

# Association between promoter methylation and gene expression of *CGB3* and *NOP56* in HPV-infected cervical cancer cells

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**Abstract.** Overexpression of the *E7* gene of human papillomavirus (HPV) type 16 is one of the primary causes of cervical cancer. The *E7* protein can bind with DNA methyltransferase I and induce methylation of tumor suppressor genes, such as cyclin-A1 (*CCNA1*), leading to suppression of their expression, and thus, cancer progression. In the present study, the confirmation of methylation-related expression of chorionic gonadotropin subunit 3 (*CGB3*) and nucleolar protein 56 (*NOP56*) genes in 5-Azacytidine (5'-aza)-treated HPV16-positive SiHa and HPV16-negative C33A cell lines was shown. Using methylation-specific-PCR and quantitative PCR, the results showed that *CGB3* and *NOP56* methylation significantly decreased as the 5'-aza concentration was increased, and this was inversely associated with their expression. Moreover, overexpression of *E7* contributed to the augmentation of *CGB3* and *NOP56* methylation levels in C33A cells, resulting in a decrease in their expression. This study extends on previous observations of *E7* HPV16 oncogenic function in terms of methylation-repressing expression in more genes, which may be wholly applied to gene therapy in cervical cancer prevention.

## Introduction

Cervical cancer is the fourth most frequent cancer amongst women worldwide, resulting in ~570,000 new cases in 2018, 90% of the 311,000 women with cervical cancer died (1). Human papillomavirus (HPV) is the most common sexually

transmitted infection and has been recognized as an important risk factor for cervical cancer (2,3). There are >100 types of HPVs, which are categorized into low- and high-risk HPV (4-7). High-risk HPVs include HPV16 and HPV18 amongst several others, and are the causative agents in ≥90% of cervical cancer cases, whilst also being linked to >50% of other types of anogenital cancer (7-9). High-risk HPV (16 and 18)-associated cervical cancer is driven by two major viral oncoproteins, E6 and E7, which are associated with the tumor suppressor genes *p53* and retinoblastoma 1, respectively, degrading them (10-15). HPV16 E6 was found to upregulate DNA methyltransferase I (DNMT1) expression and suppress *p53* in cells (16-19). Moreover, previous studies have found that E7 displayed an interaction with DNMT1, which led to aberrant methylation of the cellular genome, resulting in silencing of tumor suppressor genes (20-23).

Several groups of researchers have studied the association between HPV infection and promoter methylation. Chalertpet *et al* (24) reported that HPV16 E7 can induce promoter methylation in *CCNA1*. However, Yanatatsaneejit *et al* (25) in 2020 demonstrated that both HPV E6 and E7 induced promoter methylation in death-associated protein kinase 1 (*DAPK1*) and cell adhesion molecule 1 (*CADMI*), respectively. Moreover, their study indicated that the E7 protein can bind to the promoter region of *CADMI*, and chromatin immunoprecipitation indicated that it may bind to DNMT1 through the same mechanism as *CCNA1* (25). Na Rangsee *et al* (26), using readings from mass spectrometry followed by STRING database protein network analysis, showed that E7 possibly formed a complex with a set of transcription factors, including SP1, which links with the Yin yang 1 (YY1) transcription factor contributing to the E7 mediated hypermethylation of the genes. This highlighted the possibility of E7 combining with DNMT1 through a transcription factor to the promoter region of the *CCNA1* gene, resulting in promoter methylation. Thus, studying other tumor suppressor genes that share the same sequence as the *CCNA1* promoter where HPV16 E7 can bind may highlight novel potential targets. In order to investigate this possibility, bioinformatics analysis was performed and the results revealed that YY1 was found to bind to the promoter region of *CCNA1*. Here, other genes which shared the same

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YY1 binding site as *CCNA1* promoter were explored. *CGB3* and *NOP56* were selected to investigate the induction of promoter methylation by HPV16 E7.

The *CGB3* gene plays critical roles in increased expression of the CGB subunit in ovarian cancer through demethylation of the CGB promoter (27) and malignant transformation of non-trophoblastic cells (28,29). The *NOP56* gene is involved in several oncogenic roles in more than five types of cancers (30); however, there is no evidence linking it to cervical cancer, to the best of our knowledge. These findings lead to the investigation of the role of HPV16 E7 in cervical cancer progression through promoter methylation of these two genes.

As methylation does not alter DNA sequences, previous studies have focused on using inhibitors of DNA methyltransferases, including 5'-Azacytidine (5-aza), which can potentially be used as anticancer agents (31-33). Aberrant DNA methylation is a biochemical process that can be reversed using demethylating agents (34). 5-aza was the first DNA methyltransferase inhibitor approved by the U.S. Food and Drug Administration for use as a chemotherapeutic against myelodysplastic syndrome, a heterogeneous bone marrow disorder (35). 5-aza covalently binds to DNMT leading to a reduction in its DNA methyltransferase activity (32). This results in the loss of methylation in specific gene regions and activates the expression of the associated genes (36-38). Moreover, several studies have reported that the association between DNA methylation and repression of gene expression can lead to cancer progression (39-41). Therefore, re-expression of methylated genes in cervical cancer cell lines following treatment with a demethylating agent, such as 5-aza, should be further investigated to assess the effect of inhibiting the methylation rate and whether it can increase the expression of tumor suppressor genes (31,42).

The present study investigated whether HPV16 E7 could induce promoter methylation and decrease the expression of *CGB3* and *NOP56*. The study further assessed the effects of 5-aza treatment on promoter methylation and gene expression in cervical cancer C33A and SiHa cell lines to demonstrate the association between gene methylation and the regulation of tumor gene expression. This study may serve as an alternative strategy of drug therapy for patients with cervical cancer with aberrant gene promoter methylation (42).

## Materials and methods

**Bioinformatics analysis.** PROMO version 8.3 from the TRANSFAC database (algggen.lsi.upc.es) was used to select the genes of interest containing YY1 binding sites with *CCNA1* (43,44). JASPAR database (jaspar.genereg.net) was used to separately analyze the YY1 binding site sequences in the selected genes with reference to the *CCNA1* gene (45).

**Cell lines and culture.** Human cervical carcinoma cell lines [SiHa (HPV type 16) and C33A (HPV-)] were purchased from the American Type Culture Collection. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotic-antimycotic (Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO<sub>2</sub> in a humidified incubator.

**Transfection.** For HPV 16 E7 overexpression, 3x10<sup>5</sup> cells/ml C33A cells were seeded into a 6-well plate and incubated overnight at 37°C. Subsequently, cells were transfected with 2 µg HPV 16 E7 plasmid (E7; Invitrogen; Thermo Fisher Scientific, Inc.) and pcDNA 3.1/myc-HIS empty vector (PC; Invitrogen; Thermo Fisher Scientific, Inc.) using TurboFect reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A 72 h post-transfection, DNA and RNA were extracted from each sample to assess promoter methylation and gene expression, respectively, using specific primers for each gene.

**5-aza treatment.** To evaluate gene methylation and expression, cells were treated with 5-aza (Sigma-Aldrich; Merck KGaA). Briefly, SiHa and C33A cells (3x10<sup>5</sup> cells/ml) were seeded 1 day before 5-aza treatment. The following day fresh DMEM containing 5-aza (SiHa, 0, 20, 30 and 40 µM; C33A, 0, 3, 5 and 7 µM) was added to the cells for 5 consecutive days, being replaced every 24 h until analysis (24).

**Isolation of DNA.** DNA was extracted from SiHa and C33A cells using 10% SDS (Sigma-Aldrich; Merck KGaA), lysis buffer II (0.75 M NaCl and 0.024 M EDTA at pH 8) and 20 mg/ml proteinase K (Invitrogen; Thermo Fisher Scientific, Inc.), digested by incubating at 50°C overnight. Subsequently, phenol/chloroform extraction and 100% ethanol precipitation were performed. DNA was air-dried and resuspended in dH<sub>2</sub>O (46). DNA concentration was determined using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Inc.).

