LITERATURE REVIEWS

1. Cervical Cancer

Cervical cancer is the second most common cancer among women worldwide. At least 370,000 new cases are identified each year and 80 percent of these are in developing countries. It is killing approximately 200,000 women per annum in those developing countries (Sherris, 2000). Among Thai women, cervical cancer is the most common type of cancer. During 1988-1991, the statistical analysis of the cancer incidence from the population-based cancer registration showed that cervical cancer was the most common cancer identified in Bangkok and Songkla, while in Chiang Mai and Khon Khan, it was the second most common cancer identified. However, Chiang Mai had the highest cervical cancer incidence when compared by ASR (Vatanasapt *et al*,1993).

Concerning the cancer registration report from the Cancer Unit, Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine Chiang Mai University for women in Chiang Mai during 1991 to 1995, cervical cancer was the second most common cancer among the top five reported, with an approximate ASR of 30 (Fig. 1). Lung cancer was found to be the most common. The comparison of cervical cancer mortality with the incidence of the disease is shown in figure 2 (Chiang Mai University, 1993; 1995; 1996; 1997; 1998).

2. Cytopathology of the cervix

The epithelium that lines the ectocervix or vaginal portion of the cervix are of the stratified squamous epithelium nonkeratinizing type. Whereas, the endocervical epithelium is composed of a single layer of tall columnar cells (Fig. 3) and the small cells located at the base of the endocervical epithelium are the reserve cells that represent the source of squamous metaplasia (Fig. 4). In a normal cervix, the squamous epithelium of the ectocervix may be divided clearly into three zones. The

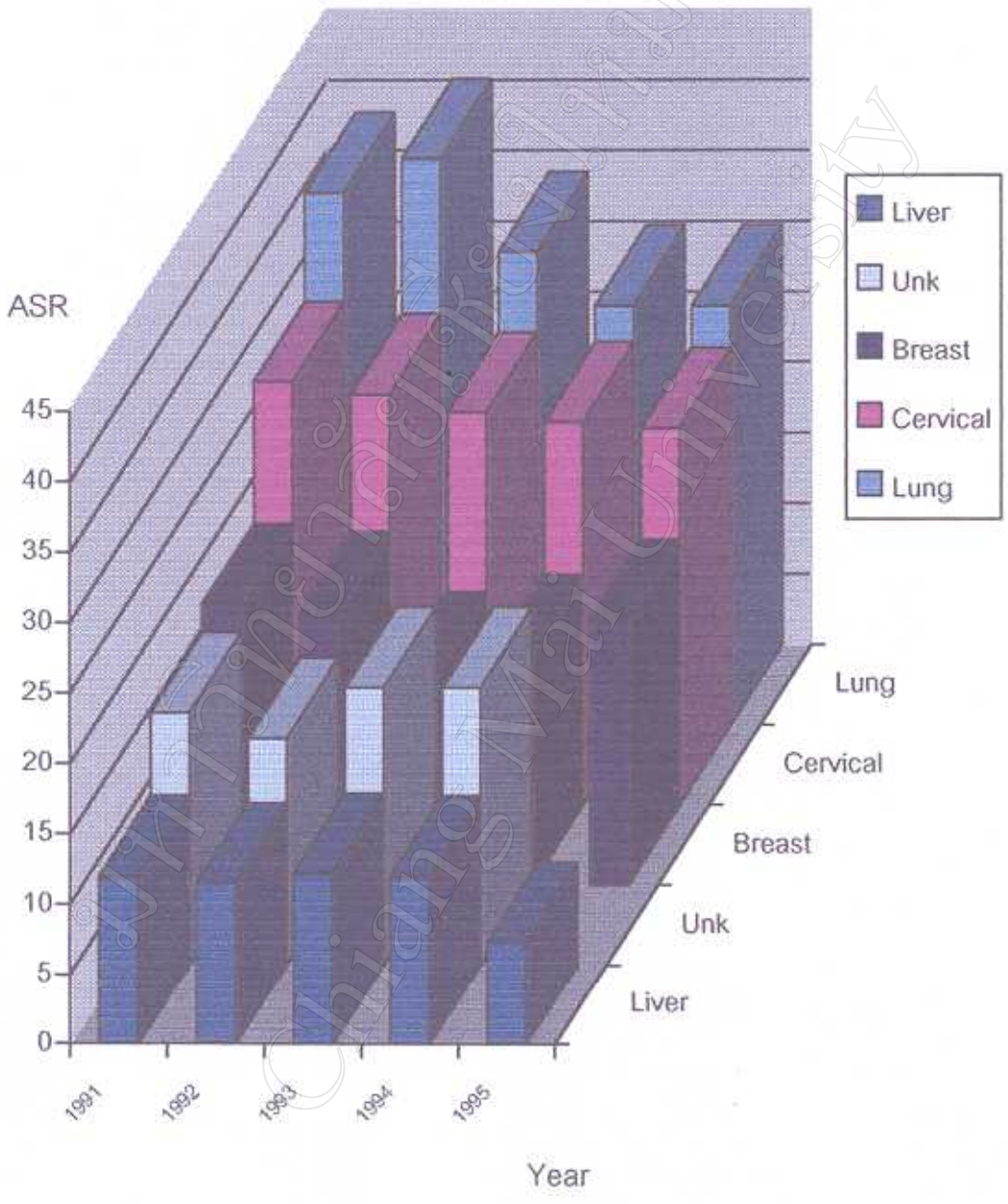


Figure 1. The bar chart shows the incidence of the top five cancers among the women of Chiang Mai during 1991-1995. (Unk = unknown primary site)

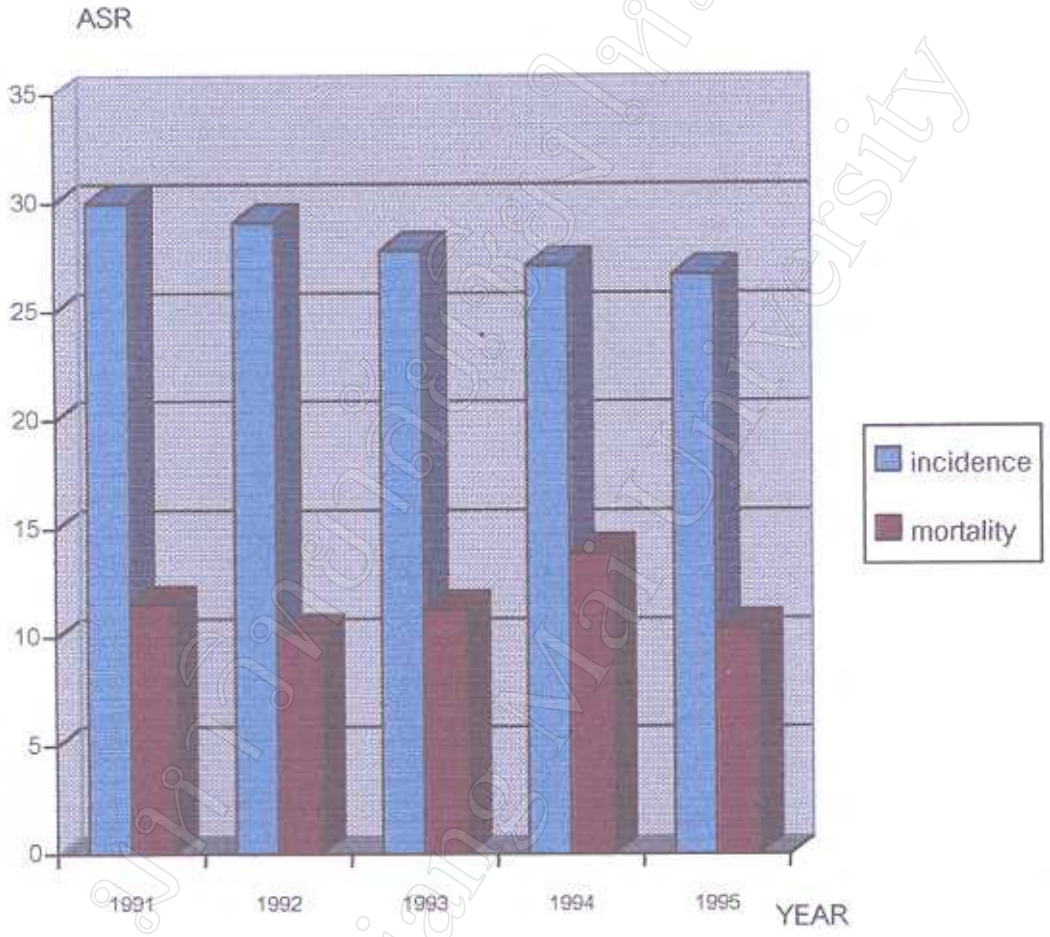
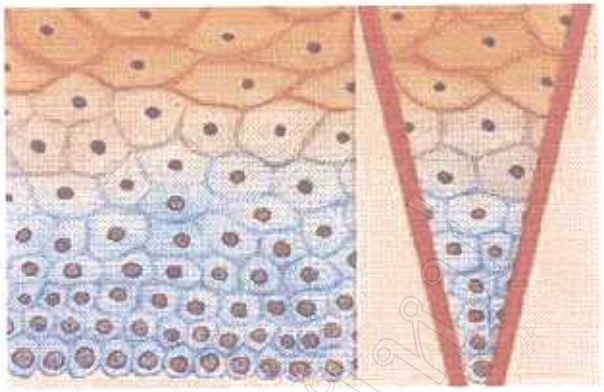
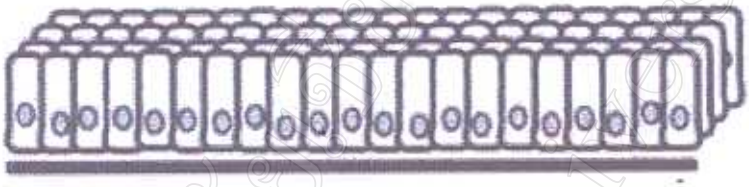


