國防醫學院生命科學研究所博士論文

A thesis submitted to Molecular and Cell Biology Program,
Taiwan International Graduate Program of
National Defense Medical Center and Academia Sinica

in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
in Molecular and Cell Biology

以甲基化生物標記作為高危險型人類乳突病毒感染婦女之子宮頸癌篩檢

Triage high risk HPV infected women with methylation biomarker for cervical cancer screening

研究生: 巴普

Student: Par Bahadur Pun

指導教授: 賴鴻政博士

Advisor: Dr. Hung-Cheng Lai

中華民國 104 年 12 月

December 2015

(國防醫學院 與 中央研究院合辦)

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授權書

本授權書所授權之論文為本人在國防醫學院 生命科學 研究所 104 年班畢業論文。

論文名稱：**以甲基化生物標記作為高危險型人類乳突病毒感染婦女之子宮頸癌篩檢**

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國防醫學院生命科學研究所博士論文

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生命科學研究所研究生 巴普 (PAR BAHADUR PUN) 所提之論文

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係由本人指導撰寫，同意提付審查。

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博士論文審定書

Verification letter from the Oral Examination Committee

論文題目 Thesis Title **Triage high-risk hpv infected women with methylation biomarker for cervical cancer screening**

本論文係巴普(Par Bahadur Pun)於生命科學研究所完成之博士論文，經考試委員審查及口試合格，特此證明。

This is to verify that the thesis written by Mr. Par Bahadur Pun has been evaluated and qualified by oral defense examination committee.

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Dedicated to

My Parents and family

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Par Bahadur Pun

December, 2015

Title **Page no**

List of Figures

List of Tables

Triage high risk HPV infected women with methylation biomarker for cervical cancer screening

Par Bahadur Pun, PhD

Abstract

High-risk HPV types of oncogenic human papillomavirus (HPV) types infects cervical epithelium, where HPV 16 and 18 are the most common type. High-risk HPV types are necessary factor that result epigenetic changes leading to progression to cervical cancer. Insufficient specificity of high-risk human papillomavirus (hrHPV) assay in distinguishing relevant high grade precancerous lesions in primary cervical cancer screening results excess unnecessary referral for colposcopy and medical burden. Additional highly specific assays to triage hrHPV-positive women are needed to overcome these drawbacks of HPV assay so as to improve the clinical efficacy of molecular cervical cancer screening. There is potential trend to utilize DNA methylation is a promising biomarker in cervical cancer. However, there are limited clinically useful biomarkers and no DNA methylation biomarkers are at clinical application phase among those biomarkers in spite of promising result. Therefore, we evaluated the clinical performance of potentially methylated candidate genes as a triage assay for hrHPV-positive women which could be translated to clinical application for cervical cancer screening.

We conducted a retrospective hospital-based case–control study in Taiwan. Cervical scrapings were collected before colposcopy for hrHPV testing and quantitative methylation-specific PCR (QMSP) of 16 genes. Five genes, *POU4F3, HS3ST2, AJAP1, PAX1*, and *SOX1,* were prioritized for the clinical performance to triage hrHPV-positive women while analyzing the clinical performance of these genes in 100 cervical scraping. In the validation phase, two

hundred cervical scrapings were randomly classified into a training set (n = 111) and testing set (n = 89). The study group in validation phase comprised 31 and 19 cervical intraepithelial neoplasm I (CIN1), 28 and 27 cervical intraepithelial neoplasia III (CIN3)/ carcinoma *in situ* (CIS), 18 and 12 squamous cell carcinomas (SCCs)/ adenocarcinomas (ACs) in training and testing set respectively. All samples were tested for hrHPV using a Hybrid Capture II (HCII) assay. HrHPV-positive women were subjected to DNA methylation analysis by real-time quantitative methylation-specific polymerase chain reaction (QMSP) analysis. In the training set, the receiver operating characteristic (ROC) curves defined the optimal methylation index (M-index) cutoff values for discriminating $CIN3^+$ from CIN1/normal, which then were applied to the testing set. Among the five genes, *POU4F3* revealed the highest area under the ROC curve (AUC) (0.86; 95 % CI, 0.78–0.95) in detecting $CIN3^+$. In the testing set, *POU4F3* revealed the best clinical performance in triage of hrHPV-positive women with a sensitivity of 74 % and specificity of 89 % for detecting $CIN3^+$.

We validated that *POU4F3* methylation analysis is a potential molecular tool for triage in detecting $CIN3^+$ in hrHPV-positive women. The combined use of broad-spectrum HPV assay and *POU4F3* methylation analysis as a new generation of molecular cervical cancer screening warrants further population-based study.

以甲基化生物標記作為高危險型人類乳突病毒感染婦女之子宮頸癌篩檢

巴普，博士

中文摘要

高危險群人類乳突病毒(hrHPV)感染子宮頸上皮細胞，造成表觀遺傳(epigenetic) 的改變為子宮頸癌發生的必要因子，而其中以 HPV 16 和 18 的類型最為常見。在子宮頸癌篩選中，對於判別 hrHPV 造成的癌前期病變 (precancerous lesions) 的測試專一性不足，而導致不必要的陰道鏡檢查轉診與醫療負擔。為了克服上述缺點，需要另外的高專一性 HPV 篩檢來分類 hrHPV 陽性女性，以提高子宮頸癌分子檢測的臨床功效。儘管研究結果顯示 DNA 甲基化有當作子宮頸癌生物標記 (biomarker) 的潛力，但目前只有有限的生物標誌且沒有以 DNA 的甲基化作為生物標記進入臨床應用階段。因此，我們評估基因的甲基化對於分類 hrHPV 陽性女性的功效，進而應用於臨床上子宮頸癌的篩檢。

我們執行一個以醫院為基礎的回溯性 (retrospective) 個案對照研究 (case-control study)。在陰道鏡檢查與 16 個基因的甲基化特異性聚合酶連鎖反應 (quantitative methylation-specific PCR, QMSP) 前收集子宮頸抹片樣品。優先選出 *POU4F3*、*HS3ST2*、*AJAP1*、*PAX1* 和 *SOX1* 等五個基因用於分類 hr-HPV 陽性女性。兩百個子宮頸抹片樣品隨機分為訓練集 (training set，n=111) 與測試集 (testing set，n=89)。所有的樣品皆利用 Hybrid Capture II 做 hr-HPV 篩檢。hr-HPV 陽性女性的樣品接著利用 QMSP 做 DNA 甲基化分析。在 training set 中，利用接受器操作特性曲線 (Receiver Operating Characteristic curve, ROC

curve) 定義最佳的 methylation index (M-index)分界值 (cutoff values) 以區分子宮頸上皮內瘤樣病變三級 (CIN3$^+$) 與一級 (CIN1)， 然後應用於 testing set。在上述五個基因中，*POU4F3* 在偵測 CIN3$^+$ 中有最大的曲線下的面積 (0.86; 95 % CI, 0.78–0.95)。在 testing set 中，*POU4F3* 在分類 hr-HPV 陽性女性的樣品有最佳的臨床表現，其敏感度 (sensitivity) 與特異度 (specificity) 分別為 74%與 89%。

我們證實了 *POU4F*3 基因甲基化是一個有潛力的分子工具，用來偵測 hr-HPV 陽性女性中 CIN3$^+$。合併廣效性的 HPV 偵測和 *POU4F3* 基因甲基化分析，將可作為新一代子宮頸癌的分子檢測，並且值得進一步做族群為基礎 (population-based) 的研究。

Chapter 1. Introduction

1.1 Cervical cancer

Cancer is a disease of aberrant uncontrolled division, proliferation and growth of abnormal cells of the body. The affected cells must acquire various biological traits to turn them as a malignant tumor [1]. Biological attributes of cancer include

a) Self-sufficiency in growth without any external growth signal.
b) Possess the potential to have unlimited replication because of break of barrier of intrinsic switch to inhibition of cell division after many cycles of cell divisions.
c) Not able to control growth by inhibition signal because of its insensitive nature for antigrowth signals
d) Acquisition of apoptosis resistance mechanisms that make cancer cells to evade apoptosis
e) Acquire properties to have continue angiogenesis that are favorable for metastatic lesions
f) Invasive nature leading to metastasize to surrounding tissues

Cancer of cervix basically involves squamous cells of the cervix that undergo rapid abnormal growth with aberrant function and phenotype during transition from normal to cancerous form.

1.1.1 Cervical cancer incidence

Cervical cancer is the third most common cancer among women worldwide and overall seventh common cancer among overall cancer as well as the fourth leading cause of death duet to caner in females globally. Cervical cancer comprises around 500000 new

cases every year worldwide that places it at as one of the important medical burden. GLOBOCAN reported overall 528,000 new cases and 266,000 deaths of cervical cancer in 2012, among them less developed countries account for major portion of the disease burden with 445,000 cases and 230,000 deaths [2]. The regions having highest incidence rates of cervical cancer are developing countries such as Africa, Melanesia, Central and South America, Caribbean, South-Central and South East Asia [2]. This figure highlighted that the intensity of disease incidence is more in developing countries and urgent need of suitable preventive intervention.

1.1.2 Cervical cancer and its precursor lesions

Lower part of uterus is cervix, which consists of the ectocervix (outer part towards vagina) and endocervix (inner part towards uterine canal). Cancer of cervix uteri take places especially lower one third of uterus composed of squamous and columnar epithelium. Squamous epithelium preliminary lines ectocervix, whereas columnar epithelium covers the endo cervical canal. The squamocolumnar junction (SCJ), the area demarcated by these two types of cells, is the region of constant metaplastic changes with transformation of columnar cells to squamous epithelium during puberty. The SCJ shifts from original place of the ectocervix to the new SCJ towards endocervix along with age. The area between these two new or old SCJ is termed transformation zone (TZ). Since this areas is in danger to subject for neoplastic alterations, abnormal TZ is consider as the region of origin of precancerous lesion and crucial for pathogenesis of cervical cancer [3, 4].

Histologically, cervical cancer can be classified in various subtypes, where majority are squamous cell carcinoma (about 80-85%) followed by adenocarcinoma (about 15% of cervical cancers) [5]. Cervical cancer is complex, progressive disease that develop through multi stages once arises from with pre-invasive precancerous precursor lesions, histologically acknowledged as cervical intraepithelial neoplasia (CIN). CIN lesions are further divided in to three groups, grade 1 to grade 3 as CIN1, CIN2 and CIN3 based on the distinct nature and progressive severity of epithelial cells of the lesions. CIN1 (equivalent to mild dysplasia) with dysplastic alterations of lower one third of squamous epithelium, CIN2 (moderate dysplasia) with changes in 2/3 of epithelium and CIN3 (severe dysplasia together with carcinoma *in-situ*) with involvement of more than 2/3 of the epithelium (Fig. 1) [6, 7]. Adenocarcinoma in situ is the precursor of adenocarcinoma. Based on the severity and associated cancer progression risk to develop invasive cancer, CIN1 is considered as low-grade CIN, whereas CIN2 and CIN3 are high-grade CIN.

A well accepted method of diagnosis of different stages of cervical cancer is based on analysis of cytological abnormalities in cervical cytology specimen so that further investigation such as biopsy for histology can be carried out for proper medical intervention and management of premalignant and malignant lesions. Bethesda system (TBS) 2001 is well accepted system of uniform classification terminology for interpretation of cervical cytology specimen and it classified squamous cell abnormalities in to following categories as negative for Intraepithelial lesion or malignancy, atypical squamous cells, low grade squamous intraepithelial lesion (LSIL) and high grade squamous intraepithelial lesion (HSIL) [8]. Atypical squamous cells (ASC) are equivocal

cellular abnormalities, which may have cervical intraepithelial neoplasia (CIN) without significantly distinguishable abnormal cells. This type of cellular abnormalities is further classified in to either ASCUS (of undetermined significance) or ASC-H (cannot exclude a high-grade lesion). Major categories of Bethesda classification are LGSIL and HGSIL where LGSIL may have CINI or II and HGSIL may show CIN II or III on biopsy. Most cases of LSIL regress, however HSIL may progress to invasive cancer [9].

1.1.3 Risk factor for cervical cancer

Following are important factors believe to associate with initiation and progression of cervical cancer.

HPV infection- Human Papilloma Virus (HPV) infection, a common sexually transmitted virus, is the most important etiology of cervical cancer that infects cervical epithelium, hijacks cellular machinery and enhances development of precancerous lesion to progression to invasive cancer. Although only around 5% HPV infected women develop cancer, persistent infection with HPV [10] along with other associated cofactors such as viral type, presence of multiple oncogenic type, viral load and host factors immune suppression, smoking, and age are responsible to precipitate development of cervical cancer.

Exposure to HPV: early age of intercourse and multiple sex partners have increased the risk of being exposure and acquisition of HPV, which is associated with development of cervical cancer [11, 12].

Other infection: *Trichomonas vaginalis* and bacterial vaginosis infection are very important factors significantly associated with persistent HPV infection and acceleration

of development and progression of cervical cancer [13].

Age: Although cervical cancer may occur at any women who ever had sex, the risk of developing cancer increased along with age and greater risk group is women with over 35 years of age [11].

Smoking: Results from long prospective study has confirmed that tobacco smoking is one of the important risk factors associated with CIN3/CIS and ICC [14].

Hormonal effect: Early pregnancy and multiple deliveries together with long term use of contraceptic pills results hormonal changes and are important factors to precipitate the cervical cancer development [13].

1.2 HPV and Cervical Cancer

Human papillomavirus (HPV) is sexually transmitted virus and one of the common infections frequently found in the young women above 20 years of age [15]. Cervical cancer is complication of persistence infection with certain type of the oncogenic human papillomavirus (HPV), which can be detected in the more than 90% of squamous cell carcinoma (SCC), cancer of protective epithelial layer, and around 80-90% of adenocarcinomas, cancer having secretary properties [16].

1.2.1 Human papillomavirus

Human papillomaviruses (HPVs) are small double-stranded DNA viruses belongs to papillomaviridae family. They consist more than 150 different types grouped in 5 different genera (α, β, γ, μ, η) based on DNA sequence analysis that share the tissue tropism and oncogenic potential within the genera (Fig. 2) [17, 18]. HPV virus can be classified in to

low-risk and high-risk type depending on the oncogenic potential of HPV. Low-risk type of HPV comprises 12 type of HPV such as 6, 11, 40, 42, 43, 44, 53, 54, 61, 72, 73 and 81 responsible for genital warts or minor cellular alternation of the cervical epithelium. Further, high-risk type of HPV consists of 13 type of HPV such as 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 [19]. Among them type 16 and 18 possess the highest oncogenic potential capable to progress in to advanced, pre-cancerous cervical cancer (CIN3) and are responsible for about 70% of cervical cancer rendering them as the most dangerous type among all. Rest of high-risk type HPVs are responsible for around 30% of cervical cancer [9, 19-21].

1.2.2 Viral genome organization

Human papillomaviruses (HPVs) are small non-enveloped icosahedral viruses of 50-60 nm in diameter with double-stranded, circular DNA (~7000-8000 bp) [18]. Based on the location, viral genome is divided in to three regions: the long control region (LCR), early region (E) and late region (L). The long control region (LCR) does not have coding potential, but critical for regulating the expression of viral gene. The early (E) region encoding six open reading frames (ORF) for six proteins (E1, E2, E4, E5, E6, E7) expressed immediately after host cell got infected with HPV virus and are critical for viral genome replication, gene expression and survival. Likewise, a late (L) region is responsible for encoding late genes (L1, L2) or viral capsid proteins. Major capsid protein L1 and the minor L2 are the two structural proteins of viral icosahedral capsid (Fig. 3). The terms “E” and “L” denote the stage of viral life cycle when first expression of these proteins occurred. Since early region encoded proteins possess significant role in viral proliferation, plethora

of studies emphasis in-depth exploration of the E2, E6 and E7 oncoproteins [22, 23]. Different viral oncoproteins have important function (Table 1) [22].

1.2.3 HPV life cycle

HPVs are intracellular parasites, which once gain entree to epithelium via micro abrasions, capable of hijacking cellular machinery of basal cell of epithelium for viral DNA synthesis critical for viral replication and propagation. L1 major capsid protein of HPV binds to heparin sulfate proteoglycans (HSPG) with the help of α6-integrin that exposes L2 minor capsid protein [24]. Once basal epithelium infected with HPV divides, the daughter cells push toward the surface triggering viral life cycle, where viral genome undergoes replication at S phase taking help of cellular DNA of these cervical epithelial cells to maintain as stable episomes [18]. Expression of viral genes increases along with viral differentiation dependent promoter upregulation. New viral particles are packed and shed during late stage of viral life cycle at upper layer of cervical epithelium (Fig. 4).

Viral oncoproteins are expressed in such a low level as to make them able to escape immune surveillance of host cells. Viral DNA replication is regulated by E2 together with E1, where E2 being transcriptional activator as in full-length protein and binds to URR of DNA to enhance early region's gene promoter and transcription of genes. Viral early protein E2 tightly regulated other early viral oncoprotein E6 and E7 via restricting expression of these oncoproteins. Viral integration with host genome abolish repressive function of E2 on E6 and E7 leading to overexpression of E6 and E7 proteins having binding partners P53 and pRb respectively and such binding disrupts the function of these cellular proteins [25]. E6 inhibits P53-mediated cellular apoptosis, whereas E7 mediates

loss of function of pRb leading to transactivation of cellular protein important for viral DNA replication. Persistent aberrant expression of viral genome and combined effect of overexpressed E6 and E7 oncoproteins abolish control of cell division leading to genomic instability and genetic changes, which thereby affect oncogenes and tumor suppressor genes for cancer progression [21, 26]. Furthermore, E6 and E7 also play significant role to induce epigenetic modification for promotion of cervical cancer development and progression (Table 1).

1.2.4 Nature history of cervical cancer

High-risk HPV (hrHPV) is associated with transformation of cervical epithelium. Persistence infection with hrHPV is well known prerequisite event for development of precancerous lesions of cervix and progression to invasive cancer. Although HrHPV infection is common among in women, not all of those hrHPV infected women develop cancer. It is because of self-regressive nature of hrHPV infection, where half of infections get clear within less than year and more than 80% of the infection clear by immune system with in few years after infection with HPV[15]. Indeed, only few of those hrHPV infected women develop cervical intraepithelial neoplasia (CIN) and SCC (Fig. 5) [26, 27].

When cervical epithelium infected with hrHPV, long sequence of multiple steps of persistence hrHPV infection mediated transformation of epithelial cells as precancerous lesions progressive in severity as CIN1, CIN2 and CIN3 (histological grade) occur, which progresses to invasive cervical cancer at the end (Fig. 6) [28]. Low-grade CIN is related to productive viral infections, whereas high-grade CIN is associated with transforming hrHPV infection and possesses the ability to progress to invasive carcinomas [29]. Basically,

present available techniques cannot predict the natural history of precancerous lesions of high-grade (CIN2 or more) as which lesions will regress from those of progressive lesions. Most of the lesions can regress, persevere, or progress, however the regression rate progressively decreases along with increasing severity of lesions. Progression of high-grade CIN to invasive cancer depends on multiple factors such as type of oncogenic hrHPV present at lesions, viral loads, duration of persistent infection, evasion of immune barrier and clonal progression of infected cervical epithelial cells. Development of high-grade CIN2 and CIN3 lesions can take long duration probably 3-5 years after cervical epithelium infected with hrHPV [6]. However, development and progression to invasive cancer might require very long time up to 20-30 years after acquisition of hrHPV infection [30, 31].

1.2.5 Methods of HPV testing

There are challenges in routinely applying the traditionally methods as culturing of HPV, serological and virology techniques like electron microscopic examination and immunohistochemistry in detecting HPV [32, 33] . Since molecular-biology techniques possess the ability of accurate detection and typing of HPVs, most HPV diagnosis is now utilized this principle [34]. Currently, available molecular techniques for diagnosis of HPV include Nucleic acid-hybridization assays, signal amplification assays and nucleic-acid amplification. Different assays under these each of these techniques are outline in Figure (Fig. 7) and benefits and weaknesses of each of them are elucidated in Table (Table 2) [35].

There is plethora of tests to detect HPV and around 125 of them are reported to be commercially available. HPV tests confers wide clinical applications indicating continue growth of number of new and efficient HPV tests in the future [36]. Effective detection of

high-grade cervical cancer associated HPV infections and differentiation of them from those of transient infection are critical for hrHPV DNA testing. Optimal clinical performance of HPV testing with balance sensitivity and specificity in detection of CIN2+ is vital for testing. HPV testing confers added advantages such as higher sensitivity and higher negative predictive value (NPV) than the cytology test, where negative HPV test reassures the absence of the clinically significant cervical cancer associated lesions. Further, HPV testing possess the advantages of having high reproducibility, no inter-observer variation, high throughput and automation. Cut-off thresholds that have been evaluated and validated to adapt for clinical application are also important. First HPV DNA test approved in 1999 by Food and Drug Administration (FDA) was the Hybrid Capture 2 (HC2) HPV DNA Test (QIAGEN Inc., Gaithersburg, MD; USA; previously Digene Corp), that is capable of detecting 13 high risk type of HPV. Since then number of HPV test has been increasing. Currently, Food and Drug Administration (FDA) has approved five tests for clinical application. DNA-based test outnumbered the RNA-based assay, where four out of 5 tests are based on HPV DNA detection such as HC2, Cervista HPV HR (Hologic, WI, USA), Cervista HPV 16/18 (Hologic Inc., Bedford, MA, USA) and Cobas 4800 (Roche Molecular Diagnostics, Pleasanton, CA, USA). The remaining one test is an RNA-based assay (APTIMA HPV assay; formerly GenProbe Inc., San Diego, CA, USA), which was approved by FDA in 2011[37]. Cervista HPV16/18 is a test useful for panel of HR HPV types and for HPV16/18 genotype [38]. Further, the Cobas 4800 HPV test, which was approved by FDA in April 2011, allows detection of HPV16/18 genotype together with testing for 12 other high-risk HPV types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) and it was evaluated in ATHENA study [39].

1.2.5.1 Nucleic acid-hybridization assays

Techniques such as southern blotting, in situ hybridization, and dot-blot hybridization are employed in assays of this category to detect HPV infection in cervical samples. Radiolabeled nucleic acid is used to hybridize with the nucleic acid of the HPV that generate high-quality information. However, there are many disadvantages of these direct-probe approaches that include low sensitivity, the need for relatively large amounts of purified DNA, and time-consuming procedures [32].

1.2.5.2 Signal-amplification assays

FDA approved two assays the Digene® HPV test using Hybrid Capture® II (HC II) technology, and the Cervista ® HPV HR assay for diagnostic testing utilized the principle of signal amplification [40]

The HC® II system (Digene, USA) is a non-radioactive signal amplification method based on the principle of hybridization of the target HPV-DNA as cocktail probes to long synthetic RNA probes complementary to the genomic sequences HPV DNA in solution. The resulting immobilized RNA-DNA hybrids are captured onto micro titer wells like 96 well plate (hence the name “hybrid capture”) and are detected with the help of specific monoclonal antibody and chemiluminiscence substrate. The intensity of emitted light, expressed as relative light units, is proportional to the amount of target DNA present in the specimen, and provides a semi-quantitative measure of the viral load. The recommended cut-off value for test-positive results is 1.0 relative light unit (equivalent to 1 pg HPV DNA per 1 mL of sampling buffer, about 5000 copies of the HPV genome). Two different probe cocktails, one containing probes for five low-risk genotypes: HPV 6, 11,

42,43 and 44 (probe cocktail A) and the other containing probes for 13 high-risk genotypes: HPV16,18,31,33,35, 39, 45, 51, 52, 56, 58, 59 and 68 (probe cocktail B) are used in two separate reactions. Since LR-HPV is not clinically significant, this assay generally utilizes only the HR-HPV probe set (probe cocktail B) in practice [41].

HC II was proved to have more advantages such as be more effective in triage of equivocal cytology (ASCUS_LSIL) than a repeated cytology examination [42]. Similarly, several randomized studies reported that incorporation of HPV testing in primary screening may diagnose 50%-70% more precancerous lesions [43-45], which reinforces application of HPV test as a screening test in addition to cytology screening for women 30 years of age and older. Since negative HPV test exclude the risk of HPV- related disease for subsequent years, this test may be very useful in increasing screening intervals to 3 to 5 years in HPV negative women. Other advantages include good inter laboratory reproducibility [46] and ease to perform the experiment. HCII confers comparable analytic sensitivity to some PCR methods for HPV DNA detection [47]. However, it also has limitation as being cable of detecting only high-risk and low-risk group and not able to distinguish specific HPV genotypes [40]. Further, cross-reactivity of the two type of probe cocktails might reduce the clinical relevance of the a positive test results [48, 49, 41].