**Sodium bisulfite treatment and methylation-specific PCR (MSP).** DNA (750 ng) for each sample was subjected to bisulfite treatment using the EZ DNA Methylation-Gold kit (Zymo Research Corp.) according to the manufacturer's protocol. Eluted DNA was used to perform MSP using methylated and unmethylated specific primers (Table I). The annealing temperature for *CGB3* was 54°C and for *NOP56* it was 52°C. The thermocycling conditions were 95°C for 15 min; followed by 27 cycles of 95°C for 45 sec, the respective annealing temperature for 45 sec and 72°C for 45 sec; with a final extension step of 72°C for 7 min. Subsequently, 10 µl PCR product was observed by gel electrophoresis using an 8% acrylamide gel and stained with SYBR reagent (Lonza Group, Ltd.). The methylated and unmethylated band intensities of each sample were visualized and measured using a Storm 840 (Amersham Biosciences) and ImageJ version 2 (National Institute of Health). The EpiTect control DNA set (Qiagen GmbH) contained positive controls for methylation and unmethylation. The experiments were performed in triplicate.

**Preparation of RNA and cDNA synthesis.** Total RNA was extracted from cells using TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, total RNA (1 µg) from each sample was reverse transcribed into cDNA using the RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

**PCR.** PCR was performed to assess the expression of each gene in SiHa and C33A cells using specific forward and reverse primers for *CGB3* expression in HPV 16 E7 transfected cell.

Table I. Primer sequences, amplicon sizes, annealing temperature and conditions for methylation-specific PCR.

| Gene                   | Sequence, 5'-3'                 | Product size, bp | Annealing temperature, °C | Number of cycles |
|------------------------|---------------------------------|------------------|---------------------------|------------------|
| <i>CGB3</i>            |                                 |                  |                           |                  |
| Methylated forward     | CGGGTTGAATTTTTTCGTTGGC          | 116              | 52                        | 27               |
| Methylated reverse     | CCCAAAAAAACGCGACTTTCG           |                  |                           |                  |
| Unmethylated forward   | GGGTTTGGGTTGAATTTTTTGTGGT       | 127              | 55                        | 27               |
| Unmethylated reverse   | CAACCTCCCAAAAAAACACAACCTTCA     |                  |                           |                  |
| <i>NOP56</i> primer I  |                                 |                  |                           |                  |
| Methylated forward     | TATTTTTTATTATATTTTTGGAATC       | 64               | 38                        | 27               |
| Methylated reverse     | ATTAAATTATTTTAACCGTCG           |                  |                           |                  |
| Unmethylated forward   | GTATTTTTTATTATATTTTTGGAATT      | 68               | 42                        | 27               |
| Unmethylated reverse   | AATATTAAATTATTTTAACCATCA        |                  |                           |                  |
| <i>NOP56</i> primer II |                                 |                  |                           |                  |
| Methylated forward     | CGTTTATTTTGATGTATTTACGAC        | 120              | 50                        | 27               |
| Methylated reverse     | ATATCTACTTACGAATCAAATCG         |                  |                           |                  |
| Unmethylated forward   | TGGAATTGTTTATTTTGATGTATTTATGATG | 132              | 52                        | 27               |
| Unmethylated reverse   | AATATTAAATTATTTTAACCATCA        |                  |                           |                  |

*CGB3*, chorionic gonadotropin subunit 3; *NOP56*, nucleolar protein 56.

Table II. Primer sequences, amplicon sizes, annealing temperatures and conditions for PCR.

| Gene                             | Sequence, 5'-3'       | Product size, bp | Annealing temperature, °C | Number of cycles |
|----------------------------------|-----------------------|------------------|---------------------------|------------------|
| Human papilloma virus 16 E7      |                       |                  |                           |                  |
| Forward                          | GGGCAATTAATGACAGCTCAG | 142              | 56                        | 30               |
| Reverse                          | GTGTGCTTTGTACGCACAACC |                  |                           |                  |
| <i>GAPDH</i>                     |                       |                  |                           |                  |
| Forward                          | CAGCCGCATCTTCTTTTG    | 96               | 56                        | 28               |
| Reverse                          | GCCCAATACGACCAAATC    |                  |                           |                  |
| <i>GAPDH1</i>                    |                       |                  |                           |                  |
| Forward                          | TGGAAGGACTCATGACCACAG | 163              | 56                        | 28               |
| Reverse                          | TTCAGCTCAGGGATGACCTT  |                  |                           |                  |
| Chorionic gonadotropin subunit 3 |                       |                  |                           |                  |
| Forward                          | CAACACCACCATCTGTGC    | 194              | 56                        | 28               |
| Reverse                          | GGCAGAGTGCACATTGAC    |                  |                           |                  |
| Nucleolar protein 56             |                       |                  |                           |                  |
| Forward                          | CAGCATCGTTCGTCTGGTGG  | 105              | 56                        | 28               |
| Reverse                          | AGGCGGAGGTCCTCATGAAC  |                  |                           |                  |

The PCR mixture contained 10X PCR buffer (Qiagen GmbH), 0.2 mM dNTPs (New England BioLabs, Inc.), 0.3  $\mu$ M forward and reverse primer (Table I) and 1 U HotStartaq DNA polymerase (Qiagen GmbH). *GAPDH* was used as an internal control (Table II). PCR products (10  $\mu$ l) were subjected to electrophoresis on an 8% acrylamide gel and stained with SYBR. The experiment was performed in triplicate.

**Quantitative PCR (qPCR).** *CGB3* and *NOP56* mRNA expression in 5-aza-treated, and HPV 16 E7 and *NOP56*

gene expression levels in HPV 16 E7 transfected cells were determined by qPCR using a 7500-fast qPCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and Power SYBR-Green PCR MasterMix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The qPCR was performed using specific primers (Table II), with *GAPDH* as the reference gene. The thermocycling condition were: 95°C for 10 min; followed by 40 cycles of 95°C for 15 sec, 56°C for 30 sec and 72°C for 45 sec. The mRNA expression levels were quantified using the  $2^{-\Delta\Delta C_q}$  method (47).

**YY1**

|                     |   |    |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|---------------------|---|----|----|----|----|----|----|----|----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
|                     | 1 | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 | 110 | 120 | 130 | 140 | 150 | 160 | 170 | 180 | 190 | 200 | 210 | 220 | 230 | 240 | 250 | 260 | 270 |  |
| CCNA1, range -499-0 |   |    |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |     |     | 0   |     |     |     | 0   |     |     |     |  |
| CGB3, range -499-0  |   |    |    |    |    |    |    |    |    |    |     |     |     | 0   |     |     |     |     |     | 0   | 0   |     |     |     |     |     |     |     |  |
| NOP56, range -499-0 |   |    |    |    |    |    |    |    |    |    |     |     |     | 0   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |

Figure 1. A YY1 transcription factor binding site detected in the DNA sequences of *CCNA1*, *CGB3* and *NOP56* genes. The binding site was predicted in 3 or more input sequences with a dissimilarity margin of  $\leq 15\%$  using the PROMO database. YY1, yin yang 1; *CCNA1*, cyclin-A1; *CGB3*, chorionic gonadotropin subunit 3; *NOP56*, nucleolar protein 56.

