BIOCHEMICAL CHARACTERIZATION OF THE INTERACTION BETWEEN CTCF/YB-1 TRANSCRIPTION FACTORS WITH HPV 16 AND 18 E7 ONCOPROTEIN AND THEIR INVOLVEMENT DURING C-MYC GENE REGULATION IN CELL PROLIFERATION PATHWAY

by

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BIOCHEMICAL CHARACTERIZATION OF THE INTERACTION BETWEEN CTCF/YB-1 TRANSCRIPTION FACTORS WITH HPV 16 AND 18 E7 ONCOPROTEIN AND THEIR INVOLVEMENT DURING C-MYC GENE REGULATION IN CELL PROLIFERATION PATHWAY

ABSTRACT

Human Papillomaviruses (HPVs) type HPV-16 and HPV-18 are known as high risk HPV, which are cause 95% of cervix cancer. The E7 oncoprotein of HPV is able to interact and inactivate cellular protein pRB which further allow progression of the cell cycle and trigger cancer development. CTCF is a multivalent transcription factor which has been found to interact directly with YB-1. Both are reported to bind synergistically on c-myc gene promoter and down regulate the gene. Interaction of E7 oncoprotein of HPV16 and 18 to CTCF and YB-1 can help to understand the biological role of this oncoprotien in c-myc gene regulation. In this study, the interactions between HPV16 and 18 E7 with CTCF and YB-1 were characterized biochemically using in-vivo and in-vitro assays. This study confirms the novel interaction between HPV16 E7 with CTCF at Zn-domain and HPV18 E7 with YB-1 at CSD domain and both interactions are direct. However CTCF and YB-1 do not interact with HPV18 E7 and HPV16 E7, respectively. EMSA has shown that interaction of HPV16 E7 protein towards CTCF~YB-1 complex have release YB-1 from the complex that binds to CTCF cognate site FpV. Meanwhile the interaction of HPV18 E7 does not disrupt the CTCF~YB-1 complex. However the introduction of HPV16 E7 to the complex of CTCF~YB-1~HPV18 E7 disassociate YB-1 from the complex but forms a new complex CTCF~HPV16 E7~HPV18 E7, this was confirmed via a pull-down assay. Therefore, we suggest interaction of E7 protein with
CTCF and YB-1 significantly reduces the formation of CTFC~YB-1 complex. Thus, enhances the up-regulation of $c\text{-}myc$ promoter and promotes tumourogenesis of cervix.
PENCIRIAN BIOKIMIA INTERAKSI ANTARA FAKTOR TRANSKRIPSI CTCF/YB-1 DENGAN ONKOPROTIIN HPV 16 DAN 18 E7 DAN KETERLIBATAN KEDUA-DUANYA SEMASA PENGAWALATURAN GEN C-MYC DALAM LALUAN PROLIFERASI SEL

ABSTRAK

bahawa interaksi onkoprotein E7 dengan CTCF dan YB-1 dapat mengurangkan pembentukan kompleks CTCF-YB-1 dengan jelas. Seterusnya, meningkatkan regulasi gen \textit{c-myc} dan menggalakkan penularan kanser serviks.
Chapter 1: Introduction

1.1 Research Background and Hypothesis

1.1.1 Human Papillomavirus and cervical cancer

Human Papillomaviruses (HPVs) are circular double-stranded DNA approximately 8000 base pairs that replicates in the nucleus and have icosahedral capsule that form non-enveloped virions. There are more than 130 subtypes of HPV and about 70 subtypes infect human and 35 of the subtypes infect genital tracts (de Villiers, 1994; Stanley, 2001). HPV is a risk factor of cervical cancer, the second most common cancer in women worldwide after breast cancer. HPV infection has been implicated in the aetiology of cervical cancer and more than 90% of cervical cancers contain HPV DNA (Bosch et al., 1995). Low-risk HPVs such as HPV-6 and HPV-11 causes benign genital warts and whereas high-risk types such as HPV 16 and 18 are associated with the development of high risk intra-epithelia squamous lesion (HSIL) and cervical cancer (Walboomers, et al., 1999). HPV 16 and 18, represent 58% and 12% in prevalence of cervical cancer, respectively (Bosch et al., 2002).

The genome of HPV contains three regions. The two encoding regions called the early and late regions encodes for six early and two late genetic open reading frames (OFR). The third region, which is both non-coding and small (1000 base pairs), is often designated as the non-coding region (NCR), long control region (LCR), or upstream regulatory region (URR) (Robboy et al., 2000). The early region covers some 69% of the genome and encodes a series of proteins E1, E2, E3, E4, E5, E6 and E7. The viral DNA
which integrates into the genome of cancer cells is truncated to various degrees. However, E6 and E7 open reading frame are consistently retained and expressed as mRNA or protein (Takabe, 1987). Protein E6 is known to bind with protein p53 that encodes oncogene $p53$ that suppress the cell growth. This process deactivate the function of $p53$ gene, thus develops cancer cells (Scheffner et al., 1990; Boyer et al., 1996).

