CHAPTER II
LITERATURE REVIEW

1. Biology of Human papillomavirus (HPV)

1.1 Classification

The classification of HPV is based on DNA nucleotide sequence differences within the L1 open reading framed (ORF). HPV is a member of the *Alpha-Papillomavirus* genus of the family *Papillomaviridae* which are officially recognized by the International Council on Taxonomy of Viruses (ICTV). An HPV isolate is described as a new genotype if the L1 sequence differs by more than 10% from any previously know HPV genotype. Within a genotype, subtypes and variants can be distinguished, which differ 2-10% and maximally 2%, respectively. At present, over 118 HPV genotypes have been classified according to their biological niche, oncogenic potential and phylogenetic position, 49 are know to infect the anogenital epithelium (14).

1.2 Virion structure

HPV is small, nonenveloped and icosahedral DNA virus. The diameter of each particle is about 55 nm (Figure 1) and its density in cesium chloride is 1.34 g/mL. The virion particles consist of a single molecule of supercoiled, closed circular double-stranded DNA that are approximately 8,000 base pair (bp) long and encased in a naked icosahedral capsid. A fine structural analysis, by cryo-electron microscopy (cryo-EM) on three-dimensional image reconstruction techniques has revealed that the viruses consist of 72 pentameric capsomers arranged on a T = 7 surface lattice. The capsomers exist in two states, one capable of making contact with six neighbors, as observed in the 60 hexavalent capsomers, and the other with five neighbors in the 12 pentavalent capsomers (Figure 2). Each pentameric capsomer is composed of five major capsid proteins, with the minor capsid protein at the center. Figure 1 Structure of the papillomavirus virion. A cryoelectromicrograph of HPV1 capsid is shown (diameter, 55 nm) (1, 15, 16).

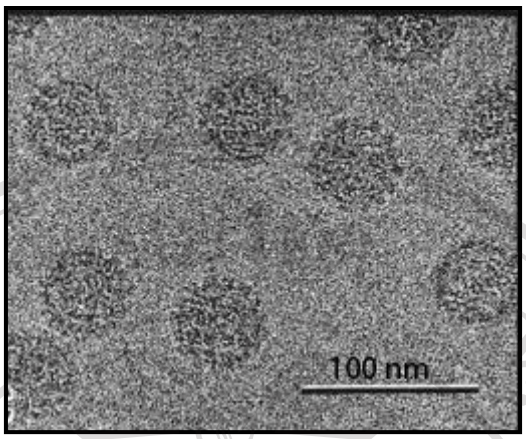

Figure 1 Electron micrograph of HPV1 virion particles (55 nm in diameter) (16).

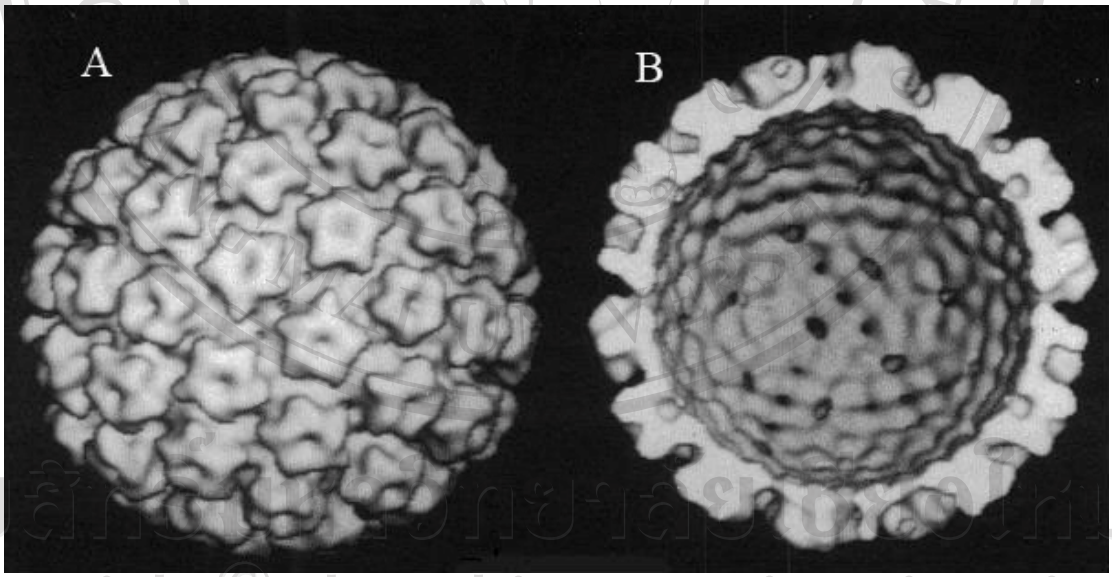

Figure 2 Surface shaded (A) and sectioned (B) displays of the HPV1 reconstructions viewed along a two-fold axis of symmetry. In B, the reconstructions was computationally sectioned at the equator and the nucleoprotein core was removed (16).

1.3 Viral genome structure and organization

The HPV genome consists of a closed circular double-stranded DNA that is approximately 8,000 bp long. The relative arrangement of the 8-10 ORFs within the genome is the same as all papillomavirus types, and a particular characteristic of papillomaviruses is that the partly overlapping ORFs are arranged on only one DNA coding strand (Figure 3). The GC content of most papillomaviruses genome is about 40-50%. The apparent molecular weight of $3x10^6$ to $5x10^6$ represents 10-13% of the virion's weight (17, 18).

The HPV genome can be functionally divided into three major regions that are separated by two polyadenylation (pA) sites: early pA (A_E) and late pA (A_L) sites.

1) The early region (E) that is about 4.5 Kb, representing about 50% of the viral genome, contains eight ORFs (E1-E8). It encodes the proteins required for viral replication, cell transformation and oncogenesis through interactions with the host cell.

2) The late region (L) that is about 2.5 Kb, covering almost 40% of the viral genome, lies downstream of the early region and contains two ORFs (L1 and L2). They encode the major (L1) and minor (L2) capsid proteins, which are expressed in only productive infected tissues.

3) The upstream regulatory region (URR) that varies from 500 to 1,000 bp between different HPVs (10% of the viral genome), which is a non-coding region and lies between the early and late regions. This has previously been termed as long control region (LCR) containing several control elements for viral replication and gene expression. The URR includes the origin of replication (*ori*), the E6/E7 gene promoter, and enhancers and silencers. The function of the URR is governed by at least several of the internal transcriptional regulatory motifs. These act as *cis*-acting elements by binding to the various cellular and viral proteins that *trans*-regulate genomic function. At least four components are shared by the URRs of the papillomavirus types examined so far: (i) a polyadenylation signal for late mRNAs at the 5′ end, (ii) E2 protein binding sites, (iii) an E1 binding site associated with the origin of replication, and (iv) a TATA box in the E6 gene promoter. The molecular mechanisms of the URR function and regulation are complex and for the most part unknow (Figure 3).

1.4 Viral gene products and their biological functions

The sizes and functions of papillomavirus proteins are shown in table 1.

1.4.1 E1 protein

The E1 protein contains about 650 amino acids (aa), encoded by the largest conserved ORF. It is involved in viral DNA replication by weakly binding to the E1 binding site (E1BS) at the *ori* in the URR (Figure 3) and forms a heterodimer with E2 protein. It interacts with several cyclin/cyclin-dependent kinase complexes, especially cyclin E DNA-dependent ATPase, and helicase activity. It also contributes to the maintenance of the viral episome and is often absent when the viral DNA is integrated (19, 20).

1.4.2 E2 protein

The E2 protein is a DNA binding protein that contains approximately 380 aa. Its main functions are viral DNA replication and viral transcription regulation. It also represses the activity of the E6/E7 promoter by binding to the E2 binding sites (E2BS) proximal to the E6 promoter (Figure 3) and permits E1 protein to initiate viral replication. In contrast, viral integration disrupts the E2 ORF and allows the free *trans*-activation of the E6/E7 promoter by several cellular transcription factors. Beside it can interact with L2 and may contribute to encapsidation of viral DNA (21).

1.4.3 E4 protein

The E4 ORF entirely overlaps the E2 ORF in a different reading frame and encodes a protein with an entirely different amino acid sequence. The E4 gene is not highly conserved among the papillomaviruses. E4 protein is approximately 100 aa long and plays a role in the maturation and release of viral particles. It is considered a late protein. After translation from spliced transcrips, E4 protein is fused with E1 protein to generate E1^E4 fusion proteins. The E1^E4 proteins from high-risk HPV types can form a filamentous cytoplasmic network that colocalizes with the cytokeratin network of intermediate filaments in the lower epithelial layers and can induce their collapse by over-expression in transient transfection assays (18, 22, 23).