**A YY1 binding site of *CCNA1***

| Matrix ID          | Name | Score   | Relative score | Sequence ID | Start | End | Strand |
|--------------------|------|---------|----------------|-------------|-------|-----|--------|
| MA0095.1           | YY1  | 7.21934 | 0.940561021535 | FP018858    | 183   | 188 | +      |
| Predicted sequence |      | GCCATG  |                | *           |       |     |        |
| MA0095.1           | YY1  | 7.21934 | 0.940561021535 | FP018858    | 185   | 190 | -      |
| Predicted sequence |      | GCCATG  |                |             |       |     |        |
| MA0095.1           | YY1  | 6.22486 | 0.889769342397 | FP018858    | 228   | 233 | -      |
| Predicted sequence |      | TCCATG  |                | **          |       |     |        |

**B YY1 binding site of *CGB3***

| Matrix ID          | Name | Score   | Relative score | Sequence ID | Start | End | Strand |
|--------------------|------|---------|----------------|-------------|-------|-----|--------|
| MA0095.1           | YY1  | 8.38313 | 0.99999988315  | FP026107    | 134   | 139 | -      |
| Predicted sequence |      | GCCATC  |                | *           |       |     |        |
| MA0095.1           | YY1  | 5.23583 | 0.839256270211 | FP026107    | 188   | 193 | +      |
| Predicted sequence |      | CCCATG  |                |             |       |     |        |
| MA0095.1           | YY1  | 5.23583 | 0.839256270211 | FP026107    | 190   | 195 | -      |
| Predicted sequence |      | CCCATG  |                |             |       |     |        |

**C YY1 binding site of *NOP56***

| Matrix ID          | Name | Score       | Relative score | Sequence ID | Start | End | Strand |
|--------------------|------|-------------|----------------|-------------|-------|-----|--------|
| MA0095.1           | YY1  | 8.19127     | 0.990201255157 | FP026665    | 411   | 416 | -      |
| Predicted sequence |      | ACCATC      |                |             |       |     |        |
| MA0095.1           | YY1  | 7.38865     | 0.949208309177 | FP026665    | 130   | 135 | -      |
| Predicted sequence |      | TCCATC      |                | **          |       |     |        |
| MA0095.2           | YY1  | 5.74428     | 0.80821831118  | FP026665    | 408   | 419 | +      |
| Predicted sequence |      | GGAGATGGTGC |                |             |       |     |        |

Figure 2. YY1 binding site in promoter region of genes as determined using JASPAR. (A) *CCNA1* with finding sequences GCCATG, GCCATG and TCCATG. (B) *CGB3* gene possessed the GCCATC sequence. *CCNA1* and *CGB3* shared 80% same sequence denoted by (\*). (C) *NOP56* possessed the TCCATC sequence. *CCNA1* and *NOP56* shared 80% same sequence denoted by (\*\*). YY1, yin yang 1; *CCNA1*, cyclin-A1; *CGB3*, chorionic gonadotropin subunit 3; *NOP56*, nucleolar protein 56.

**Statistical analysis.** To assess gene promoter methylation and expression levels in 5-aza-treated cells, a one-way ANOVA followed by a Dunnett's post hoc test was performed using GraphPad Prism version 5 (GraphPad Software, Inc.).  $P \leq 0.05$  was considered to indicate a statistically significant difference. Student's t-tests (unpaired) was used to compare *CGB3* and *NOP56* expression and methylation in SiHa and C33A cells, respectively, as well as expression and methylation of *CGB3* and *NOP56* in the HPV 16 E7- and PC-transfected cells.

**Results**

**Bioinformatics analysis.** YY1 was found to be the binding to the promoter of *CCNA1*, *CGB3* and *NOP56* based on PROMO analysis (Fig. 1). Moreover, the results from JASPAR demonstrated that the binding sequence of YY1 in the *CCNA1* gene shared 80% similarity in sequence, GCCATG and TCCATG (Fig. 2A), to that present in *CGB3* GCCATC

denoted by \* in (Fig. 2B) and in *NOP56* TCCATC denoted by \*\* in (Fig. 2C), respectively. Thus, both genes were selected for further analysis.

**Gene expression and methylation in HPV<sup>-/-</sup> cervical cancer cell lines.** To assess the expression of *CGB3* and *NOP56* genes, PCR was performed in SiHa (HPV<sup>+</sup>) and C33A (HPV<sup>-</sup>) cancer cell lines. The results showed that both *CGB3* and *NOP56* exhibited lower expression in SiHa cells (26.74 and 38.42%, respectively) compared with that observed in C33A cells (73.26 and 44.5%), respectively (Fig. 3A and C). GAPDH was used for *CGB3* gene and GAPDH1 primer was used for *NOP56* gene normalization in SiHa and C33A cells. The bar graphs shows the mean  $\pm$  standard error of the mean of *CGB3* ( $P=0.001$ ; Fig. 3B) and *NOP56* expression ( $P=0.004$ ; Fig. 3D).

Furthermore, methylation status was also observed for both the genes, and compared between SiHa and C33A cells. The results demonstrated that the methylation of *CGB3* and *NOP56*