The second FDA approved test based on the principle of signal amplification invader chemistry for the detection of specific nucleic acids is the Cervista® HPV HR Test (Cervista) (Hologic, Inc., Marlborough, MA, USA) that detects the presence of 14 HR-HPV types, consisting of -16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66 and -68. Cervista assay has a lower false-positive rate compared to the Digene assay, has an

internal control to detect the availability of sufficient HPV DNA material and requires less sample volume [40]. However, this test also could not detect individual HPV genotypes.

1.2.5.3 Nucleic-acid amplification

This technique utilizes exclusively type specific primers for amplification of nucleic-acid of a single HPV genotype present in cervical samples. In order to detect presence of multiple HPV genotypes in the sample, multiple type-specific PCR reaction must be performed separately using the same sample materials, which is expensive, time consuming, labor-intensive, requiring validation of each of type-specific PCR primers set. Broad-spectrum of HPV subtypes can amplify with the help of consensus PCR primer set such as gene fragment (MY09/MY11), which then identify with the help of specific probes [36, 41]. Diagnostic sensitivity important for clinical performance can be enhanced by using other consensual primers (PGMY, GP5+/GP6+ or SF10) [41, 37, 50].

Along with technical advancements real-time PCR methods (e.g. HPV RealTime test, Abbot, USA; Geno ID, Hungary) has been launched that can reduce reaction time considerably. Melon S et al. reviewed that even though, it can be technically difficult in multiplexing several type specific primers within one reaction, combination of type-specific PCR primers and fluorescent probes in real time detection is gaining popularity in cancer related biomarkers research [41]. The whole procedure can be automated (Cobas® 4800 HPV Test with 16/18 genotyping, Roche Diagnostics, Switzerland) with promising results in clinical diagnostic aspect.

Currently, HCII HPV DNA Test and consensus PCR assays has been employed frequently. Therefore, HCII HPV DNA Test has been applied in our study too. HPV DNA

testing has high sensitivity as much as 90% for CIN2+ lesion however, due to transient nature of HPV infection the specificity is poor for clinically significant cervical lesions. HPV DNA testing is recommended as triage test for equivocal cytology and primary test along with triage with cytology or other molecular tests.

1.3 Cervical cancer prevention

There are millions of women living with precancerous lesions. The issue of effective detection, treatment and management of those susceptible women is posing major challenge to the scientific and policy making fraternity for finding suitable strategy to overcome this challenge of cancer prevention. Cancer prevention can be divided in to two categorizes primary prevention and secondary prevention. Primary prevention, early detection through screening, proper diagnosis and intervention or treatment are critical components of cervical cancer control.

1.3.1 Primary prevention

Primary prevention of women from cervical cancer includes vaccination of young women with the prophylactic HPV vaccines. Soon after identification of association of HPV with cervical cancer, attempts have been made to use HPV particles as prophylactic vaccine. An empty non-infectious viral shells without viral DNA comprising of recombinant L1 capsid proteins are known as virus-like particles (VLPs). Immunization with VLPs can evoke immune response and offers protection by generating antibody against L1 that is 50 times higher in concentration than natural infection. L2 protein is poor immunogenic than L1 VLPs. However, multi type concatenated L2 protein not only

confers more immunogenic but also provides cross protection from HPV16, 18, 45 and 58. Viral proteins such as E1, E2 and E6 are not able to elicit antibody responses. The initial immune response is via the IgM, which decays gradually followed by the more persistent IgG1 response [51].

Two licensed prophylactic HPV vaccines are now available for primary prevention of cervical cancer. Cervarix® (GlaxoSmithKline Biologicals, Rixensart, Belgium), a bivalent vaccine containing VLPs of types 16 and 18, confers protection against those subtype that are responsible for approximately 70% of cervical cancer [52]. Another HPV vaccine Gardasil® (Merck & Co., Whitehouse Station, NJ, USA), which is a quadrivalent vaccine containing VLPs of types 6, 11, 16 and 18 [53]. However, vaccines have no therapeutic effect against well-established lesions or CIN. The best aspect of vaccination is capability of providing cross-protection against other non-vaccine HPV types such as HPV31, HPV33, HPV 52, HPV45, and HPV51 by Cervarix bivalent vaccine and HPV 31 by Gardasil quadrivalent vaccine. Since introduced of these vaccines in 2006, accessibility of these vaccines have been increasing ever [51]. The available HPV vaccine types are able to protect from only 70% of ICC cases. Therefore, it is critical for those all vaccinated women to continue participation in national cervical cancer- screening programs to have better protection from subsequent risk of acquisition of cervical cancer. Further, a new nine-valent vaccine targeting additional HPV types (31, 33, 45, 52, and 58) on top of those 6,11,16 and 18 HPV types has been developed for better protection. It is expected to have protection from this vaccine around 90% of ICC [54]. Vaccination coverage is variable in developed countries due to social and practical reasons whereas, vaccination coverage is very low in developing countries because of

expensive in cost wise, requirement of multiple doses (2-3 doses) in 6 months and especial logistic chain with cold storage facilities.

1.3.2 Secondary prevention

Progression and development of invasive cancer from precancerous lesion is very slow process taking long time. Generally, early stages of cervical cancer remain mostly asymptomatic making early detection more complicated. Cervical cancer screening at an early stage can provide opportunity for early detection and proper treatment of women with asymptomatic early stages lesions. The primary objective of secondary prevention is early detection of susceptible women having precancerous cervical lesions with the help of cervical cancer screening so that proper treatment and management of them can be possible in order to prevent cervical cancer. According to World Health Organization (WHO), screening is defined as a public health intervention applied on a susceptible or risk group of population (target group) as to identify asymptomatic individual with high probability of having or at risk for developing a diseases of interest that is, those with precancerous precursor lesions, high-grade squamous intraepithelial lesions in case of cervical cancer. Screening scenario of cervical cancer is highly dependent on the availability of resources in the areas where screening of cervical cancer at population level is being carried out.

1.4 Cervical cancer screening strategies

The current alarming situation of cervical cancer clearly indicates the requirement of developing and implementing effective cancer screening methods together with suitable, feasible intervention in properly managing those susceptible and already suffered women.

1.4.1 Cytology screening

Evaluation of usefulness of Pap smear and its subsequent application in screening strategies was major milestone towards cervical cancer prevention and had significantly reduced the mortality and morbidity of cervical cancer mainly in developed countries equipped with infrastructures [55]. Effective cytology screening requires sophisticated and costly infrastructure as to ensure high quality, adequate coverage and proper follow-up of the women at risk having precancerous lesions.

In high resources countries, Pap smears examination has been included in routine screening to evaluate cervical dysplasia or abnormalities. Women with mild or borderline abnormal cytology (dyskaryosis) are enrolled for follow up with repeated Pap smears examination at 6 and 18 months, whereas those with high-grade abnormality or CIN are referred for colposcopy evaluation as to allow early excision [56]. U.S. consensus guidelines and Canadian Task Force guidelines has also recommended that cytology examination be started as early as 21 years of age with repeated every 3 years [57]. Although cytology screening with Pap smears examination is effective, it is not ideal test because of the several drawbacks including but not limited to variable low sensitivity, poor reproducibility, diagnosis biased and fluctuation of efficacy of screening in different infrastructure [58, 59]. Further, Pap smears has limited sensitivity (around 50-70%) to

detect CIN2+ [60] and moderate specificity (around 95%) resulting over referral of women with minor abnormalities without not having high-grade lesions [61]. Additionally, there is limited attendance of susceptible women in the screening program [62]. Therefore, improvement of the screening procedure is need for effective cervical cancer prevention.

1.4.2 HPV screening

Although oncogenic human papillomavirus (HPV) infection has associated with high risk for cervical cancer, only few HPV infected women develop to invasion cancer because of the transient nature of HPV infection [9]. Vaccination against high risk HPV is not 100% effective in preventing cervical cancer indicating need of continue screening despite vaccination [63], which further emphasizes the need of extensive evaluation of the most effective screening approaches. Since development of invasive cancer is slow and lengthy process requiring persistence HPV infection, HPV testing has been utilized as an alternative approach for early detection of cervical cancer at precancerous state like CIN2 and CIN3. Although, HPV testing is less specific, it has reported to have higher sensitivity (~95%) than cytology for cervical precancerous and cancerous lesions [43, 45, 64-67], which enable negative HPV women to have lower 5-year risk of high grade lesions or cancer as compare to Pap negative women [64]. Therefore, inclusion of HPV DNA as screening test will help in improving the effectiveness of cervical cancer screening [66, 65]. Moreover, higher sensitivity and reproducibility of HPV testing are added advantages over cytology in early detection of cervical cancer, which support the inclusion of HPV testing in molecular screening [68,

69]. High-risk HPV DNA testing is considered to be useful in clinical applications [70, 71].

1. As a triage test for women with equivocal or mild cytological abnormalities for colposcopy evaluation

2. As a primary screening test in population of women over 30 years of age to detect present or absent of cervical pre-cancer lesions.

3. As a follow-up test to predict prognosis of treatment for women with high-grade CIN undergoing treatment.

Although, HPV testing has useful in clinical application, there are drawbacks of HPV testing such as lower specificity due to transient nature of HPV infection and high false positive result creating extra medical burden by increasing unnecessary colposcopy referral of HPV positive cases [72] and psychological burden by unwanted anxiety [73].

Triage testing of hrHPV-positive women is a crucial in order to reduce number of unnecessary colposcopies and associated cost, medical burden and unnecessary psychological distress. Cytology is the one of the test to triage hrHPV-positive women and those women with abnormal cytology will be referred for colposcopy, whereas women with normal cytology will recommend for repeat HVP and cytology after 6 months. Recently, molecular biomarker such as methylated gene is being intensively investigated as a potential triage test for those HPV infected women.

1.4.3 Molecular biomarker

Although hrHPV is known as vital causative agent for cervical cancer, presence of

hrHPV alone is not sufficient to initiate development of cervical pre-cancerous lesions or cancer. Development of precancerous lesions and progression to invasive cancer is very lengthy processes and this process is believed to accelerate by genetic or epigenetic changes in that milieu [6]. Many molecular changes such as mutation, deletion and epigenetic changes are associated with development and progression of cervical cancer [28]. Therefore, epigenetic modifications, an earlier event of carcinogenesis that can be detected in precancerous lesion and are gaining important in research. HPV genome and human genome methylation analysis are area of active research having the potential application in cervical cancer detection. Epigenetic changes such as DNA methylation is extensively explored area because of its association with regulation of gene expression and cervical cancer. Promoter hypermethylation of tumor suppressor gene is one of the vital molecular changes occur in cervical cancer precancerous or cancer lesions and analysis of DNA methylation of host gene could be potential molecular biomarker as to triage of hrHPV infected women [74-80].

1.5 Epigenetics

The mammalian genome basically composed of the genomic sequence consisting four basic genetic letters A, T, C and G. additional reversible chemical modifications occur on DNA and histones contribute in dynamically regulation of global gene expression leading to cell diversity. Such epigenetic medication occurs independently of changes in the genomic sequences. Epigenetics modification is important field of study in basic or clinical research including developmental biology to cancer biology.

The cells in human body comprise of the same DNA sequence, even though, the function and phenotype of these cells differ from each other's [81]. Such differences indicate existing of extra phenomenon except genetic programming that regulates phenotype. Such phenomenon is termed as epigenetics. In 1942,Weddington coined the term epigenetics and defined it as "the branch of biology, which studies the causal interactions between genes and their products, which bring the phenotype into being" [82].The term epigenetics literally means epi- (Greek for "upon", "above" or "on top of") - genetics. It refers to study of any heritable external modification of DNA leading to changes in gene expression as "on" or "off" or cell phenotype without involving changes to the DNA sequence; a change in phenotype without a change in genotype. During a cell differentiation, the cell considers the epigenomic phenotype to change the final phenomenon in the same hereditary genetics, where complexity at the DNA level provides the blue print for development. Considering the restrictiveness of heritable view, Bird redefined epigenetics as "the structural adaptation of chromosomal regions to register, signal or perpetuate altered activity states" [83]. Epigenetic change is regular or natural event that control many cellular events and is able to adapt itself in response to intrinsic and the external environmental factors, life style and disease state. Epigenetic modification commonly occurs in such a way in which cell differentiate terminally, where any irregular epigenetic changes or epigenetic disturbance may result to different phenotypes and even more damaging outcome such as disease like cancer. The epigenetics regulation controls transcription at three levels, DNA (DNA methylation), Protein (histone modifications) and RNA (non-coding RNAs). Therefore epigenetics studies include DNA methylation, histone modification, non-coding RNA and interrelation of different epigenetic mechanisms.[84].

New and ongoing research is continuously uncovering the role of epigenetics in a variety of human disorders including cervical cancer.

1.5.1 DNA methylation

DNA methylation is the best-characterized epigenetic modification, where methyl group has added to the 5 position of a cytosine (5-methylcytosine). As to epigenetics, additional information that affects phenotype can be stored in the modified base 5-methylcytosine. 5-Methylcytosine in the context of the palindromic sequence 5'-CpG-3'. There are small stretches of DNA with high CpG density as GC content >50% and observed/expected CpG >0.6 in a length>200 bp termed CpG islands. These islands are GC- and CpG-rich areas of approximately 1 kb, usually located in the vicinity of genes and often found near the promoter of widely expressed genes [85, 86]. About 60% of promotors are found with CpG islands. Most CpG islands are unmethylated, however, DNA methylation of such CGIs represses gene expression. Most CpG sites present outside the CpG islands are generally methylated whereas those of gene promoters are unmethylated to permit gene in active transcription status [87]. In highly expressed genes, DNA methylation that occurs on gene body may decrease transcriptional noise from alternative transcriptional start sites [88, 89]. Cytosine methylation occurs after DNA synthesis, by enzymatic transfer of a methyl group from the methyl donor *S*-adenosylmethionine to the carbon-5 position of cytosine. The enzymatic reaction is performed by DNA methyltransferases (DNMTs) [90, 87]. The major mammalian methyltransferases includes DNMT1, DNMT3a, DNMT3b and DNMT3L, which play significant role during embryonic

development and cell proliferation through maintenance and de novo methylation. DNMT1 is the predominant mammalian DNA methylating enzyme that prefers to target hemi-methylated DNA for the restoration of it to full methylation, called maintenance methylation. DNMT3A and DNMT3B are mainly involved in the methylation of new sites, hence called de novo Methylation [90, 91]. DNMT3L is postulated to play regulatory or mediator role in DNA methylation in stimulating Dnmt3a and Dnmt3b activity without DNA methyltransferase activity in itself [92].

The precise mechanism of transcription repression through DNA methylation is still under investigation, however, this process is known to be due to involvement of methyl-CpG-binding domain (MBD) proteins for recognizing methylated DNA [93] and interacting with histone protein modifications that regulate accessibility of DNA [94, 95]. The epigenetic effects in by DNA methylations have been shown to be important for cancer development. In cancer cells, abnormally altered epigenetic patterns such as abnormal methylation results repression of tumor suppressor genes from that of early precursor lesions of cancer

1.5.2 Histone modification

Chromatin is instructive DNA scaffold, which undergoes regulation in response to external cues to regulate various form and uses of DNA. Modification of chromatin is important in regulating expression of gene under control. Nucleosome is an important fundamental subunit of chromatin involved in regulation of the genetic sequence in the nuclear environment. The nucleosome is composed of octamer protein complex called histones consisting of two copies of each of the four core histones

(H2A, H2B, H3 and H4) arrange as such that two histone 2A (H2A) and two H2B form a tetramer that connect with H3A and H4A dimers to form a histone octamer, around which the DNA is wrapped. The N-terminal histone tail protrudes from the chromatin and that N-terminal tails of the nucleosomal histones can undergo a variety of posttranslational covalent modification including methylation, acetylation, ubiquitylation, sumoylation and phosphorylation, ADP ribosylation of selected amino acids. Such histone modification can impose chromatin structures dynamically either transcriptionally permissive or repressive state [96] by constantly changing either loose or closed configuration around DNA [97] for regulation of gene expression. A range of enzymes catalyze the histone modifications such as histone methyltransferases (HMTs), acetyltransferases (HATs), ubiquitylases and kinases. Several other enzymes such as histone demethylases, deacetylases (HDACs), deubiquitylases and phosphatases are also involved in histone modification as to remove marks or modification from that of histone affecting local or global histone structure and functions. Regulation of gene activity can be through recognizing and binding specific histone modifications by protein and forming complex. Polycomb Group (PcG) proteins are such proteins which involve in forming complex for transcriptional repression [98].

Histone is post-translationally modified, where methylation of histone is one of the important post-translation modification that help in regulation of gene expression. Trimethylation of Lys9 and Lys27 residues of histone H3 (H3K9me3 and H3K27me3) are the most studies and well characterized histone modification having repressive functions [99, 100]. Likewise, H3K4me3 and H3K9 acetylation (H3K9ac) are

involved in gene activation. Repressive function of histone modification is flexible, dynamic and short in term that easily erased by catalyzing opposing enzymes and such dynamic regulation is significant in developmental plasticity. However, DNA methylation is more stable and long-term silencing mechanism [101, 102, 81]. Furthermore, such array of post translation modifications of histone work in coordinated and orderly fashion in regulating cellular functions such as DNA replication and repair, apoptosis through condensation and segregation of chromatin. Histone modification and other modification work together in maintaining genetic activity in coherence. There are many histone-modifying molecules and protein complex. Any aberration in regulating the function of histone modifying complex deregulate histone modification and chromatin function leading to cancer progression [103, 104].

1.5.3 Non-coding RNA

Fundamental change in mRNA-centric paradigm for transcript and transcriptome landscape is undergoing along with the new finding in genetic research [105, 106]. ENCODE project estimates that non coding transcripts account 62-75% of genome [107] and RNAseq studies also show that non coding transcripts dominate the population of other RNAs such as nonribosomal and nonmitochondrial RNAs in the human cells [108]. Even though protein-coding genes are the most studied genome sequences, critically important functions of non-protein-coding genome sequences in chromatin regulation and gene expression for normal development, physiology and disease are also getting apparent [109, 110]. Many studies establish the emerging role

of ncRNAs as major source of biomarkers, targets for therapeutics and elucidating the function of ncRNAs variants in genome-wide association studies (GWAS) [111]. There is variable amount of ncRNAs present in the cells [112]. There is no distinct classification of non-coding RNA. However, these ncRNAs have been categorized based on their length or function or association with annotated protein-coding genes or protein-codding RNA resemblance or association with repeats and more [111]. Small ncRNA and long ncRNAs (lncRNAs) are the common classification based on the length of nucleotides. Biogenesis of Small ncRNAs occur through processing of longer precursors and are including but not limited to transfer RNAs (tRNAs), short interfering RNAs (siRNAs), ribosomal RNAs (rRNAs), microRNAs (miRNAs), piwi interacting RNAs (piRNAs), small nuclear RNAs (snoRNAs) [113]. Long non-coding RNAs (lncRNA) are a heterogeneous class of mRNA-like transcripts and are few hundred to several hundred thousand nucleotides (nt) long and examples of lncRNA include antisense, intronic and intergenic transcripts such as pseudogenes and retrotransposons [114]. Long non-coding RNAs (lncRNAs) are abundantly present in mammalian transcriptome, which confer array of cellular functions such as gene transcription regulation by recruiting chromatin-modifying enzymes. Cis-acting functions have been associated with macro ncRNAs or long ncRNAs and trans-acting functions with short ncRNAs. Cis-acting ncRNAs are able to regulate the expression of one or more gene on the same chromosome, whereas, trans-acting ncRNAs are cable of regulating one or more gene expression on different chromosomes as well as regulating mature RNAs in the cytoplasm [113]. Sequence specific pairing is the important mechanism of regulation of gene expression, where RNA-induced silencing

complexes (RISCs) involve in repression of gene expression.

1.6 Epigenetics and cervical cancer

Epigenetic is important biological mechanisms for regulating spatial and temporal accessibility of DNA for regulation of gene expression. Tissues and cells suffer multiple environmental and genetic stress, where epigenetic patterns of each of the cells must be precisely maintained to keep regulation of gene expression within the normal state [115]. Biological phenomenon is regulated via complex interplay between genetic composition and epigenetic regulation of sophisticated network of gene expression for various biological process including but not limited to cell renewal, gene imprinting and X-chromosome inactivation and maintaining normal gene expression for normal cellular functions [116]. Major epigenetic modifications regulating chromatin organization for controlling coding and non-coding RNAs expression include DNA methylation, histone modifications, and nucleosome remodeling and are also involved in carcinogenesis. Any aberrance in preciously regulated DNA methylation and histone modification patterns can lead to the activation of oncogenes and silencing of tumor suppressors responsible for several abnormal conditions such as cancers [115, 117, 118].

HrHPV leads to epigenetic changes of affected cervical epithelial cells through which it promotes the establishment of cancerous lesions. Viral E7 protein (HPV16 E7) binds to a transcriptional repressor E2F6-containing polycombs PRCs and that detection of these complexes is reduced in HPV16 E7-expressing cells [119]. E2F6-containing PRCs bind to H3K27me3 transcriptional repressive marks. Trimethylation at the histone H3 lysine 27 (H3K27me3) as mediated by PRCs complex possesses ability to silence

transcription. HPV16 E7 binds directly with Dnmt1 that induces methyltransferase activity of Dnmt1 in vitro [120]. Additionally, HPV16 E6 upregulates the expression of DNMT1 through repression of tumor suppressor p53 to regulate the development of cancer [121]. Epigenetics studies is gaining new height in biological studies either in elucidating disease etiology and pathogenesis or in laying foundation for developing diagnostic and prognostic tools. Overexpression of high-risk E7 leads to increase expression of p16INK4a through modification of histone architecture of its locus, which could be potential marker of hr-HPV infection. Further, epigenetic marks are responsive to non-genetic risk factors and environmental cues and such mediator's effects can offer potential targets for drug development, where DNA methyltransferase inhibitors and histone deacetylase may be significantly valuable for cancers and inflammatory diseases [117, 122].

1.6.1 Epigenetics and gene expression

As to epigenetics, additional information that affects phenotype can be stored in the modified base 5-methylcytosine. Most CpG dinucleotide pairs are methylated in mammalian cells. DNA methylation is heritable but yet reversible epigenetic mark that is the most-extensively studied epigenetic modification. CpGs are distributed in human genome non- randomly with plenty of CpGs rich areas usually located near the promoter of genes [85, 86]. Cytosine methylation occurs after DNA synthesis, by enzymatic transfer of a methyl group from the methyl donor *S*-adenosylmethionine to the carbon-5 position of cytosine. The enzymatic reaction is performed by DNA methyltransferases (DNMTs) [90]. CpGs of genome are generally methylated contrary to the unmethylated CPGs of CGIs except those of X-inactivated genes, imprinting

genes and retrotransposons [123].

DNA methylation at promoter region of gene is very potential for silencing gene expression and conferring stability in genome integrity. Methylated DNA binds methyl-binding proteins instead of transcription factor and results chromatin compaction in association with histone modification thereby leading to gene silencing [124]. The epigenetic effects in by DNA methylations have been shown to be important in cancer development. The role of epigenetics in cancer related disease is an emerging field.

1.6.2 Different methods of DNA methylation analysis

Many approaches are available for DNA methylation analysis to assess level of methylation at an individual gene or whole genome level. DNA methylation analysis techniques employed pretreatment as enzyme digestion, affinity enrichment and sodium bisulphite followed by analytical step as locus-specific analysis, gel-based analysis, array-based analysis and NGS-based analysis (Table 3) [125]. The technique of sodium bisulphite treatment and denaturation of DNA was discovered in early 1970s, where cytosine converts to uracil as deamination process and 5-methylcytosine (5mC) remained unchanged [126, 127]. Several DNA methylation analysis techniques also utilize pretreatment with sodium bisulphite to modify and permanently retain the methylation marks so that level of methylation can be quantitated subsequently by PCR methods or other suitable methods (Table 4) [128]. Among them Methyl-Light ^{TM}, which is sensitive, rapid, cost effective, can scale up in throughput analysis and quantitative technique, is gaining popularity as a common method of DNA methylation analysis in spite of it limitation as to analyze methylation at short and specific sequence.