Interaction of E7 protein with retinoblastoma protein pRb leads to disassociation of pRb-E2F complex, and stimulates the transcription of cellular genes involved in S-phase entry (Dyson, 1998; Whyte et al., 1988; Chan et al., 2001). The binding affinity of high risk E7 to pRb is 10 fold higher than the low risk (zur Hausen, 1996). E7 open reading frame is the most abundant viral protein in cells from cervical cancer and E7 is sufficient to immortalize human epithelial cell, and therefore considered as potential tumour specific antigen that could be a target of immunotherapy for cervical and precancerous (Tindle and Frazer, 1995). Besides that, few studies have indicated that an enhanced level of c-Myc was observed in cells expressing HPV E7 (Gewin and Galloway, 2001; Oh et al., 2001; DeFilippis et al., 2003). Meanwhile, Abba et al., (2004) showed $c-myc$ gene copy number increased according to the grade lesion of cervical carcinoma and the results indicate that infection with HPV 16 tightly associated with $c-myc$ gene amplification. On the other hand, Lui et al., (2007) reported that c-Myc expression in the cells transfected with E7 protein showed significant increases the level of expression. These findings have confirmed that E7 protein is essential for the activation of $c-myc$ gene, but the mechanism is yet to be discovered.
1.1.2 CTCF transcription factor

CTCF is a transcription factor which was first identified as the protein interacting with three repeats of the CCCTC sequence that regularly spaced at 12-13 base pairs interval in the chicken c-myc promoter (Lobanenkov et al., 1990). Therefore it was named CCCTC Binding Factor or CTCF. CTCF protein was later shown to bind to a number of different sequence in the human, mouse and avian c-myc promoters (Lobanenkov et al., 1990; Klenova et al., 1993; Fillipova et al., 1996). It has been found that the human CTCF gene is localized at the chromosome 16q22.1 locus, a region commonly detected in breast and prostate cancer and suggested CTCF gene is a tumour suppressor gene (Fillipova et al., 1998). CTCF is a multivalent transcriptional factor with 11 zinc-fingers which participate in the binding of DNA elements, promoters, silencer and insulator (Fillipova et al., 1996). A number of different target genes regulated by CTCF are implicated in a variety of regulatory functions, ranging from promoter repression and activation, to the criterion of hormone responsive silencers and enhancer-blocking and/or boundary elements between Igf2 and H19 genes (Ohlsson et al., 2001). Transcription of the two imprinted genes H19 and Igf2 is controlled in part by CTCF binding insulators located between them (Bell and Felsenfeld, 2000; Hark et al., 2000).

On the other hand, Loss of Imprinting (LOI) defines loss of normal pattern of expression of a specific parental allele. In cancer it can lead to activation of growth promoting imprinted genes such as H19 and Igf2. Douc et al., (1996) have reported the methylation status of H19 and Igf2 genes in 29 invasive cervical carcinomas in different clinical stages. Fourteen (48%) and 13 (45%) tumours were heterozygous for H19 and
Igf2, respectively. LOI of H19 and Igf2 was detected in 2 of 12 (17%) and 5 of 10 (15%) tumours, respectively. Therefore, suggested that H19 and Igf2 genes, via deletion and/or abnormal imprinting, could play a crucial role in a large proportion (58%) of cervical cancers where they may be associated with disease progression (Douc et al., 1996). However, until now there have been no study has been reported of CTCF interaction with HPV oncogenes correlating with the tumourogenesis of cervical cancer.

1.1.3 YB-1 transcription factor

The Y-box protein (YB-1) is the highly conserved 70 amino acid DNA domain, the so-called ‘cold shock domain’ (CSD) was defined initially in bacteria as a characteristic feature of this family (Wolffe et al., 1992). The name Y-box protein comes from the ability of the CSD to bind to the Y-box sequence [5’- CTGATTGG – 3’] of DNA, which is an inverted CCAAT box, in the promoter region of many genes (Wolffe et al., 1992 and Wolffe, 1994). It can bind to double and single stranded DNA in a sequence-specific manner (Wolffe, 1994), but shows preference for duplex DNA enriched with pyrimidines and purines on opposite strands (Ozer et al., 1990 and Sakura et al., 1988). Diverse functional roles have attributed the Y-box raging from prokaryotic cold shock response to eukaryotic transcription factors, chromatin modification proteins, DNA repair proteins, RNA packaging proteins and translational repressors (Wolffe, 1994).
A correlation has been developed between YB-1 expression and the development of malignant phenotype in several tumours such as breast cancer (Bargou et al., 1997), osteosarcoma (Oda et al., 1999), colorectal carcinoma (Shibao et al., 1999) and ovarian serous adenocarcinoma (Kamura et al., 1999). In addition, the presence of YB-1 has been detected in HeLa cells (cervical cancer cells) by Shamsuddin (2002), and HPV 18 enhancer oligonucleotide was used as the binding material to detect the presence of YB-1 (Spitkovsky et al., 1992). Besides that, Chernukhin et al., (2000) have revealed the interaction YB-1 with CTCF towards the suppression of c-myc gene. However, there were no reports indicating the mechanism of c-Myc expression in cervical carcinoma samples correlating with HPV oncogenes.

