ASSOCIATION BETWEEN GENE EXPRESSION AND PROMOTER METHYLATION OF *CGB3*, *GSTP1* AND *NOP56* GENES AND THEIR POTENTIAL IN BIOMARKER DEVELOPMENT - ASSOCIATED CERCIVAL CANCER.

Mrs. Palak Singh

A Dissertation Submitted in Partial Fulfillment of the Requirements
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ความสัมพันธ์ระหว่างการแสดงออกและการเกิดเมทิลเลชันที่บริเวณโพรโมเตอร์ของยีน*CGB3*, *GSTP1* และ *NOP56* และศักยภาพในการพัฒนาเป็นเครื่องหมายโมเลกุลสำหรับมะเร็งปากมดลูก

นางปาลัก ซิงห์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Thesis Title	ASSOCIATION BETWEEN GENE EXPRESSION AND PROMOTER METHYLATION OF *CGB3*, *GSTP1* AND *NOP56* GENES AND THEIR POTENTIAL IN BIOMARKER DEVELOPMENT - ASSOCIATED CERVICAL CANCER.
By	Mrs. Palak Singh
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ปาลัก ซิงห์ : ความสัมพันธ์ระหว่างการแสดงออกและการเกิดเมทิลเลชันที่บริเวณโพรโมเตอร์ของยีน*CGB3*, *GSTP1* และ *NOP56* และศักยภาพในการพัฒนาเป็นเครื่องหมายโมเลกุลสำหรับมะเร็งปากมดลูก. (ASSOCIATION BETWEEN GENE EXPRESSION AND PROMOTER METHYLATION OF *CGB3*, *GSTP1* AND *NOP56* GENES AND THEIR POTENTIAL IN BIOMARKER DEVELOPMENT - ASSOCIATED CERVICAL CANCER.) อ.ที่ปรึกษาหลัก : ปฐมวดี ญาณทัศนีย์จิต

การจับกันของ DNMT1 กับ E7 สามารถกระตุ้นโพรโมเตอร์เมทิลเลชันในยีนต้านมะเร็ง เช่น *CCNA1*, *CADM1* ที่นำไปสู่การยับยั้งการแสดงออก และด้วยเหตุนี้ จึงทำให้เกิดการลุกลามของมะเร็ง งานวิจัยนี้ศึกษาความสัมพันธ์ระหว่างการแสดงออกที่เกี่ยวข้องกับเมทิลเลชั่นของยีน *CGB3* และ *NOP56* ในเซลล์สายพันธุ์มะเร็งปากมดลูกที่เลี้ยงร่วมกับยา 5-Azacytidine การใช้ MSP-PCR และ qPCR พบว่า methylation ของ *CGB3* และ *NOP56* ลดลงอย่างมีนัยสำคัญเมื่อความเข้มข้นของ 5-Aza เพิ่มขึ้น และสิ่งนี้สัมพันธ์ผกผันกับการแสดงออกของยีน ซึ่งแสดงให้เห็นความสัมพันธ์ระหว่างเมทิเลชันของยีนและการควบคุมการแสดงออกของยีนจากการใช้ 5’-aza นอกจากนี้ ยีน *CGB3* และ *NOP56* สามารถใช้เพื่อระบุการเป็นมะเร็งในตัวอย่าง PAP (รวมถึง ASC-US) ตามเซลล์วิทยาและเนื้อเยื่อวิทยา จากการตรวจสอบการเกิดเมทิลเลชันของยีน *CGB3* และ *NOP56* โดยใช้ MSP-PCR จากผลทางสถิติโดยใช้การเขียนโปรแกรม Python พบว่า ยีน *CGB3* มีความไวและความจำเพาะสูงเมื่อเทียบกับ *NOP56* โดยมีความสามารถในการวินิจฉัยในการแยกแยะระหว่างตัวอย่าง "ปกติ" และ "ผิดปกติ" สิ่งนี้บ่งชี้ว่า *CGB3* มีศักยภาพมากกว่าในการแยกแยะและระบุความรุนแรงในตัวอย่าง PAP ที่คัดกรองมะเร็งปากมดลูกในระยะเริ่มแรก

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต

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Binding of DNMT1-E7 complex can induce promoter methylation in tumor suppressor genes, such as *CCNA1*, *CADM1* leading to their expression suppression and thus, cause cancer progression. This research establishes the association between methylation-related expression of *CGB3* and *NOP56* genes in cervical cell lines treated with 5-Azacytidine drug. Using MSP-PCR and qPCR, it was found that the methylation of *CGB3* and *NOP56* were significantly decreased as concentration of 5-Aza increased and this was inversely related with their expression. This demonstrated the association between gene methylation and the regulation of gene expression which were assessed by 5'-aza drug treatment. Of note, the identification of malignancy in screening PAP samples (included ASC-US) based on cytology and histology, were determined by evaluating the performance of methylation status of *CGB3* and *NOP56* genes using MSP-PCR. Based on statistical results using Python programming, *CGB3* gene showed high sensitivity and specificity compared to *NOP56* with significant diagnostic ability to distinguish between the "Normal" and "Abnormal" samples. This indicates that *CGB3* has more potential to distinguish and identify the malignancy in cervical screening PAP samples at their early stage.

Field of Study:	Biotechnology	Student's Signature .....
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Palak Singh

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CHAPTER 1
INTRODUCTION

Cervical cancer is the fourth most common cancer amongst women aged between 30-50 years. In 2020, the number of cervical cancers reported with new cases were around 604,000 along with estimated 342000 deaths out of which 90% of cases occurring amongst the lower and middle level income countries(Sung et al. 2021). In Thailand, cervical cancer is the most frequent causes for cancer related deaths (with 11.7%) after breast cancer (21.8%) amongst women. Moreover, the cervical cancer related deaths have been steadily increasing in the past (Imsamran et al. 2018) and about 90% of adenocarcinoma of the cervix occurs in the women are related to Human papillomavirus (HPV) infection (Chua and Hjerpe 1996; Ho et al. 1998)

HPV is the most common sexually transmitted infection and have been recognized as important risk factor for cervical cancer (Bosch et al. 2008; zur Hausen 2009). HPVs have circular, double-stranded DNA genomes that are approximately 8 kb in size and contain eight genes, E1, E2, E4, E5, E6, E7, L1 and L2, of which E5, E6 and E7 have transforming properties (Burd 2003). More than 100 strains of HPV are further categorized into low risk and high-risk factor. HPV types particularly 6, 11, 42, 43, and 44 are rarely associated with pre-cancer or cancer of the lower genital tract and about 90% cause genital warts knows as “Low-Risk” HPV types while HPV types such as 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 (Muñoz et al. 2003; Walboomers et al. 1999) have been identified as the causative agents of at least 90% of cervical cancers, where HPV-16 itself accounts for 60% of cervical cancers (Gultekin et al. 2018; Stany et al. 2006). HPV 16 and HPV 18 are induced by two viral oncoproteins E6 and E7, associate with the tumor suppressor genes p53 and pRB, respectively. E6 protein binds the cellular ubiquitin ligase E6AP to form a complex that targets p53 for degradation (Havre et al. 1995; Pim, Massimi, and Banks 1997). For E7, it binds to tumor suppressor retinoblastoma protein and interferes with its function, causing release of the transcription factor E2F which influences the expression of cell cycle – related genes (Caldeira, Dong, and Tommasino 2005). HPV16 - E6 oncoprotein was found to upregulate the DNMT1 and suppresses p53 in

cells (Au Yeung et al. 2010). Moreover, several studies found that E7 showed interaction with DNMT1, which maintains the methyltransferase that leads to aberrant methylation of the cellular genome, resulting in the silencing of tumor suppressor genes (Burgers et al. 2007; Chalertpet et al. 2015; Rosendo-Chalma et al. 2020). From Chalertpet study (Chalertpet et al. 2015) showed that the association between HPV infection and promoter methylation demonstrated that HPV16 - E7 can induce promoter methylation in *CCNA1* gene. However, from the recent research of Yanatatsaneejit (Yanatatsaneejit et al. 2020) it was found that HPV E6 and E7 both can induce promoter methylation in death-associated protein kinase 1 (*DAPK1*) and cell adhesion molecule 1 (*CADM1*) genes, respectively. Moreover, they also showed through chromatin immunoprecipitation that E7 protein can bind to promoter region of *CADM1* which indicated that it may also bind to *DNMT1* by the same mechanism as explained by Chalertpet et al. 2015. research using *CCNA1* as gene model. There is opportunity to study about other tumor suppressor genes for which HPV may induce promoter methylation. Based on study from (Na Rangsee et al. 2020) using readings of mass spectrometry with STRING database protein network analysis it was demonstrated that E7 possibly formed a complex with a set of transcription factors, including SP1, which linked with the Yin yang 1 (YY1) transcription factor contributing to the E7 mediated hypermethylation of the genes. This indicated the possibility that E7 could bind to DNMT1 through a transcription factor to the promoter region of *CCNA1* gene, resulting in promoter methylation. Thus, in same way there is opportunity to study other tumor suppressor genes that share the same sequence as the *CCNA1* promoter where HPV16 E7 can bind and induce promoter methylation and in order to investigate this possibility, bioinformatics analysis was performed according to which the YY1 transcription factor might bind to the area in promoter region of *CCNA1*. *CGB3* and *NOP56* genes were found to be candidate genes as they shared the same sequence as that of *CCNA1*. The *CGB3* gene plays critical roles in increased expression of the CGB subunit in ovarian cancer through demethylation of the CGB promoter and malignant transformation of non-trophoblastic cells (Bellet et al. 1997; Sohr and Engeland 2011; Śliwa et al. 2019). The *NOP56* gene is involved in more than five types of cancers (Gong et al. 2017); however, there is no evidence linking it to cervical cancer, to the best of our

knowledge. These findings led to the investigation of the role of HPV16 E7 in cervical cancer progression through promoter methylation of these two genes.

From study of (Lee et al. 2004), down-regulated proteins by E7 in C33A cell lines were analyzed by mass spectrometer followed by MALDI-TOF-MS from which the author shortlisted five genes (*GSTP1, VAV1, ING1, IL4R-alpha* and *PDI-A3*). *GSTP1* (Glutathione S-transferases P) has been reported to function as tumor suppressor gene in various types of human cancers like breast cancer and also used as biomarker for prostate cancer (Fang et al. 2015; Nakayama et al. 2004). From study of (Katzav 2009) *VAV1* (vav guanine nucleotide exchange factor 1) was first isolated as an oncogene, and its wild-type form has recently been implicated in mammalian malignancies. These properties make *VAV1* a promising target for new therapeutic approach to cancer therapy. *ING1* (Inhibitor of growth protein 1) links to DNA repair, apoptosis and chromatin remodeling to the transcriptional regulation of critical genes and *ING1* exerts its tumor suppressor functions by helping maintain genomic stability (Gong et al. 2005). *IL4R-alpha* (Interleukin 4 receptor alpha) plays an important role in regulating antibody productions and the development of effector T-cell responses (Brown and Hural 2017) and is produced only by a subset of activated hematopoietic cells, including T cells. *PDI-A3* (Protein disulphide isomerases 3) are chaperones which functions according to redox potential of the substrate (Ye et al. 2018) and also regulates the cell growth and death according to oxygen concentrations and plays a role in cytokine-dependent signal transduction, including STAT3 signaling (Santana-Codina et al. 2013). These selected genes were further analyzed for whether E7 can induce promoter methylation and suppress the expression of above-mentioned genes or not.

Based on epigenetics therapy, DNA methylation mechanism is one of the most studied methods amongst the methods used for the treatment of various cancers. Aberrant DNA methylation is the most common molecular lesion of the cancer cell, which is reversible through interventions with demethylating agents (Stresemann and Lyko 2008). 5'-azacytidine (5-aza) is a well-known demethylating agent approved by the U.S. Food and Drug Administration for using as a chemotherapeutic against myelodysplastic syndrome. 5-aza covalently binds to DNMT and thus reduce the biological activity of DNA methyltransferase (Christman 2002; Glover and Leyland-

Jones 1987). The results show the loss of methylation in specific gene regions and hence, activate the expression of the associated genes (Lande-Diner et al. 2007). Moreover, several studies have reported that the association between DNA methylation and repression of gene expression leads to cancer progression. Therefore, re-expression of methylated genes in cervical cancer cell lines after the treatment with a demethylating agent (5-aza) will be further examined to evaluate the gene expression by inhibiting methylation rate in order to increase the expression of tumor suppressor gene (Baylin 2005; Karpf and Jones 2002). This research provides the evidence that HPV16 - E7 could induce promoter methylation and decrease the expression of the selected genes. The research study further evaluates the effects of 5-aza treatment on promoter methylation and genes expression of above-mentioned genes after treatment in cervical cancer cell line C33A (negative cancer line) and SiHa (positive cancer line) which demonstrates the association between gene methylation and the regulation of tumor gene expression. The research study may also serve as an alternative strategy of drug therapy for patients with cervical cancer with aberrant gene promoter methylation (Biktasova et al. 2017).

From the national perspective, lack of screenings and treatment of abnormal cells in the pre-cancerous stages have been identified as major cause of health hazards related to cervical cancers although they could have been easily prevented (Arbyn et al. 2013; Bray et al. 2018). Pre-cancerous lesions caused by HPV can be detected using early screenings and prevent cancers from further developing (Pimple and Mishra 2019). Papanicolaou test (PAP smear), visual inspection of the cervix with acetic acid (VIA), and HPV testing (Burd 2003; Ngo-Metzger and Adsul 2019) are few of the screening tests used for cervical cancers. According to “Bethesda System guidelines” which involves colposcopy and biopsy methods (Davey 2003; Koh et al. 2019; Wright Jr et al. 2002) that can be used for examination of Women with cytology-proven severe lesions. However, there are still some opportunities for improvement when it comes to management of women with atypical squamous cells of undetermined significance (ASC-US) where more explanations can be made (Cox 2005) and this opens up an opportunity towards further study in terms of between negative and a confirmed SIL (Squamous Intraepithelial Lesion) (Wright et al. 2015). The 'U' in ASC-US stands for 'undetermined' significance which indicate that women

with cytology of ASC-US may have negative results or it may have low- or high-grade squamous intraepithelial lesion which is unclear (Melnikow et al. 1998). Followed-up by cytology, the abnormal ASC-US cells were further examined by clinicians to determine the stage of cells by performing colposcopy or biopsy. Based on further analyses of ASC-US results, they have also categorized the SIL results in to LSIL (Low SIL) and HSIL (High-SIL) (Solomon et al. 2002).

Furthermore, using selected genes as methylation marker the identification of ASC-US stages were determine without going through the painful biopsy process. Therefore, the aim of the present study is to identify the new methylation markers to detect the malignancy of PAP samples at their early stages. Based on the study of ALTS Group (Group 2003) from U.S., the cervical screening programs have shown that HR-HPV were responsible for about 50% of women with atypical squamous cells. Therefore, based on HPV typing ASC-US samples were categorized into HPV types HPV16, HPV18, HPV others and no HPV categories using Cobas® 4800 with specific identification of types HPV16 and HPV18 as described in (Rao et al. 2013). To identify malignancy in the collected liquid-based PAP test samples of ASC US, these were classified into four categories, namely LSIL, HSIL and normal samples (with or without HPV). Note that there are no cancer samples in the ASC US, as these are supposed to contain only normal or pre-cancerous samples by definition. By using methylation specific PCR (MSP), the *CGB3* and *NOP56* genes were used to differentiate between the normal and abnormal samples through detecting promoter methylation in both types of samples. The findings of the present study should help to evaluate PAP samples at an early stage, and this will be of importance in terms of the clinical diagnosis of ASC US samples.

1.1 Research Objectives:

1. To evaluate the association between promoter methylation and gene expression of *CGB3*, *GSTP1* and *NOP56* by treating with 5-azacytidine in E7 transfected C33A cell line
2. Identification of malignancy in screening cervical sample of ASCUS (Atypical Squamous Cells of Undetermined Significance) by detecting promoter

methylation of genes (*CGB3, GSTP1* and *NOP56*) and using them as candidate biomarker for early diagnosis of cervical cancer.

1.2 Research Benefits

Early diagnosis of abnormal pap smear by using candidate genes and biomarker for identification of ASC-US samples will be obtained.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

The word cancer was oriented from Greek physician named Hippocrates (460-370 BC), who is known to be a “Father of Medicine” (Grammaticos and Diamantis 2008) and in 19th century, Rudolf Virchow, the founder of cellular pathology, used a modern microscope to study the pathology of cancer. This gave better perspective to understanding the damage done by cancer and aided the development of cancer surgery by examining the removed body tissue by the surgeon or pathologist by diagnosing it. In 1915, John Hill a clinician recognized tobacco as a *carcinogen* (a substance known or believed to cause cancer in humans) (Diamandopoulos 1996).

There are many substances that are known to cause cancers, like coal tars and their derivatives (benzene), some hydrocarbons, aniline (a substance used to make dyes) and many others that should be avoided. In 1968, Peyton Rous from New York was awarded the Nobel Prize for describing a type of cancer (sarcoma) in chickens caused by the Rous sarcoma virus and also demonstrated that several viruses were also linked to cancer in humans (DeVita Jr and Rosenberg 2012). For example:

- Hepatitis B or C viruses can cause liver cancer.
- One of the herpes viruses, the Epstein-Barr virus linked to nasopharyngeal cancer.
- The human immunodeficiency virus (HIV) which has greatly increased the risk of developing several cancers in human.
- Human papilloma viruses (HPVs) have been caused of many cancers related to cervix, vulva and vagina. Some cervical cancer and head neck cancers are linked to the high-risk types of HPV, too.

At present, the characteristics of cancer cells have been explored in terms of pre-cancerous and cancerous cells by many researchers in various studies.

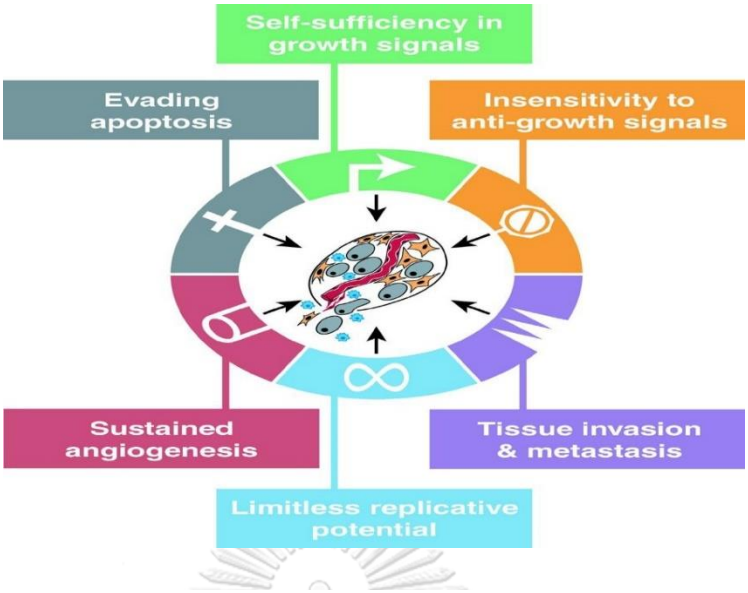


Figure 1. The six hallmarks' capabilities of cancer. Source: (Hanahan and Weinberg 2000).

The hallmark of cancer describes cancer cell genotypes that display six essential alterations in cell physiology that impact malignant growth (Fig.1). In 2000, the six characteristics of cancer which included 'self-sufficiency in growth signals', 'evading programmed cell death (apoptosis)', 'insensitivity anti-growth signals', 'tissue invasion and metastasis', 'limitless replicative potential' and 'sustained angiogenesis' (Hanahan and Weinberg 2000).

1. It grows and divides cells with its own growth signals (self-sufficiency in growth signals) without the usual stimulating signals.
2. There is a mechanism to avoid cell death programs (evading apoptosis).
3. It has the ability to avoid signals of cell growth inhibition. (Insensitivity to antigrowth signals) to be able to grow and divide cells at any time.
4. Invasion of neighboring tissues (invasion) can spread to different areas of body gain (metastasis).
5. Have the ability to try yourself or divide cells uninterrupted (limitless replicative potential)
6. New blood vessels are formed (sustained angiogenesis).

Cancer spreads beyond their usual boundaries and invade to other part of the body, travel through the blood or lymph system and form new tumor in another organ called metastasizing tumor (Fig.2).

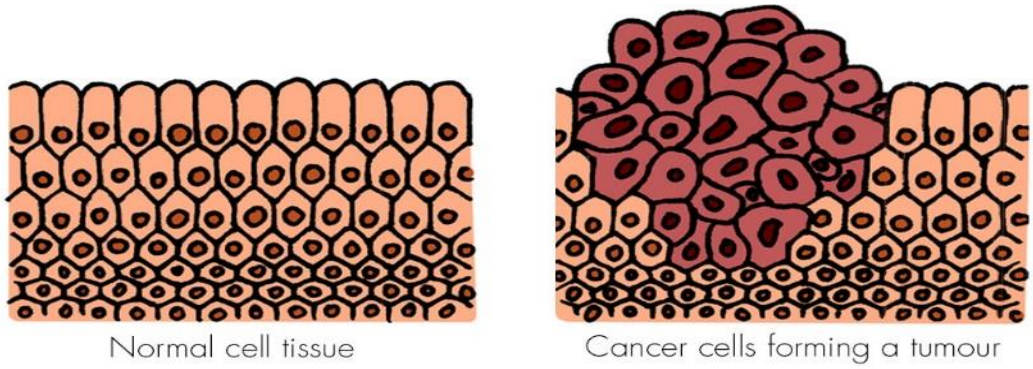


Figure 2. Growth of normal cell Vs abnormal cells (Cancer cell). Source *http://www.haleo.co.uk/the-body/cancer-cell/*

Worldwide, cancer is the second leading cause of death amongst men and women with an estimated rate of 18.1 million new cancer cases and 9.6 million cancer deaths as reported in GLOBOCAN 2018 (Fig. 3). In men the most common types of cancers are lung, prostate, colorectal, stomach and liver cancer while in women breast, cervical, lung, colorectal and thyroid cancer are the most common types amongst which breast cancer is the most commonly diagnosed cancer with death in women followed by lung and colorectal cancer for both incidence and mortality. Cervical cancer is in fourth rank for both incidence and mortality. Depending on the economy development, medical facility, infrastructure and life style factor of each country, the cancer rates and cause of death are depended (Bray et al. 2018).

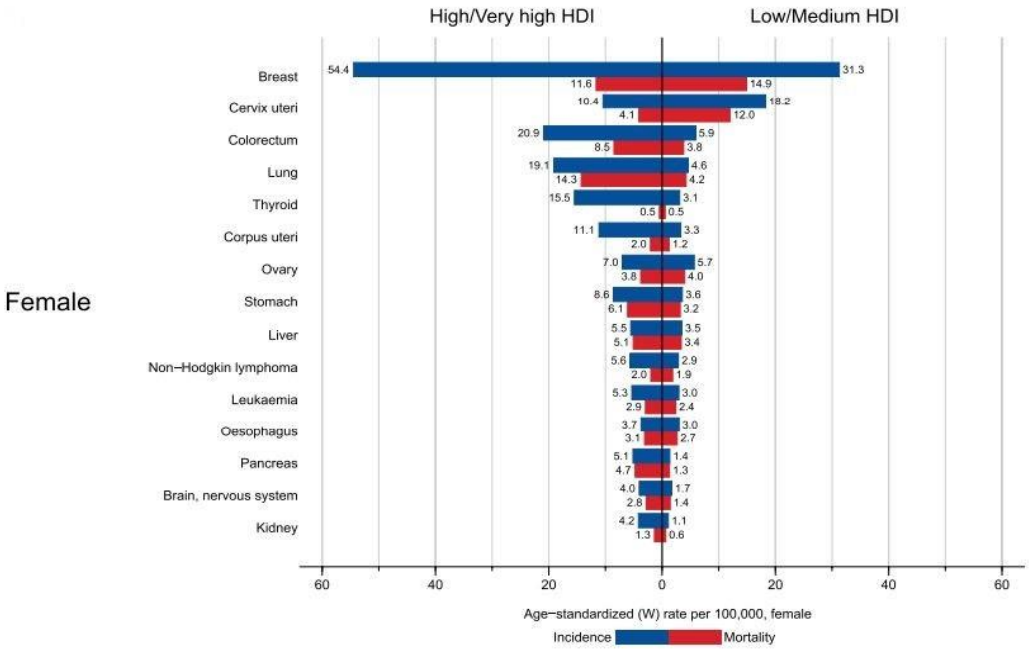


Figure 3. The 15 most common cancers world (W) in 2018 are shown in descending order of the overall age-standardized rate for both sexes combined. Source: (Bray et al. 2018)

2.2 Cervical Cancer

Amongst women in low- and middle-income countries like Bangladesh, Afghanistan, Cambodia, Central African Republic, Angola, etc , cervical cancer rate is about 90% of new cancer related cases and deaths reported in Global cancer statistics 2020 (Sung et al. 2021). Cervical cancer occurs in the cells of the cervix- the lower part of the uterus that connects to the vagina (Fig. 4) and helps to produce fluid to keep vagina healthy, pass the mensuration blood from the uterus into the vagina and it also help the sperm to travel from uterus to fallopian tube by releasing fluid to get fertilized. The cervix part has two types of cells (Levine, Lin, and Gaillard 2020). The outer surface known as ectocervix has squamous cells and inner surface known as endocervix has glandular cells. The cancer that occurs at squamous and glandular cells are called ‘squamous cell carcinoma’ and ‘adenocarcinoma’ respectively but most of the cervical cancer starts at transformation zone where squamous and glandular cells meet. The growth of abnormal cells in cervix part has the ability to

invade to other parts of the body and is called ‘cervical cancer’. Abnormal growth or precancerous cervical cells may show symptoms such abnormal vaginal bleeding after menopause, or during or after sexual intercourse, pelvic pain or pain during sexual intercourse or abnormal vaginal discharge with unusual smell or color.

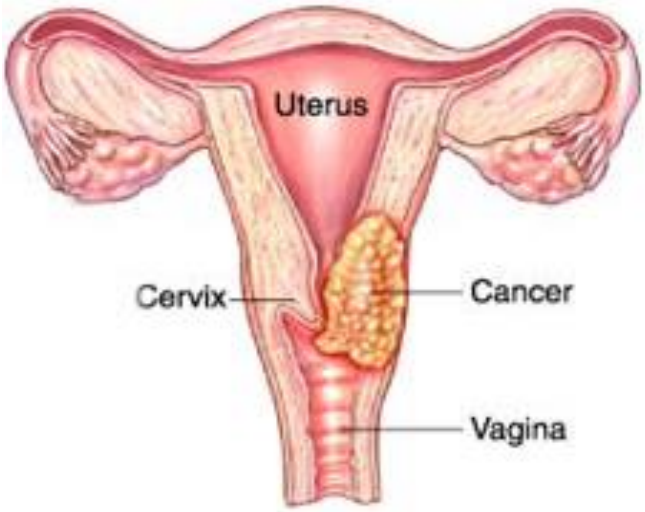


Figure 4. Position of cancer cells in the cervical area

Source: (*National Cancer Institute (US), 2002*)

In Thailand, according to data from the National Cancer Registry, cervical cancer is considered as a major public health problem with age-standardizes incidence rate 23.4 per 100,000 reported in 1990 by (Vatanasapt et al. 1995). This affects the quality of life of Thai peoples and also effect the economic resources. In 1990, cervical cancer was ranked first as most common cancer among all based on the trend analysis of the Age-Standardized incidence rate as shown in (Fig. 5A). But in 2018, now its ranked third as common cancer in women with estimated rate 11.7 per 100,000 as reported in (Imsamran et al. 2018) (Fig. 5B).

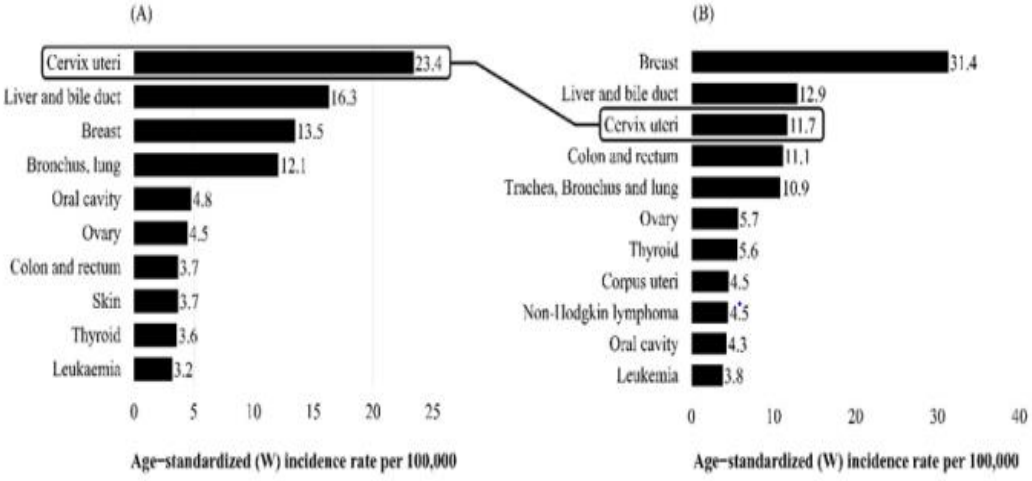


Figure 5. Comparison of Age-Standardized Incidence Rates of the ten most Frequent cancers in Thai Women. (A) In 1990 (Vatanasapt et al. 1995) and (B) In 2014 (Imsamran et al. 2018)

2.3 Human papillomavirus (HPV) and Cervical Cancer

Worldwide, Human papillomavirus (HPV) is the most common sexually transmitted viral infection of the reproductive tract (Burd 2003). The link between HPV and cervical squamous cells carcinoma has become well established since the early 80s. The account for the association between HPV and cervical cancer cells is higher than that for the association between smoking and lung cancer (Franco 1995). According to studies by (Burd 2003; Unger and Barr 2004), 200 different types of HPV exists, amongst which 15 types are known to be associated with cervical cancer. Theses HPV types are categorized as low-risk HPV types and high-risk HPV types. The high-risk HPV types are associated with cervical cancer and its precursor lesions.

The low-risk HPV types also known as ‘non-oncogenic’ HPV types includes types 6, 11, 42, 43, and 44 and high-risk HPV types also known as ‘oncogenic’ HPV types include types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 (Walboomers et al. 1999). About 99.7% of cervical cancer cases are caused by persistent genital high-risk human papillomavirus (HPV) infection. In particular, low

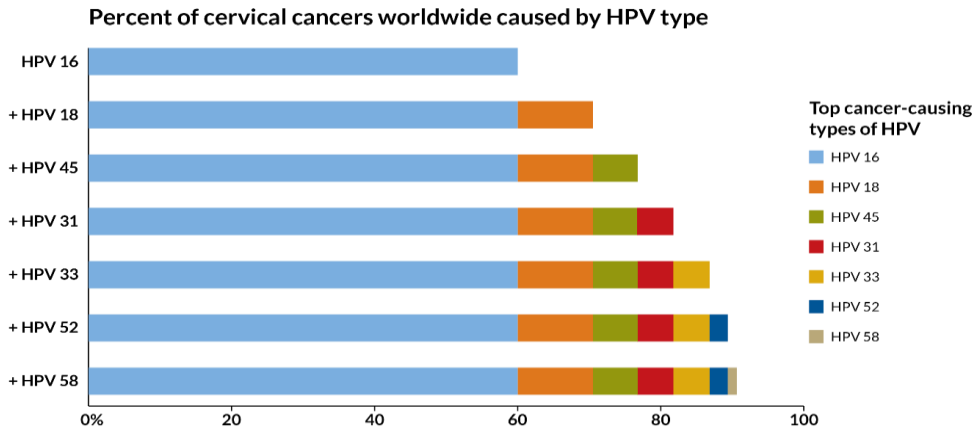


Figure 6. Bar chart represent percentage of cervical cancer cause by different HPV strain Source: *https://www.sciencenews.org/article/new-hpv-shot-fends-more-types-virus*

-risk HPV type 6 and type 11 are the most common HPV types accounting for about 90% of genital warts. For high-risk HPV types, types 16 and type 18 accounts for about 70% of HPV infections found in cervical cancer patients as shown in Fig. 6 (Li et al. 2011; Lowy and Schiller 2006).

2.4 Human papillomavirus genome

HPV is a group of DNA virus which belongs to the Papillomaviridae family whose members are known as papillomaviruses. With reference to HPV16 type, the genome of HPV is 7.9 kb in size (Fig.7) of which is segmented into three sections: the early gene-coding region (E), the late gene-coding region (L), and the long control region (LCR), also called as non-coding region (NCR) or upstream regulatory region (URR) (Graham 2010). The function of six genes - E6, E7, E1, E2, E4, and E5 involved in the replication of viral gene expression. The two late genes L1 and L2 were involved in capsid formation (Schiffman et al. 2007). The URR, located between the L1 and E6 genes contain the E6 promoter and an enhancer region with cis-responsive elements that regulate viral gene expression, replication, and packaging into viral particles (Bernard 2002; zur Hausen 2009).