This method is based on quantitative PCR of TaqMan™ system, where qPCR is carried out on bisulphite modified DNA utilizing methylation sequence specific TaqMan probe as to anneal to PCR product, which after binding emits fluorescent signal and such signal is continuously detected in whole PCR reaction so as to quantitatively measure the level of methylation at that targeted sequence of interest [129].

1.6.3 DNA methylation and cervical cancer

Numerous studies corroborated the significance of methylation of genes such as tumor suppressor genes in cancer initiation and progression. Researches regarding discovery of optimal methylated genes for clinical utility in cervical cancer screening is gaining momentum along with the advancement and utilization of technologies in the process of discovery, identification and validation of novel biomarkers. Number of aberrant methylated genes associated with cervical cancer progression are growing. This progress indicates bright future in the field of development of robust biomarker that could be applicable for cervical cancer screening at population level.

1.6.3.1 Association of aberrant DNA methylation with cervical Cancer

Since cervical cancer progresses slowly taking years to turn into invasive cancer, level of candidate genes methylation at subsequent stages of cervical cancer progression is necessary to detect the association of candidate gene methylation with carcinogenesis. Several studies reinforce the notion that sequential increased level of aberrant DNA methylation is associated with increasing severity of precancerous

lesions and is critical event for cervical cancer. Since aberrant methylation of many genes is early events of carcinogenesis before any visible cytological abnormalities, quantitative measurement of methylation level of genes could be potential surrogate biomarker for early detection of cervical cancer only if there is significant different in methylation level between normal and precancerous lesions/invasion cancer [130]. Several studies identified significantly high level of aberrantly methylated genes present in precancerous lesions while actively researching suitable biomarkers as an alternative approach that is applicable for cervical cancer screening and early detection (Table 5) [131, 132].

Promoter hypermethylation of *cyclin A1* is specific to invasive cancer with high frequency in high-grade squamous intraepithelial lesions including microinvasive and invasive cancers (36.6%, 60% and 93.3% respectively), whereas absent in normal cells and low-grade squamous intraepithelial lesions [133]. Similarly, methylation of *BLU* and *RASSF1A* genes are increased along with the severity of cervical lesions (76.9% of SCC, 57.4% of HSIL, 20.0% of LSIL and 12.5% of normal tissues for *BLU* and 15% of SCC, 17.5% of HSIL, but nil in LSIL or normal tissues for *RASSF1A*) supporting the notion that *BLU* and *RASSF1A* gene promoter methylation is associated with cervical carcinogenesis [134]. Likewise, methylation level analysis of several genes such as *DAPK1, IGSF4* genes[135], *DAPK1, IGSF4, TFPI2* [136] *CCNA1, DAPK* [137], *SFRPs* gene family[138], *ZAR1, SFRP4*[139], *TSLC1* [140], *CDH13, DAPK1, RARB, TWIST1* [141] *p16, RARb , FHIT, GSTP1, MGMT, hMLH1genes*[142], *CADM1, MAL* [143], *PAX1* [144, 145], *LMX1A* and *NKX6.1* [145] revealed that all these genes are associated with cervical cancer

development as aberrant methylation was early event having increasing trend of methylation level from low grade CIN to severe tumor. Further, analysis of methylation level of *TNFRSF10C, DAPK1, SOCS3, HS3ST2, CDH1* genes demonstrated that hyepermthylation of *HS3ST2* and *CDH1* in cancer tissues and CIN3 cytology (93% and 70% respectively for *HS3ST2* as well as 89% and 26% respectively for *CDH1*) as compared to absent of methylation in normal cervical tissues [146]. Additionally, methylated *PAX1* gene is also more specific than HPV test for cancer detection [144].

Of note, strong association of increased frequency of genes methylation with severity of cancer lesion as compared to normal lesion reinforces the possibility of utilization of methylated candidate gene as potential biomarkers for early detection of cervical cancer in screening. Although there are discrepancies in methylation level among analyzed genes in different studies, robust markers should have high sensitivity for HSIL/CIN3^{+} or cancer and high specificity for normal cervical lesions in QMSP analysis of identical setup.

1.6.3.2 Biological consequences of gene methylation in cervical cancer

Promoter hypermethylation, which is frequently detected in cancer, inactivates the genes that control normal cell growth, development, proliferation, and function that lead to progression of cervical cancer. Several studies regarding the analysis of methylated inactivated genes involved in cervical cancer revealed that many of

them are belonged to the range of cellular or signaling pathways important for biological functions.

Methylation mediated silencing of genes important for balancing oncogenic pathway may result aberrant activation of oncogenic pathway resulting tumor progress. Wnt/β-catening signaling has vital role in cell differentiation, proliferation, migration at the time of embryogenesis and tumorigenesis. Hypermethylation of negative Wnt regulators such as *DKK3, SOX17, SFRP2* [74], *SFRP, SFRP4* [138] and adenomatous polyposis coli (APC) [147] may induce irregular activation of Wnt signaling in cervical cancer. Similarly, *AJAP1, EPO, EDN3, MAGI2* and *SOX17* may also involve in β-catenin signaling mechanism for progression of cervical cancer [148]. Likewise, many hypermethylated mediated inactivated genes of cervical cancer are also involved in diverse biological functions such as *CCNA* and *FHIT* genes in cell cycle, *CADM1* in epithelial cell adhesion, E-cadherin encoded by *CDH1* gene in calcium-dependent interaction of adjacent epithelial cells for maintaining adhesion junction, *DAPK1* in apoptosis, *MGMT* in DNA repair, P73 in similar function as P53 signaling pathway, *RAR-β* in cell growth suppression function, *RASSF1A* in cell cycle, apoptosis and gene instability [149-152].

1.7 Epigenetic marks as biomarker for cancer translation studies

1.7.1 Identification of clinical relevant biomarkers

In translational or clinical prospective there are specific parameters for selecting some molecules or molecular targets as biomarkers. Such biomarkers should be sensitive

enough and easy in analyzing qualitatively and quantitatively together with reliable having satisfactory performance in various dimensions of clinical application such as to detect the presence of a disease early in advance, to predict the prognosis of treatment as well as providing suitable and personalized tailoring of the care and management of patients [153]. Different types of biochemical molecules such as protein, DNA, RNA and lipids are being used as biomarkers. Immense interest in discovery of novel biomarkers facilitates utility of DNA methylation as biomarker

Many recently developed tools for DNA methylation analysis including genome-wide screening, locus or gene-specific analysis offer wide scope in molecular diagnosis of abnormal or disease conditions by analyzing the all type of biological fluids such as urine, plasma and serum obtained via non-invasive method and biopsy specimens such as frozen tissues and FFPE samples [154, 153]. There are evidences that DNA methylation based molecular biomarkers could be the more or equivalent in specificity and sensitivity as compared to protein biomarkers that further bolsters and justify the ideas of wide application of DNA methylation as potential biomarkers. Various steps must be completed across in the course of identification of clinical relevant biomarkers beginning with genome-wide studies or candidate gene approach before identification of an optimal biomarker suitable for validation.

1.7.2 Validation of clinical performance of biomarkers

One of the most important steps towards finding clinically applicable molecular biomarker includes validation of clinical performance of such biomarkers. The principle criteria include higher sensitivity to capture maximum number of susceptible subjects and

higher specificity to reduce numbers of the susceptible subjects from being further evaluation.

Validation process includes two steps such as training set and testing set with approximately equal sample size in each set. Training set is important in generating optimal cut-off value so there should be minimum bias towards detecting normal case as being case and escaping disease case as being normal. In the training set, the receiver operating characteristic (ROC) curves defined the optimal methylation index (M-index) cutoff values for discriminating disease from normal, which then were applied to the testing set. Analysis of data of training set is followed by analysis of data of testing set. Testing set is significate in obtaining optimal sensitivity and specificity of the potential biomarker under investigation. These steps are very crucial for obtaining suitable potential biomarkers having clinical application.

1.8 Hypermethylated genes as potential biomarkers

Plethora of studies with methylation analysis have revealed that various methylated genes such as *FAM19A4* [80], *FKBP6, ZNF516* [155], *POU4F3* [156] , *SOX9* [157], *CADM1* and *MAL*[76], *DAPK1, SLIT2, WIF1, RARB* [158] could be potential biomarkers to predict precancerous lesions

Powerful genome-wide methylation array or candidate gene approaches are useful methods to discover the potential novel candidate for validation. While analyzing methylation level in different type of cervical lesions, most genes revealed excellent performance in detection of $CIN3^{+}$ that is clinically significantly (Table 6). The process of finding suitable biomarker is hampered by discrepancies of methylation analysis results

between different studies as variable, low sensitivities and specificities [131]. Many factors such as sample source, method of sample collection, sample handing, nature of sample, type of methylation analysis, reagent and instrument used may be the reasons of discrepancies of methylation analysis results. Therefore, systemic analysis using optimized protocol and sample may help to overcome these obstacles.

Cancer associated genes with consistent high DNA methylation frequencies in cancer and high sensitivity as well as optimal specificity for detection of cancer, are warranted to achieve the goal of finding suitable methylated biomarker. Intensive research with the objective of finding the robust DNA methylation biomarker suitable for screening and early detection of cervical cancer in mass level is dire need of present time [131, 28]. Numerous methylated genes have been reported having superior sensitivities, specificities and optimal area under curves (AUC) with higher degree of cancer lesions detection by markedly discriminating them from normal lesions further strengthens the potential scope of methylated genes as suitable biomarker applicable for cervical cancer screening. Methylation detection not only exhibits gene status but also provides advantages to complement recently available screening tools.

1.9 Clinical utility of DNA methylation in cervical cancer

Major challenges of current research in the field of cervical cancer biomarker discovery are not only finding the robust DNA methylation biomarker but also optimal clinical application of such biomarkers in to appropriate streamline screening strategies for early detection and management of cervical cancer in feasible way. Potential methylated biomarkers can be clinically utilized in different cervical cancer screening strategies as

triage tool with HPV testing as primary assay (Fig. 8) or as combination with cytology (Fig. 9) and as triage tool with primary cytology (Fig. 10) with the aims of optimal cervical cancer detection by complementing drawbacks of each methods and minimizing the false detection rate, optimizing sensitivity and specificity.

1.9.1 HPV testing as primary test with DNA methylation as triage test

HPV testing has many advantages such as high sensitive, high negative predicative value, reproducible, less human error and extended screening interval up to 5 years after negative test, which favor application of HPV testing as primary test [159, 57]. Since majority of HPV infection remains as transient stage leading to unnecessary referral for colposcopy. Therefore, triage of HPV test positive women by DNA methylation analysis of candidate genes, which is as sensitive as cytology, is very important to improve effective and efficient case detection rate [28] (Fig. 8). Several studies demonstrating the usefulness of DNA methylation as potential biomarker are strong rationale for inclusion of gene methylation in screening strategies to triage high-risk HPV-positive women [156, 76, 77, 74, 160, 161, 79, 78] .

DNA methylation assay to triage hrHPV infected women can be performed on self-sampled specimen with similar results as physician collected samples. [161-163]. Therefore, analysis of biomarker on self-collected samples can improve cervical cancer screening program by decreasing non-responders and enhancing enrollment for screening. HrHPV-positive women are further referred for biopsy only if they are also positive for methylated candidate gene. Otherwise, hrHPV-positive women having negative methylated genes are followed up every 6-12 months. Advantages of such

screening algorithm include the capturing more susceptible women with the test having high sensitivity such as HPV testing and triaging with methylated gene having optimal clinical performances hereby reducing the number of cases being referred for colposcopy and subsequent medical burden.

1.9.2 Pap smears and DNA methylation as co-testing

Cytology test has high specificity for cervical intraepithelial neoplasia that favors its usefulness as primary test. However, its drawbacks such as variable low sensitivity, poor reproducibility, diagnosis biased [58, 59] indicate the need of triage test or co-testing of these women with abnormal cytology. Methylated genes are suitable for cervical cancer screening as alone or in combination with cytology test to improve cervical screening and case detection [159, 164]. Likewise, *PAX1* methylation analysis in combined parallel testing with Pap smear improved the sensitivity of cytology test alone without affecting its specificity and such combination further offered improved specificity and equivalent sensitivity to the combined testing of Pap smear and HPV testing [165]. Additionally, combined analysis of cytology and DNA methylation to triage high risk HPV-positive women offered higher CIN2+/3+ sensitivities with slightly compromising specificity as compare to cytology alone [166]**,** which reinforces the important of new screening strategy (Fig. 9).

In this strategy, Pap smear and methylated gene negative women are followed up routinely 3-5 years of interval; whereas women with both tests positive are referred for biopsy. Similarly, women with negative Pap smear and positive gene methylation are either referred for biopsy or followed up every 3-6 months. Even if methylated gene is

negative, women with HSIL lesion in Pap smear are referred for biopsy; whereas, these women with LSIL, AGC and ASCUS are further triage using methylated gene analysis

1.9.3 Pap smears as primary test with DNA methylation as triage test

Aberrant methylation patterns of cervical cancerous cells, which initiate early stage and increase along with the severity of cancer, can be utilized to detect women with atypical cytology at an early stage [167]. Methylated genes could be suitable in cervical cancer screening as alone or triage tool for women having equivocally abnormal cytology (Fig.10). Methylation analysis of *PAX1* genes, a potential biomarker, has better performance to triage women with equivocal cervical lesions such as ASCUS, LSIL, and ACG than HPV testing [168, 132, 165]. Similarly, methylation analysis of *SOX1*[165] , *PCDH10* or *WT1* [169] are superior to HPV testing as to triage women with equivocal cytology. Additionally, *DAPK1*, *RAR-β2* and *MGMT* methylation analysis are effective in detection of CIN2+ from women with ASCUS and LSIL in cytology examination [170]. All these studies reinforce important of this strategy.

In this screening strategy, methylated candidate gene can be explored to triage women having equivocal results in Pap smear examination such as ASCUS, LSIL or AGC (Fig. 10). If methylated gene is positive in these women with atypical Pap smear, then they are referred for biopsy examination, whereas women with negative methylated tests are follow up every 3-6 months of interval.

1.10 Research Hypothesis, Objective and Specific Aim

1.10.1 Hypothesis

Candidate methylated genes may have better clinical efficacy in the triage of hrHPV infected women for colposcopy which might be translated in clinical application for cervical cancer screening

1.10.2 Objective

To evaluate clinical performance of candidate methylated genes in the triage of hrHPV-positive women for colposcopy.

1.10.3 Specific Aims

1. To select the candidate methylated genes having potential clinical application

2. To evaluate the clinical performance of the methylated genes for triage of hrHPV-positive women

Chapter 2. Materials and Methods

2.1 Study design

We conducted a case-control study using the hospital based patient samples. Further, all specimens were delinked from clinical information after numbering each of them until data analysis. In this study, we included the employment of a stepwise approach to find candidate methylated gene as potential biomarker for detection of cervical cancers. Novel hypermethylated genes, which were silenced in cervical cancer, were pooled. An initial step was filtering out potential hypermethylated candidate genes from a pool of 16 genes previously reported in cervical cancer. Potential hypermethyated genes were selected using the criteria of high sensitivity and specificity in detection $CIN3^{+}$. After selecting candidate genes, they were validated according to the standard validation parameters in training and testing sets. The study design of a training and testing set further allowed for additional assessment of ranking these selected candidate genes as the most potent potential biomarker.

Patients and control subjects were randomly divided in to training set and testing set, where subjects of training set were used to generate optimal cutoff values of candidate genes. Using the same optimal cut off value, sensitivity and specificity of respective genes were validated in a testing set. Candidate gene having high sensitivity and specificity in detecting $CIN3^{+}$ obtained from validation set was selected as potential biomarker. The Institutional review board of Tri-Service General Hospital, National Defense Medical Center approved this study.

2.2 Subjects and sample collection

We conducted a retrospective case–control study using hospital-based patient samples in the National Defense Medical Center, Tri-Service General Hospital (NDMCTSGH), Taipei, Taiwan, from December 2009 to November 2010. Patient aged ≥ 20 years referred for a colposcopy and cervical biopsy once cytology identified low-grade lesions and who were underwent to subsequent conization or major surgery once biopsy revealed $CIN3^{+}$ were enrolled in this study. Before the study, all the subjects were informed about the study and were enrolled after obtaining proper documented full consent from each subjects. 100 samples for selecting candidate genes and another 200 samples for analyzing clinical performance of candidate methylated genes were enrolled in total. Cervical scrapings for laboratory analysis were collected using cervical brush (PAP BRUSH, young Ou Co., Ltd., Yongin City, South Korea) before biopsy for laboratory analysis and were preserved in sterile phosphate-buffered saline at 4ºC until DNA extraction for HPV testing using a HCII hrHPV DNA assay (Digene, Silver Spring, MD, USA) and quantitative DNA methylation analysis of potential candidate genes using QMSP.

Healthy women undergoing routine Pap screening having normal Pap smear were selected as controls, only when their Pap smear showed normal pattern. Women with or suspicious Pap smears were excluded from control. The final diagnosis regarding different stages of the cancer was made by Tissue-proven histopathological examination, except those of healthy control women. Exclusion criteria applied in this study were

1. Poor or compromised quality of Pap smear,
2. Positive or abnormal Pap smear results

3. Patients with a history of anti-HPV vaccination,
4. Patients with a history of cervical neoplasia
5. Patients with a history of surgery in the uterine cervix,
6. Presence of other cancers,
7. Genital warts or
8. An immunocompromised state or pregnancy.

Further, all specimens were delinked from clinical information after numbering each of them until data analysis. In this study, all the women were tested for HPV infection and only samples from hrHPV-positive women underwent DNA methylation analysis. Cervical scrapings of 100 recruited women underwent HPV assay and hrHPV-positive women were proceeded for DNA methylation analysis to prioritize candidate genes for further analysis of clinical performance. Further, Cervical scrapings from 200 women recruited for analysis of clinical performance were randomly classified using a random number table as a 1:1 ratio into a training set (n = 111) and a testing set (n = 89). Methylation levels in the training set were used to generate optimal M-index cutoff values of candidate genes that can distinguish relevant cancerous cases from control. The clinical performance of candidate genes was validated using the optimal cutoff values in the testing set. This study was approved by the institutional review board of the Tri-Service General Hospital, National Defense Medical Center.

2.3 DNA extraction

Genomic DNA was extracted from cervical scrapings using DNeasy Blood&Tissue Kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's

recommendations [148] for quantitative methylation analysis of 14 methylated genes and HPV testing using a Hybrid Capture II assay (HC II: Digene, Silver Spring, MD,USA). The concentration of DNA was determined using NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE). Unlike other study, the quality of DNA was not a limiting factor in this study. Those samples with a DNA yield as low as 500ng or more (>500ng) were considered for further analysis in this study.

2.4 Bisulfite conversion

Bisulfite modification was conducted based on the principle that bisulfite treatment of DNA converts unmethylated cytosine residues into uracil, whereas methylated cytosine residues remain unmodified. Thus, after bisulfite conversion, methylated and unmethylated DNA sequences can be distinguished by sequence-specific primers.

CpGenome TM DNA Modification kits, S7820 (Millipore, Temecula, CA) were used for bisulfate modification of the genomic DNA sample according to the manufacturer's recommendations. Bases are first exposed by denaturing the DNA to its single stranded form with the help of heat and alkaline pH. Sodium salt of bisulfite ion (HSO_3^-) from reagent I converts unmethylated cytosine to sulfonated form which then hydrolytically deaminated to yield an uracil sulfonate intermediate. DNA is bound with micro-particle carrier (Reagent III) in the presence of salt (Reagent II). Such bound DNA is then subsequently desalted by repeatedly by 70% EtOH. The bisulphite modified DNA is finally eluted from carrier with the help of heating in TE buffer [165].

Briefly, DNA was denatured with 3 M NaOH by incubating 10 minutes at 50°C, followed by DNA modification through treatment with reagent I containing a sodium salt of bisulphite ion, HSO_3^- (sodium bisulfite) for 4-16 hours at 50°C. During initial desalting process, treated DNA was bound with micro-particle carrier (Reagent III) in the presence of salt (Reagent II) in β-mercaptoethanol while incubating at room temperature for 5-10 minutes. Pallet downed the micro-particle carrier (Reagent III) through spinning for 10 seconds at 5000 X g followed by 3 times washes with 70% EtOH and repeated spin down for 10 seconds each. At the end, drying of pallet was done with high speed spine for 2 minutes. DNA modification was followed by desulfonation, second desalting and elution for completion DNA bisulphite modification. After DNA pallet down, it was treated with 20mM NaOH/90%EtOH solution with properly vertex to mix and incubate at room temperature for 5 minutes. Spined for 10 seconds at 500 x g and the precipitated DNA was washed with 90% EtOH for 2 times. After that sample was centrifuged at high speed for 3 minutes. Precipitated DNA was dried for 10-20 minutes. The modified DNA was eluted or dissolved in around 20-25 µl of TE buffer or distilled water by incubating for 15 minutes at 50-60°C. Bisulfite modified DNA was stored at –80 °C.

2.5 Quantitative methylation-specific PCR (QMSP) and methylation index analysis

Florescence-based real time PCR was used for quantitative MSP to estimate the methylation level of the candidate genes. TaqMan-based quantitative methylation-specific polymerase chain reaction (QMSP) amplification was performed on bisulfite

treated genomic DNA [171]. While selecting potential candidate methylated genes for further performance analysis, QMSP was performed in a TaqMan probe system using the Applied Biosystems 7900HT Fast-Real-Time PCR system in total volume of 20 mL reaction mixture containing 2 µL of bisulfite template DNA, 250 nM of each primer, 225 nM TaqMan probe, and 10 µL of FastStart Universal Probe Master (Rox) (Roche Diagnostics, Roche Applied Science, Mannheim, Germany)[148]. HPLC-grade qMSP primers were synthesized by TIB Molbiol (Berlin, Germany). The 5' end of probes was labeled with 6-carboxy-fluorescein (6-FAM), and the 3' end was labeled with a quencher dye (MGB by Applied Biosystems or BHQ1 by TIB; (Table 14). The type II collagen (COL2A) gene was used as an internal reference by amplifying non-CpG sequences (iStat, New Taipei City, Taiwan). In vitro methylated genomic DNA treated with CpG methyltransferase (M.SssI; New England Biolabs, Beverly, MA) was used as a positive control, and assumed to give 100% methylation of each gene. However, for analysis of clinical performance of selected candidate genes QMSP for *AJAP1*, *HS3ST2* and *POU4F3*, and multiplex QMSP *PAX1* and *SOX1* were performed in a TaqMan probe system using the LightCycler 480 Real-Time polymerase chain reaction (PCR) System (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) [165]. Briefly, the total reaction volume of 20 µL contained 2 µL of modified template DNA, 1 µL of 20x custom TaqMan reagent, and 10 µL LightCycler® 480 Probes Master (Roche). A mixture of primers and probes was used for *PAX1* and *SOX1*. The type II collagen (COL2A) gene was used as an internal reference (iStat, New Taipei City, Taiwan), whereas in vitro methylated genomic DNA treated with CpG methyltransferase (M.SssI; New England Biolabs, Beverly, MA) was used as a positive control. The reactions were

performed using an initial incubation at 95ºC for 10 min, followed by 50 cycles of 95ºC for 10s and annealing and extension for 40 s at 60ºC (using a thermal cycler protocol in the standard mode). The DNA methylation level was assessed as the methylation index (meth-index) using the formula: 10,000 x 2^ (Cp value of gene - Cp values of COL2A) [172]. Testing results showing the very high Cp values of COL2A greater than 36 were defined as detection failures.

2.6 HPV testing

HPV DNA testing was used as primary test in this study. HR-HPV infection was detected using Hybrid capture II (HCII) test kits (Digene) according to the manufacturer's protocol which can detect HPV type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. Samples with a relative light units (RLU)/cutoff value ratio higher than 1.0 were recorded as positive.

