ความสัมพันธ์ระหว่างโปรตีน E6 ของ HPV กับการแสดงออกของโปรตีนในกลุ่ม ΔNp63

นางสาวศริตา ญาณพินิต

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ASSOCIATION BETWEEN HPV E6 PROTEIN AND EXPRESSION OF PROTEINS IN ΔNp63 FAMILY

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ศริตา ญาณพินิต : ความสัมพันธ์ระหว่างโปรตีน E6 ของ HPV กับการแสดงออกของโปรตีนในกลุ่ม ΔNp63 (Association between HPV E6 protein and expression of proteins in ΔNp63 family) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รองศาสตราจารย์ ดร. ภาวพันธ์ ภัทรโกศล 104 หน้า.

มะเร็งปากมดลูกเป็นปัญหาสุขภาพที่สำคัญของสตรีทั่วโลก โดยมะเร็งปากมดลูกส่วนใหญ่พบมีการติดเชื้อไวรัสแปปิโลมากลุ่มที่มีความเสี่ยงสูงร่วมด้วย โปรตีน E6 ของเชื้อไวรัสแปปิโลมาในกลุ่มที่มีความเสี่ยงสูงมีบทบาทสำคัญในการพัฒนาเนื้องอก

ΔNp63 ไอโซไทป์ คือโปรตีน p63 เป็นกลุ่มโปรตีนที่อยู่ในครอบครัวเดียวกับ p53 แต่ไม่มีส่วนปลายด้าน NH_2 การศึกษาก่อนหน้านี้พบว่า ΔNp63 ทำหน้าที่ตรงข้ามกับการตรึงวงจรเซลล์ (cell cycle arrest) และการเกิดภาวะเซลล์ตายแบบ apoptosis ΔNp63 มีความสัมพันธ์กับการเกิดความผิดปกติของเนื้อเยื่อบริเวณปากมดลูก จึงเป็นไปได้ว่า ΔNp63 อาจเพิ่มความรุนแรงของโรคมะเร็งปากมดลูก โดยทำหน้าที่ร่วมกับโปรตีน E6 ของเชื้อไวรัสแปปิโลมา

ในการศึกษานี้ได้มีการสร้างสายพันธุ์เซลล์ที่มีการแสดงออกของโปรตีน ΔNp63 ไอโซไทป์ และโปรตีน E6

ทำการตรวจสอบคุณสมบัติของเซลล์โดยการดูการตายและการเพิ่มจำนวนของเซลล์ที่เปลี่ยนแปลงไป จากผลการศึกษาพบว่า HPV16E6, ΔNp63α และ ΔNp63β มีบทบาทในการยับยั้งการตายของเซลล์ที่ถูกเหนี่ยวนำด้วยแคมโตเธซิน ขณะที่ ΔNp63γ มีบทบาทในการเพิ่มจำนวนเซลล์ ดังนั้น ΔNp63 ไอโซไทป์ แต่ละชนิดมีหน้าที่เกี่ยวข้องกับคุณสมบัติการตายและ/หรือการเพิ่มจำนวนเซลล์ โดยมีประสิทธิภาพสูงขึ้นเมื่อมี HPVE6 ร่วมด้วย

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2552 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

4989193120 : MAJOR INTER-DEPARTMENT MEDICAL MICROBIOLOGY
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SARITA YANPINIT: ASSOCIATION BETWEEN HPV E6 PROTEIN AND EXPRESSION OF PROTEINS IN ΔNp63 FAMILY. THESIS ADVISOR : ASSOCIATED PROFESSOR PARVAPAN BHATTARAKOSOL, Ph.D., 104 pp.

Cervical cancer is an important health care problem of women worldwide. Infection with high risk human papillomaviruses (HPV) was the commonest incidence. High risk HPV E6 protein plays the critical roles in tumor development. ΔNp63 isotypes are p63 proteins in p53 family which lacks NH_2-terminal domain. The previous studies suggested that ΔNp63 variants showed the opposite function to cell cycle arrest and apoptosis. ΔNp63 related to cervical intraepithelial neoplasia (CIN) and may increase the severity of cervical cancer when working with HPV E6 protein.

In this study the stable cell lines expressing both ΔNp63 isotypes and HPV E6 proteins were generated by transfection. The transfected cells were selection with appropriate drugs. Cell death detection and cell proliferation assays were performed. HPV16E6, ΔNp63α and ΔNp63β showed ability to inhibit apoptosis induced by camptothecin while HPV16E6 and ΔNp63γ played the role in cell proliferation. This study suggested that ΔNp63 isotypes played role in the ability of either apoptosis or proliferation of cells. In association with HPVE6, the efficiency of ΔNp63 increased.

Field of Study : Medical Microbiology Student's Signature .....
Academic Year : 2009 Advisor's Signature .....
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LIST OF ABBREVIATIONS

bax	Bcl-2–associated X protein
bp	Base pair
°C	Degree celsius
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
kDa	Kilodalton
µl	Microliter
ml	Milliliter
µg	Microgram
IgG	Immunoglobulon G
LB broth	Lauria-Bertani broth
mM	Millimolar
mdm2	Murine double minute 2
ng	Nanogram
NOXA	Gene encoding a pro-apoptotic member of the Bcl-2 protein family
PE	Phycoerythrin
PREP	Gene encoding prolyl endopeptidase
SAM	Sterile alpha motif
SDS	Sodium dodecyl sulfate
TEMED	Tetramethylethylenediamine

CHAPTER I

INTRODUCTION

Cervical cancer is an important health care problem of women worldwide. Cancer of uterine cervix was found to be the second most common incidence among female in less developed countries with an estimated 275,000 cancer death. Infection with high risk human papillomaviruses (HPV) was the commonest incidence [1]. Approximately 70% of cervical cancer worldwide are associated with the high risk HPV genotypes 16 and 18 [2]. High risk HPV E6 protein plays the critical roles in tumor development. E6 protein interacts with many cellular proteins that regulate cell cycle and interferes with both extrinsic and intrinsic pathways of apoptosis. E6 protein down-regulates activity of p53, the tumor suppressor protein by inducing p53 to the ubiquitin degradation pathway. However, E6 can interfere with p53 function through other mechanisms. Beside the degradation, its interaction prevents p53-mediated transcriptional suppression of TATA-containing promoters and represses p53's transactivation of promoters containing p53 responsive element [3-6]. The p53 protein responds to stress signals such as DNA damage then switches from an inactive state to an active state. The active p53 protein is involved in cell cycle checkpoint [7]. P63 family is the group of proteins which share the similarity of conformational structure and amino acid sequences with p53 proteins which consist of 3 domains: NH_2-terminal transactivating domain, DNA binding domain and COOH-oligomerization domain [8]. P63 proteins located on chromosome 3q27 [9, 10]. The p63 gene contains two promoters that are used for generating p63 proteins with and without NH_2-terminal transactivating domain termed TAP63 and ΔNp63. The alternative splicing of p63 generates 3 isoforms with the different COOH-oligomerization domain termed α, β, and γ. The p63α proteins contain sterile alpha motif or SAM domain. SAM domain was reported to have the function about protein-protein interaction in developmental process [11, 12]. It may involve in other processes such as apoptosis, transcriptional activation, focal adhesion, chromatin remodeling, receptor-tyrosine kinase signaling, and SUMOylation [8]. The studies about biochemical activities of p63 in transient transfection

or adenoviral infection suggested that TAp63 variants could induce cell cycle arrest and apoptosis, while ΔNp63 variants showed the opposite function [13-15]. Studies comparing TA-variants show that TAp63γ stimulates transcription as well as wild-type p53 whereas TAp63α shows little or no transactivating activity. ΔNp63α represses p53- or TAp63γ-mediated transcription, while ΔNp63γ is only able to repress p53-mediated transcription. Carboxy-terminus shows the ability to repress transcription; this evidence proposed that it interacts with the TA-domain of p63 in a region homologous to the MDM2 binding site in p53. ChIP experiments with E1A-expressing mouse embryo fibroblasts (MEFs) revealed *in vivo* p63 binding to the p53 target genes, *mdm2*, *bax*, *PERP*, and *NOXA*. These indicate that p63 involves in regulating the stability of p53 and its apoptotic functions [8, 15]. The study in p63 knockout mice demonstrated the lack of epidermal barrier and the defect in limb and craniofacial development [16]. A study in zebrafish with the specific disruption of ΔNp63α showed a lack of epidermal morphogenesis and fin truncation [17]. Mutation of the human p63 genes is associated with a variety of developmental defects. ΔNp63α was found as a primary p63 variant expressed in squamous epithelial tissue and also determined that it acts antagonistically toward p53 [15]. Over expression of ΔNp63 variant in Rat 1a cells led to increase growth of these cells [18]. The studies in normal cervix showed the expression of ΔNp63 in basal and parabasal cells, whereas the expression of ΔNp63 could be detected in superficial cells of cervical intraepithelial neoplasia (CIN) [19]. ΔNp63α expression is directly correlated with the clinical response to cisplatin in patients with head and neck tumors [20]. The evidences from studying p63 expression in mouse models and human led to two hypotheses concerning p63 function [16]. One hypothesis stated that ΔNp63 is necessary for the regenerative proliferation of epithelial progenitor cells. Another hypothesis is that TAp63 is necessary for the specification and differentiation of squamous epithelial cells. ΔNp63 may increase the severity of cervical cancer by working with HPV E6 protein. To study the correlation between HPV16 E6 protein and the expression of ΔNp63 family, the cell line expressing ΔNp63 and HPV

E6 proteins were generated. The cell death detection and cell proliferation assays were performed.

CHAPTER II

OBJECTIVE

Hypothesis

HPV E6 and Δ Np63 affect cell growth.

Objective

To study the correlation between HPV16 E6 protein and the expression of ΔNp63 family proteins in cell growth.

CHAPTER III
REVIEW OF LITERATURE

Cervical Cancer

Cancer is the uncontrolled growth and spread of cells. The growths often invade surrounding tissues and can metastasize to distant sites. Cancer is called as a modern disease. The data reported by WHO in 2008 demonstrated that there were 12.4 million new cancer cases and 7.6 million cancer deaths worldwide [21]. Cancer of uterine cervix was found to be the second most common incidence among female in less developed countries with an estimated 275,000 cancer death (Figure 1 [21]). Many risk factors of cancer were found in cervical cancer cases either human or environmental factors. However infection with high risk human papillomaviruses (HPV) was the commonest incidence [1].

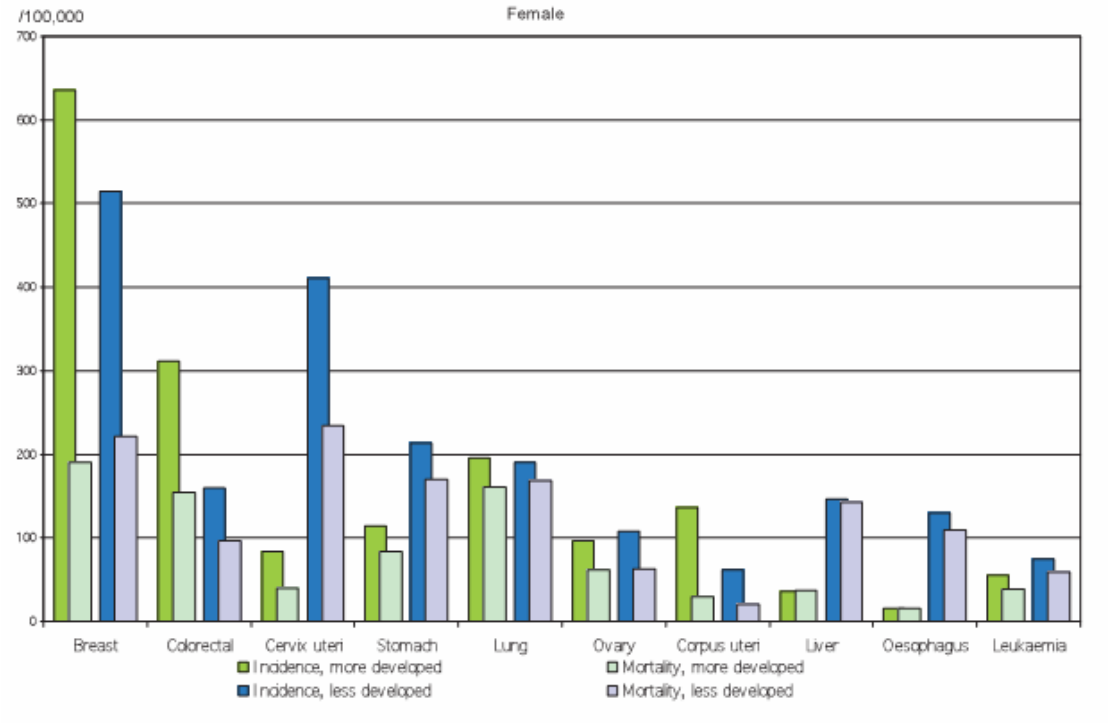

Figure1 The incidence and mortality of the most common cancers in female in developing and developed countries [21].