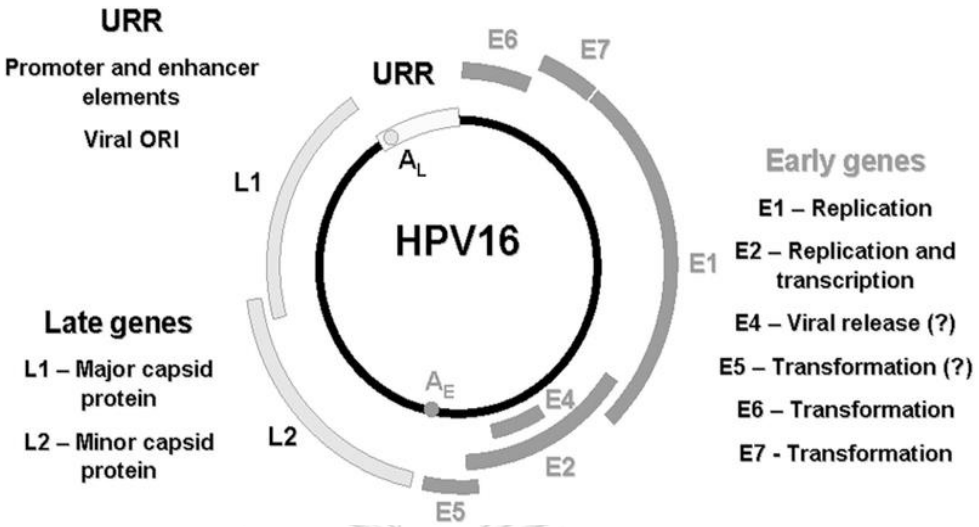


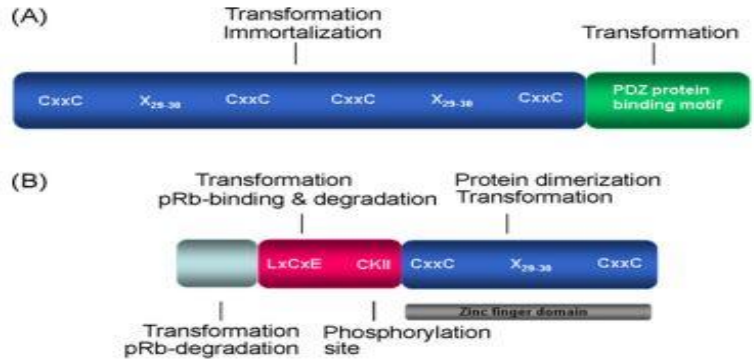
Figure 7. Genomic organization of HPV genome. Source: (Stanley, Pett, and Coleman 2007)

2.5 Structure of E6 and E7

The HPV oncoproteins, E6 and E7 play critical role in driving the cells towards oncogenesis. The E6 and E7 proteins of high-risk types are the main mediators of carcinogenesis due to their interactions with cellular tumor suppressor proteins p53 and pRb which play important role regulating cell cycle and apoptosis, respectively (Fehrmann and Laimins 2003). The E6 protein is a small basic protein of about 150 amino acids. Every E6 protein contains four CxxC motifs, which are believed to bind to zinc binding domain(Zanier et al. 2012). The important functions of four motifs, such as activation of transcription site, transformation and association with tumor suppressor protein p53 (Boulet et al. 2007).

The E6 COOH-terminal region contains PDZ domain in case of high-risk HPV types which involved in the interaction with several PDZ domain-containing proteins Fig.8A.The E7 proteins contain three conserved regions CR1 (residues 2–15), CR2 (residues16–41) are localized in disordered N-terminus and CR3 region (54–95) lies in ordered c-terminus (García-Alai, Alonso, and de Prat-Gay 2007). The CR1 region binds with the cellular targets like p600, SKp2 and p300 whereas the CR2 region binds with Rb1, TBP and p21. The E7 COOH-terminal CR3 domain consisting of two CXXC motifs, this region is used in the association of pRb and other host

cellular proteins (Hebner and Laimins 2006) . It also reported that LXCXE (where X represents any amino acid) motif of CR2 region has high binding affinity towards pRb protein. However, both CR2 and CR3 regions are needed for efficient degradation of pRb that ultimately leads to inhibition of cell cycle arrest (Todorovic et al. 2012).



2.6 Integration of HPV DNA

Figure 8. Structure of E6 and E7 oncoproteins. (A) Schematic representation of the E6 oncoprotein. (B) Schematic representation of the E7 oncoprotein. Source: (Boulet et al. 2007).

The HPV DNA is in episomal form when it effects at the basal layer of squamous epithelial cells (McGregor and Rustin 1994). During viral infection of HPV, E6 and E7 gene are expressed at very low levels due to transcriptional repression by E2 gene. HPV DNAs are found chromosomally integrated in cells of cervical cancer. Frequently, more than one copy of HPV DNA is integrated. Note that HPV DNA exists in an episomal state form without chromosomal integration in its life cycle (Fig.9). The site of integration is random with respect to the chromosome site. In contrast, the site of integration of HPV DNA is not random but it is inserted via the E1 and E2 regions of HPV DNA. Consequently, E1 and E2 genes are disrupted, but E6 and E7 genes are preserved in cancer cells (Pett and Coleman 2007). Interestingly, transcription of E6 and E7 genes is suppressed by E2 protein, which acts as a transcriptional suppressor, and is expressed at a low level in normal cells. Basically, E6 and E7 genes activity is responsible for the multiplication of the viral

genome with the help of the cellular machinery, as revealed by several interactome analyses (Neveu et al. 2012). In contrast, E6 and E7 proteins are overexpressed in cancer cells via two distinct mechanisms. First, E6 and E7 genes are overexpressed, since E2 gene, a transcriptional suppressor of E6 and E7 genes, was disrupted during integration. Secondly, E6 and E7 mRNAs are stabilized in cancer cells (Ganguly and Parihar 2009; Gupta, Kumar, and Das 2018). In fact, as the 3′ splice sites of E6 and E7 genes are lost during integration, alternative splicing sites from downstream gene are utilized. Consequently, an AU-rich sequence element, which confers RNA instability, is lost.

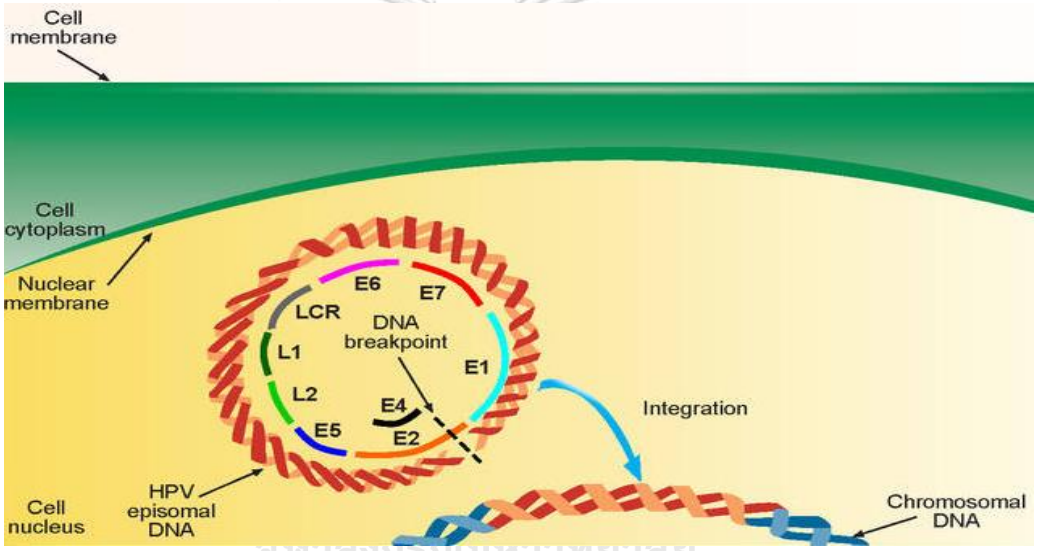


Figure 9. Replication of HPV cycle from Episomal DNA to Integrated DNA.
Source: (*https://www.immunopaedia.org.za/clinical-cases/infectious-diseases/case-of-giant-wart)*

Although there are several studies showed that integration HPV is major form of infection which led to carcinogenesis. However, there are some studies that showed that the deletion in the YY1 binding site of the long control region of HPV episomal DNA can increase E6 and E7 which can also lead to cervical cancer Thus, both physical states of HPV in a host cell can be the cause of tumorigenesis (Dong et al. 1994; May et al. 1994).

2.7 Functions of E6 and E7 Oncoproteins

According to Durzynska (Durzynska, Lesniewicz, and Poreba 2017), the high-risk types are driven by two majors viral oncoproteins E6 and E7 which

contribute towards deregulation of growth suppressor. E7 and E6 react with the tumor suppressor gene products pRb and p53 in host cell proteins, respectively, resulting in induced cellular immortalization, transformation and carcinogenesis due to them interference with cell cycle and apoptosis control Fig 10 (Thomas, Pim, and Banks 1999; Szymonowicz and Chen 2020). The HPV oncogenes, E7 and E6, have been shown to be the main contributors to the development of HPV induced cancers, probably due integration of the viral genes in the host cell genome. The tumor suppressor p53 is involved in multiple central cellular processes, including transcription, DNA repair, regulation, progression through the cell cycle, apoptosis and often called as the “guardian of the genome” (Lane 1992; Park et al. 2016).

The HPV E6 and E7 genes are thought to play causative roles since E6 promotes the degradation of p53 through its interaction with E6AP, an E3 ubiquitin ligase. The affinity of E6AP for p53 is likely to be modified by the association with E6. p53 is a tumor suppressor gene involved in apoptosis after DNA damage, and regulation of both G1/S and G2/M cell cycles. HPV-infected cells induce inhibition of apoptosis by E6 inactivation of p53 (Scheffner et al. 1990; Scheffner et al. 1993). Whereas the E7 protein can interact with pRb, an important negative regulator of entry into S phase of the cell division cycle. It is thought that the high-risk HPVs have a unique ability to induce the proteolytic degradation of pRb (Boyer, Wazer, and Band 1996). In the hypo phosphorylated state, combined E7 and pRb activates the E2F transcription factor, which triggers the expression of proteins necessary for DNA replication and cell cycle progression (Kaneko et al. 1999; McLaughlin-Drubin, Park, and Munger 2013). Phosphorylation of pRb by G1 cyclin-dependent kinases releases E2F leading to cell cycle progression into S phase. Because E7 is able to bind to unphosphorylated pRb, it may prematurely induce cells to enter S phase by disrupting pRb-E2F complexes. In addition to the inactivation of pRb family members, numerous functions of E7 have been reported (Wentzensen et al. 2005; Guo et al. 2011; Denton et al. 2010).

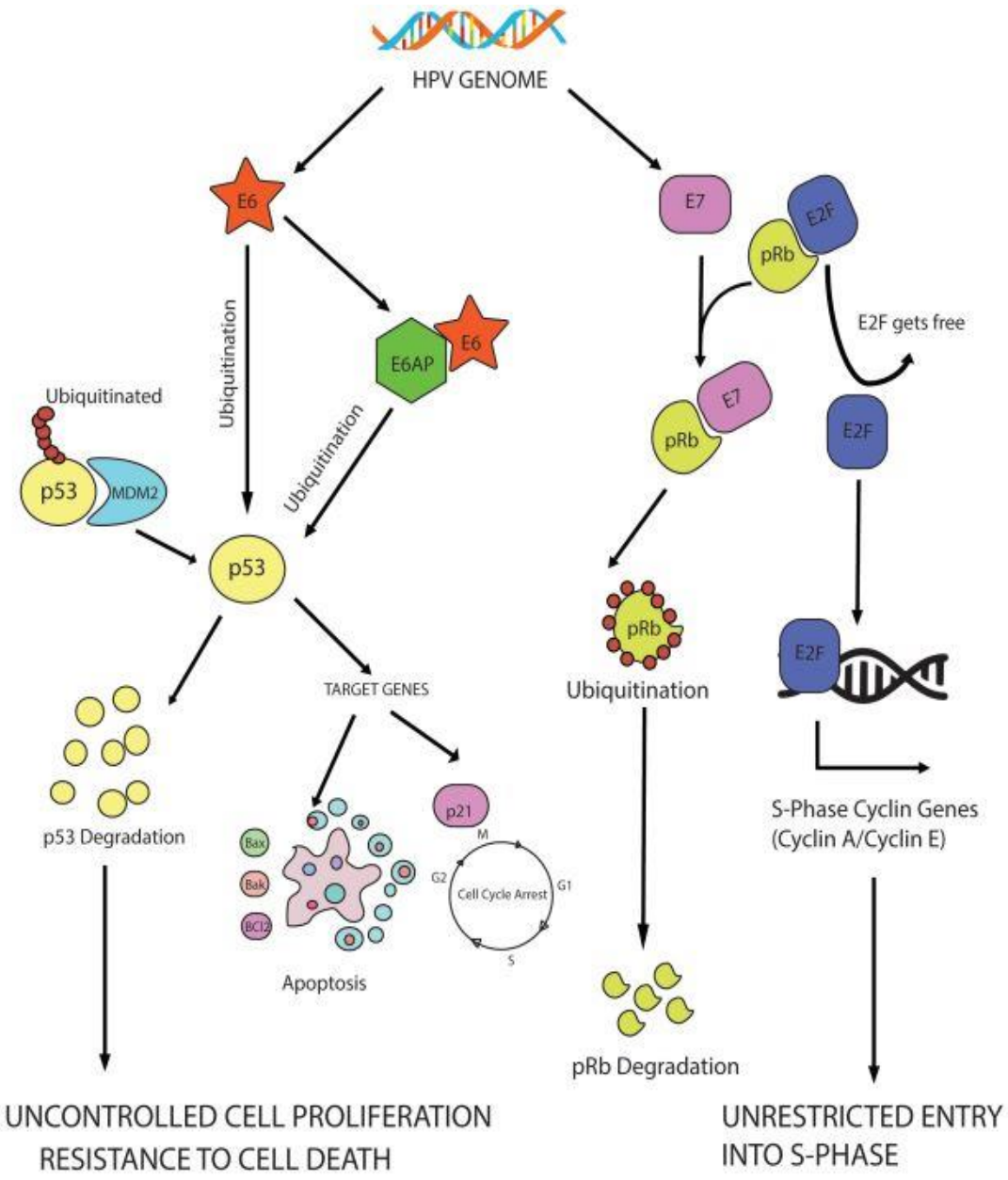


Figure 10. Mechanism of E6 and E7 in degradation of p53 and pRB tumor suppressor protein. Source: (Pal and Kundu 2020)

2.8 Screening and diagnosis for cervical cancer

Cervical screening (a smear test) also known as Papanicolaou (PAP smear) test examines the status of cervix part to find cancer in people before any symptoms appear. This screening program can help to prevent cervical cancer or detect it early

by doing regular screening tests (Pimple and Mishra 2019). In case of positive results for PAP smear screening tests, another test known as HPV testing can be performed to detect the presence of HPVs in the sample. Both the PAP smear and HPV testing can be either done alone separately or at the same time, also called a ‘co-test’. (Jans et al. 2021). According to National Cervical Screening Program, women aged between 21-25 can perform cervical screening test at intervals of 5 years up to the age of 70 (Vash-Margita et al. 2021; Ngo-Metzger and Adsul 2019). During the screening test, a clinician or doctor gently inserts an instrument called a s speculum into the vagina to get a clear view of the cervix, and then they use brush or spatula to remove some cell from cervix part for further examination (Fig. 11). Later the samples were placed into a small liquid container and sent to laboratory for further analysis.

- The collected cells are examined under the microscope to assess whether they are normal or abnormal.
- Secondly, presence of HPVs is checked in the given samples and if the HPV is present, the pathologist performs some additional tests to check for cell abnormalities which is called liquid-based cytology (LBC).
- In case samples are detected to be HPV positive, further close examinations of cervix or vaginal area (also known as ‘colposcopy’ method) are performed by a colposcopist where a lighted, magnifying instrument called colposcope is used to check the vagina and cervix for any abnormal changes in the cells (Smith 2000; Burness, Schroeder, and Warren 2020).
- In case the colposcopist finds any abnormalities or suspicious changes in the cells, a small piece of tissue is removed (also known as ‘biopsy’ method) by using a spoon-shaped instrument called a curette and sent to lab for further analysis (Perkins et al. 2021).

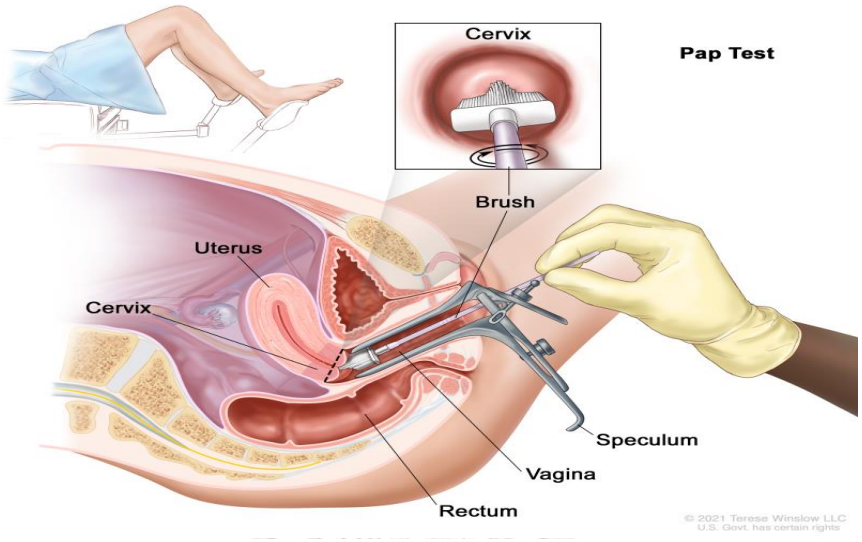


Figure 11. Cervical cancer screening (PAP smear). Source: (*https://www.ncbi.nlm.nih.gov/books/NBK65910/*)

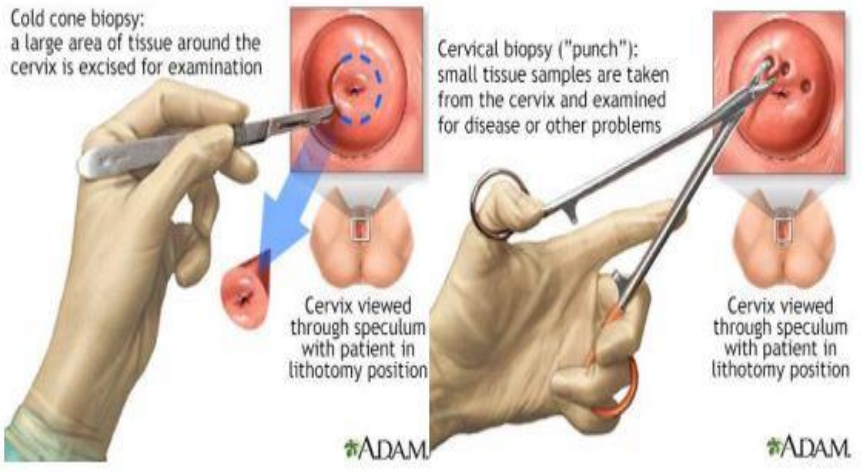


Figure 12 Cervical cancer screening "Biopsy".

Source:(*https://ssl.adam.com/content.aspx?productid=117&pid=1&gid=000893&site=riverviewssl.adam.com&login=RIDG9829*)

‘Biopsy’ is known to be one of the most accurate ways to confirm certain phytological effect (histology) or detect abnormality in cells to determine whether the cell is in pre-cancer, a true cancer, or neither state. There are many different types of biopsy procedures (Fig. 12). The most common types include:

(1) **Punch biopsy:** This biopsy involves removing of small piece of skin using circular blade like a paper hole puncher. Depending on the presence of abnormal cells in different areas of the cervix, one or two punch biopsies can be performed (Abrams 1990).

(2) **Cone biopsy:** In this biopsy procedure, a laser or scalpel instrument are used to remove a large cone-shaped piece of tissue from the cervix area and is performed in proper surgical centers or hospitals under anesthesia condition (Basu et al. 2018).

The cervical cytology, “Cervical interepithelial neoplasia” also known as CIN was compared between WHO (World Health Organization) and Bethesda system (BST). Over the past six decades, the system of reporting cervical cytology cases in WHO has undergone changes with ultimate diagnostic categories, where they categorized abnormal undetermined samples as “HPV”, while in the Bethesda system 1998, this abnormal undetermined samples were given nomenclature as ‘atypical’ squamous cells and were introduced as grey zone area which is categorized into two categories “Atypical squamous cells of undetermined significance” (ASC-US) and “Atypical squamous cells suspicious for HSIL” (ASC-H) (Fig. 13) (Nguyen and Nordqvist 1999). According to 2001, Bethesda System (TBS), the PAP test results were categorized into two parts: ‘Negative for intraepithelial lesion (Normal)’ and ‘Epithelial cell abnormalities’ (Smith 2002).

Negative for intraepithelial lesion (Normal samples)

- Normal samples (without HPV): Also known as ‘negative’ samples, indicates samples without HPV types and do not contain any signs of cancer or pre-cancer.

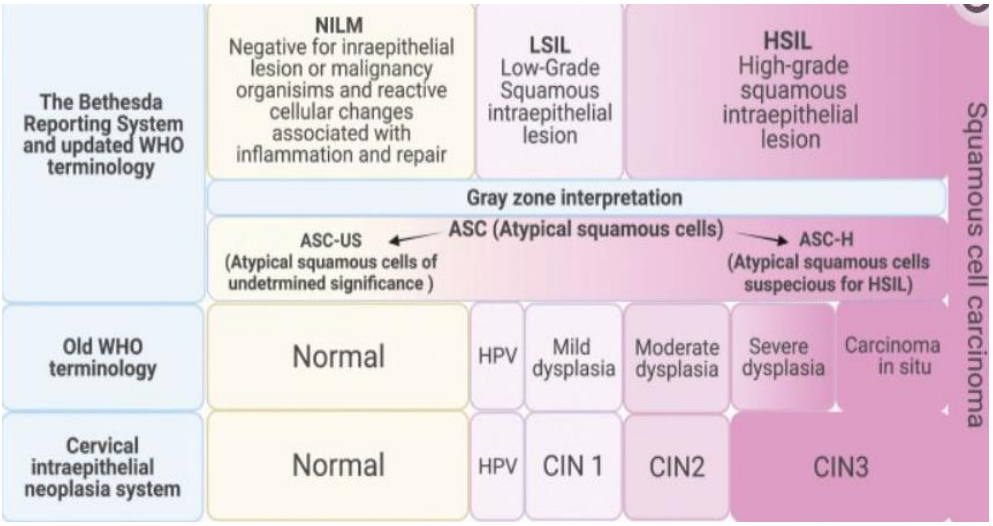


Figure 13. Introduction of ASC-US in Bethesda system and comparison of cervical examination reports Source: (Alrajjal et al. 2021)

Although no abnormalities were found, these samples may still lead to symptoms that are unrelated to cervical cancer such as signs of infection with yeast or herpes which show some "reactive cellular changes" around cervix area.

- **Normal samples (with HPV):** Also known as 'Normal samples with HPV' showed presence of HPV strains or other related signs of infections with yeast or herpes which show some symptoms around the cervix area.

The Epithelial cell abnormalities were further categorized as 'squamous' and 'glandular' cells abnormalities. The squamous intraepithelial lesion (SIL) was further categorized as (Lindeque 2005; Alrajjal et al. 2021)

- **'Atypical squamous cells of uncertain significance (ASC-US)' –** They are used to describe undetermined samples which are observed as 'abnormal' and for which it is very difficult to determine whether they cause infections, irritations or contain any pre-cancerous cells. These kinds of cells were immediately referred for HPV testing.
- **Low-grade SIL (LSIL)** – They are cells which look mildly abnormal based on PAP test results, and are also known as 'mild dysplasia' or 'cervical intraepithelial neoplasia grade 1' (CIN1).
- **High-grade SIL (HSIL)** - They are cells which look severely abnormal based on PAP test results and are also more likely to eventually develop into cancer

if they are not treated on time. They are also called ‘moderate’ to ‘severe dysplasia’ or ‘cervical intraepithelial neoplasia grade 2 or 3’ (CIN2 and/or CIN3).

- **Squamous cell carcinoma –** The cells under this category known as cancer cells or invasive cancer. For which the further diagnosis or treatment is performed immediately.

The glandular cells abnormalities were further categorized as:

- **Atypical glandular cells (AGC)-** The abnormal cells which might consider as cancerous cells and immediately need further more testing.
- **Adenocarcinoma**: Cancer occurs at endocervix area in the uterus or may also affect at other part of the uterus called adenocarcinomas.

The progression of benign cervical lesion to invasive cervical cancer is most likely due to the presence of HPV type, especially HPV 16 (Lowy and Schiller 2006). When HPV effect at the basal layer of cells, it started effecting the normal cells into abnormal cells, which cause a benign condylomas lesion, low grade dysplasia to high grade lesion (LSIL to HSIL). Once the E2 gene get disrupted, the overexpression of

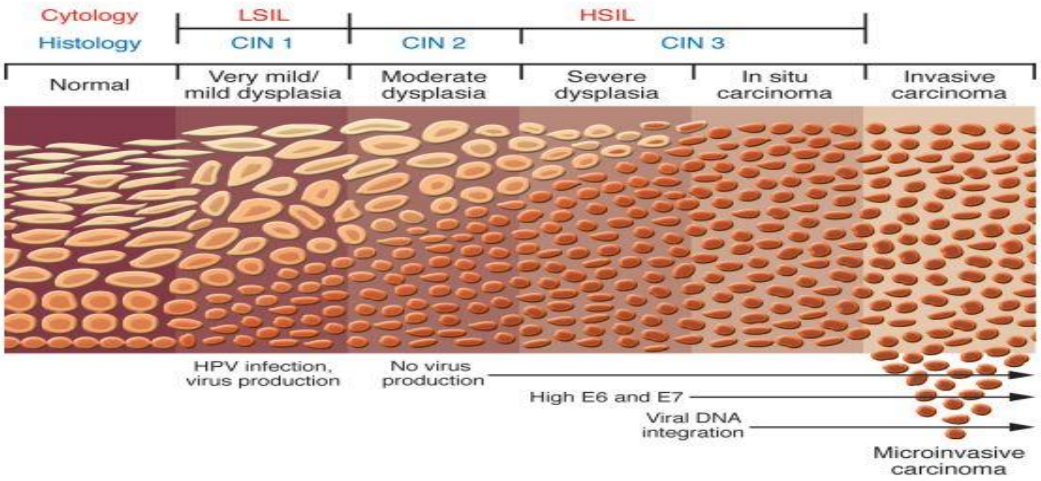


Figure 14 Progression from a benign cervical lesion to invasive cervical cancer. Source: (Lowy and Schiller 2006)

oncogenes E6 and E7 that preferentially expressed in cervical cancer, which occurs until several years after the infection (Fig.14). Once episomal DNA form changed into integration form and hence, rapidly changes in genetic and epigenetic of cellular genes (Prétet, Charlot, and Mougin 2007).

Epigenetic

The word “Epi” means above which indicate that any changes occur at “above” or “on top” called EPI- changes and any changes in genomic DNA or chromatin which resultant in alteration of gene expression without changing in sequence or coding of the gene called as an epigenetic phenomenon (Rutherford 2015; Dupont, Armant, and Brenner 2009). The two major epigenetic alteration, based on chemical modification are DNA methylation and Histone modification, that take place to cause orientational changes in DNA which led to inactivation or activation of gene expression (Bird 2007).

2.9 DNA Methylation

The initial discovery of DNA methylation was in 1925, since, DNA methylation has been vast instigated in many organisms and linked to gene regulation and to disease and aging. Research on composition of nucleic acid which is found to be a polymer chain of nucleotide growing interest in the field of Epigenetics with DNA methylation as a central actor. DNA methylation is an epigenetic mechanism which is used to control gene expression by fixing genes in the “off” position. This involved the covalent modification of cytosine at 5’-position where methyl group is mediated by presence of DNA methyltransferase enzyme, resulting in the formation of 5-methylcytosine (Bird 2002). In 1989, first methylation was occurring in CpG island of tumor suppressor gene in human (Greger et al. 1989). Methylation of cytosine occurs usually lie next to a guanine base at the CpG palindrome in DNA, resulted in two methylated cytosines which lie diagonally opposite to each strand (Illingworth and Bird 2009; Smith and Markham 1952). Due to methylation in CpG island, transcription factor was unable to bind to the promoter region caused gene expression repression (turn it off). It is the most studied epigenetic tool which play major role in cancer development, especially in cervical cancer, DNA methylation may use as a marker for the early detection or prediction of cancer (Clark et al. 1994).

2.10 DNA methyltransferase enzyme (DNMTs)

The transfer of methyl group at 5th position of cytosine is carried out by a family of enzymes called DNA methyltransferases (DNMTs) which play key role in epigenetic regulation. Due to the presence of DNMTs the methyl groups carried by SAM (S-adenosylmethionine) to SAH (S-adenosylhomocysteine); thus, 5-methylcytosine is formed (Moore, Le, and Fan 2013) (Fig. 15). DNMTs includes four member DNMT1, DNMT3a, DNMT3b and DNMT3L, among which DNMT3L does not possess any enzymatic activity as compared to other remaining DNMTs member which were active on DNA. Dnmt1 appear to be responsible for maintaining methyltransferase and DNMT3A/DNMT3B encode *de novo* DNA methylation patterns to maintain genomic methylation (Okano et al. 1999). While this maintenance vs *de- novo* division has been convenient, there is clear evidence for functional overlap between the maintenance and the de novo methyltransferases (Chédin 2011). DNMT activity is highly regulated and are associated with several human diseases, including cancer (Jin and Robertson 2013). If DNA hypermethylation occurs it causes silencing of tumor suppressor gene become tumorigenesis (Herman and Baylin 2000).

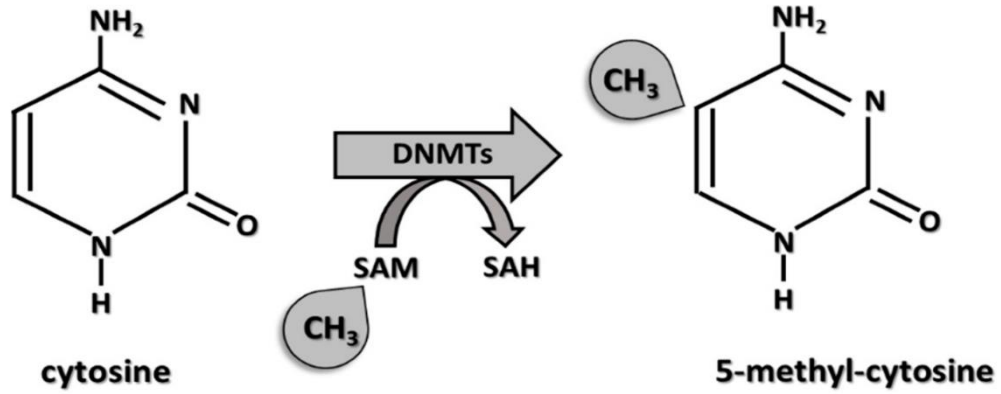


Figure 15 Mechanism of DNA methylation. Source: (Ciechomska, Roszkowski, and Maslinski 2019)

But DNMTs are not limited to gene silencing and can also include transcriptional activation and post-transcriptional regulation (Nunes et al. 2020; Kulis and Esteller 2010).

2.11 The association between DNA methylation and HPV16- E7

There are many studies have been done in field of DNA methylation and HPV 16 E7 which showed that HPV-16 E7 or E6 linked with DNMT1 which led to aberrant DNA methylation, resulted in tumorigenesis. From study of Sen P et al., (Sen, Ganguly, and Ganguly 2018) demonstrated the role of HPV oncoprotein E6 and E7 regulated the DNA methylation and disruption of the normal epigenetic process, implies to clinical applications of epigenetic based therapy for new approach towards early diagnosis or drug treatment for cervical cancer. From Chalertpet (Chalertpet 2015) studies showed that HPV16 E7 can induce promoter methylation in *CCNA1*. However, Yanatatsaneejit (Yanatatsaneejit et al. 2020) demonstrated that both HPV E6 and E7 induced promoter methylation in death-associated protein kinase 1 (*DAPK1*) and cell adhesion molecule 1 (*CADM1*), respectively. Moreover, both the studies indicating that E7 protein are capable of induing promoter methylation in particular gene which basically involved in apoptosis, cell cycle and cell adhesion, by using chromatin immunoprecipitation method, which bind to DNMT1 through the same mechanism as *CCNA1*. The study of Na Rangsee showed E7 possibly formed a complex with a set of transcription factors which included SP1 that bind to YY1 by using STRING database protein network (Na Rangsee et al. 2020). This contributing to the E7 mediated hypermethylation of the genes (Fig. 16) which highlighted the possibility of binding E7 with DNMT1 through transcription factor and induce promoter methylation in *CCNA1* gene. The clear understanding the role of these epigenetic alterations in cervical carcinogenesis will contribute to developing effective strategies in detecting and treating this disease.