Figure 2. The bar chart shows the incidence and mortality of cervical cancer cases among the women of Chiang Mai during 1991-1995.



(A)



(B)

Figure 3. Schematic drawing of A) nonkeratinizing squamous epithelium. Note gradual increase in cell sizes as the squamous epithelium matures toward the surface. There is a reduction in the size of the nuclei in the eosinophilic (pink) superficial cell layers. B) columnar epithelium lining the endocervical canal. (Koss and Gompel, 1999)

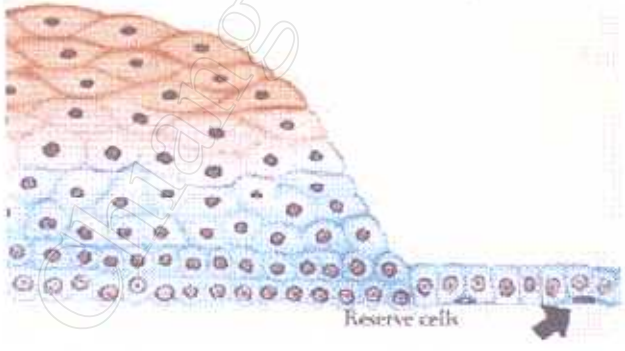


Figure 4. Schematic drawing of the transformation zone. Reserved cells, the source of squamous metaplasia, are small cells located at the base of the endocervical epithelium (arrow). (Koss and Gompel, 1999)

basal zone of the epithelium is composed of one or two layers of the basal cells, resting on the basement lamina. The cells are spherical, and approximately 15μm in diameter with a relatively large nuclei. The basal cells in this layer actively divide, which allows the renewal of the epithelium within approximately 4 days. An abnormal increase in the number of layers is known as **basal cell hyperplasia of squamous epithelium.**

The intermediate zone of the epithelium consists of several layers of cells that increase progressively in size toward the surface. The superficial zone is composed of 3 - 8 layers of larger cells. These cells are the last stage of cell maturation in the squamous epithelium. The squamous epithelium rests on the basal membrane, which separates it from the underlying basement lamina. The basement lamina is made up of collagen and other fibrillary proteins. A break in the basal membrane is important in ascertaining invasion in the case of malignant transformation of the epithelium. Thus, the malignant cells have to cross the basal membrane before cancer becomes **invasive.**

The junction between squamous and columnar epithelium is known as the squamocolumnar junction or the transformation zone where most neoplastic lesions are initiated.

The malignant lesions of the uterine cervix are histologically divided into two major stages, preinvasive and invasive, depending on the degree of invasion of the malignant transforming cells.

2.1 Preinvasive lesion of the cervix

It was well documented early in the 20th century that invasive squamous cancer of he uterine cervix was preceded by a malignant transformation of the epithelium origin. These precursor lesions are currently known as precancerous or preinvasive lesions, which are subdivided into several categories of dysplasia or intraepithelial neoplasia. The initial neoplastic events generally result from the malignant transformation of the basal cells, particularly at the transformation zone (Fig. 5). After initiation, the neoplastic events may spread to either the squamous epithelium of the ectocervix or the endocervical epithelium (Fig. 5). The morphological aspects of the

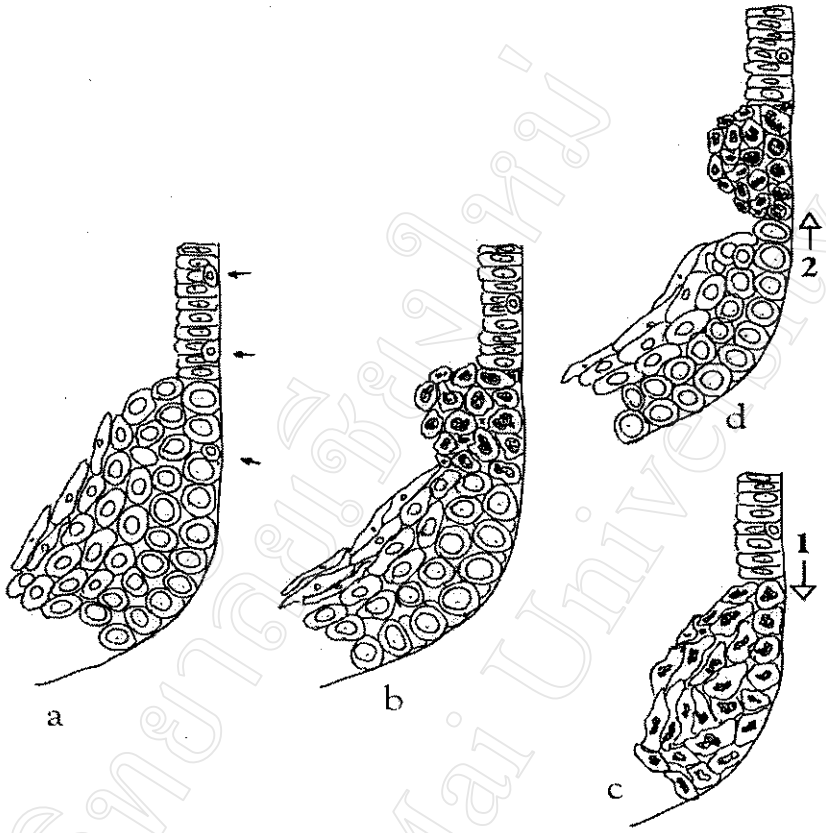


Figure 5. Schematic representation of the transformation zone (squamouscolumnar junction) and the sequence of events in early cancerous changes in the uterine cervix. **a.** Normal transformation zone with endocervical and squamous epithelium. The endocervical reserve cells are indicated by *small arrows*. **b.** The development of early cancerous changes. **c.** Lesions that progress in the direction of the squamous epithelium of exocervix (*large arrow 1*) are composes of large, keratin-forming squamous cells. **d.** Lesions progressing in the direction of the endocervical canal (*large arrow 2*) assume the basis characteristics of metaplastic cells (large-cell lesions) or endocervical cells (adenocarcinomas) or of reserve cells (small-cell carcinomas). (Koss and Gompel, 1999)

lesions developing in the squamous epithelium differ from those that develop in the endocervical epithelium. These differences are of major diagnostic significance. Lesions involving the squamous epithelium usually retain many features including the ability to synthesize keratin. Lesions involving the endocervical epithelium may be composed of cells that often retain similarities to squamous metaplasia. The lesions that derive from reserve cells of the columnar epithelium are **small-cell carcinomas**, which may also give rise to **adenocarcinomas.**

2.1.1 Classification

Over the years, several classification systems of premalignant lesions of the uterine cervix have been proposed. The most significant classification systems are those of dysplasia, carcinoma in situ (CIS) and cervical intraepithelial neoplasia (CIN).