1.1.4 Synergistic effect of CTCF and YB-1 towards c-myc oncogene

CTCF was known to bind to the CCCTC nucleotide sequence of c-myc promoter and YB-1 was known bind to CTCF and synergistically represses the c-myc expression (Lobanenkov et al., 1990; Chernukhin et al., 2000) (Figure 1.1). This repression was able to control the cell cycle progression and allow the cells to mature in natural condition before dividing. However, in cervical carcinoma cells which have been infected with HPV 16 and 18 the expression of c-myc was known to be high (Abba et al., 2004). Furthermore, the presence of E7 oncoprotein was tightly associated with increased level of c-myc expression compare to normal cells (Gewin and Galloway, 2001; Oh et al., 2001; DeFilippis et al., 2003; Lui et al., 2007). Therefore, this research
was carried out to study the effect of E7 oncoprotein of HPV 16 and 18 with CTCF~YB-1 complex correlating with the expression of c-myc.

**Figure 1.1:** The binding of CTCF~YB-1 complex to the promoter of c-myc gene and down-regulates the expression of c-myc gene (Chernukhin et al., 2000).
1.2 Cervical Cancer

Cervical cancer affects the epithelial cell of the cervix. Cervical cancer is the second most common cancer of women worldwide after breast cancer (Parkin et al., 2001). It is estimated that about 470,000 of new cervical cancer cases occur per annum, which contributes towards 1.4 million of invasive cancer of cervix. Cervical cancer is the leading cause of the death among women in the developing countries with approximately 233,000 deaths per annum (Ferlay, 2000). Meanwhile, in Malaysia cancer of the cervix is ranked as the second major cause of mortality among women after breast cancer. The annual incidence rate of cervical cancer in Malaysia is estimated at 19.7 per 100,000 population and the incidence rate increases after the age of 30 years, when a peak incidence rate at the age of 60-69 years (National Cancer Registry, 2003). For the past 20 years, the Ministry of Health, Malaysia, has recorded an average of 2,200 new cases per year (Ministry of Health Annual Reports 1980-2000).

The etiology of cervical cancer explains how normal columnar epithelial cells of the cervix changes to carcinoma. Cervical cancer develops in the lining of the cervix on the lower part of uterus that elongate to vagina. Normally the exterior of the cervix appears smooth, shiny and moist with a thin layer of mucus coating the surface. However, in appearance it looks like florid exophytic cauliflower-like growth during cancerous stage (Othman, 2003). Cervical cancer is known to progress through multiple processes by increasing severe premalignant dysplastic lesion called cervical intraepithelial neoplasia (CIN) I, II, III and carcinoma in-situ, thus progresses to invasive carcinoma. The low grade CIN and high grade CIN resembles minimal and greater
degree of abnormality (Iwasaka et al., 1998). In nature, columnar epithelial cells transform to normal squamous epithelial cells (mature metaplastic) by induction of estrogen hormone and acidic vaginal condition. During this transformation stage, exposure to the carcinogens and human papillomavirus (HPV) infection contributes towards CIN. The carcinogens are chemicals, smoking, alcohol, contraceptive, radiation and infection of human papillomavirus (HPV) (Coker et al., 1992; Parazzini et al., 1998; Franco et al., 1999). A PCR-based study showed that 99% of invasive cervical cancer contains high risk HPV DNA (Munoz, 2000). High risk HPV type 16 and 18 are known as high risk HPV because they are strongly associated with cervical carcinoma (zur Hausen, 2002).

The high risk HPVs exerts their oncogenicity by constitutively expressing two major oncoproteins E6 and E7 (Finzer et al., 2002). The interaction of E6 with p53 causes rapid p53 degradation in a ubiquitin-dependent manner resulting in cell resistance to apoptosis and hence chromosomal instability (Scheffner et al., 1993; Rapp and Chen, 1998; Finzer et al., 2002; zur Hausen, 2002). Meanwhile, E7 binds to pRB and causes the release of E2F from E2F/pRB complex, subsequently promotes cell cycle progression (Morris et al., 1993; Boyer et al., 1996; Morozov et al., 1997). It has been demonstrated that high-risk HPV E6/E7 can efficiently immortalize human primary cells (Shiga et al., 1997; Thonemann and Schmalz, 2000). Besides that, the high risk HPV E7 proteins is able to induce DNA synthesis in quiescent or differentiated cells, thus transforms primary baby rat kidney cells (Phelps et al., 1988; Crook et al., 1989; Morozov et al., 1997).
**Figure 1.2:** Pathophysiology of squamous cell carcinoma of the cervix. Hormonally induced eversion of the cervix and acidic vaginal environment encourage the development of the transformation zone. In physiological conditions, benign squamous metaplasia is the eventual outcome. In the presence HPV 16 and 18, the benign metaplasia process is diverted into a malignant transformation, resulting first in increasingly severe grades of cervical intraepithelial neoplasia (CIN) and then, progress to invasive squamous cell carcinoma. (Modified from Robboy et al., 2000).
1.2.1 Cervical cancer and cellular mechanism

