thermo Fisher Scientific, Rockford, USA) by gentle rotation at 4 °C overnight. After that, resin beads were washed for reducing non-specific proteins by using PBS with 25 mM Imidazole and centrifugation at 1000g for 5 minutes at 4 °C. Finally, expected protein was eluted by elution buffer (PBS with 250 mM Imidazole) and centrifugation at 1000g for 5 minutes at 4 °C.

### SDS-PAGE and western blot

The pulled-down proteins were separated on SDS-PAGE (using 10% polyacrylamide gel) and transferred to PVDF membrane. Proteins were detected with the appropriate specific antibodies (anti-HPV16E7 Invitrogen 8C9, Camarillo, CA; dilution 1:200, anti-DNMT1 Abcam ab13537; dilution 1:1000 and anti AP2alpha Abcam ab52222; dilution 1:1000), and developed with Chemiluminescent Substrate Kit (Merck Millipore, Billerica MA, USA)

### Statistical analysis

Independent T-test was used for comparison with the relative intensity of protein band between transfection HPV16E7 plasmid and Empty vector at *P-value* = 0.001

### Results

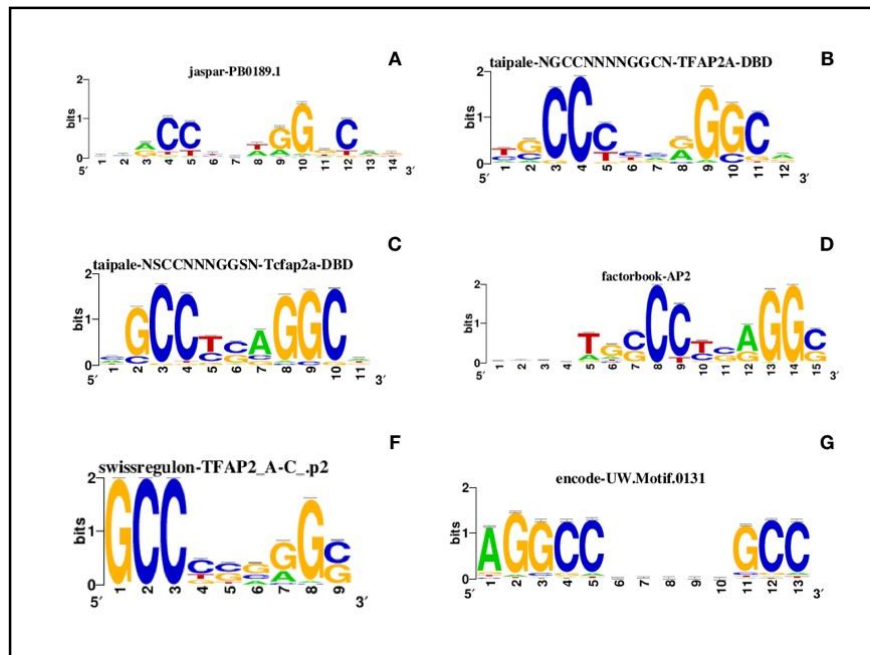
1. Bioinformatics prediction from the nucleotide sequences of transcription factor binding sites on *CCNA1* promoter was referred from a previous report (Chalertpet et al., 2015) (Figure1.) TFAP2 and EST1 2 databases were shown in Table1. After that, TFAP2A with the highest score of binding to *CCNA1* promoter, derived from the Cytoscape program (Figure2.), was selected to investigate the interaction with HPV16E7 and DNMT1 complex.