2.7 Statistical analysis

IBM SPSS Statistics for Windows, version 20.0 (Armonk, NY, USA) was used for all statistical analyses. To determine the detection rate, CIN3 was taken as a cutoff value for the QMSP analysis of each of candidate genes. ROC curves for each of the candidate genes were calculated using the data from the training set. Optimal cutoff (M-index cutoff) values of the candidate genes were generated from receiver operating characteristic (ROC) curves according to the maxima of sensitivity and specificity to distinguish $CIN3^+$ (including CIN3, CIS, SCC, AC and ASC) and $CIN2^-$ (including normal controls, CIN1 and CIN2) patients [173]. Thus obtained optimal M-index cutoff

values were used to further analyze the clinical performance of each of the candidate methylated genes in the testing set. Sensitivities, specificities, positive predictive values (PPV), and negative predictive values (NPV) of *AJAP1*, *HS3ST2*, *POU4F3*, *PAX1*, and *SOX1* for detecting $CIN3^{+}$ were calculated.

Chapter 3. Results

3.1 Characteristics of patients

Characteristics and basic data of the patients such as histopathology for stratification of sample, mean age and HPV percentage enrolled in this study are summarized in Table (Table 7 and Table 11). 67 (67%) cervical scrapping out of 100 women in set for selecting candidate genes, 68 (61.3%) cervical scrapping out of 111 women in training set, and 55 (61.8%) cervical scrapping out of 89 women in testing set were positive for hr-HPV and were proceeded with DNA methylation analysis. The set for selecting candidate genes comprised 40 histopathologically confirmed $CIN3^+$, 20 women with CIN2 and 40 women with normal/CIN1 out of 100 women. Similarly, the training set included 46 women with histopathologically confirmed $CIN3^+$ and 65 women with normal/CIN1 out of 111 women. The testing set comprised 89 women including 39 women with $CIN3^+$ and 50 women with normal/CIN1. The age of patients among set for selecting candidate genes, training set and testing set did not show any difference (47.8 ± 13.2 years, 46.8 ± 13.5 years and 44.2 ± 13.3 respectively).

3.2 Panel of cervical cancer associated candidate genes

We used candidate gene approached to find the potential methylated biomarker. The panel of cervical cancer associated candidate genes included 14 genes *ADRA1D, AJAP1, COL6A2, EDN3, EPO, HS3ST2, MAGI2, POU4F3, PTGDR, SOX8, SOX17, ST6GAL2, SYT9,* and *ZNF614.* These genes are previously reported in our laboratory as to be associated with cervical cancer and the clinical performance analysis revealed that they could be potential biomarker in detecting $CIN3^+$. Based on this rational, we used these 14 genes. We

compared the sensitivity and specificity of each gene at the maximal AUC for detection of $CIN3^+$ lesions. In clinical performance analysis of each gene at maximal AUC for detection of $CIN3^+$ lesion showed that there are 9 genes with more than 60% sensitivity, whereas all 14 genes with than 80% specificity indicating their strong association with cervical cancer (Table 8). Six genes such as *AJAP1, HS3ST2, MAGI2, POU4F3, PTGDR,* and *SYT9* demonstrated an accuracy above 0.8. Among all 14 genes single *POU4F3* gene methylation analysis showed sensitivity of 88% and a specificity of 82% at the highest AUC (0.85) (Table 8).

3.3 Candidate methylated genes selection for validation

We randomly collected cervical scrapings from 100 women (Table 7) before treatment. Analysis of hrHPV status using cervical scrapping from these women by hrHPV assay, HC II offered 83% sensitivity and 43% specificity, 49% PPV and 79% NPV (Table 9). In this study, we aimed to identify which candidate methylated genes have ability as suitable biomarker and offer potential clinical application in the triage of hrHPV-positive women. Methylation level of cervical cancer associated genes from panel of candidate genes among hrHPV-positive women in candidate gene selecting set was analyzed as to select potential candidate gene having optimal clinical performance. Cervical scraping samples from those hrHPV-positive women, 67 out of 100, were subjected to quantitative methylation-specific PCR (QMSP) analysis of 14 genes *ADRA1D*, AJAP*1*, COL*6A2*, EDN*3*, EPO, *HS3ST2*, MAGI*2*, *POU4F3*, *PTGDR*, SOX*8*, *SOX17*, *ST6GAL2*, *SYT9*, and *ZNF614* using the same cutoff values as previously described [148] (Table 10).

Among these 14 genes three genes *POU4F3*, *HS3ST2*, *AJAP1* demonstrated better sensitivity 88%, 88% and 64%, comparable specificity 82%, 82% and 100%, positive predictive value 83%, 83% and 100% and superior negative predictive value 88%, 88% and 74% respectively in detection $CIN3^+$ among hrHPV-positive women (Table 10). Specificities of *POU4F3*, *HS3ST2* and *AJAP1* for detecting $CIN3^+$ (82%, 82%, 100% respectively) were significantly superior to hrHPV test (43%) with better sensitivity for *POU4F3, HS3ST2* (88% for both genes vs 83%) and slightly compromised sensitivity for *AJAP1* (64% vs 83%) than hrHPV test. We selected candidate genes with a sensitivity of >85 % or specificity of >98 % in detecting $CIN3^+$ in hrHPV-positive women for further validation. Three genes, *POU4F3*, *HS3ST2*, and *AJAP1*, fulfilled these criteria. We prioritized these three methylated genes as potential candidate genes for further validation of clinical performance. Of note, there were no prior study of potential methylated genes *POU4F3, HS3ST2* and *AJAP1* in triaging hrHPV-positive women.

3.4 Generation of methylation index cutoff values at training set

We aimed to further tested clinical performance of the candidate genes *POU4F3*, *HS3ST2* and *AJAP1* methylation as potential biomarkers in the triage of hrHPV-positive women according to the work flow (Fig. 11). The study samples consisting of independently enrolled 200 women were randomly classified into two groups with a training-to-testing ratio of 1:1 and random number table was used during the procedure of random classification (Table 11). There was no significant difference in the age ($P = 0.17$) and diagnosis distribution in the training set and testing set. Analysis of hrHPV status using cervical scrapping from these women by HC II hrHPV assay offered 89% sensitivity and 59%

specificity, 62% PPV and 88% NPV (Table 9). The training set comprising 111 women and cervical scrapings from only women with hrHPV-positive assay were further analyzed for methylation level by qMSP.

POU4F3, *HS3ST2* and *AJAP1* methylation levels increased according to the severity of cervical lesions (Fig. 12, a-c). Receiver operating characteristic (ROC) curves of *POU4F3*, *HS3ST2* and *AJAP1* was generated to calculate accuracy of respective gene in detecting $CIN3^+$ or disease irrespective of HPV status. The areas under the ROC curves (AUC) in distinguishing $CIN3^+$ from normal/CIN1 were 0.85 (95 % CI, 0.77–0.93) for *POU4F3*, 0.80 (95 % CI, 0.71–0.90) for *HS3ST2*, and 0.73 (95 % CI, 0.63–0.83) for *AJAP1* (Fig. 12, d-f). Further, methylation levels of the *POU4F3*, *HS3ST2* and *AJAP1* in hrHPV-positive women also increased according to the severity of cervical lesions or disease (Fig. 13, a-c). We generated receiver operating characteristic (ROC) curves of *POU4F3*, *HS3ST2* and *AJAP1* to calculate accuracy of respective gene in detecting $CIN3^+$. According to receiver operating characteristic (ROC) curves of each gene the optimal methylation index (M-index) cutoff values for detecting $CIN3^+$ were defined as 38 for *POU4F3* and 2 for both *HS3ST2* and *AJAP1*. The areas under the ROC curves (AUC) in distinguishing $CIN3^+$ from normal/CIN1 were 0.86 (95 % CI, 0.78–0.95) for *POU4F3*, 0.82 (95 % CI, 0.71–0.92) for *HS3ST2*, and 0.71 (95 % CI, 0.59–0.83) for *AJAP1* (Fig. 13, d-f). Because we have previously discovered and tested *SOX1* and *PAX1* genes as potential biomarkers [165] , we also included the data of these two genes in this study to compare their clinical performance. At the optimal M-index cutoff values, the sensitivities of *POU4F3*, *HS3ST2*, *AJAP1*, *SOX1*, and *PAX1* in discriminating $CIN3^+$ among hrHPV-positive women were 79, 67, 63, 78, and

70 %, respectively, whereas the specificities were 78, 89, 64, 71, and 89 %, respectively (Table 12).

3.5 Validation of the clinical performance of methylated genes at testing set

Cervical scrapings of 55 hrHPV-positive women out of 89 women in testing set were analyzed further for estimating DNA methylation levels (Fig. 11 and Table 7). The Meth-index cutoff values of each these genes obtained from training set were applied to the testing set in order to validate the clinical performance of candidate methylated genes *POU4F3, HS3ST2* and *AJAP1* together with *SOX1* and *PAX1* genes in detecting $CIN3^{+}$ among hrHPV-positive women. The results of testing set for validating of performance of potential genes were similar to those of training set with sensitivities and specifies as 74% and 89% for *POU4F3,* 55% and 100% for *HS3ST2,* 80% and 74% for *AJAP1* 63 and 74 for *SOX1* as well as 60 and 100 for *PAX1* respectively indicating robustness of performance of our methylated genes (Table 12). The testing set validated that *POU4F3* methylation analysis conferred the best clinical performance among five potential candidates with 74 % sensitivity and 89 % specificity (Table 12). When stratified by histology, *POU4F3* and *AJAP1*methylation testing did not miss any invasive cancer patients (11/11) (Table 13). *AJAP1* methylation had better performance in detecting CIN3/CIS lesions than *POU4F3* (70.8 vs. 62.5 %). However, more CIN1 lesions were detected using *AJAP1*.

Chapter 4. Discussion

Persistent hrHPV infection leads to epigenetic changes in host genome favoring cervical cancer progression. Epigenetic modification such as DNA methylation is an important epigenetic change and has significant role in initiation and progression of cervical cancer. Extensive studies have been done about it as to discover relevant and potential biomarker to detect cervical cancer in early stages during cervical cancer screening as secondary prevention strategy. Several previous studies along with many powerful technological advancements support the concept of that DNA methylation could be a potential molecular biomarker for detection of cervical precancerous or cancer lesions [28, 131, 148, 164, 172]. An ideal methylation biomarker should have better specificity than HPV testing and better sensitivity than cytology when applied as a primary screening tool. Recent studies proposed the alternative role of methylation biomarkers as a triage method for hrHPV-positive positive women [75-77, 79, 80]. The present study demonstrated that DNA methylation analysis as a triage for hrHPV-positive women is feasible. Three potential genes *POU4F3, HS3ST2* and *AJAP1* among 16 genes have better performance in detecting $CIN3^+$ among hrHPV-positive women. Additionally, two more potential genes, *PAX1* and *SOX1* also included in this study as to compare the clinical performance of those three genes. Since no gene has absolute specificity to the relevant $CIN3^+$ lesions, M-index cutoff values was determined for $CIN3^+$ so as to obtain better sensitivity and specificity from ROC curves. The M-index cutoff values thus obtained provide the benefit as such that variable methylation level in individual women does not compromise the application of the promising biomarkers to detect $CIN3^+$. The clinical performance of methylated gene at testing set is similar to the

training set indicating the robustness of those biomarkers. Among five candidate genes the *POU4F3* methylation analysis confers the best clinical performance when combined with the HPV assay with the highest AUC (0.86), 74% sensitivity and as high as 89% specificity in detecting $CIN3^{+}$ among hrHPV-positive women. This is the first study to demonstrate that methylated *POU4F3* as the promising biomarker to triage women with HPV infection and carcinoma by reducing unnecessary treatment of innocuous lesions.

Primary screening test should be highly sensitive, broad spectrum in nature capable of detecting all HPV types [174]. More high-risk HPV genotype detection may have a better chance to include more women at risk for triage in the primary screening. In screening set up, sensitivity and specificity of HC II (pooled sensitivity 0.89 and pooled specificity 0.73) was much higher than PCR GP5+/6+ in detecting HSIL (pooled sensitivity 0.85 and pooled specificity 0.62) and there was higher accuracy of HCII and PCR based tests between European and Asia-pacific region studies [175], which might be due to the distribution of HPV type as 16, 31, 33, and 18 are prevalent in Europe, and 16, 58, 52 and 18 are prevalent in the Asia–Pacific region Asia–Pacific region [174, 175]. Hybrid capture 2 assay (HC II) is an easy, FDA approved and robust assay of detecting hr-HPV that has been recommended as ideal primary screening tool for large population based screening [176, 175] followed by triage with more specific test to avoid false positive rate and unnecessary referral [67]. Therefore, this study we used the Hybrid Capture II (HCII) assay for hrHPV testing as primary screening tool which assays 13 high-risk genotypes simultaneously [177, 175, 176]. HPV based screening strategies have broader and encouraging prospective towards clinical application [178]**.** Further hrHPV subtype analysis may clarify type-specific correlation with *POU4F3* methylation,

which may be useful in estimating the impact of molecular screening strategy using HPV detection followed by methylation triage in post-vaccination era.

POU domain, class 4, transcription factor 3 *(POU4F3)* gene (formerly known as Brn3.1 or Brn3c) is located in chromosome 5q32 and plays various biological functions such as regulation of transcription, cellular and metabolic process, organ development, cellular differentiation, nervous system development, neurogenesis and generation of neurons [179]. *POU4F3* regulates a wide array of neuroendocrine development pathways [180] and is one of the first transcription factors involved in hair cell (HC) development. It expressed in cochlear and vestibular hair cells, all hair cells in the organ of Corti, the otolith organs and the cristae as well as in spinal cord, merkel cells, subsets of retinal ganglion cells, in dorsal root and trigeminal ganglia, and in neurons of the superior and inferior colliculi in adult mice [181]. Mice with a deletion of the Pou domain gene *POU4F3* were completely deaf, "owing to a failure of HCs to appear in the inner ear, with subsequent loss of cochlear and vestibular ganglia" [182]. Mutations of human *POU4F3* gene has been related to deafness and *DFNA15*, an autosomal dominant, progressive, nonsyndromic, sensorineural hearing loss or impairment [181]. *POU4F3* is not only necessary for late HC differentiation, including their functional transduction and synaptic specialization but also plays an essential role in controlling migration, maturation, maintenance and survival of hair cells in inner ear sensory epithelia and determining or maintaining the identities of a small subset of visual system neurons [183]. *POU4F3* regulates neurotrophin gene expression; the two transcription factors Gifi-1 and Lhx3, downstream targets of *POU4F3*. Increased *POU4F3* levels positively regulate outer hair cells survival and differentiation through the regulation of Gfi1,

another transcription factor [184]. *ATOH1* cooperates with unknown factors to fully express *POU4F3*, which, in turn, cooperates with *ATOH1* to maintain HCs [185]. *ATOH1* is involved in regulating *POU4F3*, whereas and its long term expression may be dependent on *ATOH1* expression [182]. In the other hand, the function of *POU4F3* in cancer biology remains largely unknown. Class III POU genes (*POU3F1*, *POU3F2*, *POU3F3*, and *POU3F4*) and *class IV POU* genes (*POU4F1*, *POU4F2*, and *POU4F3*) share similar properties in neural development and further expression of *class III/IV POU* genes is important for the proneural/neuroendocrine differentiation of lung cancer cells [186]. *POU4F3* hypermethylated in cervical cancer and glioma suggest its suppressor role in cancer [179, 148] emphasizing the significant of this gene in cervical cancer. This study supports the concept that *POU4F3* could be a potential triage biomarker for hrHPV-positive women.

DNA methylation suitable for triage test should be highly specificity to reduce false positive rate and optimal single gene is preferable as triage test over multiple genes panel. Various research groups reported methylated genes as potential biomarker for detection of precancerous lesions [164, 172, 148, 131, 28, 75] and triage of hrHPV infected women either alone or in panel. HPV test can detect more risk population due to its higher sensitivity, whereas DNA methylation triage can have reduced the false positive rate due to its higher specificity. Methylated *DKK3* and *SFRP2* [74] as well as methylated *EPB41L3* may be valuable triage test for HPV-positive women for colposcopy [78]. Addition of methylated *CADM1-M18* and *MAL-M1* bi-marker panel to HPV test increased specificity to 78% together with a sensitivity 70% for detection of $CIN3^{+}$ [75]. Further this bi-marker panel is validated in prospective population-based

study as having 65.5% sensitivity and 83.3% specificity that clinical performance was equal as cytology (66% sensitivity and 79% specificity) or combined cytology and HPV16/18 genotyping (84% sensitivity and 54% specificity) together with AUC 0.719 to discriminate $CIN3^+$ among hrHPV-positive women [76]. Methylation panel of at least two out of five methylated markers detected more $CIN3^+$ among hr-HPV positive women with specificity 76.6% [79]. Similarly, panel with four methylated markers as triage test for hrHVP-positive women demonstrated 84% sensitivity and 69%specificity for detecting $CIN3^+$ together with correct referral compared to combination of HPV and cytology test [77]. Analysis of methylated bi-marker panel *MAL-M1* and *miR-124-2* on self-sampled cervico vaginal sample is promising triage test that is non-inferior than cytology for detecting $CIN3^+$ among hrHPV-positive women advocating for full molecular screening strategy suitable for enrolling more women for screening especially in area with less medical resources [161, 163]. All these studies have reinforced the concept of DNA methylation as promising biomarker in detecting $CIN3^+$. However, the published results are not sufficient because of moderate to high specificities in detecting $CIN3^+$ even in combination of marker panel bolstering the significance of our study for evaluation and validation of robust molecular biomarker having improved specificity than previously reported. In the present study, a single gene, *POU4F3*, has a specificity of 89 % in detecting $CIN3^+$ in hrHPV-positive women with limited compromise in sensitivity (79 to 74 %), which is better than the specificities previously published using *FAM19A4* (67%) [80] or a panel of two genes *CADM1–M18/MAL-M1* (71–83 %) [75, 76, 166, 187], or a panel of at least two out of five methylated biomarkers (77 %) [79], or a panel with four methylated biomarkers (69%) [77] or comparable to the

specificity of *JAM* (88%) [162]. Clinical performance of methylated candidate gene in training set was similar to that of testing set emphasizing the robustness of our methylated gene as biomarker. Strength of this study is that validation of applicability of *POU4F3* in the HPV based screening strategy.

We propose a scenario for the combination of HPV assay and *POU4F3* methylation analysis for cervical cancer screening (Fig. 14). However, it requires further independent validation together with additional standalone biomarker. Women without hrHPV infection undergo follow-up three to five years later [57]. Women with hrHPV infection will undertake *POU4F3* methylation analysis. Women having positive *POU4F3* methylation are referred for colposcopy. Because *POU4F3* methylation analysis did not miss any invasive cancer, *POU4F3* methylation-negative, hrHPV-positive women may repeat HPV assay and DNA methylation analysis 1 year later. This strategy may substantially reduce the referral rate. However, a longitudinal follow-up study is needed to clarify the natural history of those infected with hrHPV, but without *POU4F3* hypermethylation to determine if a longer interval between screenings is also safe. The high negative predictive value in hrHPV-negative women is well documented, which means a longer screening interval is safe. However, determining *POU4F3* methylation in HPV-negative women to assess *POU4F3* as independent from HPV as a marker for cervical neoplasia/CIN/CIN3/cancer may also be a consideration. Because HPV assay and methylation analysis can be conducted in the same self-collected cervical sample, the application of this approach may improve the participation of women for screening [163, 178], especially those in low-resource areas. The application of a DNA methylation analysis using self-collected vaginal samples warrants further

evaluation. In addition, this is a retrospective hospital-based study, which did not include long term longitudinal follow up data of those subjects having precancerous lesions other than CIN3^{+} and included only Asian subjects only. Population-based studies in different geographical and ethnic backgrounds are needed to validate these results so as to prove broad applicability of this molecular biomarker.

Some of the cervical cancer might be hrHPV-negative but *POU4F3* methylation positive. This might be due to that hrHPV infection is necessary but not only causative agent for cervical cancer. One third of HC II-negative CIN 3^{+} is related to the presence of other HPV genotypes not covered by the HC II panel or to undetectable HPV in the lesion; or other risk factors for cervical cancer including environmental factors. These rare occurrences were already described in large cancer series and partially explain the occurrence of HPV-negative CIN 3^{+} [188]. These risk factors include other infection such as *Trichomonas vaginalis*, bacterial vaginosis infection, Chlamydia infection a relatively common kind of bacteria that can infect the reproductive system and warts, that are significantly associated with acceleration of development and progression of cervical cancer [13]; the risk of developing cancer increased along with age and greater risk group is women with over 35 years of age [11]. Similarly, smoking is one of the important risk factors associated with CIN3/CIS and ICC [14]. Women who smoke are about twice as likely as non-smokers to get cervical cancer. Tobacco by-products have been found in the cervical mucus of women who smoke and these substances damage the DNA of cervix cells and may contribute to the development of cervical cancer. Smoking also makes the immune system less effective in fighting HPV infections. Additionally, Early pregnancy and multiple deliveries together with long term use of contraceptive pills results

hormonal changes and are important factors to precipitate the cervical cancer development [13]. Immunosuppression due to the human immunodeficiency virus (HIV) or due to use of drugs to suppress immune response such as in treating autoimmune disease or those who have had an organ transplant have a higher risk for cervical cancer. Further, women who had used an intrauterine device (IUD) has increased risk of infection and cervical cancer. Persistent chronic inflammation or infection can favor progression of cervical cancer. In view of these risk factors, one must be careful in evaluation of hrHPV negative subjects as to exclude further examination during cervical screening. These subjects might come from rare occurrence group with $CIN3^{+}$/CIS lesions and incorporation with other screening test such as Pap smear examination may increase specificity of case detection. This is one of the important aspects that needs further study.

In our study, sensitivity of *POU4F3* to detect CIN3/CIS was 62.5%. Improved sensitivity could enhance the detection of susceptible women for colposcopy. This sensitivity can be improved by incorporating another equal or better methylated biomarkers. Such novel better methylation biomarker having high sensitivity can be obtained from DNA methylation array using sample from those cervical scrapings having low *POU4F3* methylation. Such useful novel biomarkers could improve the sensitivity of the *POU4F3* by compensating the its low sensitive. This is an exciting area of future work for better biomarkers discovery and validation.

Although we did not include adenocarcinoma cases in our validation set with 200 samples, *POU4F3* methylation analysis assay was capable of detecting 100% adenocarcinoma cases (5/5) irrespective of HPV status in our previous study [148]. Therefore, using only hrHPV-positive adenocarcinoma subject in investigation of

applicability of methylated *POU4F3* analysis as potential biomarker in detecting adenocarcinoma among hrHPV-positive subjects is one of the important aspect that requires further study.

In this study, we adapted histopathological diagnosed $CIN3^+$ as end point because CIN2 is not only equivocal in nature with tendency to regress to normal instead of progressing to $CIN3^+$ [189], where likelihood of CIN2 to progression to invasive cancer is only 5% [15] but also heterogeneous in regard to DNA methylation profiles [145]. Further, diagnose of CIN2 is much less reproducibility than CIN3 [190] because of difference in natural history of CIN2 with that of CIN3 [189]. However, CIN3 has higher tendency to progress to invasive cancer because of it being immediate precursor and has better reproducibility [174]. Therefore, it is more appropriate to adapt $CIN3^+$ as surrogate end point for early diagnosis cervical cancer. We generated methylation index cutoff value from perspective of clinical end point using $CIN3^+$ so that variable methylation level in individual women does not compromise the application of the *POU4F3* as biomarker to detect all SCCs and adenocarcinoma.

In summary, the present study validated that methylated *POU4F3* as the potential biomarker in detection of $CIN3^+$ among hrHV-positive women. Therefore, our study reinforced the combination of HPV test and *POU4F3* methylation analysis as possible cervical cancer screening strategy.

Chapter 5. Conclusion

In conclusion, our study validates for the first time that methylated *POU4F3* as the promising biomarker for the triage of $CIN3^+$ among hrHPV-positive women, which has potential to be appropriate screening strategy. Utilization of the new optimal biomarker *POU4F3* in screening algorithm may reduce the unnecessary referral of hrHPV-positive women for colposcopy without compromising $CIN3^+$ detection. Thus our study provides significant further step towards the molecular screening of cervical cancer.

Chapter 6. Future works

- We want to further investigate the clinical performance of *POU4F3* methylation testing as triage of hrHPV infected women in population-based studies in different geographical and ethnic backgrounds to validate applicability of this gene in population base screening.

- We want to conduct a longitudinal follow-up study to clarify the natural history of those infected with hrHPV, but without *POU4F3* hypermethylation to determine if a longer interval between screenings is also safe.

- We propose a scenario for the combination of HPV assay and *POU4F3* methylation analysis for cervical cancer screening. We want to conduct further independent validation together with additional standalone biomarker.

