CHAPTER III

REVIEW OF LITERATURE

Structure

HPV belong to the *Papillomavirus* genus of the family *Papovaviridae*. The viruses are nonenveloped and have icosahedral symmetry. The genome consists circular double-stranded DNA approximately 8 kb in length. All of the open reading frames (ORFs) of the genomes are located on one strand. The viral genome is divided into early (E1 – E7) and late (L1 and L2) regions (Fig. 1). Early genes encode proteins that are involved with regulation of viral DNA replication and transcription. The late genes, L1 and L2, encode the major and minor capsid proteins. The E1 protein demonstrated ATPase and helicase activity. It functions is both to promote viral replication and to inhibit viral integration into the host genome. E2 protein significantly increases the affinity of E1 binding to the viral replication origin. Furthermore, the presence of both E1 and E2 is necessary and sufficient to induce viral DNA replication. In addition, E2 has also a role in viral transcription. Not much is known about HPV E4 protein. It is associated with collapse of the cellular cytokeratin network, perhaps enhancing virus exit from the cell. The E5 protein forms complexes with EGFR resulting in increased ligand-dependent activation of EGFR. E5 also may work together with the E7 protein to promote malignant transformation in cell lines (40). E6 and E7 enable HPV to use cellular proteins for continued viral replication. These proteins have been shown to inhibit the function of tumour suppressor proteins p53 and pRb. E6 is able to bind to p53 with great affinity, thereby significantly enhancing its degradation. Rb plays an important regulatory role in cell division. In its hypophosphorylated form, Rb releases E2F, which stimulates cell cycle progression. The binding of E7 to pRb acts in a manner similar to hypophosphorylation. Additionally, HPV type 16 E7 acts to promote the degradation of pRb (41, 42). The functions of HPV genes are listed in Table 1.

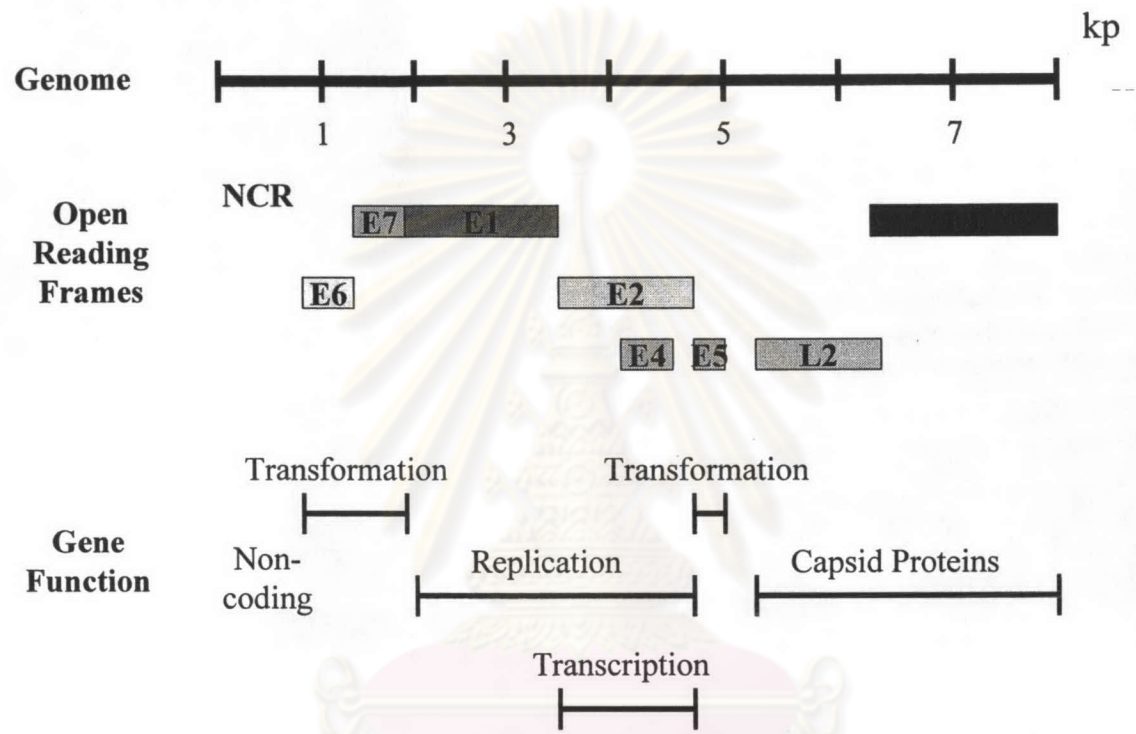

Figure 1. Map of HPV-16 genome. E1 – E7: early regions; L1 –L2: late regions; NCR: Non-coding region (4).

Table 1. A description of the functions of HPV open-reading frames (3).

Viral Protein	Functions and Properties
E1	DNA helicase activity, DNA-dependent ATP-binding, ATPase activity. Role in replication and replication repression.
E2	Regulator of viral transcription and replication, control of early region viral gene expression, necessary for efficient viral DNA replication together with E1.
E3	No known function (only present in a minority of papillomavirus).
E4	Expressed as a late gene primarily in differentiating epithelium, role in productive infection, associated with the keratin cytoskeleton of cultured epithelial cells, role in viral egress.
E5	Transforming activity in HPV-16 *in vitro*. Presumably stimulates benign cell proliferation *in vivo* but might have a role in the initiation of carcinogenesis.
E6	Role in transformation process together with E7. Transcriptional activation properties. E6 of high-risk HPVs inactivates p53 by inducing its degradation. Together with E7 provides a cellular environment for viral DNA replication.
E7	Transactivating properties similar to the adenovirus E2 promoter, induces DNA synthesis in quiescent cells, role in rodent cell transformation in co-operation with an activated ras oncogene. E7 binds to the hypophosphorylated form of the retinoblastoma protein (pRb) resulting in its function inactivation permitting cell progression to S phase of the cell cycle. E7 proteins from the low-risk HPV types 6 and 11 bind less efficiently than the E7 protein from high-risk HPVs (types 16 and 18).
E8	No known function (only present in a minority of papillomavirus).
L1	Major capsid protein.
L2	Minor capsid protein.

Classification and antigenic diversity (variation)

The classification of HPV is based on degree of genetic relatedness. HPV type is defined as having less than 90% nucleotide sequence homology within the L1 open reading frame (ORF) of all other known types. Subtypes have DNA homology between 90% and 95%. More than 100 new HPV types have been detected but to date, only 85 of these have been fully sequenced and characterised (29). Phylogenetic analyses, base on the DNA sequence of different regions of the viral genome, reveal a classification consistent with the biology of the different papillomaviruses. Thus, several supergroups of papillomaviruses, which themselves break down into groups, can be delineated (Fig. 2). Supergroup A includes all the genital papillomaviruses, supergroup B consists of the viruses isolated from epidermodysplasia verruciformis lesion, supergroup C and supergroup D obtain animal papillomaviruses such as bovine papillomavirus and deer papillomavirus and supergroup E contains HPV found in cutaneous lesion, as well as some animal papillomaviruses such as the cottontail rabbit papillomavirus and the canine oral papillomavirus (43).