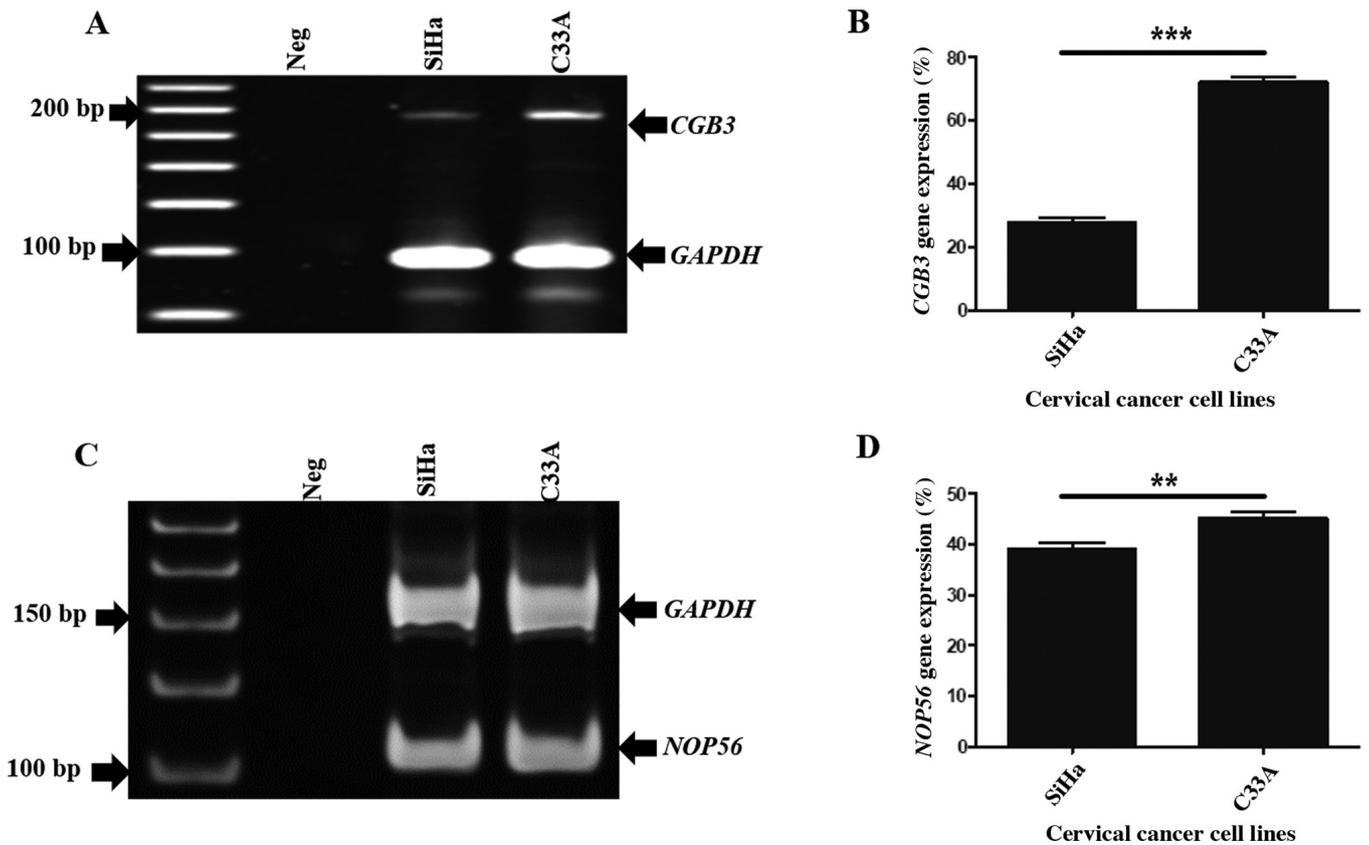


Figure 3. Expression of *CGB3* and *NOP56* in SiHa and C33A cells. (A) *CGB3* expression was observed. (B) Graphs represent the mean  $\pm$  the standard error of the mean of *CGB3* expression. (C) *NOP56* expression was observed. (D) Data are presented as the mean  $\pm$  the standard error of the mean of *NOP56* expression. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Neg, distilled water negative control; *CCNA1*, cyclin-A1; *CGB3*, chorionic gonadotropin subunit 3; *NOP56*, nucleolar protein 56.

(primer II) in SiHa cells was 61.18 and 53.94% compared with C33A cells (38.81 and 46.05%), respectively (Fig. 4A and C). The bar graph shows the mean  $\pm$  standard error of the mean of *CGB3* ( $P = 0.004$ ; Fig. 3B) and *NOP56* methylation ( $P = 0.045$ ; Fig. 3D). The results indicated that the presence of HPV may influence the expression and methylation of *CGB3* and *NOP56* genes.

*HPV 16 E7 induces promoter methylation and decreases gene expression.* To assess whether HPV E7 induced gene promoter methylation and thus decreased expression, HPV 16 E7 was overexpressed in C33A (HPV) cells. The results demonstrated that the expression of HPV 16 E7 was significantly higher in HPV 16 E7 transfected cells ( $P = 0.001$ ) compared with the PC transfected cells (Fig. 5).

Furthermore, alterations to gene promoter methylation and expression were assessed in HPV 16 E7 transfected C33A cells. At 72 h post-transfection, the band intensities of *CGB3* and *NOP56* were compared between E7- and PC-transfected cells. To measure gene expression and methylation, reverse transcription-PCR and MS-PCR were performed, respectively. The gene expression levels of *CGB3* were significantly reduced ( $P = 0.02$ ) in HPV 16 E7 transfected cells (band intensity, 37.2%) compared with that in the PC transfected cells (band intensity, 62.7%) (Fig. 6A). The bar graph shows the mean  $\pm$  standard error of *CGB3* expression (Fig. 6B). *NOP56* expression level in HPV 16 E7 transfected C33A cells was significantly decreased ( $P < 0.0001$ ) compared with that in the

PC transfected cells (Fig. 7). *GAPDH* was used as an internal control. The expression of *NOP56* was measured by qPCR.

Promoter methylation of both genes was significantly increased in HPV 16 E7 overexpressing C33A cells compared with that in the PC transfected cells. In HPV 16 E7 transfected C33A cells, the band intensities of *CGB3* and *NOP56* (Primer I) were 54.21 and 55.01%, respectively, which were significantly higher than those in the PC transfected cells (39.36 and 41.54%;  $P = 0.005$  and  $P = 0.0004$ , respectively). The methylation positive control displayed band intensities of 6.43 and 3.45%, respectively (Fig. 8A and C). The bar graph shows the mean  $\pm$  standard error of the mean of both *CGB3* and *NOP56* expression (Fig. 8B and D), respectively. The unmethylated forms of *CGB3* and *NOP56* (Primer I) in HPV 16 E7 transfected cells gradually decreased (data not shown).

*Effect of 5-aza on gene promoter methylation and gene expression of CGB3 and NOP56.* An association between promoter methylation and gene expression was confirmed by measuring mRNA expression levels in SiHa and C33A cells following 5-aza treatment. The C33A and SiHa cancer cells were treated continuously for 5 days with 5-aza at concentrations of 0, 3, 5 and 7 or 0, 20, 30 and 40  $\mu\text{M}$ , respectively. Subsequently, DNA and RNA were extracted to measure promoter methylation and expression levels of each gene in cells following treatment with different concentrations of 5-aza.

The *CGB3* and *NOP56* (Primer II) promoter methylation results demonstrated that the methylation status of both