Human papillomaviruses (HPV)

First discovery of HPV in cervical carcinioma was made by zur Housen and colleagues [1]. The first identified HPV DNA sequence was found in condyloma and cervical carcinoma biopsies and the following data confirmed the correlation of specific HPV genotypes in genital wart and cervical cancer. HPV infection was found in many sites throughout the body such as skin, anogenital tract and respiratory tract.

HPV Structure

Papillomaviruses are small, non enveloped DNA viruses with 55-60 nm in diameter. The capsid is comprised of 72 pentamers of major capsid protein (L1) arranged on a T=7 icosahedral lattice (Figure 2). An internal minor capsid protein (L2) is associated with a subset of the L1 pentamers that form the outer shell [22].

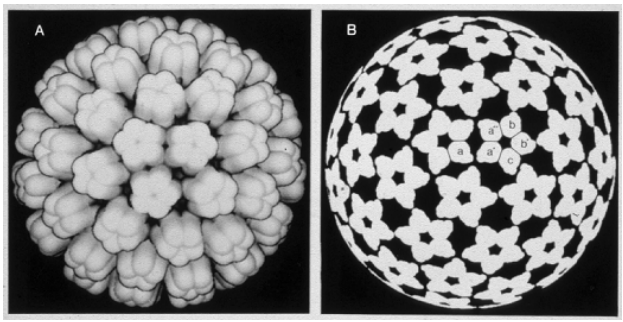

Figure 2 Schematic diagram of the papilloma capsid [22].

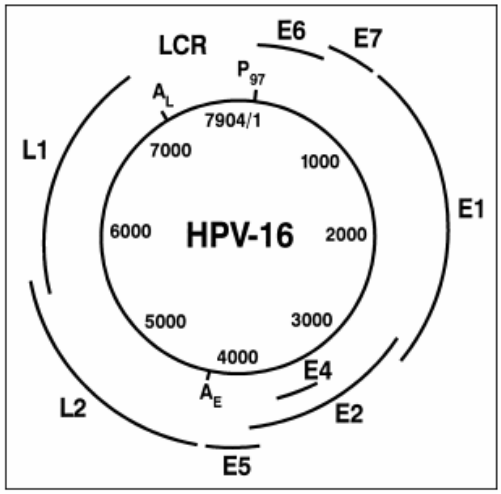

Figure 3 Genes organization in HPV genome [23].

HPV genome is double stranded circular DNA (Figure 3). It is about 8 kb in size containing two sets of genes encoding six early proteins (E1, E2 and E4-E7) that are necessary for the replication of viral DNA and for the assembly of new virus particle within the infected cells. Two late proteins (L1 and L2) are the structural proteins that form the capsid of virus particle. Both sets of genes are separated by the sequences that regulate transcription and replication named long control region (LCR). The functions of HPV proteins are briefly described in table 1.

Table 1 The functions of HPV proteins [24].

Protein	Function	Some of their partner proteins
E1	Helicase essential for viral DNA replication	E2,DNApolymerase α-primase, cyclinE-CDK2
E2	Viral transcription and factor	E1,Sp1,AMF-1,TBP,TFIIB,p53 CBP,RPA
E4	Interacts with cytoskeletal proteins; allows viral assembly	EGF receptor, PDGF receptor, vacuolar ATPase
E5	Weak transforming activity, upregulates growth factor receptor numbers	
E6	Binds p53 and direct p53 degradation by ubiquitin-targeted proteolysis	p53, E6AP, hDLG, hScrib, paxillin, IRF-3 , Bak
E7	Bind pRb with deregulation of the G_1/S check-point in the cell cycle	Rb, p107, p130, TBP, ISGF3, AP1, Mi2ß
L1	Major capsid protein	
L2	Minor capsid protein	

Classification of Human Papillomaviruses

In 1970s papillomaviruses had been classified in the family Papovaviridae based on the similarity of viral capsid that lacks envelope and double-stranded circular DNA genomes. Based on the molecular finding, many researches showed the differences in genome size, genome organization and the homologies of the proteins between polyomaviruses and papillomaviruses. In 2004 the Internationnal Committee on Taxonomy of Viruses (ICTV) was confirmed and classified papillomaviruses in new family named “Papillomaviridae”. In this family major phylogenetic branches formed by remotely related types are referred to “genus” while groups of closely related types are “species”. “Types” are separated by more than 10% of the difference of the nucleotide sequence of L1 gene with other types. Term “subtype” is separated by 2-10 % difference of the sequence of any types. Finally, independent isolates of the same type called “variants” (Figure 4).

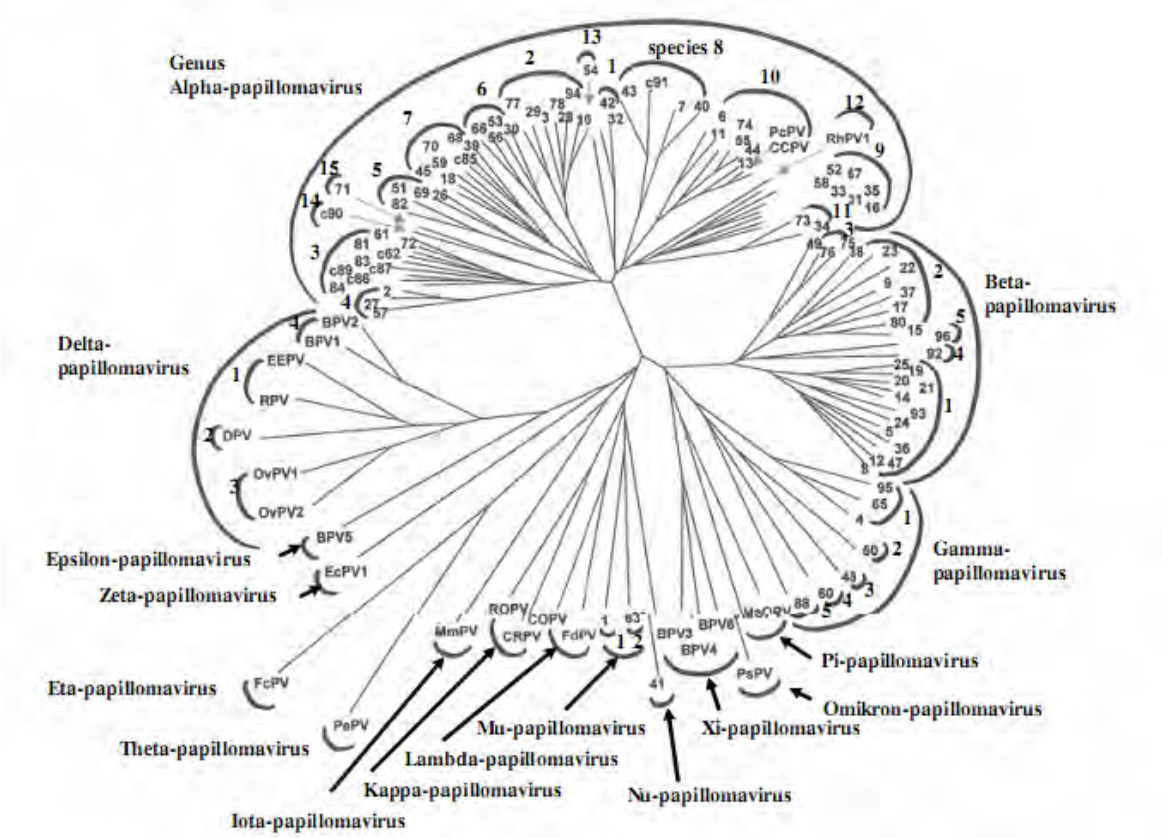

Figure 4 Phylogenetic tree of 118 papillomavirus types. All numbers refer to HPV types, c- numbers to candidate types, ie. the abbreviations including the letters refer to animal papillomaviruses. BPV, bovine PV; COPV, canine oral PV; CRPV, cottontail rabbit PV; DPV, deer PV; EcPV, Equus caballus or horse PV; EEPV, European elk PV; FcPV, Fringilla coelebs or chaffinch PV; FdPV, Felix domesticus or cat PV; HaOPV, hamster oral PV; MnPV, Mastomys natalensis or African rat PV; OvPV ovine or sheep PV; PePV, Psittacus erythacus or African grey parrot PV; PsPV, Phocoena spinipinnis or porpoise PV; RPV, reindeer PV; ROPV, rabbit oral PV. The outermost semicircular symbols identify papillomavirus genera. The inner semicircular symbols identify papillomavirus species [25].

HPV were categorized into two groups by the severity of infection. Low risk HPV genotypes such as HPV types 6, 11, 40, 54 and 61 cause low-grade cervical dysplasia while high risk HPV genotypes such as HPV types 16, 18, 31 and 52 cause cervical cancer. Approximately 70% of cervical cancer worldwide are caused by the high risk HPV genotypes 16 and 18 (Figure 5) [2].

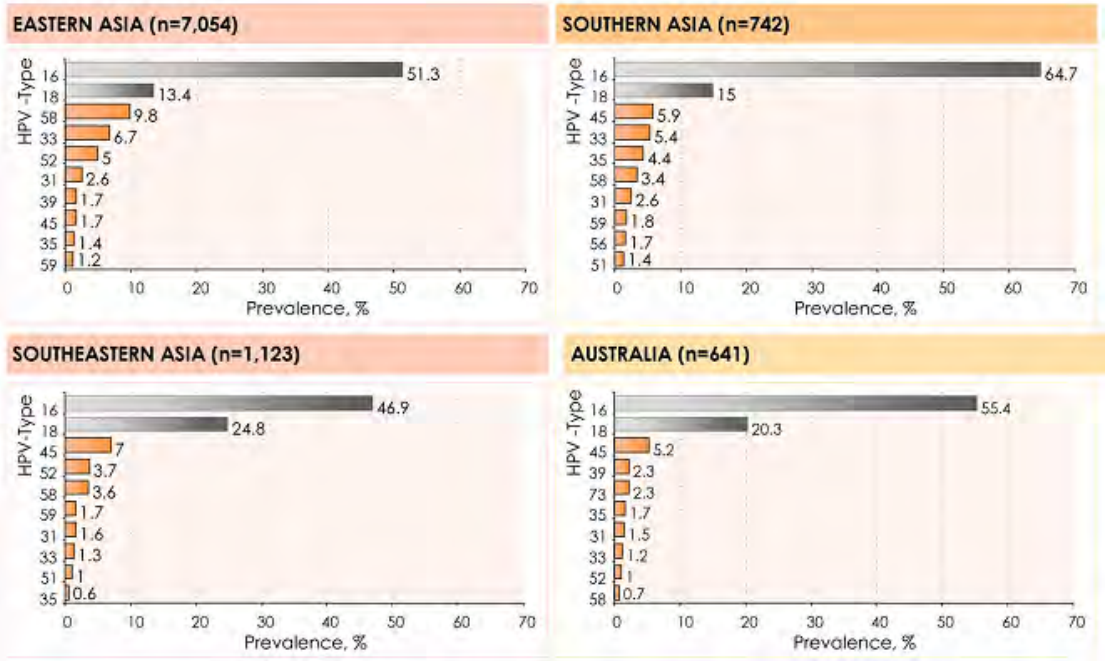

Figure 5 Ten most frequent HPV types in women with normal cytology by Asian sub-region [2].

Pathogenesis of HPV

Pathogenesis of HPV links to the differentiation program of host tissue. HPV infects basal epithelial cells through micro-abrasion. Heparan sulfate proteoglycans (HSPGs) serve as primary receptor for HPV. The virions are internalized via a clathrin-dependent endocytic pathway. Reduction of intracapsomeric disulfide bonds and acidification of endosomes trigger uncoating of the viral genome. L2 protein mediates endosomal escape and may chaperone the viral genome to the nucleus. HPV genome

maintains in the basal cell as multi carry extra chromosomal plasmid at approximately 20 to 100 copies per infected keratinocytes by using the host cell mechanism. Viral gene expression in high-risk HPV types is mediated by major promoter and a series of minor promoters. The major promoter is active in differentiated basal cells as well as most differentiated suprabasal cells. Early HPV E1, E2, E4, and E5 genes are expressed. In high-risk HPV types, differential expression of E6 and E7 is mediated through a single primary transcript that is alternatively spliced in the E6 gene while low-risk HPV have two early promoters: one initiates transcription upstream of E6 open reading frame while second is within E7 gene. In midzone and superficial zone of epithelium, the viral genome replicates and the late genes L1 and L2, and E4 are expressed. L1 and L2 are responsible for the virion assembly and DNA packaging. The shed virus can initiate a new infection called transient infection. Low-grade intraepithelial lesions support productive viral replication. The progression of untreated lesion in microinvasive and invasive cancer is associated with the integration of HPV genome into the host chromosome. To integrate into host chromosome, the viral genome most break in E1/E2 region. Disruption of E1/E2 region leads to the loss of virion production and subsequent upregulation of E6 and E7 oncogene expression, LCR, long control region. A persistent infection with a high-risk HPV type is necessary for the development of high grade intraepithelial neoplasia and invasive disease [26].