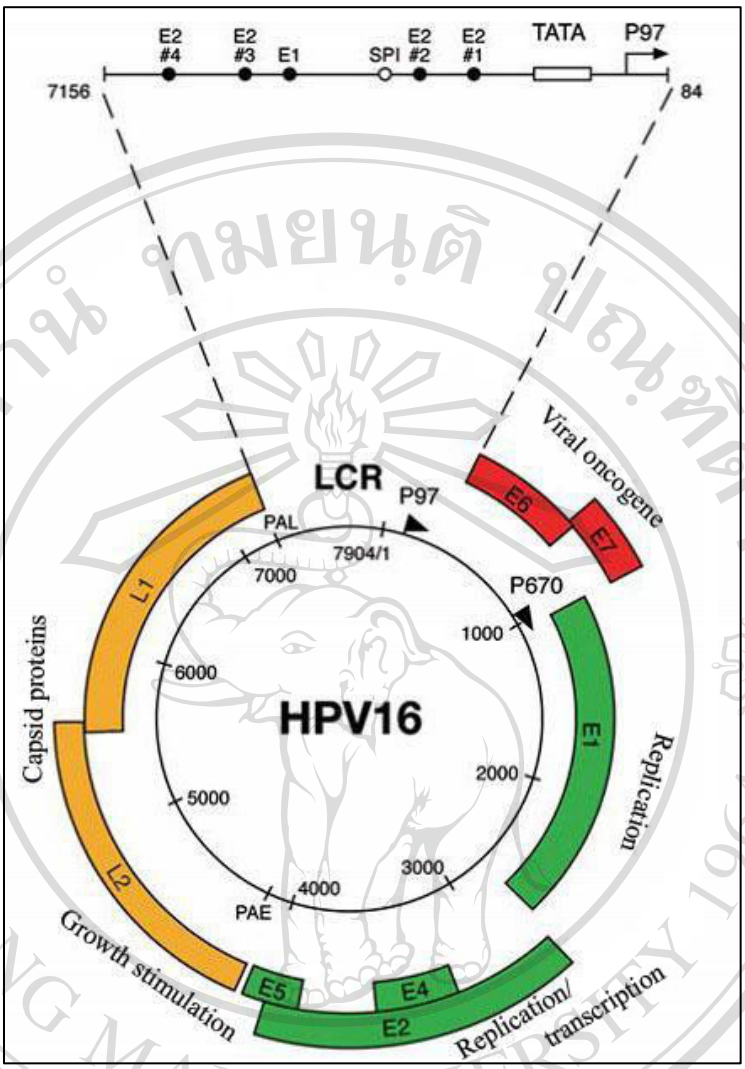

Figure 3 Organization of the HPV genome. The HPV16 genome (7904 bp) is shown as a black circle with the early (p97) and late (p670) promoters marked by arrows. The six early ORFs [E1, E2, E4 and E5 (in green) and E6 and E7 (in red)] are expressed from either p97 or p670 at different stages during epithelial cell differentiation. The late ORFs [L1 and L2 (in yellow)] are also expressed from p670, following a change in splicing patterns, and a shift in polyadenylation site usage [from early polyadenylation site (PAE) to late polyadenylation site (PAL)]. All the viral genes are encoded on one strand of the double-stranded circular DNA genome. The long control region (LCR from 7156–7184) is enlarged to allow visualization of the E2-binding sites and the TATA element of the p97 promoter. The location of the E1- and SP1-binding sites is also shown (23).

1.4.4 E5 protein

The E5 protein is approximately 90 aa long and consists of small hydrophobic proteins located in the endosomes, the Golgi apparatus, and cellular membranes. It participates in malignant transformation by interacting with cellular growth factor receptors, including the epidermal growth factor (EGF) receptor and platelet-derived growth factor (PDGF) receptors. EGF activates the phosphokinase C pathway of signal transduction, which leads through the mitogen-activated protein kinase activation (MAPK) pathway to the activation of c-*jun* and c-*fos* cellular oncogenes. c-*jun* and c-*fos* assemble in a heterodimer, activation protein 1 (AP-1), that has potent transcriptional activity. The net result is a stimulation of cell proliferation and differentiation. The E5 protein can also enhance endothelin-1-induced keratinocyte growth (24).

1.4.5 E6 protein

The E6 protein is about 151 aa in size and contains two zinc-binding domains with the motif Cys-X-X-Cys, which is important in functions, such as transcriptional activation, transformation, immortalization and association with cellular proteins. The key action of high-risk E6 proteins is their ability to inhibit the function of p53, a tumor suppressor protein, by enhancing its degradation through the ubiquitin pathway. To inhibit p53, E6 protein requires the 100 kDa cellular E6-associated protein (E6-AP), a calcium binding protein, that could be involved in terminal cell differentiation and apoptosis. Most E6 proteins from low-risk HPVs do not bind to p53, and none of them induce its degradation. Furthermore, E6 protein can inhibit degradation of SRC-family kinases by E6-AP, stimulating mitotic activity and can upregulate telomerase activity by transcriptional activation of the human telomerase reverse transcriptase (hTERT) gene, encoding the telomerase catalytic subunit, causing over proliferation of infected cells. The high-risk E6 proteins also interact with several *P*SD-95/*D*lg/*Z*O1 (PDZ) domain-containing substrates, inducing their ubiquitin-mediated degradation. These substrates are implicated in the control of cell proliferation, cell polarity and adhesion, which further supports that E6 proteins contribute to HPV-induced malignancy (17, 25).

1.4.6 E7 protein

The E7 protein comprises around 100 aa and is primarily localized in the nucleus, where it binds to and inactivates the hypophosphorylated retinoblastoma gene product (pRb), a product of the tumor suppressor gene *Rb-1*, E7 protein prevents its binding with E2F protein, an important family of transcription factors, to facilitate progression into the S-phase of the cell cycle and lead to cell replication. Besides pRb, E7 protein can interact with two other members of the pRb family, p107 and p130, which also negatively regulate E2F transcription. In addition, high-risk E7 protein stimulates the S-phase genes cyclin E and cyclin A, interacts with cyclin-kinase complexes and abrogates the inhibitory activities of cyclin-dependent kinase inhibitors (CKIs), such as $p21^{CIP-1/WAF-1}$ and $p27^{KIP-1}$. These interactions are a major factor in growth stimulation of HPV-infected cells, uncoupling cyclin-dependent kinase activity from CKIs and interfering with the ability of p53 to induce G1 growth arrest following DNA damage. Another consequence of high-risk E7 protein expression is the induction of genomic instability. By inducing centriole amplification, E7 protein also induces aneuploidy of the E7-expressing cell, which contributes to tumorigenesis.

E6 and E7 proteins are independently able to immortalize human cells, but their combined expression leads to a complementary and synergistic effect, which in turn is responsible for increased transforming efficiency (Figure 4).

It is currently unclear how low-risk papillomaviruses (whose E6 proteins are unable to interfere with p53 but whose E7 proteins bind to pRb) overcome the p53-mediated apoptosis. In addition, it has been shown that the E6 proteins of some HPV types bind to a component of the single-strand break DNA repair complex and thereby inhibit its efficiency. A cell that is persistently infected with HPV, therefore, undergoes continuous cell division, and consequently is no longer able to react in response to DNA damage with G1 arrest or apoptosis. DNA repair is also impeded, which clearly promotes the cell's pathway to malignancy (17, 18, 25).

1.4.7 E8^E2 fusion protein

A new E2 protein, consisting of a fusion of the product of the small E8 ORF with part of the E2 protein, has been described. This fusion protein is able to repress viral DNA replication as well as transcription, and is therefore believed to play a major role in the maintenance of viral latency observed in the basal cells of infected epithelium (17).

1.4.8 L1 and L2 proteins

L1 and L2 proteins are structural proteins that form the capsid. L1 protein is called the major capsid protein because it represents 83% of the viral coat. It has hemagglutinating activity. Antibodies to L1 are neutralizing. For L2 protein, it is called minor capsid protein and dispensable in the formation of the capsid but appears to be important for the encapsidation of viral DNA. They are not expressed in precancerous or tumor cells, but they are important for vaccine development (18).

1.5 Viral replication

Papillomavirus DNA replication appears to be governed by the state of differentiation of the keratinocytes in the squamous epithelium. The virus initiates its replication as an episome after entering a basal cell, generating a small number of copies (less than 20 to 50) per cell. Replication begins with displacement of the histones associated with the viral DNA and the unwinding of the supercoiled viral DNA. E1 protein binds to a receptor in the URR that incorporates the *ori*. It has helicase and ATPase activity. In cooperation with E2 protein, topoisomerase I, and replication protein A (RP-A), E1 protein displaces the histones associated with the viral DNA and unwinds the DNA supercoiled conformation. E1 protein subsequently forms a bidirectional replication fork complex with cellular proteins (polymerase α/primase, DNA polymerase δ/proliferating-cell nuclear antigen, replication factor C, topoisomerase II, and DNA ligase). HPV DNA replication progresses bidirectionally from the *ori*. Viral DNA is then encapsidated in a process that involves its association with cellular histone proteins (H2a, H2b, H3, and H4). A transient binding of L2 protein with E2 protein presumably guides the DNA into the aggregation of viral L1 and L2 proteins, which eventually form the capsid. The release of viral particles is

probably passive, resulting from the disintegration of the upper squamous epithelium, possibility facilitated by the E1^E4 protein. Desquamating cells are infectious (18).

