CHAPTER III

REVIEW OF LITERATURE

Basic virology

Vius Structure

Human papillomaviruses (HPV) are member of the *Papillomaviridae* family. HPV is a nonenveloped virus, 52-55 nm in diameter and has an icosahedral symmetry. The outer icosahedral capsid composed of 72 capsomers, which contain of two structural proteins, L1 and L2. The genome of HPV is circular double-stranded DNA and approximately 8 kb in length (1-5). The viral genome is divided into three regions: noncoding region, early region and late region. All of open reading frame (ORF) coding for six early proteins (E1, E2, E4, E5, E6 and E7) and two late proteins (L1 and L2) (Fig.1)(19). The noncoding region as a upper regulatory region (URR) or long control region, have many binding sites for repressors and activators that regulate DNA replication by controlling of the ORFs transcription. The early region encodes proteins that are involved in viral DNA replication, transcription and oncogenesis especially E5-E7. The late region encodes structural protein for the viral capsid that L1 is a major and L2 is a minor capsid protein (10, 19, 20). The E1 protein has helicase that is essential for viral DNA replication and inhibits viral integration into the host genome. E2 protein is viral transcription factor that binds E1 to facilitate of viral DNA replication and important for genome encapsidation. In addition E2 protein has also a role as a repressor of E6 and E7 proteins expression. E4 protein plays role in viral egress, productive infection and is associated with cytoskeletal proteins. E5 protein to be expressed through out the viral life cycle and is capable of enhancing tumorigenesis. And this protein is associated with epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptor, thereby constitutively activating them. However E5 is not be essential in maintaining malignant transformation of the host cell. E6 and E7 proteins play important role in the malignant transformation of cervical cancer and immotalizes human foreskin keratinocytes. These proteins have been shown to inhibit the function of tumor suppressor proteins p53 and pRb. The E6 protein can bind to 53 protein using ubiquitin

ligase E6 associated protein (E6AP) and this complex is degraded by cellular protease leading to a loss of cell control (Fig 2)(20). The E7 protein binds to phosphorylated retinoblastoma protein (Rb) which Rb plays an important regulatory role in cell division. After binding of E7 and pRb causing separation of Rb from elongation factor (E2F) complex. And then the Rb protein is degraded through the ubiquitin proteosome pathway (Fig 3.)(20). The function of HPV genes are listed in Table 1(21).

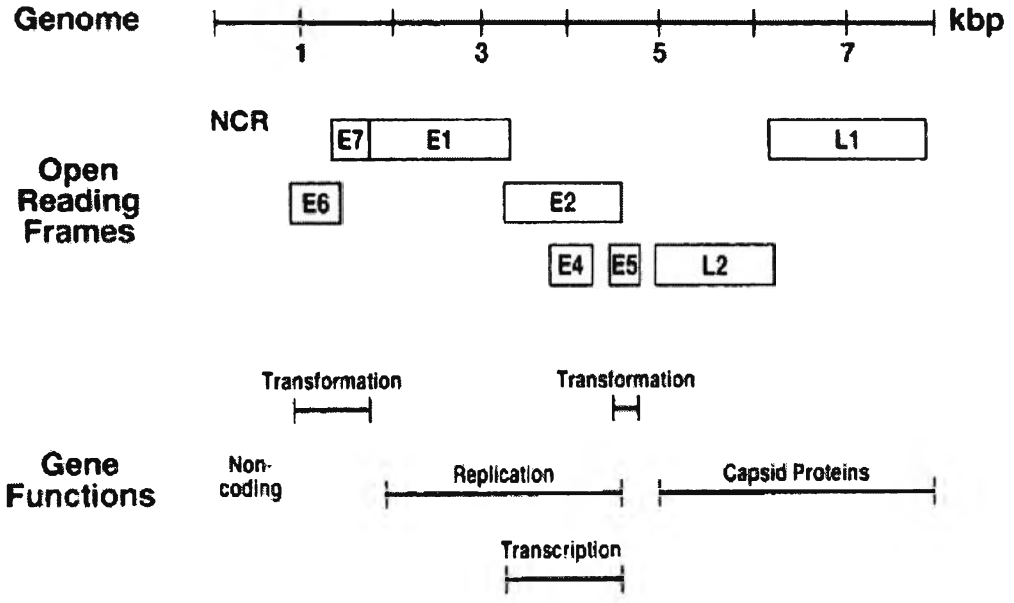

Figure1. Map of HPV type 16 genome. E1-E7 : early region; L1-L2: late region; NCR: Non-coding region (19).