2.1.2 Dysplasia

The term dysplasia, proposed by Papanicolaou in 1949, was used to describe neoplastic abnormalities of the uterine cervix with unpredictable behavior, and it did not meet the criteria of classic carcinoma in situ. Today, some cytologists use the term dysplasia to describe the lesion that may be better differentiated than the carcinoma in situ. Dysplasia was later subdivided further by Reagan into slight, moderate and severe (Reagan and Patten,1962; Patten, 1978).

2.1.3 Cervical intraepithelial neoplasia (CIN)

CIN is the term used to define cervical epithelial cell abnormalities. The epithelial cells are malignant, but confined to the epithelium layer. Richart initially subdivided the lesion into three grades, CIN I, CIN II and CIN III, each with a different invasive potential. In CIN I, the upper two thirds of the epithelium, although showing some nuclear abnormalities, has undergone cytoplasmic differentiation. The cells in the lower one third lack evidence of cytoplasmic differentiation or normal maturation. Mitotic figures are few and, if present, are normal (Fig. 6). CIN II is the abnormal change of CIN I that progresses to the lower two thirds of the epithelium (Fig. 7). The CIN III lesions

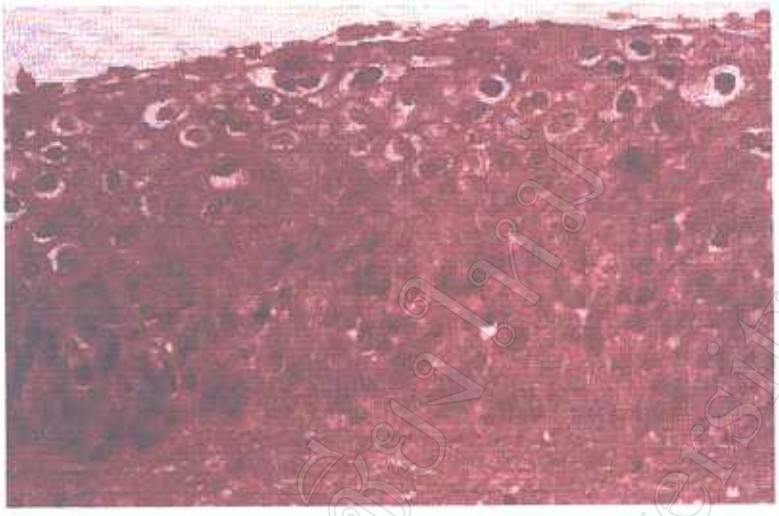


Figure 6. The histological of CIN I cervical biopsy. The cells in the bottom one third of the epithelium are smaller and have scant cytoplasm. Some have lost their normal orientation. There is nuclear pleomorphism. Mitoses are seen above the basal layer; the cells above have more cytoplasm, but still have abnormal nuclei. A clear cavity surrounds the abnormal nuclei of the cells, which are known as koilocytes. (Anderson and Atkinson, 1996)

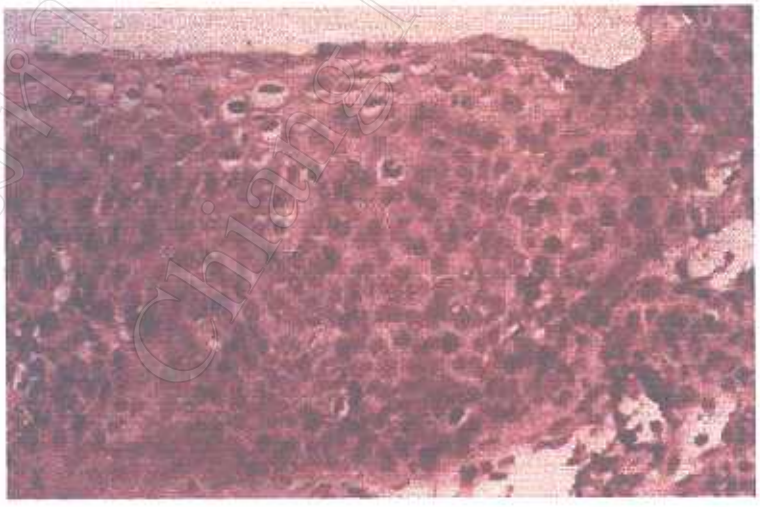


Figure 7. The histological of CIN II cervical biopsy. Basaloid cells occupy two thirds of the epithelium. Mitoses are seen high in the epithelium. Koilocytes are present near the surface. (Anderson and Atkinson, 1996)

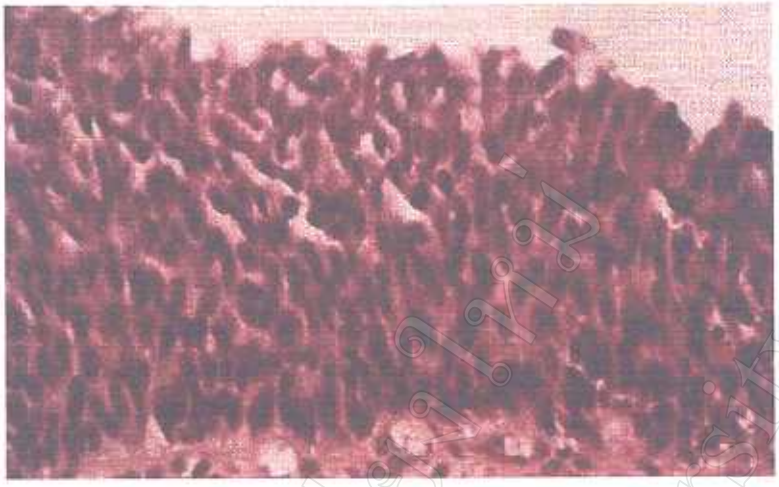


Figure 8. The histological of CIN III cervical biopsy. The basaloid cells occupy the full thickness of the epithelium. The orientation of the cells is somewhat disorganized, but many of the nuclei have their long axis perpendicular to the basement membrane. There is prominent hyperchromasia. (Anderson and Atkinson, 1996)

have full thickness changes with undifferentiated nonstratified cells. Nuclear pleomorphism is common, and mitotic figures are abnormal (Fig. 8) (DiSaia and Creasman, 1993). The CIN system has been reduced recently into two groups, low-grade CIN and high-grade CIN, which corresponds to the Bethesda system.

The Bethesda system is a cytological classification system of genital smears, proposed by a group of experts in 1988 and modified in 1991. According to this system, cytological abnormalities have been classified into two groups: low-grade squamous intraepithelial lesion (LGSIL) and high-grade squamous intraepithelial lesion (HGSIL). LGSIL comprises lesions classified as CIN I, mild dysplasia and lesions associated with HPV (including condyloma). HGSIL includes moderate and severe dysplasia, carcinoma in situ or CIN II and CIN III (Fig. 9).

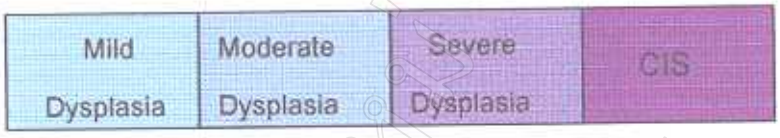
2.1.4 Carcinoma in situ (CIS)

Carcinoma in situ is composed of cells that resemble metaplastic cells that usually develop at the squamocolumnar junction and may extend toward either the exocervix or endocervix. The lesion is confined to the cervical epithelium. The mucous membrane sometimes bleeds easily on contact, and erosion or a superficial defect of the endocervix is relatively common.

2.2 Invasive carcinoma of the cervix

By definition, any cervical tumor that has broken through the basement lamina and, thus, invaded the underlying stroma is an invasive carcinoma. Staging of invasive cancer is an assessment of its spread. The lesion with cancer cells, which is still confined to the uterine cervix, is defined as stage I, while the lesion where cancer extends beyond the cervix is in stage II-IV. The invasive carcinoma is divided into two types, squamous cell carcinoma and adenocarcinoma, depending on the cell type.