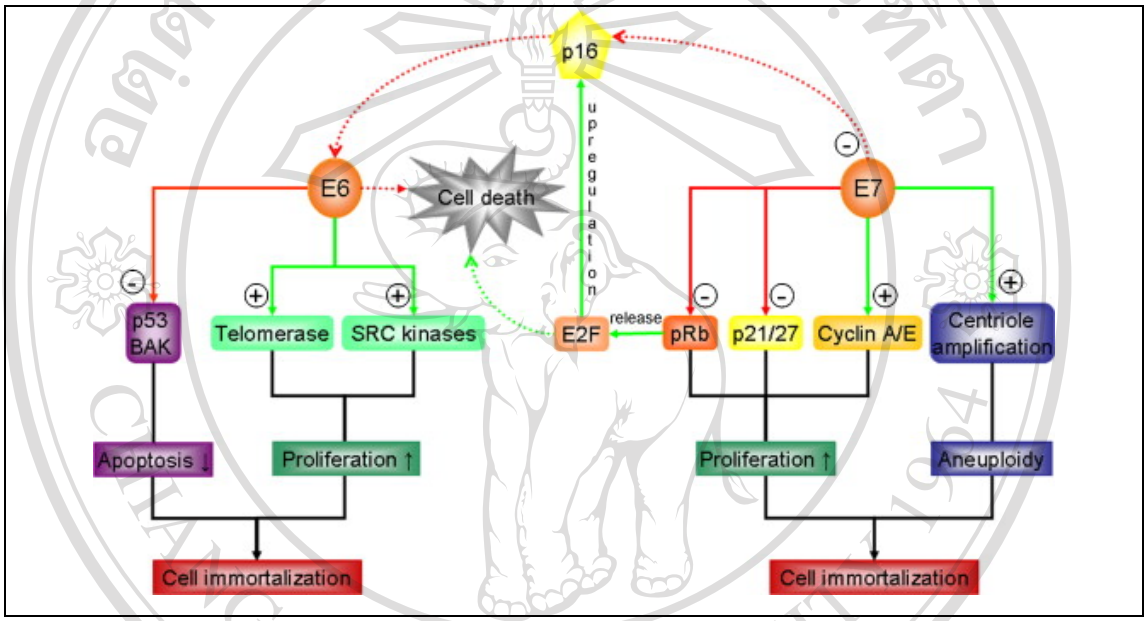

Figure 4 Cellular interactions of E6 and E7 oncoproteins and their synergy in induction of cell immortalization. E6 activates telomerase and SRC kinases, and inhibits p53 and BAK. E7 inhibits pRb, with consequent release of E2F and upregulation of p16, which is inactivated by E7. In addition, E7 stimulates cyclins A and E, inactivates CKIs p21 and p27 and induces centriole amplification. E6 and E7 synergize in cell immortalization (dotted lines); E6 prevents apoptosis induced by high E2F levels, while E7 shields E6 from inhibition by p16 (25).

Table 1 Size and function of papillomvirus proteins (17)

Viral proteins/ genomic elements	Molecular weights/ sizes	Functions
Non-coding elements		
Long control region (LCR)	500-1,000 bp	Origin of replication and regulation of HPV gene expression
Early proteins		
E1	68-85 kD	Helicase function; essential for viral replication and control of gene transcription; similar among types
E2	48 kD	Viral transcription factor; essential for viral replication and control of gene transcription; genome segregation and encapsidation
E3	Unknow	Function not know; only present in a few HPVs
E1^E4	10-44 kD	Binding to cytoskeletal proteins; viral assembly
E5	14 kD	Interaction with EGF/PDGF-receptors, stimulate cell growth
E6	16-18 kD	Oncoprotein, cell immortalization, interaction with several cellular proteins; degradation of p53, activation of telomerase, anti-apoptotic effect, induction of genomic instability
E7	~ 10 kD	Oncoprotein, cell immortalization, interaction with several cellular proteins; interaction with pRb, transactivation of E2F-dependent promoters, induction of genomic instability
E8^E2C	20 kD	Long distance transcription and replication repressor protein

Table 1 Size and function of papillomvirus proteins (continued)

Viral proteins/ genomic elements	Molecular weights/ sizes	Functions
Late proteins		
L1	57 kD	Major capsid protein, assembles into pentamers and is sufficient to form VLPs
L2	43-53 kD	Minor capsid protein, role in recruiting viral genomes for encapsidation, involvement in nuclear transport of viral DNA

1.6 Pathogenesis of HPV infection

In productive HPV infection, HPVs exploit host cell factors to regulate viral transcription and replication. The initial infection by HPV in stem cells of the basal layer of stratified epithelium of the transformation zone through microabrasion. Following infection and uncoating, the viral early proteins (E1, E2, E4, E5, E6, and E7) are expressed at low level and the viral DNA replicates from episomal DNA. The viral genome is maintained in the basal layer as a low copy number (around 10-200 copies per cell). After entry into the midzone of the epithelium, the late gene L1 and L2, and E4 are expressed, high copy numbers of the circular viral genome are amplified and capsid proteins are synthesized. In the superficial zone of the epidermis or mucosa, L1 and L2 proteins encapsidate the viral genomes to form progeny virions in the nucleus. The shed virus can then initiate a new infection (Figure 5) (23).

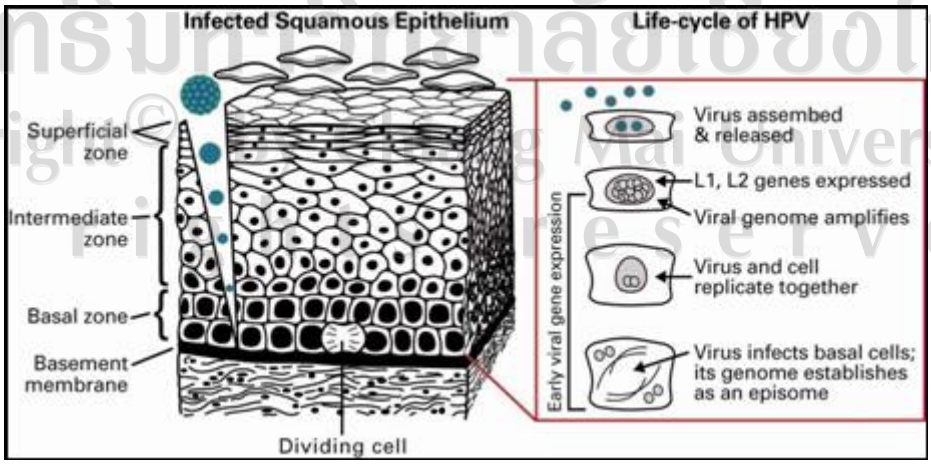

Figure 5 Productive life cycle of HPV (26).

In non-productive HPV infection, high-risk HPV is integrated randomly into the host genome and part of the E2 ORF is deleted, resulting in loss of full length E2 gene expression, leading to increased E6 and E7 genes expression. The E6 gene product binds to the tumor suppressor protein p53 and rapidly degrades it through the cellular ubiquitin pathway. This subverts the MDM2-p53 pathway and leads to degradation of G1 arrest, apoptosis and cell cycle checkpoints. The HPV E7 gene product binds to the tumor suppressor gene pRb and degrades it, releasing the transcription factor E2F-1 and upregulating $p16^{INK4A}$. Host cells increase their proliferation rate and genomic instability, finally leading to tumorigenesis (Figure 6) (28).

1.7 Clinical manifestations

Infections with HPVs may cause local cell proliferation, which can develop into plantar or common warts and condylomas. The majority of these benign tumors regress spontaneously in immunocompetent individuals. However, in those with inherited or pharmacologically induced immune deficiencies, there is a strong tendency for the infections to persist, with a high probability of malignancy in the case of infection with high-risk HPV types. The malignant potential of HPV-induced papillomas was first demonstrated in patients with the rare hereditary disease, Epidermodysplasia verruciformis (EV). In these patients, several EV-specific HPV types induce disseminated warts. Within 20 years of disease onset, 30-60% of EV patients are predicted to develop squamous cell carcinoma, primarily at sun-exposed sites. More than 90% of these carcinomas contain HPV 5 DNA, and the majority of the remainder HPV 8 DNA. The clinical associations of HPV genotypes are shown in Table 2 (17, 29-32).

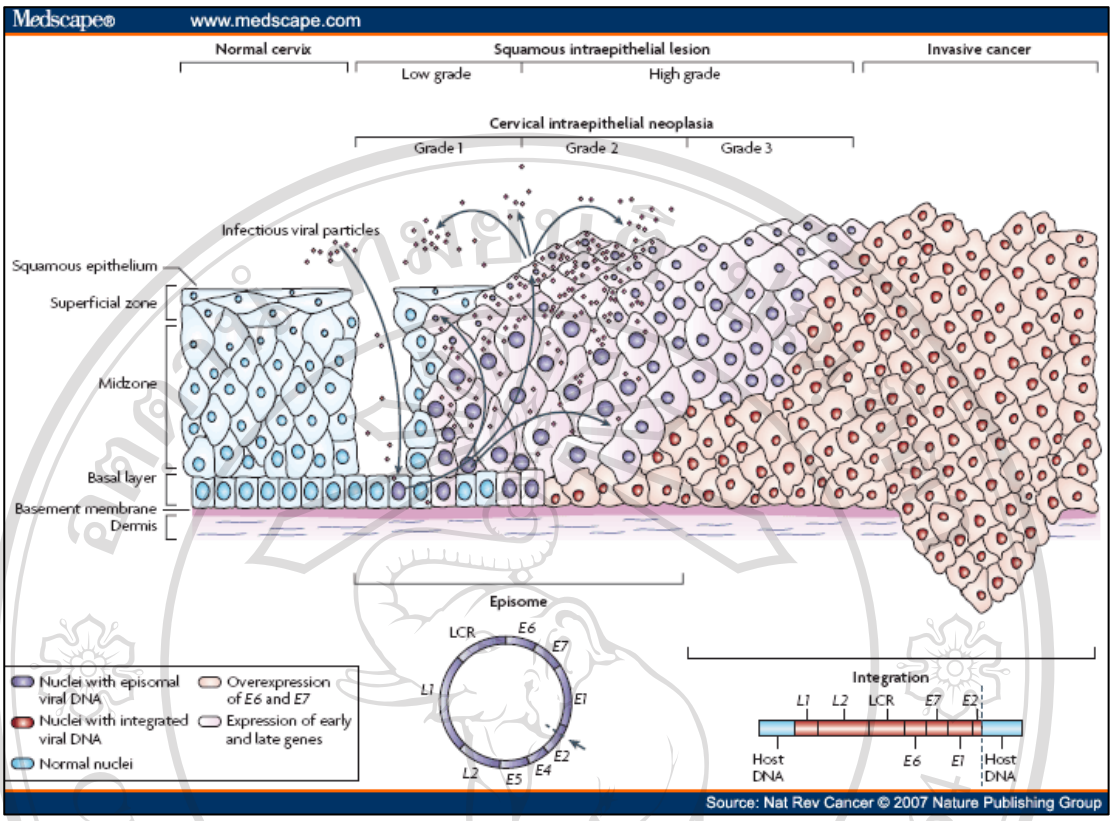

Figure 6 HPV-mediated progression to cervical cancer. Basal cells in the cervical epithelium rest on the basement membrane, which is supported by the dermis. HPV is thought to access the basal cells through micro-abrasions in the cervical epithelium. Following infection, the early HPV genes E1, E2, E4, E5, E6 and E7 are expressed and the viral DNA replicates from episomal DNA (purple nuclei). In the upper layers of epithelium (the midzone and superficial zone) the viral genome is replicated further, and the late genes L1 and L2, and E4 are expressed. L1 and L2 encapsidate the viral genomes to form progeny virions in the nucleus. The shed virus can then initiate a new infection. Low-grade intraepithelial lesions support productive viral replication. An unknown number of high-risk HPV infections progress to high-grade squamous intraepithelial lesion (HSIL). The progression of untreated lesions to microinvasive and invasive cancer is associated with the integration of the HPV genome into the host chromosomes (red nuclei), with associated loss or disruption of E2, and subsequent upregulation of E6 and E7 oncogene expression (27).