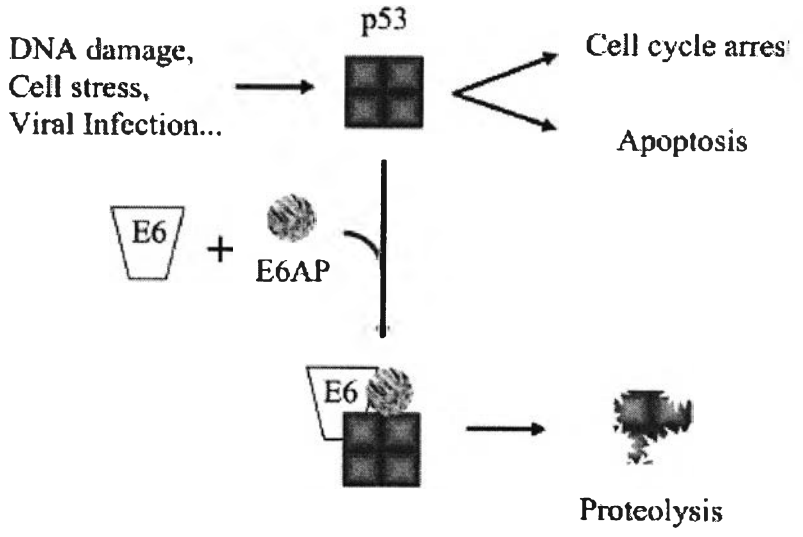

Figure 2. E6 targets p53. Activated p53 induces either cell cycle arrest or apoptosis. E6 binds to p53 in conjunction with E6AP and targets p53 for proteolysis (20).

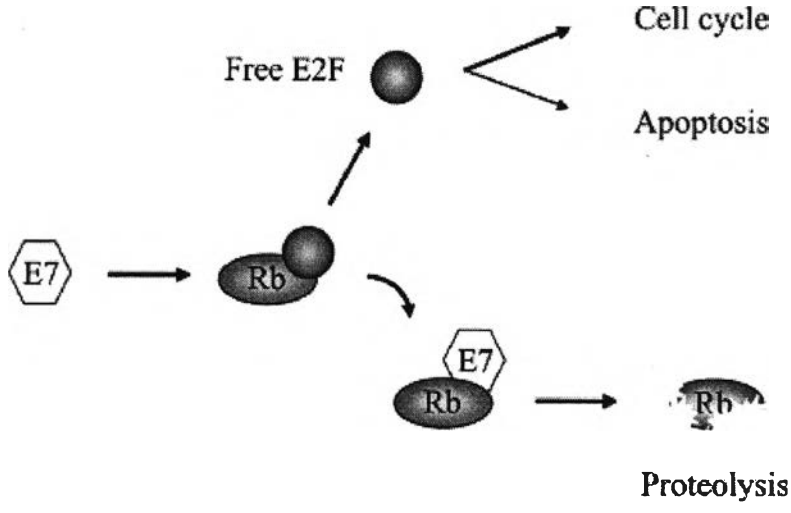

Figure 3. E7 targets Rb. E7 binds to Rb and brings about the release of E2F. Free E2F proteins promote cell cycle progression and can also induce apoptosis. The binding of E7 to Rb targets Rb for proteolysis (20).

Table 1. A description of the functions of human papillomavirus open reading frames (ORFs) (21)

ORF	Functions
E1	Helicase essential for viral DNA replication
E2	Viral transcription factor Binds E1 to facilitate the intiation of viral DNA replication Important in genome encapsidation
E4	Interacts with cytoskeletal proteins; allows viral assembly
E5	Weak transforming activity Upregulates growth factor receptor numbers
E6	Immortalizes primary human keratinocytes in co-operation with E7 Binds p53 and direct p53 degradation by ubiquitin-targeted proteolysis
E7	Immortalizes primary human keratinocytes in co-operation with E6; transcriptional, transactivator; binds pRb with degradation of the G_1/S check-point in the cell cycle
L1	Major capsid protein
L2	Minor capsid protein

Classification

The classification of human papillomavirus is based on degree of genetic similarity. HPV type was defined as having less than 90% nucleotide sequence homology within the E6, E7 and L1 open reading frame of all other known types. To date, more than 100 types of HPV have been identified on the basis of DNA sequence. Only 85 of HPV genotypes have been fully sequenced and characterized. Phylogenetic analyses based on the DNA sequence of different regions of the viral genome reveal a high diversity between viruses. Phylogenetic analyses, based on the DNA sequence of different regions of the viral genome, reveal a classification consistent with the biology of the different papillomaviruses (Fig.4). HPV have been classified into type or subtype based on percentage of homology of the nucleotide sequences of E6, E7 and L1 ORFs (Fig.5) (10, 22).

Furthermore, HPV also are generally classified in two groups according to their potential to induce malignant transformation or tissue tropism. Two groups of HPV can be separated to high-risk and low-risk group. Low-risk group of HPV includes types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP108. High-risk group of HPV includes types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82. All of the high-risk group, HPV type 16 and 18 are the most closely associated with cervical carcinoma (1).