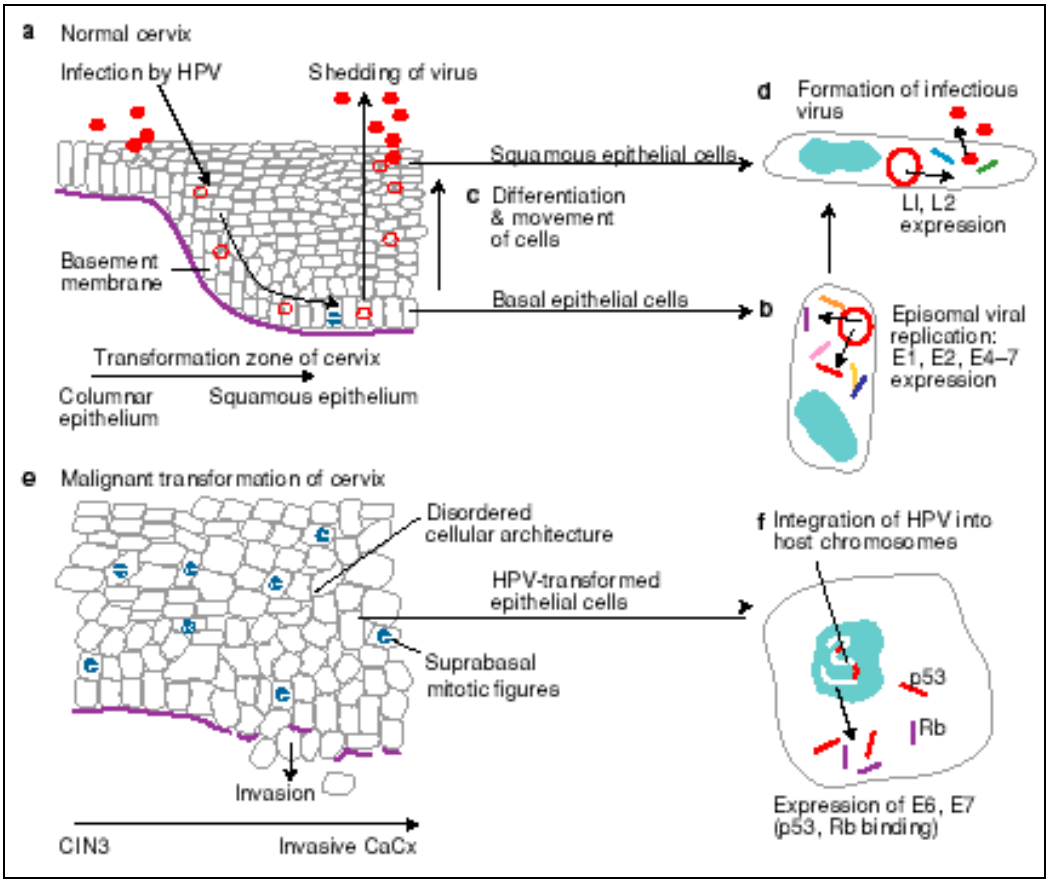

Figure 6 Human papillomavirus (HPV) infection and replication in cervical epithelial cells. (a) HPVs are probably most infectious to cells that are close to transformation zone. (b) HPV viruses gain access to the basal epithelial cells of the cervix via the vagina, where they replicate episomally and express the (early) viral genes E1, E2, E4, E5, E6 and E7. (c) The infected basal cells, which show signs of cell disruption as a result of the viral infection, continue their differentiation and migration to the epithelial surface, where (d) the squamous cells start to express the late HPV genes LI and L2. Infectious virus particles are formed and shed into the lumen of the vagina. (e) HPV infection (particularly with the high-risk types) can progress to: (1) HPV-induced mild dysplasia, (2) the final stages of cervical intraepithelial neoplasia (CIN3) and, eventually, (3) invasive cervical cancer (CaCx), when the basement membrane is breached by the cells, allowing local spread and also distant metastasis. (f) In transformed epithelial cells, HPV genes are integrated into the host chromosomes, with expression of (the oncogenic) E6 and E7 proteins, which bind to the tumour-suppressor proteins p53 and Rb [27].

HPV diseases

HPV is associated with malignant and nonmalignant disease. Normally HPV infects the squamous epithelia of the skin or the mucous membranes, but some types show specific tropisms for the distinct cell types. Due to the anatomic sites-specific HPV prevalences, HPV can be devided into three types: genital-mucosal types, nongenital cutaneous types, and types specific for epidermodysplasia verruciformis [23, 28]. (Table 2)

Table 2 Human Diseases Asssociated with HPV Infection [23].

Site	Diseases
Skin	Deep plantar warts Common warts Mosaic warts Flat warts Butcher's warts Periungual warts
Anogenital tract	Anogenital warts Bowenoid-papulosis Buschke-Lowenstein tumors Cervical, vulvar, and perianal intraepithelial neoplasia Carcinoma of the vulva Carcinoma of the cervix Carcinoma of the anus Carcinoma of the penis
Respiratory tract	Juvenile laryngeal papillomatosis Squamous cell carcinoma of the larynx Squamous cell carcinoma of sinuses

	Squamous cell carcinoma of lung
Other	Conjunctival papillomatosis Carcinoma and keratosis in epidermodysplasia verruciformis Carcinoma of periungual areas Carcinomas associated with deficits in cell-mediated immunity: genetic, iatrogenic, and HIV-related

HPV oncoproteins and cancer induced by HPV oncoproteins

An uncontrollable cell cycle progression and ability to escape the apoptosis pathway are the main factors inducing cancer cell development. The etiology of that is HPV-infection. It is believed that HPV E6 and E7 oncoproteins play important role.

HPV E6 protein

E6 is a small protein, approximately 18 kDa, consisted of 150 amino acids. HPV E6 protein has four Cys-X-X-Cys motifs which have the interaction with two zinc fingers. These motifs are strictly conserved in E6 proteins throughout the HPV family which is important to E6 function [3]. E6 interacts with many cellular proteins that regulate cell cycle. The proteins and their interactions are as follow:

1) E6-AP (E6 associated protein) E6 binds to E6-AP and p53 to form complex. The E6-E6AP-p53 complexes target p53 to degrade through the ubiquitin degradation pathway.

2) E6-BP (binding protein) or ERC-55 E6BP is a putative calcium-binding localized in the endoplasmic reticulum and may play the role in epithelial differentiation.

3) Bak E6 protein of HVP 18, 16 and 11 can bind to Bak protein (pro-apoptotic protein in Bcl-2 family) and stimulate its degradation.

4) Paxillin is a multidomain protein that localizes in the site of cell adhesion to cellular matrix called focal adhesions which form a structural link between the extracellular matrix and the actin cytoskeleton. They are the important sites of signal transduction. The binding of HPV E6 and paxillin results in the disruption of the actin cytoskeleton that impacts cell function.

5) hDlg and hScrib E6 interacts and induces the degradation of the human homologous of two Drosophila PDZ domain-containing protein, hDlg and hScrib which are the tumor suppressor proteins.

6) MAGI-1, -2 and -3 The interactions between HPV E6 and MAGI-1, -2 and -3 result in the degradation of MAGI proteins, the molecular scaffolds in the formation of multimolecular complexes.

7) IRF-3 is the interferin regulatory factor 3. Binding of HPV E6 to IRF-3 inhibits IRF-3 activity as a potent transcription activator.

8) MMP7 is the subunit of replication licensing factor M. It is the substrate of E3-ubiquitin ligase/E6-AP.

9) CBP/p300 E6 which interacts with transcription activator CBP/p300 resulting in down-regulation of p53 transcription activity.

10) Telomerase The interaction between E6 and telomerase stimulates telomerase activity and results in the development of cancer.

11) E6TP1, a Rap GTPase-activating protein homologue, the negative regulator of the mitogenic signaling pathways mediated by Rap family protein. E6 targets E6TP1 for ubiquitination through E6AP and subsequent degraded by the proteasome pathway. The degradation results in cellular immortalization.

12) PKN It is a Rho-regulate serine threonine kinase. Although PKN functions in cytoskeleton remodeling, the interaction of E6 to PKN is not sufficient for immortalizing normal mammary epithelial cells.

13) Gps2 exhibits transcriptional activation activity. E6 induces the degradation of Gps2 in vivo but not in vitro. Factors involved in a variety of cellular regulatory processes.

14) c-myc is a group of structurally related transcriptional factors. Formation of myc-E6-E6AP prevents myc-induced apoptosis and ensures the maintenance of viral infection.

15) orther proteins E6 interacts with other proteins that are important to growth or death mechanism of infected cells such as Tyk2, h-ras and INK4a. [29]

E6 protein interferes both extrinsic and intrinsic pathways of apoptosis.

In extrinsic pathway, E6 binds to FADD and TNF R1 then inhibits TRIAL-induced apoptosis by the degradation of FADD and caspase-8. In some case E6 may inhibit the mitochondrial pathway through the degradation of Bak and Bax which can trigger the releasing of cytochrome c, a small protein which acts as pro-apoptotic stimuli. [30]

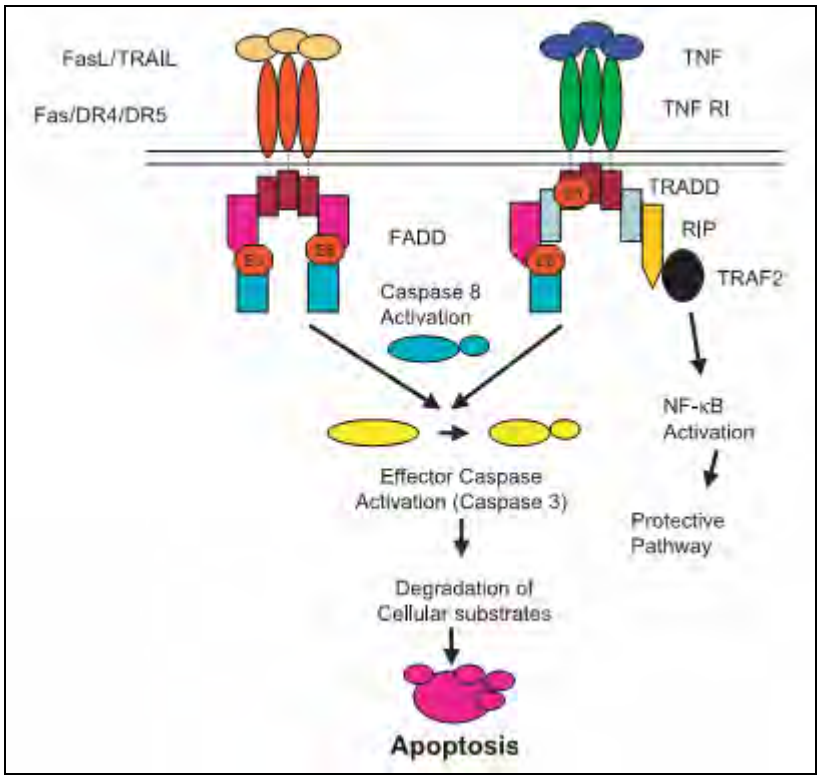

Figure 7 HPV 16 E6 exploits similarities in the apoptotic signaling pathways of TNF, FasL and TRAIL [30].

In intrinsic pathway, HPV E6 protein down-regulates p53 activity by inducing p53 to the ubiquitin degradation pathway. By the help of other cellular proteins, ubiquitin is activated by E1 ubiquitin-activating enzyme and transferred to a ubiquitin conjugating enzyme E2. E6AP and HPV E6 protein form the complex that acts as a ubiquitin ligase E3. E3 ligates ubiquitin to p53 then poly-ubiquitinated p53 targets p53 to ubiquitin degradation pathway (Figure 8) [30].