WHO & Reagan's Classification



Richart's Classification

Bethesda System

ASCUS= atypical squamous cells of unknown significance

CIS = carcinoma in situ

CIN = cervical intraepithelial neoplasia

LGSIL = low-grade squamous intraepithelial lesions

HGSIL = high-grade squamous intraepithelial lesions

Figure 9. Comparison of the various cytologic and histologic classification systems of precancerous lesions and cancer of the uterine cervix, starting with Reagan's Classification and ending with the Bethesda System. The terminology increasingly tends to be the same for both diagnostic media, thus facilitating the statistical analysis of results. (Koss and Gompel, 1999)

2.2.1 Squamous cell carcinoma

The International Federation of Obstetrics and Gynecology (FIGO) has divided squamous cell carcinoma of the cervix into microinvasive squamous cell carcinoma (stage IA1 and IA2), and invasive squamous cell carcinoma (stage IB – IVB).

2.2.1.1 Microinvasive squamous cell carcinoma (MIC)

MIC has been defined as a tumor invading the stroma for a distance of 5 millimeters or less and has a diameter of 7 millimeters or less. FIGO has divided MIC into two stages: stage IA1, which shows minimal invasion of the stroma, and stage IA2, which shows invasion up to 5 millimeters in-depth from the basement membrane from which it originated. It could be migrated from an endocervical gland or the surface epithelium (Fig. 10).

2.2.1.2 Invasive squamous cell carcinoma

Any tumor volume that exceeds the FIGO definition of stage IA cervical cancer, but is still confined to the cervix, is considered as stage IB. Stage IB tumors range from microscopic lesions to tumors measuring several centimeters in size. If the tumor extends to the vagina and involves its upper two-thirds without parametrial extension it is considered stage IIA. A tumor with unilateral or bilateral parametrial involvement, which does not reach either pelvic sidewall, is considered stage IIB. Stage IIIA exists when the cervical cancer reaches the lower third of the vagina and parametrial tumor and, if present, does not reach the pelvic sidewall. A stage IIIB tumor extends to one or both pelvic sidewalls. Hydronephrosis or a non-functioning kidney that is secondary to urethral obstruction by the cancer also places the lesion in stage IIIB. A stage IVA cervical carcinoma extends to the bladder or rectum and is proved by biopsy. Stage IVB connotes spread to distant organs. The lung and the liver are the distant organs most frequently involved with metastasis from cervical squamous cell carcinoma (Heller *et al*, 1996).

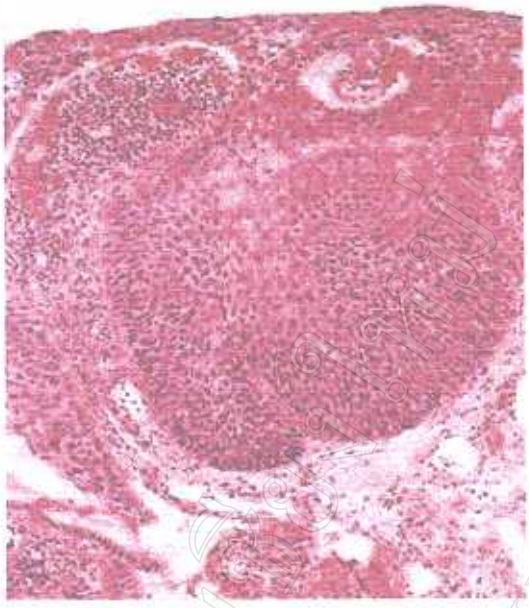


Figure 10. Cervical biopsy showing microinvasive squamous (epidermoid) carcinoma of the cervix with superficial invasion of the stroma. (Koss and Gompel, 1999)

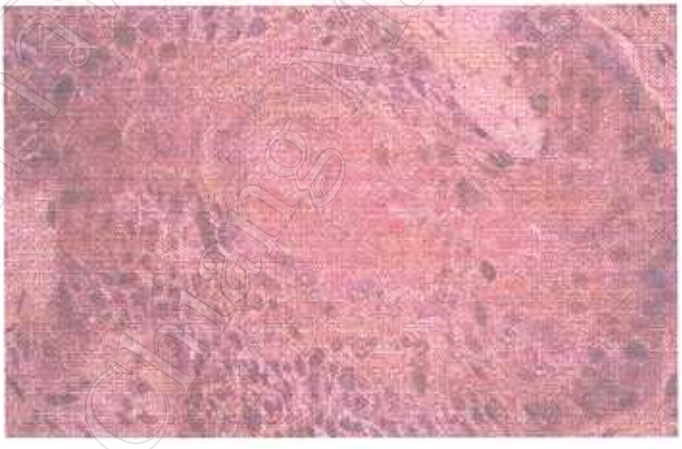


Figure 11. This well differentiated squamous cell carcinoma forms cystic spaces filled with debris. (Heller et al, 1996)

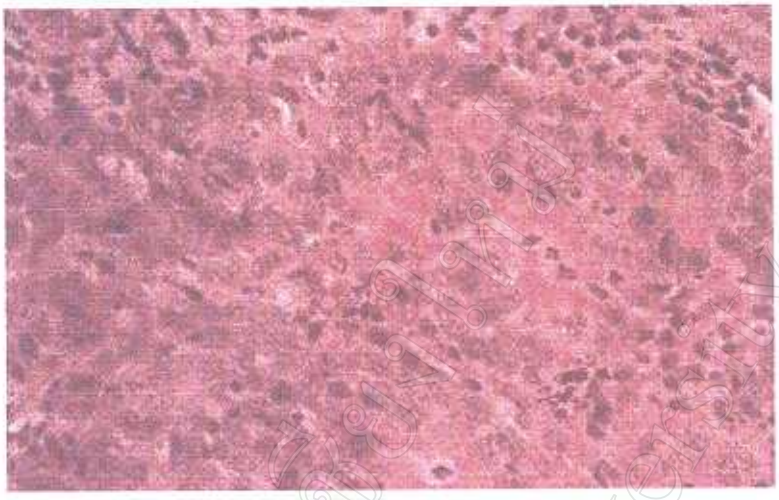


Figure 12 This poorly differentiated squamous cell carcinoma shows marked pleomorphism. Many of the cells have scant cytoplasm, and inter cellular bridges are absent. The nuclei have a vesicular chromatin pattern and prominent nuclei. (Heller et al, 1996)

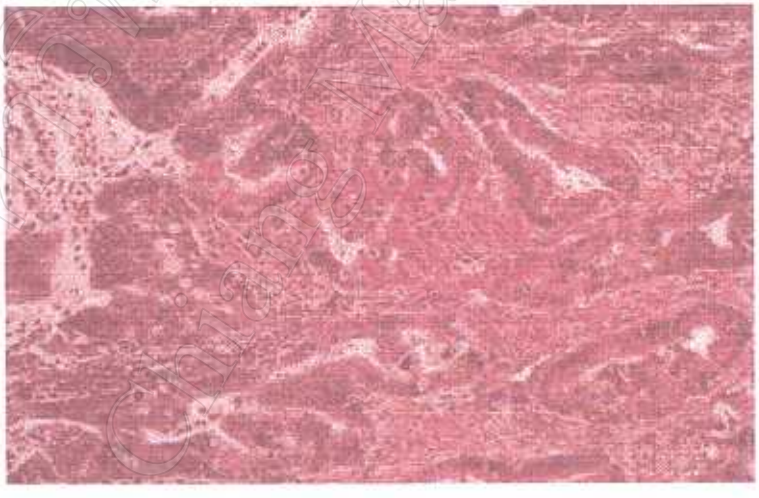


Figure 13 The moderately differentiated endocervical adenocarcinoma. Infiltrating grandular structures are present, and a cribriform pattern is prominent in the large gland. (Heller et al, 1996)

Concerning with the histological forms, invasive squamous cell carcinoma could be divided into three patterns depending on the presence and degree of keratinization in those tumor cells; a) well-differentiated keratinizing carcinoma, b) moderate-differentiation and c) poor-differentiation. A well-differentiated squamous cell carcinoma has generous amounts of keratin in the form of epithelial pearl (Fig. 11). Necrotic keratinizing debris may be present. When this necrotic keratinizing debris is found in the center of the mass of infiltrating squamous carcinoma it may produce a pseudo-grandular appearance. There are prominent intercellular bridges. The nuclei are often hyperchromatic and pyknotic, and less than two mitoses per high power microscopic field are seen. Intracellular glycogen might be noted. In a moderately differentiated tumor, there is less keratin. Cells have individual cytoplasmic keratin, but epithelial pearls are absent. The tumors are composed of large cells with fewer well-defined intercellular bridges. There is cellular pleomorphism, and two to four mitoses per high power field are seen. The nuclei are enlarge and irregular in size. The pattern of infiltration generally consists of a cellular mass with pushing and rounded borders. In poorly differentiated squamous cell carcinoma, individual cell keratinization is absent. Cell size varies and the cytoplasm is scant (Fig.12). Intercellular bridges are scant or absent. The nuclei are pleomorphic and may be hyperchromatic, or have a vesicular chromatin pattern. Nucleoli are present. The mitotic rate is more than four per high power field. The stroma is infiltrated extensively by single cells, tumor cords and a small nest (Heller *et al*, 1996).

