CHAPTER II

REVIEW OF LITERATURE

History

Papillomavirus is a group of oncogenic small DNA virus. It can induce warts (papillomas), benign tumor and malignancy in many different tissues of higher vertebrates including man [29]. Most of the papillomavirus have a specific cellular tropism for squamous epithelial cells and, there are no examples of papillomavirus from one species causing a productive infection in another species [34]. That is specie-specific. Therefore, several papillomaviruses are recognized such as cotton tail rabbit papillomavirus (CRPV), bovine papillomavirus (BPV), canine oral papillomavirus (COPV), deer papillomavirus (DPV) and the most important one is human papillomavirus (HPV) [29,35].

HPV is the first tumor virus to be transmitted experimental from one host to another. The experiment demonstrated that warts could be induced by cell-free filtrate of warts material [36]. Electron microscopic studies of Strauss, et al. (1949) [37] and Almeida, et al. (1962) [38] confirmed a viral etiology for cutaneous warts Melnick, (1962)[39] grouped the papillomaviruses together with the polyomaviruses in the family *Papovaviridae* because both were DNA tumor viruses with physically similar icosahedral capsid and circular, double-stranded DNA However, in the beginning, study of HPV was slow because the virus could not be propagated in tissue culture or transmitted to laboratory animals. In the 1970s, when techniques for molecular cloning of viral DNA became available, the first papillomavirus genome was successfully cloned in bacteria [29,34]. The clones provided reagents which allowed the sequencing and a systematic genetic analysis that led in turn to the elucidation of the functions of the specific genes coded by the papillomaviruses.

Virology

HPVs are members of family *Papovaviridae* genus *Papillomavirus*. They are small, non-enveloped, icosahedral capsid with 72 capsomers, about 55 nanometer (nm) in diameter. The virion particle consists of a single molecule of double-stranded circular DNA approximately 8,000 base pair (bp) in size. The guanosine-cytosine (G+C) content of most papillomavirus genomes is approximately 42%. The DNA constitutes approximately 12% of the virion by weight, accounting for the density cesium chloride of approximately 1.34 g/ ml [22,29].

The capsid protein is composed of 2 kinds of structural proteins. The major capsid protein (L1) has a molecular weight of approximately 55 kilodalton (kd) and represents approximately 80% of the viral proteins. L1 is a hemagglutinin and play an important role in viral attachment [40]. The minor capsid protein (L2) has a molecular weight of approximately 70 kd. L2 is larger and dispensable in the formation of the capsid, but appears to be important for the encapsidation of viral DNA [41,42].

Genome structure

All papillomaviruses have similar genetic organization. The open reading frames (ORFs) are located on one strand of DNA [43]. The genome of papillomavirus has 3 functional regions (Figure 2).

1. **Early (E) region** It consists of 8 ORFs (E1-E8), most of them involve in DNA replication, regulation of transcription and cell transformation. The E8 ORF is found only in BPV.

2. **Late (L) region** It consists of L1 and L2 ORFs, L1 codes for major capsid protein and L2 codes for minor capsid protein.

3. **Upsteam regulatory region (URR)** It is located between the 3' terminal of L1 region and 5' terminal of E region. It is composed of origin of replication, promoter and enhancer elements.

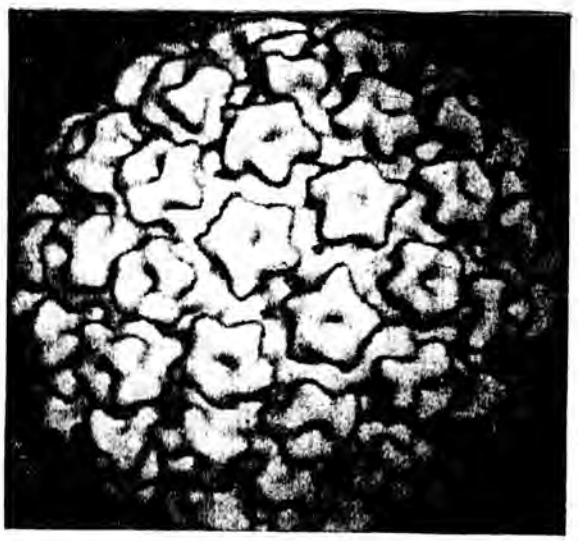

Figure 1. Structure of papillomavirus virion. Cryoelectron microscopy of HPV-1 capsid.
(from : Bonnez, W. 1997.) [42]