- *POU4F3* methylation testing is a potential molecular biomarker for the triage of hrHPV-positive women for $CIN3^{+}$ lesions. We want to further investigate the mechanisms in cervical cancer.

- Significance of *POU4F3* methylation testing as a potential molecular biomarker will be examined in other cancer types

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Chapter 8. Figures and Tables

8. a Figure

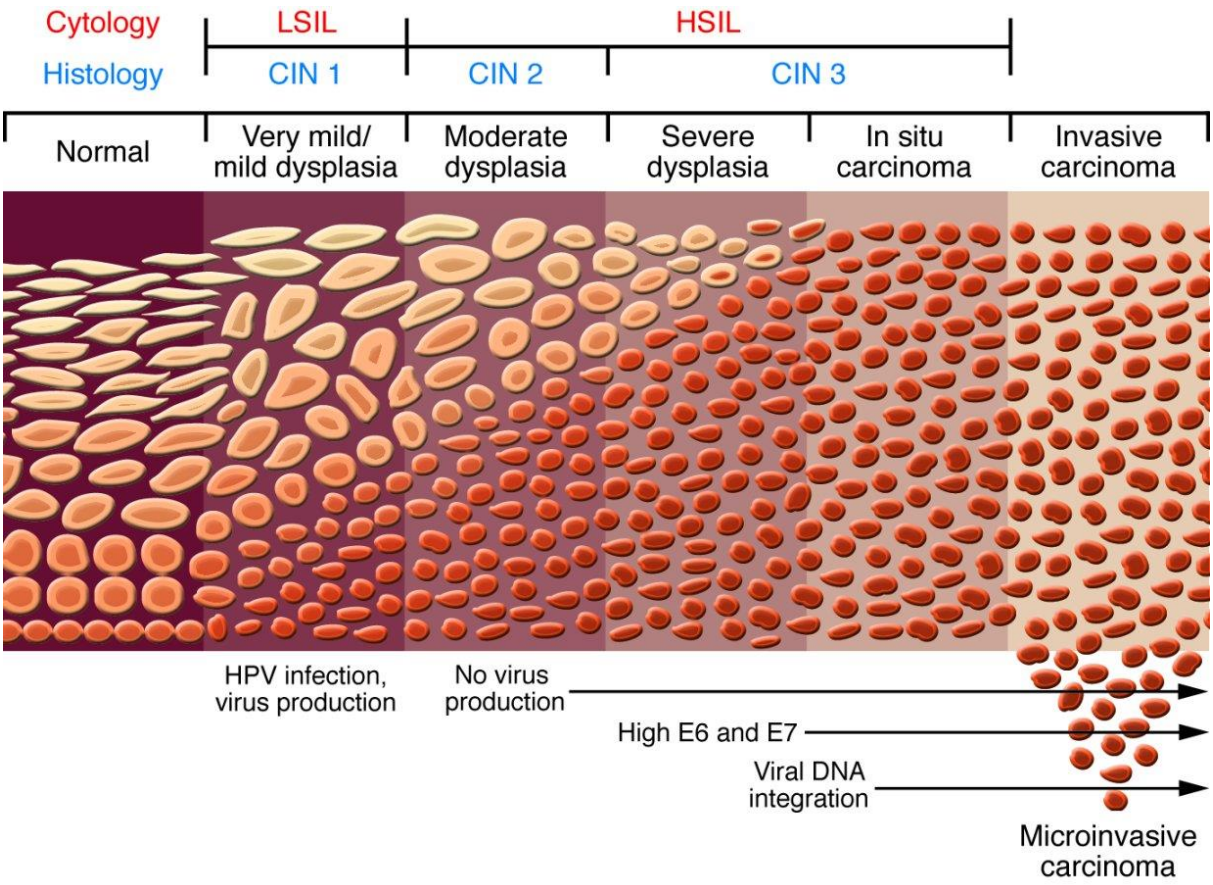

Figure 1. Schematic diagram of precancerous lesions of cervical cancer.

The morphological alterations occur due to viral infection in consecutive precancerous cervical lesions and correlation of the cervical intraepithelial neoplasia (CIN) classification with squamous intraepithelial lesions (SILs) and dysplasia. Adapted from [7].

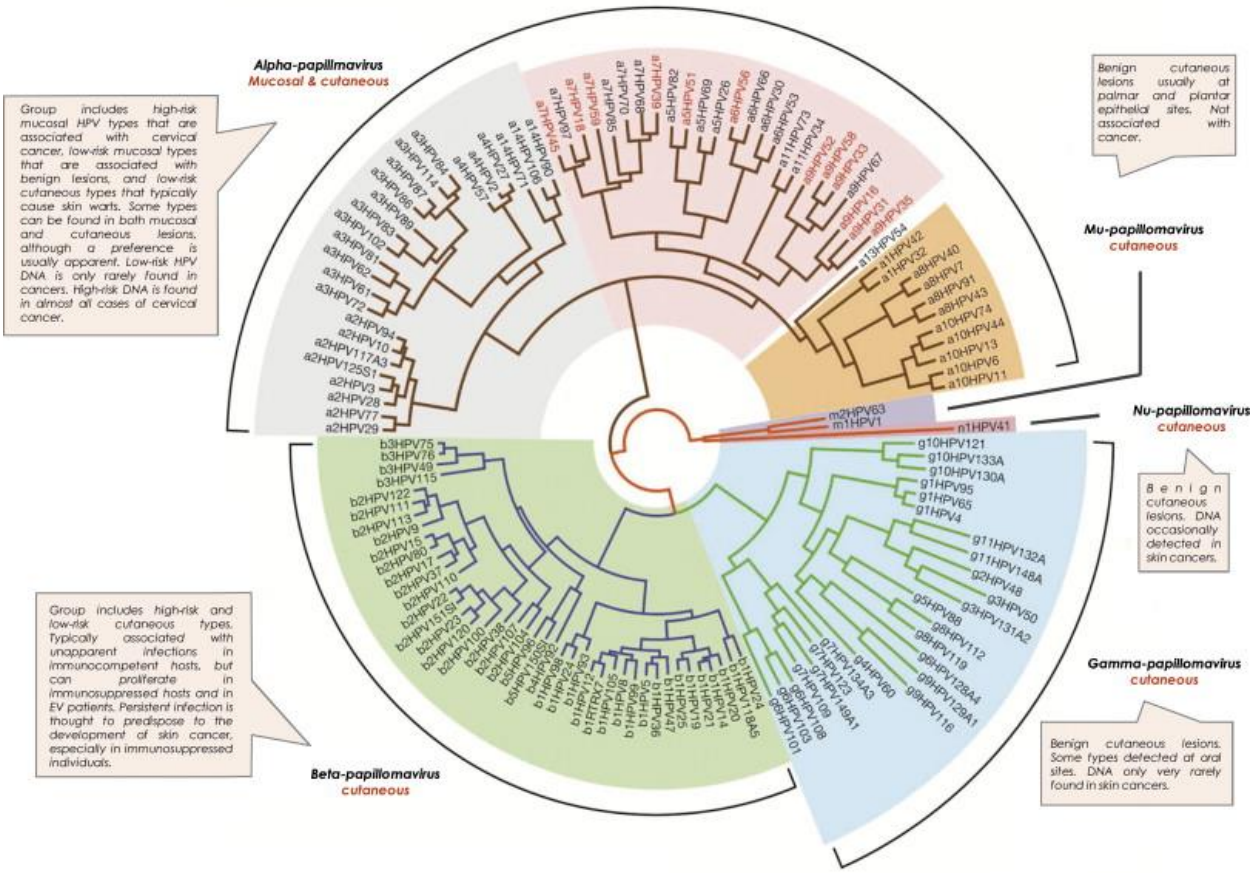

Figure 2. Evolutionary Relationship between Human Papillomaviruses.

There are five different evolutionary genera and more than 150 type of Human Papillomaviruses with various disease associations. The Alpha papillomaviruses include the low-risk mucosal types (orange shaded) responsible for genital warts, and the high-risk mucosal types (pink shading) responsible for cervical cancer. HPV types (Grey shading of Alpha), Green shading of Beta) and blue shading of Gamma) are not generally associated with cancers. Adapted from [18].

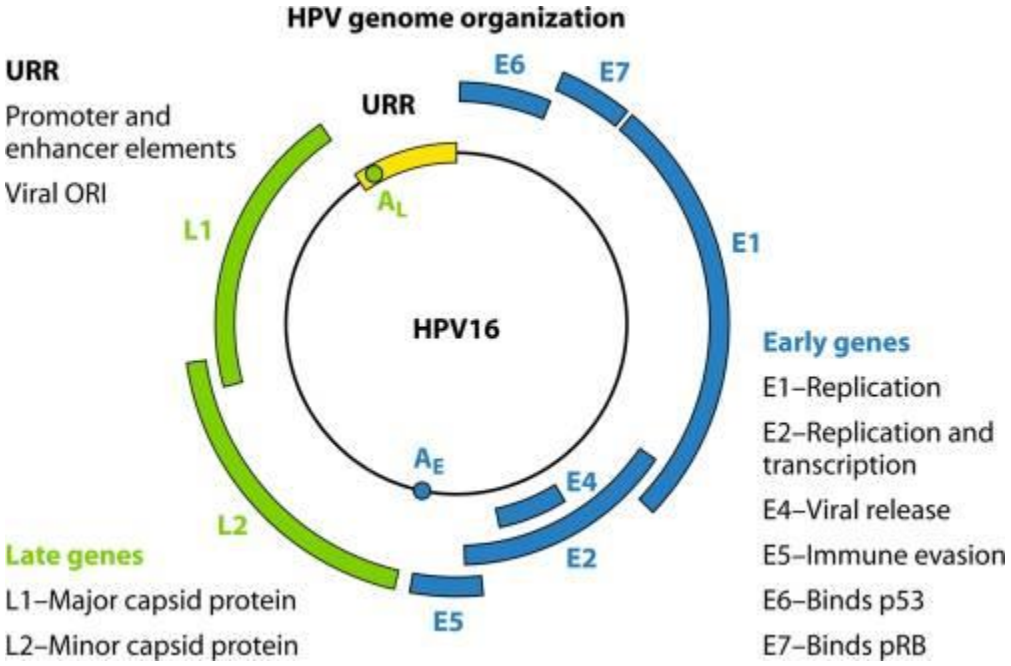

Figure 3. Schematic diagram of genomic organization of high-risk HPV.

It consists of early regions with five ORF encoding five proteins (E1, E2, E5, E6, E7) and late regions with 2 ORF encoding two proteins (L1 and L2) corresponding to the timing of expression of gene in viral life cycle. Early expressed proteins are significant for viral replication (E1), replication and transcriptional control (E2), viral release (E4), immune evasion (E5) and binding to p53 and pRB (E6 and E7 respectively). Additionally, late expressed proteins are important viral structural protein such as L1 as major capsid protein and L2 as minor capsid protein. Upstream regulatory region (URR) contains promoter, enhancer elements and the viral origin of replication important for regulation of transcription of early genes and viral replication. Adapted from [22].

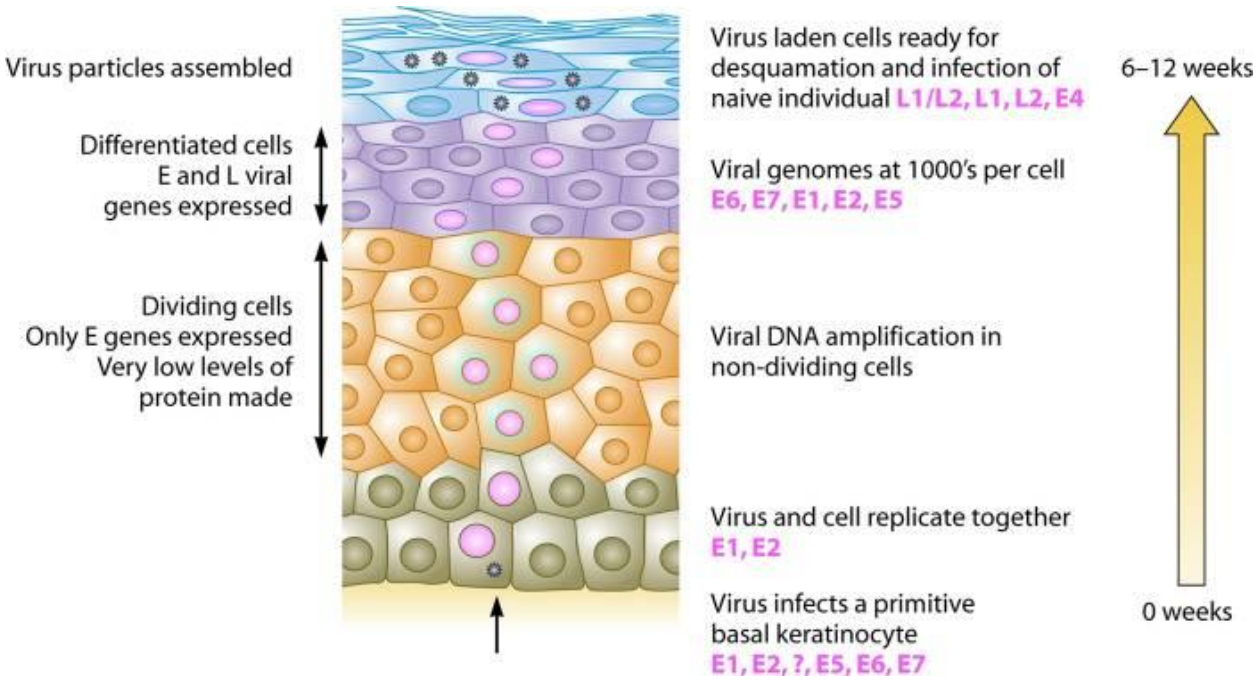

Figure 4. Human Papillomaviruses replication and assemble during viral life cycle.

Fully differentiating squamous epithelium are only cells HPV will infect and replicate inside them. Viral life cycle includes complex process of temporal and spatial viral expression. When virus gets entry in to cells of basal layer of epithelium, early regions proteins are expressed for viral replication together with cell replication. when host cells start to differentiate at the upper layer of epithelium, virus through its genes activation increases genome copy number and L1 and L2 proteins where thousands of viral genomes are exit the cells as encapsulated infectious virus particles. Adapted from [22].

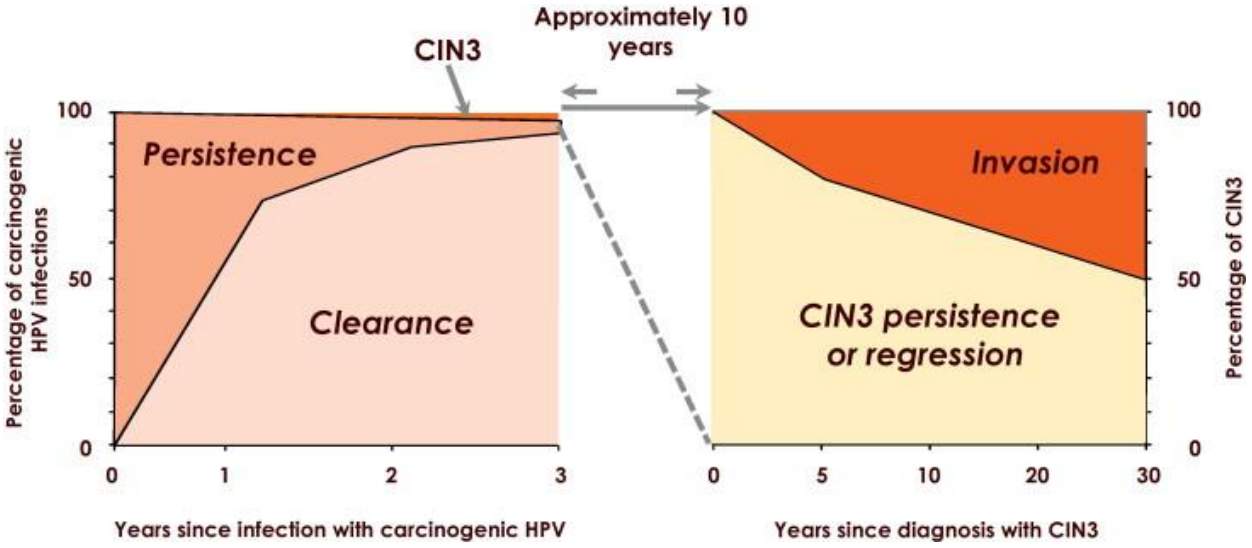

Figure 5. Natural history of HPV infection.

Majority of oncogenic HPV infection automatically clear with in first 3 years. Even major portion of persistent oncogenic HPV infection and CIN3 (Cervical intraepithelial neoplasia grade 3) also regress. Only few percentages of CIN3 becomes invasive cancer. Adapted from [27].

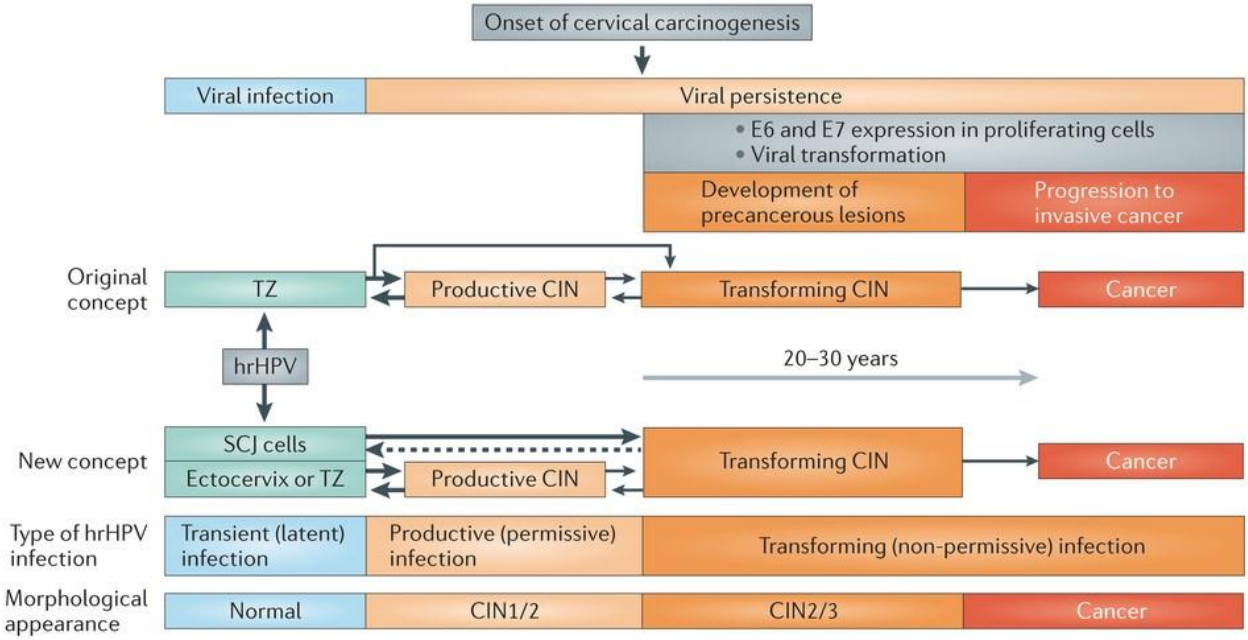

Figure 6. Schematic outline of concept of hrHPV driven cervical cancer.

Infection with hrHPV remains transient infection (normal pathology) initially, which may convert to productive infection (CIN1 and subset of CIN2) and transformation infection (remaining CIN2 and CIN3). It may take long period ranging 20-30 years for transforming CIN to develop invasive cancer. Transforming CIN is a heterogeneous mixture of CIN2 and CIN3 having different outcomes as to regress to normal or progress to cancer depends on alternation of molecular cellular mechanisms of host cells. It is advocated that exposure of hrHPV with squamo-columnar junction (SCJ), vulnerable cells for HPV infection mediated transformation, leads to origin of transforming CIN and cervical cancers. Additionally, ectocervix or transformation zone (TZ) is most susceptible region for productive CIN lesions. Adapted from [28].

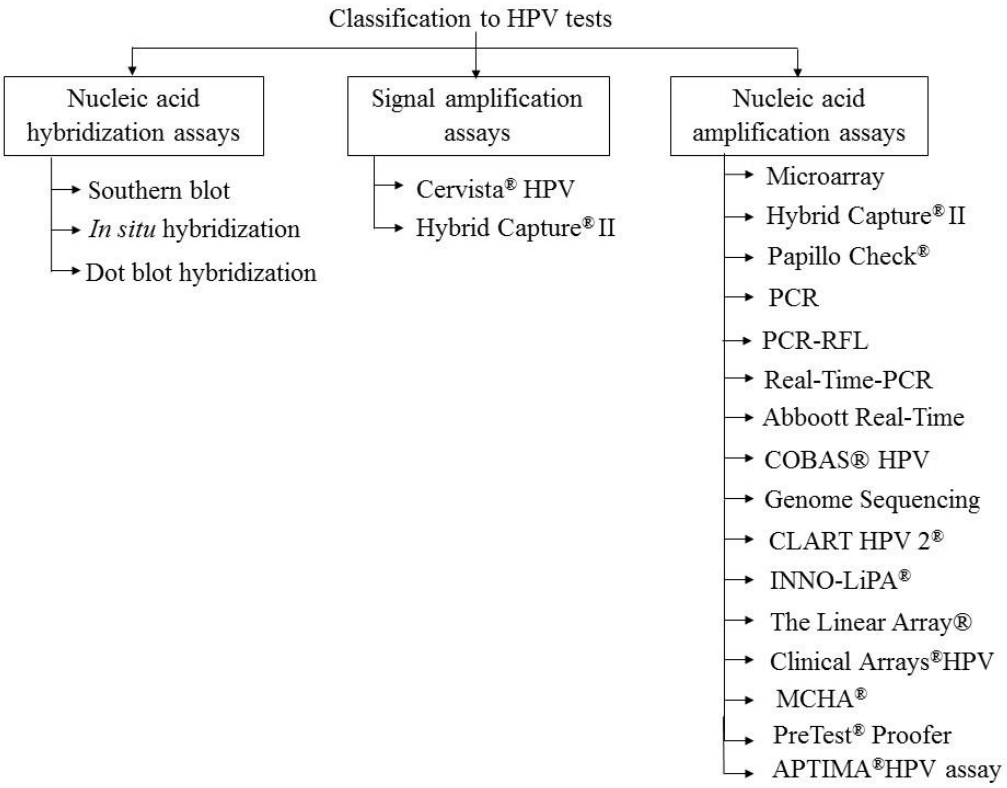

Figure 7. Outline of different techniques utilized in hrHPV detection in cervical sample.

Techniques employed in detection of HPV in cervical sample are broadly classified in to 3 types based on the principle of each of them. There are different types of techniques come under each categories of techniques. Hybrid capture II (HC II) comes under Signal amplification assays. Adapted from [35].

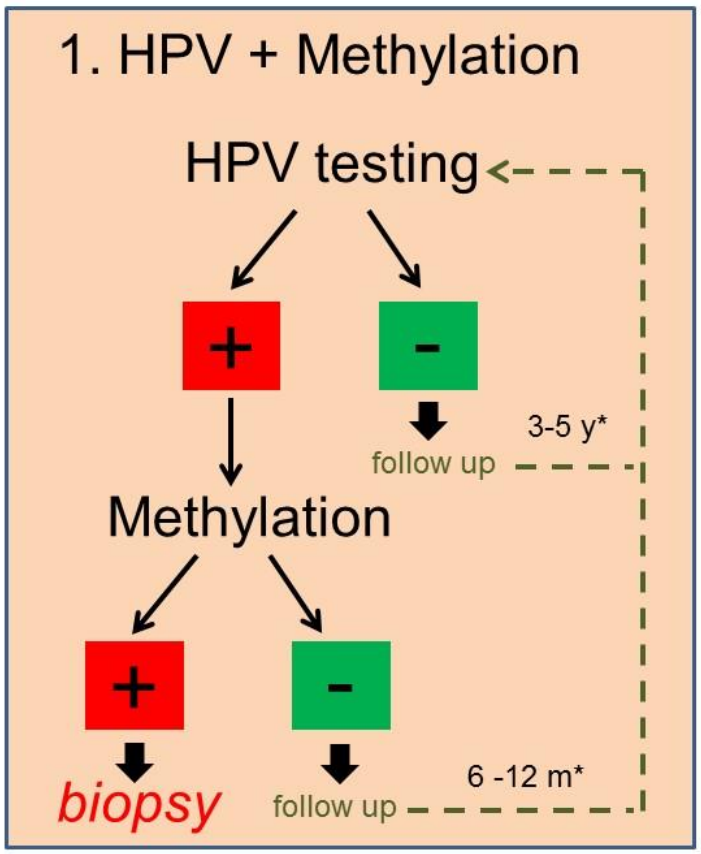

Figure 8. Flowchart of utilizing DNA methylation analysis to triage HPV infected women. HPV testing now approved as primary test. As to compensate drawbacks DNA methylation assay of candidate gene is subsequently utilized to triage HPV infected women. HPV test negative women are followed up every 3-5 years whereas, women with positive for both HPV test and methylated candidate gene are referred for biopsy. However, women having positive HPV test and negative methylated candidate gene women are further followed up for every 6-12 months.