2.2.2 Adenocarcinoma

Adenocarcinoma arises from the endocervical mucus-producing gland cells. The lesions are characteristically bulky neoplasms that expand the cervical canal and create barrel-shaped lesion of the cervix. The spread pattern of the lesion is similar to that of squamous cell carcinoma (Fig. 13).

3. Risk factors in cervical cancer development

A certain cause of cervical cancer has not been established. However, there have been many epidemiological investigations that exhibit the correlation between cervical cancer and many factors, as follow:

a. Socioeconomic

Women with a low education and economical status are at higher risk than those with high education and economical status (Briton,1992).

b. Sexual activities

Studies concluded during the past 30 years have consistently indicated that cervical cancer risk is strongly influenced by two measures of sexual activities: the number of partners and the age at which intercourse first occurred. The frequency of intercourse also increases the risk of developing cervical cancer (Briton *et al*,1987; Franco, 1996).

c. Husband or a women's male partners

The wives of patients with penile cancer are at an increased risk for cervical cancer later in life. While wives of husbands who have sexual activities with many partners may have an increased risk of developing cervical cancer earlier (Buckley *et al*, 1981; Briton *et al*, 1987; Franco, 1996).

d. Contraception

The long-term use of contraceptive pills has been implicated as one of the important factors associated with the development of cervical cancer. Several studies have shown that the rate of neoplastic intraepithelial lesions is higher in women receiving oral contraceptives than those using a barrier contraceptive. (Briton,1991; Franco, 1996).

e. Tobacco smoking

Smoking has been a well-known risk factor for cervical cancer. The direct carcinogenic action of cigarette smoking on the cervix has been implicated on the grounds that nicotine metabolites can be found in the cervical mucus of smokers. The

cervical cancer incidence from women who smoke was 2 times higher than non-smokers (Slattery *et al*, 1989).

f. Viral infection

Several seroepidemiological studies of herpes simplex virus type 2 (HSV-2) infection have revealed an association between HSV-2 and the development of cervical cancer by a presence of high antibody titers in women with cervical cancer and precanceruos lesions (Rawls et at,1968). This was also supported by an in vitro experiment where HSV DNA transformed cells in a culture. However, this suggestion still has some controversy since the viral genome was not present in the cervical cancer specimen. Human papillomaviruses, particularly high-risk types HPV-16 and -18, have been recognized recently as the major cause of cervical cancer. More than 95% of cervical carcinoma specimens contain HPV DNA. Furthermore, a genetic analysis of HPVs indicated that their transforming function resides in an early region of the viral genome and its infection alters cell growth properties in ways that resemble malignant tumor cells.

4. Human papillomaviruses

4.1 Virion Structure

Human papillomaviruses (HPVs) are a group of viruses in the genus *Papillomavirus*, *Papovaviridae* family. They are small, nonenveloped, icosachedral DNA viruses. The diameter of each particle is about 55 nm (Fig. 14) and its density in cesium chloride is 1.34 g/mL. The virion particles consist of a single molecule of closed circular double-stranded DNA that are approximately 8,000 nucleotide long and surrounded by an icosahedral capsid. A fine structural analysis, by cryoelectron microscopy on three-dimensional image reconstruction techniques has revealed that the viruses consist of 72 pentameric capsomers arranged on a T=7 surface lattice. The capsomers exist in two states, one capable of making contact with six neighbors, as observed in 60 hexavalent capsomers, and the other with five neighbors in 12 pentavalent capsomers.

The viral capsid consists of two structural proteins encoded in late genes, L1 and L2. The L1 gene product is the major capsid protein that has a molecular weight of approximately 55 kDa and represents almost 80% of the total viral protein. A minor L2 protein has a molecular size of approximately 70 kDa (Howley, 1996).

4.2 Viral genome structure and organization

All papillomaviruses have a similar genetic organization. They are a close circular double-stranded DNA containing approximately 10 open reading frames (ORFs) and all large ORFs reside on one DNA coding strand (Fig. 15). The GC content of most papillomavirus genomes is approximately 42%.

The viral genome is divided into 3 regions: 1) The early gene region that is about 4.5 Kb, containing 8 ORFs and designated E1 to E8. They encode the proteins required for viral replication and transformation. 2) The late gene region is about 2.5 Kb and located next to the early gene region containing 2 ORFs designated as L1 and L2. They encode viral capsid proteins, which are expressed in only productive infected tissues. 3) The upstream regulatory region (URR), which is non-coding and lies between the early and late regions. This has previously been termed as long control region (LCR) containing control sequences for HPV replication and gene expression. Multiple constitutive enhancer elements as well as domains that respond to some viral and host proteins, for example, AP1, NFI and cytokeratin (CK) octamer binding sequences, glucocorticoid responsive elements (GRE), interferon responsive sequences (IRS) and E2 responsive sequences (E2RS), are lie in this region (Fig. 16). The binding of enhancer sequences with factors like AP1, NFI and tissue specific factor CK octamer binding protein makes the virus be tightly regulated by cellular factors (Baker, 1990).

4.3 Viral Replication

The papillomaviruses are highly species-specific and induce squamous epithelial lesion in their natural host. Until recently, knowledge about the replication cycle of HPV was quite limited, due to the lack of a suitable tissue culture system for the

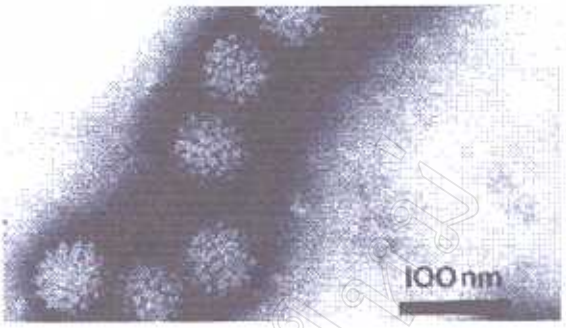
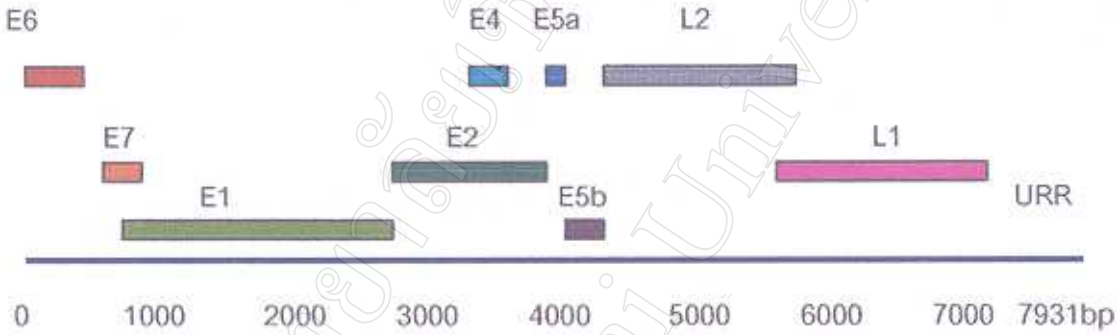
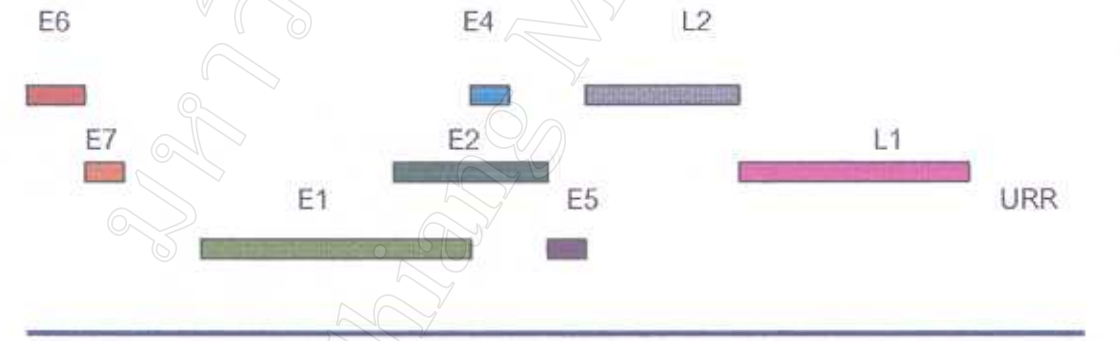