The development and progression of cervical carcinoma has been shown to be dependent on various cellular genetic and epigenetic events, especially alterations of the cell cycle machinery at various checkpoints. The precise control of the cell cycle in mammalian cells is regulated by the activity of cyclin-dependent kinases (CDK1, CDK2, CDK4, CDK6) and their essential activating co-enzymes, the cyclins (cyclins A, B, D, E). The kinase activities of these CDKs are regulated by the abundance of their partner cyclins, phosphorylation by various kinases, de-phosphorylation by cell cycle phosphatases, and interaction with CDK-inhibitory proteins (CDKIs) [Funk, 1999; Clarke and Chetty, 2001; Milde-Langosch and Riethdorf, 2003].

The CDK family is an important group of molecules that regulate cell proliferation. In addition, two classes of mammalian cyclin-dependent kinase inhibitors (CDKIs) have been described: the CIP/KIP family, comprised of p21, p27, and p57, and the INK4 family, comprised of p15, p16, p18, and p19 (Sherr and Roberts, 1999). The INK4 molecules specifically inhibit cyclin D complexes by interaction with the CDK4 and CDK6 components. The KIP family is promiscuous, affecting cyclin E, cyclin A/CDK2, and cyclin B/CDK1 by binding to both cyclin and CDK subunit (Clarke and Chetty, 2001). CDKs, cyclins, and CDKIs generally function within several defined pathways, including the p16\textsuperscript{INK4A}-Cyclin D1-CDK4/6-pRb-E2F pathway, the p21\textsuperscript{WAF1}-p27\textsuperscript{KIP1}-cyclinE-CDK2 pathway, and the
p14ARF-MDM2-p53 pathway (Semczuk and Jakowicki, 2004). Each of these components plays either a positive or a negative role in cell-cycle control mechanisms in cervical carcinogenesis. Besides that, alterations in CDKs, CDKIs, and cyclins can lead to uncontrolled proliferation and might contribute to malignant transformation of the uterine cervix (Kim and Zhao, 2005).

1.2.1.1 The p16INK4A-cyclin D1-CDK4/6- pRb-E2F pathway

1.2.1.1.1 p16 INK4A

The p16INK4A gene maps to 9p21 and contains three exons, which encodes a nuclear phosphoprotein with a molecular weight of 16 kDa. The p16 protein functions in the negative regulation of the cell cycle through the inhibition of cyclin-dependent kinases (CDK) 4 and 6, and interactions with cyclin D1 (Murphy et al., 2004). In the absence of p16, CDKs bind to cyclin D1, and the Retinoblastoma protein (pRb) is phosphorylated. Phosphorylation of pRb leads to its deregulation at the G1/S checkpoint, thus cell proliferation is switched on. In a variety of human malignant tumors and cell lines, the p16INK4A gene is inactivated by various genetic mechanisms, including point mutations, homozygous deletions, and hypermethylation of CpG islands in the p16INK4A promoter (Kim and Zhao, 2005). Kim et al., (1998) found a high percentage of p16 exon 2 mutations in cervical cancer specimens. Meanwhile, Dong et al., (2001) and Nuovo et al., (1999) described the hypermethylation of p16 promoter and
documented inactivation of the gene as a frequent epigenetic event in cervical carcinoma.

The portion of p16-positive samples increases as the tumor progresses from the CIN I to the invasive carcinoma stage (Klaes et al., 2001). This indicates over-expression of the p16 protein is a characteristic of dysplastic and neoplastic alterations of the cervical epithelium. Some reports have shown that p16 expression is detectable by immunohistochemistry in cervical neoplasia and this expression may be a direct result of HPV infection with inactivation of pRb, which is known to bind with p16 (Nuovo et al., 1999; Klaes et al., 2001). Squamous cell carcinomas (SCCs), high-grade squamous intraepithelial lesions (HSILs), and adenocarcinomas (ACs) of the cervix have shown increased p16 immunostaining (Marjoniemi, 2004). Klaes et al., (2001) postulated p16 expression was restricted to cervical cancer, CIN 2-3, and those cases of CIN 1 associated with high-risk HPV types only.