Table 2 Clinical associations of HPV genotypes (17).

Clinical features	HPV genotypes
Warts of the skin - Verruca plantaris/plantar warts, mosaic-type warts, verruca plana/flat warts, butchers' warts	1-4, 7, 10, 26-29, 41, 48, 49, 57, 60, 63, 65
Upper respiratory tract and eye - Laryngeal papilloma, recurrent respiratory, papillomatosis, nasal papilloma, oral papilloma, focal epithelial hyperplasia, conjunctiva papilloma	2, 6, 11, 13, 16, 32
Epidermodysplasia verruciformis - Macular lesions - Squamous cell carcinoma	5, 8, 9, 12, 14, 15, 17, 19, 20-25, 36, 38, 47, 50
Anogenital warts - Condylomata acuminate, flat condylomata, Bowen's disease, Buschke-Loewenstein tumors	2, 6, 11, 16, 18, 30, 40-42, 44, 45, 54, 55, 61
Anogenital carcinomas - the high-risk HPVs - Squamous cell carcinoma of the cervix, vulva and penis - Squamous cell carcinoma of the remaining anogenital tract	16, 18, 26, 31, 33, 35, 39, 45, 51-53, 56, 58, 59, 66, 68, 73, 82

2. Cervical cancer

2.1 Epidemiology

Cervical cancer is the second most common cancer in women worldwide. It is the cancer of uterine cervix and a common cause of death among middle-age women (40-60 years of age) (30). At least 493,000 new cases are identified each year and 83 % of these are in developing countries. It killed an estimated 274,000 deaths in the year 2002 (31). Among Thai women, cervical cancer is the most common type of cancer, followed by breast and liver cancer. The estimated age-standardized incidence rate (ASR) of cervical cancer in Thailand is 19.5/100,000 population. In 2003, an estimated 6,000 new cases were reported, and ASR of cervical cancer was highest in Chiang Mai (25.6/100,000), in the north, and lowest in Khon Kaen (15.0/100,000), in the northeast. Cervical cancer was the first of common cancer identified in Chiang Mai and Songkla, and the second was in Bangkok and Khon Kaen (111).

Concerning gynecologic oncology annual report form Gynecologic Oncology Unit, Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine, Chiang Mai University for women in Chiang Mai during 1997 to 2005, cervical cancer was the most common cancer followed by ovarian cancer and uterine corpus cancer. It was the highest among women 41-50 years of age. In 2004, the International Agency for Research on Cancer (IARC) reported 6,192 new cases per year which caused death 3,166 deaths (approximately 51% deaths) in Thailand (111).

Most cancers occur in the transformation zone of the cervix, the columnar cells of the endocervix form a junction with the stratified squamous epithelium of the exocervix. About 85% of cervical cancers are squamous cell carcinomas (SCCs). Most of other cases are adenocarcinomas, with a small number being small cell neuroendocrine tumors (15).

The distribution of HPV genotypes of cervical cancer in women worldwide was found that HPV16 was the most common (54.6%), HPV18 the second most common types, and followed by HPV33, 45, 31, 58, 52, and 35. HPV16 and 18 are mainly caused of cervical cancer about 70% (29, 32). In Asia, HPV16 was the most common followed by HPV18, 58, 33, 52, 45, 31, and 35 (33). In Thailand, the most

common was also HPV16 also and the second common was HPV18. Both are mainly caused of cervical cancer approximately 73-75%, and the third common was HPV58 (11-18%) (111).

2.2 Pathogenesis of cervical cancer

Cervical cancer development is a multi-step process. The major steps have been HPV infection and HPV persistence for over 1 year followed by slow progression to precancerous lesions and eventually to invasive cancer. Most HPV infections resolve spontaneously in 6-12 months and the majority of the precancerous lesions regress due to immune response (Figure 7). A small proportion of HPV infections will eventually lead to cervical cancer, then other cofactors are needed for cervical cancer development (34, 35).

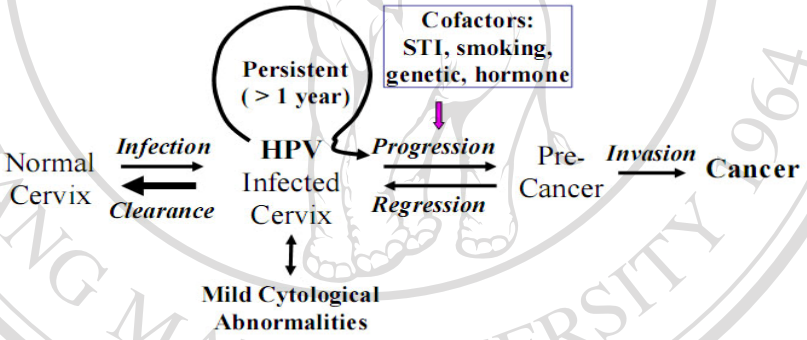

Figure 7 Natural history of cervical cancer development (35).

2.3 Cervical precancerous lesions

Cervical precancerous lesions are defined by cytological abnormalities within the cervical epithelium and are usually classified in three- and two-tier systems (Figure 8). The cervical intraepithelial neoplasia (CIN) system was introduced in 1973 and is based on tissue architecture. CIN1 refers to abnormal cells occupying the lower third of the cervical epithelial stratum, CIN2 indicates that two thirds are occupied, and in CIN3 the entire epithelial layer are occupied. CIN1, CIN2 and CIN3 describe different processes – CIN1 indicates a self-limiting HPV infection, and CIN2 or CIN3 are actual cervical cancer precursors. The other reporting system is the

Bethesda system that was introduced in 1988. This system classifies cytological abnormalities as either low-grade squamous intraepithelial lesion (LSIL) or high-grade squamous intraepithelial lesion (HSIL) (Solomon2002). LSIL corresponds to CIN1 and HPV infection; and HSIL corresponds to CIN2, CIN3 and carcinoma *in situ*. In addition, the abnormal cells with "borderline" changes are classified as atypical squamous cell of undetermined significance (ASC-US) or atypical squamous cells, which are difficult to distinguish from HSIL (35, 36).

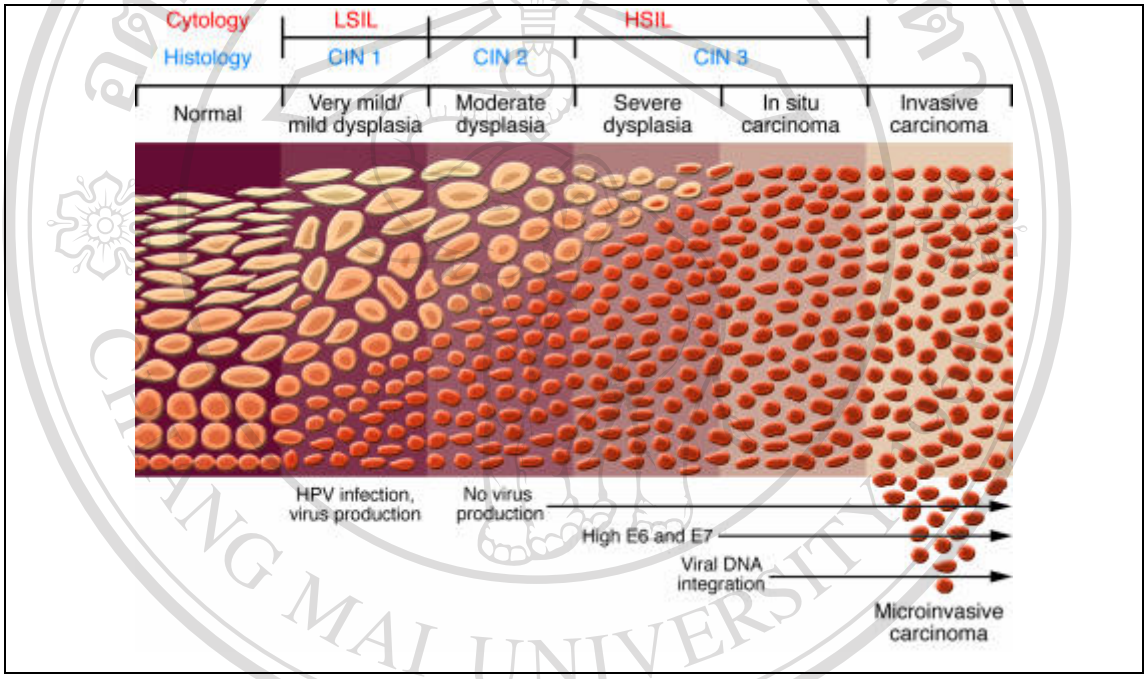

Figure 8 Progression from a benign cervical lesion to invasive cervical cancer. Infection by oncogenic HPV types, especially HPV16, may directly cause a benign condylomatous lesion, low-grade dysplasia, or sometimes even an early high-grade lesion. Carcinoma *in situ* rarely occurs until several years after infection. It results from the combined effects of HPV genes, particularly those encoding E6 and E7, which are the 2 viral oncoproteins that are preferentially retained and expressed in cervical cancers; integration of the viral DNA into the host DNA; and a series of genetic and epigenetic changes in cellular genes. HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; CIN, cervical intraepithelial neoplasia (37).