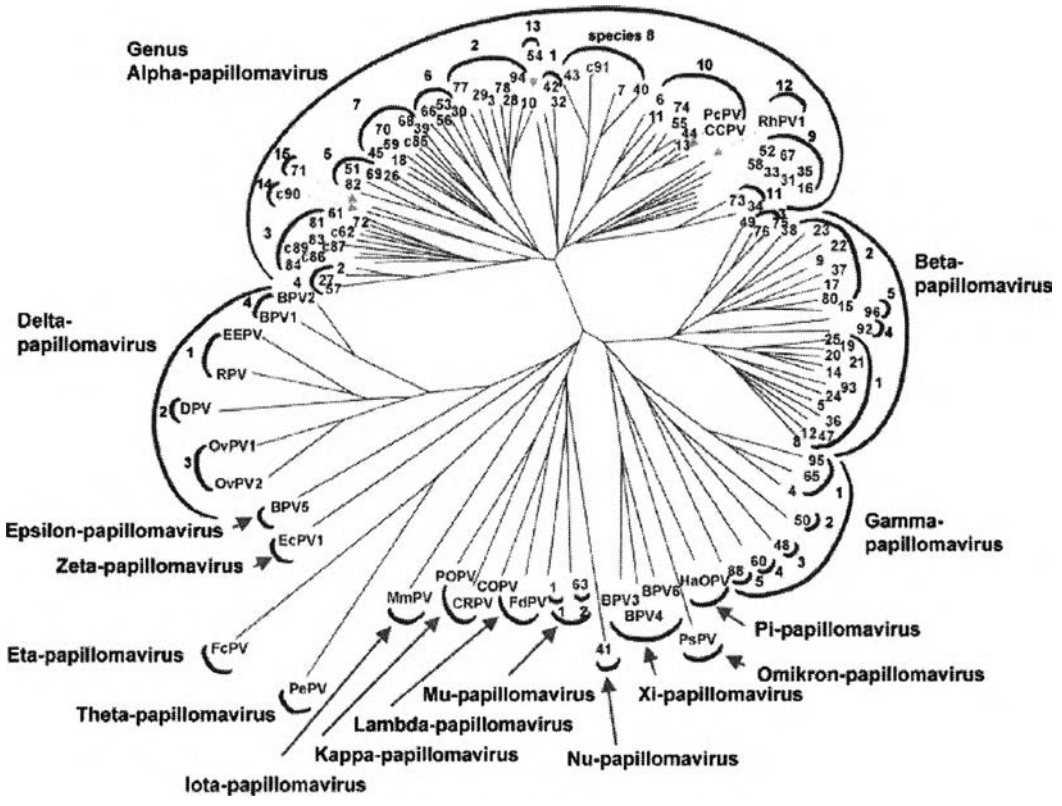

Figure 4. Phylogenetic tree containing the sequences of 118 papillomavirus types. The number at the ends of each of the branches identity an HPV type ; c – numbers refer to candidate HPV types. All other abbreviations refer to animal papilloma virus types. The outermost semicircular symbols identify papillomavirus genera, e.g.the genus alpha-papillomavirus. The number at the inner semicircular symbol refer to papillomavirus species (22).

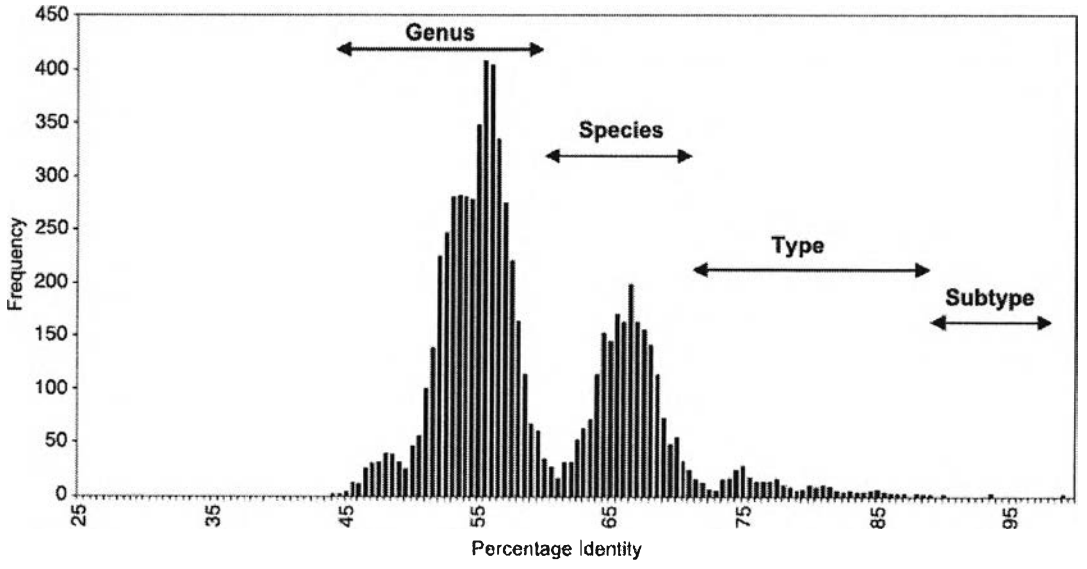

Figure 5. Frequency distribution of pairwise identity percentage from nucleotide sequence comparison of the L1 ORFs of 118 papillomavirus types (22).

Epidemiology of HPV infection

HPV infection is primarily transmitted by skin to skin contact, usually by sexual activity but nonsexual transmission via fomites can also occur. HPV infection can occur in any age especially among young women and appears to drop off with increasing age. In several international regions, cross –sectional and cohort studies have shown a peak prevalence of HPV infection in women below 25 years, a decrease among women aged 35-54 years and a second peak in women aged 55-64 years. HPV infection risk is associated with number of sex partner, current or past smoking and oral contraceptive use. In addition HPV infection also appears to very common in men.