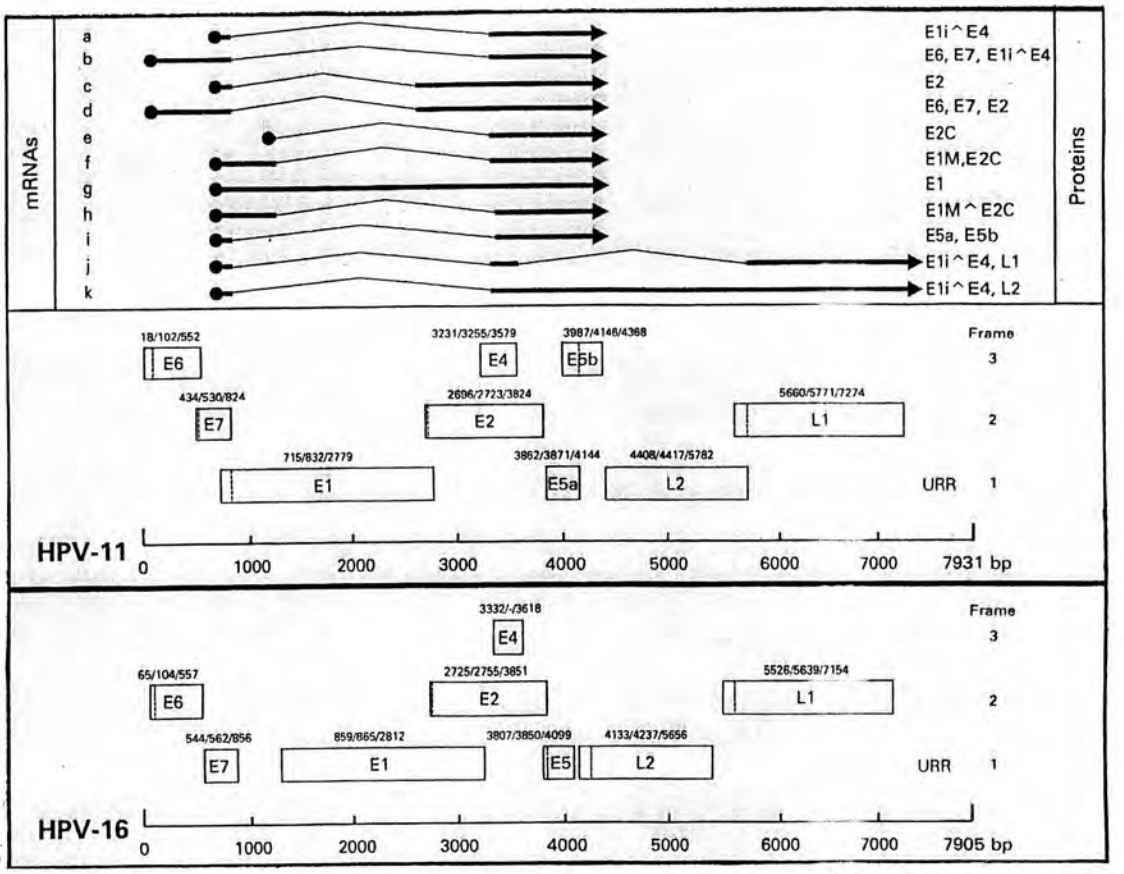

Figure 2. Structure and coding region of HPV-16 and HPV-11 genome.
(from : Bonnez, W. 1997.) [42]

Viral proteins

E1, E2 proteins

The E1 gene is the largest ORF and is relatively well conserved among all of the papillomavirus. The E1 proteins share structural similarities with the large T antigen (TAg) of simian virus (SV40), an essential replication protein of that virus [29]. In TAg, this region contains signals for nuclear localization, sites of amino acid phosphorylation, domain for ATP binding, ATPase, and DNA helicase activity [44]. These similarities suggest a common function for E1 protein in the initiation of viral DNA replication. Furthermore, Liu, et al. (1995) [45] reported that a neutralizing antiserum directed against the amino-terminal one-third of the E1 protein totally abolishes initiation and elongation of DNA replication. In addition, E1 also contributes to the maintenance of the viral episome, and is often absent when the viral DNA is integrated [42].

E2 protein significantly stimulates the DNA replication by binding with E1 protein in order to increase the affinity of E1 to its binding [46,47]. In addition, E2 protein has a transcription regulatory properties. It plays an important role in regulation the activity of the E6/E7 promoter by binding to the E2 binding sites proximal to the E6 promoter [42,48].

E4 , E5 proteins

The E4 ORF of the papillomavirus is located in the early region (Figure 2), yet it appears to be expressed as a late gene with a role in productive infection [29]. In general, the E4 gene is not highly conserved among the papillomaviruses. Recent studies have shown that the E4 protein can induce collapse of cytokeratin network suggesting that it may involves in the release of virus from the host cell [10,49].

E5 protein is a small membrane bound protein. It may involve in cell transformation because E5 protein can bind with several growth factor receptors for example epithelial growth factor receptor (EGFR), platelet derived growth factor (PDGF), thus the binding may result in interfering of signal transduction [50].

E6 , E7 proteins

E6 and E7 proteins are nuclear proteins. They have important role in malignancy transformation. E6, E7 proteins of high risk group (HPV-16,18) have a potency to immortalize several cell types such as primary human keratinocytes, human diploid fibroblast and rodent fibroblast cell line (NTH-3T3)[51,52]. Moreover, transgenic mice have been used to examine the effects of the E6,E7 genes of HPV-16 *in vivo*. Linkage of the E6, E7 genes to the α–crystallin promoter led to microphthalmia , cataracts and the formation of lens tumors in a large proportion of transgenic animals [53]. Therefore, E6 and E7 proteins are oncoprotein.

E6 protein is a zinc binding protein approximately 150 amino acids in size [19]. E6 oncoprotein of high risk HPVs (HPV-16,18) have ability to bind p53 tumor suppressor protein, this binding promotes degradation of p53 via ubiquitin protease system [54,55].

E7 protein is also a zinc binding protein. HPV-16 E7 protein shares amino acid sequence similarity with portions of the adenovirus E1A (Ad E1A) protein and the TAg antigen of SV40. The conserved regions in all of those oncoproteins bind cellular proteins, one of which is the retinoblastoma tumor suppressor protein (pRB) [29,42].