Furthermore, HPV also are grouped according to potential for malignant transformation. Two groups corresponding to an association with CIN and CaCx can be separated; high-risk groups such as HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 66, and low-risk groups such as HPV-6, 11, 42, 43 and 44. In 1995, IARC evaluated all relevant data on the carcinogenicity of HPV and concluded that there was sufficient evidence to categorize HPV types 16 and 18 as human carcinogens (44).

Amino acid differences in the E6 and E7 proteins are likely to be at least partly responsible for the differential pathogenicity of the various HPV types. However, the amino acid sequences of these proteins vary not only across HPV types but within type as well. The distribution of E6 and E7 variants have recently been reported and these mutations are different according to race and geography (31, 36, 45-49). In a study of variants in the E6 region, *in vitro* studies have been reported that there are biologic differences associated with variation in amino acid 10, 14, 78 and/or 83 of the E6 protein resulting in increase cellular transformation (35, 50-57). More than 25 sequence variants of HPV-16 have been reported and a phylogeny-based classification incorporating the E6 genes has been proposed by Yamada et al. (36). Within HPV-16, five major phylogenetic

branches have been distinguished, each predominating within specific geographical region. These main HPV-16 branches were designated European (E), Asian (As), Asian-American (AA), African-1 (Af1) and African-2 (Af2). Additional minor phylogenetic branches have been identified and designated North American 1 (NA1) (36) (Fig. 3). Evidence of E7 nucleotide and amino acid variation has been reported in previous studies (31-33, 35, 53). It has been reported the position of mutation at pRb binding resulting in increase oncogenic potential and alternated of conformation (31-33, 35, 53). Sequence variations in HPV proteins might affect virus assembly (58), immunologic recognition by the host (59), and immortalization activity (60). Recent investigations have evaluated association of specific HPV-16 variants with viral persistence and with the development of high-grade cervical lesion (61-67).

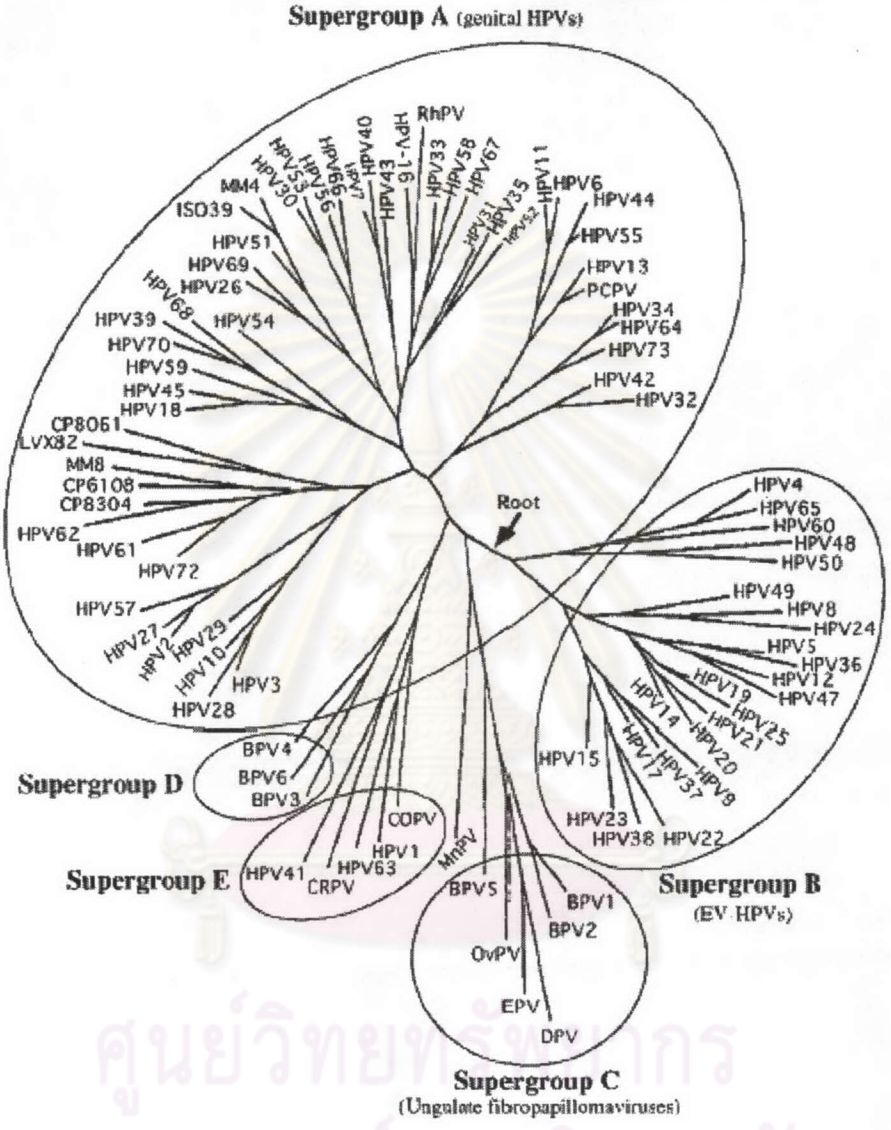

Figure 2. Phylogenetic tree of papillomaviruses. The tree was established on the basis of DNA sequence homology in the L1 open reading frame. BPV, bovine papillomavirus; COPV, canine oral papillomavirus; CRPV, cottontail rabbit papillomavirus; DPV, deer papillomavirus; MnPV, *Mastomys natalensis* (rodent) papillomavirus; PCPV, pygmee chimpanzee papillomavirus; RhPV, rhesus monkey papillomavirus (43).