2.4 Risk factors and cofactors in cervical cancer development

2.4.1 HPV risk factors

HPV is the most prevalent sexually transmitted viral infection among both men and women. It is estimated that 80% of sexually active adults have been infected with at least one HPV type (38). It is well established that high-risk HPV infection causes cervical cancer. Other factors pertaining HPV infection (see below) may modify viral-host biological interaction and play a role in the development of cervical cancer.

2.4.1.1 High-risk HPV types

Recent epidemiological and functional studies have shown that HPV DNA can be found in 99.7% of all cervical SCCs and adenocarcinoma, with HPV16, 18, 33, 45, 31, 58, 52 and 35 being the most frequent. Based on these observations, the anogenital HPVs have been divided into two groups: the first is associated with a high risk for cervical cancer development – the high-risk HPVs (HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82) are found in cervical cancer and precancerous lesions and the World Health Organization (WHO) has recognized HPV16 and HPV18 as carcinogenic agents for humans, and the second group with a low carcinogenic potential – the low-risk HPVs (HPV6, 11, 40, 42, 43, 44, 54, 61, 72, and 81) are primarily found in genital warts (17).

2.4.1.2 Viral variants

In order for an HPV type to be classified as a new HPV type, it must exhibit less than 90% sequence similarity to know HPV types in the L1 ORF. If the type shows 90% or higher sequence similarity to the prototype in these regions it can either be classified as a subtype (90-98% similarity) or as a variant (≥98% similarity). HPV variants have different biological and biochemical properties important to cancer risk (39). These variants often appear to have disparate geographical and ethnic origins. Most studies of HPV variants focus on high-risk HPV16. HPV16 has 5 know variants: European (E), Asian (As), Asian-American

(AA), African-1 (Af1) and African-2 (Af2). Asian-American variants have apparently higher oncogenic potential than European variants (35, 40).

2.4.1.3 Viral load

The term of viral load refers to the HPV virus copy number in the infected cells. It has been suggested that viral load correlates to cervical disease severity (41, 42). However, some studies suggested that a high viral load alone is insufficient to induce progression from HPV infection to CIN2, 3 and cancer (43, 44). Moreover, low levels of all types of high-risk HPVs are able to induce tumorigenesis (35, 45).

2.4.1.4 Viral integration

Viral integration has been reported to be associated with carcinogenesis. Integrated HPV is more frequently found in HSIL and cervical cancer than in LSIL (46). During carcinogenesis part of HPV E2 is deleted, and the loss of full length E2 expression leads to increased expression of E6 and E7 oncoproteins (28). High-risk HPV E6 and E7 impairs p53 and pRb functions and causes the cell to escape cell cycle check point surveillance, subsequently leading to genome instability and cell immortalization (47). Persisting HPV infection may be a consequence of viral integration (35).

2.4.1.5 Multiple HPV co-infections

Most of multiple HPV co-infections are double co-infections, but triple, quadruple and even quintuple HPV co-infections have also been detected (10, 48, 49). Multiple HPV genotypes, usually with at least one high-risk type, were found in 11.8% of patients with normal cytology and ASC-US, in 34.5% of patients at CIN1 or 2, and in 4.5% of cervical cancer tissue samples (8, 35).

2.4.2 Non-HPV risk factors

HPV infection alone is not sufficient for cervical cancer development. Some endogenous and exogenous factors can act as cofactors by influencing the risk of HPV persistence and cancer progression. Exogenous cofactors include the use of

oral contraceptives, high parity, diet, life style, tobacco smoking, cervical trauma, and co-infection with other sexually transmitted agents. Endogenous cofactors include hormonal levels, and genetic factors associated with human leukocyte antigen (HLA) (35, 50).

2.4.2.1 Long-term use of oral contraceptives (OCs)

The IARC have reported that long-term use of OCs is associated with a moderate increase in cancer risk (odd ratio = 1.4), and that there is a strong dose-response relationship with increased periods of use (51). The URR of HPV contains sequences similar to the glucocorticoid responsive elements that are induced by steroid hormones such as progesterone (the active components of OCs) (39). OC hormones may promote HPV DNA integration into the human genome, deregulating E6 and E7 expression and influencing the progression of carcinogenesis (35).

2.4.2.2 High parity

High parity has been consistently found to increase the risk of squamous cell cervical carcinoma among HPV positive women. High parity leads to direct exposure of the transformation zone in the cervix to HPV infection, hence increasing the risk of cervical cancer (52, 53). Increased levels of estrogen and progesterone during pregnancy may also modulate the immune response to HPV and influence the risk of persistence or progression (35, 53).

2.4.2.3 Smoking

Smoking shows a moderate and statistically significant association with cervical cancer in case-control studies, even after adjusting for HPV infection (odd ratio = 2) (54). Nicotine- and tobacco-specific carcinogens have been detected in the cervical mucus of cigarette smokers (55). Smoking increases a woman's susceptibility to tumorigenesis by lowering the immune surveillance in the mucosa (35, 56, 57).

2.4.2.4 Other sexually transmitted infections (STIs)

It has been suggested that other STIs may be cofactors of HPV in cervical cancer progression (58). Herpes simplex virus 2 has been suggested as a cofactor in cervical cancer development in a multi-center case-control study (59). Antibodies to *Chlamydia trachomatis* in high-risk HPV-infected women increase the risk of cervical cancer two-fold (60). Furthermore, HIV infected women have significantly higher recurrence rates (87%) of HPV-associated cervical lesions than women without HIV infection (18%) (35, 61).

2.4.2.5 Life style

An individual has an increased risk of cervical cancer with an early onset of sexual activity (<16 years), multiple sexual partners (more than four), or having partners with multiple sexual partners (62). These behaviors increase the risk of HPV infection as well as other sexually transmitted diseases (35).

2.4.2.6 Immunosuppression

HIV infection is associated with a higher prevalence of HPV-related diseases. Higher persistence rates of HPV16 and HPV18 among HIV patients have been reported (63). Furthermore, HIV infected patients and patients receiving immunosuppressive medication are at increased risk of developing cervical cancer (35, 64).

2.4.2.7 Genetic predisposition

Biological daughters of women with cervical cancer had an increased risk of the disease as compared to adopted daughters, with an approximately 50% reduced risk for half-sisters (65). These findings suggested that genetic predisposition may contribute to the risk of cervical cancer. Gene polymorphisms or genetic variations in immune-related genes might be related to HPV persistence and progression to cancer. HLAs are the most extensively studied immune-related gene. HLA molecules play an important role in immune function, through the presentation of peptides for recognition by T lymphocytes. For example, the HLA type DRB1*1301 is consistently negative associated with cervical cancer (35, 66).

3. HPV Immunology and vaccines

HPV infection is restricted to epithelial cells; therefore presentation of viral antigens to the host immune system is limited.

3.1 Innate immunity

The first line of defense against HPV infection is the innate immune system, which provides non-specific protection and also enhances the adaptive immune response. However, HPV often escape innate immune recognition and elimination. They are resistant to lysis by natural killer (NK) cells but are sensitive to cytokine-activated NK cells. Activated macrophages also kill HPV-infected cells and prevent invasive cancer development (35).

3.2 Humoral immunity

Natural HPV infection of the genital tract gives rise to a slow and modest but measurable serum antibody response in most but not all infected individuals. The intensity of this humoral response depends on viral load and persistence (67). The presence of HPV antibodies is long lasting but does not contribute to the clearance of established infections. HPV antibody is a marker of current and/or past exposure to HPV. The production of HPV antibody is important in preventing viral transmission and reinfection. Most HPV antibodies are type-specific and appear six months to a year after HPV infection (DNA) has been detected. HPV-specific immunoglobulin A (IgA) is correlated with virus clearance, however, immunoglobulin G (IgG) is associated with persistence of HPV infection (39). Viral-neutralizing anti-L1 antibodies are generated against epitopes at the surface of the viral capsid and are essentially type-specific. Anti L2-antibodies are less potent than anti-L1 antibodies but they appear to show some cross-reactivity to heterologous HPV types (68).

3.3 Cellular immunity

Clearance of a naturally acquired HPV infection is triggered by a specific cell-mediated immune (CMI) response. Dendritic cells or Langerhans' cells, present in the cervical epithelium, play an important role in recognizing HPV infected cells and stimulating Th1 helper cells, which elicit the production of cytotoxic T-

lymphocytes (CTL). These cytotoxic effector cells attack infected cells, resulting in the resolution of the infection (68). The T-cell proliferative responses to HPV16 E7 are stronger in women with persistent HPV infection and progressive disease (99% reactive) than those who cleared the infection (41% reaction) (69). These data indicates that the CD4 Th1 helper cell response is important for preventing HPV-induced diseases. However, little is known about how to modulate these immune responses (35, 68).

3.4 HPV vaccines

The vast majority of cervical cancers contain high-risk HPV type and approximately 70% contain HPV16 or 18. HPV6 or 11 are responsible for approximately 90% of genital warts. Vaccines against HPV infection are designed for use in preventing or treating cervical cancer and other HPV-related diseases. Two types of vaccines have been developed.

3.4.1 Prophylactic vaccines

Prophylactic HPV vaccines based on the use of virus-like particles (VLPs) obtained by auto-assembly of L1 viral capsid proteins. These are morphologically similar to infectious virions, present conformational epitopes that are highly immunogenic, but lack viral DNA. They can prevent HPV infections by inducing high levels of neutralizing antibodies against the virus (70).