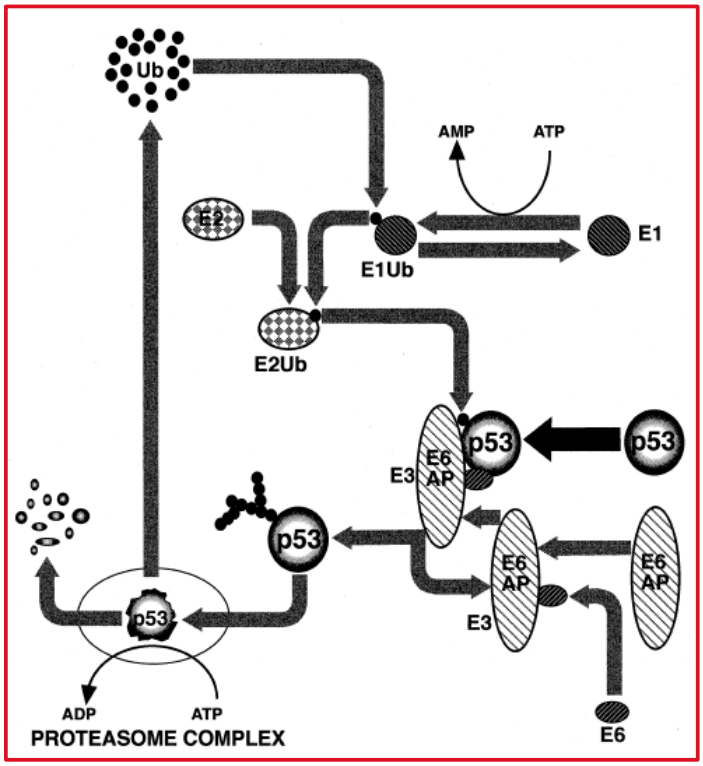

Figure 8 HPV E6 induces the degradation of p53 [3].

E6 interferes with p53 function through additional mechanism. E6 proteins from high and low risk HPV can bind to p53 without inducing the degradation. This interaction prevents p53-mediated transcriptional suppression of TATA-containing promoters and represses p53's transactivation of promoters containing p53 responsive element. This observation can be explained by the finding that when E6 binds to p53, it prevents p53 from binding to a number of its DNA recognition motif [3-6]

HPV E7 protein

The primary target of E7 is retinoblastoma (Rb) and its related proteins including p107 and p130. Rb forms a complex with histone deacetylase (HDAC) and binds to the E2F transcription factor in cell cycle. Binding of E7 to Rb and HDAC leads to the releasing of E2F then allowing cell cycle to reenter the S phase. The study by

Yuan et al., suggests that E7 can inhibit TNF-mediated apoptosis in keratinocytes by up-regulating the expression of the inhibitor of apoptosis protein, c-IAP2. In another study, the expression of E7 in fibroblasts delays Fas-mediated apoptosis and prevents TNF-mediated apoptosis by a mechanism involving the suppression of caspase-8 activation. However, the majority of studies suggest that E7 serves in pro-apoptotic role. [29, 30]

Cell cycle

Cell cycle is the fundamental mechanism of all organisms that is important for propagation and survival. In unicellular organisms, such as bacteria and yeasts, each cell cycle produces an additional organism. In multicellular organisms, cell cycle is required to make the reproductive cells and a new individual to replace the damaged or worn out cells.

Components of the cell cycle

Cyclins and cyclin-dependent kinases

Cyclins (the regulatory subunit) and cyclin-dependent kinases (CDKs, the catalytic subunit) play the important roles in cell cycle progression (Figure 9). Cyclin contains the Cyclin box, a homologous region of about 100 amino acids which can bind to an appropriate catalytic CDK subunit to form a complex (cyclin-CDK). The cyclin-CDK complex activates the transcription factors that activate the genes required for next stage of cell cycle. The regulation of CDKs activity occurs as the rate-limiting step in cell cycle progression due to changing in synthesis and degradation. The subcellular localization of cyclins into the nucleus or the cytoplasm is important in cell cycle control. The Cyclin/CDK complex requires phosphorylation on threonine for activation. The CDKs are phosphorylated and activated by CDK-Activating Kinases (CAK). A level of CDK activation depends on the dephosphorylation of its ATP-binding site by dual specific tyrosine and threonine phosphatases of the CDC25 family.

Tumor suppressor genes

Tumor suppressor genes prevent excessive growth of a cell. The most well known are p53 and the retinoblastoma (Rb) gene.

Retinoblastoma (RB) Gene

Retinoblastoma gene is involved in the G checkpoint in the following way. It binds to the transcription factors in E2F family and represses their transcription of E2F-responsive genes, such as thymidine kinase (TK), needed for DNA replication, and cyclin E and A, needed for cell cycle progression. Rb is activated by cyclin D/CDK4/6 at G1 phase. Phosphorylation of Rb allows E2F to be released.

p53

The p53 protein responds to stress signals such as DNA damage then switches from an inactive state to an active state. The p53 proteins triggers transcription of the gene for p21, which is a CDK inhibitor. An inactive CDK will cause the cycle to halt. The p53 protein is also involved at the G checkpoint in cases, for example, where DNA has been synthesized incorrectly. At this checkpoint, p53 binds to E2F and prevents it from triggering transcription of proto-oncogenes, for example *c-myc* and *c-fos*, which are required for mitosis [7].

Cell cycle consists of two main processes. The preparation for cell division occurs during interphase, the cells then divide during mitosis. In interphase, RNA is constantly being synthesized, protein is produced and the cell is growing in size. Due to the molecular level, interphase can be divided into the following stages (Figure 10) :

-Gap0 (G0). Cells may leave the cell cycle for a temporary resting period or more permanently if they have reached the end of their development. The exposure of G0 cells to growth factors re-activates cell cycle progression by inducing transcriptional activity.

-Gap1 (G1). Cells increase in size, produce RNA and synthesize proteins.

-Synthesis phase (S phase). DNA is synthesized during this stage for duplication of genetic materials.

-Gap 2 (G2). Cells continue to grow and produce new proteins.

Cells enter mitosis, which is further divided into the following stages:

-Prophase. At the beginning of prophase, the nuclear membrane breaks down and chromatin in the nucleus condenses into chromosomes. Each chromosome consists of two genetically identical chromatids. Microtubules are used to form the mitotic spindle.

-Prometaphase. Some mitotic spindle fibers elongate to specific areas on the chromosomes.

-Metaphase. Tension is applied to the spindle fibres, aligning all the chromosomes in one plane at the centre of the cell.

-Anaphase. The chromosomes are pulled away from the central plane towards the cell poles.

-Telophase. Chromosomes arrive at cell poles and decondense, and the nuclear envelope reforms around the clusters at each end of the cell, thereby forming new nuclei.

-Cytokinesis. The cell is cleaved to form two daughter cells and microtubules reform for the cells to enter interphase.

Cells formed by mitosis contain two sets of homologous chromosomes. Another form of cell division is meiosis which occurs only in reproductive cells during the formation of sex cells. A cell dividing by meiosis duplicates its DNA as with cells

undergoing mitosis, but splits into four new cells instead of two and contains only one copy of each chromosome.[7, 31]

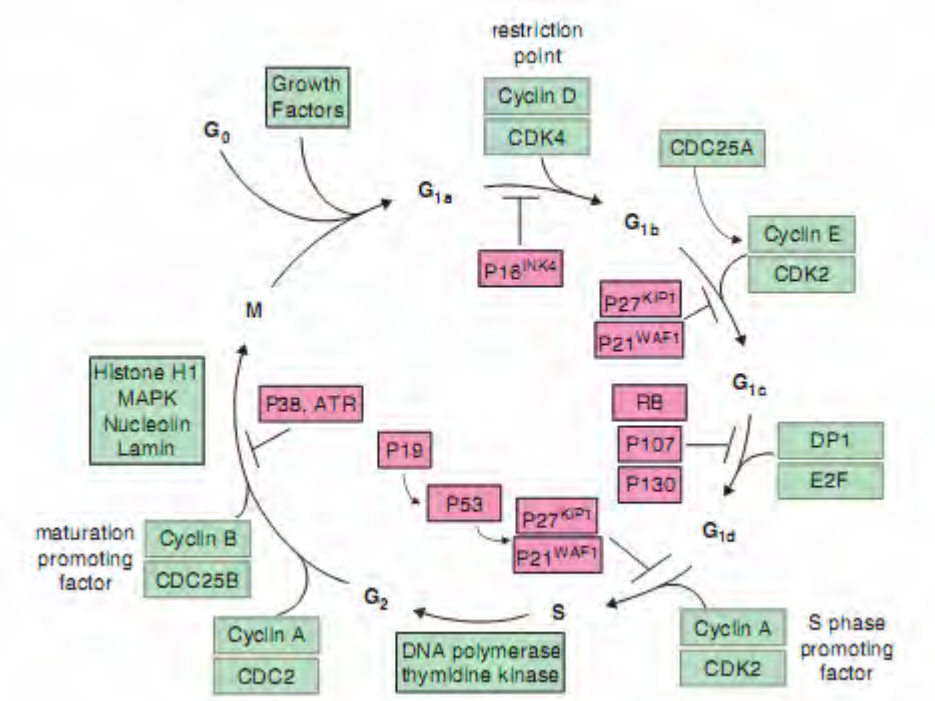

Figure 9 Components of the cell cycle. Promoting factors are depicted on green background outside the cell cycle, while inhibitory factors are shown on red background inside the cell cycle. Cells in G0 have exited the cell cycle, but can be stimulated to reenter it by growth factors [7].

Cell cycle check point (Figure 11)

Three essential components of cell cycle control are the Ubiquitin–proteasome pathway, CDK inhibitors, and checkpoint kinases. Reversal of the cell cycle is prevented by the rapid degradation of Cyclins. This is predominantly accomplished by the APC/C. Components of the APC are required for ubiquitination of substrates and

targeting to the proteasome. CDK Inhibitors block the activation of CDKs by CAKs, mostly through blocking of their ATP and Mg 2+binding sites. They comprise the CIP/KIP and INK4 families of proteins. Their expression and cell cycle control functions are largely controlled by p53 and RB. Checkpoint kinases act upstream of CDK Inhibitors. They include four con-served upstream kinases, ATM, ATR, CHK1, and CHK2. In an alternative pathway, P38 also acts as upstream kinase.

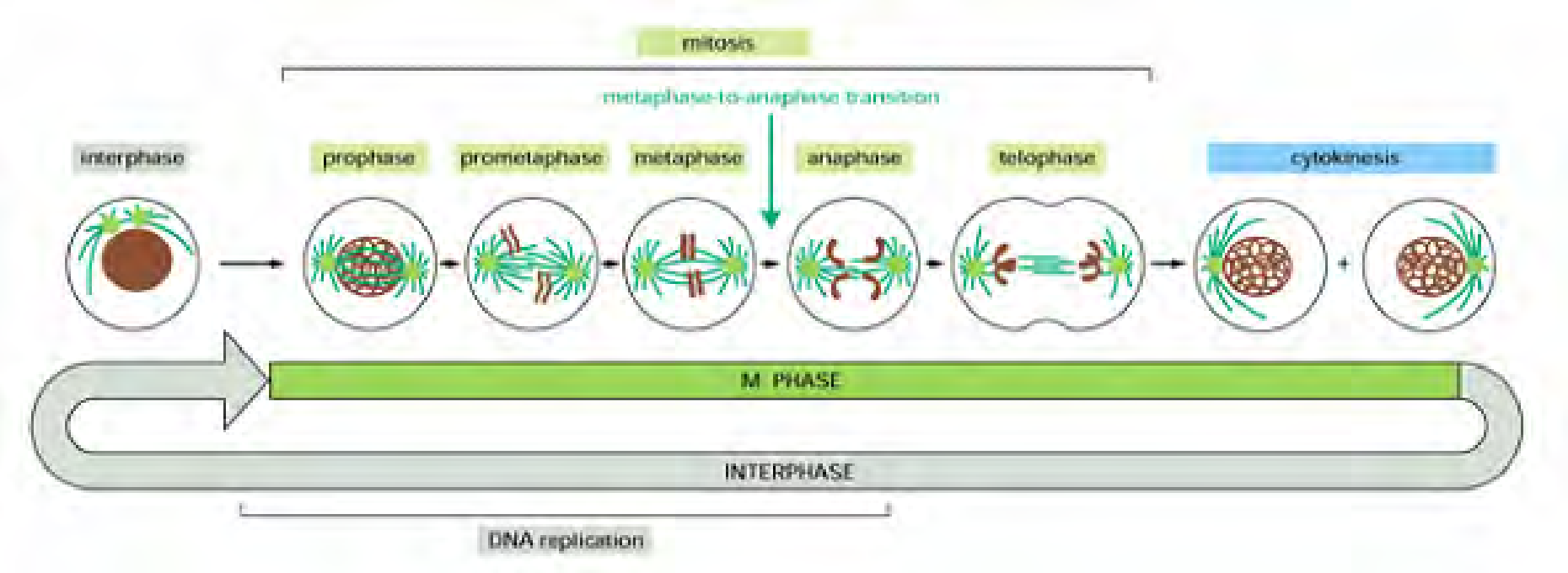

Figure 10 Cell division as seen under a microscope. M phase is the processes including nuclear division (mitosis) and cell fission (cytokinesis). During M phase, cell changes in the biochemical state at the transition from metaphase to anaphase. Cell can pause in metaphase before this transition point called “cell cycle checkpoint”, but once the point is passed, the cell will pass through cytokinesis into interphase. The longer part of the cycle is known as interphase that has the preparation process for cell division [31].