Table 1. HPV proteins and their possible functions

Viral Protein	Size (kd)	Function and properties
E1	68-76	Participation in viral DNA replication
		Has DNA-dependent ATPase, and helicase activity
		Forms a heterodimer with E2
		Has a binding site in the URR
		Maintenance of viral episome
E2	40-58	Participation in viral DNA replication
		Has several binding sites in the URR
		Forms a heterodimer with E1
		Repress transcription of E6/E7
E3		Unknown function
E4	10-17	Binding to the cytokeratin filament network
E5	10	Malignant transformation , enhances growth factor receptor signal transduction.
		Binding to and cooperates with cellular growth factor receptors (PDGF)
E6	16-19	Malignant transformation and keratinocyte differentiation
		Binds to the tumor suppressor protein p53 and accelerates its degradation (HPV-16 and HPV -18, much less with HPV-6 and HPV 11)
E7	10-14	Malignant transformation
		Interferes with the retinoblastoma protein (pRB), a tumor suppressor gene product, that negatively regulates cell proliferation (high-risk HPVs)

(modified from : Bonnez, W. 1997.) [42]

Viral replication

HPV has specific cellular tropism especially for squamous epithelial cell. The virus gains entry to basal or parabasal cell through the disruption of epithelium cell and its replication links to proliferation and differentiation of the epithelium cell [29]. Attachment of virus to the host cell membrane depends on major capsid protein (L1). Recently, Evander, et al. (1997) [56] suggests that the α-6 integrin may act as a receptor for papillomavirus. However, little knowledge of virus entrance which gain entry to the nucleus or uncoating its DNA is known. It assumes that mechanism are similar to polyomavirus. Viral DNA replication can be divided into 2 stage i.e., maintenance and vegetative stage. The first stage likely occurs in the basal or parabasal cell [57,58]. The viral DNA is apparently maintained as a stable multicopy plasmid which replicates an average only once per cell cycle, and transcription of early gene appears in this stage. This type of viral replication assures a persistent and latent infection. Patients may display no evidence of cytological or clinical abnormalities, yet may still harbor the viral genome[59]. The second is vegetative DNA replication, this stage appears in the more differentiated epithelial cells. The signals that trigger the induction of vegetative replication are unknown. However, wound healing in abrasion may then stimulate basal cell division and vascular proliferation, which may accelerate viral replication [26]. Events in this stage include the production of virion particles, expression of capsid protein L1 and L2 and assembly of virion. Therefore, viral particles and viral capsid antigens are found in the most superficial layers of the epithelium [34].

However, there is little information about the papillomavirus assembly or the release of virus from cells. The virus is not believed to be cytolytic, and the release of the virion particles occurs at the cornified layers of a keratinized epithelium [29].

Classification

As mentioned above, each of papillomavirus is specie-specific, and papillomaviruses are primary classified by their natural hosts (human, bovine, rabbit etc.) and subclassified into type by comparison the genetic relatedness with the known papillomaviruses from the same species. In the early, classification of specific HPV type was based on the extent of homology of the nucleotide sequence by liquid DNA hybridization under stringency condition. If it shows less than 50% homology with the previously described type, it was classified as a new type [29,60].

Until 1994, the new definition for classification of new HPV type is based on criteria adopted by the Papillomavirus Nomenclature Committee, the nucleotide sequences of the E6,E7 and L1 ORF. If it shows more than 10% different with the previously described type, it was classified as a new type [29]. Over 77 different HPV types have been identified [42]. However, complete nucleotide sequence data are available for some HPV types and partial sequence data In almost HPVs, the relationship of HPV types can be classified by construction the phylogenetic tree (Figure 3) based on the L1 ORF DNA sequence homology [34,42]. HPVs can also be classified on the basis of the site of infection, resulting in 2 main HPV groups : cutaneous and mucosal. The majority of cutaneous group (HPV-1-5,8,9,12,15,17,19,20 and 21) associated with skin lesion such as skin wart, common wart and patients with epidermodysplasia verruciformis (EV) [7,28,35]. The mucosal group can cause infection in oral mucosa, conjunctiva, respiratory tract, and, anogenital tract. More than 35 HPV types are involved in anogenital disease and 20 or more are associated with cervical malignancy [17]. Among those cause anogenital tract infection also be divided in 3 groups corresponding to their pathogenicity in developing of cancer i.e., high risk group (HPV-16,18,40,45 and 56), intermediate risk group (HPV-31,33 35,39,51,52,58 and 66) and low risk group HPV-6,11,42 and 44) [23,28,29,,61].