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>FP017398 CCNA1_1 :+U EU:NC; range -730 to -526.
CAGGAAGCGTAGGTGTGTGAGCCGACCCGGAGCGAGCCGCGCCCTCGGGCCAGCGTGGGC
AGGGCGCCGCAGCCTGCGCAGCCCCGAGGACCCCGCGTTCGCTCTCCCGAGCCAGGGTTCT
CAGGAGCGGGCCGCGCAGGAGACGTTAGAGGGGGTTGTTAGCGGCTGTTGGGAGAACGGG
TCACGGAAACAGTCCCTTCCAAAGC
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**Figure1** The nucleotide sequences between -730 to -526 of *CCNA1* promoter

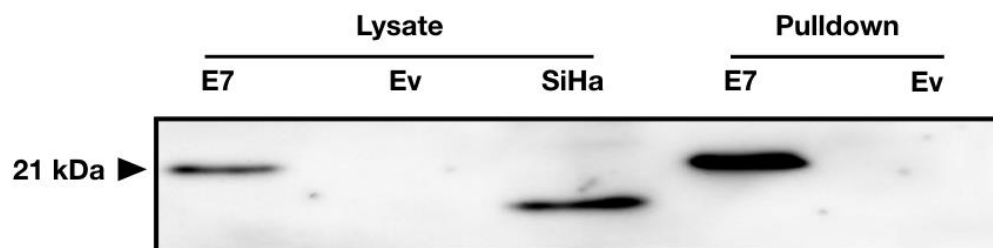
**Table1** Candidates of transcription factors

Bioinformatics	Transcription factor
<a href="http://jaspar.genereg.net">http://jaspar.genereg.net</a>	<b>TFAP2A</b> , EST1
<a href="http://alggen.lsi.upc.es">http://alggen.lsi.upc.es</a>	C/EBPbeta, PR B, PR A, ER-alpha, FOXP3, <b>TFAP2A</b> , Pax 5, p53, c-Ets-1

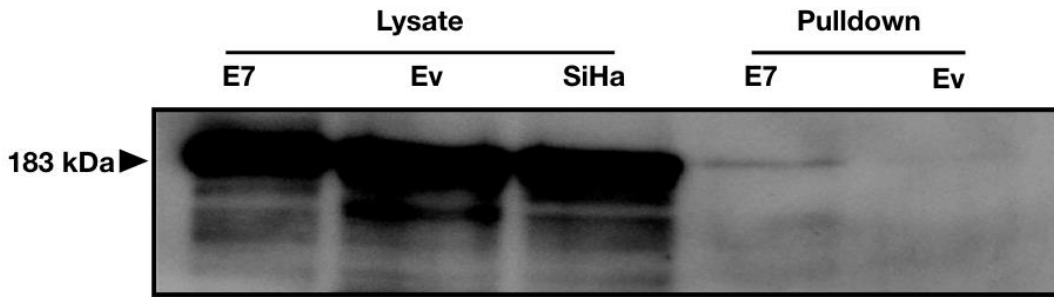


**Figure2** Conserved TFAP2A motif sequences that bind to promoter of CCNA1

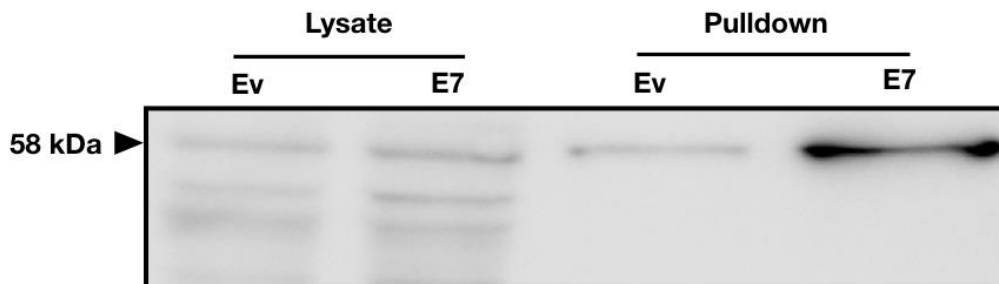
2. By pulldown assay the protein-protein interaction between HPV16E7 and TFAP2A was examined. Firstly, the interaction between HPV16E7 and DNMT1 was studied in HEK293 cells expressing 16E7 and DNMT1. 6xHistidine tagged HPV16E7 was transfected into the cells, and pulldown assay was performed by using HisPur<sup>TM</sup> Ni-NTA Resin and the complex was detected by western blot. Figure3 demonstrated HPV16E7 protein around 21 kDa was co-precipitated with DNMT1 at around 183 kDa (Figure4). The interaction with TFAP2A was detected again using anti AP2a antibody at around 58 kDa (Figure5) in E7 transfected HEK293 cells. A comparison of relative intensity of TFAP2A protein bands between HEK293 cells transfected with Empty vector and with HPV16E7 showed that this protein band (58 kDa) should not be a non-specific protein as a significant increase (p value <0.001) of band intensity was detected in E7 transfected cells as validated by independent T-test (Figure6.).