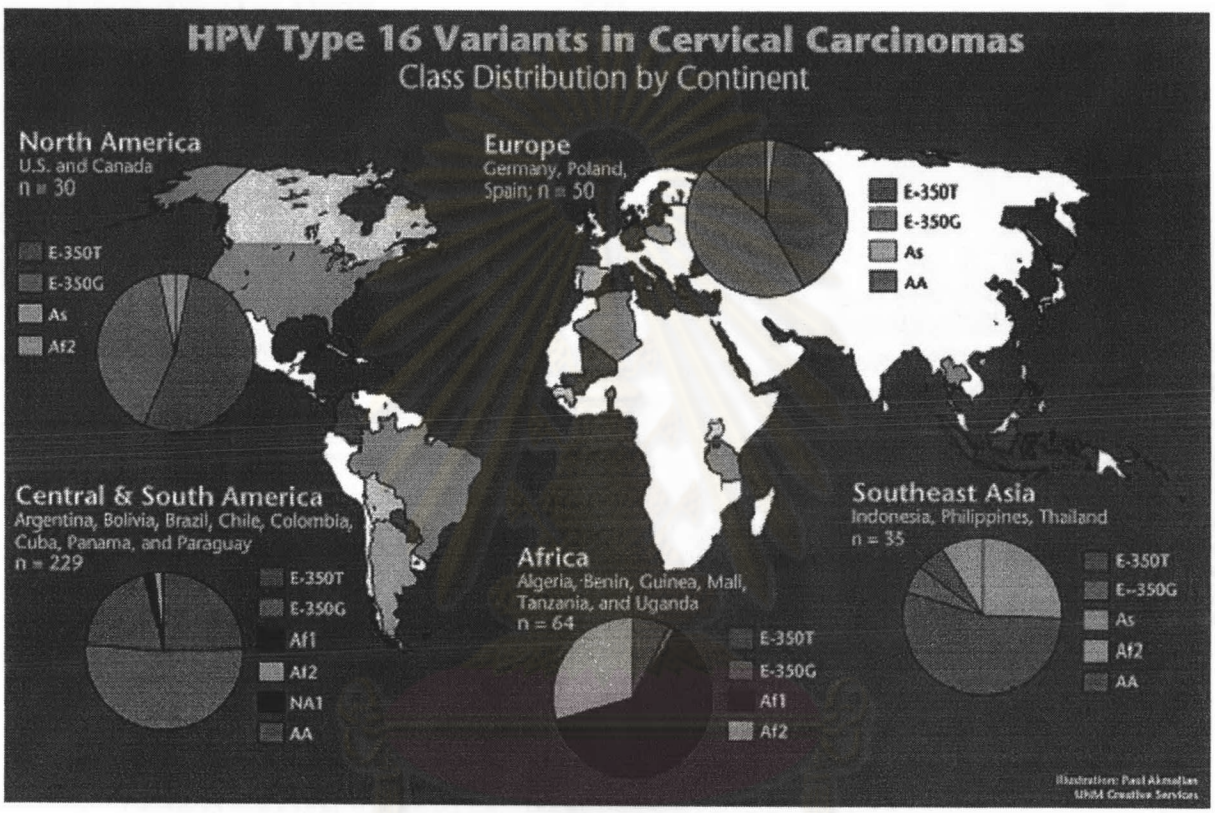

Figure 3. The classification of HPV-16 variant is based on E6 gene. Five major phylogenetic brach have been distinguished; European (E), Asian (As), Asian-American (AA), African-1 (Af1) and African-2 (Af2) and North American 1 (NA1) (36).

Epidemiology

A recent worldwide prevalence survey of HPV types has found 99.7% of CaCx specimens (16). HPV types in CaCx patients, HPV-16 was the most prevalent type (54.6%), followed by HPV-18 (11%), HPV-45 (4.4%), and HPV-31 (3.4%) (68). For CIN, the most common HPV type was HPV-16 (44%), followed by HPV-18 (39%), HPV-52 (14%), HPV-45 (7%), HPV-31 (2.8%) and HPV-33 (1.4%) (69). Moreover, in normal women could be found HPV DNA 11%. The prevalence of low-risk and high-risk in normal women were 1.6% and 13%, respectively (70). However, HPV might be attributed into geographical and racial differences, such as the detection rate of HPV-16 in Asian and African (36.6%) was lower than that reported in Europe and the U.S.A. (60 - 84%) (12, 19, 20, 22, 25, 68). Comparing the prevalence of different HPV types in different countries, Japanese studies showed a relatively lower prevalence of HPV-16 and 18, but a higher prevalence of HPV-52 and 58 (22, 71). In the United States and Germany studies showed a higher prevalence of HPV-16 and 18, but a lower prevalence of HPV-52 (68). In Thailand, HPV-16 was the most common representing 44.04%, followed by HPV-18 (15%), HPV-33 (9.33%) and HPV-11 (4%) (17, 26). Figure 4 displays the HPV DNA prevalence in eight countries in CaCx cases and controls (28, 72-77). Previous studies have reported that HPV-16 was the most prevalent type in squamous carcinoma, whereas HPV-18 was the most common type in adenocarcinoma and adenosquamous carcinoma (78, 79).

Biological characteristics of E6 and E7 gene

The high-risk HPV E6 protein together with high-risk HPV E7 protein was required for the transformation. High-risk HPV E6 protein binds p53 and has rapid degradation of p53 through the ubiquitin pathway. E6 protein of high-risk binds to a 100-kDa cellular protein, E6 associated protein (E6AP), which function as an ubiquitin protein ligase. The E6/E6AP complex then binds the zinc finger motifs or the central region of p53, which becomes rapidly ubiquitinated and is targeted to proteasomes (6, 80). The low-risk HPV E6 protein did not bind p53 and have no effect on p53 (81).

HPV E7 proteins encoded by high-risk and low-risk HPV differ in biochemical and biological properties. Low-risk HPV-6 and 11 E7 proteins bind pRb with a lower

efficiency than the high-risk HPV-16 and 18 E7 proteins (82, 83). Binding disrupts the normal function of these cellular proteins and can give rise to an increased proliferation rate and genomic instability. The HPV-16 E7 region in pRb binding has been investigated. The conserved region (CR) 2, amino acids 16 – 37, of HPV-16 E7 protein contains two independent domains; one mediates binding of pRb, while the other serves as the substrate for casein kinase II (CKII) phosphorylation (84). The amino terminus of the E7 protein has been found to be important in malignant transformation, amino acids 21 – 29 of the HPV-16 E7 protein are the most critical sites for Rb binding (84, 85). Moreover, efficient immortalization of keratinocytes requires the continuous expression of the viral E6-E7 oncogenes and the cooperation of the E6-E7 gene proteins (86, 87). However, the E7 protein alone at high levels can immortalize host cell (88).