Figure 14 The electron micrograph of human papillomavirus particles. (Mims, 1998)



(A)



(B)

Figure 15 The genomic organization of HPV 11 and HPV 16 deduced from the primary DNA sequences. Each of the genomes has been linearized for ease of presentation at a site of the ORFs. The boxes above the line represent the ORFs. All of the ORFs of the HPV genomes are located on one strand, and only that strand is transcribed. A) Linearized with HPV-11 DNA map. B) Linearized with HPV-16 DNA map

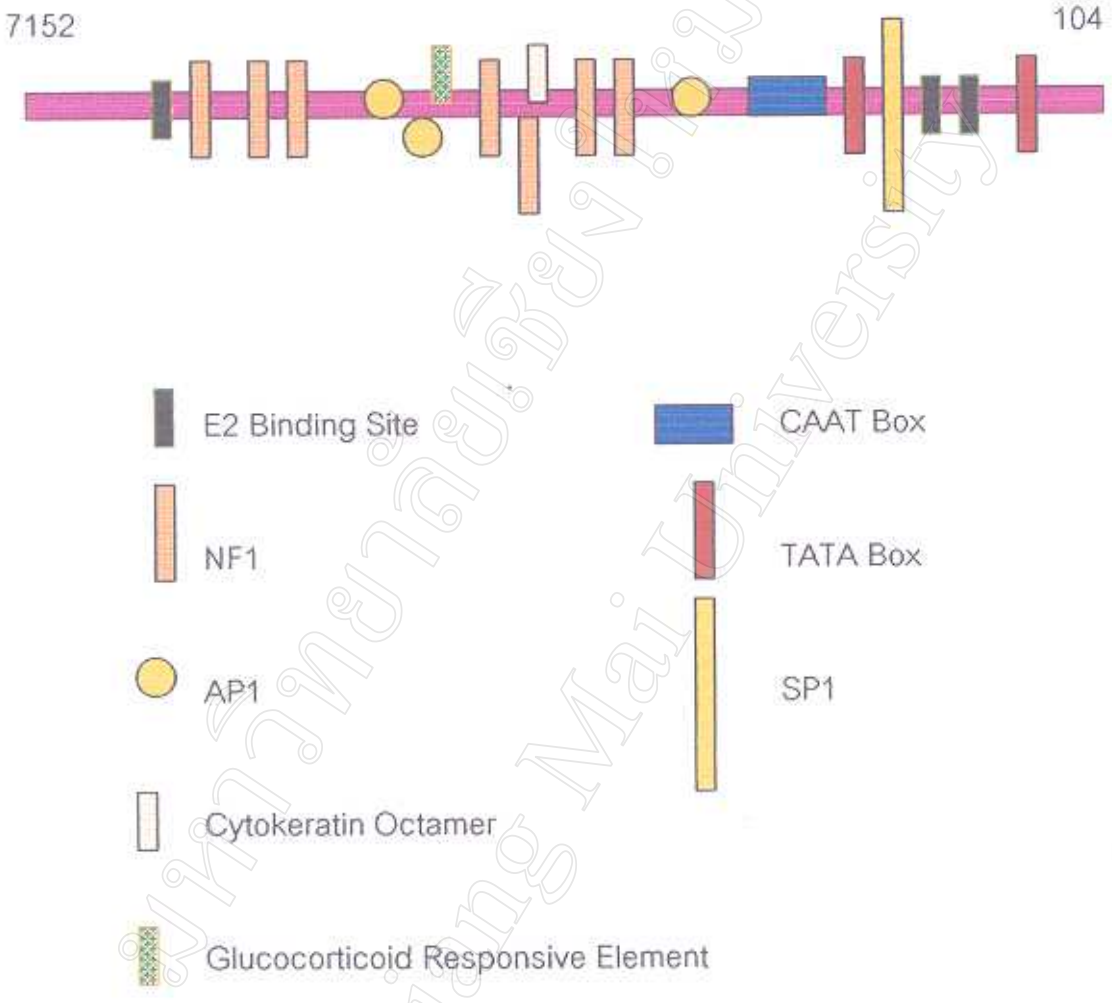


Figure 16. The constitutive enhancer elements binding site on HPV 16 URR.

propagation of the virus. Moreover, their late gene function is intimately tied into the differentiation program of the host cells. A high level of viral DNA and capsid protein synthesis were observed only in the terminal differentiated cells and the squamous epithelial cells of keratinocytes. The early study of papillomavirus replication was carried out exclusively in bovine papillomavirus type 1 (BPV1). However, in recent years, the study has been extended to some HPVs by using infected clinical tissues and cervical carcinoma cell lines. After uncoating, the viral genome replicates as an autonomous plasmid in the nuclei of infected cells, although integrated viral DNA is also observed. There is no published evidence for the mechanisms by which the papillomaviruses enter the cell and nucleus or the uncoating process. It is presumed that the papillomavirus used mechanisms similar to polyomaviruses.

A transient replication assay by transfection of the recombinant DNA into a cell culture reveal that at least two viral proteins, E1 and E2, and a viral *cis* origin of replication (*ori*) sequence in the URR are essential for the plasmid replication of BPV1 and HPV. As suggested by protein sequence homology with SV40 and polyoma T proteins, the E1 proteins have ATPase and helicase (DNA unwinding) activities. On the other hand, E2 proteins are transcriptional activator proteins that begin with unwinding supercoiled DNA by binding the E1 protein to the *ori* sequence near the E6 gene in the 3' end of the URR. However, the binding affinity of the E1 proteins for the papillomavirus *ori* elements is low, and the E2 promotes E1 attachment to the *ori* by cooperative binding. E1-*ori* complex stabilization involves different amino acid residues in the E2 activation domain from those required for transcriptional activation. The E1 complex subsequently forms a bi-directional replication fork complex with cellular proteins, DNA polymerase α / primase, DNA polymerase δ, peplication protein A (RPC), replication factor (RPC), PCNA and other undefined replication factors that are provided by the host cells. The viral DNA replication is then progressed bidirectionally from the origin of replication. After that, the newly synthesized viral DNA is encapsidated in a process that involves its association with cellular histone proteins (H2a, H2b, H3, and H4). A transient binding with E2 proteins presumably guides the DNA into the aggregation of the viral L1

and L2 proteins that eventually form the capsid. The release of viral particles is probably passive, due to the disintegration of the upper squamous epithelium (Bonnez, 1997).