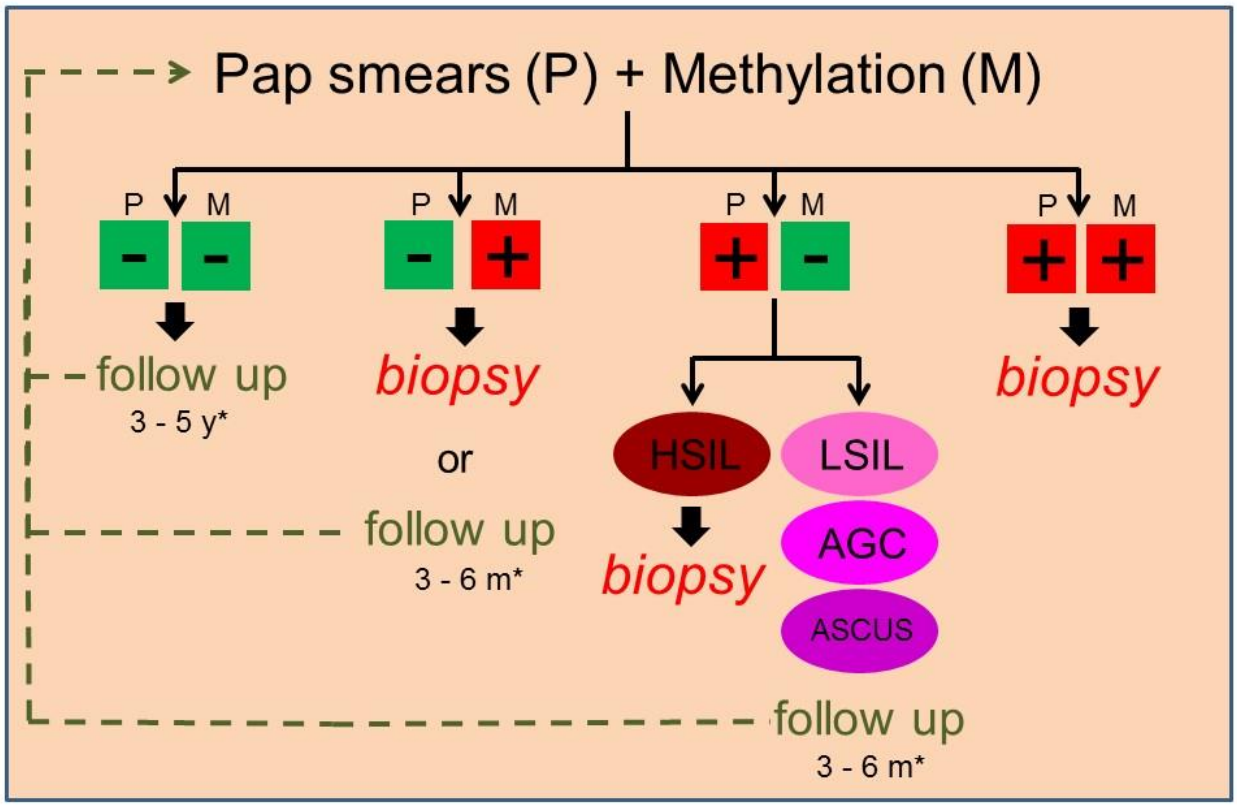

Figure 9. Flowchart of utilizing DNA methylation analysis in combination with cytology.

Cytology and gene methylation are carried out as combined parallel tests. Women with both tests negative are followed up every 3-5 years whereas, women with negative Pap smears and positive methylated genes are referred for either biopsy or follow up every 3-6 months depending on the level of methylation. However, women with negative methylated gene and HSIL lesions in Pap smears are referred to biopsy whereas, women with LSIL, AGC and ASCUS precancerous lesions in Pap smears are subsequently follow up or triage with methylated gene (Figure3). Additionally, women with positive for both Pap smears and methylated candidate gene are referred for biopsy.

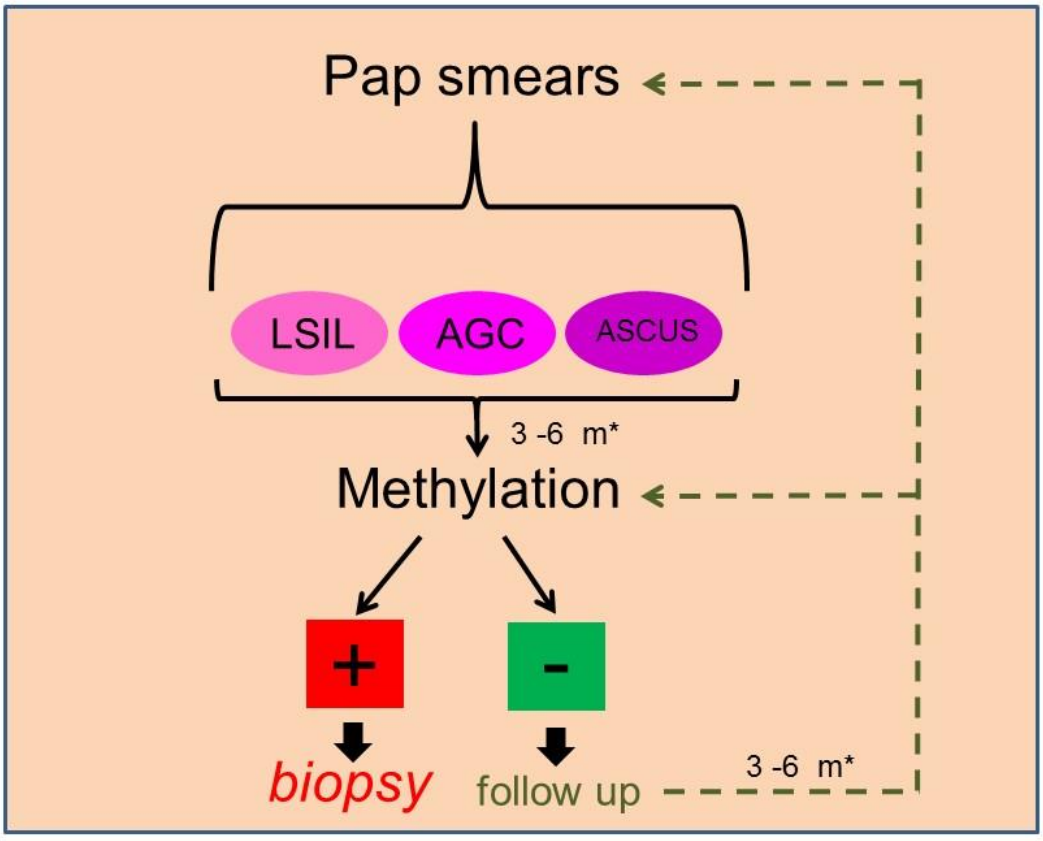

Figure 10. Flowchart of utilizing DNA methylation to triage women with abnormal cytology.

Pap smear is used as primary testing followed by subsequent triage using methylated gene testing for those women having precancerous lesions such as LSIL, AGC and ASCUS in positive Pap smears. If women having ASCUS, LSIL or AGC Pap smear also positive for methylated gene then they are referred for biopsy. However, women with atypical Pap smear and negative methylated genes are further follow up every 3-6 months.

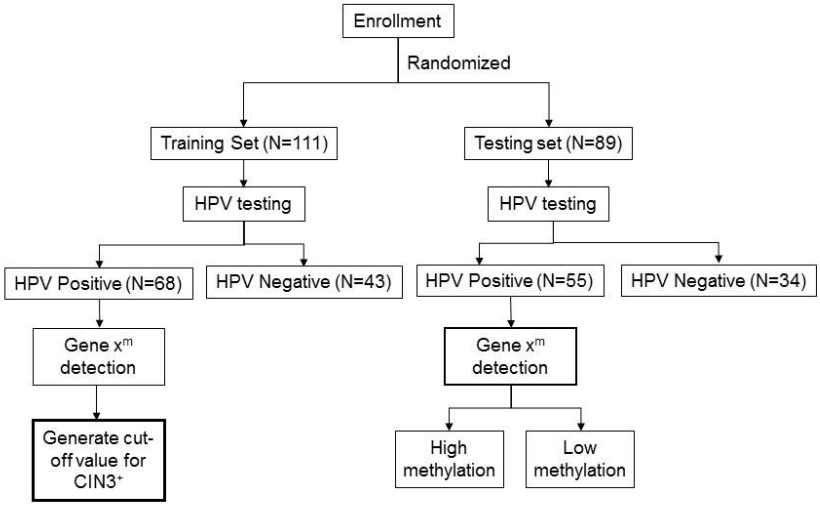

Figure 11. Work flow for analysis of clinical performance of candidate genes.

A total of 200 women were enrolled and randomly assigned to a training set and a testing set. Methylation analysis of candidate genes using cervical scrapings of hrHPV-positive women under the training set was used for generating M-index cutoff values, which were then applied for analysis of the clinical performance of the candidate genes. X^m is the level of methylation of the candidate gene.

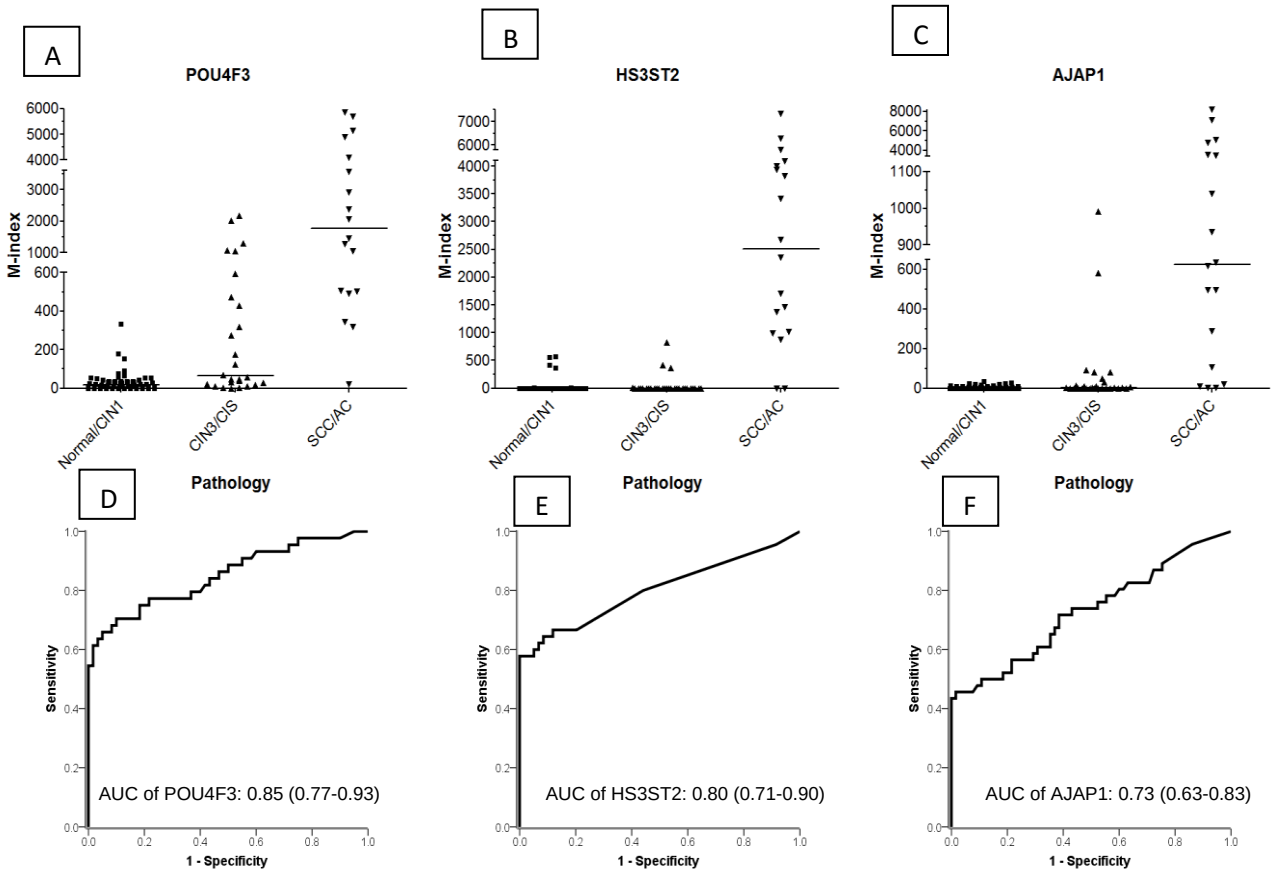

Figure 12. ROC curves of genes for M-index to trade off performance in detecting CIN3$^+$. Methylation index levels of *POU4F3* (A), *HS3ST2* (B), and *AJAP1* (C) in cervical scrapings such as normal and tumors graded as normal/CIN1, CIN3/CIS, or SCC/AC diagnosed by proven histopathology in samples irrespective of hrHPV status. Each dot in the figure represents the M-index level of an individual women. Analysis of ROC curve of *POU4F3* (D), *HS3ST2* (E), and *AJAP1* (F). The AUC of the ROC curve of an individual candidate gene was calculated to diagnose CIN3$^+$ lesions.

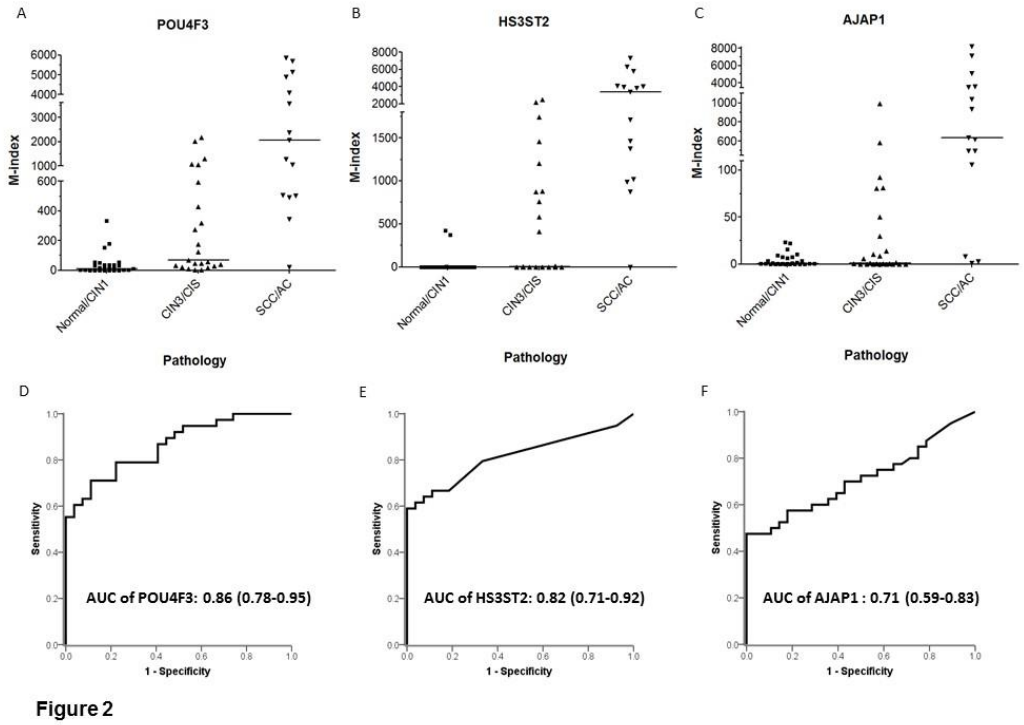

Figure 13. ROC curves of genes for M-index to trade off performance in detecting CIN3$^+$. Methylation index levels of *POU4F3* (A), *HS3ST2* (B), and *AJAP1* (C) in cervical scrapings such as normal and tumors graded as normal/CIN1, CIN3/CIS, or SCC/AC diagnosed by proven histopathology in hrHPV-positive samples. Each dot in the figure represents the M-index level of an individual women. Analysis of ROC curve of *POU4F3* (D), *HS3ST2* (E), and *AJAP1* (F). The AUC of the ROC curve of an individual candidate gene was calculated to diagnose CIN3$^+$ lesions.

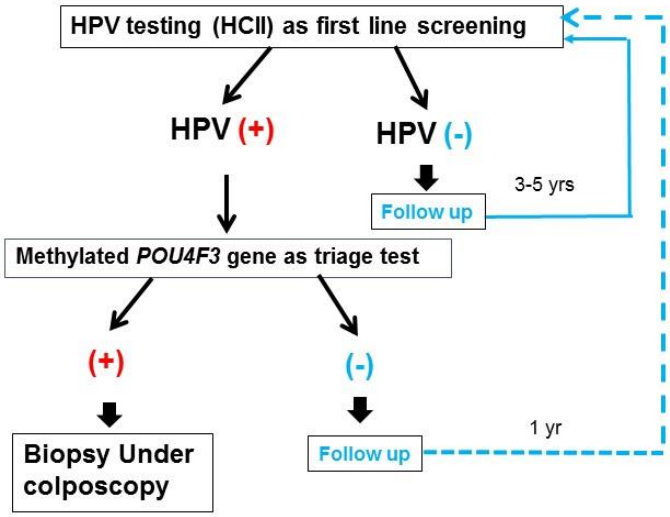

Figure 14. Proposed cervical cancer screening strategy using hrHPV assay and *POU4F3* methylation analysis as a triage test. In this proposed scenario, HCII hrHPV DNA assay is used as the primary screening test, where women without hrHPV infection undergo follow-up three to five years later. Samples from women with hrHPV infection undergo *POU4F3* methylation analysis, where women having positive *POU4F3* methylation are referred for colposcopy. Additionally, women with a positive hrHPV assay but negative *POU4F3* methylation may repeat HPV assay and DNA methylation analysis one year later.

8.b. Tables

Table 1. Main functions of the Human Papillomavirus proteins

HPV protein	Protein function
E1	Binds with E2 to form heterodimer complex, requires for controlling viral replication and viral life cycle completion.
E2	Involves in viral DNA replication together with E1, regulate early gene promotor as being transcriptional activator as in full-length protein, which binds to URR of DNA to enhance early region's transcription, transcription repression of early region through small protein, origin binding, viral DNA and host chromosomes
E4	Expressed in later stages of infection during assembling of complete virions, Significant for the maturation and replication of the virus through destabilizing cytokeratin network and enhancing release of viral particles.
E5	It mediates stimulation of mitogenic signals of growth factors via interacting with cell membrane based cellular receptor such as EGF and PDGF, may have important role in stimulating cell proliferation.
E6	One of the major oncoprotein that inactivates several cellular proteins, vital for replication of virus, immortalization of host cell and transformation, that binds with p53 and stimulates degradation of p53 via ubiquitin-dependent proteolytic pathways.
E7	One of the major oncoprotein that inactivates several cellular proteins, critical not only for viral replication, but also for immortalization of host cell and cellular transformation. Crucial for stimulating transcription of cellular genes via binding to Rb protein and dissociating E2F-Rb complex.
L1	Major capsid protein and important component of HPV prophylactic vaccine
L2	Minor capsid protein

Abbreviations: URR, upper regulatory region; EGF, epidermal growth factor; PDGF, platelet derived growth factor; Rb, retinoblastoma. Adapted and modified from [23].

Table 2. Benefits and weaknesses of the molecular methods for HPV detection

Method	Benefits	Weakness
Nucleic acids hybridization assays	Southern blot assay is considered as gold standard test for HPV genomic analysis. HPV in lesion is associated with morphology	Time consuming, Low sensitivity, requiring large amounts of purified DNA Degraded DNA cannot be used in southern blot and hybridization
Signal amplification assays	Quantitative; FDA-approved test (HC II); Lower false-positive rate; High sensitivity to genotyping	Licensed and patented technologies hindering further development. Cannot genotyping individual subtypes
Nucleic acids amplification assays	Flexible technology and useful for viral load and genotype; very high sensitivity; multiplex analysis	Lower amplification signals of some HPV genotypes. Contamination with other material leading to false positives

HPV = Human *Papillomavirus*; FDA= Food and Drug Administration; HC II = Hybrid Capture® II; PCR = Polymerase Chain Reaction. Adapted and modified from [35].

Table 3. DNA methylation analysis principles and methods

Pretreatment	Analytical step			
	Locus-specific analysis	Gel-based analysis	Array-based analysis	NGS-based analysis
Enzyme digestion	• *Hpa*II-PCR	• Southern blot • RLGS • MS-AP-PCR • AIMS	• DMH • MCAM • HELP • MethylScope • CHARM • MMASS	• Methyl–seq • MCA–seq • HELP–seq • MSCC
Affinity enrichment	• MeDIP-PCR		• MeDIP • mDIP • mCIP • MIRA	• MeDIP–seq • MIRA–seq
Sodium bisulphite	• MethyLight • EpiTYPER • Pyrosequencing	• Sanger BS • MSP • MS-SNuPE • COBRA	• BiMP • GoldenGate • Infinium	• RRBS • BC–seq • BSPP • WGSBS

AIMS, amplification of inter-methylated sites; BC–seq, bisulphite conversion followed by capture and sequencing; BiMP, bisulphite methylation profiling; BS, bisulphite sequencing; BSPP, bisulphite padlock probes; CHARM, comprehensive high-throughput arrays for relative methylation; COBRA, combined bisulphite restriction analysis; DMH, differential methylation hybridization; HELP, *Hpa*II tiny fragment enrichment by ligation-mediated PCR; MCA, methylated CpG island amplification; MCAM, MCA with microarray hybridization; MeDIP, mDIP and mCIP, methylated DNA immunoprecipitation; MIRA, methylated CpG island recovery assay; MMASS, microarray-based methylation assessment of single samples; MS-AP-PCR, methylation-sensitive arbitrarily primed PCR; MSCC, methylation-sensitive cut counting; MSP, methylation-specific PCR; MS-SNuPE, methylation-sensitive single nucleotide primer extension; NGS, next-generation sequencing; RLGS, restriction landmark genome scanning; RRBS, reduced representation bisulphite sequencing; –seq, followed by sequencing; WGSBS, whole-genome shotgun bisulphite sequencing. Adapted and modified from [125].

Table 4. Quantitative DNA methylation techniques using bisulphite-modified DNA

Technique	CpG sites interrogated per assay (n)	Advantages	Inconveniences
Bisulphite sequencing	5–50 (target sequence up to 600 bp)	'Gold standard'	Sub cloning required; labor-intensive
MS-DBA	1	Sensitivity; (internal oligo probe); affordable	Alkaline-phosphatase development difficult to normalize
MS-HRM SMART-MSP	2	Scalable for large scale screening; commercial kit available (Qiagen)	Requires a real-time PCR instrument
COBRA	1	Sensitivity; affordable	Limited to specific restriction targets
MS-SnuPE	Up to 6 (if multiplexed)	Sensitivity	Requires a capillary electrophoresis instrument
Q-MSP; MethylQuant	1	Scalable for large-scale screening	Requires a real-time PCR instrument
MethyLight	1-2	Scalable for large-scale screening	Requires a real-time PCR instrument
Mass spectrometry	5–50 (target sequence up to 600 bp)	Commercial service available (Sequenom)	Cost of custom assay
Pyrosequencing	5–20 (target sequence up to 120 bp)	Low amount of bisulphite-modified DNA required; commercial service available	cost
Illumina Golden Gate	Up to 1536	Powerful technology	Cost

COBRA: Combined bisulfite restriction analysis of DNA; MS-DBA: Methylation sensitive dot blot assay; MS-HRM: High-resolution melting technology; MS-SnuPE: Methylation sensitive single nucleotide primer extension; Q-MSP: Quantitative methylation-specific PCR; SMART-MSP: Sensitive melting analysis after real-time methylation-specific PCR. Adapted and modified from [128].