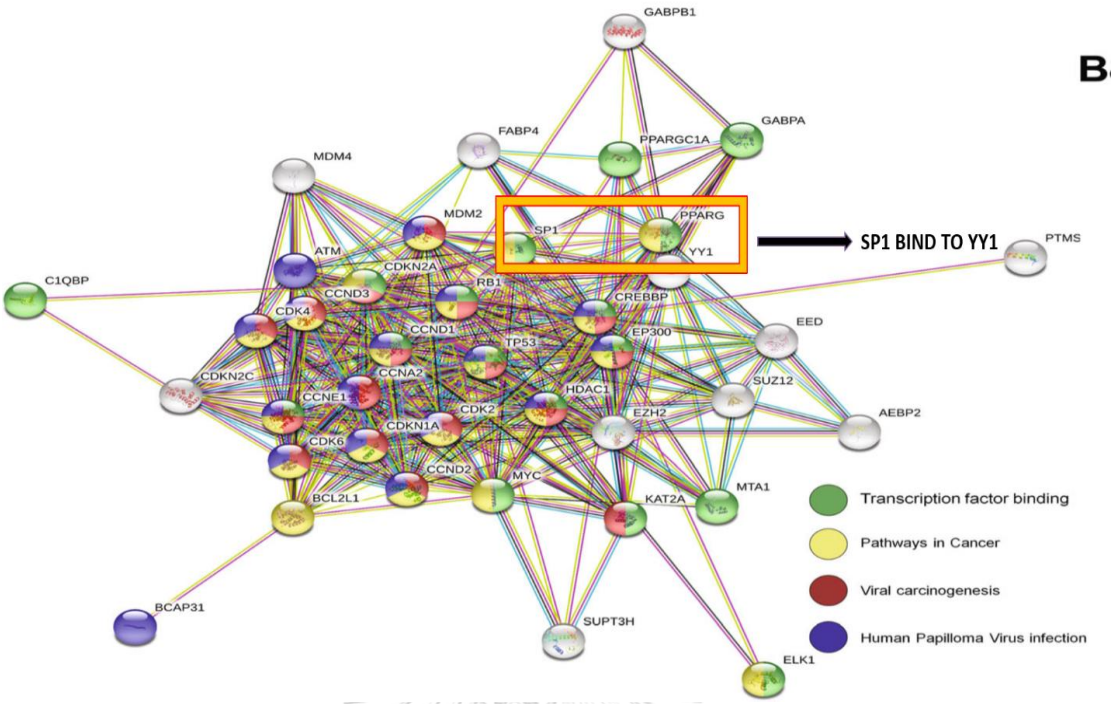


Figure 16. SP1 bind to YY1 transcription factor by STRING database protein network analysis. Source: (Na Rangsee et al. 2020)

2.12 Sodium bisulfite treatment

Bisulfite genomic sequencing was first introduced by Frommer et al (Frommer et al. 1992)and it is based on the finding that the amination reactions of cytosine and 5- methylcytosine (5mC) proceed with very different consequences after the treatment of sodium bisulfite. Treatment of DNA with sodium bisulfite leads to the deamination of cytosine residues and converts them to uracil and recognized as thymine, while 5-mC residues remain the same to be distinguished from unmethylated cytosines (Clark et al. 1994). This was performed by using a gold-standard technology for detection of DNA methylation because it provides a qualitative, quantitative and efficient approach to identify 5-methylcytosine at single base-pair resolution with optimized accuracy (>99%) (Taylor et al. 2007). The procedure of bisulfite treatment is explained in three processes based on chemical reaction of single DNA strand with sodium bisulfite ((HSO 3 ⁻) at low pH and high temperature as follow: **Sulfonation** is the first step where cytosine gets converted into cytosine sulphonate at the carbon-6

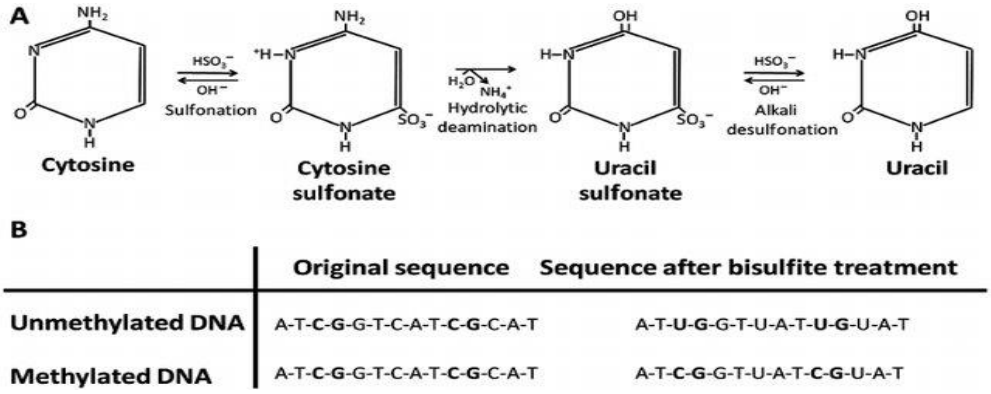


Figure 17 Chemistry of Sodium bisulfite treatment DNA. (A) Mechanism of cytosine converted to uracil. (B) Conversion of cytosine to Uracil. Source: (Kristensen and Hansen 2009)

position of cytosine. **Hydrolytic deamination** is the second stem which is irreversible and gets converted from cytosine sulfonate to uracil sulfonate. Finally, subsequent **Desulfonation** step which occurs in the presence of alkaline condition were Uracil is finally generated Fig. 17A (Herman et al. 1996; Kristensen and Hansen 2009).

Bisulfite sequencing analysis can detect even the single methylated allele from one thousand unmethylated alleles. From the results, unmethylated cytosine of original DNA gets converted to uracil, whereas methylated DNA remain as same Fig. 17B. Bisulfite sequencing not only identify DNA methylation status along the DNA single strand, but also detect the DNA methylation patterns of DNA double strands since the converted DNA strands are no longer self-complementary and the amplification products can be measured individually (Warnecke et al. 2002).

2.13 Methylation specific PCR (MSP-PCR)

MSP-PCR is the most common method for analysis of DNA methylation status of particular genes (Herman et al. 1996).In this method two methylation specific primers are named as “Methylated primer” and “Unmethylated primer”. Though there are many publications like (Davidović et al. 2014), internet resources or software are available for the construction of methylated or unmethylated primer and

here in this research also, author has picked the selected promoter sequence of genes from ENSEMBL database *(https://asia.ensembl.org/index.html)* and NCBI *(http://www.ncbi.nlm.nih.gov/)*. After bisulfite treatment of DNA samples, unmethylated cytosine

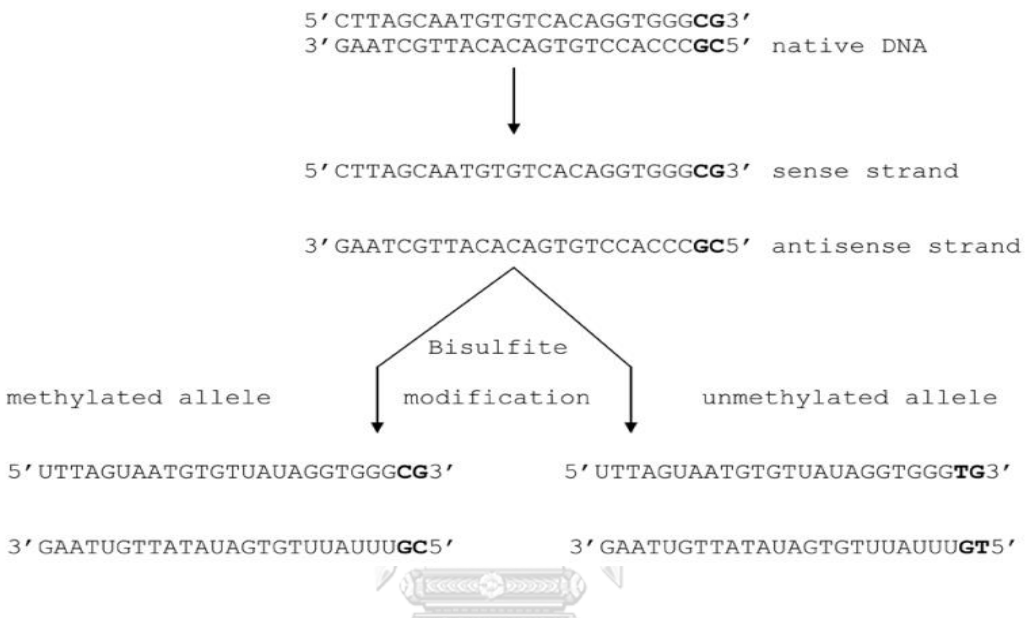


Figure 18 MSP based bisulfite modification (Uracils to Thymines). *Source:* (Davidović et al. 2014)

converted into uracil while 5-methylcytosine remain the same, thus there is no longer any DNA complementary strands to each other (Fig. 18). MSP-PCR machine has ability to amplify or distinguish between the methylated or unmethylated cytosine by using specific primer of methylated or unmethylated gene, based on bisulfite modified DNA samples (Ku, Jeon, and Park 2011).

2.14 Action of 5-Azacitidine drug

The chemical name of 5-azacitidine (5-aza) is 4-Amino-1-β-D-ribofuranosyl-1,3,5-triazin-2(1H)-one, which is well known as DNMT1 inhibitor. The molecular weight is 244.21and can be store at room temperature upto 12 months. 5-Aza is a chemical ring analog of cytosine nucleoside showed interest in DNA and RNA level which related to gene expression, DNA synthesis, cell differentiation and metabolism (Veselý 1985). The high concentration of 5-aza (varying from 150 to 750 mg/m2),

was first appeared and used for the treatment of acute leukemia in 1970s as novel chemotherapeutic agents with pronounced toxicity (Saiki et al. 1978). At low dose, 5-aza may induce antineoplastic activity or severe phenotypic changes by inhibition of DNMTs function. Later from many research studies have been shown that 5-aza may use as epigenetic drug by reducing the level of DNA methylation (Christman 2002).In order to achieve cytotoxicity, 5-aza used at higher dose through incorporate into RNA and DNA. 5-aza first approved by the Food and Drug Administration (FDA) for the treatment of patients with myelodysplastic syndrome (MDS) proving both response and survival rate (Buckstein et al. 2011). This was first marked as an approval of 5-aza used as epigenetic drug in cancer therapy.

On a molecular level, azacitidine is phosphorylated to 5-azacytidine monophosphate by uridine-cytidine kinase, then to diphosphate by pyrimidine monophosphate kinases and triphosphate by diphosphate kinases. 5-Azacytidine diphosphate is reduced to 5-aza-deoxycytidine diphosphate by ribonucleotide reductase. The resultant metabolite is phosphorylated to 5-azadeoxycitidine triphosphate by nucleoside diphosphate kinases. 5-azadeoxycitidine triphosphate is then incorporated into DNA, leading to inhibition of DNA synthesis. Once 5-aza was incorporated into DNA, they covalently bind to DNMT1, led to loss of methylation in specific gene regions and activation of associated genes as shown in Fig 19. In addition, 5-Aza causes DNA hypomethylation after cells divide in the absence of DNMT1. The antineoplastic activity of azacitidine resulting in the hypomethylation of genes leading to the reactivation of tumor suppressor gene (TSG) expression or downregulation of cancer oncogenes. Hypomethylation may restore normal function to genes that are critical for differentiation and proliferation. Therefore, 5-Aza may use as a therapeutic agent for cancers in which epigenetic silencing of critical regulatory genes has occurred (Christman 2002; Müller and Florek 2010; Dimopoulos and Grønbæk 2019).

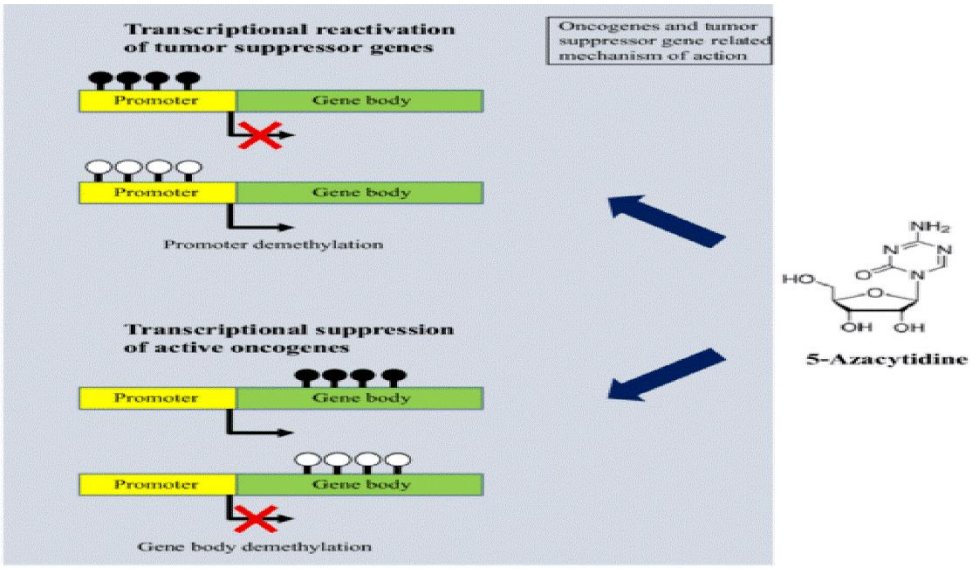


Figure 19 Mechanisms of actions of 5-azacytidine. Source:(Christman 2002)

2.15 REAL TIME PCR (qPCR)

Real - time PCR or quantitative PCR (qPCR) is a technique used to measure the amount of PCR product at the end of each amplification cycle. With each amplification cycle, as the amount of the template DNA increases, the number of fluorophores bound, and therefore, the intensity of the fluorescent signal, increase. Their primary advantage is cost, but they are non-specific and bind all dsDNA and not just the target. DNA measurements can use various types of dye. Dye, which is a fluorescent color that can bind to the DNA straightened minor groove of double-wire DNA. It can absorb blue light (488 nm) and emit green light (522 nm), during denaturation. The dual DNA cables are separated into single wires, and when a new DNA synthesis is started, SYBR begins. Inserts itself into dual-wire DNA and glows when triggered by UV light, which detects signals- Light with a light receiver located in a real time thermocycler (Jozefczuk and Adjaye 2011; Ponchel et al. 2003).

Recently Wu et al. (Wu et al. 2018) quantitatively detected E6 and E7 mRNAs using qPCR in cervical brushing cells from HPV patients overcoming limitations of low sensitivity and accuracy of traditional PCR methods. This method can be used for cytological diagnosis of cervical neoplasia and can also help to determine the severity of the lesions of viral infection. Many companies (e.g., Qiagen,

Sigma, Thermofisher etc.) have developed specific qPCR-based miRNA assays that are now widely used for diagnosis and prognosis of many diseases including cancer. Recently, Gong et al. established miR-221 overexpression and its correlation with tumor stage, metastatic status, and response to chemotherapy pretreatment in Osteosarcoma patients (Gong and Gong 2018). Over the last decade, the advent of technologies like next generation sequencing, real-time PCR technique etc have undergone considerable modifications which are more sophisticated and customized to user needs. Automation of gene profiling methods and imparting it a high-throughput edge, have a huge potential to take expression-based studies to a broader range of clinical applications and expediting the drug discovery process. High-throughput automated qPCR-based transcriptome analysis, quantitating the mRNA abundance has provided new dimensions to the personalized medicine research and has also contributed to the process of development of novel therapeutics (Lim et al. 2015).

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Equipment's used in research

1. Optical Microscope (Olympus, Japan)
2. Hot plate stirs (Labtech, Korea)
3. Vortex (Scientific Industries, USA)
4. Sonicator (Sonics & materials, USA)
5. Automatic suction controller (pipette aid) (Technorama, Switzerland)
6. Digital Scales (Mettle Toledo, USA)
7. Gel Imaging Machine (Storm 840 Imaging System) (Amersham Biosciences)
8. Gel doc (Bio-Rad, USA)
9. Electrophoresis (Bio-Rad, USA)
10. Nano drops meter (Thermo Scientific, USA)
11. Air Compressor (Hirayama, Japan)
12. 1.5 ml tube centrifuge (microcentrifuge) (Boeco, Germany)
13. Polypropylene conical tube centrifuge (Boeco, Germany)
14. High quality pure water generator (ultrapure water type I) (Millipore, France)
15. Thermal cycler (Eppendorf, Germany)
16. Real time PCR DNA booster (Applied biosystems, UK)
17. Microwave (Sanyo, Japan)
18. Heat blocker (Stuart, UK)
19. Centrifuge (Microcentrifuge) (Thermo Scientific, USA)
20. Freezer temperatures of -20oC and -80oC (Revco, Japan)
21. Fume hood (S.K.Powerable, Thailand)
22. Class II sterile cabinet (laminar flow cabinet) (Gelman sciences, Singapore)
23. Cell cabinet in conditions with 5% CO2 (Shel lab, USA)
24. Refrigerator (Hitachi, Japan)
25. Pressure steamer for sterilization (High pressure steam sterilizer, Autoclaves) (Tomy, Japan)
26. Incubator (Memmert, Germany)
27. Micropipet (Eppendorf, Germany)

28. Water bath (Memmert, Germany)

3.2 Material used in research

1. Measuring cylinder (Witeg, Germany)
2. Duran bottles 50 ml, 100 ml, 250 ml, 500 ml and 1,000 ml (Duran, Germany)
3. T25 cell bottle (Nest, China)
4. Scoop spoon
5. Latex Gloves (Sri trang group, Thailand)
6. Tips for micropipes (Nest, China)
7. 2 ml, 5 ml, 10 ml and 25 ml sterile pipettes (Nest, China)
8. Parafilm (Parafilm M, USA)
9. Tube size 0.2 ml, 0.5 ml, 1.5 ml and 2 ml (Nest, China)
10. Cryovial tube (Corning, USA)
11. Polypropylene conical tube size 15 ml and 50 ml (Nest, China)
12. Slide Mirror (Chance, UK)
13. Thermometer (Precision, Germany)
14. Magnetic Rod (Agimatic-e, China)
15. Cell Count Slide (Haemocytometre) (Boeco, Germany)
16. Cell Scraper (Jet biofill, China)
17. Cryovial tube (Corning, USA)
18. Polypropylene conical tube size 15 ml and 50 ml (Elkay, USA)
19. 6 well plate (Jet biofill, China)
20. 96 well plate (Thermo, USA)

3.3 Chemicals used in research

1. Common chemicals
   1.1. Absolute ethanol (Merck, Germany)
   1.2. Acetic acid (Merck, Germany)
   1.3. Agarose (Cambrex, USA)
   1.4. Ammonium acetate (Merck, Germany)
   1.5. Ampicillin (Sigma, USA)
   1.6. Bromophenol blue (USB, Germany)
   1.7. Chloroform (Merck, Germany)

1.8. Dimethyl sulfoxide (DMSO) (Sigma, USA)
1.9. EDTA (USB, Germany)
1.10. Formaldehyde (Merck, Germany)
1.11. Formaldehyde (Merck, Germany)
1.12. Glycine (Amersham Bioscience, Sweden)
1.13. Glycogen (USB, USA)
1.14. Hydrochloric acid (Merck, Germany)
1.15. Hydroquinone (Merck, Germany)
1.16. Isopropanol (VWR, France)
1.17. Methanol (SK chemicals, Korea)
1.18. Phenol (USB, USA)
1.19. Proteinase K (USB, USA)
1.20. RNase A (Applichem, Germany)
1.21. Sodium chloride (Merck, Germany)
1.22. SYBR green I nucleic acid gel stain (Lonza, USA)
1.23. Tris (Tris[hydoxymethyl] aminomethane) (Omnipur, Germany)
1.24. TritonX 100 (Bio-RAD, USA)
1.25. Trizol reagent (Invitrogen, USA)
1.26. Trypan blue (Sigma, USA)
1.27. Tris base (USB, USA)
1.28. Xylene (Merck, Germany)
1.29. 100 base pair DNA ladder (Promega, USA)
1.30. 25 base pair DNA ladder (Promega, USA)

2. Chemical for cell culture

2.1. Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA)
2.2. Fetal Bovine Serum (FBS) (Gibco, USA)
2.3. Anti-anti (100X) antibiotic-antimyotic (Gibco, USA)
2.4. Trypsin EDTA (Gibco, USA)
2.5. Phosphate Buffered Saline (PBS) (Gibco, USA)
2.6. Tryphan blue (Gibco, USA)

3. Chemical for transfection

   3.1. Opti-MEM reduced serum medium (Gibco, USA)

   3.2. Turbofect (Thermo, USA)

4. Chemicals for PCR

   4.1. Deoxynucleotide (dNTP) Solution Mix (New England Biolabs, USA)

   4.2. 10X PCR buffer (Qiagen, USA)

   4.3. Hot start Taq DNA polymerase (Qiagen, USA)

   4.4. Oligonucleotide primers (Integrated DNA Technologies, Singapore)

5. Chemicals for Bisulfite sulfite treatment

   5.1. EpiTect control DNA set (Qiagen GmbH)

   5.2 Hydroquinone (Sigma-Aldrich, USA)

   5.3. Sodium metabisulfite (Sigma-Aldrich, USA)

6. Chemical for REAL TIME PCR

   6.1. Oligonucleotide primers (Bioneer, Korea)

   6.2. Power SYBR® Green PCR master mix (Applied biosystems, UK)

7. Chemical for bacterial culture

   7.1. Agar bacterial powder (Conda, Spain)

   7.2. Tryptone (Bio Basic Inc, Canada)

   7.3. Yeast extract powder (Bio Basic Inc, Canada)

8. Chemical for 5-aza treatment

   8.1. 5-Azacytidine (abcam, USA)

9. Series of chemical kits used

   9.1. EZ DNA Methylation-Gold Kit (Zymo Reserch, USA)

   9.2. GeneJET Plasmid Miniprep Kit (Fermentas, USA)

   9.3. Revert Aid First Strand cDNA synthesis Kit (Fermentas, USA)

Research procedure

This research can be divided into five main areas:

1. Cell lines and cultures
2. Selection of genes based on promoter methylation regulated by E7 oncoprotein
3. To prove methylation and gene expression in E7 transfected C33A cell line
4. Analysis to observe whether methylation controls the gene expression
5. Detection of promoter methylation in clinical samples: Normal samples (with and without HPV), ASC-US and Cervical cancer.

3.4 Cell line and cultures

3.4.1 Thaw cells

The cells were quickly thaw after removing from the liquid nitrogen storage tank and place the lower half of the vial into a 37°C water bath while agitating gently for 60sec. The vial was clean by 70% of alcohol and placed in the biological safety cabinet in aseptic conditions to avoid any contamination. The re-suspended cells were gently transfer into a 15 mL sterile conical tube which contained 5 mL of pre-warmed Dulbecco's Modified Eagle's Medium (DMEM) with Fetal Bovine Serum (FBS) 10% and antibiotic 1%. Further the cells were centrifuge at 200x g for 3 minutes. The supernatant was discarded and the pellet was dislodged by flicking the tube with finger. Later the resuspended cells were added in the 5 ml of growth medium in T25 flask and incubated at 37°C with 5% CO2 and 95 – 99% humidity for overnight (Chalertpet et al. 2015).

3.4.2 Trypsinization (Cell-dissociation)

Microscopically the cells morphology was observed and checked for confluency percentage whether it reached up to 70 to 80% or not. Further, the trypsinization (Trypsin-EDTA) process was proceed. Firstly, the old media was removed and the cells were rinse with 3ml of PBS. Next, the PBS was removed using aspiration and 1ml of Trypsin-EDTA was added to entire surface of the cells. Later, the flask was incubated at 37°C (in the incubator) at least for 5min until cells appear

rounded and start detaching from the substratum. The cells were detached from the surface of the flask by tapping it and added 2ml of fresh DMEM serum-containing medium to inactivate the Trypsin action. Later the suspended cells were collected in centrifuge tube and centrifuged at 200g for 5min in room temperature. Resuspend the cells in an appropriate DMEM medium and determined the number/viability of cells by using a haemocytometer. An aliquot of cells were subcultured into a new T25 flask with fresh DMEM growth medium and placed in incubator at 37°C with 5% CO2 (*Cell Dissociation Protocol using Trypsin (sigmaaldrich.com).*

3.5 Selection of genes based on promoter methylation regulated by HPV16-E7

GSTP, *PDI-A3*, *IL4R alpha*, *ING1*, *VAV1*, *CGB3* and *NOP56* genes were analyzed based on their expression and promoter methylation in SiHa and C33A samples by using specific primer from Table 10 and Table 3.

3.5.1 Analysis and comparison of gene expression in cervical cell lines

3.5.1.1. Transformation

The pcDNA3.1-E7 (E7), pcDNA3.1 empty vector (PC) plasmid and DH5α competent cell were thawed on ice for 5 min. The 100 µL of DH5α competent cell was aliquoted into each 1.5 micro-centrifuge tube. Then, 5 µL of E7 and PC plasmid was added separately into each tube which containing competent cell followed by gently mixed. Then, tubes were incubated on ice for 5 min. Heat shock method was performed, the tubes were placed in 42 °C thermomixer (Eppendorf, USA) for 45 s, immediately the tubes were placed on ice for 2 min. Next, 900 µL of SOC medium was added (Biolabs, USA) into each tube and gently incubated the tubes in thermomixer machine for 45 min at 37 °C, 400 rpm. Bacterial cells were collected by spinning down at 8000 rpm for 5 min (Song et al., 1993). The 900 µL of supernatant was discarded and the cell pellet 100 µL was resuspended by pipetting. The 100 µL competent cell contained plasmid was spread on Luria-Bertani (LB) agar (Titan Biotech, India) contained ampicillin antibiotic final concentration 0.1 mg/mL (Merck, Germany). The agar plates were incubated at 37 °C in 5% CO2 incubator (Na Rangsee et al. 2020).

3.5.1.2 Plasmid extraction and purification

The positive colonies on Luria-Bertani (LB) agar (TitanBiotech, India) contained ampicillin antibiotic final concentration 0.1 mg/mL (Merck, Germany) were selected and continued cultured in 10 mL Luria-Bertani (LB) broth with 10 µL ampicillin (final concentration 0.1 mg/ mL) at 37 °C, 250 rpm shaking incubator overnight. Next day, 1000 mL of LB broth with 1 mL of ampicillin (final concentration 0.1 mg/mL) was prepared, amongst which 5 mL of broth were taken and cultured bacteria was added which kept for overnight on shaking incubator at 37 °C, 250 rpm. Plasmid extraction (E7, PC) was done afterward using Maxi Plasmid Kit Endotoxin Free according to the manufacturer's instructions (Geneaid™ Taiwan) (Bimboim and Doly 1979).

3.5.1.3 Transfection of HPV16-E7 hybrid plasmids into C33A cells

For HPV 16 E7 overexpression, $3x10^5$ C33A cells were seeded with 2ml of DMEM growth media into each well of 6-well plate and incubated overnight at 37˚C in incubator. On the next day, old media was discarded with new DMEM (without antibiotics) media by volume 1800µl per well. Subsequently, 2µg of HPV 16 E7 plasmid (E7) DNA and pcDNA 3.1/myc-HIS empty vector (PC) DNA were diluted with 200µl of opti-MEM in separate 1.5ml of tube. Prior to transfection, 6µl of TurboFectTM transfection reagent (Thermo Scientific, USA) mixed with 184µl of Opti-MEM in another 1.5ml of tube for 5min at room temperature. After 5min of incubation, the diluted DNA and diluted TurboFect reagent were combined and incubated for 20 min at room temperature. Then, 20 µL of the transfection reagent/DNA mixture were added drop-wise to each well and the plate was gently rock to achieved even distribution of the complexes. Later, plate was placed in 37 °C with 5% CO2 incubator for 72 hrs (Chalertpet 2015). The plasmids enter the cell and then collected for the next stage of experimentation.

3.5.1.4 RNA extraction

The total RNA extraction was performed using TRIzol® reagent after once HPV16- E7 and empty plasmid PC DNA was transfected into C33A cells for 72hrs of incubation. The old media was removed and trypsinization was performed through

which we collected the cells into siliconized 1.5mL of microcentrifuge tube centrifuge at 500 g for 4 minutes and supernatant was removed. The cells sediments were washed with 1ml of PBS buffer twice at same speed of centrifugation 500g for 4min and later supernatant was removed. The 1ml of TRIzol was added into the cells and pipet the lysate up and down several times to homogenized cells completely and incubated at room temperature for 10min. Next, 200µl of chloroform was added (for 1ml of TRIzol) and the samples were for 15sec and further incubated at room temperature for 2-3min. Later, the samples were centrifuge at speed 12,000 g for 15 minutes at 4°C. Following centrifugation, the mixture was separated into a lower red phenol chloroform phase, an interphase and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase (which is about 60% of the volume of TRIzol reagent used for initial homogenization). Hence, the upper layer of aqueous phase was collected in the new 1.5 microcentrifuge tube without disturbing the interphase. RNA precipitation was done by adding 500µl of 100% cold isopropanol (for 1ml of TRIzol) and incubated at room temperature for 10 minutes in aqueous phase, also labelled the samples accordingly. Later, the samples were centrifuged at 12,000 g for 10 minutes at 4°C. The precipitated RNA formed a translucent gel-like pellet on the side and bottom of the tube which was washed twice with 1ml of 75% ice-cold ethanol for 1 mL of TRIzol reagent. The pellet was mixed with ethanol by gently up and down of tube and centrifuged at 7,500 g at 4°C for 5 minutes. Following the centrifugation, the supernatant was removed and air dried the RNA pellet for approximately 15-30 minutes by leaving the tubes cap open at the counter. When the pellet was dried completely and there was no visible ethanol in the tube appeared then the RNA pellet was resuspended with 20-50 µL of nuclease-free water depended on the RNA pellet, by pipetting up and down gently. Incubated the RNA pellet in 37°C water bath for 15 minutes to dissolved completely. RNA concentration was measured by nanodrop and kept in freezer -80°C till the further step of experiment was proceed (Na Rangsee et al. 2020).

3.5.1.5 cDNA synthesis

After the extraction of RNA, cDNA synthesis was performed from RNA template by using RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific, Inc. UK). All the component of the kit was briefly centrifuged, mixed and kept on ice before starting the experiment. The RNA concentration was used 1µg in total volume 11µl of diethylpyrocarbonate (DEPC) treated water in 0.2ml sterile, nuclease-free tube. 1µl of Oligo(dT) primer with concentration 0.5g was added in the same tube and incubated at 70°C for 5minutes in PCR. Oligo(dT) primes cDNA synthesis from the poly(A) tail present at the 3'-end of mRNA. Following incubation, 4µl of 5x reaction buffer, 1µl of ribonuclease inhibitor (20ug/µl), 2µl of 10mM dNTP mix and 1µl of reverse transcriptase (200u/ul) were added and mix well by pipetting. Next, the samples were incubated at 42°C for 60 minutes followed by 70°C for 10 minutes using reverse transcriptase PCR machine. Once the synthesis of cDNA was completed the samples were stored in freezer at -80°C (Chalertpet 2015).

3.5.1.6 Polymerase chain reaction (PCR)

The cDNA of the samples was used to detect the gene expression of *CGB3*, *GSTP1* and *NOP56* by using specific primer of each gene were designed by Ensembl database with band size 194 bp, 96 bp and 105 bp, respectively. The Condition for monitoring *CGB3*, *GSTP1* and *NOP56* gene expression as shown in Table 1 and 2. To evaluated the expression of *CGB3*, *GSTP1* and *NOP56* genes in transfected HPV 16-E7 in C33A and SiHa cell lines and also checked in treated 5-aza in SiHa and C33A cell lines by using specific primer as mentioned in Table 3. GAPDH used as internal control while checking the expression of any gene. To check *CGB3* and *GSTP1* expression GAPDH primer was used while for *NOP56* expression GADPH I primer was used.

Table 1 Ingredients used to apply PCR to determine the gene expressions

Chemical ingredients	Volume (µl)	Final concentration
10X PCR	1.0	1X
10 mM dNTPs	0.2	0.2 mM
20 uM Forward primer	0.15	0.3 uM
20 uM Reverse primer	0.15	0.3 uM
250 U HotStarTaq polymerase	0.1	0.25 U/reaction
Distilled water	7.4	
DNA	1	
Total volume	10	

Table 2 PCR condition to determine the expression of *CGB3, GSTP1* and *NOP56.*

Step	Temperature (˚C)	Time
1. Initial denaturation	95	15min
2. PCR cycle (28 cycle) -Denature -Annealing -Extension	 95 56 72	 1min 1min 1sec

3. Final extension	72	7min
4. Holding	4	

3.5.1.7 Electrophoresis

The PCR product was examined with 6% non-denaturing acrylamide gel. The gel was run at 120 volts for 45 min. After that, the gel was immersed in 1X TBE + 1X SYBR green I nucleic acid gel stain for 30 min. Then the gel was examined by Gel imaging and recording machine Storm 840 (Amersham Biosciences), to visualized the band size of particular gene.

Table 3 Primer sequence of *CGB3*, *GSTP1*, *NOP56* and *GAPDH* for gene expression

Genes	Sequence, 5'-3'	Product size, bp
CGB3	Forward Primer: CAACACCACCATCTGTGC Reverse Primer: GGCAGAGTGCACATTGAC	194
GSTP1	ForwardPrimer: CCTGGTGGACATGGTGAATGAC ReversePrimer: ATGGCTCACACCTGTGTCCATC	252
NOP56	Forward Primer: CAGCATCGTTCGTCTGGTGG Reverse Primer: AGGCGGAGGTCCTCATGAAC	105
E7	ForwardPrimer:GGGCAATTAAATGACAGCTCAG Reverse Primer GTGTGCTTTGTACGCACAACC	142
E6	ForwardPrimer:GCAAGCAACAGTTACTGCTGCGAC Reverse Primer: GCTTTTCTTCAGGACACAGTGG	243
GAPDH	Forward Primer: CAGCCGCATCTTCTTTTG Reverse Primer: GCCCAATACGACCAAATC	96
GAPDH I	ForwardPrimer: TGGAAGGACTCATGACCACAG Reverse Primer: TTCAGCTCAGGGATGACCTT	163

3.6 Detection of promoter methylation in the candidate genes

3.6.1 Transfection of HPV16 E7 and PC in C33A cell lines

After completing the step of transformation and plasmid extraction of E7 and PC plasmids as shown in step number 3.5.1.1 and 3.5.1.2, the transfection of E7 and PC (empty plasmid) in C33A cells using TurboFectTM transfection reagent (Thermo Scientific, USA) as explained briefly in 3.5.1.3 step.

3.6.2 DNA extraction

According to step number 3.5.1.3, when HPV-16 E7 plasmid was transfected into C33A and SiHa cells for 72hrs, followed by trypsinization from step 3.4.2, when cells were scrapped and collected in 1.5ml microcentrifuge tube for centrifugation at 500g for 4minutes. The cells sediments were washed twice with 1ml of PBS to remove the serum of media completely by centrifuge at 500g for 4minutes. The supernatant was discarded and 950µl of lysis buffer II was added. Later, the samples were mixed with 50µl of 10%SDS, also 30µl of proteinase K was added and incubated in water bath at 50°C for overnight. The next day, phenol:chloroform:isoamyl alcohol (25:24:1) was added to the samples, mixed well and then centrifuged at 14,000 rpm for 5min. The upper layer of mixer was transfer into the new 1.5ml of centrifuge tube and 100% of ethanol volume was added into the upper layer and also 7.5 M of CH3COONH4 was added. The samples were mixed by upside down 3-4 times and then centrifuged at 14,000 rpm for 15 min. The supernatant was poured out and washed the DNA pellet with 500µl of 75% ethanol and then centrifuged with the same speed of 14,000 rpm for 15min. The supernatant was removed and the pellet was kept at room temperature by inverting tube on the paper for air dried. Later, the resuspend of DNA pellet as done by adding 20-30µl of double distilled water ddH_2O depends on the amount of DNA pellet and further, incubated at 37°C in water bath for 30min. The DNA concentration was measured by nano drop machine and kept at -20°C in freezer until the next step of the experiment was performed (Maniatis 1989).