The interaction of p16 with pRb is thought to be central role of p16 in controlling cell cycle progression. It has been suggested that HPV infection leads to HPV-E7 binding to pRb, which in turn results in increased p16 expression. Some studies have suggested that p16 expression is induced by E2F factor, which disassociates from pRb due to the interaction of pRb with high risk HPV E7 oncoprotein (Giarre et al., 2001). The p16 has been used as a biomarker for dysplasia in the diagnosis of cervical squamous lesions and has the
potential to be used as an additional screening tool (Von Knebel, 2001; Murphy et al., 2003; Murphy et al., 2004). The overexpression of p16 is closely associated with high-risk HPV infection and high-grade CIN (Guo et al., 2004). A recent report showed that in cervical biopsy specimens, the staining pattern of p16 and a high percentage of p16-positive cells are closely related to infection with high-risk HPV types 16 and 18, and with CIN 2/3 (Saqi et al., 2003; Hu et al., 2005).

1.2.1.1.2 Cyclin D1

The cyclin D1 (PRAD-1, CCND-1) gene maps to 11q13 and shows the characteristics of a cellular oncogene. Expression of cyclin D1 moderately oscillates throughout the cell-cycle, reaching peak levels in G-phase (Semczuk and Jakowicki, 2004). Cyclin D1 serves as a key sensor and integrator of extracellular signals in early to mid-G1 phase, mediating its function through binding with CDKs, histone acetylase, and histone deacetylases to modulate local chromatin structure around the genes that are involved in regulation of cell proliferation and differentiation. Genetic aberrations in the regulatory circuits that govern transit through the G1 phase of the cell cycle occur frequently in human cancer, and overexpression of cyclin D1 is one of the most commonly observed alterations (Fu et al., 2004).
The role of cyclin D1 in cervical carcinogenesis is not clearly understood, and controversial results have been described. Cho et al., (1997) found that cyclin D1 levels were significantly lower in HPV-positive HSIL, invasive SCC, or adenocarcinoma compared to HPV-negative cases and normal cervical epithelium, consistent with previous findings (Southern and Herrington, 1998; Bae et al., 2001). Contrary to this, the results of Nichols et al., (1996) described elevated cyclin D1 mRNA levels in invasive cervical cancer that were not associated with increased protein amounts. In addition, there was no significant increase in cyclin D1 protein levels were detected in cervical carcinoma, despite overexpression of cyclin D1 mRNA demonstrated by in-situ hybridization (Cho et al., 1997; Bae et al., 2001).

Few studies showed that the level of cyclin D1 was significantly lower in HPV-positive LSIL, HSIL, invasive SCC and AC compared to HPV-negative cases and normal cervical epithelium (Cho et al., 1997; Southern and Herrington, 1998; Psyrri et al., 2004). Cyclin D1 and HPV E7 possess similar binding regions for pRb and pRb-related pocket proteins, and inactivation of pRb either by the cyclin/CDK complexes in G1 phase or by interaction with the high-risk HPV E7 oncoprotein may result in a decreased of cyclin D1 expression (Kim and Zhao, 2005).
1.2.1.3 CDK4

The D-type cyclins (D1, D2, and D3) and their catalytic partners CDK4 and CDK6 act in the early G1 phase of the cell cycle (Clarke and Chetty, 2001). Mitogen-induced signal transduction pathways promote the activation of cyclin D/CDK complexes at different levels such as gene transcription, cyclin D translation and stability, assembly of D cyclins with their CDK partners, and import of the holoenzymes into the nucleus (where they ultimately phosphorylate their substrates). Besides that, cyclin D-dependent kinases (CDK4 and CDK6) can phosphorylate pRb family members (pRb, p107, and p130), thus inactivate their transcriptional corepressor activities (Kim and Zhao, 2005).

Aberrantly expressed CDK4 could play an important role in cervical tumorigenesis. It is postulated that CDK4 oscillates between the INK4 and KIP inhibitors by blocking their suppressor activity. In cervical cancer, it was demonstrated that the level of INK4 is low compared to CDK4, which would favor the binding of more abundant KIP inhibitors to these kinases, and their ability to inhibit of cyclin E is disrupted (Kim and Zhao, 2005). The high risk E7 oncoprotein would deregulate pRb initially and unleashing E2F-induced cyclin E expression, then the over-expressed CDK4 binds to KIP molecules, thus allowing cyclin E to become sufficiently active to phosphorylate and inactivate pRb and p27KIP1 (Grana and Reddy, 1995; Milde-Langosch and Riethdorf, 2003; Sheer and Roberts, 2004).
Yoshinouchi *et al.*, (2000) have found overexpression of CDK4 in 72.6% of cervical cancer specimens and correlates with previous studies of CDK4 expression in cervical carcinoma (Skomedal *et al.*, 1999; Cheung *et al.*, 2001). Phosphorylation of pRb by CDK4 was found to be non-critical in the carcinogenesis or establishment of HPV-positive cervical cancer cell lines, since the HPV oncoproteins E6 and E7 inactivate p53 and pRb tumor suppressor functions, respectively, thus resulting in deregulated progression of the cell cycle (Yoshinouchi *et al.*, 2000). Interestingly, very recent work has indicated that cyclin D/CDK4 complexes phosphorylate Smad3, thus negatively regulates the functions of transcriptional complexes that mediate cell growth inhibition by TGF family proteins (Matsuura *et al.*, 2004). Importantly, several lines of evidence indicate that cyclin D/CDK complexes play a second non-catalytic role in G1 progression by sequestering proteins of the Cip/Kip family, including p27KIP1 and p21WAF1/CIP1, two potent inhibitors of CDK2 (Toyoshima and Hunter, 1994; Hall *et al.*, 1995).