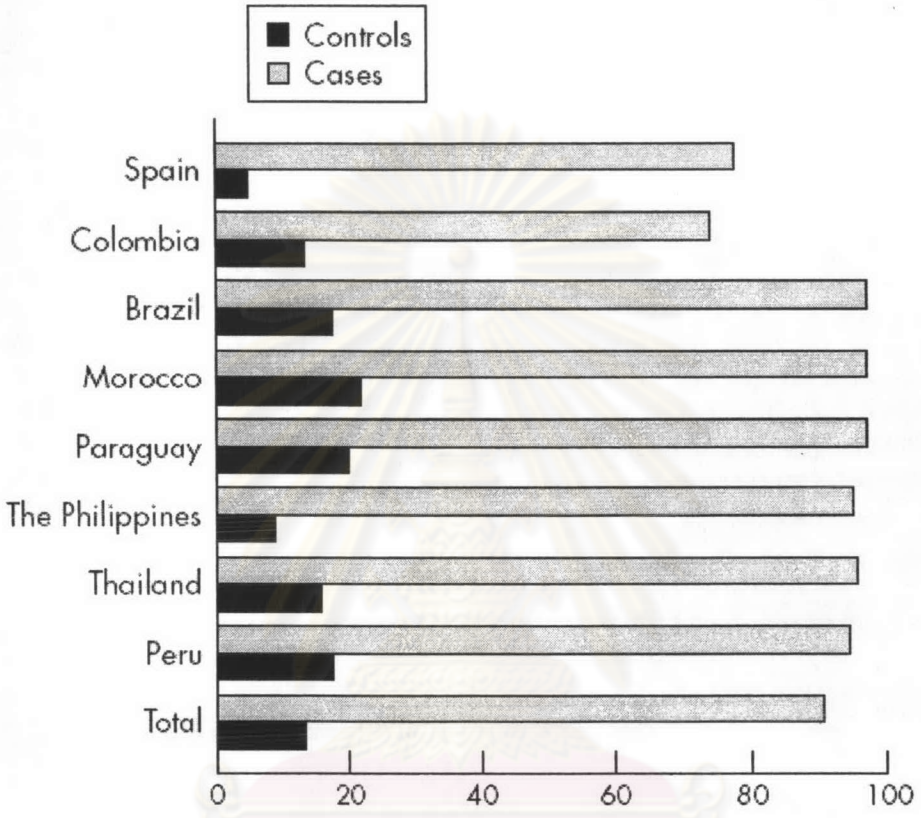

Figure 4. Prevalence of HPV DNA in CaCx cases and controls in the IARC multicentre case-control study (28, 72-77).

HPV life-cycle

The replication cycle of papillomavirus, HPV DNA is often maintained as a multicopy episome and integration of the viral is not required for either the initiation or maintenance of the transformed state. Furthermore, transformation is dependent upon the continued expression of viral DNA. HPV entry into basal epithelial cells, there are two modes of viral DNA replication. There is an initial non-productive stage in which the viral genome is apparently maintained as a stable multicopy episome. This mode of DNA replication ensures a persistent and latent infection in the epithelial cell. The second mode of DNA replication is vegetative DNA replication, which occurs in the more differentiation of epithelium cells. The viral genome is amplified a high-copy number, synthesizes capsid proteins and packaged into progeny virions (Fig. 5) (89-91).

Pathogenesis

HPV have a high degree of cellular tropism or tissue specificity. HPV infects only surface squamous epithelia of the skin or mucosa producing for the most part benign epithelial tumours (2). HPV infection has been associated with benign and malignant lesion. Benign lesions include common warts, CIN. Malignant lesions associated with HPV include oral and respiratory squamous cell carcinomas, anogenital cancer and CaCx. When HPV are associated with benign skin lesion, the viral genome replicates as an extrachromosomal episome separately from the host cell's DNA. In contrast, in most malignant lesion, the viral DNA can be integrated into the host cell chromosome (92). When integration occurs, a break in the viral genome occur leading to loss of function of E1/E2 genes. This in turn causes deregulation of E6 and E7 gene resulting in cellular transformation (89-91, 93) (Fig. 5). The E6 and E7 gene products deregulate the host cell growth cycle by binding and inactivating tumour suppressor proteins (94). The high-risk HPV encoded E6 proteins form complex with p53, and resulting degradation of p53 via the ubiquitin pathway (95, 96). The high-risk HPV E7 protein binds to the pRb. This binding disrupts the complex between pRb and the cellular transcription factor E2F, resulting in the release of E2F, which allows it to function as a transcriptional activator of cellular genes involved in cellular DNA synthesis (5, 8).

Clinical and histopathologic evidence of HPV infection usually develops 1 to 8 months after initial exposure. Untreated, the lesion may regress to precancerous lesion and eventually, cancer (97). However, the progress and outcome of an HPV infection depend on HPV type, anatomical location, and the nature and timing of local cellular and tissue influences. In HPV infection, it has not been possible to clearly distinguish from viral persistence with a low level of virus replication (98).