3.6.3 Sodium bisulfite treatment

The sodium bisulfite treatment was performed for the analysis of the methylation status of cytosines in DNA, followed by extraction of DNA step. In this technique, sodium bisulfite was deaminated the cytosine nucleotide into uracil, but does not affect 5-methylcytosine, a methylated form of cytosine with a methyl group attached to carbon 5. Bisulfite treatment was performed by using the EZ DNA Methylation-Gold kit (Zymo Research Corp.), According to protocol, 750ng concentration of DNA for each sample was prepared in dH2O with 20µl of total volume in PCR tube. The CT conversion reagent was prepared by adding 900µl of dH2O, 300 µl of M-Dilution buffer, and 50 µl M-Dissolving buffer in same CT conversion tube, which was available in kit box. The reagent was mixed at room temperature with frequent vertexing process for 10 min. (Note: The CT Conversion Reagent is light sensitive, so minimize its exposure to light by cover it with foil). Later, 130 µl of the CT Conversion Reagent was added to the 20 µl of the sample DNA in a PCR tube. The samples were mixed by flicking the tube and vertexing the liquid to the bottom of the tube. The sample tube was placed in thermal cycler and following step was performed: a. 98°C for 10 minutes b. 64°C for 2.5 hours c. 4°C, at this stage the samples were kept for storage up to 20 hours. With continuation the above step, 600 µl of M-Binding Buffer was firstly added to the Zymo-Spin™ IC Column and placed the column into a provided collection tube. Later, the sample prepared in CT conversion tube was loaded to the same Zymo-Spin™ IC Column which contained the M-Binding buffer. The cap of the column was closed and mixed the sample by inverting the column several times. Later, the sample was placed in the centrifugation machine and spin at speed more than 10,000 g for 30 seconds and then the flow-through was discarded. The 100 µl of M-Wash buffer was added to the same column and further spin at speed more than 10,000 g for 30 seconds. The 200 µl of M-Desulphonation buffer was added and kept at room temperature for 20 minutes. After the incubation, centrifugation was done at speed 10,000 g for 30 seconds. Next, 200 µl of M-Wash buffer was added to the column and centrifuged again with the same speed 10,000 for 30 seconds. This step was repeated one more time with the same condition. In the last step, the new lower 1.5ml centrifuge tube was replaced with the old one but with same column. Further, the 10 µl of M-Elution buffer was

directly added to the column and centrifuged at speed 30 sec to elute the DNA. Collected the DNA for performing the next experiment or store in freezer at -20°C (Chalertpet 2015). .

3.6.4 Methylation specific PCR (MSP-PCR)

The MSP-PCR was used to analyzed the DNA methylation patterns in CpG islands by modifying the genomic DNA with sodium bisulfite treatment. Once the sample DNA was treated by sodium bisulfite, the two independent primer sets for *CGB3*, *GSTP1*, *NOP56* I and *NOP56* II genes was designed for PCR amplification, as one pair designed to recognized the methylated sequence and the other pair the unmethylated versions of the bisulfite-modified sequence (as mentioned in Table 5, 6, 7 and 8 respectively). The condition to monitoring promoter methylation status of *CGB3*, *GSTP1* and *NOP56* as discuss below:

Table 4 Ingredients used to apply MSP- PCR to determine promoter methylation

Chemical ingredients	Volume (µl)	Final concentration
10X PCR	1.0	1X
10 mM dNTPs	0.2	0.2 mM
20 uM Forward E7 primer	0.15	0.3 uM
20 uM Reverse E7 primer	0.15	0.3 uM
250 U HotStarTaq polymerase	0.1	0.25 U/reaction
Distilled water	8.4	
DNA	1	
Total volume	10	

Table 5 Primer sequence of *CGB3* for methylation and unmethylated genes

Gene	Sequence, 5'-3'	Product size, bp	Annealing temperature, ˚C	Number of cycles
CGB3				
Methylated forward	CGGGTTGAATTTTTCGTTGGC	116	52	27
Methylated reverse	CCCAAAAAAAACGCGACTTCG			
Unmethylated forward	GGGTTTGGGTTGAATTTTTTGTTGGT	127	55	27
Unmethylated reverse	CAACCTCCCAAAAAAAACACAACTTCA			

Methylated PCR monitor the changes in methylation levels in the promoter region of *CGB3*, *GSTP1* and *NOP56* genes. The methylated and unmethylated band of *CGB3* is 116 and 127, respectively. The methylated and unmethylated size of *GSTP1* is 231 and 236 bp, respectively. For *NOP56*, the two methylated and unmethylated primer was used as follow: to determine the methylation status of *NOP56* in SiHa and C33A, the *NOP56* II primer was used while to checked the methylation status in transfected HPV 16-E7, the *NOP56* I was used. In the same way, to checked the methylation status of *NOP56* in treated 5-aza in SiHa and C33A cells *NOP56 II* primer was used. Furthermore, for analysis of PAP samples *NOP56* II

primer was used with base pair 120bp to determine the promoter methylation. The amplicons of methylated and unmethylated are visualized using ethidium bromide staining followed by agarose gel electrophoresis. Amplicons of the expected size

produced from either primer pair are indicative of the presence of DNA in the original sample with the respective methylation status.

Table 6 Primer sequence of *GSTP1* for methylation and unmethylated genes

Gene	Sequence, 5'-3'	Product size, bp	Annealing temperature, ˚C	Number of cycles
GSTP1				
Methylated forward	GTCGTCGAGATCGCGTTATTG	231	56	27
Methylated reverse	GAAACTTACACACCCGCTTCAC			
Unmethylated forward	GTGAGTTGTTGAGATTGTGTTATTG	236	55	27
Unmethylated reverse	CAAAACTTACACACCCACTTCAC			

Table 7 Primer sequence of *NOP56-I* for methylation and unmethylated genes

Gene	Sequence, 5'-3'	Product size, bp	Annealing temperature, ˚C	Number of cycles
NOP56 primer I				
Methylated forward	TATTTTTTATTA TATTTTGGAATC	64	38	27
Methylated reverse	ATTAAATTATTT TAACCGTCG			
Unmethylated forward	GTATTTTTTATT ATATTTTGGAA TT	68	42	27
Unmethylated reverse	AATATTAAATT ATTTTAACCATC A			

Table 8 Primer sequence of *NOP56* II for methylation and unmethylated genes

Gene	Sequence, 5'-3'	Product size, bp	Annealing temperature, ˚C	Number of cycles
NOP56 primer II				
Methylated forward	CGTTTATTTTGATGTATTTACGAC	120	50	27
Methylated reverse	ATATCTACTTACGAATCAAAATCG			
Unmethylated forward	TGGAATTGTTTATTTTGATGTATTTATGATG	132	52	27
Unmethylated reverse	AATATTAAATTATTTTAACCATCA			

3.7 To prove methylation and gene expression in E7 transfected C33A cell line

3.7.1 Transfection of HPV 16 E7 and PC in C33A cell line

C33A cells was seeded into two 6-well plates and incubated for overnight. Next day, 2μg of E7 and PC (empty vector) plasmid was transfected in the C33A cells using TurboFectTM transfection reagent for overnight as explained in 3.5.1.3 step.

3.7.2 Extraction of DNA and RNA from the samples

After the 72 hrs of transfection, DNA and RNA were extracted separately from each of the samples to carried out gene expression and methylation by using the same protocol as explained in 3.5.1.4 and 3.6.2 step respectively. Further, the band intensities of each gene *CGB3*, *GSTP1* and *NOP56* in each sample of E7 and PC were measured and calculated by using same Storm840 and ImageJ software respectively. For the confirmation of results, three times the transfection was carried out to check the expression and methylation of each gene in the samples.

3.8 Analysis to observe whether methylation controls the gene expression

3.8.1 Treatment with 5-azacytidine

For evaluation of gene expression and methylation in cancer cell lines treated by 5-aza from Sigma-Aldrich; Merck KGaA. Firstly, the 10mg of 5-aza as a stock was prepared in 50ul of water and can be stored in -20˚C (wrapped with aluminum foil as this drug is very sensitive to light or any radiation). The SiHa and C33A cells were counted and seeded ($3x10^5$ cells/ml) in 6-well plate, prior to 1 day before of 5-aza treatment. The next day fresh DMEM media was prepared containing 5-aza with different concentration for SiHa and C33A. As for SiHa cells, the concentration of 5-aza was 0, 20, 30 and 40 μM whereas for C33A the concentration of 5-aza was 0, 3,5 and 7 μM added in both 6 well plate and labelled as SiHa with different concentration and C33A with different concentration number on the plate. The same amount of 5-aza concentration in fresh DMEM media was added for both SiHa and C33A consecutive 5 days after replacing with old media in every 24 h until the analysis was done (Chalertpet et al. 2015; Singh et al. 2022).

3.8.2 Observation of gene expression after treatment with 5-aza by real-time PCR

The over-expression of E7 in C33A cells were calculated by ΔΔCT method. ΔΔCT method is a study of the relative expression of genes in order to calculate the relative fold gene expression of samples while performing real-time polymerase chain reaction.

Table 9 Primer sequence of E7 and GAPDH for Real time PCR expression

Genes	Sequence, 5'-3'	Product size, bp
E7	Forward Primer GGGCAATTAAATGACAGCTCAG Reverse Primer GTGTGCTTTGTACGCACAACC	142 bp
GAPDH	Forward Primer GTGGGCAAGGTATCCTG Reverse Primer GATTCAGTGTGGTGGGGGAC	96 bp

The quantification based on the threshold cycle (Ct) obtained from the intersection between amplification curve and threshold lines of both the target gene (E7 gene) and reference gene (*GAPDH* gene), was used in the calculations according to the Livak and Schmittgen equations. (2001) in which the resulting gene expression is proportional to the fold change as follows:

Fold change = $2^{-\Delta\Delta CT}$

$= 2^{-(\Delta Ct(test) - \Delta Ct(reference))}$

$= 2^{-[(Ct(target,test)-Ct(ref,test))-(Ct(target,control)-Ct(ref,\ control))]}$

Where, Ct(target,test) = Ct of the E7 gene in C33A

E7 hybrid plasmid

Ct(ref,test) = Ct of the GAPDH gene

Ct (target, control) = Ct of the PC (empty plasmid) in C33A.

empty plasmid

Ct (ref, control) = Ct of the GAPDH gene exposed to the bare plasmid.

Table 10 Ingredients used to apply Real Time PCR to determine the gene expressions.

Chemical ingredients	Volume (µL)	Final concentration
1. 2X SYBR (Applied biosystems, USA)	5.0	1X
2. 20 uM target Forward primer	0.05	0.1 uM
20 uM target Reverse primer	0.05	0.1 uM
20 uM GAPDH (Ref) Forward primer	0.05	0.1 uM
20 uM GAPDH (Ref) Reverse primer	0.05	0.1 uM
3. Distilled water	3.8	
4. cDNA	1	
Total volume	10	

Table 11 Real Time-PCR condition to determine the gene expression

Step	Temperature (˚C)	Time
1. Initial denaturation	95	15min
2. PCR cycle (28 cycle) -Denature -Annealing -Extension	 95 56 72	 45 min 45 min 45 sec
3. Final extension	72	7min
4. Holding	4	

The expression of *NOP56* gene in transfected E7 and PC plasmid in C33A cells were analyzed by using the real time PCR with reference to E7 gene as explained above. GAPDH used as reference gene for this experiment. The ingredients amount and concentration were same as shown in Table 10. By using the specific expression primer of *NOP56* from Table 3 with band size 105 bp the real time PCR was performed. The expression of *CGB3* and *NOP56* genes in treated 5-aza were also analyzed by Real time PCR by using the same conditions of applied with reference to E7 overexpression and by using same primers as mentioned in Table 3 with band intensity of 194 for *CGB3* and 105 for *NOP56* gene.

3.8.3 Observation of methylation rate after treatment with 5-aza by MSP-PCR

DNA was extracted from the 5-aza treated samples by the same procedure as explained briefly in 3.6.2 step. Each sample was subjected to bisulfite treatment by using EZ DNA Methylation kit as explained in 3.6.3 step. Later the methylation status of each gene *CGB3*, *GSTP1* and *NOP56* were observed by MSP-PCR by using the specific methylated primer from Table 5, 6 and 8, respectively.

3.8.4 MTT assay in azacytidine-treated C33A and SiHa cells

Cells were seeded at 2x103 cells/well in a 96-well plate before azacytidine treatment. After 24 hrs, azacytidine was treated to C33a and SiHa every day for 5 days, follows the concentration of 0, 3, 5 and 7 µM and 0, 20, 30, 40 µM, respectively. MTT was measured by using a microplate reader (Thermo Fischer Scientific, MA, USA) at 570 nm as a primary wavelength and 630 nm as a reference wavelength.

3.8.5 Statistics for gene expression and methylation

To assess gene promoter methylation-expression levels and cells viability 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) were used to compare *CGB*3, *GSTP1* and *NOP56* expression and methylation in SiHa and C33A cells, respectively, as well as expression and methylation of *CGB3*, *GSTP1* and *NOP56* in the HPV 16 E7- and PC-transfected cells.

3.9 Detection of promoter methylation in clinical samples: Normal samples (with and without HPV), ASC-US and Cervical cancer.

This study is conducted to evaluate the performance of *CGB3* and *NOP56* genes methylation in Pap samples and types of HPV to distinguish between the normal and abnormal samples. The diagnostic ability was measured for both the genes with respect to 200 PAP samples.

3.9.1 Collection of clinical samples

The total of 200 PAP test samples were identified from the pathology archive of the Chulalongkorn Medical Hospital and from National Research Institute of Cancer (NCI), Thailand. The HPV typing had already been performed using Cobas®4800 HPV genotyping assay that targets 14 different types of HPV genotypes which included type 16 and 18 with specific primer of identification and "Other" type of HPV which consist of (12HR HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) (Heideman et al. 2011) mentioned as "Other HPV". All the samples were

approved by the ethical committee (IRB no. 477/61) of Faculty of Medicine, Chulalongkorn university.

3.9.2 Classification of 200 PAP samples based on cytology and histology

The 200 PAP samples were divided into two groups as “Determined” and “Undetermined” PAP samples. Based on cytology (microscopic observation) 78 samples were categorized as Determined PAP samples and remaining 122 samples were categorized as undetermined ASC-US samples were selected on the basis of histology which were performed and confirmed by the clinical pathologist. In the same way, the cytology-based samples included LSIL, HSIL, Cancer and Normal (with and without HPV) were confirmed by clinician pathologist. Therefore, the total PAP samples were classified as ASC-US (Undetermined) based on histology (n= 122) and remaining samples named as Determined PAP samples (n=78). Further, the 78 Determined PAP samples were categorized into cancer (20/78 samples), high grade squamous intraepithelial lesion - HSIL (24/78 samples), low grade squamous intraepithelial lesion – LSIL (12/78 samples) and normal (with HPV - 8/78 and without HPV – 14/78 samples).

Total 200 PAP samples classified as:

Determined samples (Cytology based, N= 78)	Undetermined “ASC-US” samples (Histology based, N=122)
Cancer (20/78 samples),	High grade squamous intraepithelial lesion - **HSIL** (15/122 samples)
High grade squamous intraepithelial lesion - **HSIL** (24/78 samples)	Low grade squamous intraepithelial lesion – **LSIL** (33/122 samples)
Low grade squamous intraepithelial lesion – **LSIL** (12/78 samples)	**Normal** (with HPV -66/122 and without HPV – 8/122 samples)
Normal (with HPV - 8/78 and without HPV – 14/78 samples)	

Figure 20 Classification of the total 200 PAP samples

3.9.3 Detection of genes methylation in 200 PAP samples

To evaluate the performance of *CGB3* and *NOP56* genes methylation in total number of PAP samples were performed by extracting the DNA from each sample of PAP screening by same methods as explained step 3.6.2 DNA extraction part. Subsequently, 500ng of each sample was subjected to bisulfite treatment by using EZ DNA Methylation-Gold kit as same as explained in step 3.6.3 Sodium bisulfite methods. The Eluted DNA of each of the samples obtained from the bisulfite kit was used to perform the detection of methylation using methylated and unmethylated specific primers of *CGB3* and *NOP56* from Table 5 and 8. Subsequently, 10µl of PCR product was loaded in 8% acrylamide gel and observed by gel electrophoresis. Then the gel was stained with SYBR reagent (Lonza Group, Ltd.) and were visualized using a Storm 840 (Amersham Biosciences) for the detection of methylated and unmethylated band intensities of each sample. The positive controls for methylation and unmethylation was contained in EpiTect DNA set control (Qiagen GmbH). The detection of promoter methylation of *CGB3* and *NOP56* were able to distinguish the PAP samples between normal and abnormal.

3.9.4 Statistics for detecting gene methylation in PAP samples

The categorical data sets of abnormal or pre-cancerous samples were compared with the normal samples, and methylation in the various HPV types was analyzed using GraphPad Prism software, version 5 (GraphPad Software, Inc.) χ^2 tests, using the two-tailed test, were used for statistical analysis, where $P \leq 0.05$ was considered to indicate a statistically significant value. The diagnostic abilities of the *CGB3* and *NOP56* genes in the PAP samples were measured, and receiver operating characteristic (ROC) curves were generated using Python programing language (version 3.8) and the Jupyter Notebook Integrated Development Environment (IDE)(Kluyver et al. 2016; McKinney 2010). The area under the curve (AUC) values were subsequently calculated (AUC values ≥0.5 were considered to be significant).

CHAPTER 4

RESULTS

4.1 Selection of genes

4.1.1 Selection of genes based on review of literature

The list of genes was selected from the study of Lee et al., 2004 which focused on genes retrieved from *MALDI-TOF-MS* shown downregulation by HPV 16 E7 in C33A cells lines, using the "ExPASY PEPTIDENT" search program from which the author picked up a list of down-regulated genes whose functions were related to tumor suppressor genes. The Primer was constructed using NCBI and Ensembl database as mentioned in Table 12 with sequence of genes, amplicon sizes and annealing temperatures.

Table 12 Selecting genes from review of literature

Genes	Sequence, 5'-3'	Product size, bp	Annealing temperature, ˚C
GSTP1	FW: CCTGGTGGACATGGTGAATGAC RW: ATGGCTCACACCTGTGTCCATC	252	57
PDI-A3	FW: ACGCTAAAGGTTCCAACTACTG RW: GAATCTCTCCAGCAGTGCTC	152	55
VAV1	FW: CAACCAACCCCTGATCCCAG RW: CTCTCAGGAGCTGGTGAAACAC	275	57
IL4R alpha	FW: GCGTTTTATTCCGAGACCAA RW: GCCCAGTGACCTTTGAAGAG	238	58

ING1	FW: GACTCCATCGAGTCCCTGC RW: GCCAGGTCTGCAGAGAACGA	261	56

4.1.2 Selection of genes based on bioinformatics

Based on the bioinformatics study of Na Rangsee et al., 2020 as discussed in the introduction section where E7 formed a complex with some transcription factors including SP1 which binds to YY1 as shown in Fig 16. With reference to the above study, *CCNA1* was used as gene model in this research as it shared the same promoter sequence for binding of YY1 gene. Based on this, the author picked those genes which predicted the binding sequence of YY1 which were similar to *CCNA1*. The promoter sequence of *CCNA1* (Fig. 21), *CGB3* (Fig. 22) and *NOP56* (Fig. 23) were retrieved from Eukaryotic promoter database (EPD) from *https://epd.epfl.ch//index.php* as follow:

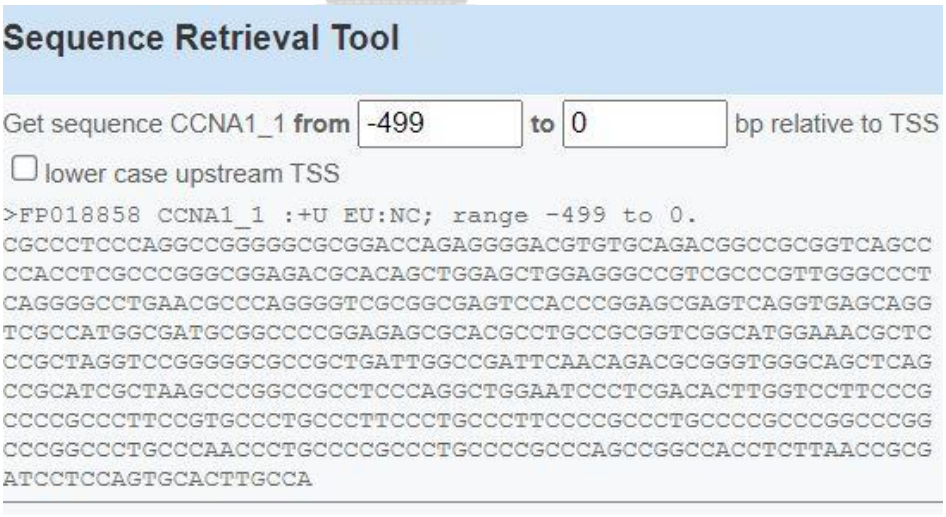


Figure 21 Sequence of *CCNA1* promoter from EPD database. Source: *https://epd.epfl.ch//index.php*

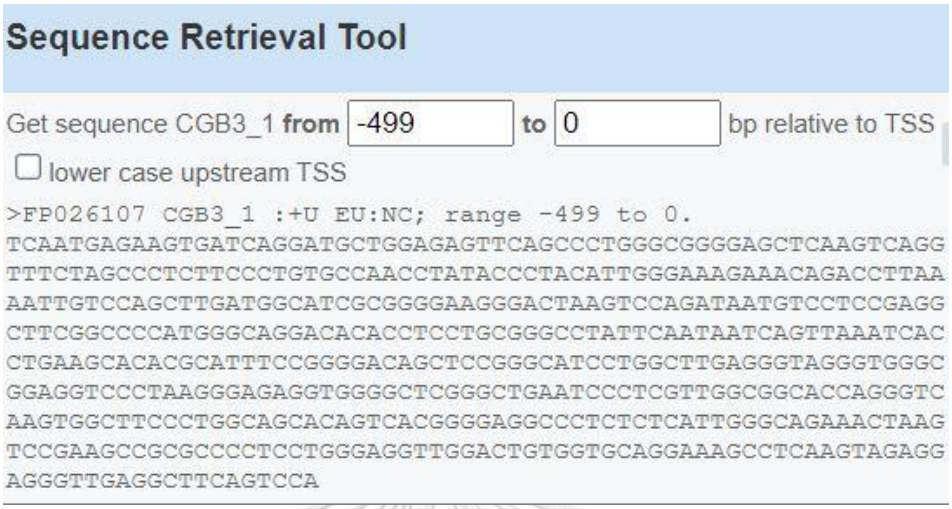


Figure 22 Sequence of *CGB3* promoter from EPD database. Source: *https://epd.epfl.ch/cgi-bin/get_doc?db=hgEpdNew&format=genome&entry=CGB3_1*

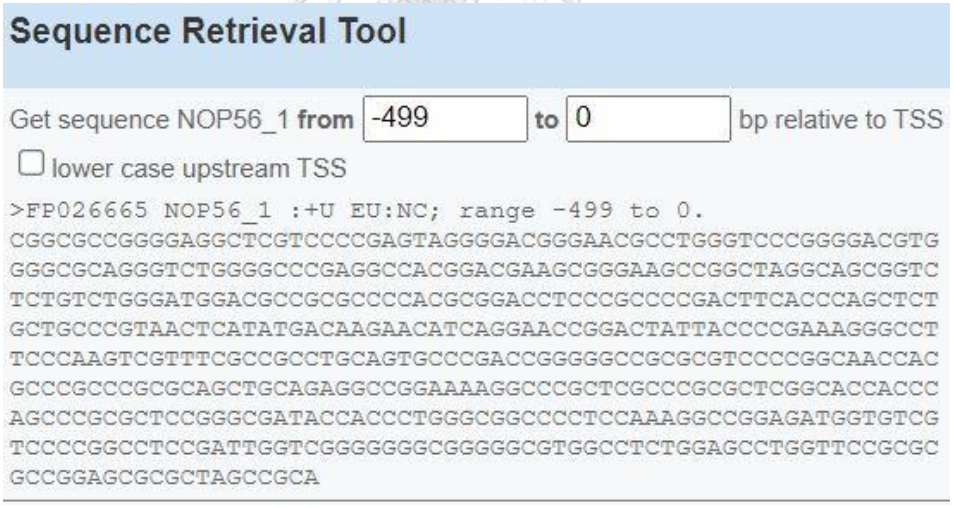


Figure 23 Sequence of *NOP56* promoter from EPD database. Source: (*https://epd.epfl.ch//index.php*)

In addition to the above findings, PROMO version 8.3 from the TRANSFAC database (alggen.lsi.upc.es), were also used to select the genes of interest containing YY1 binding sites similar to *CCNA1*. Similarly, other genes which shared similar

sequences as that of *CCNA1* were analyzed. On the basis of above-mentioned gene selection processes - *CGB3 and NOP56* genes were selected as shown in Fig 24.

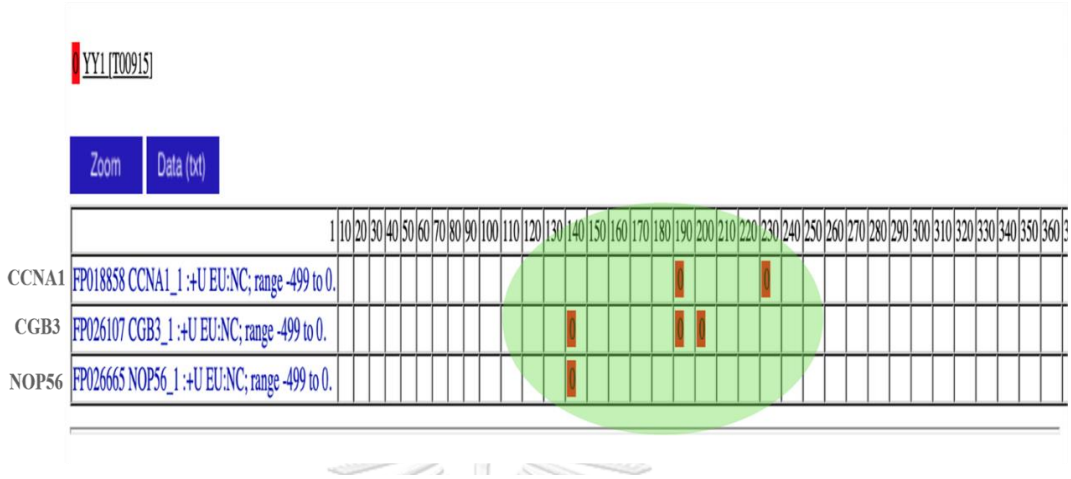


Figure 24 Binding prediction of YY1. In the promoter region of *CCNA1*, *CGB3* and *NOP56* genes.

CCNA1 gene predicted three sequences as follows:

- GCCATG (183-188), GCCATG (185-190) and TCCATG (228-233) in the promoter region where YY1 can bind as shown in Fig. 25A. These sequences were highlighted as GCCATG (+) with blue highlighter, GCCATG (-) with red highlighter and TCCATG (-) with green highlighter (Fig 25B).

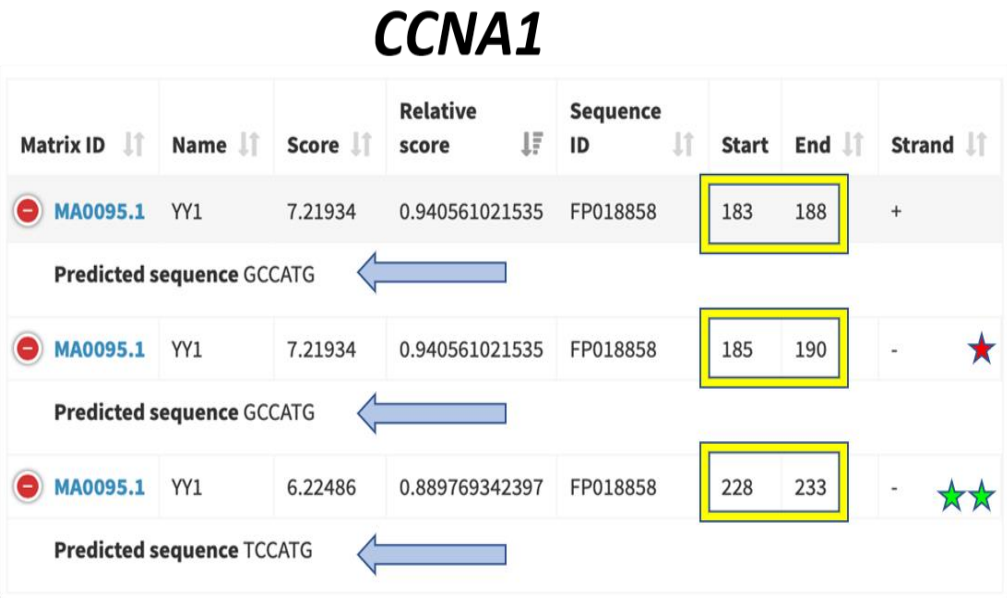
Similarly, YY1 also predicted 3 sequences in *CGB3* and NOP56 as follows as shown in Fig. 26A and 27A, respectively:

- *CGB3*: GCCATC (134-139), CCCATG (188-193), CCCATG (190-195)
- *NOP56*: ACCATC (411-416), TCCATC (130-135), GGAGATGGTGTC (408-419)

From the analysis 80% similarity was found between the sequence GCCATG (-) from *CCNA1* and *CGB3* as showed in Fig. 25A and 26A, respectively. Hence this sequence GCCATG (-) was highlighted in the promoter region of *CGB3* as shown in Fig. 26B. Similarly, for *CCNA1* and *NOP56* - 80% similarity was found between the sequence

TCCATG (-) as showed in Fig 25A and 27A, respectively. As all the predicting sequences were highlighted the promoted region of *NOP56* as showed in Fig 27B.

A



B

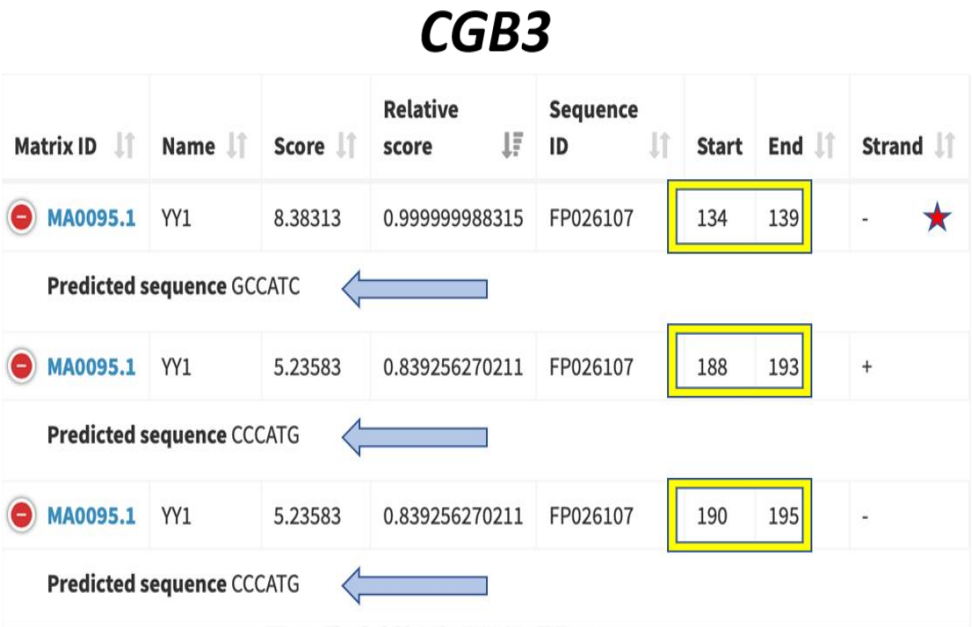
SEQUENCE PRIDICTED: GCCATG (+, GCCATG (-), TCCATG (-)

CCNA1 PROMOTER SEQUENCE

CGCCCTCCCAGGCCGGGGGCGCGGACCAGAGGGGACGTGTGCAGACGGCCGCGGTCAGCC
CCACCTCGCCCGGGCGGAGACGCACAGCTGGAGCTGGAGGGCCGTCGCCCGTTGGGCCCT
CAGGGGCCTGAACGCCCAGGGGTCGCGGCGAGTCCACCCGGAGCGAGTCAGGTGAGCAGG
TCGCCATGGCGATGCGGCCCCGGAGAGCGCACGCCTGCCGCGGTCGGCATGGAAACGCTC
CCGCTAGGTCCGGGGGCGCCGCTGATTGGCCGATTCAACAGACGCGGGTGGGCAGCTCAG
CCGCATCGCTAAGCCCGGCCGCCTCCCAGGCTGGAATCCCTCGACACTTGGTCCTTCCCG
CCCCGCCCTTCCGTGCCCTGCCCTTCCCTGCCCTTCCCCGCCCTGCCCCGCCCGGCCCGG
CCCGGCCCTGCCCAACCCTGCCCCGCCCTGCCCCGCCCAGCCGGCCACCTCTTAACCGCG
ATCCTCCAGTGCACTTGCCA

Figure 25 YY1 binding site in promoter region of *CCNA1* represents region from 0-499. (A) Predicted three sequence GCCATG (+) highlighted with blue color, GCCATG (-) highlighted with green color and TCCATG (-) highlighted with red color. (B) *CCNA1* promoter sequence represent all the predicted three sequences in the region.

A



B

SEQUENCE PRIDICTED: GCCATC (-), CCCATG (+), CCCATG (-)

CGB3 PROMOTER SEQUENCE

TCAATGAGAAGTGATCAGGATGCTGGAGAGTTCAGCCCTGGGCGGGGAGCTCAAGTCAGG
TTTCTAGCCCTCTTCCCTGTGCCAACCTATACCCTACATTGGGAAAGAAACAGACCTTAA
AATTGTCCAGCTTGATGGCATCGCGGGGAAGGGACTAAGTCCAGATAATGTCCTCCGAGG
CTTCGGCCCCATGGGCAGGACACACCTCCTGCGGGCCTATTCAATAATCAGTTAAATCAC
CTGAAGCACACGCATTTCCGGGGACAGCTCCGGGCATCCTGGCTTGAGGGTAGGGTGGGC
GGAGGTCCCTAAGGGAGAGGTGGGGCTCGGGCTGAATCCCTCGTTGGCGGCACCAGGGTC
AAGTGGCTTCCCTGGCAGCACAGTCACGGGGAGGCCCTCTCTCATTGGGCAGAAACTAAG
TCCGAAGCCGCGCCCCTCCTGGGAGGTTGGACTGTGGTGCAGGAAAGCCTCAAGTAGAGG
AGGGTTGAGGCTTCAGTCCA

Figure 26 YY1 binding site in promoter region of *CGB3* represents region from 0-499. (A) Predicted three sequence GCCATC (-) highlighted with red color, CCCATG (+) highlighted with blue color and CCCATG (-) highlighted with blue color. (B) *CGB3* promoter sequence highlighted all the three predicted sequence included GCCATC same as *CCNA1* (highlighted with red color).