4.4 Viral gene products and their biological functions

4.4.1 E1 proteins

The E1 protein is encoded from the E1 ORF, the largest continuous sequence present in the genome of papillomaviruses. The E1 sequence is relatively well conserved among all the papillomaviruses. In the BPV1 system, two replication functions could be assigned to this ORF; the 5' one third encodes a modulator (E1-M) function and the remaining 3' part encodes a positive replicator function (E1-R) (Lusky and Botchan, 1984; 1986). In transient replication assays in C127 cells, mutation in the 3' portion of the ORF E1 results in replication defect (Lusky and Botchan, 1986a; 1986b). In contrast to the replication defect caused by the 3' mutations, mutants with lesions in the 5' half of ORF E1 undergo a normal transient replication in C127 cells (Berg *et al*, 1986). However, the cotransfection of plasmids with mutations in the 5' and 3' portion of ORF E1 allow the normal replication of both plasmids (Berg *et al*, 1986). These experiments indicated that the 5' end of the ORF E1 encodes a negative *trans*-acting replication activity, whereas, the 3' end encodes a distinct positive one. According to this model, the replication of BPV1 has been explained on the basis that incorporates these two E1 activities, when the virus enters the cell it initially produces a protein encoded by the 3' E1 ORF, which is necessary for the DNA to amplify to a high copy number of around 100. This phase of replication ends when the product of the 5' E1 ORF is expressed and the replication regulates negatively, resulting in the cessation of amplification and maintenance of a constant copy number. In addition, in the absence of modulator activity, BPV1 replication continues unchecked and may result in toxicity of the host cells. The E1-M protein of BPV1 was recently identified as a 23 kDa phosphoprotein, whose coding sequence was located precisely between the 5' end of ORF E1 and the splice donor at position 1235 (Thorner *et al*, 1988). This N-terminal portion E1-M protein is generally less conserved. Whereas, the highest degree of

homology was found within the C-terminal 190 amino acid of the putative replicator proteins, E1-R. The homology has been mapped to a region of the SV40 and polyoma large T antigen, which corresponds to their ATPase active site (Clertant and Seif, 1984).

4.4.2 E2 protein

The E2 protein was first characterized in the case of BPV as a transcriptional activator protein that potentiated viral DNA replication and regulated viral gene expressions particularly in E5, E6, and E7 transforming genes. The role of the E2 protein in viral DNA replication may be either cooperative binding with the E1 protein, thus, stabilizing the E1-*ori* complex or by directly contributing to the viral *ori* replication by *cis* acting. However, it is possible that the unique contribution of the E2 activation domain is to recognize the E1 protein (Ham *et al*, 1991).

The E2 ORF encodes multiple transcriptional regulatory proteins. Three E2 proteins have been identified in BPV1-transformed C127 cells by immuuno-precipitation with antibodies raised to E2 proteins that have been synthesized in bacteria (Androphy *et al*, 1987; Hubbert *et al*, 1998, Lambert *et al*, 1989). The major species has a molecular weight of around 31 kDa and two minor ones have a molecular weight of around 48 kDa and 28 kDa. All three share common determinants from the 3' E2 ORF. Only the 48 kDa protein has N-terminal determinants and its size is consistent with a protein encoded by the full-length E2 ORF. The 31-kDa protein is translated from an unspliced mRNA that is transcribed from P_{3080} by using the translation initiation codon at n3091 (Lambert *et al*, 1989). The 28 kDa has recently been shown as the product of an E8/E2 fusion ORF and is probably translated from a P_{890} transcript that utilizes an n1235/3225 splice. The ratio of the 48-, 31-, and 28 kDa proteins in the transformed cell has been estimated at 1:10:3 (Hubbert *et al*, 1988). The tenfold predominance of the truncated E2 over the full-length E2 protein is probably responsible for the low level of transcription in the BPV1 transformed C127 cell. However, studies in the biological functions of those proteins revealed that the full-length E2 protein is a *trans*-activator, protein while the truncated C-terminal E2 protein and the E8/E2 fusion protein

antagonize the effect of the full-length E2 protein and they, therefore function as a *trans*-repressor (Lambert *et al*, 1987; 1989; Hubert *et al*, 1988). The DNA binding domain of the E2 proteins has been mapped to the C-terminal 101 amino acid that is shared by all E2 proteins.

4.4.3 E4 protein

The papillomaviruses E4 ORF entirely overlaps the E2 ORF in a different reading frame and encodes a protein with an entirely different amino acid sequence. The E4 gene is not highly conserved among the papillomaviruses. E4 proteins are expressed primarily in the differentiating layers of the epithelium and the E1^E4 protein has been co-localized with L1. There are multiple species of E4 proteins in infected cells. E4 proteins are expressed at high levels in infected tissues, but their precise role in the viral life cycle is unclear. E4 proteins are not found in the virion particles. Mutational analysis of the E4 gene in BPV-1 showed that E4 was not essential for viral transformation or viral DNA replication (Neary *et al*, 1987). The expression of HPV-16 E4 protein was localized in the nucleus, primarily in the superficial layers of the squamous cervical epithelium (Palefsky *et al*, 1991). The expression of the HPV-16 E1-E4 protein in human keratinocytes resulted in the total collapse of the cytokeratin matrix. (Doorbar *et al*, 1991; Roberts *et al*, 1993).

4.4.4 E5 protein

The BPV-1 E5 ORF encodes a 44 amino acid protein (Schlegel *et al*, 1986) and is sufficient for the transformation of certain established rodent cells in culture (DiMaio *et al*, 1986; Schiller *et al*, 1986). The BPV-1 E5 protein is composed of two protein domains; a very hydrophobic segment at its amino terminus anchored in the cell membrane (Schlegel *et al*, 1986), and a carboxy-terminal hydrophilic domain located outside. The overexpression of E5 proteins alone can modify cell growth and result in overt neoplastic transformation in some experiment systems (Leechanachai *et al*, 1992; Martin *et al*, 1989; Schlegel *et al*, 1986). Major targets of the E5 are transmembrane

growth factor receptors, the epidermal growth factor (EGF) receptor and the platelet-derived growth factor (PDGF) receptor. It is likely that the E5 protein promotes receptor association and retards their removal and degradation. The HPV 16 E5 stimulates the transforming activity of the EGFR by enhancing a growth factor-mediated signal transduction to the nucleus. However, E5 cannot be expected to have a major role in the maintenance of the malignant phenotype of cancer cells because these genes are frequently destroyed by the integration process of the viral genome into the cellular DNA (Pfister *et al*, 1996).

4.4.5 E6 and E7 proteins

The E6 and E7 proteins of HPVs have been shown to involve a variety of different functions such as cell-transforming activity, *trans*-activation of transcription and induction of the DNA synthesis. Furthermore, these proteins contain repeated Cys-X-X-Cys motifs, which have been found in a number of nucleotide binding proteins and are presumably DNA-binding zinc finger proteins (Barbosa *et al*, 1989). The E6 and E7 proteins of HPV 16 and 18 could immortalize primary human keratinocytes under experimental conditions. Recent evidence points to a cooperation between E6 and E7 proteins to achieve a fully transformed state of cells (Halbert *et al*, 1991; Hudson *et al*, 1990; Munger *et al*, 1989). However, the overexpression of either E6 or E7 protein may overcome this requirement. The aminoterminal sequence of the HPV E6 and E7 proteins have a significant homology to a portion of other DNA viral transforming proteins; the AdE1A of the adenovirus and the SV40 large T Ag of simian virus 40. Similar to those DNA viral transforming proteins, the E6 and E7 proteins of HPV interfere with the function of several cellular proteins. They are the product of the retinoblastoma tumor suppressor gene (pRB) and p53 (Dyson *et al*, 1989; Munger *et al*, 1989). E6 proteins are capable of binding and degrading the p53, which has transformation suppressing properties. Whereas, the E7 proteins form a complex with pRB resulting in inactivation of its normal function. However, E7 proteins from the low-risk HPV types 6 and 11 bind pRB with lower efficiency than the E7 proteins of high-risk HPV types 16 and 18 (Munger *et al*,

1989). The HPV 16 E7 mutants that do not bind pRB are no longer able to immortalize primary human keratinocytes (Jewers *et al*, 1992).

The E6 and E7 transcripts, which putatively encode the E6 and E7 proteins respectively, initiate from the same p97 promoter that is located in the URR. There are two transcripts that generate by processing at a splice donor site at nucleotide position 266. The E7 transcripts, designated as E6*I have a splice acceptor site at nt 408, while the E6*II transcripts, which encode one of the two potential E6 proteins, have a splice acceptor site at nt 526. The unspliced transcripts putatively encode a second full-length E6 protein species.