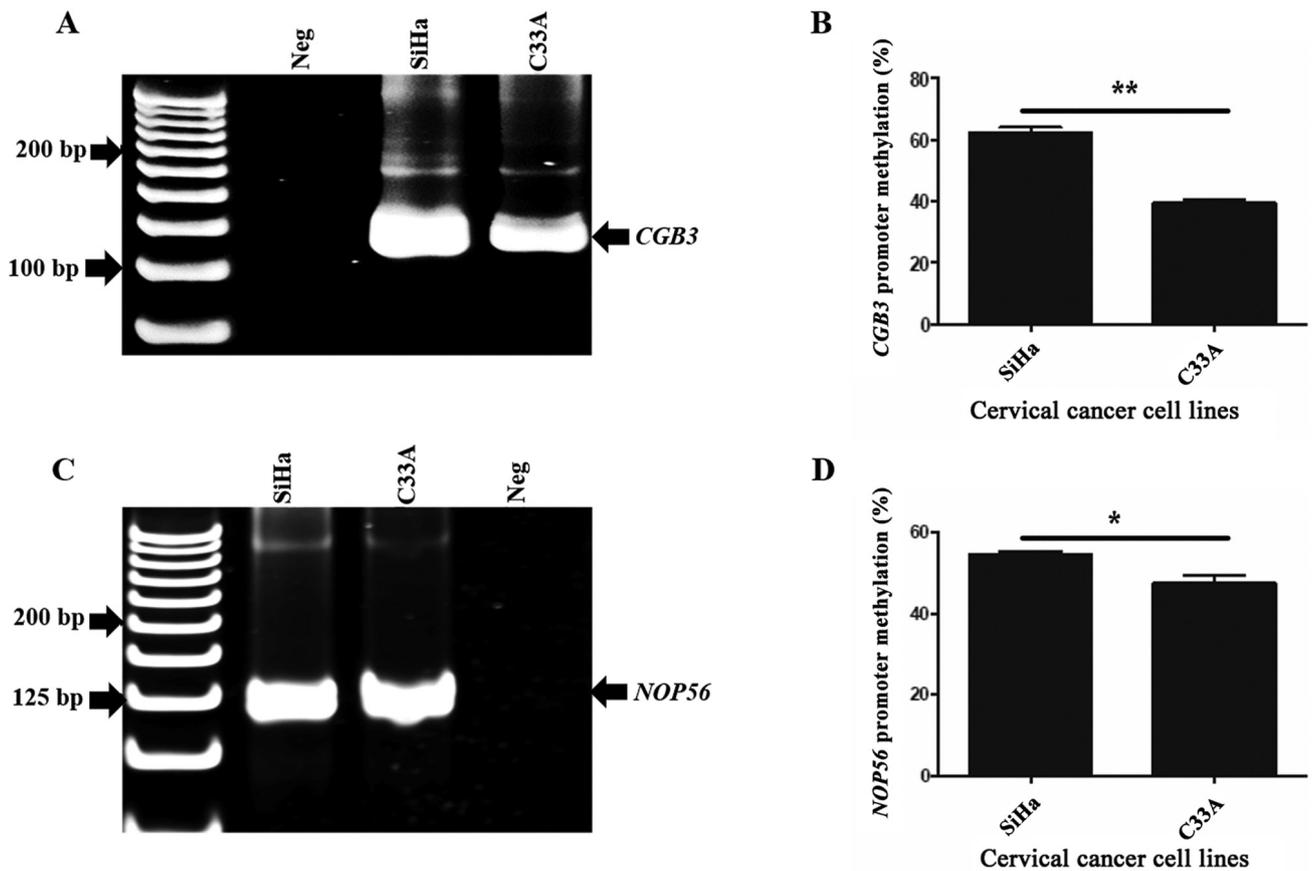


Figure 4. Methylation of *CGB3* and *NOP56* in SiHa and C33A cells. (A) *CGB3* methylation was observed. (B) Graphs represent the mean  $\pm$  the standard error of the mean of *CGB3* methylation. (C) *NOP56* methylation was observed. (D) Data are presented as the mean  $\pm$  the standard error of the mean of *NOP56* methylation. \* $P < 0.05$ , \*\* $P < 0.01$ . Neg, distilled water negative control; *CCNA1*, cyclin-A1; *CGB3*, chorionic gonadotropin subunit 3; *NOP56*, nucleolar protein 56.

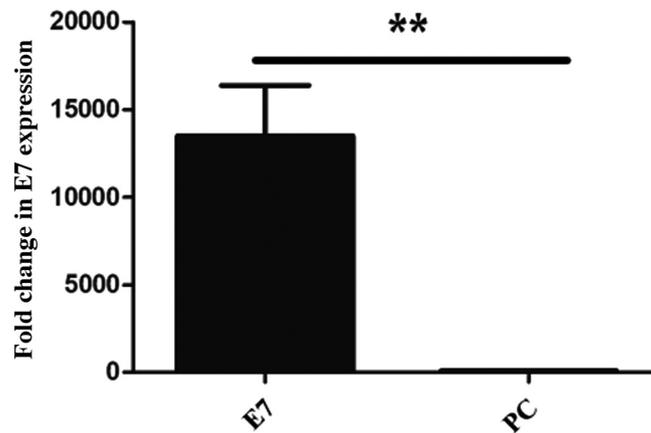


Figure 5. HPV 16 E7 recombinant plasmid and PC were overexpressed in C33A cells. The graph represents the fold change of HPV 16 E7 expression at the mRNA level. \*\* $P < 0.01$ . Neg, distilled water negative control; PC, empty plasmid; HPV 16, human papillomavirus type 16.

gene promoters in C33A and SiHa cells were significantly decreased following treatment with different concentrations of 5-aza. The band densities of *CGB3* and *NOP56* methylation in C33A cells gradually decreased as the 5-aza concentration increased (0-7  $\mu\text{M}$ ; Fig. 9A and C), whereas the unmethylated forms of both genes in C33A cells increased with increasing 5-aza concentrations (data not shown). In parallel, the band densities of *CGB3* and *NOP56* methylation (Primer II) in SiHa cells decreased with increasing 5-aza concentrations

(0-40  $\mu\text{M}$ ; Fig. 9B and D). The decrease in the methylation status of both *CGB3* and *NOP56* following treatment with different concentrations of 5-aza in C33A ( $P < 0.0001$  and  $P = 0.0002$ ; Fig. 10A and C) and SiHa ( $P < 0.0001$  and  $P = 0.0002$ ; Fig. 10B and D) cells was significant.

Gene expression was shown by using reverse transcription-PCR and qPCR. The band densities of *CGB3* and *NOP56* expression in C33A cells gradually increased with increasing 5-aza concentrations (0-7  $\mu\text{M}$ ; Fig. 11A and C). The

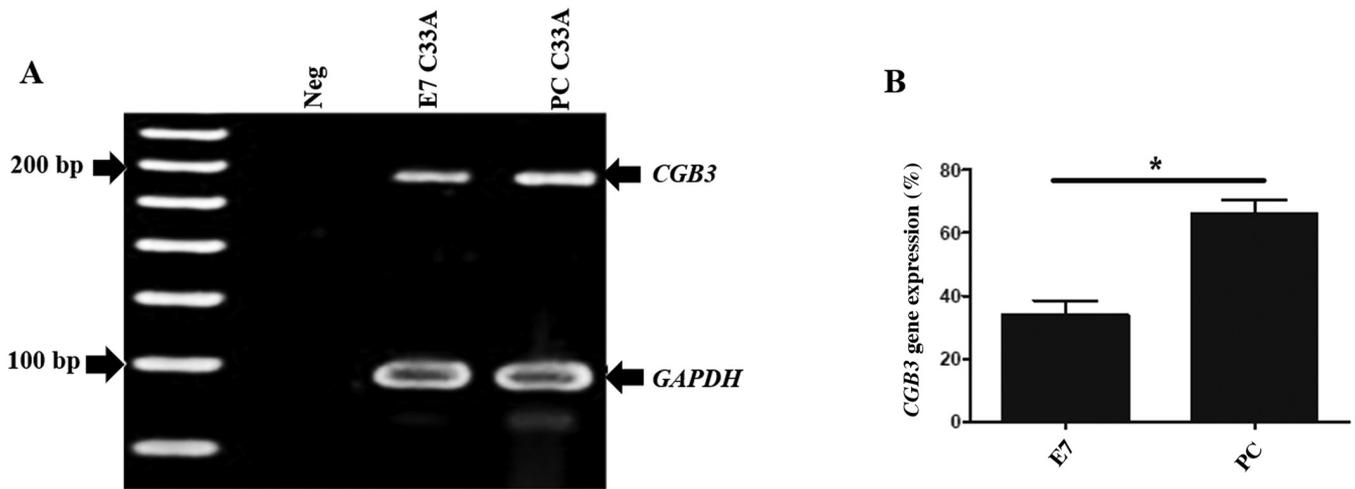


Figure 6. Downregulation of *CGB3* expression following HPV 16 E7 transfection (A) Expression of *CGB3* following HPV 16 E7 transfection in C33A cells. (B) Data are presented as the mean  $\pm$  the standard error of the mean of three repeats. \* $P < 0.05$ . Neg, distilled water negative control; PC, empty plasmid; *CGB3*, chorionic gonadotropin subunit 3; HPV 16, human papillomavirus type 16.

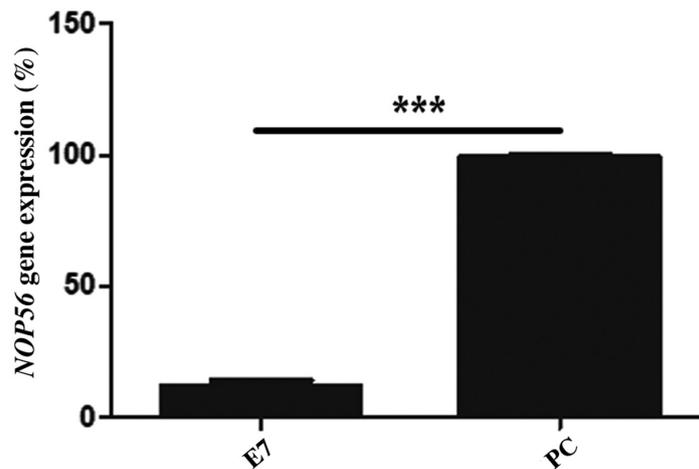


Figure 7. Expression of *NOP56* at mRNA level in E7 recombinant plasmid and PC transfected in C33A cells. Data are presented as the mean  $\pm$  the standard error of the mean of three repeats. \*\*\* $P < 0.001$ . PC, empty plasmid; HPV 16, human papillomavirus type 16; *NOP56*, nucleolar protein 56.

expression of *CGB3* and *NOP56*, as determined using qPCR, in C33A cells also significantly increased with all concentrations of 5-aza treatment (both  $P < 0.0001$ ; Fig. 11B and D). Similarly, the band densities of *CGB3* and *NOP56* observed in SiHa by RT-PCR gradually increased with increasing 5-aza concentration (0-40  $\mu\text{M}$ ; Fig. 12A and C). Expression of *CGB3* and *NOP56* measured by qPCR in SiHa cells were significantly higher when treated with 40  $\mu\text{M}$  5-aza (both  $P < 0.0001$ ; Fig. 12B and D).