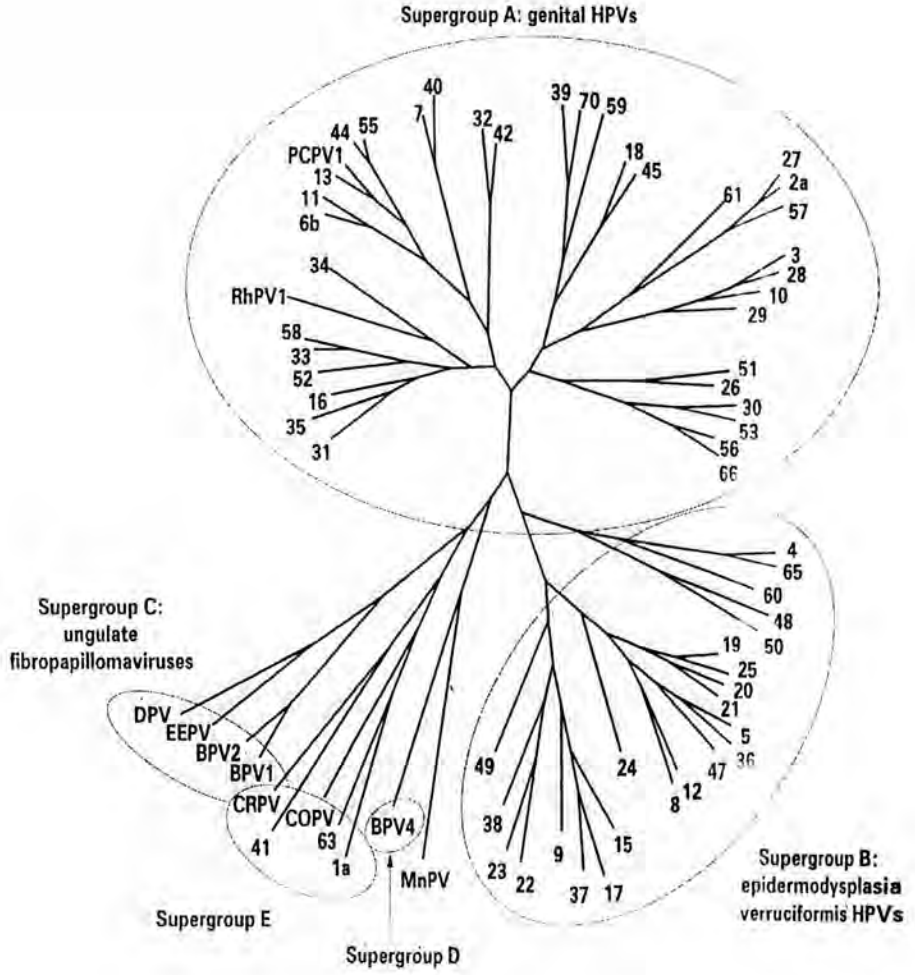

Figure 3. Phylogenetic tree of papillomaviruses. The tree was established on the basis of DNA sequence homology in the L1 ORF. BPV, Bovine papillomavirus; COPV, Canine oral papillomavirus; CRPV,Cottontail rabbit papillomavirus; DPV, Deer papillomavirus; EEPV, European elk papillomavirus; MnPV, *Mastomys natalensis* (rodent) papillomavirus; PCPV, Pygmee chimpanzee papillomvirus; RhPV Rhesus monkey papillomavirus.

(from : Bonnez, W. 1997) [42]

Route of transmission

Most of cutaneous HPV infection is transmitted by direct contact from the lesion or by indirect contact for example, through formites present in communal baths or swimming pools [34]. Melchers, et al. (1993) [62] reported the spread of HPV infection, cutaneous warts, among meat handlers is possible by protection glove and professional equipments. In addition, formites transmission of non-genital HPV types to the genital area has been described from tanning beds [29]. An auto inoculation from the lesions may be possible. According to observation, most of genital HPV-infection is transmitted by sexual contact and the risk of HPV infection increases with the number of sexual partners [34,63,64,65]. However, some investigators have reported that the HPV seropositivity in children and occasional genital HPV-DNA could be detected in virgin [26,66]. Thus the possibility of vertical transmission of HPV may be appeared. Moraes, et al (1994) [67] reported the presence of HPV-DNA in amniotic fluids of pregnant women with cervical lesion. Nevertheless, the significance to vertical transmission remains unproved. Moreover, the transmission of HPV from mother with cervical lesion to newborn may occur during childbirth [26]. An incubation period of disease is unclear. An experiment on animal model showed that the incubation period was about 3-18 months, however, the incubation period in human by sexual transmission was around 3 weeks to 8 months by average [26].

Role of HPVs in human diseases

Cutaneous warts

Cutaneous warts may occur at any sites on the skin. They are 3 major types of cutaneous warts and the morphology of the warts are variable depend on the type of warts. Cutaneous warts are dome-shaped with multiple conical projection (papillomatosis). The surface of the warts are velvety appearance, most often multiple and are found on the hands[34,68]. Plantar warts are characterized by

a highly thickened corneal layer (hyperkeratosis) generally single and are found on the plantar surface of feet. Flat warts exhibit minimal or no papillomatosis. They are almost always multiple and found frequently on arms, face and around the knees. HPV types recovered from skin at different sites are list in Table 2.

Epidermodysplasia verruciformis (EV)

EV is a disease in which patients are incapable to resolving the warts virus infection because of genetic defects especially in cell mediated immunity. The disease is characterized by warts like lesion on the face and trunk. Two clinical types of warts occur in the patients, flat warts and red or reddish-brown macular plaques. An approximately 30% of the cases may progress to skin tumor arising in the reddish-brown macular plaques on the skin after exposure to sunlight [35]. Several HPV types are often found in EV such as HPV-5,8,9,12,14,15,17,19,20 and 25 [34].

Oral cavity and larynx

Single oral papillomatosis is the most benign epithelial tumors of the oral cavity. They are pedunculated with a fibrovascular stalk and usually have a rough papillary appearance to their surface. They can occur in any age group, are usually solitary, and rarely recur after surgical excision [68]. Laryngeal papillomas are commonly associated with HPV-11 and most common benign epithelial tumors of the larynx. It is usually considered a life threatening condition in children because of the danger of air way obstruction. Multiple papillomas are clinically and pathologically similar to condylomata, are commonly associated with HPV-11 and HPV-6. They occur more frequently in oral cavity of children than adults, and typically multiple [69].

Focal epithelial hyperplasia is commonly associated with HPV-13 and HPV-32, and mostly occurs only in the oral cavity, appearing clinically to be flat or slightly elevated and either white or the color of the surrounding mucosa.

Table 2. Association of HPV types to human diseases

Disease	HPV types	
	frequent	**rare**
Skin warts		
Deep plantar warts	1,2	4, 63
Common warts	2,4	4,,26, 27, 29, 41, 57, 65,
Flat warts	3,10	27, 38, 41, 49, 75, 76
Epidermodysplasia verruciformis (EV)	2,3,5,8	19, 20, 21, 22, 23, 24, 25,
	9, 10	36, 37, 38
Condylomata accuminata	6, 11	30, 42, 43, 44, 45, 51, 54,
Oral papilloma	6 , 11	
Laryngeal papilloma	6 , 11	
multiple papilloma	6 , 11	
Focal epithelial hyperplasia	13, 32	
Conjunctiva papillomas and carcinoma	6, 11, 16	
Cervical intraepithelial neoplasia		
CIN-I	6, 11	16, 18, 31, 33, 35, 42, 43,
		44, 45, 51, 52,
CIN-II , CIN-III	16,18	6, 11, 31, 33, 35, 39, 42,
		44, 45, 51, 52
Cervical carcinoma	16, 18	31, 33, 35, 39, 45,
		51, 52, 58. 59, 66, 68, 70