A recent worldwide prevalence of HPV DNA has found more than 99.7% of cervical cancer. HPV type in cervical cancer, HPV-16 was the most prevalent type (54.4%), followed by HPV-18 (16.5%), HPV-58 (5.1%) and HPV-33 (4.7%) (Fig.6). For the women with low grade (LSIL) and high grade lesion (HSIL) the data was shown in figure 6. Moreover, normal women could be found HPV DNA 11.4% (23). The prevalence of low-risk and high-risk in normal women were 1.6% and 13%, respectively. 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%). In addition previous studies have reported that HPV-16 was the most prevalent type in squamous carcinoma, while HPV-18 was the most common type in adenocarcinoma.

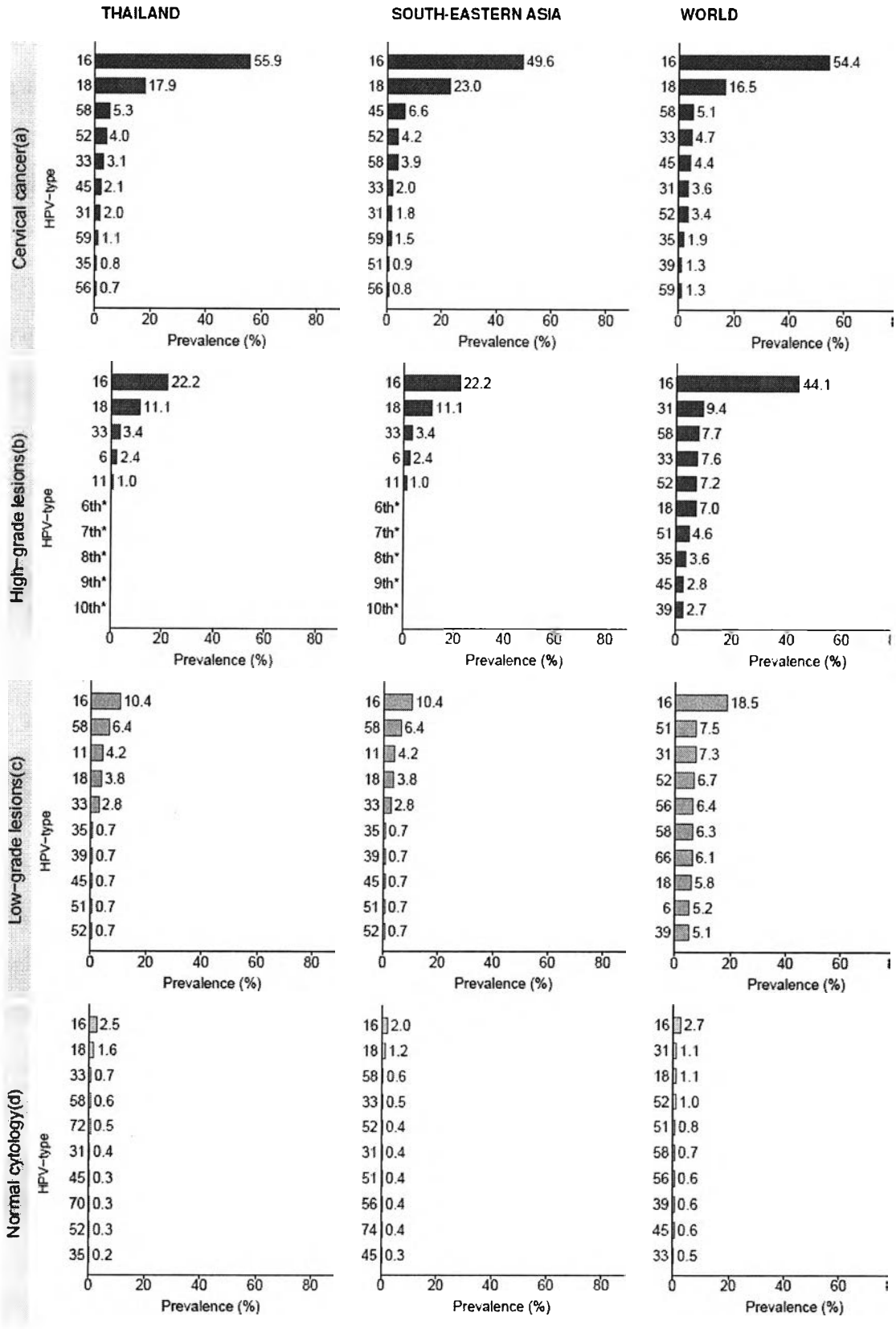

Figure 6. Ten most frequent HPV types among women with and without cervical lesions in Thailand compared to South-Eastern Asia and the World (23).

Life cycle of HPV

The replication cycle of papillomavirus, the viral genome is replicated to a multicopy episome and maintained for varying periods of time. At the initially infected state is maintained to low copy number, but still replicating. And integration of the viral is not required for either the initiation or maintenance of the transformed state. For this basal DNA replication E1 and E2 viral proteins are essential. HPV infect basal epithelial cells which can be divided into two modes of viral DNA replication. First, 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. Second, HPV DNA replication is vegetative DNA replication in differentiation program of epithelial cells which the basal cells are pushed to the suprabasal compartment. The viral genome is replicated further, and the late proteins L1 and L2 and E4 are expressed. The capsid proteins encapsidate the viral genome packed into progeny virions and release into the environment (Fig. 7) (4, 8, 10, 21, 24, 25).