G1 checkpoint. This checkpoint depends on the RB and p53 pathways, and on CDK Inhibitors of the CIP/KIP and INK4 families. The tumor suppressor LATS-2 also plays a role.

S checkpoint. Ionizing radiation and UV light lead to CDC25A degradation, the key of S checkpoint. CDC25A is important during the initiation and progression of S phase. In the absence of CDC25A, the activation of the CDK2 is prevented and DNA synthesis dose not take place.

G2/M checkpoint. Cyclins A and B in association with CDC2 and CDK1 (CDC2, P34) regulate the entry into M phase. Protein phosphatase 2A synergizes with CDC25C in activating CDK1 and facilitating cell cycle progression at this stage. DNA damage leads to cell cycle arrest either at the G2 /M border or in metaphase.

Spindle assembly checkpoint. (Figure 12) (Spindle checkpoint, mitotic checkpoint, and kinetochore checkpoint) The alignment of the chromosomes on the metaphase spindle is checked before the initiation of anaphase. The mitotic checkpoint detects the orientation of the chromosomes, ensuring that all the pairs of sister chromatids establish bilateral attachments to the mitotic spindle and become aligned on the metaphase plate. Any failure of the spindle fibers to attach to the kinetochores leads to cell cycle arrest in metaphase. A single unattached kinetochore can delay the segregation of the already aligned chromosomes. Consistent with the effect of chromatid separation on genomic stability, many of the spindle checkpoint proteins also play roles in DNA repair [7].

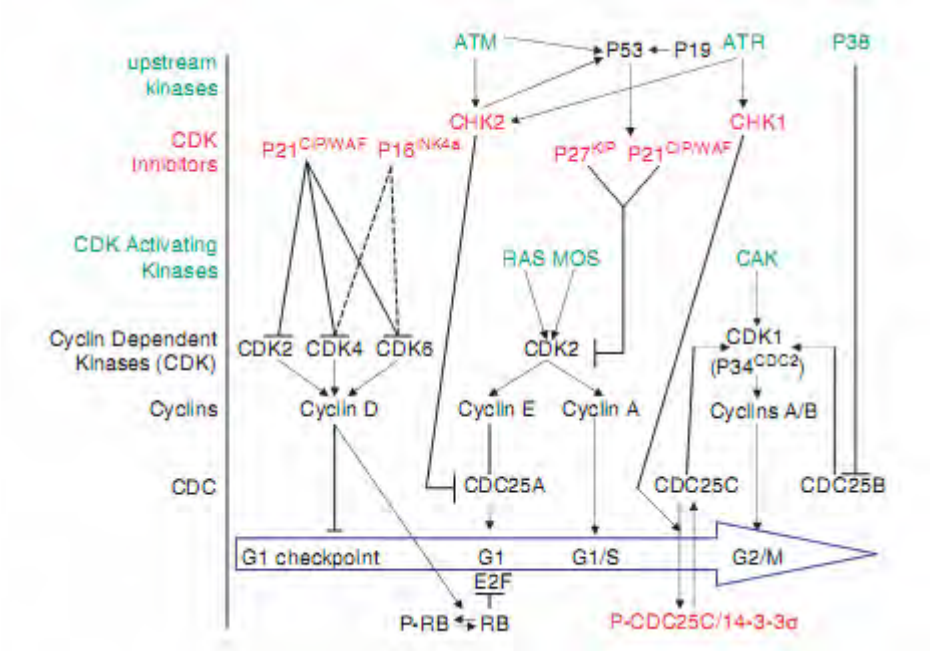

Figure 11 Checkpoints of the cell cycle. The passage past cell cycle checkpoints depends on Cyclin/CDK complexes. Their activity is tightly regulated. Various kinases (shown in green) activate Cyclin/CDK complexes and facilitate progression through the cell cycle. Cyclin-Dependent Kinase Inhibitors (shown in red) prevent this activation and cause cell cycle arrest. In addition, the roles of RB and CDC25C in the cell cycle are also determined by their phosphorylation status [7].

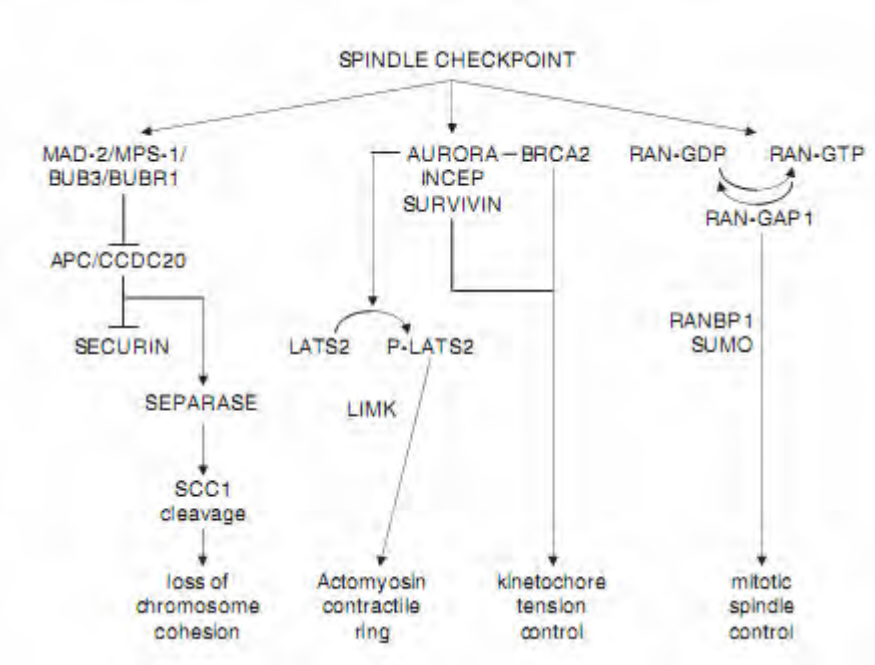

Figure 12 Spindle checkpoint. The mitotic checkpoint detects the orientation of the chromosomes, ensuring that all the pairs of sister chromatids establish bilateral attachments to the mitotic spindle and become aligned on the metaphase plate. The molecular components involved include the mitotic spindle, the kinetochore, the contractile actomyosin ring that separates the daughter cells, and chromosomal Cohesins. Any failure of the spindle fibers to attach to the kinetochores leads to cell cycle arrest in metaphase [7].

Apoptosis

Apoptosis plays an important role in organism development, aging, cellular homeostasis and also occurs as a defense mechanism in immune reaction, infection, and the abnormalities of cells by exposing toxic agent. There are two main mechanisms of apoptosis, extrinsic and intrinsic pathways (Figure 13). Extrinsic pathway is triggered by transmembrane receptor-mediated interactions such as FasL/FasR, TNF-α/TNFR1 and Apo3L/DR3.[32-34] Intrinsic pathway is initiated by the stimuli either in negative fashion such as absence of growth factors, hormones and cytokines that can lead to failure of suppression of apoptosis or in positive fashion such as toxin, radiation, free radicals and viral infections. These stimuli cause changes in the inner mitochondrial membrane that results in an opening of mitochondrial permeability transition (MPT) pore, loss of mitochondrial transmembrane potential and release pro-apoptotic proteins involve cytochrome c. Smac/DIABLO, HTrA2/Omi, AIF, endonuclease G and CAD into the cytosol. Induction of extrinsic and intrinsic pathways leads to the biochemical modification such as protein cleavage, protein cross-linking, DNA break down, and phagocytic recognition. Apoptosis requires caspase-dependent pathways to regulate cell destruction.[35-37]

Caspases are cysteine protease that is always expressed in an inactive proenzyme form. These proteases are able to cleave their targets at aspartate residue. Caspases work as a proteolytic cascade, ten major caspases have been catagorized into initiators (caspase-2, -8, -9, -10), effectors or executioners (caspase-3, -6, -7) and inflammatory caspases (caspase-1, -4, -5). The other caspases that have been identified including caspase-11, which is reported to regulate apoptosis and cytokine maturation during septic shock, caspase-12 which mediated endoplasmic-specific apoptosis and cytotoxicity by amyloid-β, caspase-13, which is suggested to be a bovine gene, and caspase-14 which is highly expressed in embryonic tissue but not in adult tissue. In an extrinsic pathway, the formation of a death-inducing signaling complex (DISC) leads to the activation of caspase-8 and the following cascade while in

an extrinsic pathway, the action of cytochrome c induces the activation of caspase-8, -3, -7, and -9 [38-40].

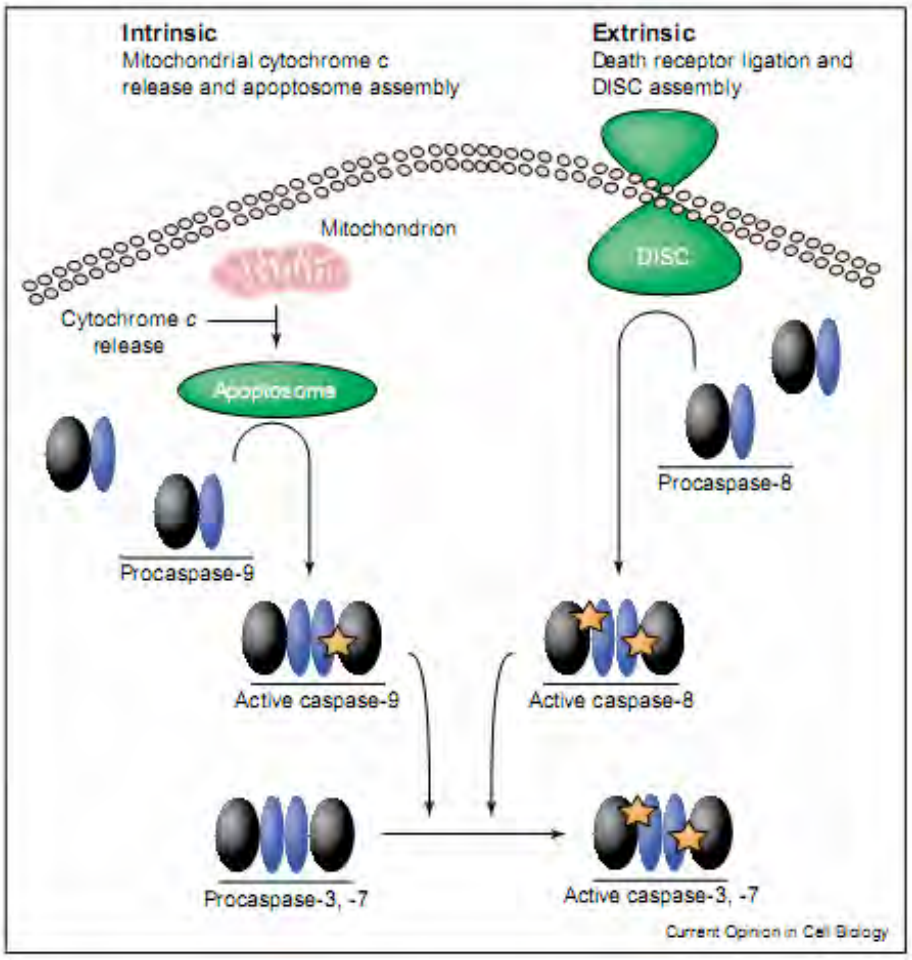

Figure 13 Representation of the two molecular mechanisms of pro-caspase activation [38].

P53 Family

The transcription factor p53 responds to the cellular stresses to regulate many target genes that induce cell cycle arrest, apoptosis, senescence, DNA repair or alter metabolism. In addition, p53 induces apoptosis by non-transcriptional regulation. The human p53 gene is composed of 19,200 bp spanning over 11 exons on chromosome 17p13.1. The p53 protein is widely studied because mutations of p53 gene

are highly associated with tumorgenesis. Human p53 consists of 393 amino acids, with 5 domains. The transactivation domain (TAD) is required for transcriptional activation. The proline-rich domain (PRD) contains 5 PXXP motifs that enable protein–protein interactions.[41] The DNA-binding domain (DBD) binds to DNA consensus recognition elements in the promoters of target genes. The tetramerization domain (4D) and the C-terminal regulator domain (CTD) bind DNA nonspecifically and might regulate specific DNA binding by the DBD.[42, 43] A nuclear localization signal (L) located between the DBD and the 4D, and a nuclear export signal (E) embedded in the 4D.