4.4.6 L1 and L2 genes

The HPV L1 ORF, which represents the major structural component of the capsid protein of the virus, and appears to disulfide cross-linked virus particles, encodes proteins of approximately 55-kDa. The HPV L2 ORF, which is a minor component of the virion and does not be disulfide the link to either L1 protein or itself, encodes proteins of approximately 70 kDa (Doorbar and Gallimore, 1987; Howley, 1996). The cross-reaction between antiserum prepared against HPV-1 L1 ORF HPV-2 and BPV-1 was found by immunofluorescence (Doorbar and Gallimore, 1987). A study by immunostaining showed that the expression of HPV-16 L1 ORF is dependent upon viral replication and epithelial differentiation (Crum *et al*, 1990). Also, the HPV capsid protein L1 and L2 are detected only in terminally differentiated cells, which indicate that the expression of L1 and L2 genes is blocked in dividing cells.

The human papillomavirus type 16 L2 ORF encodes stronger cis-acting inhibitory sequences than human papillomavirus type 1, and observations have been made of a higher virus production in HPV-1 infected tissue than in HPV-16 infected epithelium. This suggests that the inhibitory sequences in L1 and L2 genes may aid the virus to avoid the host immunosurvillance and establish persistent infection (Socolowski *et al*, 1998).

The N-terminal of the L2 protein could bind HPV-DNA that do not required a specific DNA sequence. This study proposed that HPV L2 protein might play a major role in papillomavirus capsid assembly by introducing HPV DNA to the virus particles formed by the self assembly of the L1 major structure protein (Zhou *et al*, 1994). Immunofluorescent stain and confocal microscopy was used to examine the subcellular localization of BPV L1 and L1 proteins in cultured cells. When expressed separately, the L1 protein showed a diffuse nuclear distribution. The L1 protein was found to localize and punctate the nuclear region that is identified as promonocytic Leukemia protein (PML) oncogenic domains (PODs). The coexpression of L1 and L2 induced a relocation of L1 in the PODs, leading to the colocalization of L1 and L2. This finding probably serves as a mechanism to promote the assembly of papillomavirus by either increasing the local concentration of virion constituents or providing the physical architecture necessary for efficient packaging and assembly (Day *et al*, 1998).

5. Role of Human papillomaviruses in cervical carcinogenesis

The causal link between HPV and cervical cancer arose from many experimental findings such as 1) HPV DNA, particularly from high-risk types, were found in approximately 90% of cancer samples. 2) Most tumors contained integrated viral DNA, although only a small number of malignant tumors harbored a nonintegrated episomal form. Integration is an essential step in malignant transformation by most tumor viruses. In malignant cells, HPV DNA is randomly integrated into various chromosomes resulting in substantial deletions or disruption of the viral genome. However, two oncogenic E6 and E7 ORF are always retained. 3) The E6 and E7 ORF were consistently expressed in cervical tumor tissue and cervical tumor cell lines. In view of these findings, many current papillomavirus researches are mainly focused on the role of HPV, particularly the E6 and E7 ORF, during malignant conversion.

HPV infection occurs in the basal epithelial cells probably because of wounding or trauma. In these cells, HPV DNA is maintained as an episome with highly restricted replication that occurs only once per cell cycle (Wright and Richard, 1990).

Replication is increased during the stages of cell differentiation, and viral production is critically dependent on the terminal differentiation of the epithelium. It is likely that the malignant conversion of the epithelium is not part of the normal viral life cycle and cancer cells do not undergo normal differentiation. It is believed that the malignant conversion process needs a long latency period of probably a decade for HPV infection in cervical cells, during which, some viral DNA becomes integrated with the host chromosomes. Viral integration, however, usually leads to the disruption of the E2 ORF, which encodes the major proteins controlling viral gene transcription, especially in E6 and E7 ORFs. Thus, the E6 and E7 ORFs are no longer under control. The products of E6 and E7 ORFs can also be detected in cervical cancer cells. An *in vitro* experiment revealed that the transcription from a p97 promoter, the promoter for E6 and E7 ORFs, responds to the full-length E2 *trans*activator protein in a concentration dependent fashion. This was also confirmed by an *in vivo* observation that the HPV16 with E2 ORF mutant is more efficient in extending the lifespan of cultured human keratinocytes (Turek and smith,1996).

The roles of HPV E6 and E7 ORFs in cervical carcinogenesis have been supported by a number of experiments including those on rodents and human cells. In rodent cells, the principal transforming gene of HPV-16 and -18 is the E7, which can immortalize normal rodent cells and cooperate with *ras* to transform these cells. Since assays based on rodent cells may not reflect the transformation process accurately in human epithelial cells, and as a result may not allow a complete understanding of the involvement of HPV in neoplasia, the primary human epithelial cells are also being used as recipients in gene-transfer assays. The common finding is that an expression of HPV-16 or -18 immortalizes these cells and blocks their differentiation, but not sufficiently for malignant transformation of human keratinocytes. E7 alone is much less effective in immortalizing activity than E6 and E7 together. No such activities are found in E6 alone. Thus, HPV alone may not be sufficient for the development of human cervical cancer, and other factors including cellular gene mutation or interaction with some cellular factors are yet to be identified.

Current observations strongly support the possibility that DNA tumor viruses utilize common mechanisms to transform cells (Dyson *et al*, 1989). The oncogenes of those DNA viruses encode proteins that bind to the cellular regulatory proteins, the pRB and p53 proteins. The E7 protein of HPVs, E1A of Adenoviruses and large T antigen of SV40 bind to the pRB, whereas, the E6 protein of HPVs, E1B of Adenoviruses and antigen of SV40 bind to p53. The pRB was reported to restrict cell proliferation. It acts as a negative regulator of the cell cycle at the G1/S border. The hypophosphorylated pRB is an active form that normally binds to the E2F transcription factor and inhibits cellular transcription. The phosphorylation of the pRB is regulated throughout the cell cycle. It becomes hypophosphorylated in G0 to G1 and then phosphorylated during the S and M phase. The phosphorylation occurs at multiple serine residues by the action of cyclin dependent kinase (CDK) at the G1/S boundary and remains phosphorylated until late M. Then it is hypophosphorylated through the action of specific phosphatase. The mechanism of pRB in regulation to cell proliferation could be explained through its binding to the E2F transcription factor. In the arrested cell growth, hypophosphorylated pRB binds to the E2F. When the normal cell cycle progresses. Then the pRB is phosphorylated and becomes inactivated, thus releasing the E2F transcription factor and allowing the expression of genes necessary for DNA replication and cell proliferation. HPV E7 proteins, which preferentially bind to the active form of pRB, may cause the dissociation of the E2F and allow cell proliferation. However, the E7 proteins encoded by the low-risk types, HPV-6 and -11, bind to pRB with a lower affinity than those encoded from the high risk types, HPV-16 and -18. This finding may confirmed the differences in transforming ability of those two groups of viruses.

The E6 proteins from the high-risk, but not the low-risk, HPVs are capable of binding to the cellular p53 protein that functions in the suppression of cell growth. The p53 protein was found to accumulate in response to cell stress or DNA damage which may contribute to either the arrest of the cell cycle, thus allowing the DNA to be repaired, or the death of the cell through apoptosis. The binding of the E6 protein to p53 is mediated by a 100kDa cellular protein that is designated to an E6-associated protein

(E6AP), which functions as a ubiquitin ligase (Scheffner, 1990). The consequence of the interaction between the HPV E6 protein and p53 is the rapid degradation of p53 through ubiquitin-directed proteolysis that resulted in the depletion of the cellular p53 pool (Scheffner, 1990; Hubbert, 1992).

Some recent studies, however, suggested that both E6 and E7 proteins share the same regulatory pathway via the *waf*-1 protein in the alteration of cell growth. The *waf*-1 protein is a cyclin-dependent kinase (CK1) inhibitor that could be activated by p53 dependent genes. Thus, the HPV would be, involved in the alteration of the cell growth in two separate points. The E7 protein in competition with E2F in binding to the pRB resulted in the release of unbound E2F to drive the cell cycle. Whereas, the E6 protein together with the cellular protein, E6AP, form a ternary complex with p53 that is rapidly degraded by the ubiquitin dependent pathway and, thus, prevents the transcription of *waf*-1. The low concentration of *waf*-1 protein leads to increase the level of the active cyclin-dependent kinase, which inactivates the pRB through posphorylation.