Pathogenesis of HPV infection

All HPV types have a high degree of cellular tropism or tissue specificity. Squamous epithelial cells are target of HPV infection. Infection begins with viral entry through traumatized epithelium cells (10, 19). Following infection, the early HPV proteins E1, E2, E4, E5, E6 and E7 are expressed and the viral DNA replicates from episomal DNA . In upper layers epithelium the viral genomes were formed progeny virions in nucleus and shed to infect new cells (8, 26). HPV infection has been associated with benign and malignant lesion. In benign lesion, the viral genome replicates as an extrachromosomal episome, while in most malignant lesion, the viral DNA can be integrated into the host cell chromosome (26). When viral DNA integrated into host cell, a break in the viral genome occur leading to loss function of E1/E2 genes. This in turn causes deregulation of E6 and E7 gene resulting in cellular transformation (Fig.7) (10, 24, 25, 27). The E6 and E7 proteins can bind and inactivate tumor suppressor proteins, p53 and pRB respectively. The high-risk HPV E6 protein binds to p53 and targets it for rapid degradation via a cellular ubiquitin ligase. In contrast low-risk HPV E6 protein

does not bind p53 at detectable levels and has no effect on p53 *in vitro*. The high-risk HPV E7 binds to the pRb resulting in disruption complex between pRb and cellular transcription factor E2F. The outcome is the stimulation of cellular DNA synthesis and cell proliferation (10, 28, 29). Clinical and histopathologic evidence of HPV infection usually develop 1 to 8 months after initial exposure. However the progress and outcome of HPV infection depend on HPV type, anatomical location, and the nature and timing of local cellular and tissue influences. Untreated, the lesion may progress to precancerous lesion and eventually, cancer (19).

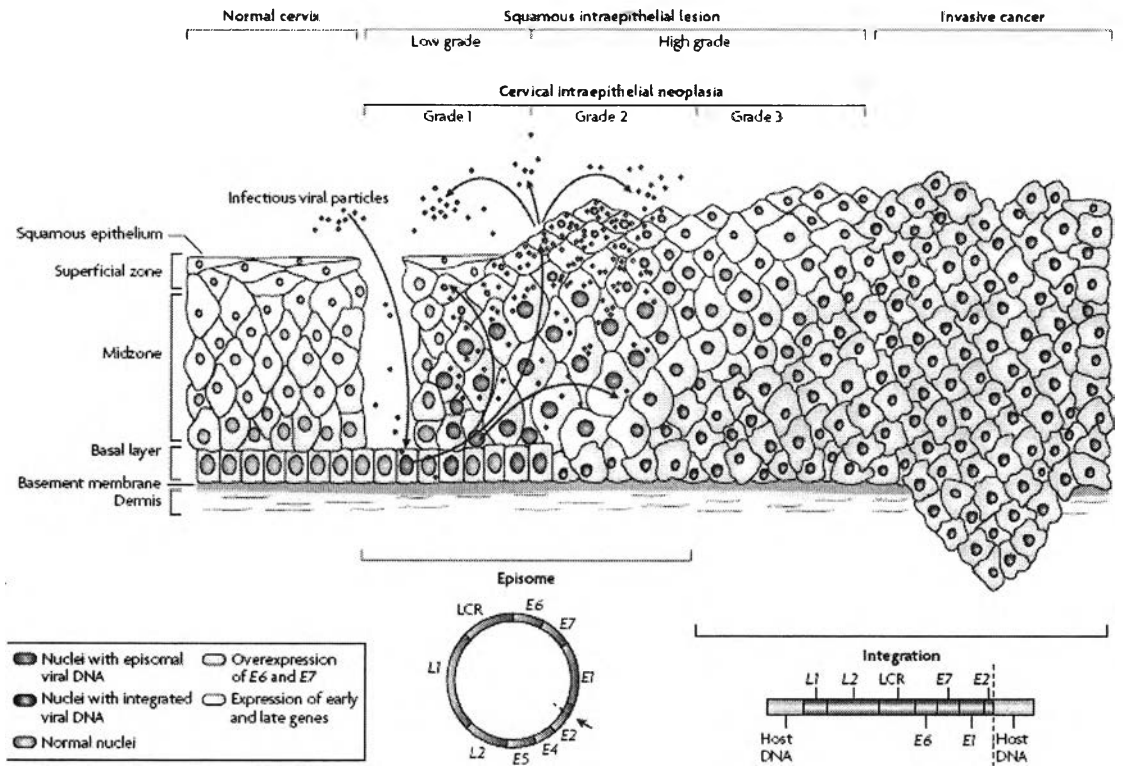

Figure7. HPV-mediated progression to cervical cancer. HPV is thought to access the basal cells through micro-abrasions in the cervical epithelium. Following infection, the early HPV genes are expressed and the viral DNA replicates from episomal DNA. In the upper layers of epithelium the viral genome is replicated further, and the late genes 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 (8).

Clinical manifestations

HPV is associated with a wide range of disease processes, depending on HPV type (Table 2) (19). Most HPV infections are benign, and first recognized as the cause of cutaneous warts. HPV can induce epidermodysplasia verruciformis, nongenital warts, anogenital warts and cervical cancer.