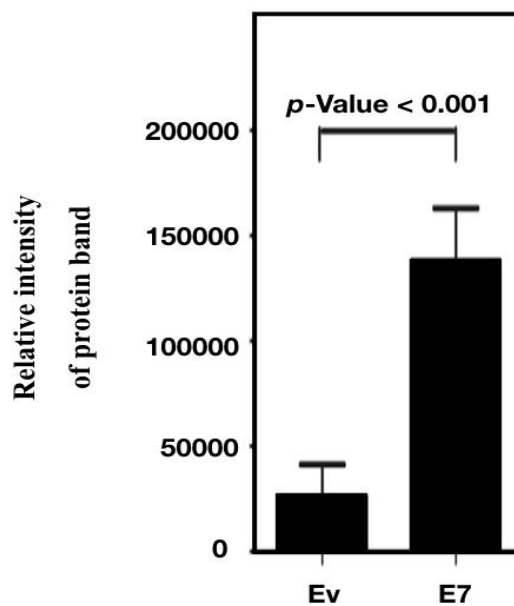
**Figure3** Western Blot analysis of HPV16E7 protein with Ni-NTA resin bead and detecting HPV16E7 with HPV16E7 antibody. Protein lysate from HEK293 transfected HPV16E7 (E7) was used as positive controls and HEK293 transfected Empty vector (Ev) was used as a negative control. Protein from HEK293 transfected HPV16E7 was found at 21 kDa because of HPV16E7 plasmid contain 6x histidine tag while protein from SiHa which is a positive HPV16 cervical carcinoma cell line was used as a control of HEK293 transfected HPV16E7 was found at 17 kDa.



**Figure4** Western Blot analysis of HPV16E7 protein with Ni-NTA resin bead and detecting HPV16E7-DNMT1 with DNMT1 antibody at 183 kDa. Protein lysate from HEK293 transfected HPV16E7 (E7), SiHa and HEK293 transfected Empty vector (EV) were used as positive controls.



**Figure5** Western Blot analysis of pulldown assay of HPV16E7 protein by Ni-NTA resin bead and TFAP2A was detected with TFAP2A antibody that expected interaction with HPV16E7 protein at 58 kDa.



**Figure6** Relative intensity of TFAP2 bands in Western Blot analysis of pulldown assay of HPV16E7 transfected cells (E7) compared with empty vector control transfected cells (Ev) as analyzed by t-test at P value <0.001.

### Discussion and Conclusion

HR- HPV E7 drives the oncogenic function by modifying cellular growth- regulator pathways as well-known the interaction with the retinoblastoma protein (Dyson et al., 1989). Nevertheless, the interaction between HR- HPV E7 and pRb is just one of the many distinct interactions. In this study, we demonstrate protein-protein interaction between HPV16 E7-DNMT1 complexes and TFAP2A from pulldown assay of HPV16E7 and detection with TFAP2A antibody, it could presumably be concluded that HPV16E7 binds to TFAP2A and possibly by forming complex with DNMT1. TFAP2A was chosen from predicted candidates of transcription using <http://jaspar.genereg.net> and <http://alggen.lsi.upc.es>. programs. TFAP2A plays a role in regulating the expression of genes which are involved in tumor progression (Su et al., 2014).

Our study suggested that forming the complex of HPV16E7 with DNMT1 and TFAP2A might trigger methylation of *CCNA1* promoter which is targeted gene of TFAP2A and might consequently induce the suppression of *CCNA1*. However, we are determining other putative target genes, especially TSGs of TFAP2A and examining methylation to support our hypothesis. Moreover, the results will be examined and validated in positive and negative cervical cancer cell, SiHa and C33A, respectively to convince our hypothesis.

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