A

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 GGAGATGGTGTC							

B

SEQUENCE PRIDICTED: ACCATC (-), TCCATC (-), GGAGATGGTGTC (+)

NOP56 PROMOTER SEQUENCE

CGGCGCCGGGGAGGCTCGTCCCCGAGTAGGGGACGGGAACGCCTGGGTCCCGGGGACGTG
GGGCGCAGGGTCTGGGGCCCGAGGCCACGGACGAAGCGGGAAGCCGGCTAGGCAGCGGTC
TCTGTCTGGGATGGACGCCGCGCCCCACGCGGACCTCCCGCCCCGACTTCACCCAGCTCT
GCTGCCCGTAACTCATATGACAAGAACATCAGGAACCGGACTATTACCCCGAAAGGGCCT
TCCCAAGTCGTTTCGCCGCCTGCAGTGCCCGACCGGGGGCCGCGCGTCCCCGGCAACCAC
GCCCGCCCGCGCAGCTGCAGAGGCCGGAAAAGGCCCGCTCGCCCGCGCTCGGCACCACCC
AGCCCGCGCTCCGGGCGATACCACCCTGGGCGGCCCCTCCAAAGGCCGGAGATGGTGTCG
TCCCCGGCCTCCGATTGGTCGGGGGGGCGGGGGCGTGGCCTCTGGAGCCTGGTTCCGCGC
GCCGGAGCGCGCTAGCCGCA

Figure 27 YY1 binding site in promoter region of *NOP56* represents region from 0-499. (A) Predicted three sequence ACCATC (-) highlighted with green color, TCCATC (-) highlighted with blue color and GGAGATGGTGTC (+) highlighted with blue color. (B) *NOP56* promoter sequence highlighted all the three predicted sequences included TCCATC (highlighted with green color) as same as *CCNA1*.

The selected genes *CGB3* and *NOP56* along with other genes selected from literature review (*PDI-A3*, *GSTP1*, *VAV1*, *ING1* and *IL4R alpha*) were further assessed for gene expressions in cervical cell lines SiHa and C33A, to find out whether HPV 16 - E7 can induce promoter methylation in the above-mentioned genes similar to *CCNA1*.

4.2 Gene expression and methylation of *CGB3*, *GSTP1* and *NOP56* measured in cervical cell lines

4.2.1 Detection of HPV16-E6 and HPV16-E7 expression in SiHa and C33A cell lines

The expression of HPV genes E6 and E7 were analyzed by RT-PCR in cervical cell lines using specific primer as mentioned in Table 3 with the base pair size of 243bp and 142bp respectively and C33A cell line used as control. From the results, we observed that there was expression of HPV16-E6 (Fig 28A) and the HPV16-E7 (Fig 28B) in SiHa cell line but no observations of expressions were made in C33A cell line, which indicated that SiHa had HPV infection.

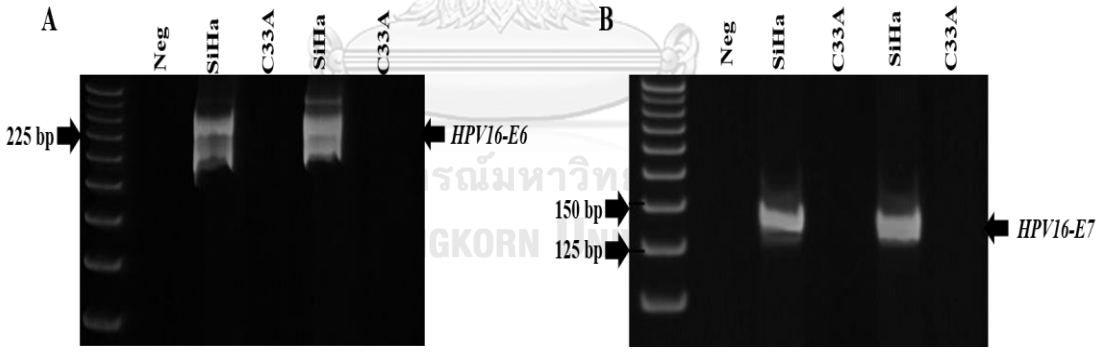


Figure 28 Expression of HPV gene E6 and E7 in SiHa and C33A cells. In figure A, where Neg- Double distilled water used as negative control, SiHa - Positive cervical cell line showed presence of HPV 16-E6 band with 243bp, C33A- Negative cervical cell line. In figure B, where Neg-Double distilled water used as negative control, SiHa- Positive cervical cell line showed presence of HPV 16-E7 band with 142bp, C33A- Negative cervical cell line.

4.2.2 Comparison of expression and methylation of CGB3 and NOP56 genes in SiHa and C33A cells

To examine the level of expression of *CGB3* gene, RT-PCR was performed in SiHa and C33A cell lines by using specific primer as mentioned in Table 3 with base pair size of 194bp. From the results, we observed that *CGB3* expression in SiHa had lower band intensity (26.74%) as compared to C33A (73.26%) as shown in Fig. 29A, with statistically significant difference level (*P*=0.001***) performed by using unpaired t-test with three replicates. The *GAPDH* was used as a reference gene with band intensity 50.3% and 49.7% for SiHa and C33A respectively with band size of 96bp. In contrast, the methylation status of *CGB3* was measured by MSP-PCR using specific primer mentioned in Table 5. The methylation band intensity of *CGB3* in SiHa cells (61.18%) and was found to be higher compared to C33A cells (38.81%) as shown in Fig. 29B with statistically significant difference level (*P*=0.004**) performed by using unpaired t-test.

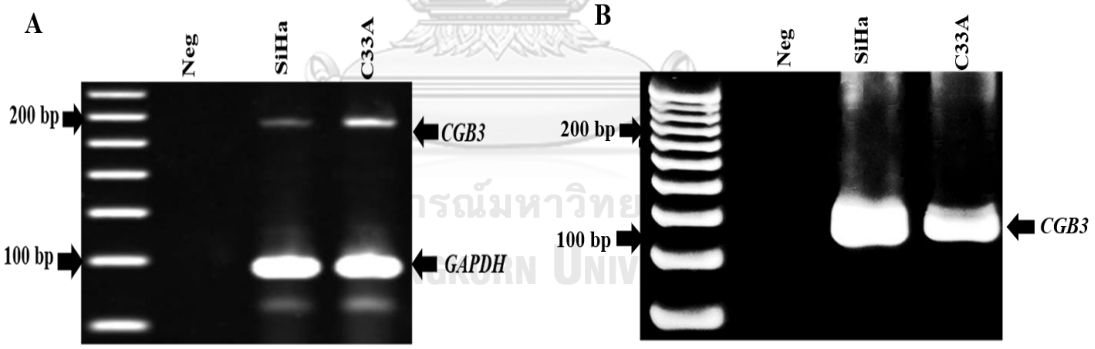


Figure 29 Observation of *CGB3* in SiHa and C33A cell lines. Figure A represents *CGB3* expression, where Neg- Double distilled water used as negative control, SiHa- Positive cervical cell line showed low expression of *CGB3* with 194bp, C33A- Negative cervical cell line showed high expression of *CGB3* with 194bp, *GAPDH* - Used as internal control with 96bp. Figure B represents *CGB3* methylation, where Neg- Double distilled water used as negative control, SiHa- Positive cervical cell line showed high band intensity of *CGB3* methylation with 116bp, C33A- Negative cervical cell line showed low band intensity of *CGB3* methylation with 116bp.

Similarly, the level of expression of *NOP56* gene was analyzed using RT-PCR in SiHa and C33A cell lines by using specific primer as mentioned in Table 3 with base pair size of 105bp. From the results, we observed that *NOP56* expression in SiHa had lower band intensity (38.42%) as compared to C33A (44.5%) as shown in Fig. 30A, with statistically significant difference level (*P*=0.004**) performed by using unpaired t-test with three replicates. The *GAPDH I* was used as a reference gene with band intensity 46.6% and 49.8% for SiHa and C33A respectively with band size of 163bp. In contrast, the methylation status of *NOP56* was measured by MSP-PCR using specific primer from Table7. The methylation band intensity of *NOP56* in SiHa cells (53.94%) and was found to be higher compared to C33A cells (46.05%) as shown in Fig. 30B with statistically significant difference level (*P*=0.045*) performed by using unpaired t-test.

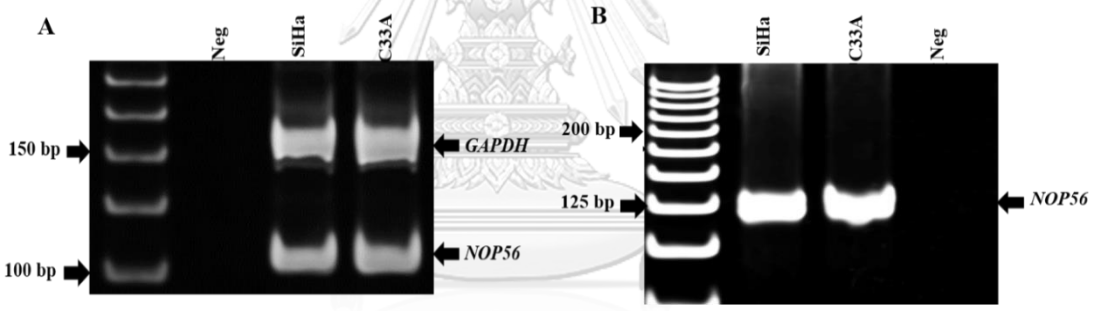


Figure 30 Observation of *NOP56* in SiHa and C33A cell lines. Figure A represents *NOP56* expression, where Neg- Double distilled water used as negative control. SiHa- Positive cervical cell line showed low expression of *NOP56* with 105bp, C33A- Negative cervical cell line showed high expression of *NOP56* with 105bp, *GAPDH I*- Used as internal control with 163bp. Figure B represents *NOP56* II methylation, where Neg- Double distilled water used as negative control, SiHa- Positive cervical cell line showed high band intensity of *NOP56* II methylation with 120bp, C33A- Negative cervical cell line showed low band intensity of *NOP56* II methylation with 120bp.

Furthermore, expression of *GSTP1, PDI-A3, IL4R-alpha, ING1 and VAV1* genes were analyzed in SiHa and C33A cell lines by using RT-PCR with specific primer as mentioned in Table 12. *GSTP1* showed lower band intensity in SiHa (29.7%) compared to C33A (53.4%) as shown in Fig. 31, with statistically significant difference level (P = 0.002**) performed by using unpaired t-test with three replicates. The *GAPDH* was used as a reference gene with band intensity of 49.8% and 50.14% in SiHa and C33A cell lines respectively with base pair size 96 bp. Further, the *GSTP1* gene was directly used in transfected E7 and PC cells to examine the expression and methylation status of *GSTP1*. Hence, there was no data to show *GSTP1* methylation in SiHa or C33A cell lines.

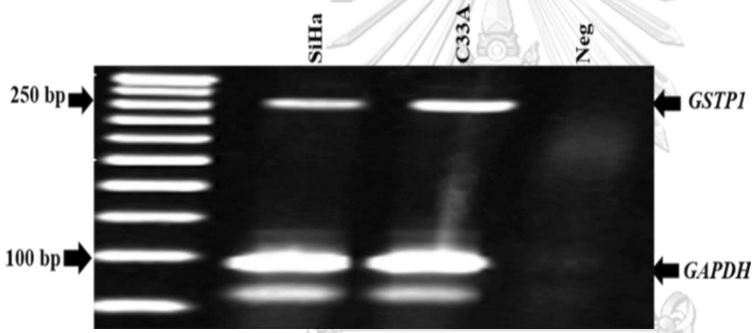


Figure 31 Expression of *GSTP1* in SiHa and C33A cell lines, where SiHa- Positive cervical cell line showed low expression of *GSTP1* with 252bp, C33A- Negative cervical cell line showed high expression of *GSTP1* with 252bp, Neg- Double distilled water used as negative control, *GAPDH* - Used as internal control with 96bp as represented in figure.

The expression level of *PDI-A3* and *ING1* genes in SiHa and C33A cell lines were found to be very close. The expression levels for *PDI-A3* and *ING1* ins SiHa cell lines were 51.6% and 50.71% respectively as compared to 48.3% and 49.29% in C33A cell lines with product size 152 bp and 261bp (Fig 32A and 32B) respectively. The *GAPDH* was used as reference gene for both *PDIA-3* and *ING1* genes, which showed band intensity 50.3% and 49.39% in SiHa, and 49.1% and 50.61% in C33A

cell lines respectively with base size 96. Thus, the results indicated that there was no significant difference in expression of genes between SiHa and C33A cell lines.

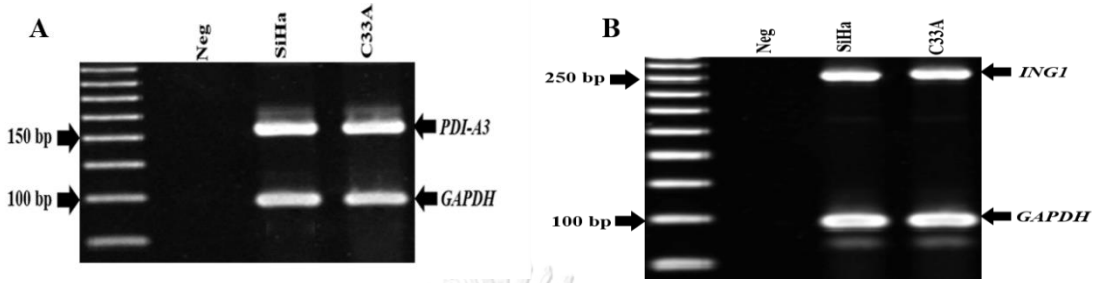


Figure 32 Expression of *PDI-A3 and ING1* in SiHa and C33A cell lines. Figure A represents expression of *PDI-A3,* where Neg- Double distilled water used as negative control, SiHa- Positive cervical cell line showed low expression of *PDI-A3* with 152bp, C33A- Negative cervical cell line showed high expression of *PDI-A3* with 152bp, *GAPDH*- Used as internal control with 96bp. Figure B represents the expression of *ING1* gene, where Neg- Double distilled water used as negative control, SiHa- Positive cervical cell line showed low expression of *ING1* with 261bp, C33A- Negative cervical cell line showed high expression of *ING1* with 261bp, *GAPDH*- Used as internal control with 96bp.

The expression level of *IL4R-alpha* and *VAV1* genes in SiHa and C33A cell lines were found to be low band intensity in C33A. The expression levels for *IL4R alpha* and *VAV1* in SiHa cell lines were 61.51% and 52.61% respectively as compared to 38.49% and 47.39% in C33A cell lines with product size 238 bp and 275 bp (Fig 33A and 33B) respectively. The *GAPDH* was used as reference gene for both *PDIA-3* and *ING1* genes, which showed band intensity 50.1% and 52.8% in SiHa, and 47.39% and 48.17% in C33A cell lines respectively with base size 96. Due to lower expression in C33A cell lines, *IL4R-alpha* and *VAV1* genes were not considered for further experiments as there was no effect observed by the presence of HPV in SiHa. which indicated that HPV16-E7 does not affect or induce promoter methylation in *IL4R-alpha* or *VAV1* genes. Similarly, the remaining genes selected from review of literature such as *PDI-A3* and *ING1* did not show any differences in SiHa and C33A.

The gene expression sequences for each of the genes retrieved from review of literature are mentioned in Table 12.

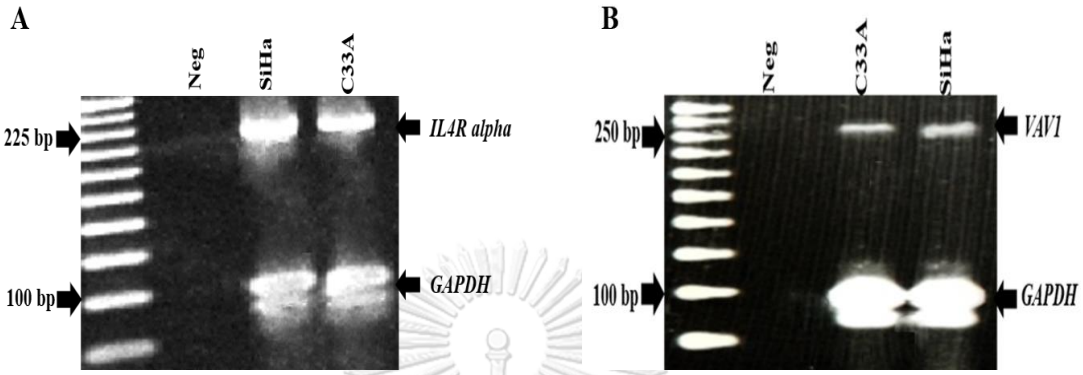


Figure 33 Expression of *IL4R alpha* and *VAV1* in SiHa and C33A cell lines. The figure A represents the expression of *IL4R alpha* in SiHa and C33A cell lines, where Neg- Double distilled water used as negative control, SiHa- Positive cervical cell line showed low expression of *IL4R alpha* with 238bp, C33A- Negative cervical cell line showed high expression of *IL4R alpha* with 238bp, *GAPDH*- Used as internal control with 96bp. The Figure B represents the expression of *VAV1* in SiHa and C33A cell lines, where Neg- Double distilled water used as negative control, SiHa- Positive cervical cell line showed low expression of *VAV1* with 275bp, C33A- Negative cervical cell line showed high expression of *VAV1*with 275bp, *GAPDH*- Used as internal control with 96bp.

From the above findings, the expression of *CGB3*, *GSTP1* and *NOP56* were observed in cervical cell lines demonstrating the presence of HPV in positive cancer cell line “SiHa” which might have been responsible for the effect of expression and methylation in genes. Therefore, we further analyze the expression and methylation of *CGB3*, *GSTP1* and *NOP56* genes in HPV 16- E7 by transfecting HPV-16 E7 plasmid into C33A (HPV-negative) cancer cell lines.

4.3 Gene expressions and methylation of *CGB3*, *GSTP1* and *NOP56* in transfected HPV 16 E7 and PC

4.3.1 Over expression E7 gene in C33A cells

Over the period of 72hrs of transfection, the transfected HPV-16 E7 were subjected to check the expression by qPCR by using *GAPDH* as a reference gene. From the results it was found that the expression of HPV-16 E7 was significantly high and overexpressed in C33A cell lines as compared to PC (empty plasmid) transfected cells with statistically significant difference level (*P*=0.01**) using t-test with three replicates. The graphs represent mean ± standard error for overexpression E7 (1420±140) and control PC (1±0.1) as shown in Fig. 34.

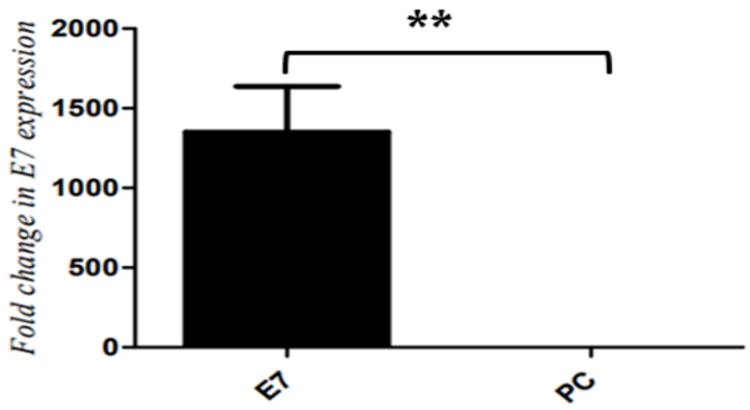


Figure 34 HPV 16 E7 recombinant plasmid and PC were overexpressed in C33A cells. The graph represents the fold change of HPV 16 E7 expression at the mRNA level. Neg, distilled water negative control. The expression of HPV16-E7 was measured by qPCR.

4.3.2 HPV-16 E7 induces promoter methylation and decrease expression of genes

The gene expression and promoter methylation for each of the genes were evaluated in HPV16 - E7 transfected C33A cell lines and PC. The gene expression levels for *CGB3* were measured by RT-PCR and the band intensity was found to be lower (37.2%) in HPV16 - E7 transfected cells as compared to PC transfected cells

(62.7%) as mentioned in (Fig. 35A). *GAPDH* was used as internal control with band intensity of 49.4% and 50.5% in SiHa and C33A cell lines respectively, with product size of 96bp. The statistically significant value (*P*=0.02*) was calculated by using unpaired t-test. The bar graph shows the mean ± standard error of *CGB3* expression for E7 (32.1±5.1) and for PC (61.9±3.2), respectively with three replicates (Fig. 35B).

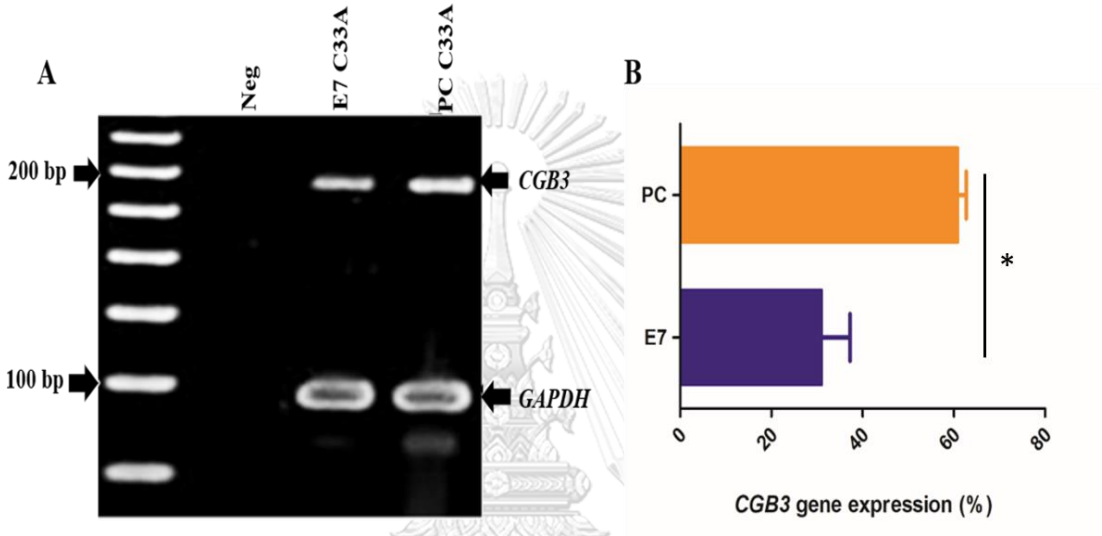


Figure 35 *CGB3* expression compared between transfected E7 and PC. Figure A represents the expression of *CGB3* in transfected E7 in C33A cell lines, where Neg- Double distilled water used as negative control, E7 C33A- Transfected E7 in C33A represents low expression of *CGB3* gene with 194bp, PC C33A- Transfected PC in C33A represents high expression of *CGB3* gene with 194bp, *GAPDH* - Used as internal control with 96bp. Figure B, data are presented as the mean ± the standard error of the mean of three repeats. The expression of *CGB3* was measured by RT-PCR.

Similarly, *GSTP1* expression was evaluated in transfected HPV 16 E7 and PC using RT-PCR. From result we observed that *GSTP1* gene had lower band intensity in HPV 16 E7 transfected cells with band intensity 31.5% as compared to PC transfected with 69.1% band intensity as shown in Fig. 36A, with significant value

(*P*=0.004**) performed by unpaired t-test. *GAPDH* used as internal control with band intensity 50.3% and 49.6% in SiHa and C33A, respectively with product size 96bp. The bar graph shows the mean ± standard error of *GSTP1* expression for E7 (32.5±0.75) and PC (71.5±2.4) with three replicates Fig. 36B.

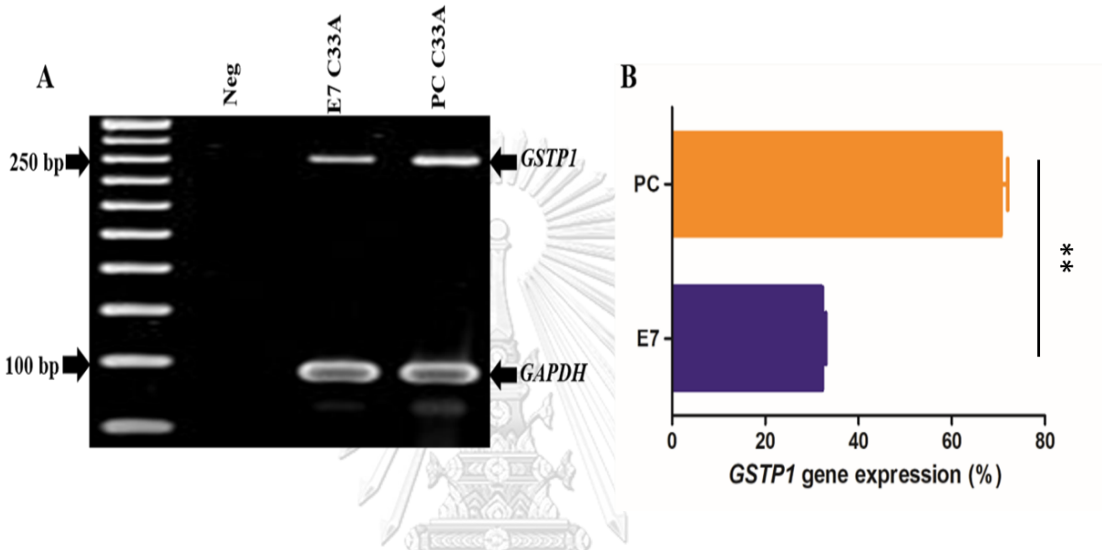


Figure 36 *GSTP1* expression compared between transfected E7 and PC. Figure A represents the expression of *GSTP1* in transfected E7 in C33A cell lines, where Neg- Double distilled water used as negative control, E7 C33A- Transfected E7 in C33A represents low expression of *GSTP1* gene with 252bp, PC C33A- Transfected PC in C33A represents high expression of *GSTP1* gene with 252bp, *GAPDH* - Used as internal control with 96bp. Figure B, data are presented as the mean ± the standard error of the mean of three repeats. The expression of *GSTP1* was measured by RT-PCR.

The expression level of *NOP56* gene was measured by qPCR in HPV 16 - E7 transfected C33A cell line. The results showed that the expression of *NOP56* were significantly decreased with *P* value (*P*<0.0001***) compared with that of PC transfected cells, performed using unpaired t-test (Fig. 37). *GAPDH* was used as an internal control. Graph represent the mean ± the standard error of *NOP56* expression for E7 (12.04±1.29) and for PC (98.7±1.1) the mean of three repeats. This experiment

was conducted by (Sukbhattee, 2019) using qPCR and the final result are shown as (Fig. 37). Therefore, the gel picture is not available to the author.

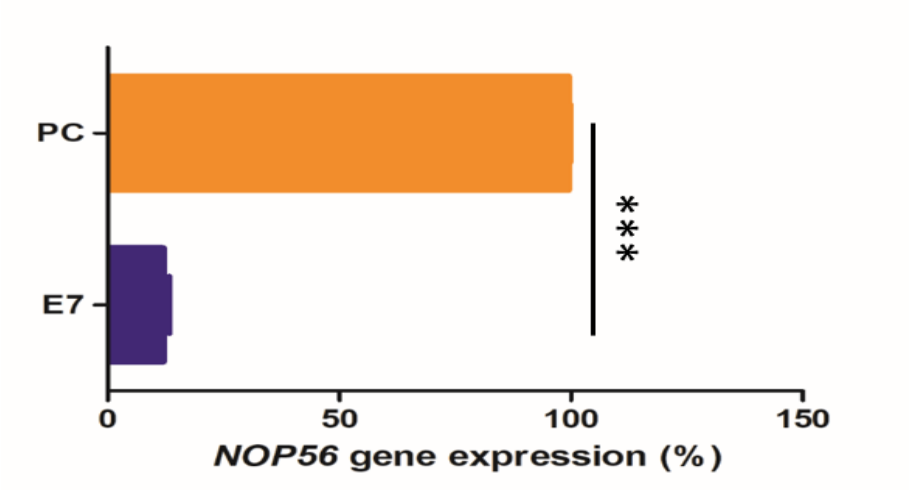


Figure 37 Expression of NOP56 at mRNA level in E7 recombinant plasmid and PC transfected in C33A cells. Data are presented as the mean ± the standard error of three repeats.

4.3.4 Promoter methylation of genes in transfected HPV-16 E7 and PC

The promoter methylation of *CGB3*, *GSTP1 and NOP56* were observed in overexpressed HPV-16 E7. According to the results, there was an increase in the band intensity for *CGB3* methylation in E7 (54.21%) as compared to PC (39.36%) with significant value (*P*=0.005**) using unpaired t test by three replicates as mentioned in Fig. 38A. The methylation positive control (M+) displayed band intensity of 6.43%. In contrast, the band intensity of unmethylated *CGB3* in HPV-16 E7 had lower band intensity (42.2%) as compared to PC (61.01%) with unmethylated control (UM+) had band intensity of 3.6% as shown in Fig. 38B.

The methylation of *GSTP1* gene band intensity was increased in E7 with 55.01%, as compared to PC 32.5%, with significant value (*P*=0.004**) using unpaired t test with three replicates as mentioned in Fig. 39A. The methylation

positive control (M+) displayed band intensities of 8.5%. In contrast, the band intensity of *GSTP1* unmethylation in HPV-16 E7 had lower band intensity 43.04% as compared to PC 52.69%, with unmethylation control (UM+) band intensity 4.17% as shown in Fig. 39B.

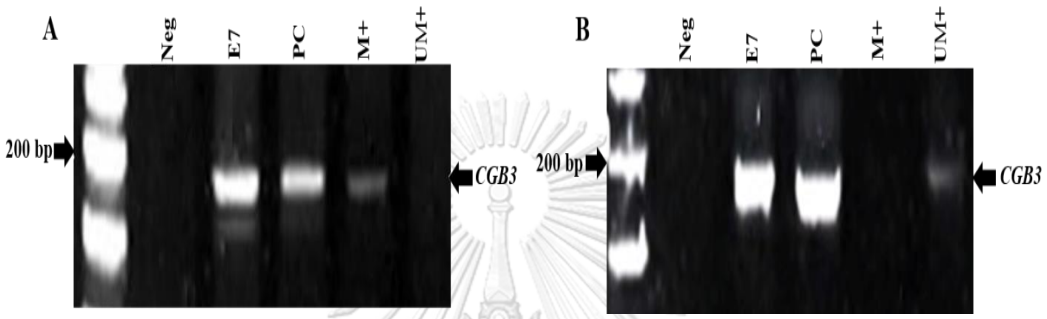


Figure 38 *CGB3* methylation and unmethylation status in transfected E7 and PC. Figure A represents *CGB3* methylation in transfected E7 and PC, where Neg- Double distilled water used as negative control, E7- Transfected E7 showed high band intensity of *CGB3* Meth with 116bp, PC- Transfected PC (empty plasmid) showed low band intensity of *CGB3* Meth with 116bp, M+- Methylation control, UM+ - Unmethylation control. Figure B represents *CGB3* unmethylation in transfected E7 and PC, where Neg- Double distilled water used as negative control, E7- Transfected E7 showed low band intensity of *CGB3* Unmeth with 127bp, PC- Transfected PC (empty plasmid) showed high band intensity of *CGB3* Unmeth with 127bp, M+- Methylation control, UM+ - Unmethylation control.

Similarly, *NOP56* I methylation band intensity was increased in E7 (59.01%) as compared to PC (41.54%) with significant value (*P*=0.003**) using unpaired t-test with three replicates as mentioned in Fig. 40A. The methylation positive control displayed band intensity of 5.3%. In contrast, the band intensity of *NOP56* I unmethylated in HPV-16 E7 was so low that it was unmeasurable for comparison between E7 and PC (Fig. 40B).

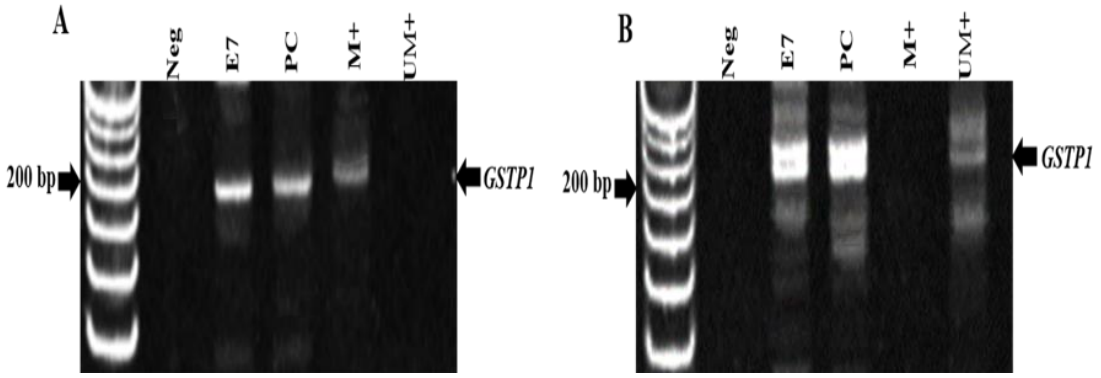


Figure 39 *GSTP1* methylation and unmethylation status in transfected E7 and PC. Figure A represents *GSTP1* methylation in transfected E7 and PC, where Neg- Double distilled water used as negative control, E7- Transfected E7 showed high band intensity of *GSTP1* Meth with 231bp, PC- Transfected PC (empty plasmid) showed low band intensity of *GSTP1* Meth with 231bp M+- Methylation control, UM+ - Unmethylation control. Figure B represents *GSTP1* unmethylation in transfected E7 and PC, where Neg- Double distilled water used as negative control, E7- Transfected E7 showed low band intensity of *GSTP1*Unmeth with 236bp, PC- Transfected PC (empty plasmid) showed high band intensity of *GSTP1* Unmeth with 236bp, M+- Methylation control, UM+ - Unmethylation control.