Two vaccines are commercially developed: Cervarix™ (GlaxoSmithKline Biologicals, Rixensart, Belgium), a bivalent vaccine against HPV 16 and 18, and Gardasil™ (Sanofi Pasteur MSD/Merck, Paris, France) a quadrivalent vaccine against HPV 16, 18, 6, and 11. Both vaccines produced from baculovirus-infected insect cells and from yeast, respectively (68, 71).

Results from the phases-II and -III trials published thus far indicate that the L1 VLP HPV vaccine is safe and well tolerated. It offers HPV naïve women a very high level of protection against HPV persistent infection and cervical intra-epithelial lesions associated with the types included in the vaccine (68).

3.4.2 Therapeutic vaccines

Therapeutic vaccines are aimed at generating specific T cells targeted at the high-risk HPV E6 and/or E7 oncogenes and are designed for treatment for existing HPV infection and HPV-related diseases. Various forms of therapeutic HPV vaccines are presently undergoing testing in clinical trials, including those based on peptide, protein, plasmid DNA, viral vector, and administration of E7 pulsed dendritic cells. However, these therapeutic vaccines are in an earlier stage of development than prophylactic vaccines (35).

4. Cervical cancer screening

4.1 Pap smear screening

Since its discovery in 1949 by the pathologist George Papanicolaou, cervical cancer screening by cytology is based on Papanicolaou (Pap) staining of epithelial cells sampled from the cervix in the expectation that detectable nuclear abnormality (dyskaryosis) will be representative of histologically defined underlying lesions (2). The Pap smear screening has helped to decrease cervical cancer incidence and mortality rates by 70% (72). While the Pap smear has been useful in the detection of pre-cancerous lesions of the cervix, this method has several limitations especially the sensitivity is relatively low, which ranges from 47 to 62% (73), is reflected by the false negative rate (5-50 %). The major factors contributing to the false negative rate are inadequate sampling of the transformation zone of the cervix, failure to detect abnormal cells and failure to correctly interpret the significance of abnormal cells due to cervical cells being clumped or overlaid with other cervical cells or inflammatory cells, and presence of obscuring material (blood or mucus) (74, 75). However, this method has relatively high specificity (60-95%) (73). Recently, a new technique, liquid-based cytology (LBC), has emerged to improve the quality of cervical preparations thus leading to improved sensitivity and specificity. Unlike the conventional Pap smear preparation, specimens are collected in a suspension of cells in preservative solution to produce a monolayer of cells on the slide with well-preserved cellular morphology and absence of air-drying, cell crowding, and obscuring cells, with minimal cell overlap. LBC not only reduces the proportion of

specimens classified as technically unsatisfactory for evalution, but also provides representative cell suspensions for HPV, *Chlamydia trachomatis*, and other molecular biological tests (74, 76). There are three Food and Drug Administration (FDA) approved liquid-based tests: PreservCyt® ThinPrep® Pap test (Cytyc Corporation, Boxborough, MA, USA), SurePath™ (TriPath Care Technologies, Burlington, NC, USA) and MonoPrep® (MonoGen, Inc., Vernon Hills, IL) (77).

4.2 Visual inspection with acetic acid (VIA)

VIA (synonyms: direct visual inspection, cervicoscopy, aided visual inspection) was explored as an adjunct to the Pap smear to decrease the false negative rate of cytology. It involves naked eye examination of 3-5% acetic acid-swabbed uterine cervix without any magnification, usually by nurses and other paramedical health workers, with illumination provided by a bright light source. A positive test is the detection of well-defined, dull acetowhite lesions on the cervix, leading to the early diagnosis of HSIL and early preclinical, asymptomatic invasive cancer. Many aspects of VIA make it an attractive test for use in low-resource setting. It is a simple, inexpensive, low-technology test that requires minimal infrastructure for use (2). The sensitivity of VIA ranges from 71-77%, comparing with Pap smear, but specificity is relatively low (64-80%), may result in over-investigation and possible over-treatment in test and treat conditions (78, 79). The test positivity rate that varies from 10-35% so adequate training of health workers is important to reduce false-positive referrals. However, VIA is currently being investigated for its efficacy in reducing incidence of and mortality from cervical cancer (2).

4.3 HPV DNA detection

Due to the difficulties of propagating HPV virus in tissue culture and the unreliability of serological tests to diagnose HPV infection, testing for HPV relies on the detection of viral DNA. Since all HPV types are closely related, assays can be designed to target conserved regions of the genome or regions whose sequences can best be used to discriminate between different HPV types (3). Two test that have been validated in large trials and epidemiological studies include hybridization-based assay and polymerase chain reaction (PCR) – based assays.

4.3.1 Hybridization-based assay

The Hybrid Capture II® system (HC2®, Digene Corporation, Gaithersburg, USA) is a non-radioactive signal amplification method based on the hybridization of the target HPV DNA to labeled ribonucleic acid (RNA) probes in solution (4, 80). The resulting RNA-DNA hybrids are captured onto microtiter wells and are detected by a specific monoclonal antibody and a chemiluminescent substrate, providing a semi-quantitative measurement of HPV DNA. Two different probe cocktails are used, one comprising probes for 5 low-risk genotypes (HPV6, 11, 42, 43, and 44) and the other containing probes for 13 high-risk genotypes (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68), in two separate reaction. Following approval by FDA in 2003, HC2® became the standard procedure for HPV DNA detection. However, HC2® has some limitations. It distinguishes between the high-risk and low-risk groups but does not permit identification of specific HPV genotypes. The detection limit of approximately 5,000 genome equivalents, makes it less sensitive than PCR (81) and cross-reactivity of HC2® high-risk probe to HPV types, that are not represented in the probe mix (*e.g.* HPV53, 66, 67 and 73) (11, 82, 83), have a significant risk for cervical cancer maybe considered beneficial, but cross-reaction with low-risk types causes false positive results and may decrease the specificity of the test (5, 6). Nevertheless, HC2® has been widely used in clinical trials worldwide and has been shown to be robust and reproducible as a screening assay. Trials of the automated third generation HC assay were recently reported (1).

4.3.2 Polymerase chain reaction – based assays

The PCR has been used as the new gold standard for detecting a wide variety of templates across a range of scientific specialties, including virology. The method utilizes a pair of synthetic oligonucleotides or primers, each hybridizing to one strand of a double-stranded DNA (dsDNA) target, with the pair spanning a region that will be exponentially reproduced. The hybridized primer acts as a substrate for a DNA polymerase, which creates a complementary strand via sequential addition of deoxynucleotides. The process can be summarized in three steps: (i) dsDNA separation at temperature >90°C, (ii) primer annealing at 50-75°C, and (iii) optimal

extension at 72-78°C (84). HPV DNA can also be selectively amplified by a series of reactions that lead to an exponential and reproducible increase in the viral sequences present in the biological specimen. PCR-based methods have the highest analytical sensitivity and can detect as few as 10-100 copies of HPV genomes in the specimen being tested. Two approaches for detection of HPV DNA by PCR are relevant (3, 7).

4.3.2.1 Type-specific PCR versus broad-spectrum PCR

Type-specific primers design to amplify exclusively a single HPV genotype can be used, but to detect the presence of HPV DNA in a single specimen, multiple type-specific PCR reactions must be performed separately. This method is labor-intensive, expensive and the type-specificity of each PCR primer set should be validated. Alternatively, consensus or general PCR primers can be used to amplify a broad spectrum of HPV genotypes. Such primers target a conserved region in different HPV genotypes. Since the L1 region is the most conserved part of the genome, several consensus PCR primer sets are aimed at this region (85) (Figure 9). General primers in the E1 region had also been described (86) and several other broad-spectrum PCR primers were reported, but had not been extensively used in clinical situations.

Three different designs of general PCR primers can achieve broad-spectrum detection of HPV DNA. The first incorporates one forward and one reverse primer aimed at a conserved region, but fully complements only one or a few HPV genotypes, the PCR is performed at a low annealing temperature. The GP5+/6+ PCR system is an example of this approach (87, 88). The second class of general PCR primers comprises forward and reverse primers, which contain one or more degeneracies to compensate for the intertypic sequence variation at the priming sites. These primers do not have to be used at a lower annealing temperature. The MY09/11 is an example of a degenerated PCR primer set which comprises a complex mixture of many different oligonucleotides (85). The disadvantage of this design is that synthesis of oligonucleotides containing degeneracies is not highly reproducible and results in high batch-to-batch variation. Therefore, each novel batch of primers should be carefully evaluated to check the efficacy of amplification for each HPV genotype (89). The third option is to combine a number of distinct forward and

reverse primers, aimed at the same position of the viral genome. These primers do not contain random degeneracies, but may contain inosine, which matches with any nucleotide. Using a defined mixture of non-degenerate primers has the advantage that the oligonucleotides can be synthesized with high reproducibility, and PCR is performed at optimal annealing temperatures. Examples of such primer sets are the PGMY09/11 primers (89) and the SPF_{10} primers (90).

Besides the choice of primers, the size of the PCR product is also important. In general, the efficiency of a PCR reaction decreases with increasing amplimer size. Subjecting clinical samples to treatments, such as formalin-fixation and paraffin-embedding, degrades DNA. Consequently, the efficiency of PCR primers generating a small product is considerably higher than primer sets yielding larger amplimers (1, 90, 91).