Nongenital cutaneous warts

HPV associated with benign cutaneous lesions which include common warts, plantar warts and flat warts. Deep plantar warts are usually lesions located on weight-bearing surfaces of the foot. Common wart are usually multiple, well-circumscribed, exophytic, hyperkerotic and found on the dorsum of the hand between the fingers around the nail bed. Flat warts present as multiple, flat, small, asymmetric, smooth papules with a pink to tan colour and found on the face and neck (30-33). Moreover HPV infection can induce malignant mucosal lesions of the oral cavity, respiratory tract, esophagus, and eyes. The HPV type 1-4, 6 and 11 most commonly found in benign skin lesions, whereas type 16 and 18 most commonly found in cancers (19).

Epidermodysplasia verruciformis

Epidermodysplasia verruciformis (EV) is a genetic disease that characterized by the appearance of flat warts liked lesions, red to brown plaques. EV commonly found in childhood with lesions on the back, chest and limbs. More than 20 HPV type have been isolated from EV lesions (34-38).

Genital cutaneous warts

Genital HPV infection was recognized as the cause of genital warts (anogenital warts). Anogenital warts usually are found on the penis and around the anus in men,

whereas in women were on vulva, vagina introitus, perineal area and cervix. The most common HPV types found in lesions of the genital area are type 6,11, 16 and 18 (39-45).

Table2. HPV types and Clinical manifestations associated (8, 19)

Manifestation	HPV types	
	Frequent associated	Less-frequent associated
Nongenital		
Plantar warts	1, 2	4, 63
Common warts	2, 1, 7	4, 26, 27, 29, 41, 57, 65, 77, 1, 3, 4, 10, 28
Flat warts	3, 10	26, 27, 28, 38, 41, 49, 75, 76
Recurrent respiratory papillomatosis	6, 11	-
Focal epithelial hypoplasia of Heck	13, 32	-
Conjunctival papillomas and carcinomas	6, 11, 16	-
Other nongenital lesions	-	6, 7, 11, 16, 30, 33, 36, 37, 38, 41, 48, 60, 72, 73
Epidermodysplasia verruciformis	2, 3, 10, 5, 8, 9, 12, 14, 15, 17	19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 50
Anogenital		
Condyloma acuminata	6, 11, 16, 18	30, 42, 43, 45, 51, 54, 55, 70
Cervical intraepithelial neoplasia		
Unspecified	-	30, 34, 39, 40, 53, 57, 59, 61, 62, 64, 66, 67, 68, 69,
Low grade	6, 11	16, 18, 31, 33, 35, 42, 43, 44, 45, 51, 52, 74
High grade	16, 18	6, 11, 31, 33, 34, 35, 39, 42, 44, 45, 51, 52, 56, 58,66
Cervical carcinoma	16, 18	31, 45, 33, 35, 39, 51, 52, 56, 58, 66, 68, 70

Cervical cancer

Cervical cancer is the second most frequent gynaecological malignancy in the world. Cervical cancer worldwide are infected with specific types of HPV DNA mainly high-risk HPV type16 and 18 (6, 9). The clinical appearance of cervical cancer based on cytological and colposcopic analyses.

Cervical intraepithelial neoplasia (CIN) system is a classified of abnormalities of cervical epithelium that detected by histological examination of cervical biopsies. These CIN system classified into three grades; grades 1 to 3 are used to describe the proportion of the thickness of abnormal epithelium cells (Table 3) (46, 47). The Bethesda system classified by cytological examination of cervical smear can detect abnormal growth of squamous cells. These Bethesda system classified into 4 grades based on how much of the cervical epithelium is affected and how abnormal cells appear: atypical squamous cells (ASC), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL) and carcinoma (Table 3) (46, 47).

Cervical cancer occurs primarily through sexual contact. Factor that may influence the acquisition of disease process included three groups cofactors. First group cofactors are environmental or exogenous cofactors including smoking, oral contraceptives, parity and co-infection with other sexually transmitted agents. Second groups cofactors are viral cofactor such as infections with specific high-risk type of HPV. And host cofactors including genetic factor, hormone factor and immune response factor (48).

Table3. The Bethesda classification system for cervical squamous cell dysplasia (10)

Bethesda System	CIN system	Interpretation
Negative for intraepithelial lesions or malignancy	Normal	No abnormal cells
ASC ASC-US (atypical squamous cells of undetermined significance) ASC-H (atypical squamous cells, cannot exclude HSIL)		Squamous cells with abnormalites greater than those attributed to reactive changes but that do not meet the criteria for a squamous intraepithelial lesion
LSIL (low-grade squamous intraepithelial lesions)	CIN 1	Mildly abnormal cells; changes are almost always due to HPV
HSIL (high-grade squamous intraepithelial lesions with features suspicious for invasion)	CIN 2/3	Moderately to severely abnormal squamous cells
Carcinoma	Invasive squamous cell carcinoma, Invasive glandular cell carcinoma (Adenocarcinoma)	The possibility of cancer is high enough to warrant immediate evaluation but does not mean that the patient definitely has cancer

Diagnosis of HPV and cervical cancer

The high-risk types of HPV are associated with cervical cancer which found 95-100% HPV DNA in cervical cancer (49). The data show that high-risk HPV infection is a good marker for at risk progress of cervical neoplasia (10). HPV cannot routinely be cultured from clinical specimens and immunology assays are not established for detection of HPV infection (10). The conventional methods for detection of cervical cancer have been cytology and histology. And methods principally available for laboratory diagnosis of HPV infections can be divided as follows: molecular methods for detection of HPV DNA, immunocytochemistry for detection of HPV proteins, electron microscopy, and HPV serology (10).