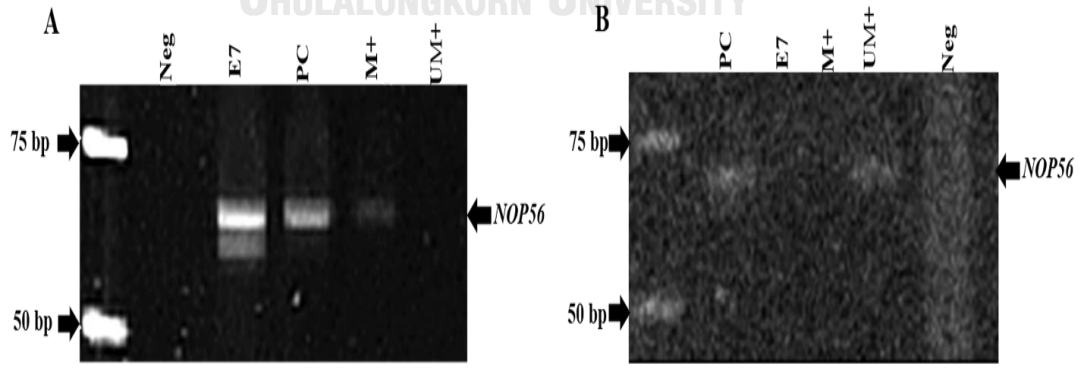


Figure 40 *NOP56* I methylation and unmethylation status in transfected E7 and PC. Figure A represents *NOP56* I methylation in transfected E7 and PC, where Neg- Double distilled water used as negative control, E7- Transfected E7 showed high band intensity of *NOP56* I Meth with 64bp, PC-Transfected PC (empty plasmid) showed

4.4 Gene expression and methylation of *CGB3*, *NOP56* and *GSTP1* in treated cell lines by 5-Aza drug

4.4.1 Effect of 5-Aza on gene methylation of *CGB3*, *NOP56* and *GSTP1*

The relationship of promoter methylation and the mRNA expression of genes was tested in C33A (HPV- negative) and SiHa (HPV-postive) cell lines by administering 5-Aza. Re-expression of *CGB3*, *GSTP1* and *NOP56* were analyzed in C33A with concentration of 0, 3, 5, 7 µM and SiHa with 0, 20, 30, 40 µM respectively (Chalertpet et al. 2015). The promoter methylation level of *CGB3* in C33A and SiHa cell lines were analyzed by MSP-PCR. The results showed that the methylation level in both cell lines C33A and SiHa were gradually decreased as the 5-aza concentration increased from 0-40 µM and 0-7 µM, respectively. By using one-way ANOVA, the *p* value showed significant difference in both cell lines of C33A (*P*<0.0001****) and SiHa (*P*<0.0001****). The graph represents the mean ± standard error for *CGB3* in C33A at (0µM- 39.15±0.3; 3µM- 24.67 ± 0.9; 5µM- 19.19 ± 0.41; 7µM- 15.25 ±1.3) as shown in Fig 41A and for *CGB3* in SiHa at (0µM- 21.7±2.7; 20µM- 6.4 ± 1.4; 30µM- 4.5 ± 0.5; 40µM- 3.7 ±0.05) as shown in Fig. 41B.

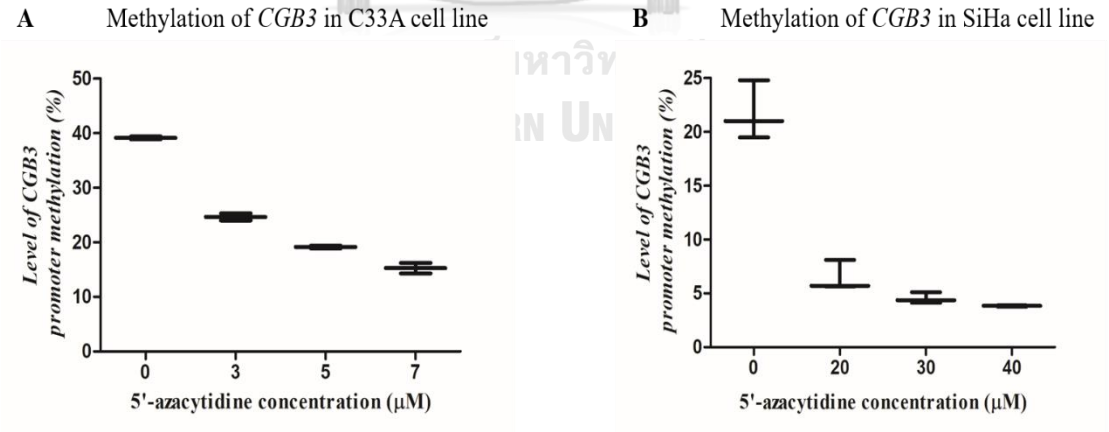


Figure 41 Changes in promoter methylation following after 5-azacytidine treatment by MSP-PCR. (A) *CGB3* methylation in C33A cells, (B) *CGB3* methylation in SiHa cells. Data are presented as the mean ± the standard error of the mean with three repeats.

Similarly, methylation level of *NOP56* in C33A and SiHa cells were analyzed by MSP-PCR. The result showed that the methylation level in both cell lines C33A and SiHa were gradually decreased as the 5-aza concentration increased from 0-40 µM and 0-7 µM, respectively. By using one-way ANOVA, the *p* value showed significantly different in both cell lines C33A (*P*=0.0002***) and SiHa (*P*=0.0002***). The graph represents the mean ± standard error for *NOP56* in C33A at (0µM- 45.9±1.8; 3µM- 22.9± 2.6; 5µM- 18.11±0.007; 7µM- 12.36±1.6) as shown in Fig 42A and for *NOP56* in SiHa at (0µM- 66.8±6.3; 20µM- 17.78±0.6; 30µM- 10.12±1.2; 40µM- 9.2±1.8) as shown in Fig. 42B.

Furthermore, the promoter methylation level of *GSTP1* were also measured in C33A and SiHa cell lines using MSP-PCR after 5-Aza treatment. The result showed that the methylation level in both the cell lines C33A (0 to7 µM) and SiHa (0 to 40 µM) were non-significant as there were no evident increasing or decreasing patterns for methylation level as the 5 aza concentration were altered. The methylation level at 7 µM and 40 µM were high as compared to control 0 µM. The graph represents the mean ± standard error for *GSTP1* in C33A at (0µM- 31.79±2.5; 3µM- 22.2±0.40; 5µM- 21.40±0.4; 7µM- 31.35±1.7) as shown in Fig 43A and for *GSTP1* in SiHa at (0µM- 23.25±3.0; 20µM- 24.4±0.8; 30µM- 20.28±1.2; 40µM- 28.18±1.6) as shown in Fig. 43B.

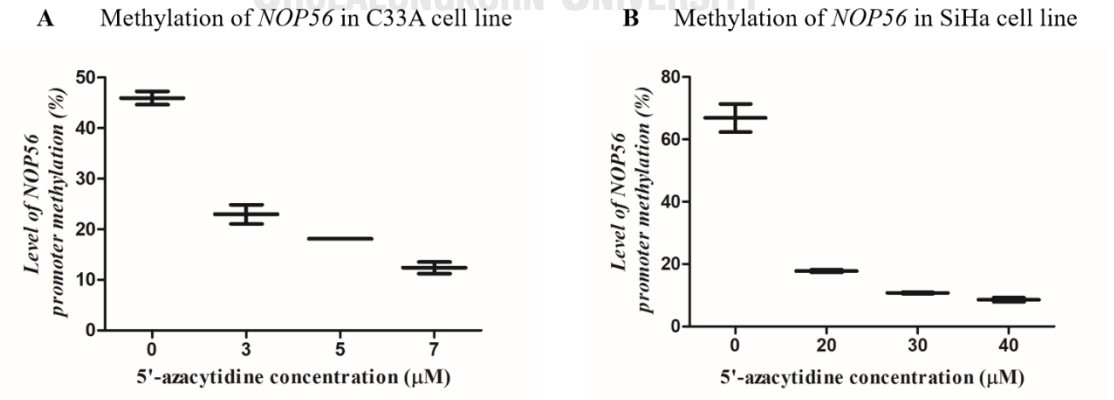


Figure 42 Changes in promoter methylation following after 5-azacytidine treatment by MSP-PCR. (A) *NOP56* methylation in C33A cells, (B) *NOP56* methylation in

SiHa cells. Data are presented as the mean ± the standard error of the mean with three repeats.

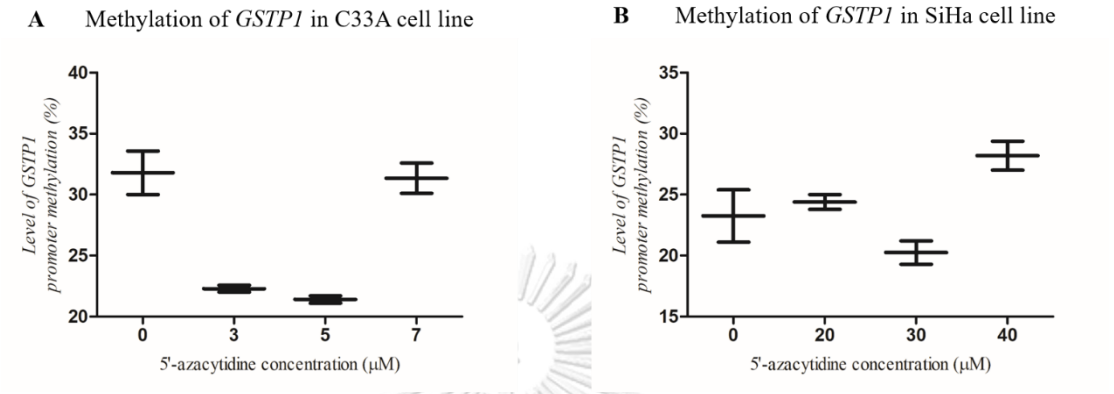


Figure 43 Changes in promoter methylation following after 5-azacytidine treatment by MSP-PCR. (A) *GSTP1* methylation in C33A cells, (B) *GSTP1* methylation in SiHa cells. Data are presented as the mean ± the standard error of the mean with three repeats.

4.4.2 Effect of 5-Aza on gene expression of *CGB3, NOP56* and *GSTP1*

In order to analyze the level of expression of *CGB3* and *NOP56* genes, the extraction of RNA treated with 5-Aza in SiHa with concentration of 0, 20, 30, 40 µM and C33A cell lines with concentration 0, 3, 5, 7 µM were measured by qPCR. According to the results, expression of *CGB3* and *NOP56* in C33A cells were gradually increasing as the 5 aza- concentration increased from 0-7 µM with significant *p* value (*P*<0.0001****) using one-way ANOVA as shown in Fig, 44A and 45A. The graph represents the mean ± standard error for *CGB3* in C33A at (0µM- 99.45±13.8; 3µM- 161.6±32.5; 5µM- 184.16±54.5; 7µM- 227.8±62.5) as shown in Fig 44A and for *NOP56* in C33A at (0µM- 100.1±6.0; 3µM- 218.53±66.4; 5µM- 299.7±42.9; 7µM- 227.8±62.5) as shown in Fig. 45A. In parallel, the expression level of *CGB3* and *NOP56* in SiHa cells were increasing from 0-40 µM with significant *P* value (*P*<0.0001****) using one-way ANOVA. *GAPDH* was used as internal control for analyzing expression of both the genes *CGB3* and *NOP56*. The graph represents

the mean ± standard error for *CGB3* in SiHa at (0µM- 92.11±25.5; 20µM- 41.8±12.9; 20µM- 74.9±23.9; 40µM- 271.2±128.9) as shown in Fig 44B and for *NOP56* in SiHa at (0µM- 100±4.0; 20µM- 48.2±24.8; 30µM- 99.3±99.6; 40µM- 194.19±89.5) as shown in Fig. 45B.

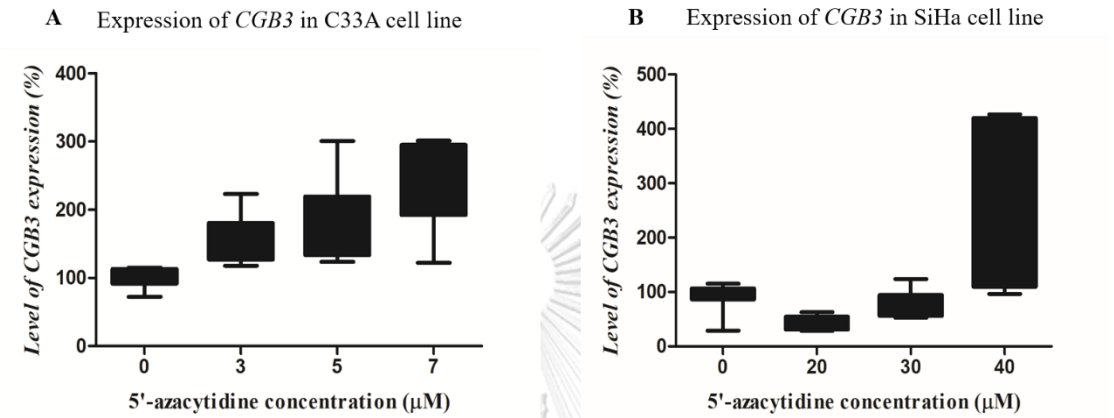


Figure 44 Changes in expression level of *CGB3* following after 5-azacytidine treatment with six replicates. (A) qPCR analysis of expression levels of *CGB3* in C33A cells. (B) qPCR analysis of expression levels of *CGB3* in SiHa cells.

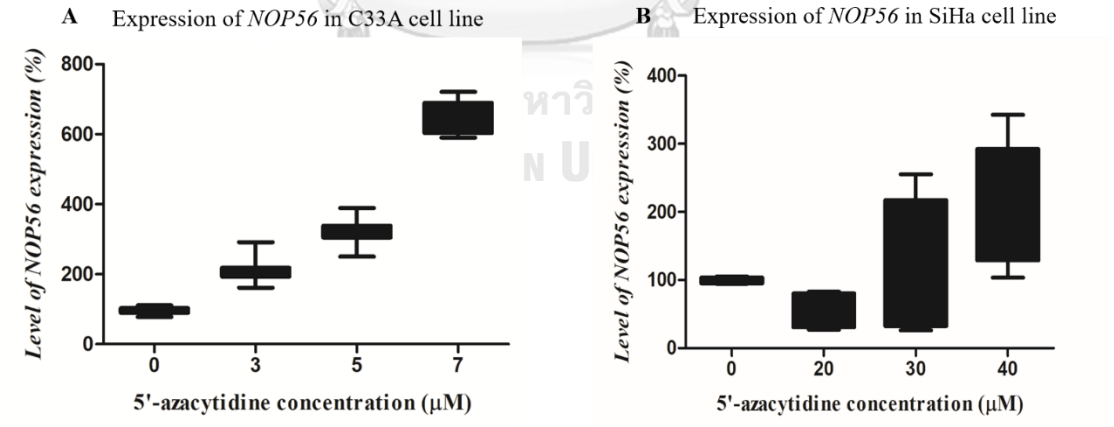


Figure 45 Changes in expression level of *NOP56* following after 5-azacytidine treatment with six replicates. (A) qPCR analysis of expression levels of *NOP56* in C33A cells. (B) qPCR analysis of expression levels of *NOP56* in SiHa cells.

In the same way, m-RNA expression level of *GSTP1* gene were measured by RT-PCR in C33A and SiHa cells treated with 5-Aza drug with 0-7 µM and 0-40 µM, respectively. From the result we observed that although the expression of *GSTP1* in C33A cells at 7 µM was highest but there was no consistent increasing pattern for expression as the concentrations levels were increased from 0 to 7 µM. Similarly, there was no consistent pattern observed for expression of *GSTP1* in SiHa cells as the concentration levels were increased from 0 to 40 µM. The graph represents the mean ± standard error for *GSTP1* in C33A at (0µM- 21.8±2.6; 3µM- 27.30±1.27; 5µM- 19.65±0.9; 7µM- 26.4±1.8) as shown in Fig 46A and for *GSTP1* in SiHa at (0µM- 19.05±0.7; 20µM- 23.15±1.6; 30µM- 19.4±0.8; 40µM- 17.3±0.9) as shown in Fig. 46B.

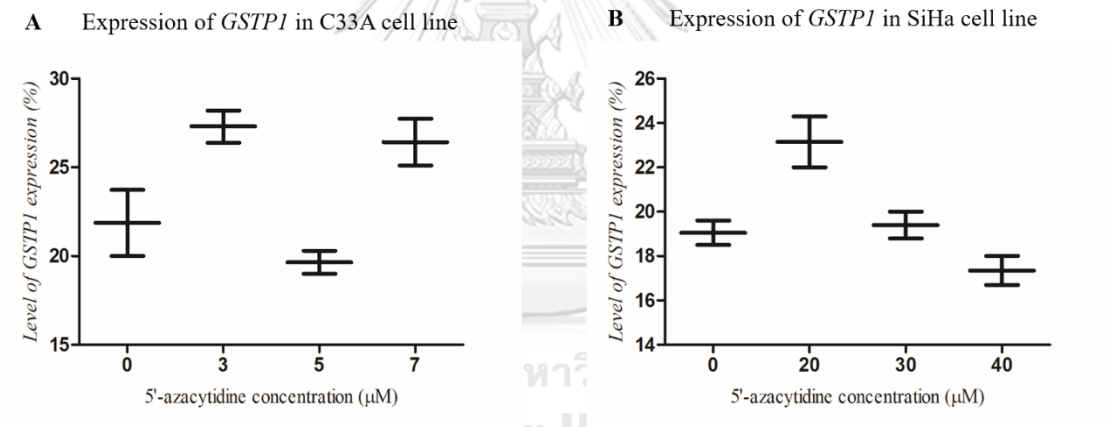


Figure 46 Changes in expression level of *GSTP1* following after 5-azacytidine treatment with three replicates. (A) RT-PCR analysis of expression levels of *GSTP1* in C33A cells. (B) RT-PCR analysis of expression levels of *GSTP1* in SiHa cells.

4.4.3 MTT assay in azacytidine-treated C33a and SiHa cell lines.

C33A and SiHa cell viability were observed by MTT assay after treatment with 5-Aza at different concentration. Cell viability of azacytidine treatment at 3, 5, and 7 µM in C33A (Fig 47A) and 20, 30 and 40 µM in SiHa (Fig 47B) was not

significantly different. However, the cells viability was compared between untreated-(0 µM) and treated-group for both cell line C33A and SiHa, result showed significantly different with (*P*=0.0008***) and (*P*=0.0029**), respectively. The graph represents the mean ± standard error in C33A at (0µM- 0.25±0.08; 3µM- 0.14±0.06; 5µM- 0.13±0.05; 7µM- 0.13±0.08) as shown in Fig 47A and in SiHa at (0µM- 0.45±0.25; 20µM- 0.20±0.05; 30µM- 0.11±0.03; 40µM- 0.14±0.1) as shown in Fig. 47B. Statistically analysis was performed using one-way ANOVA followed by a Dunnett's post hoc test.

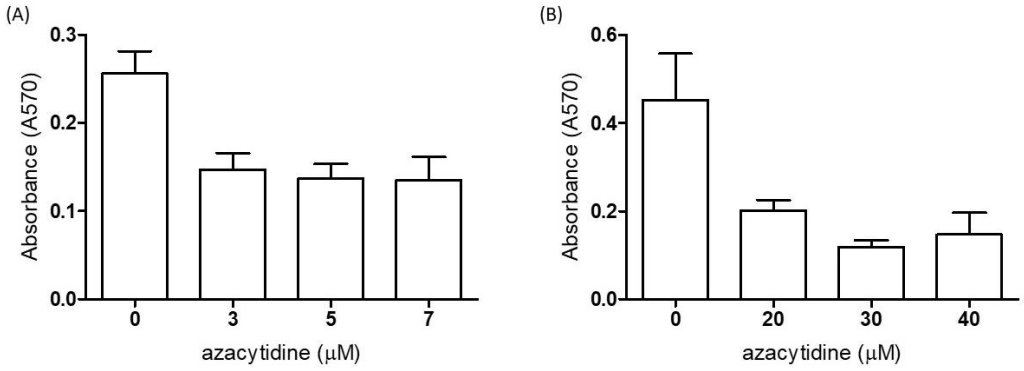


Figure 47 MTT assay in azacytidine-treated C33a and SiHa. (A) Cell viability of azacytidine treatment at 3, 5, and 7 µM in C33a and (B) 20, 30 and 40 µM in SiHa. Graphs represent mean ± SE. Statistically analysis was performed using one-way ANOVA.

4.5 Detection of *CGB3* and *NOP56* genes promoter methylation in PAP samples

4.5.1 Evaluating methylation (%) of *CGB3* and *NOP56* genes in PAP samples

The presence of methylation of *CGB3* and *NOP56* in PAP samples were Determined by using MSP- PCR with specific primer of genes. Based on the experiment, detection of methylation in the promoter region of the *CGB3* and *NOP56* can be used to differentiate between normal cervical cells and abnormal cervical cells. With regards to this, the ASC-US PAP samples and Determined PAP samples were

analyzed at individual level. In the ASC-US PAP samples *CGB3* showed 43 samples with methylation in abnormal samples out of the total size of 48 samples (89.5%) and out of the total 74 normal samples *CGB3* gene showed 17 samples (22%) with a prediction of close to 80% in terms of the accuracy of the results.

The 48 abnormal ASC-US PAP samples were further divided into HSIL (which showed 80% of *CGB3* promoter methylation from a total sample size of 15 samples), LSIL (which showed 93% of *CGB3* methylation from a total sample size of 33 samples). For the remaining 74 normal samples, the normal samples with HPV showed 25% of *CGB3* methylation from a total sample size of 66 and the normal samples without HPV showed 0% methylation from a total sample of 8) shown in Fig. 48. The 25% methylation in normal samples with HPV could have been due to the presence of HPV types which might have led to the methylation.

Similarly, based on result of *NOP56* gene showed 36 samples methylation in abnormal samples out of the total size of 48 samples (75%) and out of the total 74 normal samples, *NOP56* gene showed 24 samples (32%) with methylation predicting close to 70% results accurately. The 48 abnormal ASC-US PAP samples were further broken down into HSIL (which showed 73% of *NOP56* promoter methylation from a total sample size of 15 samples), LSIL (which showed 76% of *NOP56* methylation from a total sample size of 33 samples). For the remaining 74 normal samples, the normal samples with HPV showed 35% of *NOP56* methylation from a total sample size of 66 and the normal samples without HPV showed 12% methylation from a total sample of 8) shown in Fig. 49. The 35% methylation in normal samples with HPV could have been due to the presence of HPV types which might have led to the methylation. This signifying that abnormal samples (LSIL+HSIL) showed more methylation by *CGB3* and *NOP56* in ASC-US PAP samples compared to that of normal samples.

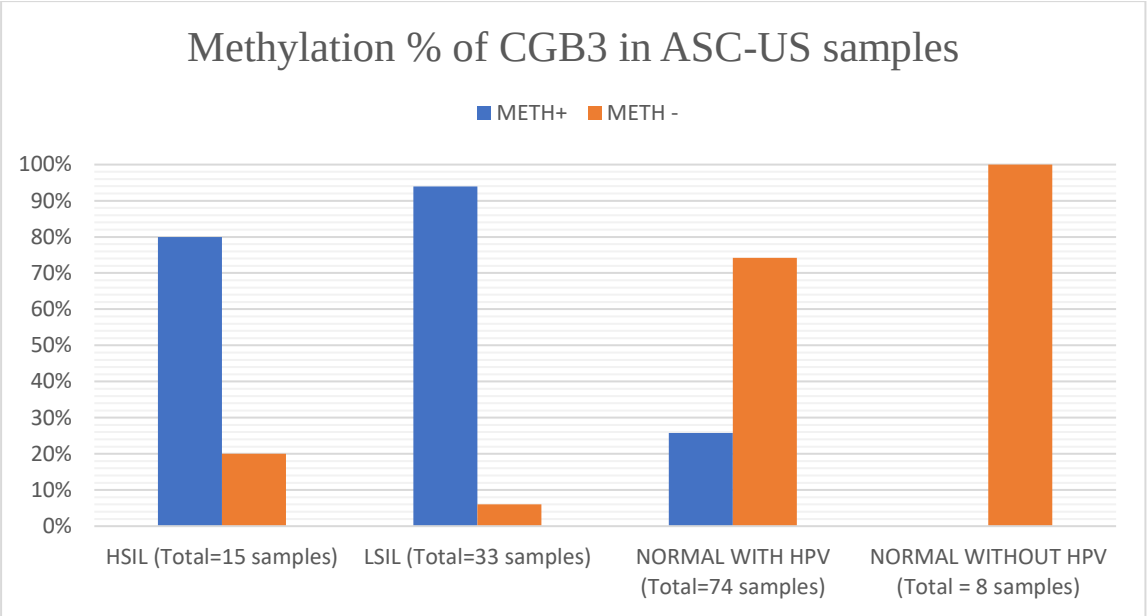


Figure 48 Distribution of methylation percentage of CGB3 in ASC-US samples

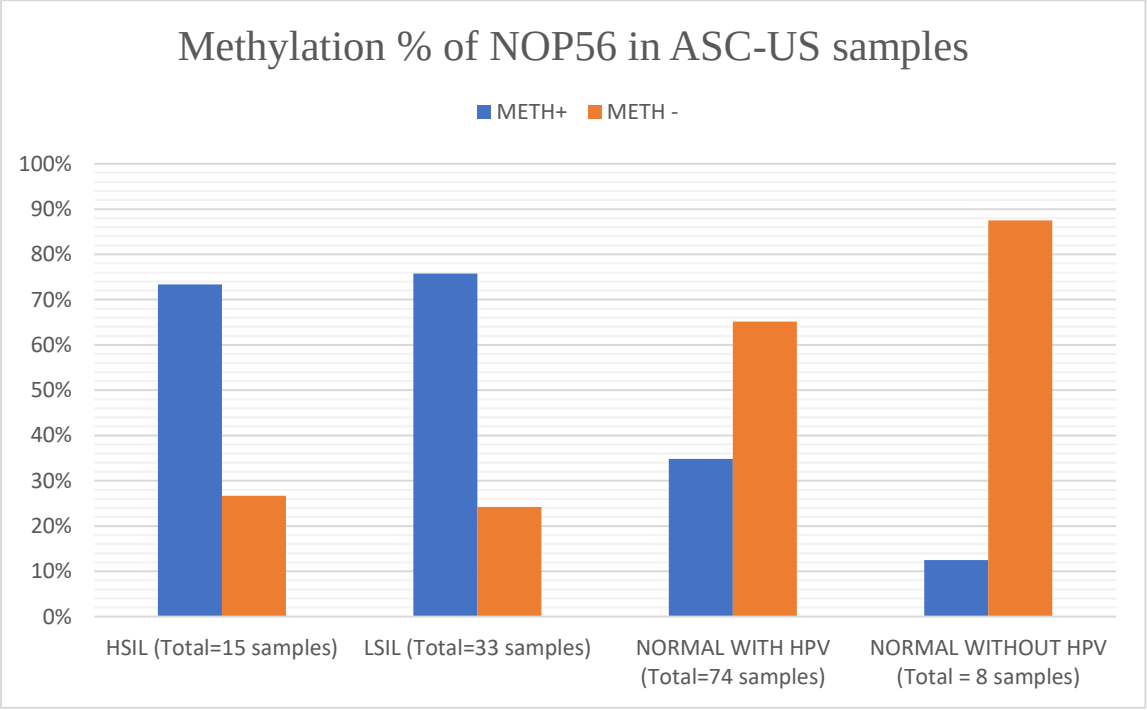


Figure 49 Distribution of methylation percentage of NOP56 in ASC-US samples

Parallelly, the effect of presence and absence of *CGB3* and *NOP56* genes promoter methylation were also analyzed in cytology based Determined PAP samples

that consisted of LSIL, HSIL, cancer and normal (with and without HPV) by using the same MSP technique. Based on findings from the result of *CGB3* methylation, we found that *CGB3* gene showed 48 samples methylation in abnormal samples out of the total size of 56 samples (85%) and out of the total 22 normal samples *CGB3* gene showed 8 samples (36.3%) with methylation predicting close to 65% results accurately. The 56 abnormal Determined PAP samples were further broken down into cancer (which showed 80% *CGB3* promoter methylation from a total size of 20 samples), HSIL (which showed 92% promoter methylation from a total sample size of 24 samples), LSIL (which showed 83.3% methylation from a total sample size of 12 samples). For the remaining 22 normal samples, the normal samples with HPV showed 62.5% methylation from a total sample size of 8 and the normal samples without HPV showed 21% methylation from a total sample of 14) shown in Fig. 50

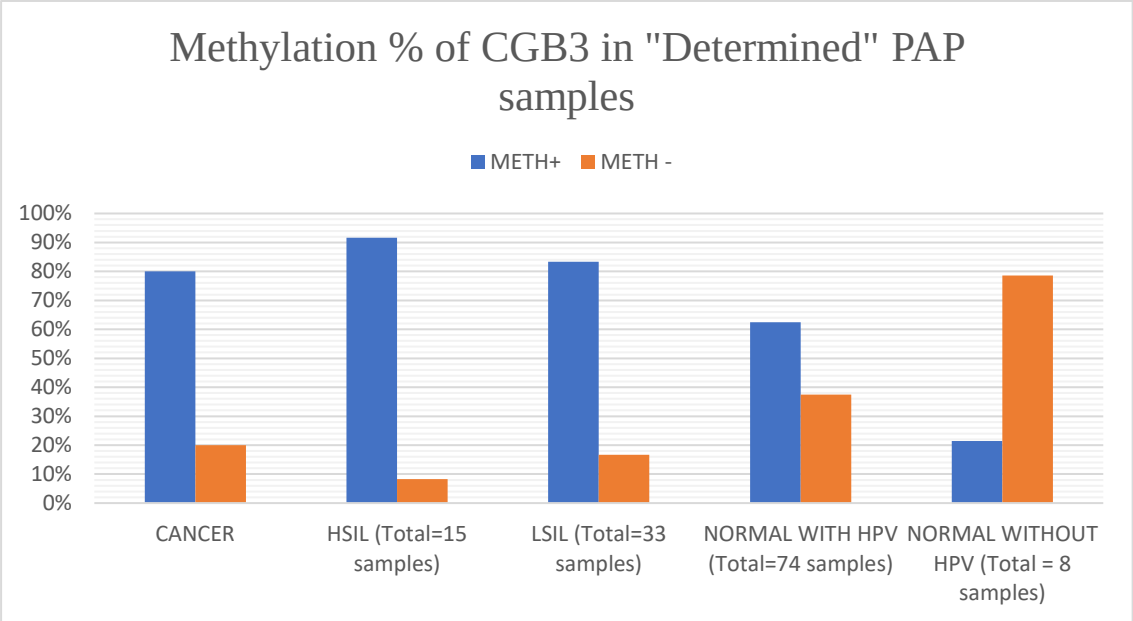


Figure 50 Distribution of methylation (%) of *CGB3* in Determined PAP samples

Similarly, based on the results of *NOP56* methylation, we found that 38 samples methylation in abnormal samples out of the total size of 56 samples (68%) and out of the total 22 normal samples *NOP56* gene showed 6 samples (27.2%) without methylation predicting close to 73% results accurately. The 56 abnormal

Determined PAP samples were further broken down into cancer (which showed 70% of *NOP56* promoter methylation from a total size of 20 samples), HSIL (which showed 62.5% promoter methylation from a total sample size of 24 samples), LSIL (which showed 67% methylation from a total sample size of 12 samples). For the remaining 22 normal samples, the normal samples with HPV showed 37.5% methylation from a total sample size of 8 and the normal samples without HPV showed 21% methylation from a total sample of 14) shown in Fig. 51.

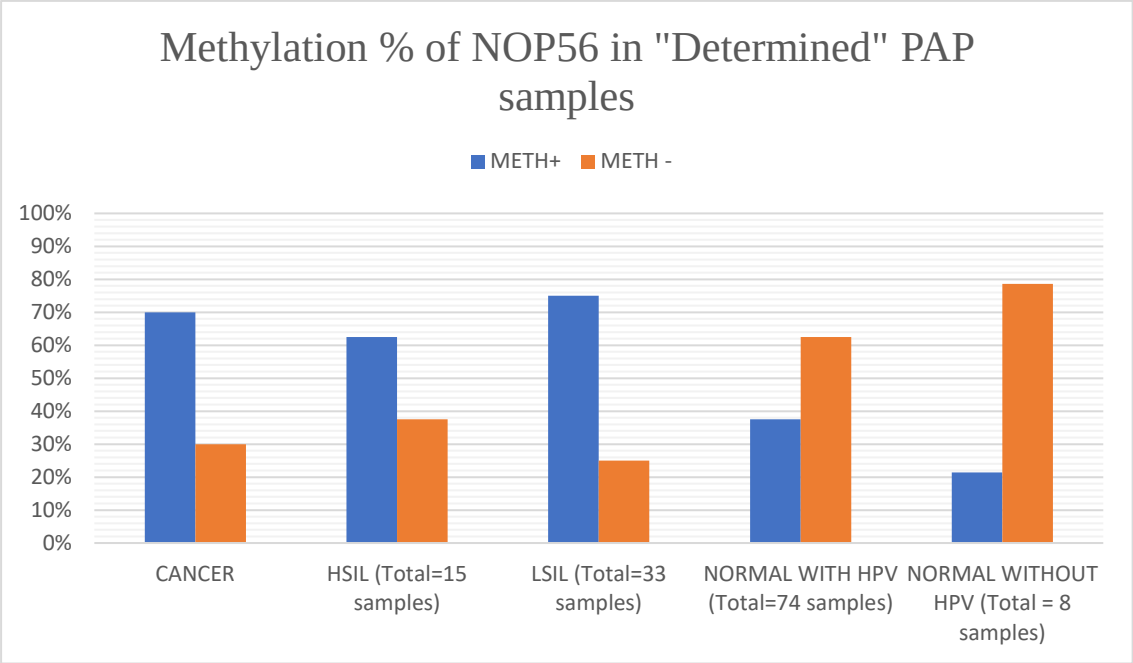


Figure 51 Distribution of methylation (%) of *NOP56* in Determined PAP samples

The distribution of the results from methylation from *CGB3* and *NOP56* genes shows that there is higher level of methylation in the “Abnormal” samples for both histology-based ASC-US and cytology based Determined PAP samples. On the contrary, the level of methylation was less detected in “Normal” samples for both histology and cytology samples (with and without HPV) indicating the effectiveness of *CGB3* and *NOP56* genes as methylation markers for early detection of Cervical cancer.