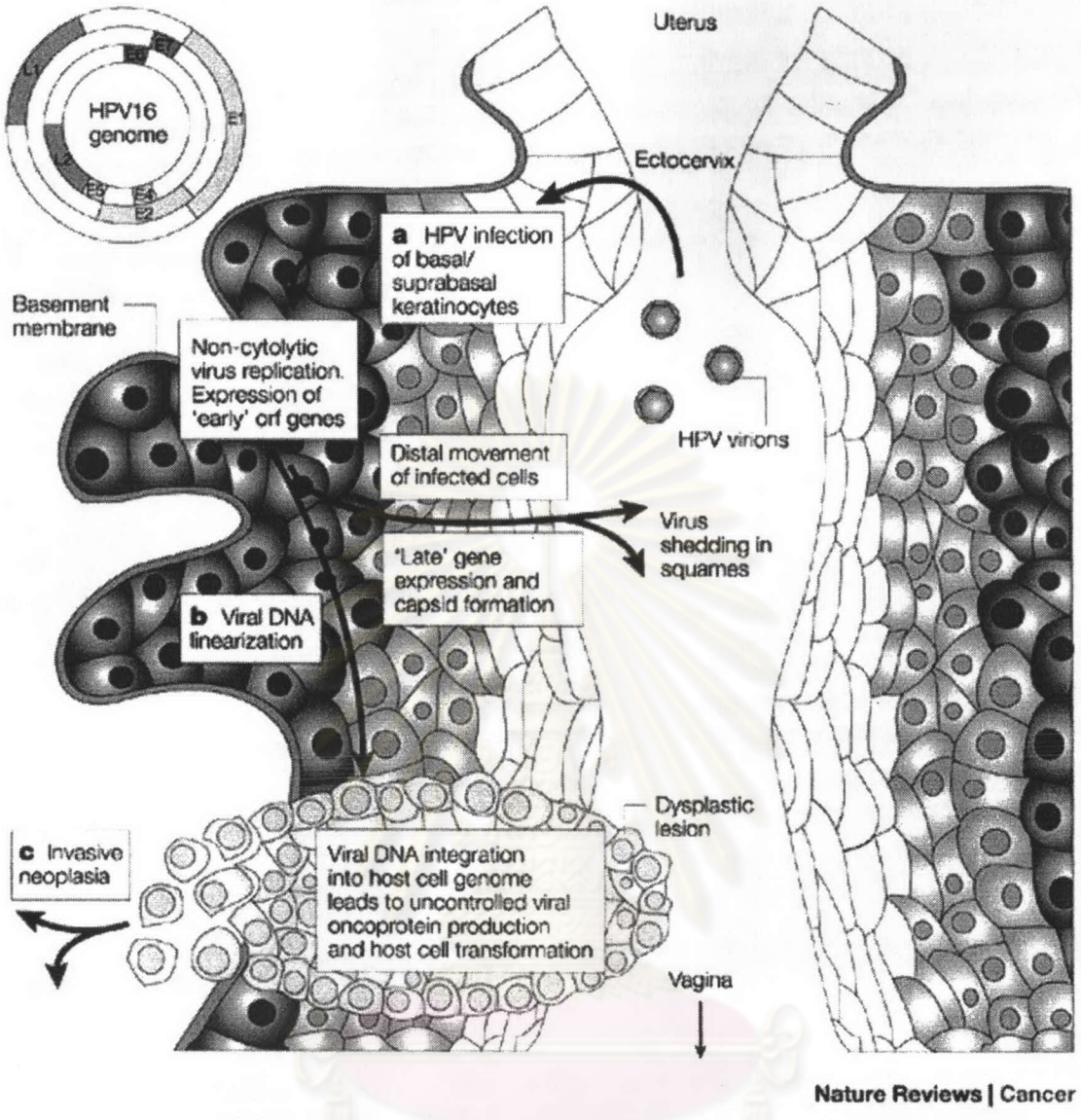

Figure 5. The HPV life-cycle. **a.** HPV infect basal epithelial cells. **b.** HPV-DNA integrates into host cell genome. When integration occurs, a break in the E1/E2 region, leading loss of control E6 and E7 oncogenes, resulting in cellular transformation. **c.** Invasive tumour ruptures the basement membrane and invades the subepidermal tissue (91).

Clinical manifestations

HPV cause a wide range of disease processes, depending on HPV type (Table 2) (4). HPV can be induced cutaneous warts, epidermodysplasia verruciformis, anogenital warts and CaCx.

Cutaneous warts

Three major types of cutaneous warts are recognised; deep plantar warts, common warts, and flat or plane warts. Deep plantar warts are usually solitary lesions preferentially located on the weight-bearing surfaces of the foot. Common warts are usually multiple, well-circumscribed, exophytic, hyperkeratotic, round papules with a coarse surface that range in size from 1 mm to 1 cm. They are normally found on the dorsum of the hand, between the fingers, and around the nail bed. Plane or flat warts present as multiple, flat, small, asymmetric, smooth papules with a pink to tan colour. They are mostly found on the face, neck, and hands of children (4).

Epidermodysplasia verruciformis

Epidermodysplasia verruciformis (EV) is characterised by the appearance of flat wart-like lesions, red to brown plaques. The disease generally begins in infancy or childhood with multiple, disseminated, polymorphic. Over 20 different HPV types have been isolated from EV lesion (Table 2). HPV-3 and HPV-10 found in flat warts of normal individuals, are also recovered from in flat warts of EV patients (4).

Anogenital warts

Genital warts are the most commonly recognized clinical lesion of genital HPV infections. Condylomas are florid and condyloma acuminatum. In males, the exophytic condylomas occur on the penis, around the anus, on the perineum. In females, they involve the vaginal introituses, the vulva, the perineum, the anus. Several HPV types have been associated with condylomas. HPV-6 and 11 are responsible for the large majority of exophytic condylomas at all location (4).

Table 2. HPV types and their disease association (4).

Disease	HPV types	
	Frequent association	**Less-frequent association**
Deep plantar warts	1, 2	4, 63
Common warts	2, 1	4, 26, 27, 29, 41, 57, 65, 77
Common warts of meat, poultry and fish handlers	7, 2	1, 3, 4, 10, 28
Flat warts	3, 10	27, 38, 41, 49, 75, 76
Intermediate warts	10	26, 28
Epidermodysplasia verruciformis	2, 3, 10, 5, 8, 9, 12, 14, 15, 17	19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 50
Condylomata acuminata	6, 11	30, 42, 43, 44, 45, 51, 54, 55, 70
Intraepithelial neoplasia (IN), unspecified		30, 34, 39, 40, 53, 57, 59, 61, 62, 64, 66, 67, 67, 68, 69, 71
Low grade	6, 11	16, 18, 31, 33, 35, 42, 43, 44, 45, 51, 52, 74
High grade	16, 18	6, 11, 31, 33, 35, 39, 42, 44, 45, 51, 52, 56, 58, 66
Bowen's disease	16	31, 34
Bowenoid papulosis	16	34, 39, 42, 45
Cervical carcinoma	16, 18	31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 70
Recurrent respiratory papillomatosis	6, 11	
Focal epithelial hyperplasia	13, 32	
Conjunctival papillomas and carcinomas	6, 11, 16	
Other: epidermoid cysts, keratoacanthoma, benign and malignant lesion of the conjunctiva, lacrimal sac, nasal passages, bronchi, esophagus and bladder, malignant melanoma, neoplastic prostatic tissues		6, 11, 16, 30, 33, 36, 37, 38, 41, 48, 60, 72, 73

Cervical cancer

HPV is the main etiological factor of benign and malignant genital neoplasia. CaCx worldwide are infected with specific types of HPV DNA mainly HPV-16 and HPV-18 (12, 36). The clinical appearances of HPV-related premalignant and malignant cervical lesions are defined by colposcopic criteria that attempt to match histopathology. All CaCx develop in the transformation zone which is located at the stratified squamous epithelium of the vagina (99).

The classification of abnormalities of the cervical epithelium is based on the CIN system (Table 3). These abnormalities are classified as CIN grades I, II, III (100). The Bethesda system was developed for understanding of cervical neoplasia and to introduce uniform descriptive diagnosis histology. The Bethesda system 2001 classifies squamous cell abnormalities into 4 categories: atypical squamous cells (ASC), low-grad squamous intraepithelial lesion (LSIL), high-grad squamous intraepithelial lesion (HSIL), and squamous carcinoma or adenocarcinoma. Furthermore, the ASC category contains two subcategories: atypical squamous cells of undetermined significance (ASC-US) and atypical squamous cells, cannot exculued HSIL (ASC-H) (Table 3.) (101-107).

Asymptomatic cervical HPV infection can be detected in 5 – 40 % of women of reproductive age (108). Diagnosis of CIN I, about 1% progress to invasive carcinoma (100). The progression from CIN I to CIN II occurs very quickly, about 5% on CIN II progressing to invasive cancer (100). Evidence indicates that CIN III have a significant risk (>12%) of progressing to invasive carcinoma if untreated (100).