## Discussion

HPV is the primary cause of cervical cancer. There are several studies showing that HPV 16 E7 is involved in promoter methylation (22,24-25). Chalertpet *et al* (24) showed that HPV 16 E7 can interact with DNMT1 on the *CCNA1* promoter leading to its methylation. Moreover, a study by Yanatatsaneejit *et al* (25) showed that HPV 16 E7 could induce *CADMI* promoter methylation through the same mechanism as *CCNA1*. In contrast, *DAPK1* promoter methylation could not be induced

by HPV 16 E7 (25). Therefore, it was hypothesized that there would be genes other than *CCNA1* and *CADMI* whose promoters are methylated by HPV 16 E7, and that there would be specific transcription factors binding with HPV 16 E7 on the gene promoter. A study by Na Rangsee *et al*, used STRING database protein network analysis, showed that E7 possibly forms a complex with the set of transcription factors containing a YY1 domain (26). In the present study, the binding site of HPV 16 E7 on the *CCNA1* promoter was analyzed to identify the putative transcription factors. According to bioinformatics analysis, a list of genes was analyzed based on the presence of the promoter sequence found in the *CCNA1* gene where HPV 16 E7 binds. Interestingly the genes *CGB3* and *NOP56* showed positive results. Thus, in addition to *CCNA1* (24) and *CADMI* (25), several other tumor suppressor genes may be affected by HPV 16 E7 through the same mechanism as that observed with *CCNA1* and *CADMI*. To assess this, *CGB3* and *NOP56* methylation and expression were observed in two cervical cancer cell lines, HPV 16-positive SiHa and HPV-negative C33A cells. Both *CGB3* and *NOP56* expression

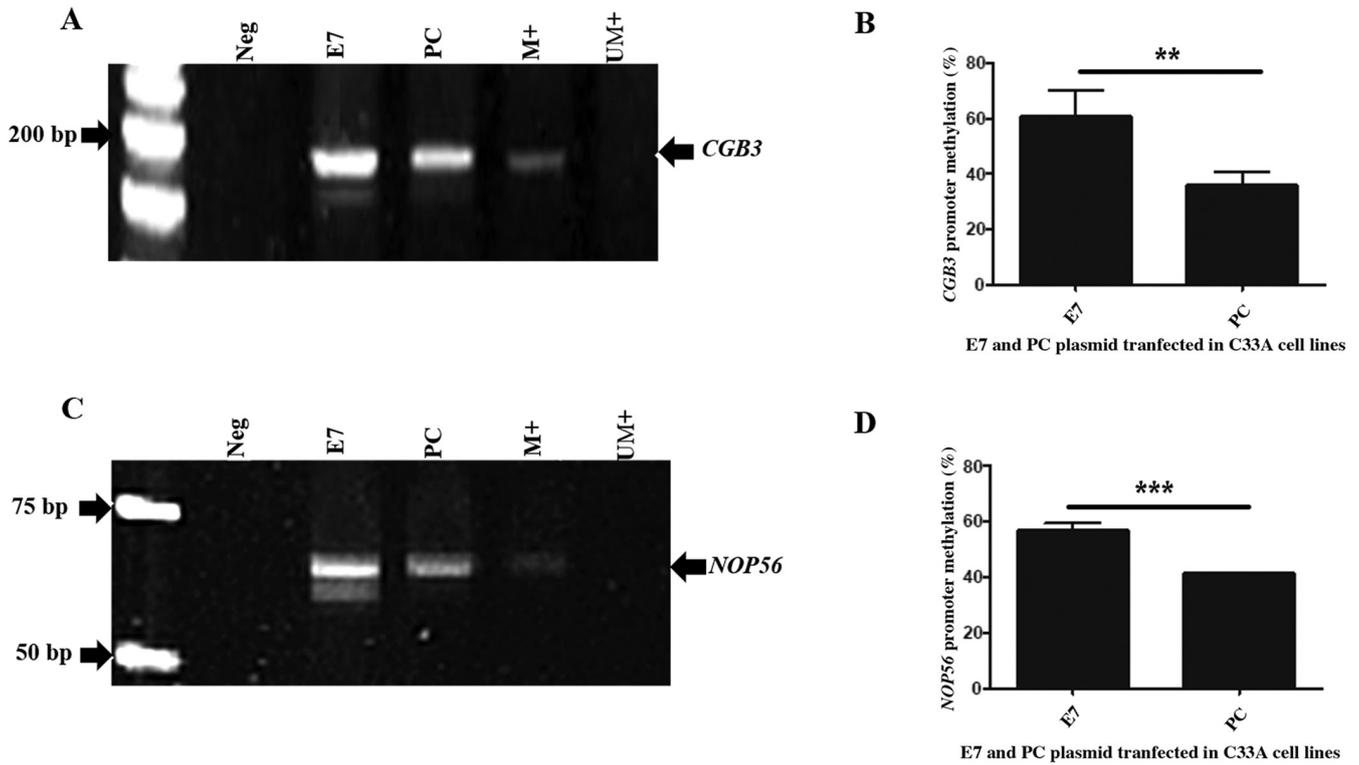


Figure 8. Methylation status of *CGB3* and *NOP56* genes in C33A cells transfected with HPV 16 E7 or PC. Methylation of (A and B) *CGB3* and (C and D) *NOP56* (Primer I). Data are presented as the mean  $\pm$  the standard error of the mean. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . HPV 16, human papillomavirus type 16; *CCNA1*, cyclin-A1; *CGB3*, chorionic gonadotropin subunit 3; *NOP56*, nucleolar protein 56; PC, empty plasmid; M<sup>+</sup>, methylated positive control; UM<sup>+</sup>, unmethylated negative control.