(modified from : Bonnez, W. 1997) [42]

HPV infection of the genital tract

Genital warts

Genital warts are also known as condylomata accuminata, characterized by slightly hyperkeratotic, firm, exophytic papules. In females, most condylomas occur exclusively on the squamous epithelium of the vulva, perineum, anus and rarely in the cervix. In male, the great majority of lesions occur on the penis, around the anus, on the perineum. Several types of HPV are involved with condylomas. However, many studies found that approximately 90-100% of condylomas are caused by HPV-6 and HPV-11, whereas HPV-16 may be responsible for about 10% [16,70,71]. Anogenital lesions infected with these HPV-6,11 are rarely progress from benign to malignant condition in otherwise healthy individual [72].

Cervical cancer

Squamous cell carcinoma of the cervix accounts for about 90% of cervical cancer. Adenocarcinoma accounts for approximately 10 % and the remainders are sarcomas and primary or secondary lymphomas [4,6]. Nearly all cervical cancers originate in the "transformation zone" which locates at the lower end of the cervix where the columnar cells of the endocervix form a junction with the stratified squamous epithelium of the vagina. Cells of the transformation zone undergo a rapid turnover and appear to be particularly susceptible to the action of carcinogens [34]. Several epidemiologic studies in cervical cancer have demonstrated that about 90-100% of such cancer have HPV-DNA [11,16,17,19-22].

Invasive cervical cancer is originate from precancerous lesion known as CIN. CIN is defined as a spectrum of intraepithelial changes begins with minimal atypia and progresses through stages of more marked intraepithelial abnormalities to invasive cancer. CIN have been classified to 3 grades according to the extent of basal-like cell proliferation [73]. CIN-I a little changes in epithelial cell appears in one third or less of the distance from the basement membrane to the surface. When more than one third but less than or equal to two thirds is CIN-II, the dysplasia become more disorderly and may be associated with some variation in cells and nuclear size and with normal looking mitose above the basal layer. The superficial layer of cell is still well-differentiated but in some case shows koilocytotic change. CIN-III is marked by greater variation in cell and nuclear size disorderly orientation hyperchromasia and the abnormal change occurs in full thickness of the epithelium cell but it does not invade into the under layer of stromal cell.

Association of HPV and CIN

HPVs are found in a large majority of the lesions spanning the entire spectrum of cytological abnormalities from CIN-I to CIN-III and cervical cancer. The prevalence of HPV infection within each lesion group varied widely, the prevalence rate of HPV infection increased with the severity of the lesion approximately 25-54% in CIN-I, 60-90% in CIN-II and 80-90% in CIN-III [13,16,17]. However, the prevalence of HPV infection increases with the sensitivity of detection technique. Depending on histologic grade, CIN lesion may regress, persist, or progress at various rates. Approximately 60% of CIN-I are regression, 30 % are persistence, 11% are progression to CIN-III and only 1% progresses to invasive cervical cancer. Forty percent of CIN-II are regression, 40 % are persistence, 20% are progression to CIN-III and 5% are

progression to invasive cervical cancer. CIN-III, approximately 3% are regression and more than 12% are progression to invasive cervical cancer [42].

In a follow up study of CIN patients demonstrated that the progression of CIN-I to CIN-III and invasive cervical cancer associate with HPV types. Campion, et al. (1986) [74] has showed that 56% of low grade CIN patients with HPV-16 infection have been progressed to CIN-III in over 14 months follow up and when compared with patients who have HPV-6 infection, only 4% have been progressed. Koutsky, et al. (1992) [75] reported that appearance 39% of patients with HPV-16 or 18 infection with normal cytological have been progressed into CIN-II or CIN-III within 2 years follow up while, only 3% of patients with negative for HPV infection have been progressed. In addition, progression of low grade CIN (CIN-I) to high grade CIN (CIN-II, CIN-III) may associate with the viral level. Cox, et al. (1995)[76] and Cuzich, et al. (1994)[77] have showed that the likelihood of detection of existing high grade increased with increasing viral load.

Role of HPV in developing of cervical cancer

The mechanism of HPV induction of cancer became better understood when the viral genome organization was known. The HPV-DNA consists of an early region, composed of 7 genes (E1-E7), encoding for proteins, among other functions, play a role in viral replication and have transforming properties. In benign or preneoplastic lesions, the HPV-DNA is not integrated to the host DNA but rather persists as an extrachromosomal DNA (episome). However, in malignant lesions or in different cell lines that are derived from cervical cancer, the HPV-DNA can be found integrated into the host chromosome. Integration of HPV-DNA in host chromosome may occur in one or many copies, and no sites specificity for integration to host

chromosome. However, most of integration occurs in the E1/E2 region of HPV genome [48,78].

E2 protein acts as a regulator protein and plays an important role in regulating the expression of E6/E7 protein. E2 protein is a DNA binding protein that binds to the consensus sequence ($ACCN_6$ GGT) which is found in both URR and in the E6 and E7 promoter [79]. The whole E2 gene product can function as transactivator whereas the truncated have been described as repressor because the shorter E2 protein contains the DNA binding domain of the C terminus but lacks the transactivation domain. The balance between the two possible transcriptions seems to favour that of the truncated form [48]. Once when the HPV integrated into the host chromosome and E1/E2 region is broken, this balance may change giving rise to uncontrolled expression of the viral protein especially E6 and E7 proteins.