1.2.1.4 pRb/E2F

The retinoblastoma tumor suppressor gene (Rb) encodes a nuclear phosphoprotein known as pRb, which has been found mutated or deleted in several types of human cancer. The pRb and other pRb family members such as p107 and p130 regulate the activity of E2F transcription factors (Lukas *et al.*, 1994). Complexes consisting of E2F and hypophosphorylated pRb repress the transcription of genes that are required for cell cycle progression, and repression is relieved by CDK-mediated phosphorylation of pRb (Salcedo *et al.*, 2002). The pRb protein is subjected to regulation by many
factors, including E2F and cyclin D1. The hypophosphorylated pRb, complexed with a transcription factor, serves as a transcriptional activator of cyclin D1 by binding to its promoter (Muller et al., 1994). On the other hand, inactivation of pRb by phosphorylation via the cyclin D/CDK complex in late G₁ would not only unleash E2F transcription factors, but would also decrease cyclin D1 expression (Muller et al., 1994; Clarke and Chetty, 2001). High risk HPV E7 oncoprotein was found to interact with hypo- or hyper-phosphorylated pRb (Milde-Langsosch and Riethdorf, 2003). Therefore, this oncoprotein occupies the pRb pocket and displace E2F factors, thus preventing pRb/E2F from inducing cyclin D1 transcription and undermining its normal growth-suppressive function (Salcedo et al., 2002; Fiedler et al., 2004).

Both binding and degradation of the Rb proteins by the HPV E7 protein are essential for sustained proliferation of HeLa cervical carcinoma cells, and E7 repression triggers senescence at least in part by activating the Rb pathway in both HeLa and HT-3 cells (Lukas et al., 1994). Besides that, pRb immunostaining using invasive cervical lesions was frequently lower than in SIL. This low level of Rb expression might be resulted from Rb gene mutations or down-regulation mechanisms, but may also be related to pRb inactivation resulting from complex formation with high-risk HPV E7 oncoproteins (Salcedo et al., 2002). On the other hand, mutations in the Rb gene seem to be rare events in cervical cancer (Choo and Chong, 1993; Sano et al., 1998). In most studies, Rb gene expression did not strictly correlate with the HPV status (Chetty et al., 1997).
1.2.1.2 The p21\textsuperscript{WAF1/CIP1}-p27\textsuperscript{KIP1}-cyclin E-CDK2 pathway

1.2.1.2.1 p21\textsuperscript{WAF1/CIP1}

The p21\textsuperscript{WAF1/CIP1} is a cyclin-dependent kinase inhibitor that associates with a class of CDKs and inhibits their kinase activities, leading to cell cycle arrest and the dephosphorylation of pRb. A large body of evidence suggests that p21\textsuperscript{WAF1/CIP1} plays an important role in cell fate decisions during growth and differentiation (Kim and Zhao, 2005). The p21\textsuperscript{WAF1/CIP1} protein is a p53-inducible protein that inactivates the cyclin/CDK complexes, blocking the cell cycle progression in the G\textsubscript{1}-S transition. However, p21\textsuperscript{WAF1/CIP1} is expressed in cells undergoing either G\textsubscript{1} arrest or apoptosis by p53-dependent or -independent mechanisms (El-Deiry \textit{et al.}, 1994; Michieli \textit{et al.}, 1994).

The p21\textsuperscript{WAF1/CIP1} expression usually correlates with favorable prognosis in ovarian, gastric, colorectal, and superficial bladder cancers and in esophageal squamous cell carcinoma (Kim and Zhao, 2005). However, in cervical cancer, the conclusions about p21 expression and its prognostic importance vary considerably. Many authors found increased p21 expression in invasive carcinomas (Skomedal \textit{et al.}, 1999) and elevated number of p21-expressing cases during the progression from normal epithelia through precancerous lesions to invasive cervical cancer (Lie \textit{et al.}, 1999; Bae \textit{et al.}, 2001). However, others have detected under-expression of p21 in micro-invasive and invasive cervical cancer compared to normal cervical epithelium (Kim \textit{et al.}, 1998; Huang \textit{et al.}, 2001).
Meanwhile, Van de Putte et al., (2004) did not find any expression of p21 in normal squamous cervical epithelium, in agreement with Giannoudis and Herrington (2000) and Skomedal et al., (1999) but in contrast with other reports (Kim et al., 1998; Bae et al., 2001). However, the p21 level might be increased in a futile attempt to overcome its impaired or bypassed function. In squamous cell carcinoma, its function could be impaired through the inactivation of p21 by the HPV-16 E7 oncoprotein (Bae et al., 2001). On the other hand, (Lu et al., 1998) reported that expression of p21WAF1/CIP1 was correlated with a favorable prognosis in adenocarcinoma of the uterine cervix.