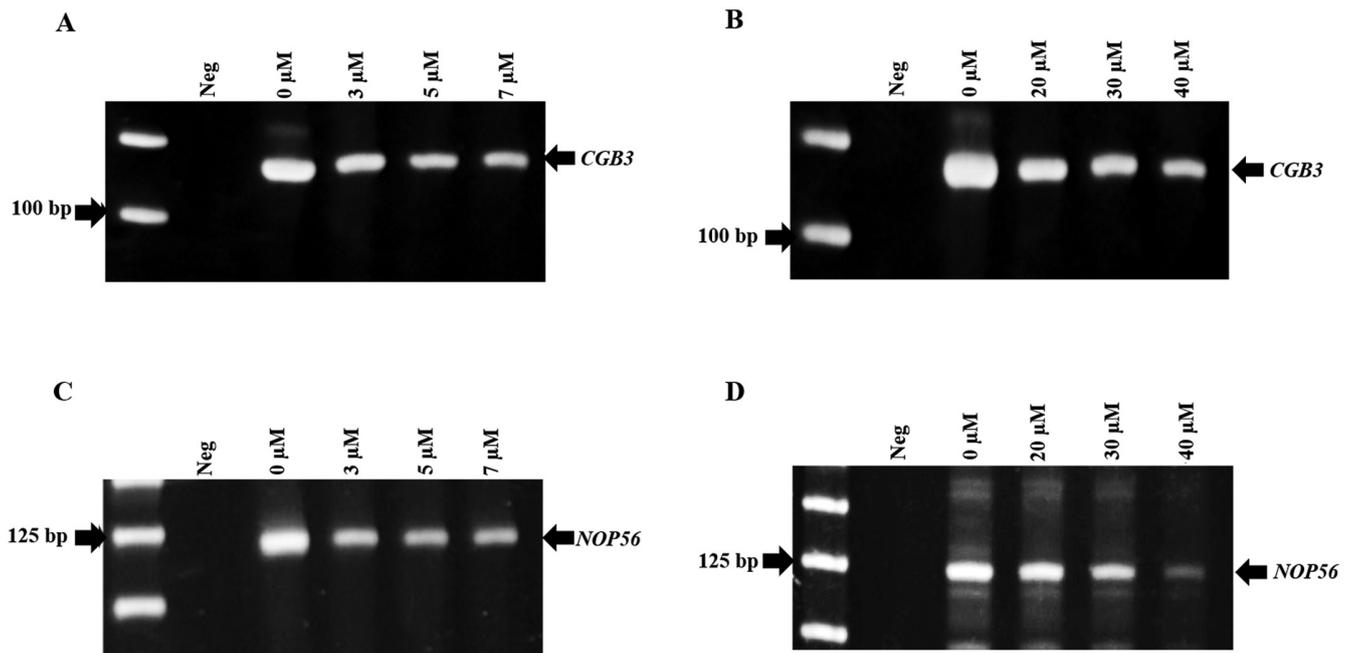


Figure 9. Treatment of C33A and SiHa cells with 0, 3, 5 and 7  $\mu$ M or 0, 20, 30 and 40  $\mu$ M 5-Azacytidine, respectively. Methylation status of (A) *CGB3* in C33A cells, (B) *CGB3* in SiHa cells, (C) *NOP56* in C33A cells and (D) *NOP56* in SiHa cells. Neg, distilled water negative control; *CCNA1*, cyclin-A1; *CGB3*, chorionic gonadotropin subunit 3; *NOP56*, nucleolar protein 56.

in SiHa cells had lower band intensities compared with the C33A cells, suggesting that the presence of HPV in SiHa may induce promoter methylation and decrease the expression of the genes. Moreover, the expression of *CGB3* and *NOP56* in

cells transfected with HPV 16 E7 and PC plasmids to assess the effect of HPV 16 E7 on the expression of these genes. Both genes exhibited lower expression in HPV 16 E7 transfected cells compared with the PC transfected cells, which

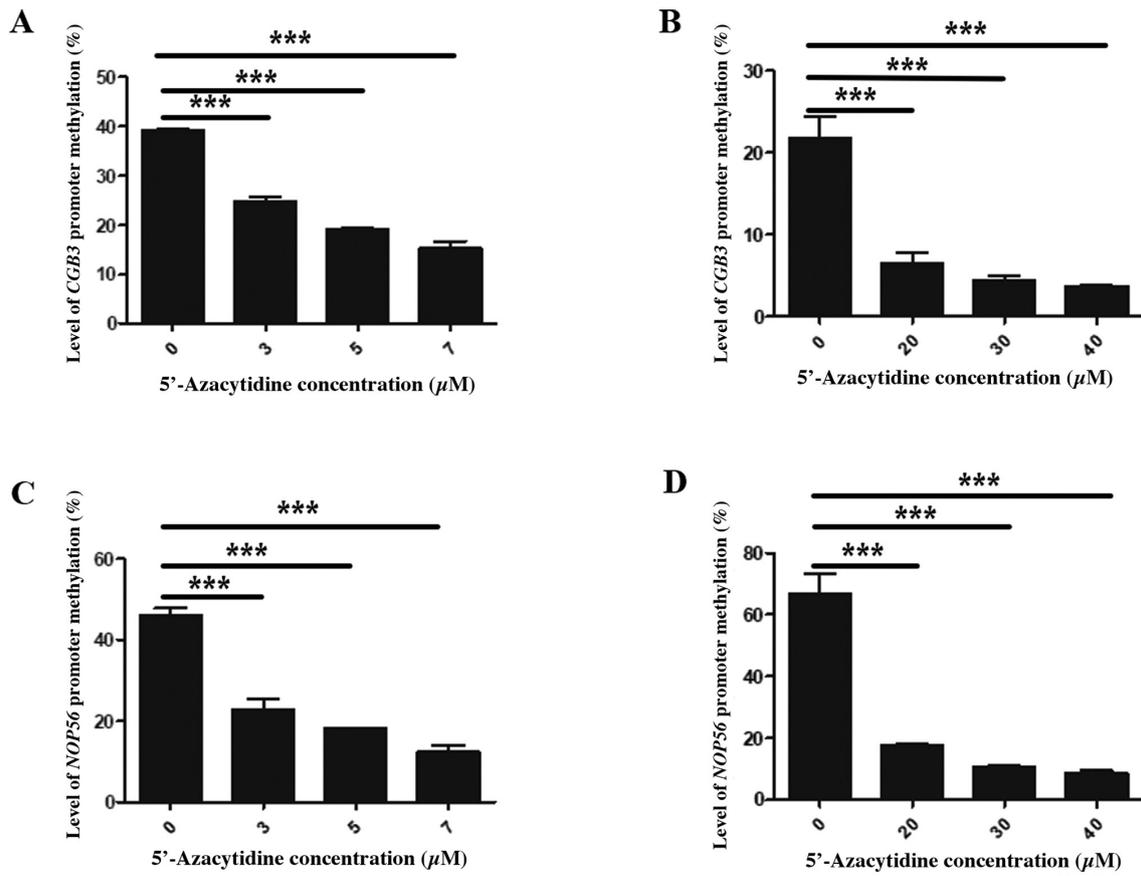


Figure 10. Changes in promoter methylation following after 5-azacytidine treatment. (A) *CGB3* methylation in C33A cells, (B) *CGB3* methylation in SiHa cells, (C) *NOP56* methylation in C33A cells and (D) *NOP56* methylation in SiHa cells. Data are presented as the mean ± the standard error of the mean. \*\*\*P<0.001. *CCNA1*, cyclin-A1; *CGB3*, chorionic gonadotropin subunit 3; *NOP56*, nucleolar protein 56.