In unstressed cells, p53 is inactive and at low levels because of the action of MDM2, which inhibits p53 in two main ways: it quenches p53 transcriptional activity by occluding the p53 TAD (thereby preventing p53 from recruiting transcriptional co-activators such as p300) and, through its ubiquitin-ligase activity, can ubiquitylate lysines in the p53 CTD to promote p53 degradation by the proteasome. (Figure 14a)

In stressed cells, the stresses induce phosphorylation in TAD that reduce the binding of MDM2. Modifications in the PRD can also participate in reduced MDM2 binding. The reduced MDM2 binding leads to tetramerization of p53 and accumulation in the nucleus.[44] The phosphorylation of the p53 TAD favours the interaction with histone acetyl transferases such as p300, which bind the PRD through PXXP motifs. This leads to the acetylation of lysines in the p53 CTD to promote p53 stabilization and increase specific DNA binding at 5 target genes. p300 can acetylate histones at the promoters of target genes, therefore inducing promoter opening and enabling transcription activation to induce different cellular responses and also creates a negative-feedback loop. A fraction of the p53 molecules could remain in the cytoplasm then bind anti-apoptotic BCL2 or BCL-X and promote apoptosis through mitochondrial outer membrane permeabilization.[7, 45] (Figure 14b)

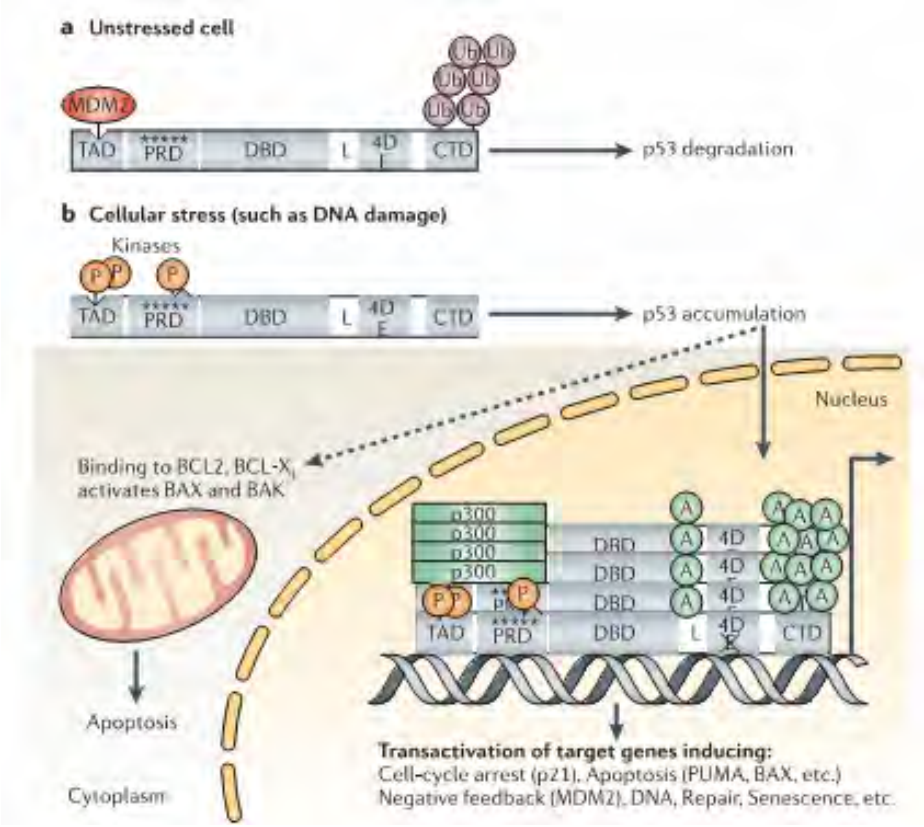

Figure 14 A model for p53 regulation based on in vitro and transfection data [45].

P63 Family

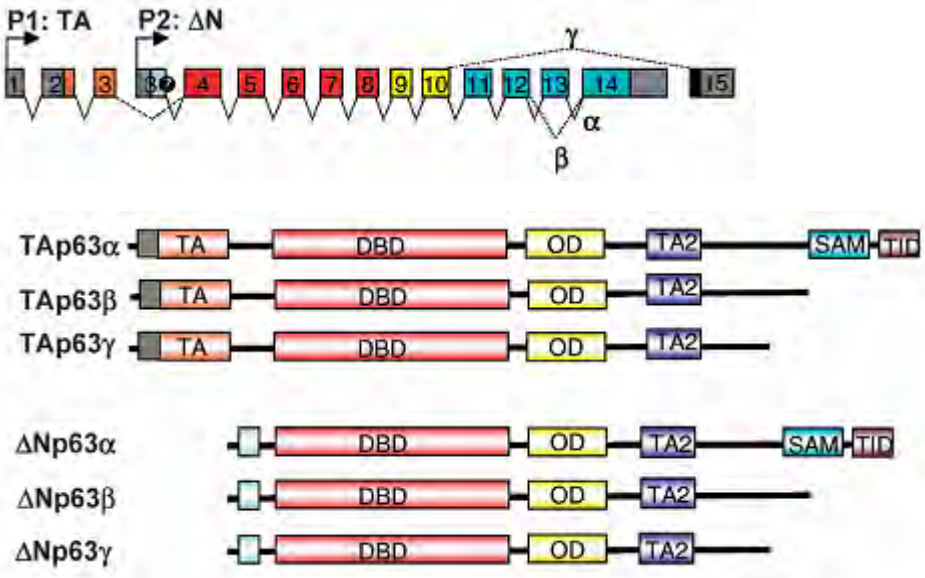

Figure 15 Structure of p63 isoforms [46].

The p53 family, p63 was discovered in 1998 [15]. The p63 gene has 270 kbp size contains 14 exons locate on chromosome 3q27 [9, 10]. P63 protein shares the similarity of protein structure and amino acid sequences with p53 protein which consist of 3 domains: NH_2-terminal transactivating domain (amino acids 1-59), DNA binding domain (amino acids 142-321) and COOH-oligomerization domain (amino acids 353-397).[8] The p63 gene contains two promoters that are used to generate p63 proteins with and without NH_2-terminal transactivating domain termed TAP63 and ΔNp63 (Figure 15). The alternative splicing of p63 generates three isoforms with the different COOH-oligomerization domain termed α, β, and γ. The p63α proteins contain sterile alpha motif or SAM domain. SAM domain was reported to have the function about protein-protein interaction in developmental process [11, 12]. It may be involved in other processes such as apoptosis, transcriptional activation, focal adhesion chromatin remodeling, receptor tyrosine kinase signaling, and SUMOylation [8].

The studies about biochemical activities of p63 in transient transfection or adenoviral infection suggested that TAp63 variants could induce cell cycle arrest and apoptosis while ΔNp63 variants show the opposite function [13-15]. As predicted from their structures, this result may be due to the interaction of transactivation domain with co-activators or the apoptosis stimulating protein of p53 family. Studies comparing TA-variants show that TAp63γ stimulates transcription as well as wild-type p53 whereas Tap63α shows little or no transactivating activity. In contrast, ΔNp63α represses p53- or TAp63γ mediated transcription, but ΔNp63γ is only able to repress p53-mediated transcription. Carboxy-terminus showed the ability to repress transcription this evidence proposed that it interacted with the TA-domain of p63 in a region homologous to the MDM2 binding site in p53. ChIP experiments with E1A-expressing mouse embryo fibroblasts (MEFs) revealed in vivo p63 binding to the p53 target genes *mdm2*, *bax*, *PERP*, and I indicate that p63 involved in regulating the stability of p53 and its apoptotic functions [8, 15].

The study in p63 knockout mice demonstrated a lack of epidermal barrier and the defect in limb and craniofacial development.[16] The zebrafish with the specific disruption of ΔNp63α showed a lack of epidermal morphogenesis and fin truncation.[17] In human, mutation in the human p63 genes are associated with a variety of developmental defect such as EEC syndrome (the triad of Ectrodactyl, Ectodermal dysplasia, and Clefting of lip and palate) with the missense mutations in the p63 binding domain and acro-dermato-ungual-lacrimal-tooth (ADULT) syndrome with missense of frameshift mutation in SAM domain. [47]

In cancer ΔNp63α was found as a primary p63 variant expressed in squamous epithelial tissue and also determined that it acts antagonistically toward p53.[15] Over expression of ΔNp63 variant in Rat 1a cells leads to increased growth of these cells.[18] The studies in normal cervix showed the expression of ΔNp63 in basal and parabasal cells whereas the expression of ΔNp63 could be detected in superficial cells of cervical intraepithelial neoplasia (CIN).[19] Moreover, ΔNp63α expression is directly correlated with the clinical response to cisplatin in patients with head and neck tumors.[20]

The evidences from studying p63 expression in mouse model and human lead to two hypotheses about p63 function.[16] One hypothesis stated that ΔNp63 is necessary for the regenerative proliferation of epithelial progenitor cells and another hypothesis is that TAp63 is necessary for the specification and differentiation of squamous epithelial cells. Due to the suppressive function of ΔNp63 onto p53, ΔNp63 may increase the severity of cervical cancer by working with HPV E6 protein.

CHAPTER IV

MATERIALS AND METHODS

Cell culture

RKO cells, a colon carcinoma cell lines containing wild-type p53 but lacking endogenous human thyroid receptor nuclear receptor (h-TRbeta1), were purchased from The American Type Culture Collection (ATCC CRL-2577). Cells were cultured in growth medium which is Minimal Essential Medium (MEM) supplemented with 20% fetal bovine serum (PAA; Austria), 100 units/ml penicillin G and 100 µg/ml streptomycin, 2 mM L-glutamine and 0.01M HEPES (N-2-hydroxyethylpiperaine-N'-2-ethan sulfonic acid) (Sigma, USA). In order to maintain the cells, the culture medium was removed and the cells were washed twice with 5 ml of pre-warmed (37°C) 0.01 M phosphate buffered saline (PBS), pH 7.5 (see Appendix B). One ml of pre-warmed (37°C) trypsin versine (see Appendix B) was added and the cells were incubated for 1 to 2 minutes at 37°C followed by discarding of trypsin versine. Cells were detached by tapping. Growth medium were added. The cell suspension was mixed by pipetting and subcultured at a splitting ratio of 1:3 every 3 days. The cells were cultured at 37°C with 5% CO_2 in atmosphere. The cells were subpassaged every 3 days.

Plasmids

pCI-Neo (Promega, USA) containing HPV16 E6 was kindly provided by Prof. Paul Harmonat, the University of Arkansas, USA.

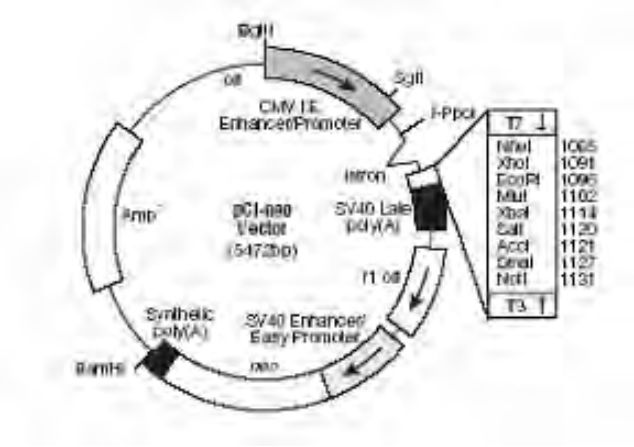

Figure 16 pCI-Neo structure.

Three plasmid constructs, pCep4 *(Invitrogen, USA)* containing deltaNp63 alpha, beta, and gamma were kindly provided by Prof. Jennifer A. Petenpol, Vanderbilt University Medical Center, USA.

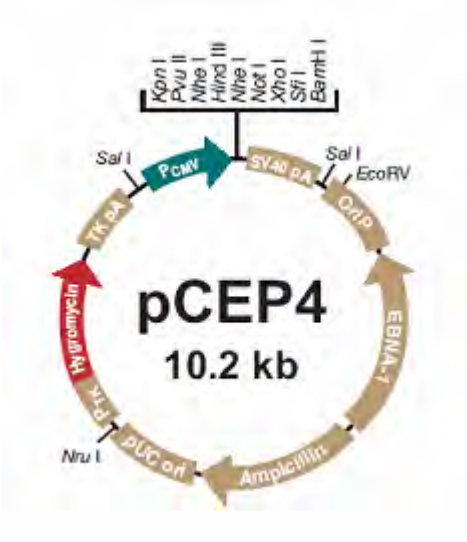

Figure 17 pCEP4 structure.