1.2.1.2.2 p27KIP1

The p27KIP1 is a negative regulator of the G1 phase of the cell cycle. The p27KIP1 gene is a tumor suppressor gene and is frequently lost in tumor cells. It has been implicated in the negative regulation of cell proliferation in response to extracellular signals and is induced upon serum deprivation. In normal epithelial cells, increased expression of P27KIP1 mediates the arrest of cells in the G1 phase of the cell cycle when induced by TGF-β (Sgambato et al., 2000). It was found that p27KIP1 associates mainly with the cyclin E/CDK2 and this complex inhibits pRb phosphorylation, thus, p27KIP1 blocks the cell from entering S phase (Shiozawa et al., 2001). Although p27KIP1 is a putative tumor-suppressor gene, mutation or homozygous deletion of this gene is rarely found in human cancer (Huang et al., 2002). Expression of p27KIP1 protein correlates with human tumor progression and Huang et al., (2002) demonstrated that decrease or
absences of $p27^{\text{KIP1}}$ protein expression was associated with more aggressive clinical behavior in a variety of human tumors, including breast, lung, colon, gastric, and ovarian carcinoma (Huang et al., 2002)

Goff et al., (2003) found that $p27^{\text{KIP1}}$ was strongly expressed in both the epithelium and stroma of normal cervix, which correlate with other investigators. Shiozawa et al., (2001) revealed that the expression of $p27^{\text{KIP1}}$ in the normal squamous epithelium of the uterine cervix was observed mainly in the intermediate and superficial layers cells, but enlighten weaker expression in the parabasal cells, which replicate most actively. This is consistent with a previous study conducted by Troncone et al., (1999). The trend of reduced $p27^{\text{KIP1}}$ expression in microinvasive and invasive carcinomas further supports the notion that $p27^{\text{KIP1}}$ plays a tumor suppressor function during neoplastic transformation in cervical epithelium. Sgambato et al., (2004) demonstrated that a decrease in $p27^{\text{KIP1}}$ expression is associated with the development of cervical cancer and may play an important role in the early stage of cervical tumorigenesis (Sgambato et al., 2004). In fact, $p27^{\text{KIP1}}$ expression has been reduced in pre-invasive lesions of the cervix and became progressively more evident during the progression from low- to high-grade SIL and from SIL to carcinoma. This result of $p27^{\text{KIP1}}$ expression in cervical carcinomas is in agreement with similar findings in several other types of human malignancies (Sgambato, et al., 2000) and with previous reports of cervical cancers (Troncone et al., 1999; Shiozawa, 2001).
1.2.1.2.3 Cyclin E

Cyclin E exists in two isoforms with high homology, designated cyclin E1 and E2. There are no major differences in expression or functions between both have been found, and their expression has been assumed to be governed by the same molecular circuitry (Geng et al., 2001; Erlandsson et al., 2003). Cyclin E, whose catalytic partner is CDK2, is another rate-limiting regulator of the G₁ phase of the cell cycle (Dirks and Rutka, 1997). Appropriate regulation of this cyclin is essential for S-phase transition and numerous processes that determine the accuracy of chromosome replication. It can play a role similar to that of cyclin D1, driving the cell cycle by phosphorylation of Rb, p107, and p130 and the subsequent release of E2F and transcription of key proteins. Cyclin E appears in late G₁ after passage through the restriction point and disappears again in early S phase (Kim and Zhao, 2005).

Even though gene amplification and post-transcriptional modification are the common causes of aberrant cyclin E expression in different malignancies, high-risk-HPV oncoprotein-associated mechanisms might contribute to the aberrant cyclin E expression in cervical lesions. Releasing of E2F factors from pRb by the action of the HPV E7 protein could result in the over-expression of cyclin E. This connection, however, was not enough to explain the highly expressed cyclin E in cervical epithelium. The HPV E7 oncoprotein was demonstrated in complexes with cyclin E and cyclin A, thus eliminating phase-dependent variation in activity. Also, the HPV E7 was found to interact with p21 and thereby block p21-mediated inhibition of cyclin E-associated kinase activity (Zerfass-Thome et al., 1996).
In normal cervical squamous epithelium, cyclin E expression was not immunohistochemically detectable or was very weak (Cho et al., 1997; Quade et al., 1988; Kim et al., 2000; Shiozawa et al., 2001; Southern et al., 2001). However, cyclin E expression was increased in both low- and high-risk HPV-infected squamous and glandular lesions (Cho et al., 1997; Dellas et al., 1998). Cyclin E expression correlated strongly with morphologic features of the HPV-infected lesions, and has been observed in intermediate, and partly in superficial, cells from LSIL and HSIL, implying sequential dysregulation (Southern et al., 2000). Cyclin E over-expression or over-activity may be the direct result of the presence of the HPV E6 and E7 proteins in invasive disease. The HPV E7 oncoprotein inactivates p27 and leads to decrease inhibition of cyclin E, thus promotes its expression (Southern et al., 2001).