As mentioned above, E6 and E7 are oncoproteins, they have malignant transformation activities. E6 protein of high risk HPV is able to bind with p53 tumor suppresser protein . This binding promotes degradation of p53 via the ubiquitin protease system[54,55]. p53 protein is the regulator of cell proliferation that is activated by DNA damage. The effects of wild type p53 on cell proliferation include regulation of the transition from G1 to S phase of cell cycle [80]. It transactivates the gene *WAF/CIP* 1 which encodes for p21 protein[81]. This p21 protein directly interacts with cyclin / cyclin dependent kinase (cdk) / proliferation cell nuclear antigen (PCNA) complex, that is responsible for phosphorylation and inactivation the retinoblastoma (pRB) protein. The final result is the arrest of the cell cycle in G1 phase (Figure 4). Moreover, p53 also induces apoptosis through the down regulation of the *bcl* 2 gene and activation of the *bax* gene [42,80].

E7 protein has been shown to bind to the pRB, a product of the tumor suppressor gene Rb-1. During the transition to the S phase, pRB is phosphorylated by cdk, resulting in the inactivation of its cell cycle regulatory functions. The cellular transcription factor E2F-1 is preferentially bound to the dephosphorylated form of pRB, and in complex with pRB can not activated transcription. The complex formation of E7 and pRB results in the release of E2F-1, allowing it to function as a transcriptional activator of cellular gene involve in cellular DNA synthesis and progression into the S phase of the cell cycle [29]. Therefore, binding of E6 and E7 proteins to p53 and pRB, respectively, offers an appealing , direct biochemical mechanism to explain HPV oncogenesis.

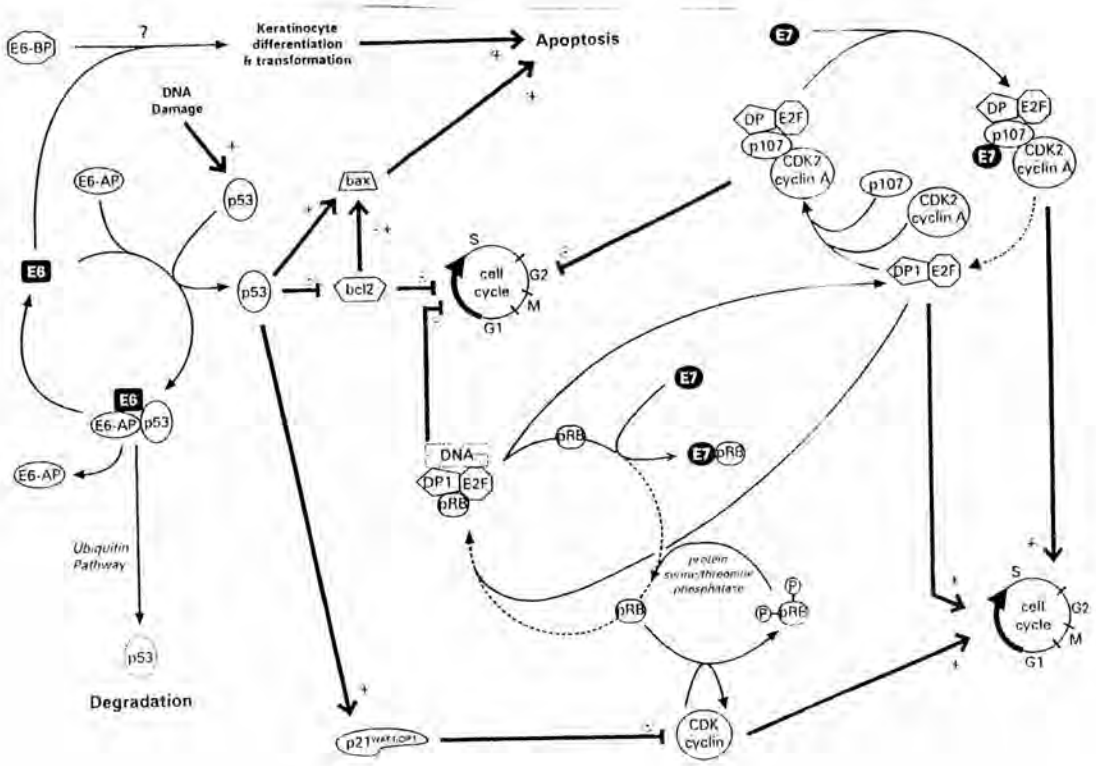

Figure 4. Model of the biologic interactions of high risk HPV E6 and E7 proteins, ⊕ , activation ; Θ, inhibition ; thick lines with open arrow heads (⟶) indicate up-regulation,the same lines with broken end (⟞) denote down-regulation; thin arrows (⟶) shows direct interactions.
(from · Bonnez, W. 1997.) [42]

Immune response to HPV infection

Humoral immunity

Humoral immune response to HPV infection may play an important role in protection against recurrent papillomavirus infection. For example, immunization of calves with BPV-L2 protein can be induced the protective antibodies against the development of mucosal warts [82]. For HPV, the L1 major capsid protein is an important epitope, the antibodies to L1 protein are neutralizing antibodies. Immunization of mice with L1 major capsid protein which expressed by recombinant plasmid known as viral like- particles (VLPs) have been shown to protect against viral challenge [83,84]. Therefore, the L1 viral like-particles are excellent substitute antigens for the virions.

Cellular immunity

Cell mediated immune response is an important aspect of the host response to HPV infection. Patients with altered cellular immunity (i.e., immunosuppression, immunodeficient state or AIDS patients) have increased incidence of HPV infection[85,86]. Patients who receive immunosuppressive drug such as organ transplant patients are also likely to increase the risk of HPV infection. Porreco, et al. (1975) [87] and Halpert, et al. (1986) [88] showed that the renal transplant patients had a 16 fold increased incidence of CIN, genital wart, and other lower genital tract neoplasia. Feltkamp, et al. (1993) [89] have demonstrated that immunization of mice with HPV-16 E7 rendered mice insensitive to a subsequent challenge with HPV-16 transformed tumor cells *in vivo*, and induced a CTL response which lysed the tumor cell *in vitro*.