Preparation of *E. coli* DH5α competent cells

Competent cells were prepared by calcium chloride method [48]. A single colony of *E. coli* DH5α was transferred to 5 ml LB (Lauria Bertani) broth in 15 ml falcon tube. Cells were grown overnight at 37 °C. One ml of cell culture was transferred to 100 ml of LB in 250 ml flask and shaken at 37 °C for 4 hours. Flask containing cell

culture was incubated on ice for 10 minutes and then the culture was transferred to 50 ml falcon tube. Cells were collected by centrifugation at 3000 rpm for 10 minutes. The supernatant was decanted and cells were gently resuspended in 10 ml cold 0.1 M $CaCl_2$. The suspension was incubated on ice for 20 minutes then centrifuged again at 3000 rpm for 10 minutes. The supernatant was discarded and gently resuspended in 5 ml cold 0.1 M $CaCl_2$ containing 15%Glycerol. Cell suspension was dispensed in microtubes (100 µl per tube) and frozen at -80 °C.

Transformation

The plasmid constructs were delivered in dry form on the filter paper. They were transformed to the competent cells, *E. coli* DH5α, by heat shock transformation method in order to generate the stock culture. In short, the plasmids in filter paper were eluted out by 500 µl TE buffer (see Appendix B). 10 µl of plasmid dilution was added to competent cells, *E. coli* DH5α. After incubating on ice for 30 minutes, the transformation mixture was dropped into pre-warmed (42°C) water for 30 seconds. 900 µl of LB broth was immediately added into the mixture followed by incubating in 37°C for 1 hour. The clones containing pCEP4-ΔNp63 alpha, beta, and gamma were selected on LB agar containing 100 µg/ml ampicillin (Bio Basic Inc., Canada). The clones containing pCI-Neo-HPV16 E6 were selected on LB agar containing 16 µg/ml neomycin (Sigma Chemical, USA).

Plasmid purification for detecting the inserted genes

In order to confirm the presence of the insert gene, plasmids were purified from the selected *E. coli* DH5α clones by using QIAGEN Plasmid Mini Kit (Qiagen, Germany). The procedure was performed according to the manufacturer's instruction. In brief, a single colony from a freshly streaked selective plate was picked and inoculated into 5 ml LB medium containing 100 µg/ml ampicillin. The culture was incubated overnight at 37 °C with vigorous shaking (approximately 300 rpm). The

bacterial cell solution was transferred into the microcentrifuge tube and harvested by centrifugation at 10000 rpm for 1 minute. The pelleted bacterial cells were resuspended in 250 µl of Buffer P1 and transferred to a microcentrifuge tube. 250 µl of Buffer P2 was added and mixed thoroughly by inverting the tube 4–6 times. 350 µl of Buffer N3 was added and mixed immediately and thoroughly by inverting the tube. The supernatant was applied to the QIAprep spin column and centrifuged for 1 minute and discarded the flow-through. The QIAprep spin column was washed by adding 0.5 ml Buffer PB and centrifuged for 30–60 seconds. The flow-through was discarded. QIAprep spin column was washed by adding 0.75 ml Buffer PE and centrifuged for 30–60 seconds. The flow-through was discarded and the column was centrifuged for an additional 1 minute to remove residual wash buffer. The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl Buffer EB (10 mM Tris-HCl, pH 8.5) was added to the center of the QIAprep spin column, standed for 1 minute, and centrifuged for 1 minute.

Polymerase chain reaction (PCR)

PCR method was used to confirm the specific genes in plasmid constructs before transfection. The selectable clones from transformation step were confirmed by PCR method with the primers specific to alpha, beta, and gamma tails of Np63 and HPV16E6. The PCR conditions were performed according to the following table (table 1). 10 µl of PCR products were analysed by 1 % agarose gel electrophoresis.

Table 3 PCR primer and condition.

Target	Sequences	Product size (bp)	Ref.
ΔNp63	Np63F (sense; 5'-CAGACTCAATTTAGTGAG -3') Np63R (antisense; 5'-AGCTCATGGTTGGGGCAC-3')	440	[15]
. p63α-tail	SKO28 (sense; 5-GAGGTTGGGCTGTTCATCAT-3) SKO29 (antisense; 5-AGGAGATGAGAAGGGGAGGA-3)	189	[49]
p63β-tail	SKO30 (sense; 5-AACGCCCTCACTCCTACAAC-3) SKO31 (antisense; 5-CAGACTTGCCAGATCCTGA-3)	206	[49]
p63γ-tail	SKO22 (sense; 5-ACGAAGATCCCCAGATGATG-3) SKO23 (antisense; 5-GCTCCACAAGCTCATTCCTG-3)	214	[49]

reaction (20 ul)	PCR condition
1X buffer (100 mM. KCl, 20 mM Tris) 1.5 mM MgCl2 200 uM dNTPs 25 pmole primers 1.25 units of Taq 1 ul of DNA sample	Start 95 °C 10 minutes 1 cycle Denature 95 °C 1.30 minutes Annealing 40 °C 1.30 minutes 40 cycels Extension 72 °C 2 minutes Extension 72 °C 10 minutes

HPV16 E6	E6F (sense; 5'-GAC CCAGAAAGTTACCACAG-3') E6R (antisense; 5'-GCAACAAGACATACATCGAC-3')	397	[50]

reaction (20 ul)	PCR condition
1X buffer (100 mM. KCl, 20 mM Tris) 1.5 mM MgCl2 200 uM dNTPs 25 pmole primers 1.25 units of Taq 2 ug/ul of DNA sample	Start 95 °C 10 minutes 1 cycle Denature 95 °C 1.30 minutes Annealing 40 °C 1.30 minutes 40 cycles Extension 72 °C 2 minutes Extension 72 °C 10 minutes

Quantitation of purified plasmid DNA

Concentration of plasmid DNA solution was determined by OD measurements under ultraviolet light at 260 nm wave length. Purity of the plasmid DNA was determined as the ratio of OD 260 nm / OD 280 nm. The plasmid solution was diluted in distilled water at the ratio of 1:10. 20 ul of the plasmid solutions were measured the absorbance of ultraviolet light at 260 and 280 nm. The absorbance value of ultraviolet light at 260 nm should be between 0.2 and 0.8. The concentration of DNA was calculated by using the following fomula.

$$\text{DNA concentration (ug/ml)} = \frac{OD_{260} \times 100 \text{ (dilution factor)} \times 50\ \mu g/ml}{1000}$$

The ratio of the absorbance values of ultraviolet light at 260:280 nm was between 1.8 and 2.0 indicating purity of DNA.

Transfection

Preparation of plasmid for transfection

The plasmids for transfection were prepared according to the manufacturer's instruction (Qiagen, Germany). A single colony from a freshly streaked selective plate was picked and inoculated into 5 ml selective LB broth (LB broth containing 100 µg/ml ampicillin) in order to generate the starter culture. The starter culture was incubated overnight at 37 °C with vigorous shaking at 300 rpm. 200 µl of starter culture was tranferred to 100 ml of selective LB broth. The bacterial cells was grown for 4 hours and then harvested by centrifugation at 6,000 x g for 15 minutes at 4 °C. The bacterial pellets were resuspended in 4 ml Buffer P1. Buffer P2 (4 ml) was added. The bacterial pellets were mixed thoroughly by vigorously inverting the sealed tube 4–6 times, and incubated at room temperature for 5 minutes. After that, 4 ml of chilled Buffer P3 was added, the tube was mixed immediately and thoroughly by vigorously inverting 4–6 times, and incubated on ice for 15 minutes. The tube was

centrifuged at maximum speed for 30 minutes. The supernatant containing plasmid DNA was collected and centrifuged again at 20,000 x g for 15 minutes at 4 °C. The supernatant containing plasmid DNA was collected promptly. A QIAGEN-tip 100 was equilibrated by applying 1 ml Buffer QBT, and allowed the column to empty by gravity flow. The supernatant was transferred to the QIAGEN-tip 100 and allowed it to enter the resin by gravity flow. The QIAGEN-tip was washed with 2 times with 10 ml Buffer QC. DNA was eluted with 5 ml Buffer QF. DNA was precipitated by adding 3.5 ml room temperature isopropanol. The eluted DNA was mixed and centrifuged immediately at 15,000 x g for 30 minutes at 4 °C. The supernatant was carefully decanted. DNA pellet was washed with 2 ml of room-temperature 70% ethanol, and centrifuged at 15,000 x g for 10 minutes. The supernatant was carefully decanted without disturbing the pellet. The pellet was air-dried for 5–10 minutes and redissolved in 50 µl of TE buffer, pH 8.0.

The condition for transfection

Before the transfected cells for the experiment were established, the ratio 3:2, 4:2, 5:2, 6:2 and 7:2 of reagent (µl):total DNA (µg) had been used for finding the suitable ratio of reagent:total DNA for transfection by the following protocol. The efficiency of transfection was estimated by using indirect immunofluorescent assay.

The protocol for transfection

In this study 7 transfected cell types were generated (Table 4). RKO cells were transfected with each plasmid according to details in table 4 by using FUGENE HD® Transfection kit (Roche, USA). The transfection method was performed according to the manufacturer's instruction. 5 x 10^4 RKO cells were plated on 24-well plate and then cultured at 37°C with 5% CO_2 in atmosphere for 24 hours. FuGENE® HD Transfection Reagent, DNA, and diluent were allowed to adjust to the room temperature. The FUGENE® HD Transfection Reagent vial was mixed. DNA was diluted with Opti-MEM (GIBCO, USA) at the concentration of 0.02 µg/µl. 100 µl of the diluent containing 2 µg DNA was placed into the sterile tube. The transfection complex was formed by adding FuGENE HD® Transfection Reagent to tubes containing diluted DNA. 4 µl of the

FuGENE HD Transfection Reagent was directly added into the medium containing the diluted DNA without allowing contact with the walls of the plastic tubes, then mixed and incubated the transfection complex for 15 minutes at room temperature. The transfection complex of each plasmid constructs was transferred to the culture and incubated for 24 hours. The culture medium was changed and re-incubated for 3 days. In the case of co-transfection, the same total reagent:total DNA ratio as that used for a single plasmid was maintained.

Table 4: A set of transfected RKO cells which was used in this study.

RKO	RKO+ΔNp63 alpha	RKO+ΔNp63 beta	RKO+ΔNp63 gamma
RKO+E6	RKO+E6+ΔNp63 alpha	RKO+E6+ΔNp63 beta	RKO+E6+ΔNp63 gamma

Optimization of antibiotics under cell selections

Since pCI-Neo and pCEP4 transfected RKO cells required neomycin and hygromycin selection medium, the appropriate drug concentrations for selection were needed to be analyzed. $5x10^4$ cells/ml were grown in 24-well plate for 4 hours. After that the medium containing drugs were added. At the indicating time of each test, drug susceptibilities of RKO cells were determined by viable cell count and crystal violet staining.

Neomycin susceptibility test

Dose-dependent RKO cells selection was done. The neomycin concentration of 500, 1000, 1500, 2000 and 2500 µl/ml were tested. The alteration of RKO cells was observed every 48 hours under inverted microscopy. The number of viable cells were counted in hemocytometer chamber and determined by crystal violet assay.

Hygromycin susceptibility test

Dose-dependent RKO cells selection was also tested. The hygromycin concentration of 50, 100, 150, 200, and 250 µl/ml were analyzed and the alteration of the cells was observed every 72 hours under inverted microscopy. The number of viable cells were counted in hemocytometer chamber and determined by crystal violet assay.

Viable cell count

For detecting the effect of drugs to RKO cells in any concentrations, cells in each well were trypsinized and added with the culture medium at the final volume of 1 ml. Cells were mixed throughly. 20 µl of cell suspension was added with 20 µl of 1% trypan blue and gently mixed. The mixture was left at room temperature for 5 minutes followed by counting in hemocytometer chamber. The dead cells were stained blue based on the fact that the chromopore is negatively charged and does not interact with the cell unless the membrane is damaged. The viable cells were colourless.

Crystal violet staining

To determine the drug susceptibility of RKO cells, the crystal violet assay was used. Preparation of cells was similar to the viable cell count experiment. The culture medium of RKO cells with various concentration of selected drug was removed. Cells were fixed with 500 µl of methanol for 10 minutes followed by staining with 0.5 ml of 0.5% crystal violet solution in 25% methanol for 10 minutes. Plates were washed several times with distilled water and air dried overnight. The incorporated dye was solubilized in 1 ml of 0.1 M sodium citrate in 50% ethanol and 100 µl aliquots were transferred to 96-well plate and measured at 630 nm in ELISA plate reader (Human Egypt, Egypt). The number of viable cells related to the measured OD.