The epidemiological research in recent years has focused on the role of risk factors that influence acquisition of persistent high-risk HPV infection or of coexisting factors that mediate progression in the continuum of lesion grade (Fig. 6) (109). Cervical cancer occurs primarily through sexual contact. Factor that may influence the acquisition of disease include the number of sexual partners, the presence of genital warts on sexual partners, a history of sexually transmitted disease and smoking. More important risk factors include the use of oral contraceptives, high parity and immunosuppression (109, 110).

Table 3. The Bethesda classification system for cervical squamous cell dysplasis (101-107)

Bethesda System	CIN System
Negative for intraepithelial lesion or malignancy	Normal
ASC ASC-US (atypical squamous cells of undetermined significance) ASC-H (atypical squamous cells, cannot excluded HSIL)	
LSIL (low-grad squamous intraepithelial lesion)	CIN I
HSIL (high-grad squamous intraepithelial lesion) with features suspicious for invasion (if invasion if suspected)	CIN II/III
Carcinoma	Invasive squamous cell carcinoma Invasive glandular cell carcinoma (adenocarcinoma)

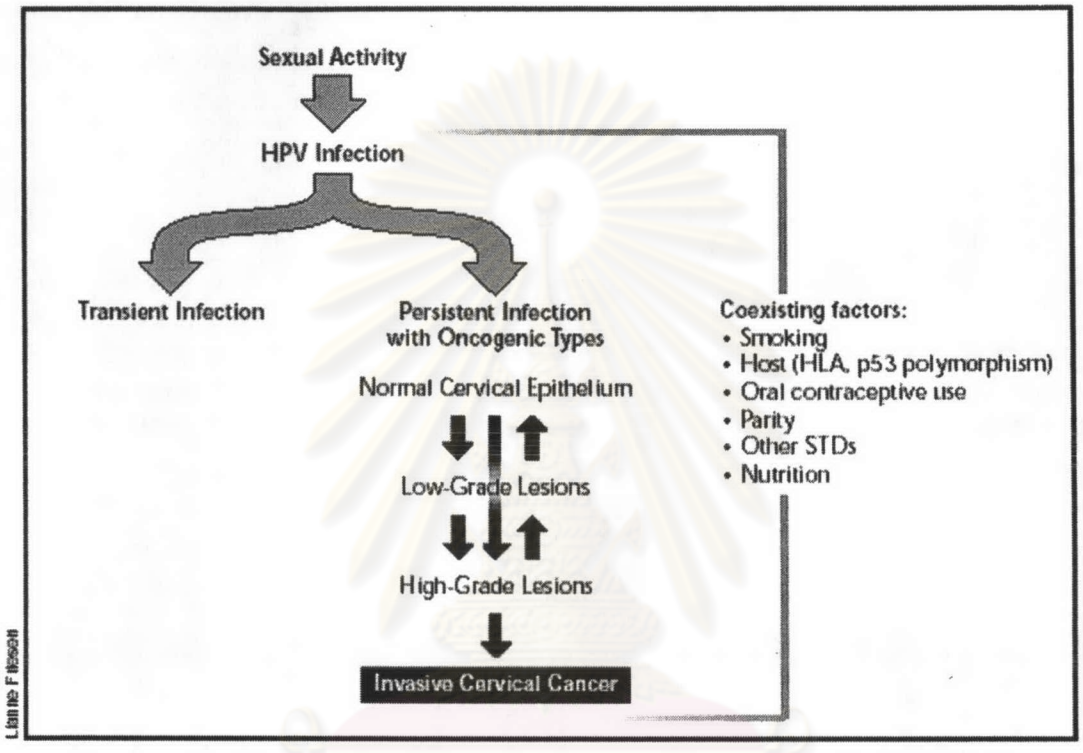

Figure 6. Aetiological model of human papillomavirus (HPV) infection and CaCx, illustrating probable role of remote behavioural risk factors for persistent infection and of coexisting factors that mediate lesion progression (109).

Immune response

Humoral immune response (HIR)

To date, epidemiologic studies have found a positive association between the detection of HPV antibodies and the risk of CaCx, in line with the thinking that HPV antibody detection is a marker of current and/or past exposure to HPV (111-114). Antibodies against HPV have been shown to be largely type specific. Although these antibodies, particularly those that target the protein comprising the virion capsid (L1 and L2 proteins), might be effective at preventing infection, it is commonly accepted that antibodies are not important effectors of regression of established HPV infections and related cervical lesions (114). Less clear is whether antibodies against one HPV type protect against subsequent reinfection with the same or another closely related type and this protection is related to specific antibody subsets (IgG and IgA). Epidemiologic studies that have begun to address this question have focused largely on antibodies against L1 virus-like particles (VLP). Future studies could benefit by expanding the scope of inquiry to include antibodies against antigens other than L1 such as L2, E2, E6 and E7 (115).

Cell-mediated immune response response (CMIR)

A strong association between persistence or variation of HPV infections and the development of CIN has been describe previously. In CMIR, CTL has been shown in protection HPV-associated disease. HPV-16 E6 and E7-specific CTL have been demonstrated in women with CaCx and CIN. Previous study have reported that CD8+ T cell against HPV antigen were more prevalence in women without CIN than in women with CIN (116). The antibody levels in persistent HPV were increased in chronic HPV infection and there is evidence of T-cell recruitment to the site of HPV infection (116). Alexander, et al. (117) stimulated peripheral blood mononuclear cell (PBMC) from CaCx patients with HLA-A2-restricted HPV-16 E7 peptide (E7 amino acids 11 – 20) and showed that CTL were capable of lysing HLA-marched and Caski cervical cancer cell line. These data indicated that the peptide E7 11 – 20 is endogenously processed and presented on the cell surface. In the study by Nakagawa, et al. (1997) HPV-16 E6 and E7-specific CTL have also been demonstrated in patients who had evidence of HPV-16

infection without CIN. The percentage of patients who demonstrated HPV-16 E6 and E7-specific CTL was higher in a group of women had HPV-16 infection without CIN than in a group of women had HPV-16 infection with CIN suggested that CTL responses played a role in disease protection (37). The effectors cell phenotypes in these assays have been shown to include both CD4+ and CD8+ T lymphocytes (38). Furthermore, CTL response is important in clearance of HPV-16 infection. In a similar subject population, Nakagawa, et al (39) found that E6 and E7-specific CTL responses of women without CIN who cleared HPV-16 infection was higher than in a group of women who had HPV-16 persistence.

Several study to identify antigenic epitopes of HPV, using both mouse model system and human system. Kast, et al. (118) identified potential CTL epitopes of HPV-16 E6 and E7 proteins for five common HLA types (HLA-A1, A2.1, A3, A11 and A24) by measuring binding affinity of 240 monomer peptides, result shown as in Table 4. Immunogenic of nine of these potential antigenic epitopes for HLA-A2.1 was tested. Using HLA-A2.1 transgenic mice four immunogenic peptides were identified in vivo (E6 29-38, E7 11-20, E7 82-90, E7 86-93). In addition, CTL induction of PBMC from human confirmed immunogenicity, in vitro, of three of the four peptides (E7 11-20, E7 82-90, E7 86-93) (119). CTL to one of these peptides (E7 11-20) have been demonstrated in CIN and cancer patients (117, 120).