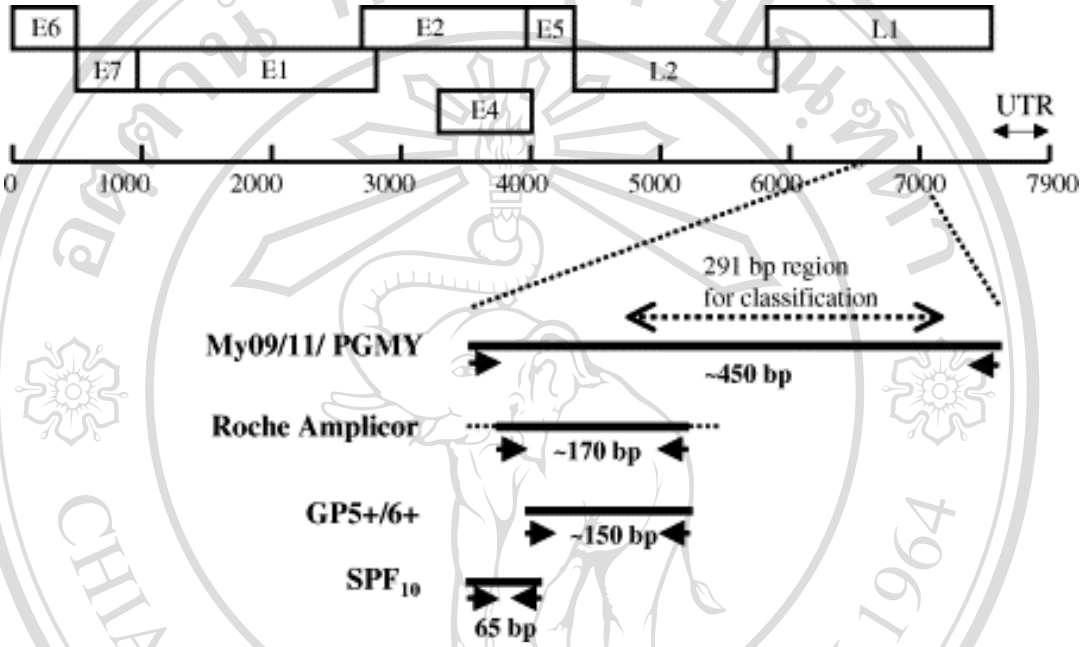

Figure 9 Outline of the HPV-DNA genome, presented in a linear form. The position of the early (E), late (L) genes and the untranslated region (UTR) is indicated, as well as the positions of the four most widely used primer sets MY09/11, PGMY09/11, GP5+/6+, SPF_{10} and Roche Amplicor HPV assay with their respective amplimer sizes (the precise location of the primers, used in the Roche assay is unknown). The 291 bp fragment used for formal classification of HPV genotypes is shown in the L1 region (1).

4.3.2.2 Real-time PCR

Recently, Real-time PCR has already proven itself valuable in laboratories around the world. In contrast to conventional assays, the amplicon detection could be visualized as the amplification progressed. The monitoring of accumulating amplicon in real-time PCR has been made possible by the labeling of primers, probes or amplicon with fluorogenic molecules. This chemistry has clear benefits over radiogenic oligoprobes that include an avoidance of radioactive emission, ease of disposal and an extended shelf life. There are several major chemistries currently in used, and they can be classified into amplicon sequence specific or non-specific methods of real-time PCR detection. The most commonly used fluorogenic oligoprobes rely upon fluorescence resonance energy transfer (FRET; Figure 10) between fluorogenic labels or between one fluorophore and a dark or 'black-hole' non-fluorescent quencher (NFQ), which disperses energy as heat rather than fluorescence. FRET is a spectroscopic process by which energy is passed between molecules separated by 10-100 Å that have overlapping emission and absorption spectra. The efficiency of energy transfer is proportional to the inverse sixth power of the distance (R) between the donor and acceptor ($1/R^6$) fluorophores. In this study, we will focus only the amplicon detection by 5'nuclease (hydrolysis or TaqMan®) oligoprobes (Figure 11), which is classified into amplicon sequence specific real-time PCR detection (84).

Nuclease oligoprobes have design requirement that are applicable to the other linear oligoprobe chemistries to ensure the oligoprobe has bound to the template before extension of the primers can occur, it including: i) A length of 20-40 nucleotides, (ii) a GC content of 40-60%, (iii) no runs of a single nucleotide, particularly G, (iv) no repeated sequence motifs, (v) an absence of hybridization or overlap with the forward or reverse primers and (vi) a T_M at least 5°C higher than that of the primers, to ensure the oligoprobe has bound to the template before extension of the primers can occur (84).

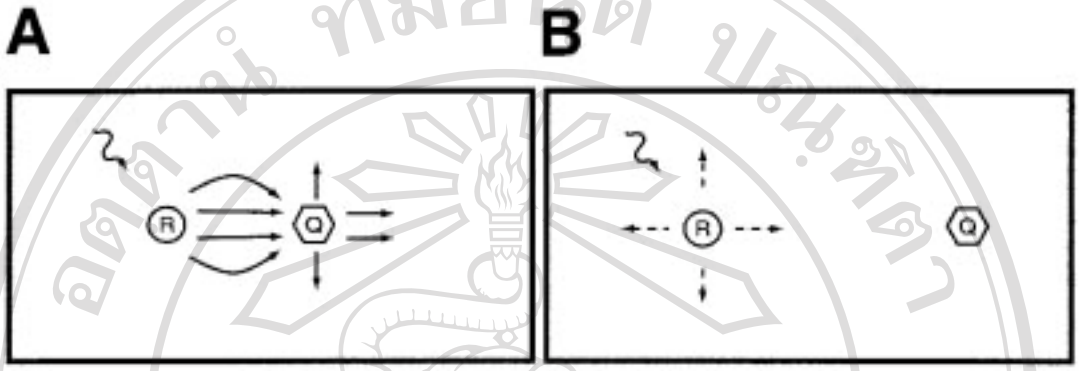

Figure 10 Fluorogenic mechanisms. When a 5'nuclease probe's reporter (R) and quencher (Q, open) are in close proximity as in (A), the quencher 'hijacks' the emissions that have resulted from excitation of the reporter by the instrument's light source. The quencher then emits this energy (solid arrows). When the fluorophores are separated beyond a certain distance, as occurs upon hydrolysis as depicted in (B), the quencher no longer exerts any influence and the reporter emits at a distinctive wavelength (dashed arrows) which is recorded by the instrument (84).

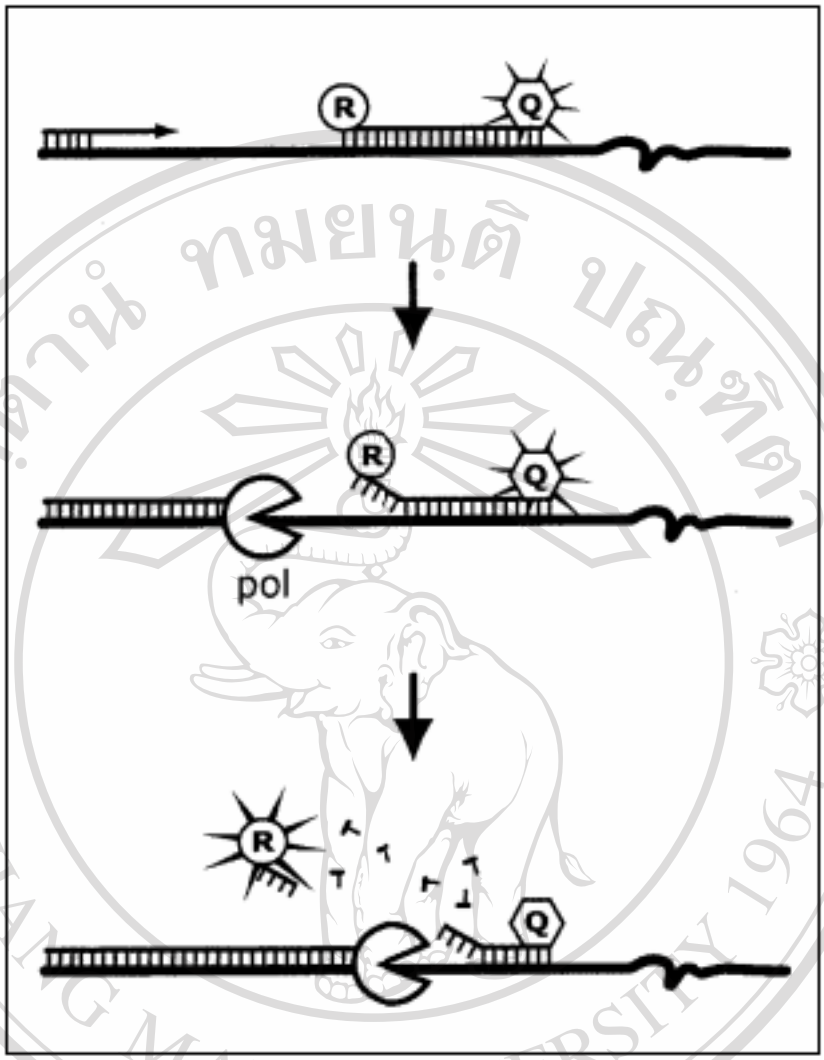

Figure 11 Amplicon detection by 5' Nuclease oligoprobes. As the DNA polymerase (pol) progresses along the relevant strand, it displaces and then hydrolyses the oligoprobes via its 5'→3' endonuclease activity. Once the reporter (R) is removed from the extinguishing influence of the quencher (Q), it is able to release excitation energy at a wavelength that is monitored by the instrument and different from the emissions of the quencher (84).

The fluorescence signal mirrors progression of the reaction above the background noise was used as an indicator of successful target amplification. This threshold cycle (C_t) is defined as the PCR cycle at which the gain in fluorescence generated by the accumulating amplicon exceeds 10 standard deviations of the mean baseline fluorescence, using data taken from cycle 3 to 15. The C_t is proportional to the number of target copies present in the sample (84).