Laboratory diagnosis

There are various methods for detecting of HPV infection (Table 3) and those used in purpose of clinical practices are cytology, histology, and colposcopy. While these techniques continue to represent the state of the art in term of detecting HPV associated lesion. They do not detect the virus or any symptoms that are necessarily correlated to or caused by virus. The cytology or Papanicolaou (Pap) smear detects cell with abnormal nuclei or with structurally distinct halos around the nuclei (koilocytes). Although it is likely that these properties normally appear in even of HPV infection but evidence for a strict correlative relationship is lacking. Similarly, colposcopic detection of cervix, vagina, vulva is suggestive but not formal proof of HPV infection [90]. Whereas, viral particles and capsid protein are often detectable within benign lesions by electronmicroscopy or immunochemistry, neither methods shows adequate sensitivity, nor can viral infection be typed [30]. In addition, a large variety of serological assay have developed to detect specific antibodies in warts, CIN and cervical cancer patients, for examples about half of patients with cutaneous warts develop antibodies to HPV-1 virions as detected by enzyme linked immunosorbent assay (ELISA)[91]. Fisher, et al. (1996) [92] used the HPV-16 E6 and E7 peptides as antigens for detection of specific antibodies by ELISA and radio immunoprecipitation assay (RIPA). In ELISA technique, antibodies to HPV-16 E6 and E7 were detected 16.8% and 32.8% respectively, for RIPA, antibodies were detected 46.7% and 38.7% respectively. Therefore, the limitation of serological assay is low sensitivity and difficulties in preparation of antigen for such test.

As consequence of this, diagnostic and epidemiological research can be based on molecular biological techniques. There are two main methods, non-amplification and amplification methods [31]. Non-amplification methods are

Southern blot hybridization, Dot blot hybridization and *in situ* hybridization. Whereas amplification method is polymerase chain reaction (PCR).

1. Southern blot hybridization (SH)

DNA with a specific base sequence may be identified through a procedure developed by Edward Southern in 1975 known as Southern blotting [93].The principle of this technique is illustrated in Figure 5. The method starts with purification of the total DNA extracted from clinical specimens. This DNA is cut with restriction endonuclease and the DNA fragments are separated according to their size by agarose gel electrophoresis, then transferred to a nitrocellulose membrane and hybridized with complementary radioactive or non- radioactive probes. Using this method, HPV typing can be identified by type specific probes. The limitations of this method are lack of sensitivity [at least 0.1 copy of HPV-DNA per cell when 10 μg of cellular (10^6 cells) DNA is assayed], and time consuming [94].

2. Dot blot hybridization (DH) [30]

Dot blot are rapidly method of analyzing samples for HPV-DNA. Time and effort are saved by omitting the restriction enzyme digestion and gel electrophoresis steps. Instead, the extracted cellular DNA are heat or alkali denatured applied directly to the membrane and fixed by baking then, hybridization can be done. Limitation of dot blot is more prone to false-positive results. In Southern blot analysis, the gel electrophoresis represents an extra purification step. Moreover, a banding pattern appropriate to that particular HPV types confirms the validity of a positive banding pattern, dot blot can not be used for low stringency analysis of total genomic samples

because large amounts of cellular DNA can appear and produce high background signals that obscure the true hybridization.

3. *In situ* hybridization (ISH)

In situ hybridization is used to test histologic material that had been placed on specially treated glass slides. It uses nucleic acids within cell as the targets. Cells are usually made permeable by proteinase K digestion to give the probes enhanced access to the intracellular nucleic acid. This technique is simple and rapid, but suffers from several limitation such as low sensitivity (an average 50 copies of the target sequence per cell can be detected), and high background [42].

Table 3. Diagnostic methods of anogenital HPV infection

HPV Diagnostic Method	Tested Material	HPV Type Determination	Sensitivity	Specificity
Cytology				
Cervical	Swab washing	No	+[a]	++
Histology				
Light microscope	Tissue	No	++	+++
Electron microscope	Tissue	No	+	++++
Immunocytochemistry	Swab washing	No	++	++++
Serology	Serum	?	++	+++
Nucleic acid hybridization				
Non amplification				
Southern blot	Swab washing	Possible	++++	+++++
Dot blot hybridization	Swab washing	Possible	+++	+++
In Situ hybridization	Tissue washing	Possible	++	++++
Amplification				
PCR	Swab washing	Possible	++++++	+++/+++++

(a) + to +++++ : from lowest to the highest magnitude.

(modified from Bonnez, W 1997) [42]