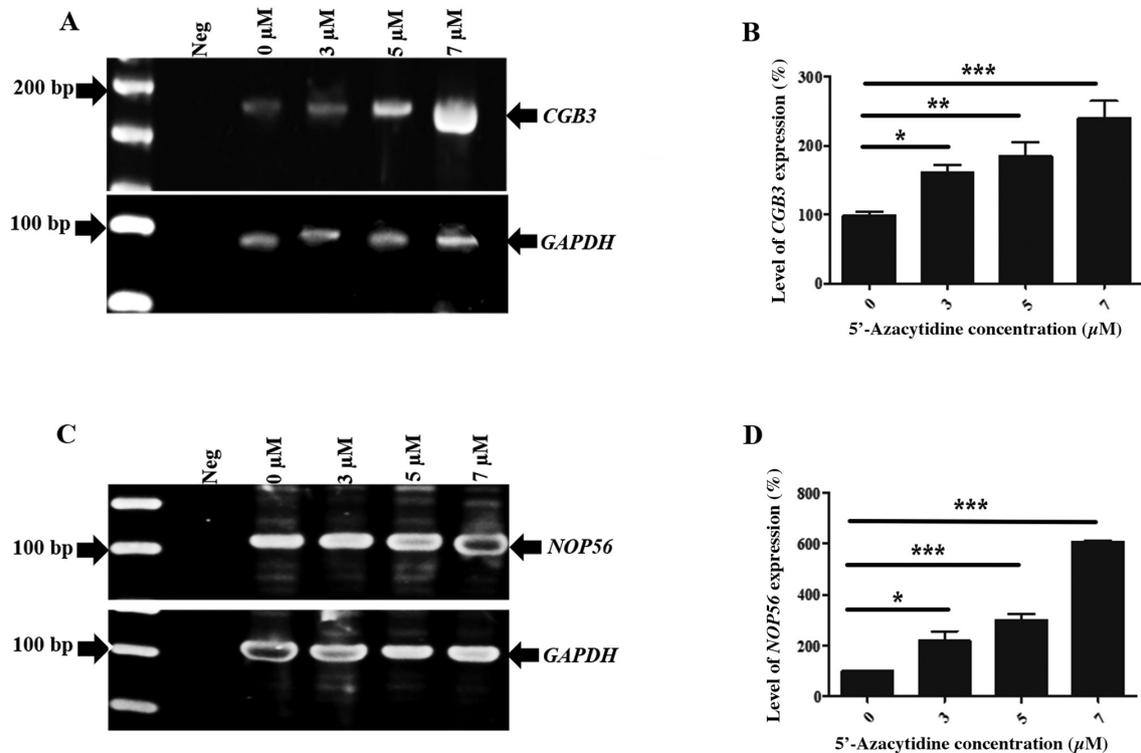


Figure 11. Changes in expression following treatment with 0, 3 and 7 μM 5-Azacytidine in C33A cells. (A) Agarose gel and (B) qPCR analysis of expression levels of *CGB3* in C33A cells. (C) Agarose gel and (D) qPCR analysis of expression levels of *NOP56* in C33A cells. Graphs represent mean ± SE between 0 μM to 40 μM, \*\*\*P<0.001. *CGB3*, chorionic gonadotropin subunit 3; *NOP56*, nucleolar protein 56; Neg, distilled water negative control; qPCR, quantitative PCR.

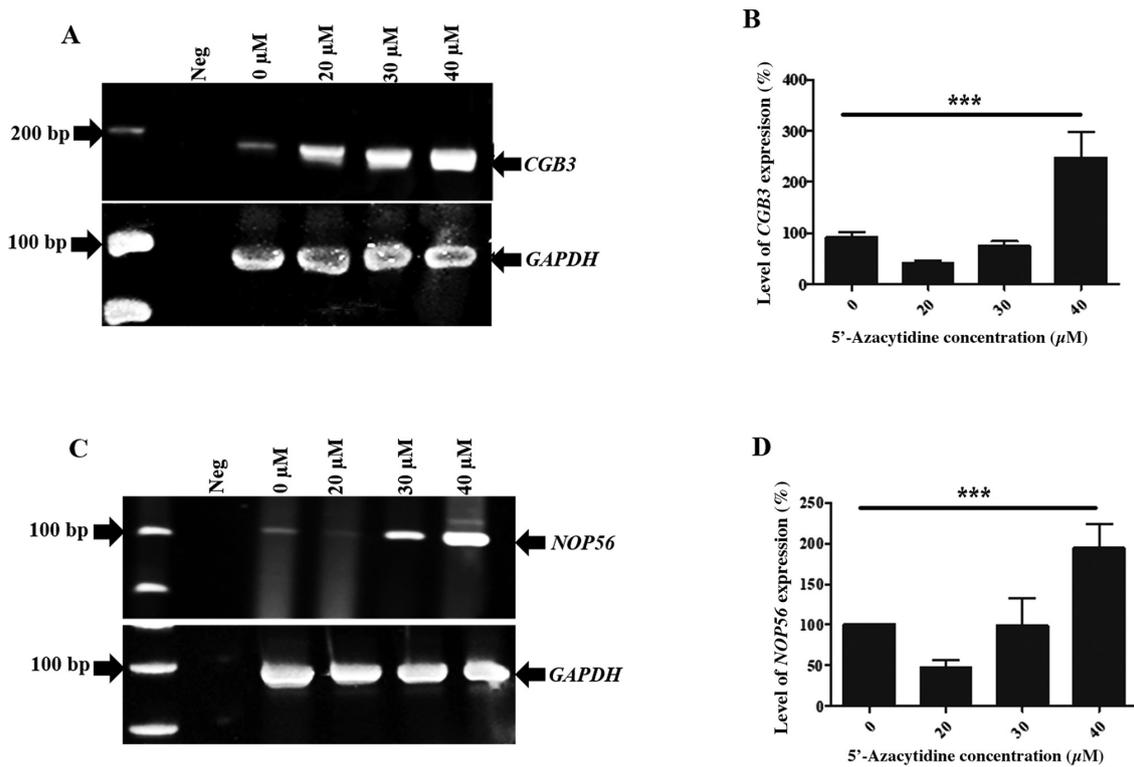


Figure 12. Changes in expression following treatment with 0, 20, 30 and 40  $\mu\text{M}$  5-Azacytidine in SiHa cells. (A) Agarose gel and (B) qPCR analysis of expression levels of *CGB3* in SiHa cells. (C) Agarose gel and (D) qPCR analysis of expression levels of *NOP56* in SiHa cells. Graphs represent mean  $\pm$  SE between 0  $\mu\text{M}$  to 40  $\mu\text{M}$ , \*\*\* $P < 0.001$ . *CGB3*, chorionic gonadotropin subunit 3; *NOP56*, nucleolar protein 56; Neg, distilled water negative control; qPCR, quantitative PCR.

demonstrated that HPV16 E7 induced promoter methylation of *CGB3* and *NOP56*, resulting in the downregulation of these genes. Thus, the expression changes in *CGB3* and *NOP56* following methylation inhibition using the demethylating agent 5-aza was next assessed.

5-aza interferes with DNA methylation and can be used to reactivate silenced genes in cancer (31-34). Examples of genes reactivated following 5-aza treatment include *CD44*, *GSTP-1*, *BRCA1*, *MDR1*, *MUC2* and *GPC3*, which possess hypermethylated promoter CpG islands in tumors (31). Wong *et al* (48) showed that methylation-mediated silencing of gene expression can be reversed by treatment with the demethylating agent 5-aza. Reactivation of methylation-silenced genes indicates that the gene is functional, and that DNA methylation regulates its transcription (39,40). With regard to the present study, it was necessary to treat SiHa cells with higher concentrations of 5-aza compared with the C33A cells, as SiHa is a HPV positive cancer cell line, meaning that promoter methylation was more prominent in them. Therefore, the lower concentration of 5-aza may not overcome HPV-induced methylation in SiHa cells. Following treatment with 5-aza at higher concentrations of 20, 30 and 40  $\mu\text{M}$  in SiHa cells, and at lower concentrations of 3, 5, and 7  $\mu\text{M}$  in C33A cells for 5 days, it was found that the methylation levels of *CGB3* and *NOP56* were decreased leading to increased expression of both genes. At 40  $\mu\text{M}$ , the re-expression of *CGB3* and *NOP56* genes was observed.

In conclusion, the results of the present study suggest that patients with cervical cancer with HPV infection should be treated with a higher concentration of 5-aza than patients without HPV infection (42,49). Taken together, the findings of

the present study showed that 5-aza may serve as an alternative strategy of drug therapy for patients with cervical cancer with aberrant gene promoter methylation. Although the present study demonstrated that HPV16 E7 decreased the expression of *CGB3* and *NOP56*, and that 5-aza treatment increased the expression of these genes, it cannot be concluded that these genes are tumor suppressor genes in cervical cancer. Further study of the function and effects of these genes in cervical cancer is thus required.

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#### Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

## Authors' contributions

PSi performed the experiments, analyzed the data and wrote the manuscript. KC, JS and NW performed the experiments and analyzed the data. PSu performed the experiments. PY wrote the proposal for grants, designed the experiments, analyzed the data and wrote the manuscript. All authors read and approved the final manuscript. PSi and KC confirm the authenticity of all the raw data.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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