4.5.2 HPV typing and methylation distribution of *CGB3* and *NOP56* in PAP samples

Another dimension of analysis was based on the level of methylation shown for selected HPV strains which were known to have causal effect of cervical cancer (Burd 2007). All the samples based on HPV strains were classified into no-HPV, HPV 16, HPV 18, combined-HPV (containing both HPV 16 and HPV 18) and others HPV (HPV genotypes (HR 12) includes: 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). In this study, different types of HPV's strain were analyzed using the 200 PAP samples included both ASC-US and Determined PAP samples as mentioned in (Fig. 52). The number of ASC-US samples with HPV16 type had a total sample of 31 samples, with HPV18 type had a total of 11 samples, combined-HPV had a total sample size of 4 samples, others had a sample size of 65 samples and no-HPV had a sample size of 11. Likewise other remaining Determined PAP samples which included LSIL, HSIL, cancer, normal (with or without HPV) were classified as HPV 16 with a sample size of 49 samples, HPV 18 with a sample size of 15 samples and no-HPV with a sample size of 14 samples. Among which total HPV 16 had 80 samples 40%), HPV 18 had 26 samples (13%), HPV 16_18 had 4 samples (2%), HPV Others had 65 samples (32%), No-HPV had 25 samples (13%).

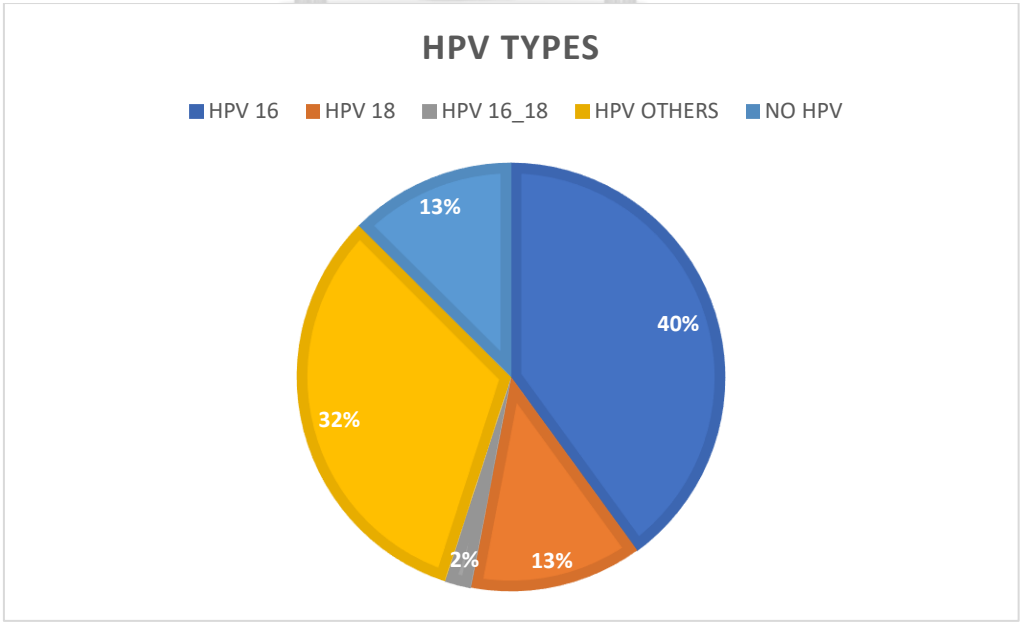


Figure 52 Categorization of 200 PAP samples into types of HPV strains.

The distribution of methylation percentage of *CGB3* and *NOP56* genes in all the 200 PAP samples were calculated in different type of HPV strains. From the results we observed significant methylation differences of *CGB3* and *NOP56* in the distribution of HPV genotyping with *P* value 0.0003 and 0.019, respectively. The methylation percentage of *CGB3* were distributed as HPV 16 strain showed 70% methylation, HPV 18 strain showed 61% methylation, combined HPV (16_18) strain showed 75% methylation, other HPV strains showed 50% methylation and no-HPV showed 20% methylation. Similarly, in *NOP56* gene HPV 16 strain showed 70% methylation, HPV 18 strain showed 62% methylation, combined HPV (16_18) strain showed 75% methylation, other type of HPV strains showed 49% methylation and no-HPV strain showed 30% methylation. Based on the findings the methylation percentages of samples containing either or both HPV 16 and HPV 18 strain showed higher percentage of methylation (Fig. 53).

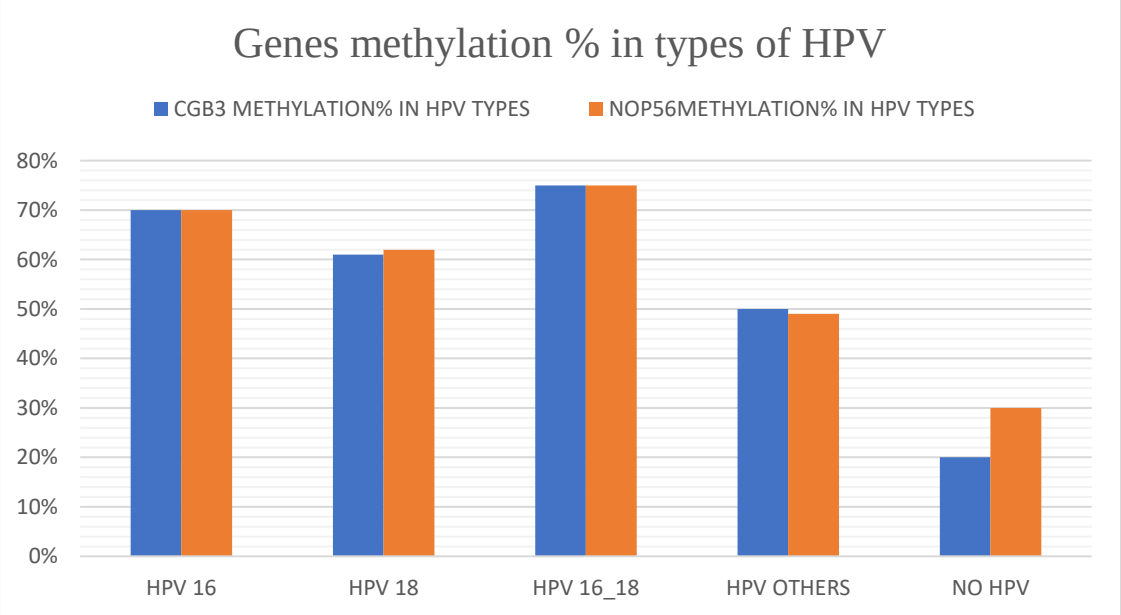


Figure 53 Methylation (%) of *CGB3* and *NOP56* genes on the basis of HPV types

4.5.3 Statistical comparing *CGB3* and *NOP56* methylation in ASC-US PAP samples

The statistical comparison of methylation in the promoter region of the *CGB3* and *NOP56* genes in the ASC-US samples (122/200) between the stage of

normal cervical cells and abnormal cells were calculated. Experiments have shown that when comparisons between different combinations of normal samples (with and without HPV) and pre-cancerous (“LSIL”, “HSIL” and “LSIL+HSIL”) were made separately for *CGB3* and *NOP56* genes, it was found that both *CGB3* and *NOP56* genes were able to distinguish between the normal samples and precancerous samples statistically (with *P* value ≤ 0.05) as in Table 13 and 14 respectively.

Table 13 Statistical comparison of *CGB3* in ASC-US stages.

Stages of Normal samples compared with abnormal samples with level of [LSIL, HSIL and (LSIL+HSIL)]. The asterisk symbol (*) denoted the significant *p* value, where *P*≤0.05 was considered to indicate a statistically significant value.

Stage compared with normal (without HPV)	**Gene**	**Chi-Square *p*-value**
LSIL	*CGB3*	0.0001 (****)
HSIL	*CGB3*	0.0003 (***)
LSIL+HSIL	*CGB3*	0.0001 (****)
Stage compared with normal (with and without HPV)	**Gene**	**Chi-Square *p*-value**
LSIL	*CGB3*	0.0001 (****)
HSIL	*CGB3*	0.0001 (****)
LSIL+HSIL	*CGB3*	0.0001 (****)

Table 14 Statistical comparison of *NOP56* in ASC-US stages.

Stages of Normal samples compared with abnormal with level of [LSIL, HSIL and (LSIL+HSIL)]. The asterisk symbol (*) denoted the significant p value, where P≤0.05 was considered to indicate a statistically significant value.

Stage compared with normal (without HPV)	**Gene**	**Chi-Square *p*-value**
LSIL	*NOP56*	0.0009 (***)
HSIL	*NOP56*	0.0038 (**)
LSIL+HSIL	*NOP56*	0.0005 (***)
Stage compared with normal (with and without HPV)	**Gene**	**Chi-Square *p*-value**
LSIL	*NOP56*	0.0001 (****)
HSIL	*NOP56*	0.0031 (**)
LSIL+HSIL	*NOP56*	0.0001 (****)

4.5.4 Statistical comparing of *CGB3* and *NOP56* methylation in Determined PAP samples

Similar to as shown for ASC-US samples with reference to histology, the statistical comparison of methylation in the promoter region of the *CGB3* and *NOP56* genes in the cytology-based Determined PAP samples (78/200) between the stage of normal cervical cells and pre- cancerous cells were calculated. Experiments have shown that when comparisons between different combinations of normal samples (with and without HPV) and pre-cancerous (“LSIL”, “HSIL” and “LSIL+HSIL”) were made separately for *CGB3* and *NOP56* genes, it was found that both *CGB3* and *NOP56* genes were able to distinguish between the normal samples and precancerous samples statistically (with *P* value ≤ 0.05) as in Table 15 and 16 respectively.

Table 15 Statistical comparison of *CGB3* in Determined PAP samples.

Stages of Normal samples compared with pre-cancerous samples with level of [LSIL, HSIL, Cancer and (LSIL+HSIL)]. The asterisk symbol (*) denoted the significant *p* value, where P≤0.05 was considered to indicate a statistically significant value.

Stage compared with normal (without HPV)	**Gene**	**Chi-Square *p*-value**
LSIL	*CGB3*	0.001 (***)
HSIL	*CGB3*	0.0001 (****)
Cancer	*CGB3*	0.0007 (***)
LSIL+HSIL+CANCER	*CGB3*	0.0001(****)
Stage compared with normal (with and without HPV)	**Gene**	**Chi-Square *p*-value**
LSIL	*CGB3*	0.008 (**)
HSIL	*CGB3*	0.0001 (****)
Cancer	*CGB3*	0.004 (**)
LSIL+HSIL+CANCER	*CGB3*	0.0001 (****)

Table 16 Statistical comparison of *NOP56* in Determined PAP samples.

Stages of Normal samples compared with pre-cancerous samples with level of [LSIL, HSIL, Cancer and (LSIL+HSIL)]. The asterisk symbol (*) denoted the significant *p* value, where $P \leq 0.05$ was considered to indicate a statistically significant value.

Stage compared with normal (without HPV)	**Gene**	**Chi-Square *p*-value**
LSIL	*NOP56*	0.006(**)
HSIL	*NOP56*	0.01(*)
Cancer	*NOP56*	0.005 (**)
LSIL+HSIL+CANCER	*NOP56*	0.001 (***)
Stage compared with normal (with and without HPV)	**Gene**	**Chi-Square *p*-value**
LSIL	*NOP56*	0.007 (**)
HSIL	*NOP56*	0.01 (*)
Cancer	*NOP56*	0.005 (**)
LSIL+HSIL+CANCER	*NOP56*	0.001(***)

4.5.5 Screening efficiency and diagnostic ability of *CGB3* and *NOP56* methylation marker in ASC-US PAP samples

The effectiveness of methylation in the promoter region of the *CGB3* and *NOP56* genes to be used to isolate and screen cervical cells were used as measurement methods. The performance calculations were performed between “true label” x “predicted label” where “True Positives (TP)”, “False Positives (FP)”, “True Negatives (TN)” and “False Negatives (FN)” of samples were calculated. On the basis

of these 4 indices, “Sensitivity”, “Specificity”, Positive Predictive Value “(PPV)” and Negative Predictive Value “NPV” were calculated with following formula.

Sensitivity = True Positives / (True Positives + False Negatives)

Specificity = True Negatives / (False Positives + True Negatives)

PPV (Positive Predictive Value) = True Positives / (True Positives + False Positives)

NPV (Negative Predictive Value) = True Negative / (False Negatives + True Negatives).

Accuracy = (True Positives + True Negatives) / (Total number of samples)

From the results of histology- based ASC-US PAP samples, *CGB3* gene showed the number of True Positives (TP) were 43, number of False Positives (FP) were 17, number of True Negatives (TN) were 57 and number of False Negatives (FN) negatives were 5. It was found that based on the screening with methylation testing methods in the promoter region of the *CGB3* gene, Sensitivity was 90%, Specificity was 77%, PPV was 72%, NPV was 92% and accuracy was found to be 82% respectively. Similarly, the efficiency of *NOP56* gene the in ASC-US PAP samples the true positives samples was 36, false positives was 24, true negatives was 50 and false negatives was 13 samples were calculated. From the result the Sensitivity showed 73%, specificity 68%, PPV and NPV values are 60% and 79%, respectively. The method of testing for methylation in the promoter of *NOP56* gene is 70% effective, as shown in Table 17.

Table 17 *CGB3* and *NOP56* screening efficiency in ASC-US samples

SCREENING TYPES / GENES	***CGB3***	***NOP56***
HISTOLOGY BASED PAP SAMPLES	ASC-US PAP SAMPLES	ASC-US PAP SAMPLES
SENSITIVITY (%)	90	73
SPECIFICITY (%)	77	68

POSITIVE PREDICTIVE VALUE (PPV) (%)	72	60
NEGATIVE PREDICTIVE VALUE (NPV) (%)	92	79
ACCURACY OF METHYLATION MARKER (%)	82	70

Based on the findings, “Sensitivity” values for *CGB3* and *NOP56* genes in ASC-US (histology-based) samples showed high values for methylation (90% and 75% respectively), showing higher level of capability for precise identification of abnormal samples correctly.

The other dimension of measuring diagnostic ability of *CGB3* and *NOP56* methylation marker is in terms of “Area Under the Curve -AUC” (which represents the measure of separability) and “Receiver Operating Characteristic – ROC” (which represents the diagnostic ability of a binary classifier system which in this case is “Methylated” and “Non-Methylated”). The AUC-ROC value above 0.5 were set to be significant value through which the classifier was able to distinguish between the normal and the abnormal samples (Carrington et al. 2022). From the analysis of *CGB3* and NOP56 methylation marker in ASC-US PAP samples, the AUC values were observed as 0.83 (Fig. 54A) and 0.71 (Fig. 54B) respectively, indicating that *CGB3* has 83% and *NOP56* has 71% ability to detect between the normal and abnormal PAP samples correctly.

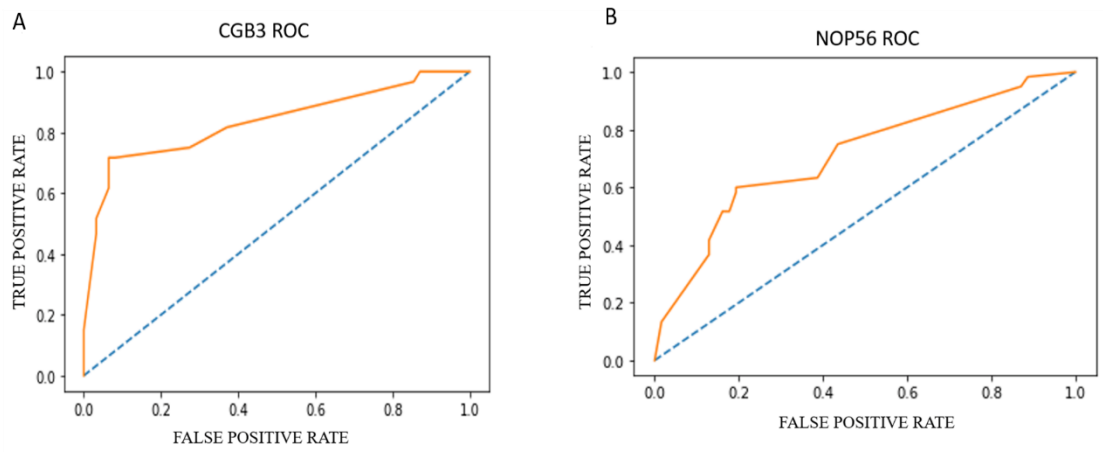


Figure 54 ROC analysis of methylation ratio per gene in ASC-US samples. Figure A represents the *CGB3* gene shows significantly value of AUC 0.83. Figure B represents *NOP56* gene shows 0.71 (which is greater than 0.5) signifying that the analysis was able to significantly distinguish between the positive and negative samples.

From the analysis of data for ASC-US samples, *CGB3* and *NOP56* individually showed sensitivity of 90% and 73 % respectively, and specificity of 77% and 68% respectively. Moreover, by combining the sensitivity and specificity of these two markers the diagnostic power of the cervical cancer detection further increased (combined sensitivity of 97% and combined specificity 93%making the testing results more precise. From study of (Parikh et al. 2008) the combined sensitivity was calculated as [1- (1 - sensitivity of CGB3) x (1 – sensitivity of *NOP56*)] and combined specificity was calculated as 1- (1 - specificity of CGB3) x (1 – specificity of *NOP56*). With a higher percentage of combined sensitivity and combined specificity, the methylation level of *CGB3* and *NOP56* in ASC-US samples showed high potential for use as a screening test for early stage of cervical cancer detection.

4.5.6 Screening efficiency and diagnostic ability of *CGB3* and *NOP56* methylation marker in Determined PAP samples

Similar to as shown for ASC-US samples the remaining cytology based - Determined PAP samples were also calculated on the basis of these 4 indices, sensitivity, specificity, PPV and NPV were calculated. For the efficiency of *CGB3* gene the true positives were 48, false positives were 8, true negative were 14 and false negative were 8. It was found that screening with methylation testing methods in the promoter region of the *CGB3* gene showed sensitivity 86%, specificity 64%, PPV and NPV values are 86% and 64%, respectively. The method of testing for methylation in the promoter of *CGB3* gene was 79.4% (approximately 80%) accuracy. Similarly, the efficiency of *NOP56* gene the true positives 38, false positives 6, true negative 16 and false negative 18 samples were calculated. From the result the Sensitivity showed 68%, specificity 73%, PPV and NPV values are 86% and 47%, respectively. The method of testing for methylation in the promoter of *NOP56* gene is 69.2% (approximately 70%) accuracy, as shown in Table 17.

Table 17 *CGB3* and *NOP56* screening efficiency in Determined PAP samples.

SCREENING TYPES / GENES	***CGB3***	***NOP56***
HISTOLOGY BASED PAP SAMPLES	Determined PAP SAMPLES	Determined PAP SAMPLES
SENSITIVITY (%)	86	68
SPECIFICITY (%)	64	73
POSITIVE PREDICTIVE VALUE (PPV) (%)	86	86
NEGATIVE PREDICTIVE VALUE (NPV) (%)	64	47
ACCURACY OF METHYLATION MARKER (%)	80	70

The diagnostic ability of *CGB3* and *NOP56* methylation marker observed in Determined PAP samples were 0.74, Fig. 55A and 0.63, Fig. 55B respectively (which is greater than 0.5) signifying that the analysis was able to significantly distinguish between the positive and negative samples.

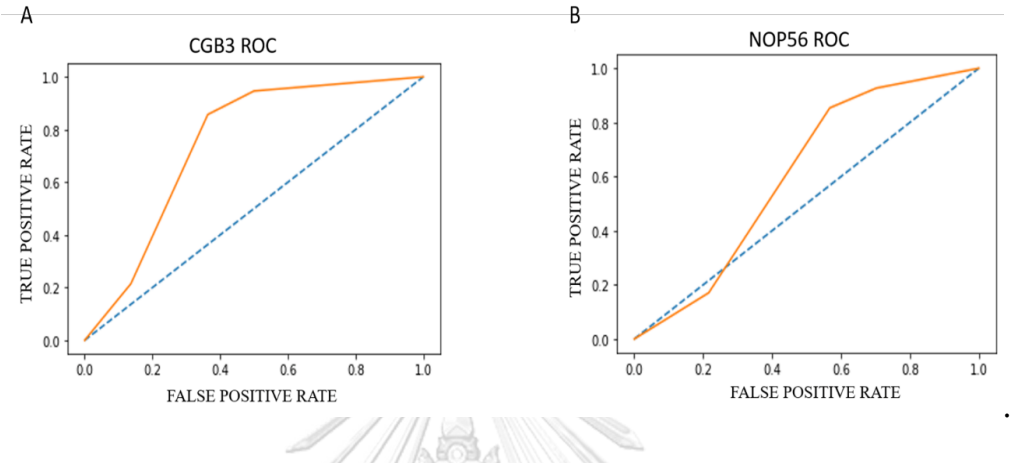


Figure 55 ROC analysis of methylation ratio per gene in Determined PAP samples. Figure A represents the *CGB3* gene shows significantly value of AUC 0.74 and figure B represents the *NOP56* gene shows 0.63 (which is greater than 0.5) signifying that the analysis was able to significantly distinguish between the positive and negative samples.

From the analysis of data for determined samples, *CGB3* and *NOP56* individually showed sensitivity of 86 % and 68 % respectively, and specificity of 64% and 73% respectively. Moreover, by combining the sensitivity and specificity of these two markers the diagnostic power of the cervical cancer detection further increased (combined sensitivity of 91% and combined specificity 90%making the testing results more precise. The combined sensitivity was calculated as [1- (1 - sensitivity of CGB3) x (1 – sensitivity of *NOP56*)] and combined specificity was calculated as 1- (1 - specificity of CGB3) x (1 – specificity of *NOP56*). With a higher percentage of combined sensitivity and combined specificity, the methylation level of *CGB3* and *NOP56* in determined cytology-based samples showed high potential for use as a screening test for early stage of cervical cancer detection.

CHAPTER 5

DISCUSSION AND CONCLUSION

Cervical cancer is by far the most common HPV-related disease. About 99.7% cervical cancers are caused by HR-HPV (Burd 2003). HPV 16 - E7, also known as oncoproteins leads to uncontrolled proliferation through deregulation of tumor suppressor gene. This research further explores HPV 16 - E7 oncogenic function in terms of methylation-repression in genes. Chalertpet et al., (Chalertpet 2015) showed that HPV 16 - E7 induces promoter methylation in *CCNA1* by the mechanism of binding to DNMT1 enzyme through process of chromatin immunoprecipitation. Moreover, Yanatatsaneejit et al., (2020) showed that HPV 16 - E7 cannot induce promoter methylation in all the genes as it induces methylation in selective cells such as *CADM1* and *CCNA1* genes using the same mechanism however it failed to to induce methylation in *DAPK1*. Therefore, it was hypothesized that in addition to *CCNA1* and *CADM1*, HPV 16 - E7 should also induce methylation in other tumor suppressor genes whose promoter region could be methylated by HPV 16 - E7 by the same mechanism as that of *CCNA1* or other specific transcription factors that could bind to E7 at the promoter region the gene. The author analyzed previous studies (Na Rangsee et al. 2020) in order to identify other tumor suppressor genes which were regulated by HPV 16 - E7 and to find transcription factor linkage between gene promoter binding to HPV 16 E7 and DNMT1, including selecting genes for further study and experimentations based on the review of literature. List of selected genes were selected from the above-mentioned approach (*GSTP1, PDI-A3, VAV1, ING1, IL4R alpha, CGB3* and *NOP56*) for observing the gene expression in two cervical cancer lines SiHa (HPV positive) and C33A (HPV negative) cell lines. From the results of experimentations, the expression of *PDI-A3*, *VAV1*, *ING1* and *IL4R alpha* showed higher band intensities in SiHa cell lines compared to C33A cell lines indicating possibility if up-regulation of genes instead of down-regulation of genes as expected. Interestingly, the genes *CGB3, GSTP1* and *NOP56* showed lower band intensities in SiHa cell lines compared to C33A cells indicating possibility if down-

regulation of genes as expected suggesting that the presence of HPV in SiHa may induce promoter methylation and decrease the expression of the genes. To prove that E7 can induce promoter methylation, further experiments were performed to analyze the expression of *CGB3, GSTP1* and *NOP56* in transfected HPV16-E7 and PC plasmid to prove the effect of E7 on gene expression and from the results, and it was observed that all the three genes had lower expression in E7 plasmid-transfected cells compared to PC which demonstrated that HPV16 - E7 induced promoter methylation of *CGB3, GSTP1* and *NOP56* resulting in the down-regulation of these genes. Even though there are studies (Gong et al. 2017; Yang et al. 2022) showing that *NOP56* functions as proto-oncogene in lung cancer, colon cancer and prostate cancer. Interestingly, the findings from the present study confirmed that the band intensity of E7 plasmid in *NOP56* had higher expression compared to PC, which demonstrated that HPV 16-E7 induces promoter methylation in *NOP56* suppressing its expression and not exhibiting its characteristic as a proto-oncogene in cervical cancer.

The expression changes in *CGB3, GSTP1* and *NOP56* following methylation inhibition using the demethylating agent 5-aza were next assessed. Therefore, the present study aimed to evaluate *CGB3, GSTP1* and *NOP56* gene expression by inhibiting the methylation rate via treatment with the demethylating agent 5-aza on the basis of aforementioned results. In the present study we aimed to evaluate the gene expressions by inhibiting methylation rate in order to increase the expression of tumor suppressor genes by treating it with demethylating agent 5-Aza which inhibits or interferes with DNA methylation, and can be used to re-activate the silenced genes in cancer (Christman 2002; Stresemann and Lyko 2008). Due to the substitution of the 5’ nitrogen atom in place of the carbon, the DNMTs are trapped on the substituted DNA strand and methylation is inhibited. The study conducted by Wong et al., (Wong et al. 2011) showed that methylation-mediated silencing of gene expression can be reversed by treatment with the demethylating agent 5-aza. There were also other genes *CD44*, *BRCA1*, *MDR1*, *MUC2* and *GPC3* with promoter CpG islands hypermethylated in tumors which were reported to be re-activated with 5-azacytidine (Christman 2002). Reactivation of methylated-silenced genes indicates that the gene is functional and that DNA methylation regulates its transcription (Karpf and Jones 2002). Therefore, the present study also aimed to determine whether 5-aza increased

the expression levels of genes resulting from decreased promoter methylation in cervical cancer SiHa and C33A cell lines. In addition to promoter methylation, SiHa cells lines are required to be treated at higher concentrations compared with C33A cells, as SiHa is an HPV^{+} cancer cell line, which induces promoter methylation. Therefore, the lower concentration of 5 -aza may not overcome HPV for demethylation in SiHa cells. Following treatment with 5-aza at higher concentrations of 0, 20, 30 and 40 µM in SiHa cell, and at lower concentrations of 0, 3, 5, and 7 µM 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. Following treatment with 5-aza at higher concentrations of 0, 20, 30 and 40 µM in SiHa cell, and at lower concentrations of 0, 3, 5, and 7 µM 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 However, at concentrations of 20 µM and 30µM of 5-aza in SiHa cell lines, the expression level of *CGB3* and *NOP56* were lower compared to that of 0 µM (untreated) and 40 µM highest concentration. The possible reason for lower expression at 20µM and 30 µM might be due to the presence of HPV in SiHa cells lines which at lower concentration for 5-aza had weak effects on *CGB3* and *NOP56* mRNA upregulation. Previous study (Biktasova et al. 2017) showed that demethylating agent (5-aza) altered the expression of HPV genes (E6 and E7) present in SiHa cell lines which has been explained using the flowchart below:

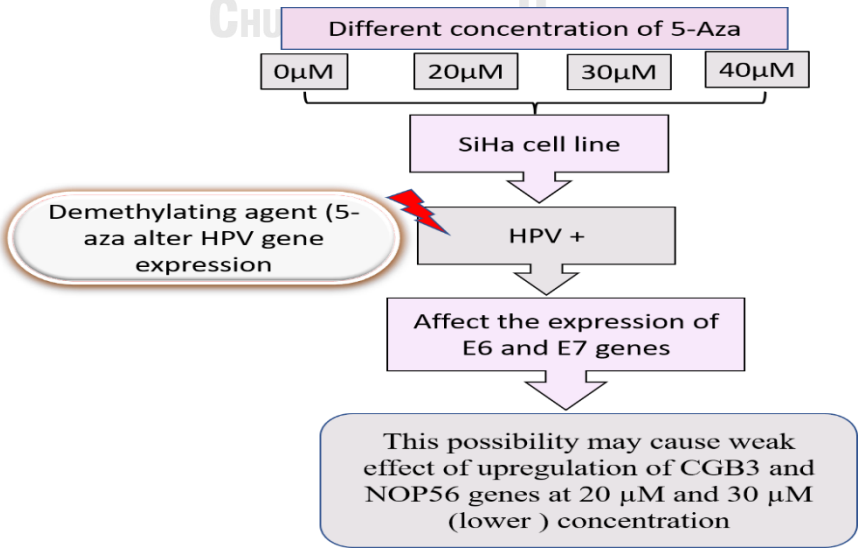


Figure 56 Possibility of weak effect of upregulation of *CGB3* and *NOP56* at 20 µM and 30 µM 5-aza concentration in SiHa cell line

This mechanism was further explored (Perrard et al. 2020) using TBX2 transcription factor in cervical cancer cell lines using 5-aza treatment which resulted in upregulation of TBX2 in both SiHa and Ca Ski cell lines at higher concentration levels, however at lower concentrations for 5-aza the effect of TBX2 mRNA upregulation was found to be weak and the regulation patterns were substantially different in both cell lines. Based on the above findings, the author deduced that there might be unknown transcription factors which mediates the expression of HPV genes present in SiHa cell line inhibiting the upregulation of *CGB3* and genes at lower concentration of 5-aza drug. Therefore, when treated with higher (40µ) concentrations of 5-aza the level of re-expression of genes observed for *CGB3* and *NOP56* were significantly higher. Furthermore, the re-expression of genes following treatment with different concentration of 5-aza drug in different cancer lines may affect the pattern of genes expression (Biktasova et al. 2017; Ren et al. 2018; Siebenkäs et al. 2017).

However, *GSTP1* gene did not show any consistent (increasing or decreasing) patterns for re-expressions or methylation after 5-Aza treatment respectively. Previous, studies (Biktasova et al. 2017) have shown that the expression of *GSTP1* is not a lone deterministic factor for the methylation or re-expression after 5-Aza treatment. There may be some other factors which influence the effect of 5-Aza treatment on *GSTP1* methylation and re-expression (Porter et al. 2017). However, the expression of *GSTP1* might have been effected by other proteins such as MPK8 (Wang et al. 2001), which mediates stress response and also with Fanconi anemia, complementation group C (Cumming et al. 2001). The above stated factors led to the decision of not choosing *GSTP1* for further analysis in the research as the level of re-expression could not have been used as factor to determine the 5-Aza treatment. Collectively, the results suggested that patients with cervical cancer with HPV infection should be treated with a higher concentration of 5-aza than patients without HPV infection.

HR-HPV infection in women is the major cause of almost all cervical cancers. Cytology-based screening, also known as the PAP test remains the mainstay of cervical cancer prevention worldwide. They have known to be reducing the cervical cancer related incidents and deaths in countries where screening is common.

However, the success of these programs relies on many other factors such as infrastructure, financial resources and medical equipment that are often not available in the developing countries. Although screening programs have greatly contributed towards to reducing the prevalence of cervical cancer in developed countries, but still there are some diagnostic tests cannot predict if mild lesions may progress into invasive lesions or not. ASC-US is the most frequent type of abnormal cells found during cervical PAP screening and can indicate either sign of benign (not cancer) or potential malignancy (Caprara et al. 2001). Thus, those women who were found to be positive for HR-HPV (High Risk HPV) types or had PAP results of ASC-US (Atypical squamous cells of undetermined significance) or higher stages were considered to have positive screening test results indicating exposure to the risk of cervical cancer and were referred for colposcopy and biopsy (Stany et al. 2006). In our study, the 200 PAP samples were divided on the basis of histology (target biopsy with confirmed diagnosis) i.e., ASC-US (Undetermined) samples and cytology (under microscopic observation for whom the diagnosis are not 100% confirmed) known as Determined PAP samples. *CGB3* and *NOP56* genes were used to analyzed the methylation in all the 200 PAP samples with hypothesis of showing methylation in abnormal samples (included LSIL, HSIL and cancer) and no methylation in normal (with or without HPV) samples. From the result of histology-based ASC-US PAP samples for distribution percentage of *CGB3* and *NOP56* genes, the result showed more methylation in abnormal PAP samples compared to normal samples with significant differences statistically.

Based on further analysis, the CGB3 gene exhibited high sensitivity/specificity in both the histology- and cytology-based samples, with the percentages of methylation determined to be 90% and 77%, and 86% and 64%, respectively, which indicated that the *CGB3* gene had the ability to detect the presence of disease with high levels of accuracy (82% and 80%, respectively). Similarly, the *NOP56* gene showed high levels of sensitivity/specificity, with values for the percentages of methylation of 75% and 68%, and 68% and 73%, with respect to the histology- and cytology-based samples, respectively. These results indicated that the *CGB3* gene was able to act as a more effective marker compared with the *NOP56* gene, via analyzing the methylation process for both the histology (confirmed

stage) and cytology (not confirmed stage) based samples. Although *CGB3* and *NOP56* genes individually already showed high sensitivity and specificity values in histology and cytology samples, by combining the value of these two genes (*CGB3* and *NOP56*) the resultant combined sensitivity and specificity values (97% and 93% respectively) were found to be higher than individual sensitivity and specificity of these two genes. Similarly for cytology-based samples the combined sensitivity and specificity of *CGB3* and *NOP56* (91% and 90% respectively) were also higher than the than individual sensitivity and specificity of these two genes. The higher values for combined sensitivity and specificity for *CGB3* and *NOP56* genes demonstrated increased diagnostic power of these two genes acting as a combined marker for detecting cervical cancer with higher precision during the early stage (Puttipanyalears et al. 2013).