Table 5. Methylated cervical cancer associated genes in precancerous CIN lesions

Gene name	Gene name Frequencies of methylation positivity in ≤LSIL (≤BMD)	Frequencies of methylation positivity in ≥HSIL (>BMD)	References
APC	0%-88%	32%-83%	[141, 191, 192, 137, 193]
C13ORF18	2%-3%	37%-70%	[194, 195]
CADM1/TSLC1/IGSF4	5-100	26-100	[137, 193, 136, 196, 140, 75, 76]
CALCA	0-100	68-100	[192, 137]
CCNA1	4-18	37-75	[137, 194]
CDH1	13-80	17-91	[141, 193, 130]
CDH13	8-17	13-82	[141, 130]
CDKN2A /$p16^{INK4A}$	25	36	[196]
DAPK1	0-65	17-90	[136, 141, 191, 192, 137, 196, 130, 197, 198]
DLX1	25-36	41-84	[79]
EPB41L3	14	50-67	[195]
ESR1	12	11-50	[192, 137]
FAM19A4	3	45	[199]
GSTP1	0	7-18	[130]
HIC1	18-100	27-100	[141, 193, 196, 197]
HIN1	18-100	27-100	[197]
HOXA9	100	100	[193]
HOXA10	68	81	[193]
HOXA11	95	100	[193]
HOXC9	61	62	[193]
HOXD9	100	100	[193]
HSPA2	0	3-73	[130]
hTERT	0-52	17-88	[195, 130, 198, 200]
ITGA4	7-14	27-68	[79]
JAM3	5	49-67	[195]
LHFPL4	-	25	[198]
LMX1A	10-13	16-36	[164, 145]
MAL	5-12	53-83	[75, 201]
MLH1	0-7	11-36	[192, 196, 130]
MGMT	2-15	11-26	[141, 191, 196, 197]
MT1G	5-18	19-55	[193, 202]
MTHFR	68	100	[203]
NKX6-1	33-79	55-76	[193, 164, 145]
NMES1	0	36	[202]
NOL4	-	42	[198]
ONECUT1	3-100	7-92	[193, 164]
$P14^{ARF}$	7	18	[196]
P73	14	18	[196]
PAX1	1-10	23-100	[193, 164, 145,

			144, 169, 168]
PCDH10	0-8	22-100	[204, 205]
PHACTR3	6	55	[199]
PRDM14	36	91	[199]
RAR-β	0-95	4-100	[141, 192, 137, 193, 197]
RASSF1A	8	0-46	[130, 197]
ROBO3	0	10	[205]
RRAD	43	68	[202]
RXFP3	9-18	41-84	[79]
SFN	93	99	[141]
SFRP1	1-5	8-59	[206]
SFRP2	10	16-81	[206]
SFRP4	2	37-68	[206]
SFRP5	9	4-10	[206]
SHP1	14	33	[197]
SLIT1	0	10	[205]
SLIT2	1	25	[205]
SOCS1	0	7-50	[130]
SOCS2	23	45-64	[130]
SOX1	3-76	9-95	[193, 164, 145]
SOX17	6-7	27-84	[79]
SPARC	5-88	49-100	[137, 136, 202]
SYK	5	4-15	[141]
TFPI1	3-38	23-82	[137, 136, 202]
TIMP3	0-8	8-100	[192, 193, 130, 198]
TWIST1	0-13	4-43	[141]
WT1	8-21	35-100	[193, 164, 206]
ZNF671	11	59-89	[79]

Adapted with permission and modified from [28].

Table 6. Clinical performances of potential biomarker in detecting cervical (pre-) cancer as analyzed by QMSP

Significant genes	Detecting methods	Classify of detecting	AUC	Sensitivity (%)	Specificity (%)	Potential biomarker in other cancer	Reference
ADRA1D	TaqMan QMSP	$CIN3^+/CIN2^-$	0.77	60	93	na	[148]
AJAP1	TaqMan QMSP	$CIN3^+/CIN2^-$	0.82	65	98	glioblastoma, gastric Cancer	[148]
C13ORF18	SYBR QMSP	$CIN2^+/CIN1^-$ $CIN3^+/CIN2^-$	na	40% (27/68) 59% (23/39)	100% (75/75) 96% (100/104)	na	[77]
CADM1	TaqMan QMSP	$CIN3^+/CIN2^-$	0.64	na	na	Meningioma; Lung and esophageal cancer	[161]
CADM1 and MAL	TaqMan QMSP	$CIN3^+/CIN2^-$	0.72	61	83	eningioma; Lung and esophageal cancer	[76]
COL6A2	TaqMan QMSP	$CIN3^+/CIN2^-$	0.69	45	93	na	[148]
DAPK1	pyrosequencing	Cancer	0.72	43	100	n/a	[158]
DAPK1, SLIT2, WIF1, RARB	pyrosequencing	Cancer	0.95	90	100	na	[158]
DBC1	TaqMan QMSP	$CIN3^+/CIN2^-$	0.6	37	83	Na	[172]
EDN3	TaqMan QMSP	$CIN3^+/CIN2^-$	0.77	55	98	Breast and colon cancer	[148]
EPB41L3	SYBR QMSP	$CIN2^+/CIN2^-$ $CIN3^+/CIN2^-$	na	50% (34/68) 67% (26/39)	89% (67/75) 85% (88/104)	Breast and esophageal cancer	[77]
EPO	TaqMan QMSP	$CIN3^+/CIN2^-$	0.75	63	87	na	[148]
FAM19A4	Multiplex QMSP	$CIN3^+$	0.77	76	67	na	[80]
FKBP6	TaqMan QMSP	Neoplasia	0.80	73	79	na	[155]
HS3ST2	TaqMan QMSP	$CIN3^+/CIN2^-$	0.82	83	82	ovarian cancer	[148]
JAM3	SYBR QMSP	$CIN2^+/CIN1^-$ $CIN3^+/CIN2^-$	na	49% (33/68) 67% (26/39)	89% (67/75) 86% (89/104)	na	[77]
JAM3, EPB41L3, TERT, C13ORF18	SYBR QMSP	$CIN2^+/CIN1^-$ $CIN3^+/CIN2^-$	na	65% (44/68) 82% (32/39)	79% (59/75) 73% (76/104)	na	[77]
LMX1A	TaqMan QMSP	$CIN3^+/CIN2^-$	0.9	77	88	Ovarian; bladder; gastric cancer	[145]
MAGI2	TaqMan QMSP	$CIN3^+/CIN2^-$	0.83	70	97	na	[148]
MAL	TaqMan QMSP	$CIN3^+/CIN2^-$	0.77	na	na	lung cancer	[161]
miR-124-2	TaqMan QMSP	$CIN3^+/CIN2^-$	0.76	na	na	bladder cancer	[161]
NKX6.1	TaqMan QMSP	$CIN3^+/CIN2^-$	0.97	93	97	na	[145]
PAX1	TaqMan QMSP	$CIN3^+/CIN2^-$	0.89	78	91	oral cancer	[145]
POU4F3	TaqMan QMSP	$CIN3^+/CIN2^-$	0.85	88	82	na	[148]
	SYBR QMSP	$CIN3^+$	0.86	74	89		[156]
PTGDR	TaqMan QMSP	$CIN3^+/CIN2^-$	0.82	65	98	na	[148]
RARB	pyrosequencing	Cancer	0.61	22	100	na	[158]
SFRP4	TaqMan QMSP	Cancer	0.75	71	65	na	[207]
SLIT2	pyrosequencing	Cancer	0.81	61	100	na	[158]

SOX1	TaqMan QMSP	$CIN3^+/CIN2^-$	0.95	88	82	ovarian cancer	[145]
SOX17	TaqMan QMSP	$CIN3^+/CIN2^-$	0.78	60	97	esophageal cancer	[148]
SOX8	TaqMan QMSP	$CIN3^+/CIN2^-$	0.7	48	93	na	[148]
SOX9	TaqMan QMSP	$CIN2^+/CIN1^-$	0.96	92	90	bladder cancer	[157]
ST6GAL2	TaqMan QMSP	$CIN3^+/CIN2^-$	0.77	58	97	na	[148]
SYT9	TaqMan QMSP	$CIN3^+/CIN2^-$	0.81	68	95	na	[148]
TERT	SYBR QMSP	$CIN2^+/CIN1^-$ $CIN3^+/CIN2^-$	na	37% (25/68) 54% (21/39)	89% (67/75) 88% (92/104)	lung; leptomeningeal metastases; gastric cancer	[77]
WIF1	pyrosequencing	Cancer	0.73	46	100	na	[158]
ZAR1	TaqMan QMSP	Cancer	0.83	77	80	na	[207]
ZNF516	Taqman QMSP	Neoplasia	0.95	90	95	na	[155]
ZNF582	TaqMan QMSP	$CIN3^+/CIN2^-$	0.82	70	82	colorectal cancer	[172]
ZNF614	TaqMan QMSP	$CIN3^+/CIN2^-$	0.75	53	98	na	[148]

na: not available

Table 7. Histopathology, mean age and HPV percentage of the patients in set for selecting genes

Variable		Set for selecting genes
Age	Mean ± SD	47.8 ± 13.5
		No (%)
Result of Pathology	Normal	20 (20.0)
	CIN1	20 (20.0)
	CIN2	20 (20.0)
	CIN3	8 (8.0)
	CIS	12 (12.0)
	SCC	15 (15.0)
	AC	5 (5.0)
HPV	Negative	33 (33.0)
	Positive	67 (67.0)
Total		100

Abbreviations: CIN: cervical intraepithelial neoplasia; CIN1: CIN grade 1; CIN2: CIN grade 2; CIN3: CIN3 grade 3; CIS: carcinoma in situ; SCC: squamous cell carcinoma; AC: Adenocarcinoma; HPV: Human Papilloma Virus

Table 8. Clinical performance of cervical cancer associated genes to detect CIN3$^+$ women

Gene name	M-index [a] cut off value	Sensitivity (%)	Specificity (%)	Negative predictive value (%)	Positive predictive value (%)
ADRA1D	15.73	60	93	78	86
AJAP1	24.63	65	98	81	96
COL6A2	2.22	45	93	72	82
EDN3	3.79	55	98	77	96
EPO	1.82	63	87	78	76
HS3ST2	2.88	83	82	88	75
MAGI2	16.40	70	97	83	93
POU4F3	9.67	88	82	91	76
PTGDR	11.15	65	98	81	96
SOX8	2.20	48	93	73	83
SOX17	6.93	60	97	78	92
ST6GAL2	9.56	58	97	77	92
SYT9	5.86	68	95	81	90
ZNF614	3.07	53	98	76	95

a

Methylation-Index (M-Index): the value cut off for precancer/cancer patent (cervical intraepithelial neoplasia grade 3 and worse, CIN3$^+$) or not (CIN2−, including normal specimens, and CIN1 and CIN2 specimens).

Table 9 Clinical performance of hrHPV assay in selection set and validation set

Detection modality test used	Parameters	
hrHPV assay (HC II)	Sensitivity (%)	83
(100)	Specificity (%)	43
	Positive predictive value (PPV) %	49
	Negative predictive value (NPV) %	79
hrHPV assay (HC II)	Sensitivity (%)	89
(200)	Specificity (%)	59
	Positive predictive value (PPV) %	62
	Negative predictive value (NPV) %	88

Table 10. Sensitivities and specificities of candidate genes in hrHPV-positive women in the selection set

Detection modality or test used		Sensitivity (%)	Specificity (%)
HPV (+) (N=67)	*ADRA1D*	61	97
	AJAP1	64	100
	COL6A2	42	91
	EDN3	58	97
	EPO	67	97
	HS3ST2	88	82
	MAGI2	70	94
	POU4F3	88	82
	PTGDR	67	97
	SOX8	46	91
	SOX17	64	94
	ST6GAL2	64	97
	SYT9	73	94
	ZNF614	58	97

Table 11. Histopathology, mean age and HPV percentage of the patients in validation set

Variable		Training set		Testing set	
Age	Mean ± SD	46.8 ± 13.5		44.2 ± 13.3	
		HPV Positive	HPV Negative	HPV Positive	HPV Negative
		No (%)		No (%)	
Result of Pathology	Normal	7 (6.3)	27 (24.3)	5 (5.6)	26 (29.2)
	CIN1	21 (18.9)	10 (9)	14 (15.7)	5 (5.6)
	CIN3	11 (9.9)	3 (2.7)	20 (22.4)	1 (1.1)
	CIS	14 (12.6)	0 (0)	5 (5.6)	1 (1.1)
	SCC	15 (13.5)	3 (2.7)	11 (12.3)	1 (1.1)
Sub total		68 (62.2)	43 (38.7)	55 (61.8)	34 (38.2)
Grand total		111		89	

Abbreviations: CIN: cervical intraepithelial neoplasia; CIN1: CIN grade 1; CIN2: CIN grade 2; CIN3: CIN3 grade 3; CIS: carcinoma in situ; SCC: squamous cell carcinoma; AC: Adenocarcinoma; HPV: Human Papilloma Virus

Table 12. Performance of methylation biomarkers to detect CIN3$^+$ in hrHPV-positive women at training and testing sets

	Gene name	POU4F3	HS3ST2	AJAP1	SOX1	PAX1
	M-Index cut-off value	38	2	2	4	4
Training set (N=68)	Sensitivity (%)	79	67	63	78	70
	Specificity (%)	78	89	64	71	89
	PPV (%)	83	90	71	80	90
	NPV (%)	72	65	55	69	68
Testing set (N=55)	Sensitivity (%)	74	55	80	63	60
	Specificity (%)	89	100	74	74	100
	PPV (%)	93	100	85	82	100
	NPV (%)	64	56	67	52	58

Abbreviation: PPV: positive predictive value; NPV: negative predictive value; CIN3$^+$: including CIN3/CIS, SCC/AC

Table 13. Clinical performance of methylation biomarker in hrHPV-positive women of testing set stratified by histology

Total number of detectable		53	52	54
Detection modality or test used -QMSP		Gene Name		
		POU4F3	*HS3ST2*	*AJAP1*
		Methylation positive/total number (%)		
Result of Pathology	Normal	0/5 (0%)	0/5 (0%)	0/5 (0%)
	CIN1	2/13 (15.4%)	0/14 (0%)	5/14 (35.7%)
	CIN3	13/20 (65.0%)	6/18 (33.3%)	14/20 (70.0%)
	CIS	2/4 (50%)	2/4 (50%)	3/4 (75%)
	SCC	11/11 (100%)	10/11 (90.9%)	11/11 (100%)
	Total	28	18	33

Abbreviations: CIN: cervical intraepithelial neoplasia; CIN1: CIN grade 1; CIN3: CIN3 grade 3; CIS: carcinoma in situ; SCC: squamous cell carcinoma; AC: Adenocarcinoma

Table 14 QMSP primers and probes utilized in this study

Gene	Forward Primer Sequence	Reverse Primer Sequence	Probe Oligo Sequence
ADRA1D	GGT TAG GTA GTT TCG TTT TCG GAT AGT C	AAA CAC AAA ACG AAC GAC CGA CA	6FAM-TAACCGCGAAACTCCAA-MGB
AJAP1	TTT GGT AGA GTT TTT CGA TTC GGT AGC	ACC GAA ACT CCG CGC CGA TAA	6FAM-AACGAACGCGAATCCCCTC-MGB
COL6A2	TTT TAG GGT TTT CGT CGG TTT TGC	CGC CAA CCC CTA CCC GCT AC	6FAM-ACTCGAACTTCGCGTCCC-MGB
EDN3	GGT AGC GCG TTT TGA AAG TTT ATG ATC	TAA ATC AAA CGC CGA AAC ACC GA	6FAM-CGACCGCTTATAACCGC-MGB
EPO	GTT TTT GGG TTA TTT CGG TCG TTC	AAA AAA CGA CTA TCC AAA AAA CGC	6FAM-CGATAACCCCGATCCGACTCCGAA-BHQ1
HS3ST2	GTA AGA GTT TGG GAG CGT TCG AGT C	CAA AAA ATC CCG AAA ACA ACG AC	6FAM-CCAACATCTCCCGATCC-MGB
MAGI2	CGT AGA GTT CGA GAT GTG GTA TTA GGC	AAA CTC CTA TAC GAA AAA AAC GCG CTA	6FAM-CCGATAAAACAAAAATAACG-MGB
POU4F3	AGC GCG GGC GTT GAG TAG C	CGC GCT CCT AAC AAA ATA ACA ACG AA	6FAM-TAACTTACTTACCGCTCTCC-MGB
PTGDR	TTG TTT CGC GTT TTT TAA TGT TAG C	AAA AAA ACT CCG AAA ACG ACG AAA T	6FAM-CACGACAAAAACCTCCTAT-MGB
SOX8	GGG TTT CGT TTT CGG GGG ATC	ATC CCG CTA CGA AAC CCG CAA CA	6FAM-CCCCGCGCCCGACTA-MGB
SOX17[1]	GGA GAT TCG CGT AGT TTT CG	AAC CCG ACC ATC ACC GCG	6FAM-CGCGCTCTAAATCTAACTC-MGB
ST6GAL2	GGT GGG GGT TTT TAG GGA GTA TCG TTA TC	CGA CTA AAT AAA ACG CAA AAA AAC GCA AC	6FAM-CCGACTCGCTAACTCC-MGB
SYT9	TGG GGT CGT CGT TAT TTT ATT TTG C	CCG CCC GAT CCC TCC GTC	6FAM-AACGATACCGCACAACCCA-MGB
ZNF614	TTT CGG GTA GGT TTC GTG GTT TTC	CTA ACA ACG ACA AAA CGC CGC ATC	6FAM-CCGAAAACGCTTCCTATT-MGB
COL2A1[2]	GGG AAG ATG GGA TAG AAG GGA ATA T	TCT AAC AAT TAT AAA CTC CAA CCA CCA A	6FAM-TTCATTCTAACCCAATACCT-MGB

[1] *SOX17* primer was referenced from Fu DY et al [208] .

[2] *COL2A1* was used as an internal reference gene by amplifying non-CpG sequences.

Note: QMSP primers and probe sequences for *PAX1* and *SOX1* will be provided upon request.

Chapter 9. Publication

Par Bahadur Pun et al. **"Triage of high-risk human papillomavirus-positive women by methylated *POU4F3*"** Clinical Epigenetics 2015, **7**:85 doi:10.1186/s13148-015-0122-0

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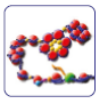

CLINICAL EPIGENETICS

RESEARCH Open Access

Triage of high-risk human papillomavirus-positive women by methylated *POU4F3*

Par Bahadur Pun[1,2], Yu-Ping Liao[3], Po-Hsuan Su[4], Hui-Chen Wang[3], Yu-Chih Chen[5], Yaw-Wen Hsu[2], Rui-Lan Huang[4], Cheng-Chang Chang[6] and Hung-Cheng Lai[1,2,3,4*]

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Triage of high-risk human papillomavirus-positive women by methylated *POU4F3*

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Abstract

Background: Insufficient specificity of the high-risk human papillomavirus (hrHPV) assay in primary cervical cancer screening results in unnecessary referral. Additional assays to triage hrHPV-positive women are needed to improve molecular cervical cancer screening. DNA methylation is a promising biomarker in cervical cancer. We evaluated the clinical performance of potentially methylated genes as a triage assay for hrHPV-positive women.

Results: We conducted a retrospective hospital-based case–control study in Taiwan. Cervical scrapings were collected before colposcopy for hrHPV testing and quantitative methylation-specific PCR (QMSP) of 16 genes. Five genes, *POU4F3*, *HS3ST2*, *AJAP1*, *PAX1*, and *SOX1*, were prioritized for the clinical performance to triage hrHPV-positive women. Two hundred cervical scrapings were randomly classified into a training set ($n = 111$) and testing set ($n = 89$). All samples were tested for hrHPV using a Hybrid Capture II (HCII) assay. HrHPV-positive women were subjected to DNA methylation analysis by QMSP. In the training set, the receiver operating characteristic (ROC) curves defined the optimal methylation index (M-index) cutoff values for discriminating $CIN3^+$ from CIN1/normal, which then were applied to the testing set. Among the five genes, *POU4F3* revealed the highest area under the ROC curve (AUC) (0.86; 95 % CI, 0.78–0.95) in detecting $CIN3^+$. In the testing set, *POU4F3* revealed the best clinical performance in triage of hrHPV-positive women with a sensitivity of 74 % and specificity of 89 % for detecting $CIN3^+$.

Conclusions: *POU4F3* methylation analysis is a potential molecular tool for triage in detecting $CIN3^+$ in hrHPV-positive women. The combined use of broad-spectrum HPV assay and *POU4F3* methylation analysis as a new generation of molecular cervical cancer screening warrants further population-based study.

Keywords: DNA methylation, HrHPV test, QMSP, Biomarker, Cervical intraepithelial neoplasia (CIN), Cervical cancer screening

Background

Cervical cancer is a common medical problem in women, with 528,000 new cases and 266,000 deaths globally in 2012 indicating the need to develop and implement an effective cancer screening strategy [1]. The Papanicolaou (Pap) smear for cytological examination has been used for the detection of precancerous cellular abnormalities of cervical cells for decades and has lessened the disease burden by reducing the mortality and morbidity of cervical cancer [2]. The Pap smear or cytology test has high specificity for cervical intraepithelial neoplasia; however, it has many drawbacks such as suboptimal sensitivity [3] and moderate accuracy to detect relevant lesions and subjective diagnosis of cervical abnormalities with poor reproducibility [4]. Oncogenic high-risk human papillomavirus (hrHPV) infection is a well-known etiology of cervical cancer [5]. Because the duration of the initial hrHPV infection until the development of invasive cancer is long, assay of HPV DNA as a screening tool is appealing [6, 7]. However, HPV infection is transient in nature, and only few infected lesions further progress as invasive cancer [8]. Insufficient specificity of the HPV DNA assay results in a high false-positive rate and extra medical burden because of the consequent high colposcopy referral rate [6]. Findings of HPV-positive assay results also cause adverse psychosocial impact [9]. Therefore, an additional

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triage assay is required to improve HPV-based molecular cervical cancer screening [10, 11].

Persistent oncogenic hrHPV infection causes genetic and epigenetic changes [12]. Promoter hypermethylation-mediated silencing of tumor suppressor genes is common in cervical carcinogenesis [12, 13]. Because DNA methylation can be easily quantitated using molecular methods, it is gaining attraction as a molecular assay for detecting cervical cancer [14]. Several studies including our group have revealed that numerous aberrantly DNA-methylated cervical cancer-related genes could be potential biomarkers to improve cervical cancer detection [12, 15–17], to triage women with atypical squamous cells [18, 19] and low-grade squamous intraepithelial lesions (LSILs) in Pap smears [20]. Methylated genes could be potential markers for the triage of hrHPV-positive women [21–27]. However, the sensitivity and specificity are not satisfactory even if combining two or more genes [23, 24, 26], highlighting the need for novel methylation biomarkers.

Using methylomic approaches, many methylated candidate genes have been revealed, including *ADRA1D*, *AJAP1*, *COL6A2*, *EDN3*, *EPO*, *HS3ST2*, *MAGI2*, *POU4F3*, *PTGDR*, *SOX8*, *SOX17*, *ST6GAL2*, *SYT9*, *ZNF614* [28], *SOX1*, and *PAX1* [29]. The performance of these methylated genes to triage hrHPV-positive women remains unexplored.

Results

Selection of potential candidate genes in hrHPV-positive women

We randomly collected cervical scrapings from 100 women including 20 normal, 20 CIN1, 20 CIN2, 20 CIN3/CIS, and 20 SCC/AC before treatment. Those samples from hrHPV-positive women, 67 out of 100, were subjected to quantitative methylation-specific PCR (QMSP) analysis of 14 genes, *ADRA1D, AJAP1, COL6A2, EDN3, EPO, HS3ST2, MAGI2, POU4F3, PTGDR, SOX8, SOX17, ST6GAL2, SYT9*, and *ZNF614*, and used the same cutoff values previously described [28] (Table 1). We selected candidate genes with a sensitivity of >85 % or specificity of >98 % in detecting $CIN3^+$ in hrHPV-positive women for further validation. Three genes, *POU4F3*, *HS3ST2*, and *AJAP1*, fulfilled these criteria.