Table 4. CTL epitopes of HPV-16 E6 and E7 proteins for five common HLA types (118).

HPV-16 E6 & E7 peptide binding to	Origin	Position	Sequence	Presence of HLA-A motif
HLA-A1	E6	80	ISEYRHYCY	+
	E7	19	TTDLYCYEQ	-
	E7	37	EIDGPAGQA	-
	E7	44	QAEPDRAHY	+
	E7	73	HVDIRTLED	-
HLA-A2.1	E6	7	AMFQDPQER	-
	E6	18	KLPQLCTEL	+
	E6	26	LQTTIHDII	-
	E6	52	FAFRDLCIV	+
	E7	7	TLHEYMLDL	+
	E7	11	YMLDLQPET	- (+)
	E7	12	MLDLQPETT	- (+)
	E7	82	LLMGTLGIV	+
	E7	85	GTLGIVCPI	+
	E7	86	TLGIVCPIC	-
HLA-A3	E6	7	AMFQDPQER	+
	E6	33	IILECVYCK	+
	E6	42	QQLLRREVY	-
	E6	59	IVYRDGNPY	+
	E6	75	KFYSKISEY	+
	E6	89	SLYGTTLEQ	-
	E6	93	TTLEQQYNK	+
	E6	125	HLDKKQRFH	+
	E6	143	AMSAARSSR	+
	E7	89	IVCPICSQK	+
HLA-A11	E6	7	AMFQDPQER	+
	E6	33	IILECVYCK	+
	E6	42	QQLLRREVY	-
	E6	59	IVYRDGNPY	- (+)
	E6	80	ISEYRHYCY	- (+)
	E6	93	TTLEQQYNK	+
	E7	89	IVCPICSQK	+
HLA-A24	E6	1	MHQKRTAMF	-
	E6	26	LQTTIHDII	-
	E6	44	LLRREVYDF	+
	E6	49	VYDFAFRDL	+
	E6	87	CYSLYGTTL	+
	E6	131	RFHNIRGRW	+
	E7	49	RAHYNIVTF	-

+ : peptides yield IC_{50} < 500 nM (high binding), - (+) : IC_{50} 500 - 5000 nM (week binding), - : IC_{50} > 5000 nM (negative binding)

In addition, more recently it has been shown that HPV can directly subvert the immune response. First, HPV disrupt the IFN pathway, HPV-16 E7 inhibits the induction of IFN-α inducible genes via loss of the IFN-stimulated gene factor (ISGF)-3 transcription complex, which binds the IFN-stimulated response element in the nucleus, resulting in initiation of transcription (121). E7 has also been shown to have an interaction with IFN regulatory factor (IRF)-1 which inhibits activation of the IFN-β promoter (122). Moreover, IFN-α, IFN-β and STAT-1 are down-regulated by HPV-16 E6 (123). Second, interference with antigen processing and presentation, the HPV E7 protein of the highly oncogenic strains HPV-16 and 18 is able to repress the major histocompatibility complex (MHC) class I heavy chain promoter. HPV E7 also represses a second promoter that regulates expression of the genes that encode the TAP-1 and LMP-2 proteins, which are involved in transport of the peptides/MHC class I complex to the cell surface (124). Recently study shown that the HPV E5 protein induces the down-regulation of MHC class I (125). Third, inhibition of interleukin-18 (IL-18) activity, both HPV-16 E6 and E7 inhibit IL-18 induced IFN-γ production in peripheral blood mononuclear cells (PBMC) and NK cells, by reducing the binding of IL-18 to the α-chain of its receptor (126).

Today, two divergent immunologic approaches have evolved for development of prophylactic and therapeutic vaccines. In general, prophylactic vaccines elicit HMIR because they induced the production of antibodies capable of neutralizing a viral antigen before it entered the host cell. In study *in vitro* and *in vivo* assays, L1 and L1/L2 virus-like particle (VLP) were capable of inducing high titer neutralizing antibodies to L1 (127-129). Therapeutic vaccines, on the other hand, were intended to induce cellular components of the immune system to recognise and attack cells infected with HPV. The categories of therapeutic vaccine strategies were peptide-based (130), protein-based (131-133), nucleic acid-based (134) and cell-based (135).

Diagnosis of HPV infections

HPV cannot routinely be cultivated in human or animal cell or tissue culture systems. Methods principally available for laboratory diagnoses of HPV infections can be divided as follows: (i) cytology and histology; (ii) methods for the detection of HPV DNA; (iii) immunocytochemistry; (iv) electron microscopy and (v) serology.

Cytology and histology of HPV infections

The conventional method for detection of genital HPV infection is the Papanicolaou-stain (Pap) smear. The Pap smear is a screening for changes in cells of the transformation zone of the cervix. However, this method has some limitations. False-negative rates as high as 20 - 30% have been reported. New method of collection and processing of specimens for Pap smear have recently been developed to help reduce the number of false-negative results. In monolayer cytology, the specimen is collected in preservative solution such as ethanol and alcohol. Cellular structure is better preserved because the cells are immediately fixed. In several study, the samples collected using cervical brushes were used to compare monolayer cytology to conventional Pap smear cytology. The results showed that statistically significant improvement in the diagnostic sensitivity of monolayer cytology, with increases detection of epithelial cell abnormalities (136, 137). In histopathology, patients with abnormal Pap smear finding who do not have a gross cervical lesion are usually evaluated by colposcopy and colposcopy-directed biopsy. Colposcopy can detect low-grade and high-grade dysplasia but dose not detects microinvasive disease. Biopsy can be used to confirm most diagnoses by observing characteristic pathologic features of HPV infection such as epithelial hyperplasia (101).

Detection of HPV DNA

HPV DNA can be demonstrated in biopsy tissues. Type-specific polymerase chain reaction (PCR) assays are based on the L1 gene of HPV subtypes. The analytical sensitivity of these assays is between 10 – 200 HPV copies per sample, depending on the HPV type. Type-specific PCR are currently used primarily in research application since throughput is limited by the need to use multiple PCR amplifications for each sample (101). The majority of studies using consensus primers to amplify a broad spectrum of

HPV types in a PCR amplification. These primers target conserved regions of the L1 capsid gene. These primers, GP5+/GP6+, SPF10, MY09/11 and PGMY to detect and genotype a broad spectrum of HPV types (138-143). Various methods have been used to identify HPV types after amplification with consensus primers such as sequence analysis, restriction fragment length polymorphism (RFLP), and hybridization with type-specific probes using dot blot (DB) (17, 144). The current commercial HPV detection kit, Digene's Hybrid Capture II kit, detects all high-risk and low-risk HPV types. Several specimen types can be used in the Hybrid Capture assay such as cervical swabs and cervical biopsy (101, 145).