Real-time PCR can also be used to detect HPV DNA by using type-specific or broad-spectrum PCR primers. Type-specific PCR primers can be combined with fluorescent probes although multiplexing several type specific primers within one reaction can be technically difficult (44, 92, 93). Broad-spectrum PCR primers (94, 95) are less amenable to quantitation than a type-specific primer system. Due to the sequence heterogeneity of different HPV genotypes, genotyping of PCR products from broad-spectrum PCR requires a mixture of probes and since these will all have different hybridization characteristics, standardization is difficult (1, 96).

4.3.3 HPV genotyping analysis

After amplification, the amplified products can be analyzed by several HPV genotyping assays including restriction enzyme analysis (REA), reverse hybridization analysis, and ultimately can be coupled to direct DNA sequencing.

4.3.3.1 PCR and restriction enzyme analysis (PCR-REA)

After amplification using consensus primers, the sequence composition of a PCR product can be investigated by restriction enzymes. Digestion of PCR products with restriction endonuclease generates a number of fragments, which can be resolved by gel electrophoresis, yielding a particular banding pattern. PCR using consensus primers with subsequent REA has been demonstrated to be a sensitive and a financially advantageous methodology to detect and characterize HPV DNA in clinical specimens (97). However, this method is straightforward but labor-intensive. More importantly, the method depends on availability of restriction enzymes capable of detecting specific mutations. Consequently, detection of multiple HPV genotypes, present in different quantities in a clinical sample by PCR-REA is usually complex and the sensitivity to detect minority genotypes is limited (1, 98).

4.3.3.2 Direct DNA sequencing analysis of PCR products

DNA sequencing is the gold-standard method for HPV genotyping. It provides sequence information for uncharacterized HPV genotypes as well as mutation information. Other methods include PCR-REA, primer-specific PCR, and reverse hybridization analysis, they only detect mutations at certain locations within a specific genome. These techniques yield information on the presence or absence (and possibly the relative amounts of mutant vs. wild type) of mutations at one or more specific locations but they give no information about other nucleotide positions in the genome (99). But conventional DNA sequencing technique is often not effective for the detection of multiple HPV co-infections in a single sample, because the sequence signals generated are mixed and the results are difficult to interpret. In contrast to traditional Sanger sequencing, Pyrosequencing is a real-time DNA sequencing method. Integrating the multiple sequencing primers method with Pyrosequencing allows for accurate detection of multiple HPV co-infections in a one sample (35, 100).

The genotype can be deduced from an HPV sequence by two methods. First, the sequence can be used to interrogate a sequence database using a homology search. Extensive databases are available on the Internet and can be freely accessed at http://www.ncbi.nlm.nih.gov. BLAST software (101) permits fast homology searches of a sequence within a continuously updated sequence database. Secondly, phylogenetic analyses can be performed. The novel sequence can be used in a multi-sequence alignment with a set of known HPV sequences, representative of different HPV genotypes. Based on the sequence alignment, a phylogenetic tree can be constructed, providing a graphical representation of the evolutionary relationships between the detected sequence and reference sequences, and a genotype can be deduced. It should be noted that formal classification of genotypes is entirely based on sequence analysis of the viral genome, whereas genotyping of clinical samples is performed by analysis of only a limited, but representative part of the genome (1).

4.3.3.3 Microtiter plate hybridization analysis

To increase the throughput of a diagnostic assay, hybridizations to oligonucleotide probes can be performed in microtiter plates (88, 90, 102). Biotin labeling of one of the primers generates labeled PCR products that are then captured onto streptavidin-coated microtiter wells. Double-stranded DNA is denatured under alkaline conditions and the unattached strand is removed by washing. A labeled oligonucleotide probe is added, which hybridizes to the captured strand. Hybrids can be detected following binding of conjugate and substrate reaction. The Roche Molecular Systems Amplicor HPV MWP assay was recently described. This method is based on the detection of 13 high-risk genotypes by a broad-spectrum PCR in the L1 region, amplifying a fragment of approximately 170 bp. The heterogeneous interprimer region is detected with a cocktail of probes for high-risk genotypes. An advantage of this method is the high throughput of the microtiter format. Therefore, this method is suitable for distinguishing HPV DNA positive and negative samples as a first step in HPV diagnosis (1).

4.3.3.4 Reverse hybridization analysis

Reverse hybridization analysis provides an attractive tool for simultaneous hybridization of a PCR product to multiple oligonucleotide probes. This method comprises immobilization of multiple oligonucleotide probes on a solid phase and addition of the PCR product in the liquid phase. Hybridization is followed by a detection stage. The most frequently used reverse hybridization technology comprises a membrane strip containing multiple probes immobilized as parallel lines, called line probe assay (LiPA™; *e.g.* INNO-LiPA HPV Genotyping v2 test, Innogenetics Inc, Ghent, Belgium); line blot assay (LBA) or linear array (LA; *e.g.* Linear Array HPV Genotyping Test (LA-HPV), Roche Molecular Systems Inc, Pleasanton, Calif, USA). A PCR product is generated, usually using biotinylated primers. The double-stranded PCR product is denatured under alkaline conditions and added to the strip in a hybridization buffer. After hybridization and stringent washing, the hybrids can be detected by addition of a streptavidin-conjugate and a substrate, generating color at the probe line, which can be visually interpreted. This method permits multiple HPV type detection in a single step and requires only a

limited amount of PCR product. An example of the HPV LiPA (8-10) is shown in Figure 12.

Alternative reverse hybridization methods for HPV and genotyping are the line blot assay using PGMY primers (11-13, 103, 104) and reverse line blot for GP5+/6+ (105). HPV DNA micro arrays work on the same principle (91, 106). Reverse hybridization methods are particularly useful for the detection of type specific infections and multiple genotypes (1).

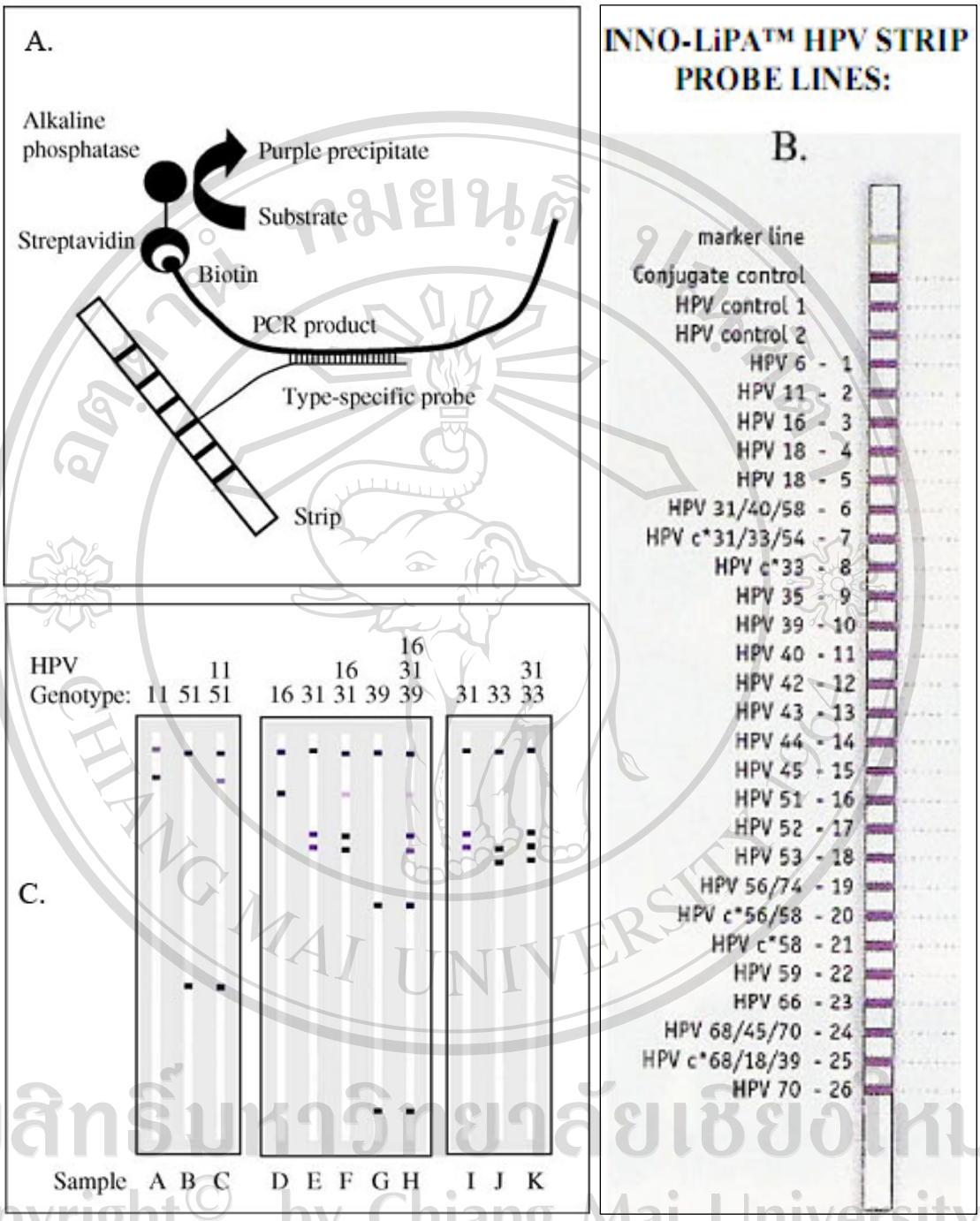

Figure 12 Outline and example of the reverse hybridization HPV line probe assay. (A) Amplimers are denatured and hybridized to probes immobilized as parallel lines on a strip. After stringent washing, the hybrids are detected by enzyme-conjugated streptavidin and a substrate, yielding a color reaction. (B and C) Hybridization patterns which can be interpreted visually after alignment with a probe line template from specimens containing single or multiple HPV genotypes (1).