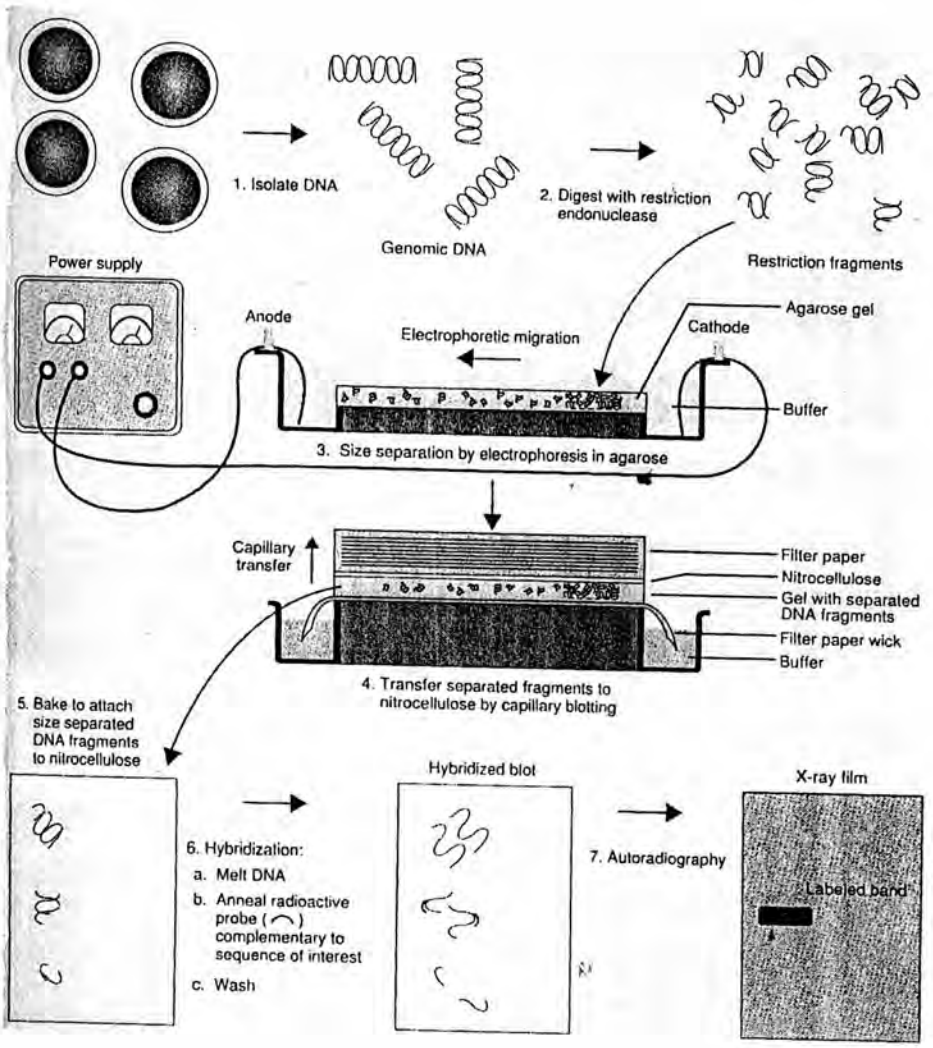

Figure 5. Principles of Southern blot hybridization using radiolabelling probes.

4. Polymerase chain reaction (PCR)

An amplification method, PCR, the method that destined by Saiki, et al. (1988) [95] is the most sensitive technique by utilizing the DNA polymerase and specific oligonucleotides which synthesized complementary to the flanks of target DNA. The specific oligonucleotides can be used as primers for replication of the DNA synthesis (Figure 6), so that the target DNA can be amplified. This method is the most sensitive and precise which can detect HPV DNA even if only one copy of HPV exists in the cell [31,32].

The validity of each methods has been done by different studies . One study compared the dot filter hybridization, Southern blot hybridization and PCR, showed that PCR can detect 87% of the samples while dot filter hybridization and Southern bolt hybridization were 57% and 49%, respectively [96]. Another study compared of immunohistochemistry, *in situ* hybridization and PCR, and the results are 69%, 77% and 100%, respectively [70]. Thus, it is clearly demonstrated that PCR is the most sensitive method .

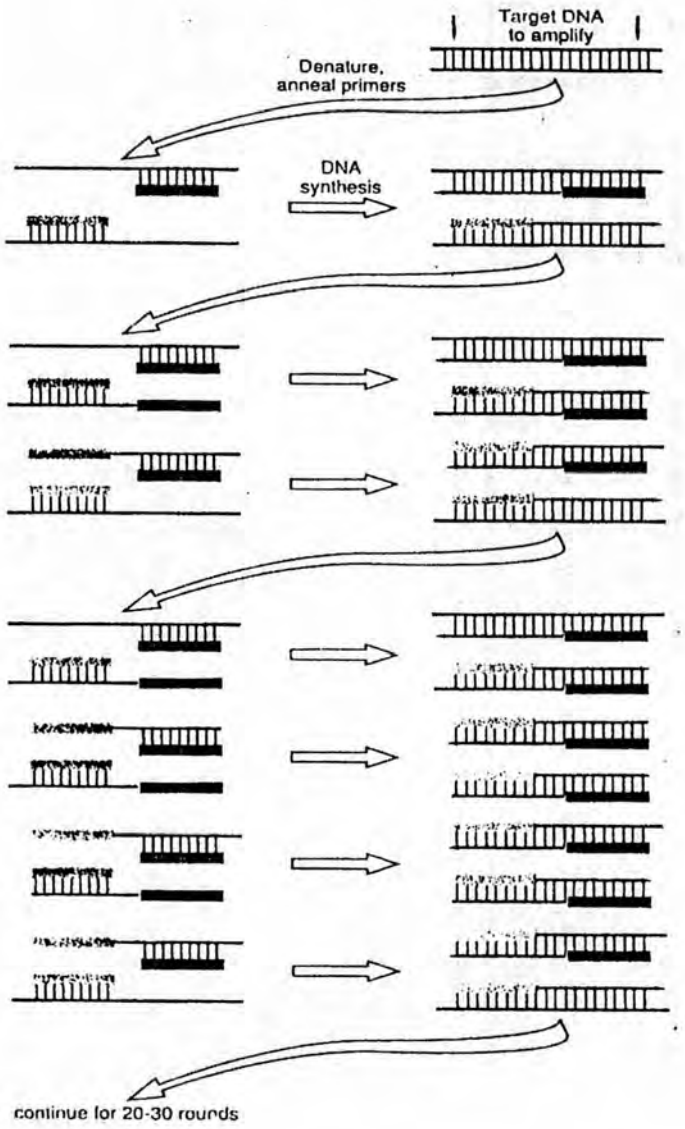

Figure 6. Principle of polymerase chain reaction (PCR).