Conventional cytology

The Papanicolaou-stained (Pap) smear is the primary method for detection of cervical cell dysplasia. The Pap smear is a screening for changes in cell of transformation zone of the cervix. The reported of Pap smear are classified in the Bethesda system or CIN system (Table 3.). However, this method has some limitations. False negative rates as high as 20-30% have been reported which can occur from clumping of cell (10).

Monolayer cytology

Monolayer cytology is a new method of collection and processing of specimens for Pap smear have been developed to help reduce the number of false negative result. In these method, the specimen is collected in preservative solution such as ethanol and alcohol. Cellular structure is better preserved because the cell are immediately fixed. In addition the samples collected using cervical brushes were used to compare with conventional Pap smear. These methods are approved by Food and Drug Administration (FDA) such as: the PrepStain system (formerly the AutoCyte PREP system) and the ThinPrep Pap smear method (Cytyc Corp.,Boxborough, Mass). The

results showed that statistically significant improvement in the diagnostic of sensitivity of monolayer cytology with increase detection of epithelial cell abnormality (50, 51).

Histopathology

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 does not detects microinvasive disease. Whereas biopsy can be used to confirm most diagnoses by observing epithelial hyperplasia (10).

Detection of HPV DNA

Molecular methods for detection of HPV DNA can be divided into amplified techniques and non-amplified techniques. The amplified techniques have been target amplified and signal amplified technique. Target amplified technique such as type-specific primer polymerase chain reaction (PCR) and general primer PCR. Type-specific PCR assays are based on the E6 or E7 gene of HPV subtypes. The sensitivity of these assays is 10-200 HPV copies per sample depending on HPV type (10). General primer PCR is a majority of studies using PCR by used consensus primer to amplify a broad spectrum of HPV type in a PCR amplification. These primers target conserved the L1 regions of HPV genome. These primers, MY09/11, GP5+/GP6+, SPF10 and PGMY, were used to detect and genotype a broad spectrum of HPV type (52-57). Various methods have been used to identify HPV types after amplification with consensus primer such as sequence analysis, restriction fragment length polymorphism (RFLP), and hybridization with type-specific probes using dot bot (DB) (48, 58). The signal amplified technique such as probe hybridization and hybrid capture. The current commercial HPV detection kit, Digene's Hybrid Capture II kit, detects all high-risk and low-risk HPV types. In theses assays have been many steps such as DNA extraction, mixing of probe and hybridization, and signal amplification assay that uses chemiluminescese to detect the presence of HPV. Several specimen types can be used in these assays such as cervical

swabs and cervical biopsy (59). The Hybrid Capture II assay can be distinguished between infection with low-risk and high-risk HPV types. Moreover these assays supported the Pap smear results to assist in assessing the risk for development of cervical cancer (10).

Non-amplified technique such as 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 (60). Dot blot hybridization is an easy and fast to perform than Southern blot. Digested and denaturized cellular DNA is fixed as a dot or spot onto nylon filter and hybridized with labeled HPV specific DNA probes. This test had a lower sensitivity than PCR (61).

Detection of HPV antigen

Detection of HPV antigen usually detected HPV L1 capsid protein by immunocytochemistry. Immunocytochemistry can be applied to tissue biopsies. Immunoperoxidase staining with antibodies using the peroxidase-antiperoxidase or avidin-biotin complex. These methods are highly specific for the detection of HPV L1 capsid protein in superficial epithelial cells of HPV infected tissue. The sensitivity of these method is less than HPV DNA detection methods.

Detection of HPV antibody

Several studies 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 detection of IgM, IgG or IgA antibodies against HPV early or late proteins are Western blot, enzyme linked immunoassay (EIA), and radioimmunoprecipitation assay (RIA).

Nanotechnology in clinical diagnosis

Nanotechnology is the study of controlling of matter on the nanometer-length scale. Nanotechnology is the creation and utilization, devices with a vast range of applications, such as in life sciences, medicine, electronics and energy production. Application in life sciences are described in detail under term of nanobiotechnology. Nanobiotechnology used in molecular diagnostics fall under the broad category of biochips/microarrays, nanoparticles labelled, nanoscale visualization, nanopore, and nanobiosensor (Table 4)(11, 12). Molecular diagnostic are used in biological research, drug discovery and development, clinical laboratory setting. Some important areas of clinically application of nanodiagnostics are immunohistochemistry, genotyping, biomarker research, early detection of cancer and detection of infectious microorganism. Nanocrystals used in immunohistochemistry by conjugated with specific antibodies, that increase sensitivity, photostable labels for detection. Biobarcode assays are nanotechnologies devices for detection of disease biomarker. The biobarcode assay can detect PSA at 30 attomolar concentration, whereas conventional assay have sensitivity limit of 3 picomolar (62). This assay was used to measure the concentration of amyloid ß-derived diffusible ligands(ADDLs) in the cerebrospinal fluid (CSF) as a biomarker for Alzheimer's disease. This test can detect ADDLs in CSF which have low concentration, whereas commercial emzyme linked immunosorbentassay (ELISA) can only detect in brain tissue where is most highly ADDLs (63). Some examples nanotechnology devices for detection of microorganisms, such as bacteria and virus are ferrofluid magnetic nanoparticle, ceramic nanospheres, nanowell, and nanowire. Conclusion of advantage nanotechnologies for clinical laboratory diagnostics are listed in table 5.