In hybridization assay, Southern blot (SB) hybridization is used to be the "gold standard" method for HPV detection. HPV types are identified according to the size of the hybridized fragments and according to the hybridization efficiency of different type-specific probes. The probes are labelled with either radioactive or non-radioactive compounds. However, the technique is labour-intensive, time-consuming performance, use of radioactive probes and low sensitivity (Table. 5) (146). DB hybridization is an easy and fast to perform than southern blot. Digested and denaturized cellular DNA is fixed as a dot or spot onto a nylon filter and hybridized with labelled HPV-specific DNA or RNA probes. This test had a lower sensitivity than PCR as shown in Table 4 (147, 148).

In filter *in situ* hybridization (FISH), exfoliated cells are filtered onto a nitrocellulose-lose membrane, denatured and hybridized at a high effective temperature. FISH is a fast and easy method and its analytical sensitivity is slightly better than southern blot. However, FISH is a low sensitivity and specificity, background problems due to cell debris, poor reproducibility and high interlaboratory variability. *In situ* hybridization (ISH) is a technique for the localization of specific nucleic acid sequences within individual cells, either in tissue sections or in whole cell preparations (149). The lower detection limit of ISH is 20 – 25 HPV copies/cell (150). ISH is a labour-intensive and technically difficult method (Table 5).

Detection of HPV protein by Immunocytochemistry

Immunocytochemistry can be applied to tissue biopsies. Immunoperoxidase staining with antibodies using the peroxidase-antiperoxidase or the avidin-biotin complex peroxidase method is highly specific for the detection of HPV L1 capsid proteins in superficial epithelial cells of productively HPV-infected tissue. The overall sensitivity of immunocytochemistry is not more than 50% compared to HPV DNA detection methods. Even in well-differentiated lesion as cutaneous warts, HPV capsid antigen cannot be detected by immunocytochemistry, because only small amount of antigen are expressed in many case. Thus, immunocytochemistry is only of limited value for the detection of HPV in clinical samples.

Electron microscopy

Electron microscopy can be used for the identification of viral particles in tissue biopsies. The sensitivity of electron microscopy for HPV detection is low, because viral particles are not regularly present in HPV-induced lesion. Furthermore, electron microscopy is expensive, labour-intensive and does not allow the identification of specific HPV types. (146)

HPV serology

Serologic assays for the diagnosis of HPV infection are not commercially available. Several study showed that HPV antibodies are generally more frequently detectable in patients with HPV-associated lesion than in controls, but HPV antibodies are not detectable in all patients with HPV pathology. These assays used for the detection of IgM, IgG or IgA antibodies against HPV early or late proteins are Western blot, enzyme linked immunoassay (EIA), and radioimmunoprecipitation assay (RIA). However, HPV serology is not yet sensitive enough to detect in patients with CIN or CaCx.

Table 5. Comparison of method for the detection of HPV nucleic acids (146)

Method	Sensitivity	Specificity	Detection limit (HPV genomes)	Perfor mance	Main advantage	Commercially available
PCR	0.90-1.0[a]	Excellent when combined with hybridization	<10-100	2	Method with highest sensitivity	Yes
HC	0.74-0.93[a]	0.61-0.71[a]	5×10^3	1	Standardized, easy to perform	Yes
SB	0.35[b]	0.94[b]	10^5	3	Differentiation between episomal and integrated HPV DNA	No
DB	0.39-0.54[b]	0.94-1[b]	1-10 /cell	2	Faster and easier than SB	Yes
ISH	≤0.45[a]	good	20-25 /cell	3	Topographic localization of HPV-specific DNA or mRNA	yes

Performance: 1 = technically easy, fast result; 2 = moderately difficult, fast result; 3 = technical difficult, labour-intensive, time-consuming.

a With histology as the reference test.

b With PCR as the reference test.

Analysis of CD8+ T cell response

Limiting dilution analysis (LDA)

The theory of limiting dilution analysis is quantitative of the precursor frequency in a population of cells. This assay used differentiates between the presence of a positive and a negative response in the individual wells of the experiment. If the result showed positive, indicated that the presence of antigen-specific precursor cells in the cell population. The effectors function of cytotoxic precursors is monitored by measuring release of chromium from labelled target cells, and proliferative cells are monitored by measuring tritiated thymidine incorporation (151, 152).

Enzyme-linked immunospot (ELISpot) assay

The ELISpot measure the cytokines that are released from an activated T cell. The cell populations stimulated with antigen that were incubated in nitrocellulose plate, which are coated with anti-cytokine antibody. After that, the responder cell product cytokines around cells can be detected by a second antibody. The spots are developed with substrate. Each spot represents cytokine produced by a single cell. Advantages of ELISpot can be measure in a short times, sensitivity was more than chromium-release assays (152, 153).

Intracellular-cytokine staining (ICS)

Cytokine production at the level of the single T cell can be analysed by flow cytometry to detect of intracellular cytokines. T cells are stimulated with antigen. After that, to block the transport of cytokines with monensin or brefeldin A therefore prevent the secretion of cytokines and the reaction was stopped by EDTA. T cells are fixed, permeabilised, and stained for the presence of intracellular cytokines. Advantages of ICS: large numbers of T cells can be analysed in a short time, use of multiple antibodies, and determination of phenotype of the cells (152).

Chromium-release assay

The chromium-release assay was gold standard assay for cytotoxic. The target-cells were labelled with sodium chromate and co-incubated with effectors-cell population. The amount of chromium released into supernatant is quantified, to provide a measure of target-cell lysis. The technique is labour-intensive, required the effectors-cell at range concentration, up to 100 times the number of target cells. Because of limitation in the total number of effectors-cell that can be placed in the microtitre plate (152).

MHC/peptide tetramer analysis

Recent technological advance to identify individual T cell is based on the specificity of binding to the MHC-peptide complex. Direct visualisation and quantification of antigen-specific cytotoxic T cells measure by MHC/peptide tetramer complex. This technique increased avidity of soluble MHC/peptide complex for the peptide-specific T cell receptor (TCR) using biotinylated MHC/peptide monomer bound to avidin or streptavidin, which has four biotin-binding sites. The binding of the tetramer to the TCR can be detected by flow cytometry. The limitation of the tetramer assay, CD8+ T cells that are quantified recognize a well-defined peptide epitope in conjugation with a known MHC class I molecule. Advantage of this assay was not required the radioisotopes, fast, and large number of samples can be processed (154).