Furthermore, the study compared the diagnostic accuracies of the *CGB3* and *NOP56* genes in histology-based ASC-US PAP samples and cytology-based 'determined' PAP samples by determining the ROC and AUC values. Usually, the ROC curve analyzes the probability of classifying the TP and FP rates by setting the thresholds based on the AUC value. However, for the majority of biomarkers, a cut-off value needs to be set above a certain threshold of the methylation level in order to detect high specificity (Boers et al. 2016). The advantage of our methylation markers was that no cut-off threshold value was used. In this case, if the PCR product was negative (i.e., no amplification of specific product), the samples were categorized as "negative", and any ratio above zero was categorized as "positive", as has been reported in Boers et al., 2016. Based on the analysis of the diagnostic ability results, the AUC values for the *CGB3* and *NOP56* genes in the histology-based samples were found to be 0.83 and 0.71 respectively, which indicated that the *CGB3* and *NOP56* genes have an 83% and a 71% capability, respectively, to distinguish between the ASC-US and normal samples. Similarly, in the cytology-based samples, the *CGB3* and *NOP56* genes were shown to have AUC values of 0.74 and 0.63 respectively, which indicated that *CGB3* and *NOP56* respectively have a 74% and a 63% ability to distinguish between abnormal pre-cancerous samples and normal samples.

A subsequent analysis was performed to validate the *CGB3* and *NOP56* methylation levels in different HPV types, which were categorized as HPV16, HPV18, combined HPV (HPV16/18), 'HPV others and no HPV. HPV infection in patients with abnormal cytology is an underlying cause of cervical cancer, which accounts for 28.8-61.3% of the reported cases (Çilingir et al. 2013; González-Bosquet et al. 2008; Levert et al. 2000). At present and future, HPV genotyping positive for strains 16 and/or 18 signals high risk for cervical neoplasia with high or low grade of squamous cells (Huh et al. 2015; Einstein et al. 2011). From previous studied data, HPV type 16 is accounted for approximately 60% of all cervical cancers as compared to HPV18 and other HR HPV types which are responsible for15% and 35% of all cervical cancers, respectively as reported in (Gultekin et al. 2018; Dursun et al. 2009). From this study, the overall HPV types infections was found to be 87.5%/ 175 of total 200 PAP samples which were further subdivided into the incidents of HR-HPV types (HPV16 -40% / 80 samples, for HPV 18 -13% / 26 and 2% / 4 samples for combined HPV16_18 strain) and other HPV types strain were 32.5% / 65 samples. The remaining no HPV type of strain were 12.5% /25 out of total 200 samples. The overall methylation percentage was found to be the highest in HR-HPV types (HPV16 andHPV18) with methylation of 68% and 87.5%, respectively. Therefore, the present study demonstrated that *CGB3* and *NOP56* showed higher methylation percentage in HR-HPV strains which indicated higher probability of cervical cancers and should be taken as a precautionary measure to further prevent the progression of cervical cancer infection to more dangerous levels.

CONCLUSION

The overall objective of this research was to identify the methylation markers for treatment of cervical cancer patients. Th findings from the study reveals that out of the three genes shortlisted (*CGB3, NOP56 and GSTP1*) from the list of genes selected (*GSTP1, PDI-A3, VAV1, ING1, IL4R alpha, CGB3* and *NOP56*) from literature review and bioinformatics, only 2 genes i.e *CGB3* and *NOP56* had characteristic to be used as DNA methylation markers based on methylation detection.

Based on the first objective of this study, the findings reveal that HPV 16-E7 induces promoter methylation in *CGB3, GSTP1* and *NOP56* genes, thereby showing association between gene expression and promoter methylation for *CGB3, GSTP1* and *NOP56* regulated by HPV 16 E7. Furthermore, the re-expression of *CGB3* and *NOP56* genes were accessed by the treatment of 5-aza drug with high concentration in SiHa (HPV+) and low concentration in C33A (HPV-) cell lines which demonstrated that HPV+ samples may need to serve or be treated with higher concentrations of 5-aza drug to induce re-expression. However, *GSTP1* gene did not show re-expression with identifiable patterns after being treated with 5-aza drug therefore this gene was not considered further for the research after that step.

Based on the second objective of this study, the findings reveal that, *CGB3* and *NOP56* promoter methylation were detected in PAP smear samples which were classified into cytology and histology-based samples. Among which *CGB3* showed higher sensitivity and specificity to distinguish between the abnormal and normal samples at a statistically significant level for both histology (biopsy based) and cytology (observation based). This demonstrated that *CGB3* have potential to detect abnormal samples at their early stage and may serve as good DNA methylation marker, it can also be helpful for clinician to diagnose malignancy in the ASC-US samples in their early stages.

This research opens up new opportunities for academicians to perform further research in the domain of identifying base characteristics of DNA methylation markers and the at the commercial level these findings can be used in future to further

test and develop large scale methylation markers for detecting abnormal samples for preventing further spread of cervical cancers.

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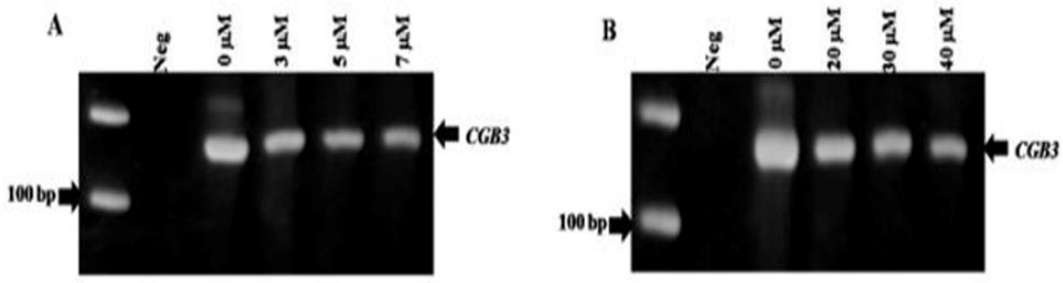
APPENDIX

1. Effect of 5-Aza on promoter methylation of *CGB3 NOP56* and *GSTP1* represented by Gel pictures

The promoter methylation status of *CGB3, NOP56* and *GSTP1* were analyzed in SiHa (0-40 µM) and C33A (0-7 µM) cell line treated with 5-Aza represented by gel picture. The band density of *CGB3* in C33A and SiHa cells were gradually decrease as 5-Aza concentration increases shown in Fig 57A and Fig 57B, respectively. Similarly, the methylation band intensity of *NOP56* gene was gradually decreases in both cell lines C33A and SiHa as 5-Aza increases shown in Fig. 58A and Fig. 58B. However, methylation band intensity of *GSTP1* gene was not get decreases while 5-Aza increases shown in Fig 59A and Fig 59B.



CGB3 METHYLATION IN C33A CGB3 METHYLATION IN SiHa

Figure 57 Methylation of *CGB3* in C33A (A) and SiHa (B) after 5-Aza treatment with band intensity %.

Figure A

- Neg – Negative control
- 0 µM C33A – control, 38.9%
- 3 µM C33A – 25.34%
- 5 µM C33A – 19.49%
- 7 µM C33A -16.24%

Figure B,

- Neg – Negative control
- 0 µM SiHa – control,19.4%
- 20 µM SiHa- 5.6%
- 30 µM SiHa- 4.3%
- 40 µM SiHa- 3.6%

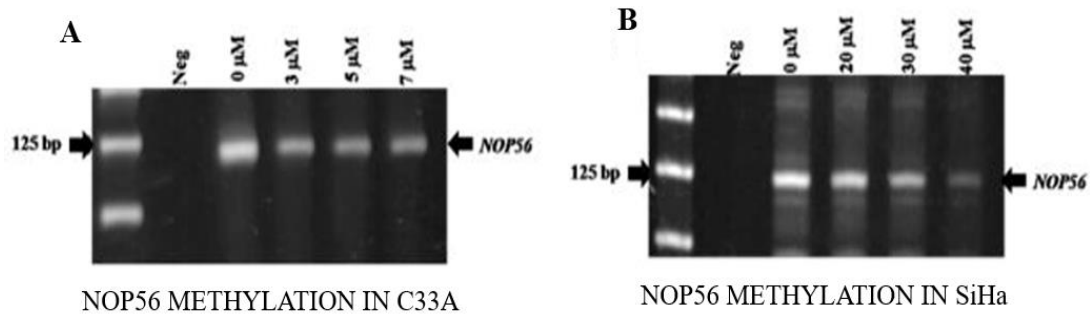


Figure 58 Methylation of *NOP56* in C33A (A) and SiHa (B) after 5-Aza treatment

Figure A,

- Neg – Negative control
- 0 µM C33A- control,47.2%
- 3 µM C33A- 21.06%
- 5 µM C33A- 18.1%
- 7 µM C33A- 13.5%

Figure B,

- Neg – Negative control
- 0 µM SiHa -control, 62.3%
- 20 µM SiHa- 17.3%
- 30 µM SiHa- 11.0%
- 40 µM SiHa- 9.2%

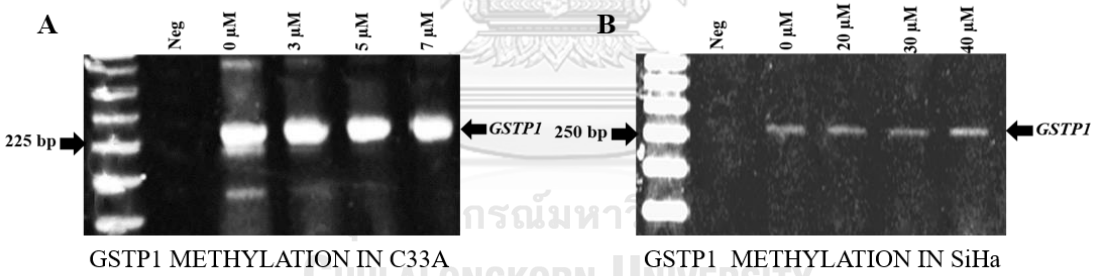


Figure 59 Methylation of *GSTP1* in C33A (A) and SiHa (B) after 5-Aza treatment

Figure A,

- Neg – Negative control
- 0 µM C33A- control, 33.5%
- 3 µM C33A- 22.5%
- 5 µM C33A- 21.7%
- 7 µM C33A- 32.6%

Figure B,

- Neg – Negative control
- 0 µM SiHa- control, 25.4%
- 20 µM SiHa- 23.8%
- 30 µM SiHa- 19.3%
- 40 µM SiHa- 29.3%

2. Effect of 5-Aza on expression of *GSTP1* represented by Gel pictures

The expression level of *GSTP1* was analyzed in SiHa (0-40 µM) and C33A (0-7 µM) cell line treated with 5-Aza represented by gel picture Fig 60A and Fig 60B.

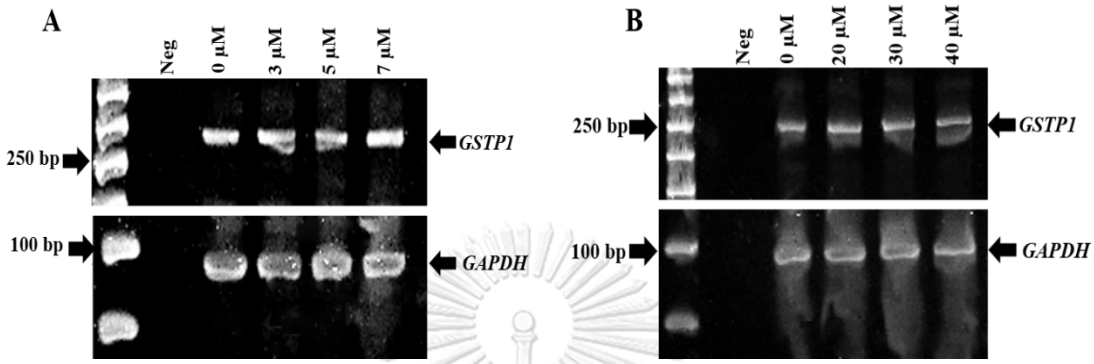


Figure 60 Expression of *GSTP1* in C33A and SiHa cell lines

Figure A,

- Neg – Negative control
- 0 µM C33A-control, 23.7%
- 3 µM C33A- 28.2%
- 5 µM C33A- 20.3%
- 7 µM C33A- 27.7%

Figure B,

- Neg – Negative control
- 0 µM SiHa- control, 18.5%
- 20 µM SiHa- 24.3%
- 30 µM SiHa- 18.8%
- 40 µM SiHa- 16.6%

3. Excel sheet of 200 PAP samples

Detection of methylation band of CGB3 and NOP56 in PAP samples PAP samples were categorized into two parts on basis of cytology (Determined PAP samples) histology (ASC-US) samples which were shown by Table 1 and Table 2, respectively. CGB3 and NOP56 promoter methylation band were detected in all the 200 PAP samples among which some of the samples were mentioned by gel picture which included normal samples (without HPV), normal samples with HPV, low-grade samples (LSIL), high-grade samples (HSIL) and cancer, shown after the Table 19 and 20.

Table 2 Excel sheet of Determined PAP samples.

The Table includes specimen (sample number), Types of HPV present, Coloposcopic result, Classification of samples and presence of CGB3 and NOP56 band (Yes or No)

Specimen	HPV TYPES	Cytology	CGB3	NOP56
B835	No-HPV	Normal with no HPV	Y	Y
B836	No-HPV	Normal with no HPV	Y	Y
B838	No-HPV	Normal with no HPV	Y	Y
B841	No-HPV	Normal with no HPV	N	N
B849	No-HPV	Normal with no HPV	N	N
B850	No-HPV	Normal with no HPV	N	N
B848	No-HPV	Normal with no HPV	N	N
B847	No-HPV	Normal with no HPV	N	N
B846	No-HPV	Normal with no HPV	N	N
B845	No-HPV	Normal with no HPV	N	N
B844	No-HPV	Normal with no HPV	N	N
B842	No-HPV	Normal with no HPV	N	N
B839	No-HPV	Normal with no HPV	N	N
B837	No-HPV	Normal with no HPV	N	N
S828	16	HSIL	Y	Y
S823	16	HSIL	Y	Y
S822	16	HSIL	Y	Y
T476	16	Cancer	Y	Y
T481	18	Cancer	Y	Y
T483	16	Cancer	Y	Y
T489	18	Cancer	Y	Y
T491	18	Cancer	Y	Y
T492	16	Cancer	Y	Y
T494	18	Cancer	Y	Y
T518	16	Cancer	Y	Y
T515	16	Cancer	Y	Y

T514	16	Cancer	Y	Y
T513	16	Cancer	Y	Y
T511	18	Cancer	Y	N
T507	18	Cancer	Y	N
T505	18	Cancer	N	N
T504	16	Cancer	N	N
T503	18	Cancer	N	N
T501	16	Cancer	N	N
T499	16	Cancer	Y	Y
T495	16	Cancer	Y	Y
S1202	18	LSIL	Y	Y
S1195	16	LSIL	Y	Y
S1193	16	LSIL	Y	Y
S1192	16	Cancer	Y	Y
S1181	16	LSIL	Y	Y
S1176	16	LSIL	Y	Y
S1173	16	LSIL	Y	Y
S1168	18	LSIL	N	N
S1138	16	LSIL	Y	N
S1127	18	LSIL	Y	Y
S1090	16	LSIL	Y	Y
S1060	16	LSIL	N	N
CU584	16	Normal with HPV	Y	Y
CU569	16	Normal with HPV	Y	Y
CU499	16	Normal with HPV	Y	Y
CU332	16	Normal with HPV	Y	N
CU159	16	Normal with HPV	Y	N
CU149	16	Normal with HPV	N	N
CU145	16	Normal with HPV	N	N
CU124	16	Normal with HPV	N	N
S1203	16	HSIL	Y	Y

S1185	16	HSIL	Y	Y
S1178	16	HSIL	Y	Y
S1174	16	HSIL	Y	Y
S1161	16	HSIL	Y	Y
S1152	16	HSIL	Y	Y
S1144	16	HSIL	Y	Y
S1143	16	HSIL	Y	Y
S1134	16	HSIL	Y	Y
S1130	16	HSIL	Y	N
S1097	16	HSIL	Y	N
S1094	16	HSIL	Y	N
S1089	16	HSIL	Y	N
S1086	16	HSIL	Y	N
S1082	16	HSIL	N	N
S1064	16	HSIL	Y	N
S1059	16	HSIL	N	N
S1057	18	LSIL	Y	N
S924	18	HSIL	Y	Y
S923	18	HSIL	Y	N
S914	18	HSIL	Y	N
S909	16	HSIL	Y	N

Table 3 Excel sheet of ASC-US samples.

The table includes specimen (sample number), Types of HPV present, Coloposcopic result, Classification of samples and presence of CGB3 and NOP56 band (Yes or No)

Specimen	**Ascus HPVType**	**Classification**	**CGB3**	**NOP56**
AS3	Other	Normal with HPV	N	N
AS5	No-HPV	HSIL	N	Y
AS22	Other	Normal with HPV	N	N

AS24	Other	Normal with HPV	N	Y
AS25	16	HSIL	Y	Y
AS28	18	Normal with HPV	Y	Y
AS33	No-HPV	Normal with no HPV	N	Y
AS 35	Other	Normal with HPV	N	N
AS 38	18	Normal with HPV	Y	Y
AS 41	Other	Normal with HPV	N	N
AS 43	16	HSIL	N	N
AS 44	Other	LSIL	Y	Y
AS 46	Other	LSIL	Y	Y
AS 47	18	HSIL	Y	N
AS 48	Other	HSIL	Y	Y
AS51	Other	HSIL	Y	N
AS52	16	LSIL	Y	Y
AS53	Other	Normal with HPV	N	N
AS56	Other	Normal with HPV	N	N
AS59	No-HPV	LSIL	Y	Y
AS60	16_18	Normal with HPV	N	N
AS61	Other	Normal with HPV	Y	Y
AS62	Other	Normal with HPV	N	N
AS63	No-HPV	Normal with no HPV	N	N
AS64	Other	Normal with HPV	N	N
AS65	Other	Normal with HPV	N	N
AS66	Other	LSIL	Y	Y
AS67	No-HPV	LSIL	Y	Y
AS68	18	Normal with HPV	N	Y
AS69	Other	Normal with HPV	N	N
AS70	16	Normal with HPV	N	N
AS72	16_18	Normal with HPV	Y	Y
AS73	18	Normal with HPV	N	Y
AS74	Other	Normal with HPV	N	Y

AS75	Other	LSIL	Y	Y
AS80	No-HPV	Normal with no HPV	N	N
AS81	Other	LSIL	Y	Y
AS82	Other	Normal with HPV	N	N
AS83	No-HPV	Normal with no HPV	N	N
AS84	No-HPV	Normal with no HPV	N	N
AS85	16	Normal with HPV	N	N
AS86	Other	LSIL	Y	N
AS87	Other	HSIL	Y	Y
AS89	Other	LSIL	Y	Y
AS91	No-HPV	Normal with no HPV	N	N
AS92	Other	HSIL	Y	Y
AS93	16	Normal with HPV	N	N
AS94	Other	Normal with HPV	N	N
AS95	18	Normal with HPV	Y	Y
AS97	16	LSIL	Y	Y
AS98	No-HPV	Normal with no HPV	N	N
AS180	Other	LSIL	Y	Y
AS181	Other	Normal with HPV	Y	N
AS183	16	Normal with HPV	N	Y
AS193	16	LSIL	Y	Y
AS184	16	Normal with HPV	N	N
AS194	Other	LSIL	Y	Y
AS195	Other	LSIL	Y	N
AS196	Other	Normal with HPV	Y	Y
AS197	16	HSIL	Y	Y
AS101	16	HSIL	Y	Y
AS102	Other	Normal with HPV	N	N
AS103	16	LSIL	Y	Y
AS104	16	LSIL	Y	Y
AS105	Other	Normal with HPV	N	Y

AS106	16_18	LSIL	Y	Y
AS109	16_18	Normal with HPV	Y	Y
AS113	16	Normal with HPV	N	N
AS115	16	LSIL	Y	Y
AS120	Other	Normal with HPV	N	N
AS121	18	Normal with HPV	N	N
AS122	18	Normal with HPV	N	N
AS123	Other	HSIL	Y	Y
AS124	Other	Normal with HPV	N	N
AS125	Other	Normal with HPV	N	N
AS128	16	HSIL	Y	Y
AS130	Other	LSIL	Y	N
AS131	18	Normal with HPV	Y	Y
AS132	Other	LSIL	Y	Y
AS133	Other	Normal with HPV	N	N
AS135	16	HSIL	Y	Y
AS136	Other	Normal with HPV	Y	Y
AS137	16	LSIL	Y	Y
AS138	16	Normal with HPV	N	N
AS139	Other	Normal with HPV	N	N
AS140	Other	Normal with HPV	Y	Y
AS142	Other	Normal with HPV	N	N
AS144	Other	Normal with HPV	N	Y
AS145	Other	Normal with HPV	N	N
AS146	16	Normal with HPV	N	N
AS147	Other	Normal with HPV	N	N
AS149	Other	Normal with HPV	N	N
AS151	Other	LSIL	Y	Y
AS152	16	HSIL	Y	Y
AS154	Other	LSIL	Y	N
AS155	Other	Normal with HPV	Y	Y

AS156	16	Normal with HPV	N	N
AS160	Other	Normal with HPV	N	N
AS161	16	HSIL	N	N
AS162	16	Normal with HPV	N	N
AS164	Other	LSIL	N	N
AS166	Other	LSIL	Y	Y
AS168	Other	LSIL	Y	Y
AS170	Other	Normal with HPV	Y	Y
AS173	Other	LSIL	Y	N
AS175	Other	Normal with HPV	N	N
AS177	16	Normal with HPV	Y	Y
AS207	No-HPV	Normal with no HPV	N	N
AS210	Other	LSIL	Y	Y
AS211	Other	Normal with HPV	Y	N
AS213	18	Normal with HPV	N	N
AS214	18	Normal with HPV	N	Y
AS215	Other	Normal with HPV	Y	Y
AS219	Other	Normal with HPV	N	N
AS221	16	LSIL	Y	N
AS223	16	Normal with HPV	N	N
AS225	Other	Normal with HPV	N	Y
AS226	16	LSIL	Y	Y
AS227	Other	LSIL	N	N
AS230	16	Normal with HPV	Y	N
AS233	16	Normal with HPV	N	N
AS240	Other	LSIL	Y	Y

4. Detection of *CGB3* and *NOP56* methylation in PAP samples

The detection of *CGB3* and *NOP56* methylation gene in ASC-US and determined PAP sample were shown by gel picture using specific primer of *CGB3* and *NOP56* with band size 116 bp and 120 bp, respectively.

1. *CGB3* detection in Normal samples (without HPV)

   The classification of each sample was mentioned in Table 1 and 2 in detail. This gel pictures of normal samples (without HPV) showed that there was no meth band were detected in the mentioned samples. AS33, AS63, AS80, AS83- ASC-US (normal samples without HPV) and B848, B849, B850, AS207- Determined (normal without HPV), SiHa Bisulfite DNA were used as meth control and water was used as negative control.

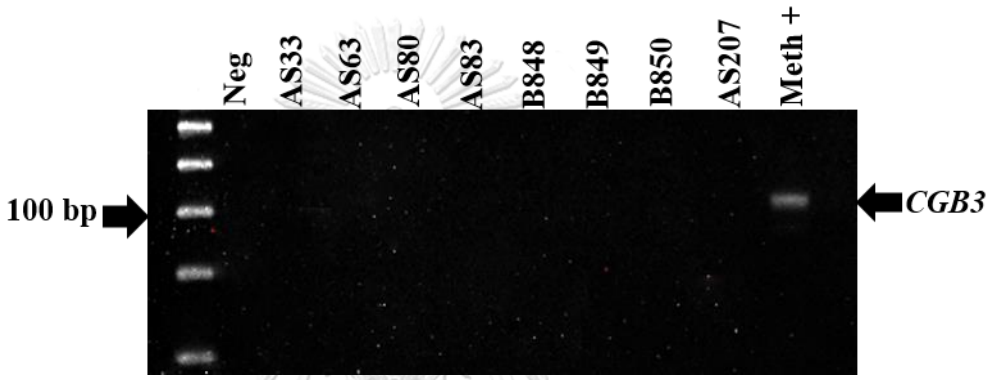


Figure 61. CGB3 detection in Normal samples (without HPV)

2. *CGB3* detection in Normal samples (with HPV)

   The presence of band shows methylation of CGB3 in samples and if there was no meth band detected it means there was no methylation by this gene in the particular samples. Samples AS3, AS53, AS68, AS73 show no band intensity while samples AS61, AS72, AS28, AS95 showed meth band. SiHa Bisulfite DNA were used as meth control and water was used as negative control.

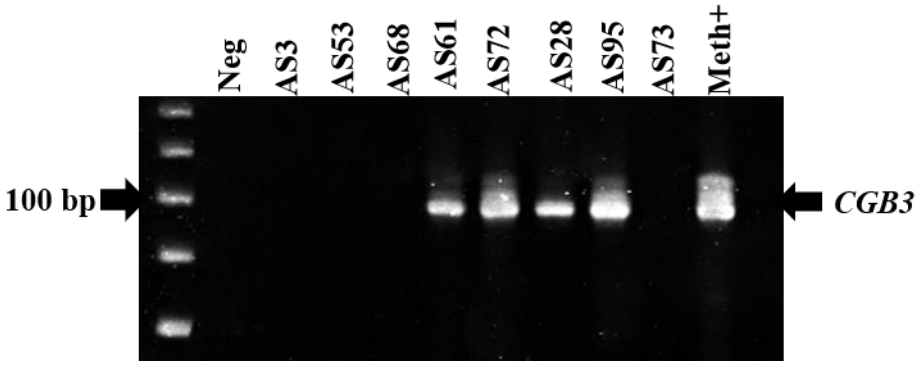


Figure 62. CGB3 detection in Normal samples (with HPV)

3. CGB3 detection in LSIL and HSIL samples

   Specimen AS44, AS89, AS195, S1127, S1181were LSIL from both ASC-US and PAP samples detected by CGB3 methylation gene. Moreover, in sample number S164 and S1168 there was no CGB3 meth band were detected. Sample AS51,S1178,S1161 were HSIL and get detected by CGB3 methylation gene.

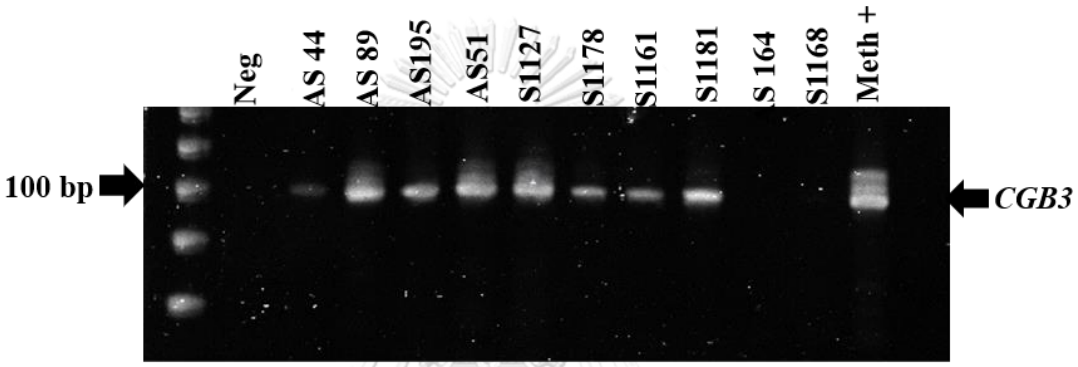


Figure 63. CGB3 detection in LSIL and HSIL samples

4. CGB3 detection in HSIL and Cancer samples

   CGB3 meth band were 80% detected by HSIL and cancer samples as shown by giving few examples of the samples.

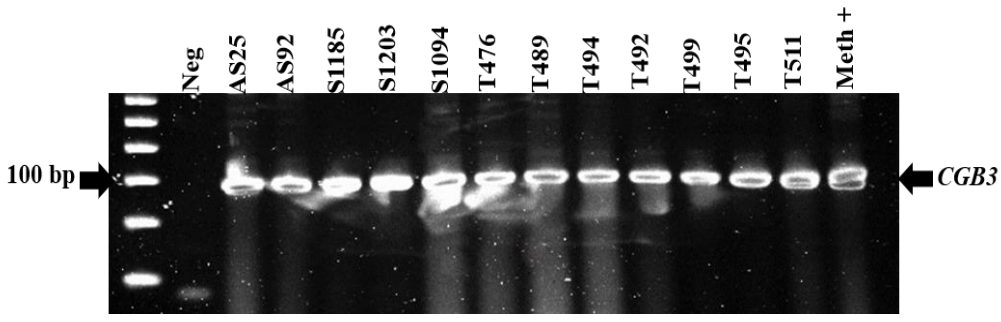


Figure 64. CGB3 detection in HSIL and Cancer samples

5. NOP56 detection in normal samples (without HPV)

The NOP56 meth band were detected in both ASC-US (AS33) and Determined PAP samples (B835 and B838). The other specimen AS63, AS83, AS84, B846, B849, B843, B837, B845 had no band detection of NOP56 gene.

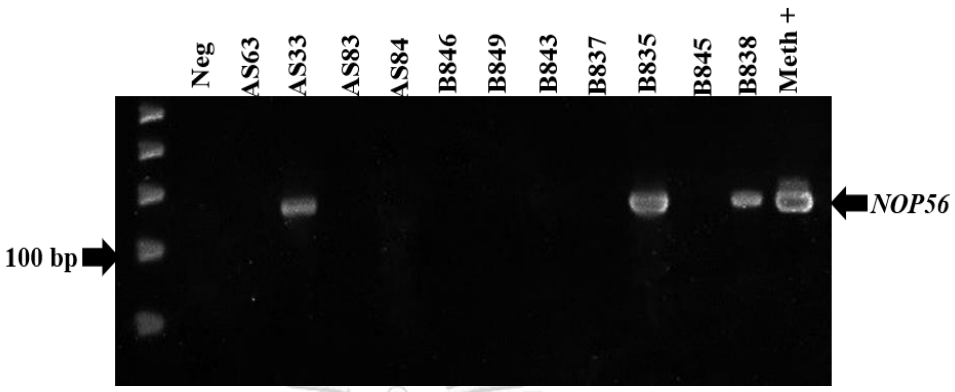


Figure 65. NOP56 detection in normal samples (without HPV)

6. NOP56 detection in normal samples (with HPV)

   The detection of NOP56 meth band were analyzed in specimen AS24, AS28, AS68, AS73 were belongs to ASC-US (normal with HPV) category while specimen CU584, CU569, CU499 were belongs to Determined (normal with HPV sample). The specimen AS65, AS64 and AS82 had no meth band of NOP56 gene.

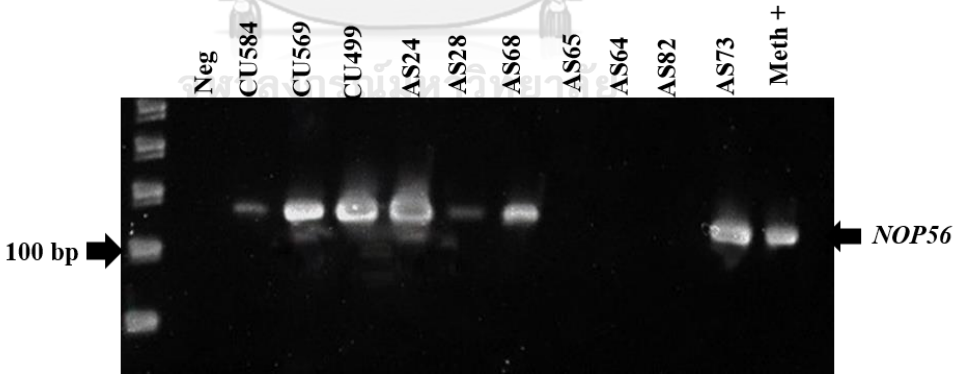


Figure 66. NOP56 detection in normal samples (with HPV)

7. NOP56 detection in LSIL samples

   All the low-grade (LSIL) specimens were detected by NOP56 gene except AS75 belongs to ASC-US category doesn’t show any NOP56 gene band.

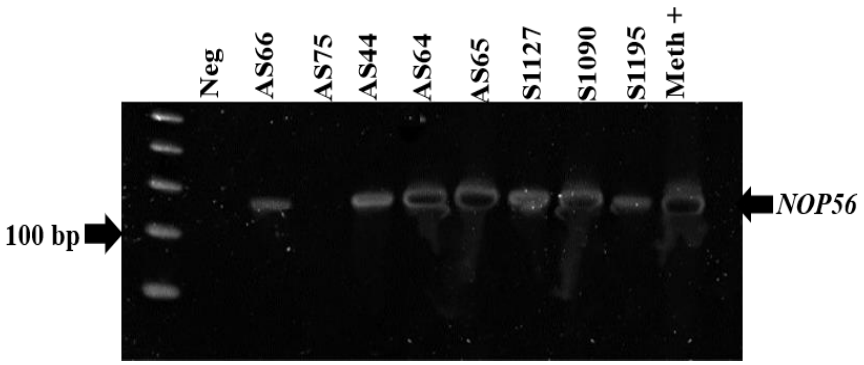


Figure 67. NOP56 detection in LSIL samples

8. NOP56 detection in HSIL and cancer samples

   NOP56 meth band detected in HSIL and cancer samples.

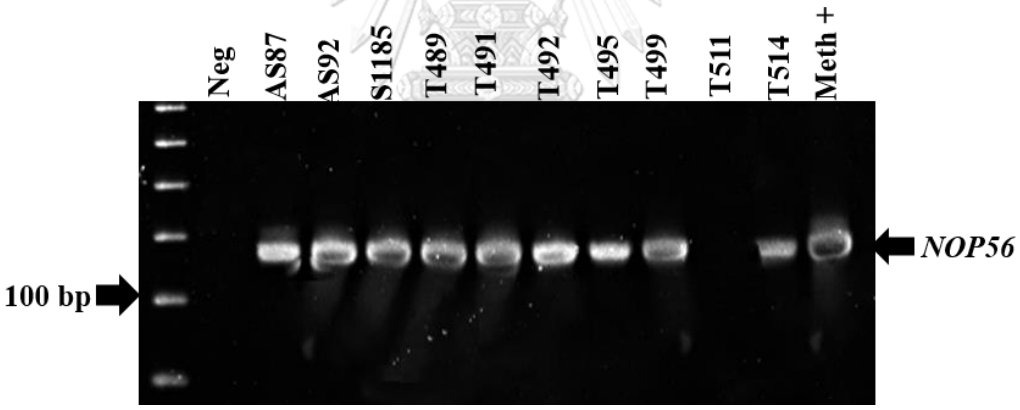


Figure 68. NOP56 detection in HSIL and cancer samples

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