Generation of methylation cutoff values for triage of hrHPV-positive women in the training set

We tested the clinical performance of *POU4F3*, *HS3ST2*, and *AJAP1* methylation for the triage of hrHPV-positive women (Fig. 1). The independently enrolled 200 women were randomly classified into two groups with a training-to-testing ratio of 1:1 (Table 2). There was no significant difference in the age ($P = 0.17$) and diagnosis distribution in the training set and testing set. Methylation levels of *POU4F3*, *HS3ST2*, and *AJAP1* in hrHPV-positive women increased with disease severity (Fig. 2a–c). The optimal methylation index (M-index) cutoff values for detecting $CIN3^+$ were 38 for *POU4F3* and 2 for *HS3ST2* and *AJAP1* as defined by receiver operating characteristic (ROC) curves. The areas under the ROC curves (AUC) were 0.86 (95 % CI, 0.78–0.95) for *POU4F3*, 0.82 (95 % CI, 0.71–0.92) for *HS3ST2*, and 0.71 (95 % CI, 0.59–0.83) for *AJAP1* (Fig. 2d–f). Because we have previously discovered and tested *SOX1* and *PAX1* genes as potential biomarkers [29], we also included the data of these two genes in this study to compare their clinical performance. At the optimal M-index cutoff values, the sensitivities of *POU4F3*, *HS3ST2*, *AJAP1*, *SOX1*, and *PAX1* in discriminating $CIN3^+$ among hrHPV-positive women were 79, 67, 63, 78, and 70 %, respectively, whereas the specificities were 78, 89, 64, 71, and 89 %, respectively (Table 3).

Table 1 Sensitivities and specificities of candidate genes in hrHPV-positive women (N = 67) in the selection set

Detection modality	Sensitivity (%)	Specificity (%)
ADRA1D	61	97
AJAP1	64	100
COL6A2	42	91
EDN3	58	97
EPO	67	97
HS3ST2	88	82
MAGI2	70	94
POU4F3	88	82
PTGDR	67	97
SOX8	46	91
SOX17	64	94
ST6GAL2	64	97
SYT9	73	94
ZNF614	58	97

Validation of the clinical performance of methylated genes in the testing set

Cervical scrapings of 55 hrHPV-positive women out of 89 women were analyzed further in the testing set for DNA methylation levels (Fig. 1). The testing set validated that *POU4F3* methylation analysis conferred the best clinical performance among five potential candidates with 74 % sensitivity and 89 % specificity (Table 3). When stratified by histology, *POU4F3* and *AJAP1* methylation testing did not miss any invasive cancer patients (Table 4). *AJAP1* methylation had better performance in detecting CIN3/CIS lesions than *POU4F3* (70.8 vs. 62.5 %). However, more CIN1 lesions were detected using *AJAP1*.

Discussion

Previous studies support the concept that DNA methylation could be a potential molecular biomarker for

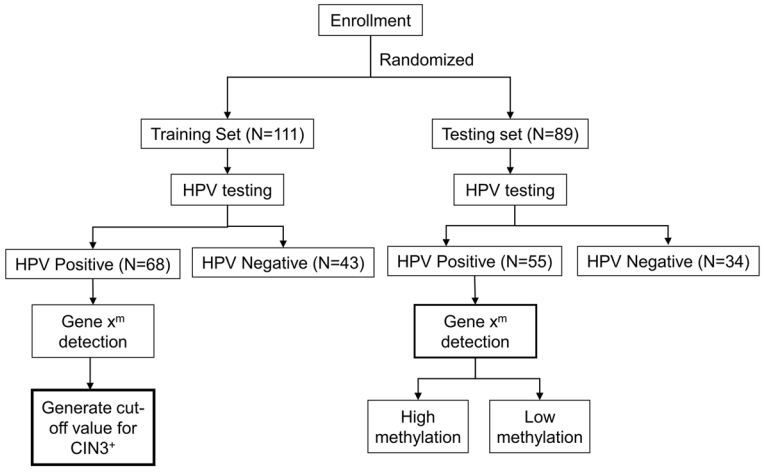

Fig. 1 Work flow for analysis of clinical performance of candidate genes. A total of 200 women were enrolled and randomly assigned to a training set and a testing set. Methylation analysis of candidate genes using cervical scrapings of hrHPV-positive women under the training set was used for generating M-index cutoff values, which were then applied for analysis of the clinical performance of the candidate genes. X^m is the level of methylation of the candidate gene

detection of cervical lesions [12, 15, 16, 28, 30]. An ideal methylation biomarker should have better specificity than HPV testing and better sensitivity than cytology when applied as a primary screening tool. Recent studies proposed the alternative role of methylation biomarkers as a triage method for hrHPV-positive women [22–24, 26, 27]. More high-risk HPV genotype detection may have a better chance to include more women at risk for triage in the primary screening. In addition, the distribution of HPV type varies across continents because 16, 31, 33, and 18 are prevalent in Europe, and 16, 58, 52 and 18 are prevalent in the Asia–Pacific region [31, 32]. We used the Hybrid Capture II (HCII) assay for hrHPV testing, which assays 13 high-risk genotypes simultaneously [7, 32, 33]. The present study demonstrated that DNA methylation analysis as a triage for hrHPV-positive women is feasible. The *POU4F3* methylation analysis confers the best clinical performance when combined with the HCII assay. In this study, the primary objective was to use broad-spectrum hrHPV testing capable of detecting more susceptible women for further triage with *POU4F3* methylation to achieve a better sensitivity. Further hrHPV subtype analysis may clarify type-specific correlation with *POU4F3* methylation, which may be useful in estimating the impact of molecular screening strategy using HPV detection followed by methylation triage in post-vaccination era.

Table 2 Histopathology, mean age, and HPV percentage of the patients

Variable		Training set	Testing set
Age	Mean ± SD	46.8 ± 13.5	44.2 ± 13.3
		No (%)	No (%)
Result of pathology	Normal	34 (30.6)	31 (34.8)
	CIN1	31 (27.9)	19 (21.3)
	CIN2	0 (0)	0 (0)
	CIN3/CIS	28 (25.2)	27 (30.3)
	SCC/AC	18 (16.2)	12 (13.5)
HPV	Negative	43 (38.7)	34 (38.2)
	Positive	68 (61.3)	55 (61.8)
Total		111	89

CIN cervical intraepithelial neoplasia, *CIN1* CIN grade 1, *CIN2* CIN grade 2, *CIN3* CIN3 grade 3, *CIS* carcinoma in situ, *SCC* squamous cell carcinoma, *AC* adenocarcinoma, *HPV* human papillomavirus

POU4F3 is located on chromosome 5q32 and plays various biological functions, such as regulation of transcription, cellular and metabolic processes, organ development, cellular differentiation, nervous system development, neurogenesis, and generation of neurons [34]. The function of *POU4F3* in cancer biology remains largely unknown. *POU4F3* hypermethylation in cervical cancer and glioma suggests its suppressor role in cancer [28, 34]. This study supports the concept that *POU4F3* could be a potential triage biomarker for hrHPV-positive women.

In the present study, a single gene, *POU4F3*, has a specificity of 89 % in detecting $CIN3^+$ in hrHPV-positive women with limited compromise in sensitivity (79 to 74 %), which is better than the specificities previously published using *FAM19A4* (67 %) [27], or a panel of two genes *CADM1–M18/MAL-M1* (71–83 %) [22, 23, 35, 36], or a panel of at least two out of five methylated biomarkers (77 %) [26], or a panel with four methylated biomarkers (69 %) [24] or comparable to the specificity of *JAM* (88 %) [37]. We propose a scenario for the combination of HPV assay and *POU4F3* methylation analysis for cervical cancer screening

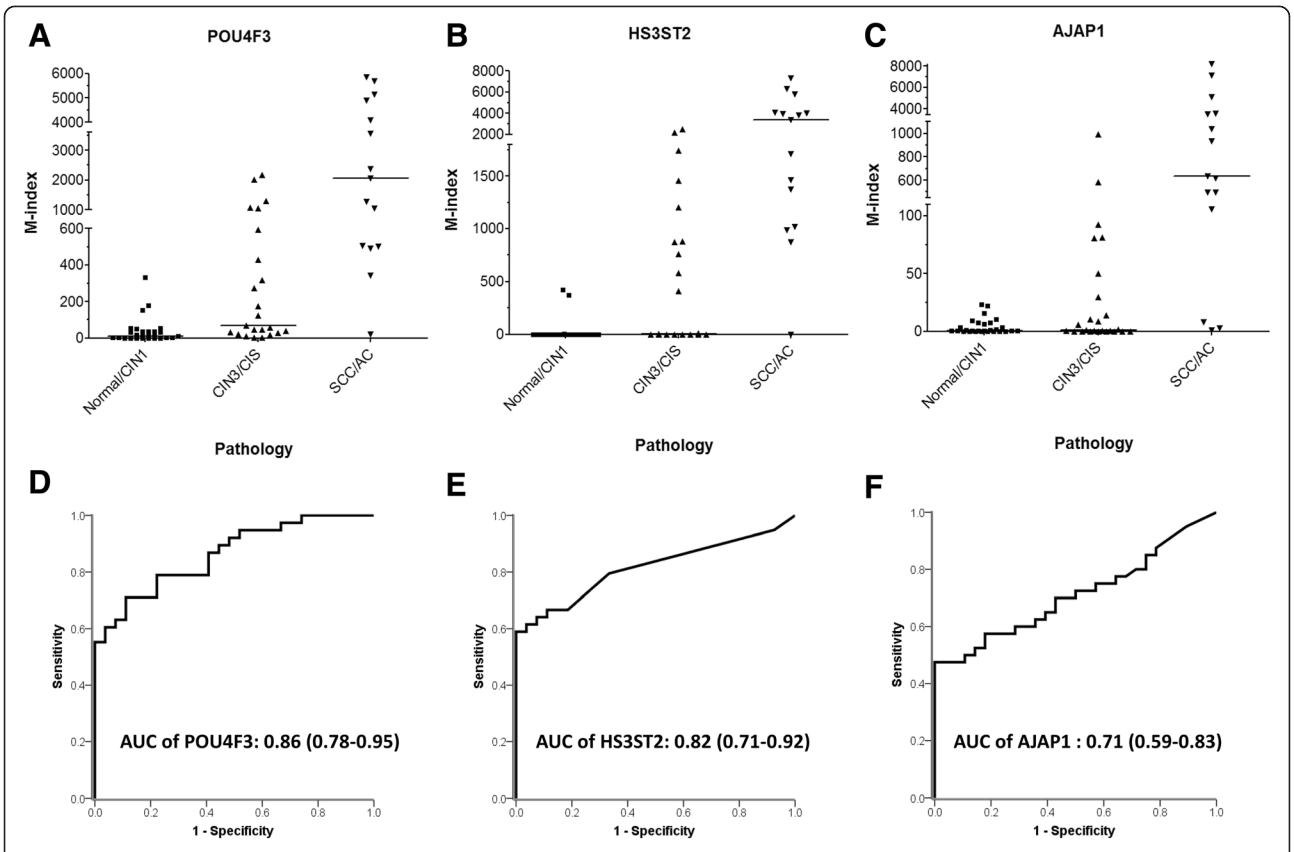

Fig. 2 ROC curves of genes for M-index to trade off performance in detecting CIN3$^+$. Methylation index levels of *POU4F3* (**a**), *HS3ST2* (**b**), and *AJAP1* (**c**) in cervical scrapings such as normal and tumors graded as normal/CIN1, CIN3/CIS, or SCC/AC diagnosed by proven histopathology in hrHPV-positive samples. Each *dot* in the figure represents the M-index level of an individual woman. Analysis of ROC curve of *POU4F3* (**d**), *HS3ST2* (**e**), and *AJAP1* (**f**). The AUC of the ROC curve of an individual candidate gene was calculated to diagnose CIN3$^+$ lesions

(Fig. 3). However, it requires further independent validation together with additional standalone biomarker. Women without hrHPV infection undergo follow-up 3 to 5 years later [38]. Women with hrHPV infection will undertake *POU4F3* methylation analysis. Women having positive *POU4F3* methylation are referred for colposcopy. Because *POU4F3* methylation analysis did not miss any invasive cancer, *POU4F3* methylation-negative, hrHPV-positive women may repeat HPV assay and DNA methylation analysis 1 year later. This strategy may substantially reduce the referral rate. However, a longitudinal follow-up study is needed to clarify the natural history of those

Table 3 Performance of methylation biomarkers to detect CIN3$^+$ in hrHPV-positive women at training and testing sets

		Gene name				
		POU4F3	HS3ST2	AJAP1	SOX1	PAX1
	M-index cutoff value	38	2	2	4	4
Training set ($N = 68$)	Sensitivity (%)	79	67	63	78	70
	Specificity (%)	78	89	64	71	89
	PPV (%)	83	90	71	80	90
	NPV (%)	72	65	55	69	68
Testing set ($N = 55$)	Sensitivity (%)	74	55	80	63	60
	Specificity (%)	89	100	74	74	100
	PPV (%)	93	100	85	82	100
	NPV (%)	64	56	67	52	58

PPV positive predictive value, *NPV* negative predictive value, *CIN3*$^+$ including CIN3/CIS, SCC/AC

Table 4 Clinical performance of methylation biomarker in hrHPV-positive women of testing set stratified by histology

Total number of detectable		53	52	54
Detection modality		Gene name		
		POU4F3	*HS3ST2*	*AJAP1*
		Methylation positive/total number (%)		
Result of pathology	Normal	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)
	CIN1	2/13 (15.4 %)	0/14 (0 %)	5/14 (35.7 %)
	CIN3/CIS	15/24 (62.5 %)	8/22 (36.3 %)	17/24 (70.8 %)
	SCC/AC	11/11 (100 %)	10/11 (90.9 %)	11/11 (100 %)
	Total	28	18	33

CIN cervical intraepithelial neoplasia, *CIN1* CIN grade 1, *CIN3* CIN3 grade 3, *CIS* carcinoma in situ, *SCC* squamous cell carcinoma, *AC* adenocarcinoma

infected with hrHPV, but without *POU4F3* hypermethylation to determine if a longer interval between screenings is also safe. The high negative predictive value in hrHPV-negative women is well documented, which means a longer screening interval is safe. However, determining POU4F3 methylation in HPV-negative women to assess *POU4F3* as independent from HPV as a marker for cervical neoplasia/CIN/CIN3/cancer may also be a consideration. Because HPV assay and methylation analysis can be conducted in the same self-collected cervical sample, the application of this approach may improve the participation of women for screening [39, 40], especially those in low-resource areas. The application of a DNA methylation analysis using self-collected vaginal samples warrants further evaluation. In addition, this is a retrospective hospital-based study, which did not follow up the participants. Population-based studies in different geographical and ethnic backgrounds are needed to validate these results.

In the present study, we adapted histopathologically diagnosed CIN3$^+$ as the end point because CIN2 is equivocal in nature with a tendency to regress to normal instead of progressing to CIN3$^+$, where the likelihood of CIN2 progression to invasive cancer is only 5 % [41]. Further, diagnosis of CIN2 is much less reproducible than CIN3 because of the difference in the natural history of CIN2 from that of CIN3 [42]. However, CIN3 has a higher tendency to progress to invasive cancer because it is an immediate precursor with a similar virological profile and has better reproducibility [31]. Therefore, it is more appropriate to adapt CIN3$^+$ as a surrogate end point for early diagnosis of cervical cancer.

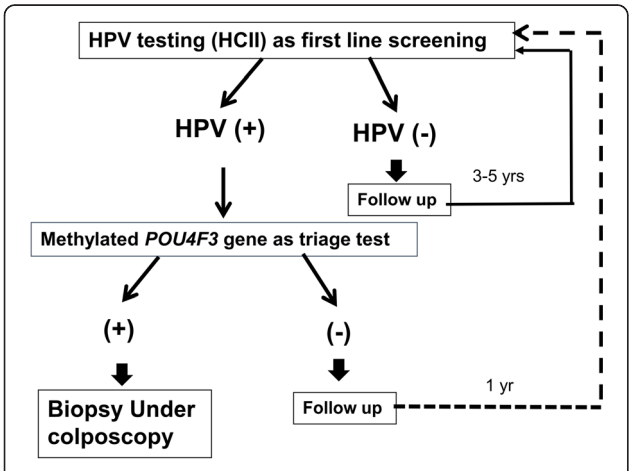

Fig. 3 Proposed cervical cancer screening strategy using hrHPV assay and *POU4F3* methylation analysis as a triage test. In this proposed scenario, HCII hrHPV DNA assay is used as the primary screening test, where women without hrHPV infection undergo follow-up 3 to 5 years later. Samples from women with hrHPV infection undergo *POU4F3* methylation analysis, where women having positive *POU4F3* methylation are referred for colposcopy. Additionally, women with a positive hrHPV assay but negative *POU4F3* methylation may repeat HPV assay and DNA methylation analysis 1 year later

Conclusions

POU4F3 methylation testing is a potential molecular biomarker for the triage of hrHPV-positive women for CIN3$^+$ lesions. We envision an era of molecular screening for cervical cancer.

Methods

Patients

We conducted a retrospective case–control study using hospital-based patient samples in the Tri-Service General Hospital, Taiwan, from December 2009 to November 2010. Patients aged ≥20 years referred for a colposcopy and cervical biopsy and who were managed with conization or surgery after biopsy revealing CIN3$^+$ were enrolled in this study. Cervical scrapings for laboratory analysis were collected in sterile phosphate-buffered saline before biopsy using a cervical brush and were stored at 4 °C until DNA extraction for HPV testing using a HCII hrHPV DNA assay (Digene, Silver Spring, MD, USA) and quantitative DNA methylation analysis of potential candidate genes using QMSP. Healthy women undergoing routine Pap screening were selected as controls, only when their Pap smears showed normal pattern. Women with positive

or suspicious Pap smears were excluded from control. Before the study, all the subjects were informed about the study and were enrolled after obtaining documented full consent. Final diagnosis regarding different stages of cancer was performed by tissue-proven histopathological examination, except in healthy control women. Exclusion criteria applied in this study were compromised quality of Pap smears, patients previously vaccinated with anti-HPV vaccine, cervical neoplasia or existence of other malignancies, surgery related to the uterine cervix, an immunocompromised state, genital warts, or pregnancy. Further, all specimens were delinked from clinical information after numbering each of them until data analysis. In this study, all the women were tested for HPV infection and only samples from hrHPV-positive women underwent DNA methylation analysis. Cervical scrapings of 67 hrHPV-positive women among 100 recruited women underwent DNA methylation analysis to prioritize candidate genes for further analysis of clinical performance. Cervical scrapings from 200 women recruited for analysis of clinical performance were randomly classified using a random number table as a 1:1 ratio into a training set ($n = 111$) and a testing set ($n = 89$). The training set included 46 women with histopathologically confirmed $CIN3^+$ and 65 women with normal/CIN1. Cervical scrapings from 68 hrHPV-positive women of the 111 underwent DNA methylation analysis. Methylation levels in the training set were used to generate optimal M-index cutoff values of candidate genes that can distinguish relevant cancerous cases from control. The clinical performance of candidate genes was validated using the optimal cutoff values in the testing set. The testing set comprised 89 women including 39 women with $CIN3^+$ and 50 women with normal/CIN1. Of the 89 women, cervical scrapings from 55 hrHPV-positive women were analyzed further for quantitative assay of DNA methylation. This study was approved by the Institutional Review Board of the Tri-Service General Hospital, National Defense Medical Center.

Extraction of DNA followed by bisulfite modification

Genomic DNA was extracted as previously described using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations [28]. Those samples with a DNA yield as low as 500 ng or more (>500 ng) as measured by NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, USA) were considered for further analysis in this study. Bisulfite modification of genomic DNA samples was performed using a CpGenome DNA Modification Kit (Millipore, Temecula, CA, USA) according to the manufacturer's recommendations, and the samples were dissolved in 70 μL of nuclease-free water [29]. Bisulfite-converted DNA was stored at −80 °C until further use.

Methylation assays of potential candidate genes

QMSP used for analysis of the methylation status of the candidate genes was based on the principle of fluorescence-based real-time PCR. TaqMan-based QMSP amplification was performed on the bisulfite-treated DNA [43]. The type II collagen gene (*COL2A*) was used as an internal reference. In vitro methylated genomic DNA treated with CpG methyltransferase (M.SssI; New England Biolabs, Beverly, MA, USA) was used as a positive control. While prioritizing potential candidate methylated genes for further performance analysis, QMSP was performed in a TaqMan probe system using an Applied Biosystems 7900HT Fast Real-Time PCR System in a total volume of 20 μL reaction mixture containing 2 μL of bisulfite template DNA, 250 nM of each primer, 225 nM TaqMan probe, and 10 μL of FastStart Universal Probe Master (ROX) (Roche Diagnostics, Roche Applied Science, Mannheim, Germany) [28]. 6-Carboxy-fluorescein was used to label the 5′ end of probes, while a quencher dye (MGB by Applied Biosystems, or BHQ1 by TIB) was used to label the 3′ end of the probes (Additional file 1: Table S1). However, for analysis of clinical performance of candidate genes, QMSP for *AJAP1*, *HS3ST2*, and *POU4F3* and multiplex QMSP for *PAX1* and *SOX1* were performed in a TaqMan probe system using a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Roche Applied Science) [29]. Briefly, the total reaction volume of 20 μL contained 2 μL of modified template DNA, 1 μL of 20× Custom TaqMan reagent, and 10 μL LightCycler 480 Probes Master (Roche Diagnostics, Roche Applied Science). A mixture of primers and probes was used for *PAX1* and *SOX1*. The reactions were conducted using an initial incubation at 95 °C for 10 min, followed by 50 cycles of 95 °C for 10 s, and annealing and extension for 40 s at 60 °C (using the thermal cycler protocol in the standard mode). The level of DNA methylation was measured in terms of M-index [30]. Results showing the very high Cp values of *COL2A* (>36) were defined as detection failures.

HPV DNA assay

The HCII hrHPV DNA assay (Digene) was used as the primary assay in this study following the manufacturer's protocol to detect hrHPV infection. This HCII assay can detect 13 high-risk HPV subtypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. Samples with a ratio of relative light units (RLU)/cutoff value higher than 1.0 were recorded as positive.

Statistical analysis

ROC curves for each of the candidate genes were calculated using the data from the training set. Optimal M-index cutoff values of the candidate genes were generated from ROC curves and were used to further analyze the clinical performance of the candidate methylated

genes in the testing set. Sensitivities, specificities, positive predictive values (PPV), and negative predictive values (NPV) of *AJAP1*, *HS3ST2*, *POU4F3*, *PAX1*, and *SOX1* for detecting CIN3$^+$ were calculated. IBM SPSS Statistics for Windows, version 20.0 (Armonk, NY, USA) was used for all statistical analyses.

Additional file

Additional file 1: Table S1. QMSP primers and probes in this study.

Abbreviations

ADRA1D: adrenoceptor alpha 1D; *AJAP1*: adherens junctions-associated protein 1; AUC: area under the receiver operating characteristic curve; CIN: cervical intraepithelial neoplasia; CIN3$^+$: CIN grade 3 or worse; *COL6A2*: collagen, type VI, alpha 2; *EDN3*: endothelin 3; *EPO*: erythropoietin; HrHPV: high-risk human papillomavirus; *HS3ST2*: heparan sulfate (glucosamine) 3-*O*-sulfotransferase 2; *MAGI2*: membrane-associated guanylate kinase, WW and PDZ domain-containing 2; *PAX1*: paired box gene 1; *POU4F3*: POU class 4 homeobox 3; *PTGDR*: prostaglandin DP receptor; QMSP: quantitative methylation-specific PCR; *SOX1*: sex-determining region Y box 1; *SOX17*: SRY-box 17; *SOX8*: SRY (sex-determining region Y)-box 8; *ST6GAL2*: ST6 betagalactosamide alpha-2,6-sialyltranferase 2; *SYT9*: synaptotagmin IX; *ZNF614*: zinc finger protein 614.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PBP and HCL conceived and designed the experiments. PBP, HCW, YCC, YWH, and RLH performed the experiments. PBP drafted the manuscript. PBP, HCL, YPL, and PHS reviewed and edited the manuscript. CCC and HCL recruited and collected the clinical samples. PBP and YPL analyzed and interpreted the data. All the authors read and approved the final manuscript.

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