1.2.1.2.4 CDK2

The CDK2 functions as an effector through phosphorylation of key substrate such as the transcription factor p53, the cell cycle regulating phosphatase cdc25A/cdc25C, MDM2, BRCA1 and the transcription factor E2F1 (Castedo et al., 2004). E-type cyclins (E1 and E2) govern the activity of the single catalytic subunit of CDK2. Unlike various combinations of D-type cyclins that are expressed in different cell types, cyclin E-CDK2 complexes are periodic and maximal at the G1 to S phase transition (Dulic et al., 1992). Cyclin E-CDK2 also preferentially phosphorylates pRb at different sites from the cyclin D-dependent kinases, and these modifications may differentially impact on the interactions of pRb with E2Fs, histone deacetylases,
and other chromatin-remodeling proteins (Harbour and Dean, 2000). One of the most significant consequences of pRb inactivation is activation of cyclin E/CDK2 subunits, often as a result of increased cyclin E expression (Kim and Zhao, 2005).

The HPV oncoprotein E7 may abrogate p21-mediated sequestration of cyclin E and render its catalytic partner CDK2 resistant to p27 (Jones et al., 1997). Shiozawa et al., (2001) revealed that no p27 expression was observed in atypical epithelial cells of SIL with elevated expression of Ki-67, cyclin E, and CDK2. The expression of cyclin-dependent kinases (CDK2 and cdc2) was also increased in a considerable number of invasive cervical carcinomas (Kanai et al, 1998; Shiozawa et al., 2001). In those cases, p27 expression was also high and retained the ability to bind to CDK2, but the p27~cyclin E/CDK2 complex still possessed phosphorylating activity (Shiozawa et al., 2001). CDK2 also regulates the cell cycle through its interactions with cyclin A2, thus its functions extend beyond G1 regulation to govern events in S and G2/M as well. Whereas maximal periodic cyclin E-CDK2 activity is detected at G1/S, low levels of cyclin A-CDK2 activity are first detected in late G1 phase. Consistent with crucial roles for cyclin E/CDK2 downstream of pRb, many tumor cells are extremely sensitive to inactivation of cyclin E/CDK2 whether or not they express pRb. This conclusion has been drawn from a multitude of studies demonstrating antiproliferative effects of overexpression of p27\textsuperscript{KIP1}, a protein inhibitor of CDK2, or of dominant-negative CDK2 subunits (Knockaert et al., 2002; Tetsu and McCormick, 2003).
1.2.1.3 The p14\textsuperscript{ARF}-MDM2-p53 pathway

1.2.1.3.1 P14\textsuperscript{ARF}

The CDK\textsuperscript{N2A} gene on human chromosome 9p21 encodes two distinct proteins, p14\textsuperscript{ARF} and p16\textsuperscript{INK4A}, which arise from the same gene by alternative mRNA splicing (Kanao et al., 2004). The p14\textsuperscript{ARF} has a mass of 15 kDa and is translated from mRNAs bearing a unique first exon, called 1-beta, and located 15 kb upstream of the exon 1-alpha of p16\textsuperscript{INK4A}. Both transcripts share common exons 2 and 3. The p14\textsuperscript{ARF} functions as a cell cycle regulator, stopping cell growth at the G\textsubscript{1}-S border and also at G\textsubscript{2}-M (Quelle et al., 1995). The p14\textsuperscript{ARF} interaction inhibits the MDM2 oncoprotein, thereby blocking formation of the MDM2-p53 complex and preventing MDM2-induced p53 degradation (Zhang et al., 1998). Kanao et al., (2004) reported that the over-expression of p14\textsuperscript{ARF} and p16\textsuperscript{INK4A} is strongly associated with HPV-positive cervical cancers and that reduced expression of p14\textsuperscript{ARF} and p16\textsuperscript{INK4A} is correlated with HPV-negative cervical cancers. These findings may indicate that impaired p14\textsuperscript{ARF} and p16\textsuperscript{INK4A} mRNA expression contribute to tumor development in HPV-negative cervical cancers by failure to support p53 and Rb instead of their inactivation by HPV E6 and E7.

In human fibroblasts, it has been shown that p14\textsuperscript{ARF}-induced cell cycle arrest is p53-dependent and is abrogated by the co-expression of the HPV E6 protein (Stott et al., 1998; Midle-langosch et al., 2001). Therefore, p14\textsuperscript{ARF} up-regulation was a consequence of inactivation of p53 rather than a sign of cell cycle inhibition. Brooks et al., (2002) demonstrated that the expression levels of