Table4. Nanotechnologies with potential applications in molecular diagnostics (11, 12)

Nanotechnology on a chip
Microfluidic chips for nanoliter volume: Nanochip
Optical readout of nanoparticle labels
Nanoarray
Protein nanoarray
Nanoparticle technologies
Gold particles
Nanobarcode
Magnetic nanoparticles: ferrofluids, supramagnetic particles combine with MRI
Quantum dot technology
Nanoparticle probes
Nanopore technology
Measuring length of DNA frangments in high-throughput manner
DNA fingerprinting
Haplotyping
Cantilever arrays
Multiple combined test (such as protein and DNA) to be performed on the same disposable chip
Prostate specific antigen binding to antibody
DNA nanomachines for molecular diagnostics
Nanoparticle-based immunoassays
DNA-protein and nanoparticle conjugates
Resonance light scattering technology
Nanosensor
Living spore as nanodetector
Nanosensor glucose monitor
Optical biosensors: surface plasmon resonance technology
Probe encapsulated by biologically localized embedding (PEBBLE)
Photostimulated luminescence in nanoparticles

Table5. Rationale of nanotechnology for clinical laboratory diagnostics (11)

- Nanoscale particle, used as tags or labels, increase the sensitivity, speed and flexibility of biological tests measuring the presence or activity of selected sunstances.

- Nanotechnology enables testing of relatively small sample volumes.

- Many of the technologies described are applicable both to DNA and proteins although some are better suited for proteins in body fluids.

- Nanotecnology enables detection of few microorganism or molecules that would not be possible with conventional techniques.

- Early detection of disease such as cancer improves the chances of cure.

- Some of the techniques describe obviate the need for PCR.

- Nanotechnology, by reducing the time required for test, has a positive impact on both clinical decision-marking and treatment costs.

Gold nanoparticles for the development of clinical diagnosis methods

Nanoparticle-based assays are usually used in field of biodiagnostics, such as for biomole detection, with DNA or proteins-functionalized gold nanoparticles used as the target-specific probe. Nanoparticles have high surface areas and unique physicochemical properties that making them ideal candidates for developing biomarker platforms. There are several nanoparticles used in diagnostics. Gold nanoparticles, quantum dot, magnetic nanoparticles are the most frequently used. Goldnanoparticles based diagnostics can be broadly advantages over conventional detection methods used in clinical diagnostics. The advantages of goldnanoparticles can be divided into three different approaches (12, 13, 64, 65). The first approach based on color changes of goldnanoparticles upon aggregation. For example to support this approach is Au-nanoprobes method that used of thiol-linked ssDNA or RNA modified gold nanoparticles for the colorimetric of DNA target. These assays based on a non cross-linking hybridization method, where aggregation of the Au nanoprobes is induced by an increasing salt concentration. The method consists in visual comparision of solution before and after salt induced Au-nanoprobes aggregation. The presence of a complementary target prevents aggregation and the solution remains red whereas non-complementary target do not prevent aggregation the solution change color from red to blue (66). The second approach is the goldnanoparticle can be tailored with a wide variety of surface functionalization to selectively bind biomarkers. And the last approach used in electrochemical based methods that can be coupled with metal deposition for signal enhancement. Functionalization of goldnanoparticles with biomolecule other than nucleic acid such as antibodies for signal enhancement in immunoassays, carbohydrate functionalization to study specific molecular interactions and direct peptides and proteins to the goldnanoparticle surface. For example methodologies based on gold nanoparticle for detection biomarker directly in clinical samples were shown in table 6. Goldnanoparticle shows easily, rapid, sensitive and in expensive for development of clinical diagnosis methods.

Table6. Methodologies based on gold nanoparticle for detection directly in clinical sample.

Detection	Detection limit	Clinical biological targets/samples	Reference
Naked-eye (dye-reagent dipstick)	2-25 fmol	Hepatitis C virus/ RNA, human plasma	(67, 68)
Naked-eye (lateral-flow strip)	0.138 μg/kg	Enrofloxacin/ chicken muscle	(69)
Naked-eye (Aggregation-based immunoassay)	1 μg/ml	Anti-proteinA/ human serum	(70)
Naked-eye (electrostatic interactions of unmodified AuNPs)	100 fmol	SNPs associated with long QT syndrome-KCNEI gene/genomic DNA	(71)
Light-scattering imaging(cross-linging aggregation)	33 zmol	Methicillin-resistant *S.aureus* and *S.epidermidis*,mec A gene/ DNA cultured bacteia	(72)
Naked-eye or UV-visible spectroscopy (non-cross-linking aggregation)	375 zmol-4.2 pmol	K-ras oncogene/ genomic DNA, colorectal adenocarcinoma cell line;β-thalassemia mutation/ genomic DNA, human whole blood	(66, 73, 74)
Naked-eye (lateral-flow strip)	10-100 ng	*M.tuberculosis*/ DNA, clinical specimens Human papillomavirus type 16/ genomic DNA	(18)