Establishment of transient and stable cell lines

Transient cell lines

Transient cell lines were derived from the cells transfected with the sets of plasmids for 1 week without transfected cells selection (no antibiotic added). Protein expression of transfected cells were confirmed by IFA on one week after transfection.

Stable cell lines

The stable cells lines were generated by selecting the transfected cells with suitable drugs at 1 week after transfection. The selected cells were subcultured every 3 days and selected by suitable drugs every 2 months. After culturing for 6 months, the transfected cells were detected protein expression by IFA.

Western blot analysis

Preparation of protein

Transfected cells were trypsinized. Cells were counted to the final concentration of 10^7 cells/ml and transferred to 1.5 ml microcentrifuge tube. Cells were washed twice with 1x PBS followed by centrifugation at 800 rpm for 15 seconds. The supernatant was removed by pipetting. Cell pellet was resuspended in 30 µl of sample buffer (see Appendix B). Cell suspension was boiled at 94 °C for 5 minutes. Cell suspension was centrifuged at 14,000 rpm for 10 minutes.

SDS PAGE and Western blot

10 µl of the supernatant of cell suspension was loaded into SDS gel (see Appendix B). Protein was separated in the chamber containing 1X running buffer (see Appendix B) at 200 V until the dye ran to the bottom of the chamber. PVDF membrane (Bio-rad, USA) was soaked in absolute methanol for 1 minute followed by incubating in running buffer for 10 minutes at room temperature. Proteins were transferred from SDS

gel to PVDF membrane in ice-cold condition at 400 mA for 3 hours. The membrane was blocked with blocking buffer (see Appendix B) overnight at 4°C. After that, membrane was washed 3 times with TTBS (see Appendix B) on the horizontal shaker at 200 rpm for 10 minutes each. The membrane was incubated with the primary antibody [dilution 1:200 mouse anti-HPV16 E6/18 E6 (C1P5); (Santa cruz, USA) in TBS containing 0.1% BSA] for 1 hour. The membrane was washed 3 times with TTBS on the horizontal shaker at 200 rpm for 10 minutes each. The membrane was incubated with the secondary antibody [dilution 1:200 goat anti mouse alkaline phosphatase (APS); (Santa cruz, USA)] in TBS containing 0.1% BSA for 1 hour. The membrane was washed again 3 times with TTBS on the horizontal shaker at 200 rpm for 10 minutes. The membrane was soaked with alkaline phosphatase buffer (see Appendix B) and incubated in substrate buffer (see Appendix B) until the specific band could be observed. Finally, the membrane was rinsed with distilled water.

Indirect immunoflurescence assay

Detect the expression of the protein of interest in transfected cells, indirect immunofluorescence staining was performed.

-For pCI-neoE6 transfected RKO cells, the primary antibody was mouse monoclonal anti-HPV16 E6/18 E6 IgG (Santa cruz) and the secondary antibody was goat polyclonal anti-mouse IgG PE (Abcam, UK).

-For pCEP4-ΔNp63 alpha, beta, and gamma transfected RKO cells, the primary antibody was goat polyclonal anti-deltaNp63 IgG (Santa cruz, USA) and the secondary antibody was rabbit polyclonal anti-goat IgG FITC (Abcam, UK).

Transfected cells were trypsinized and washed twice with 1x PBS pH 7.4. 10 µl of 10^7 cells/ml transfected cells were dried on a glass slide, fixed with cold acetone for 10 minutes, and placed at room temperature until the slide dried. 20 µl of 1:100 primary antibody dilution was added to the transfected cells and incubated at

37°C for 30 minutes. The slide was washed twice with 1x PBS, pH 7.4 for 5 minutes. 20 µl of 1:20 secondary antibody dilution was added and re-incubated at 37°C for 30 minutes. The slide was washed twice with 1x PBS, pH 7.4 for 5 minutes. The mounting fluid (see Appendix B) was dropped onto the slide then the coverslip was applied. Finally, the slide was viewed under the fluorescence microscope (Olympus, USA) at the magnification of 40x.

Cell death detection assay

Determination of the suitable concentration of camptothecin.

The suitable concentration and time of camptothecin were tested by using 10^4 and 10^5 cells/well of RKO cells incubated with various concentrations of camptothecin (1, 2, 3 and 4 µg/ml). The incubation period was fixed to 3 hours in this experiment, after that cell death detection assay was performed according to the following protocol.

Determination of the suitable incubation period for cell death detection assay.

10^5 cells/well of RKO cells were incubated with 2 µg/ml of camptothecin in various incubation periods (1, 2, 3 and 4 hours) followed by cell death detection assay.

The protocol for cell death detection assay.

In order to determine the ability of transfected cells to apoptosis resistance, Cell Death Detection ELISA plus (Roche, USA) was used. This assay is based on the quantitative sandwich-enzyme-immunoassay-principal using mouse monoclonal antibodies directed against DNA and histone, respectively. This allows the specific determination of mono- and oligonucleosome in the cytoplasmic fraction of cell lysates. The samples are placed into a streptavidin-coated plate. A mixture of anti-

histone-biotin and anti-DNA-peroxidase (POD) are added and incubated. The anti-histone antibody binds to the histone-component of the nucleosomes and captures the immunocomplex to the streptavidin-coated microplate (MP) via its biotinylation. Additionally, the anti-DNA-POD reacts with the DNA component of the neucleosome. Quantitative determination of nucleosomes by the reaction of POD retained in the immunocomplex with 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) substrate (Figure 18).

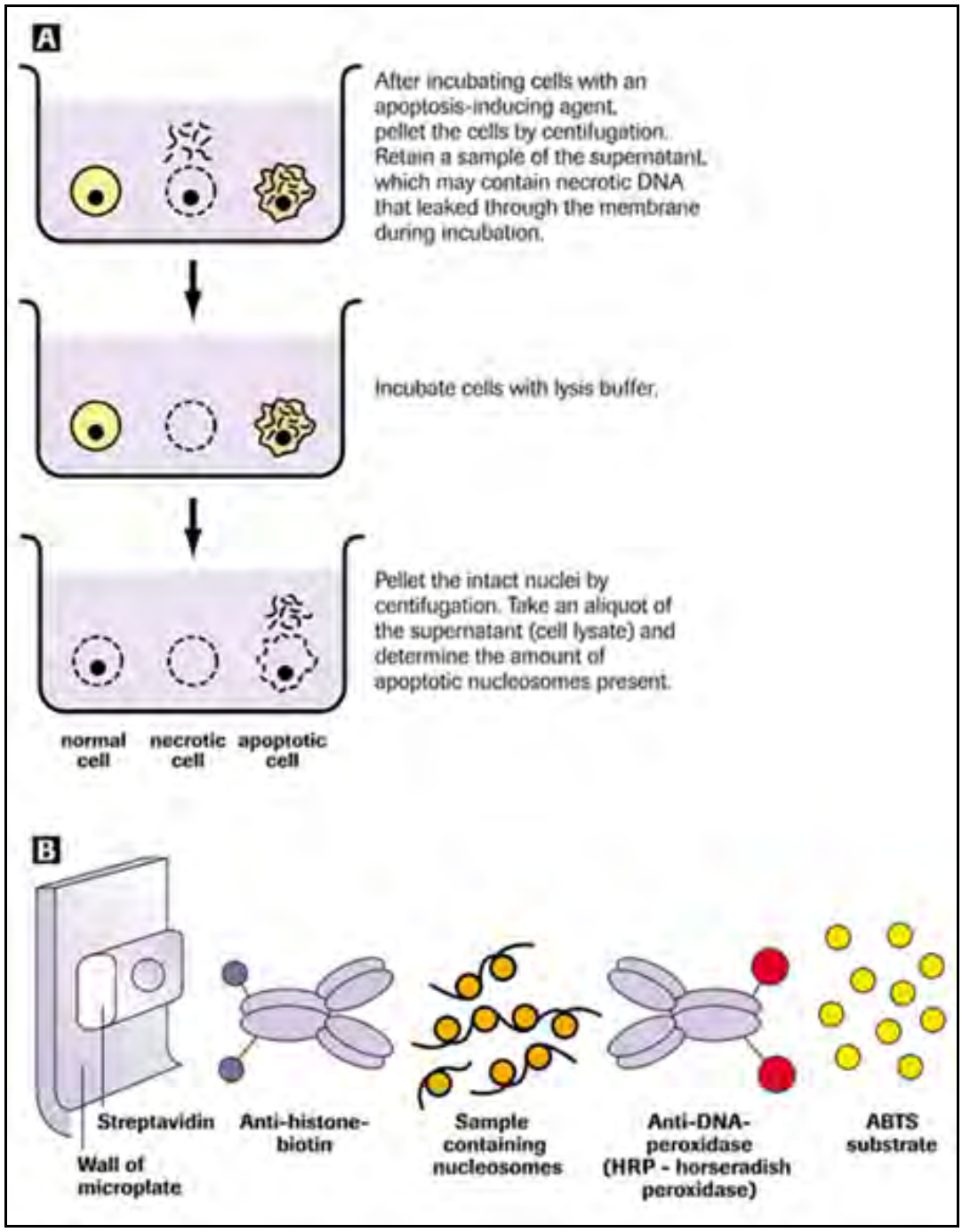

Figure 18 The principle of cell death detection ELISA plus.

The test was performed according to the manufacturer's instruction. The transfected cells at the concentration of 10^4 cells/well were cultured in 96-well plate for 24 hours at 37°C with 5% CO_2 in atmosphere. 100 µl of the suitable concentration of camptothecin (Sigma) was added into each well. The transfected cells with camptothecin were incubated for 4 hours at 37°C with 5% CO_2 in atmosphere and then centrifuged for 10 minutes at 200x g. Cells were resuspended in 200 µl lysis buffer and incubated for 30 minutes at room temperature. Cells were centrifuged again at 200 x g for 10 minutes. 20 µl of culture supernatant and controls were transferred to the microplate. 80 µl of the immunoreagent was added to each well. The microplate was covered with foil and then incubated on microplate shaker at 300 rpm for 2 hours at room temperature. The solution was removed by tapping. The microplate was rinsed 3 times with 250 µl of incubation buffer. The solution was removed carefully. 100 µl of the ABTS solution was pipetted to each well and incubated on a plate shaker at 250 rpm for 20 minutes. Then 100 µl of the ABTS stop solution was applied to each well. Finally, the solution was measured at 405 nm and 490 nm (the reference wavelength). The average value of the sample was used to calculate the enrichment factor by using the following formula:

$$\text{Enrichment factor} = \frac{\text{mU of the sample (dying/dead cells)}}{\text{mU of the corresponding negative control (cells without camptothecin treatment)}}$$

$\text{mU} = \text{absorbance} [10^{-3}]$

Cell proliferation assay

Proliferation assay was preformed by using Cell Proliferation ELISA, BrdU (colorimetric) (Roche, USA). This method quantitated cell proliferation based on the incorporation of the pyromodine analogue 5- bromo-2'-deoxyuridine (BrdU) during DNA

synthesis. The incorporated BrdU could be detected through the anibody specific to BrdU which was tagged with peroxidase. The reaction of peroxidase (POD) with tetramethyl-benzidine (TMB) was detected by measuring absorbance at OD_{450}. (Figure 19)

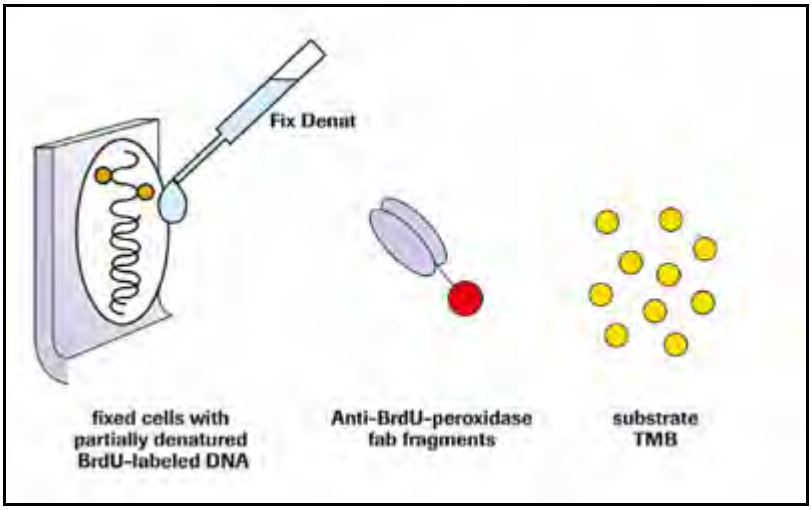

Figure 19 The principle of cell proliferation ELISA, BrdU (colorimetric).

In brief, transfected cells were cultured in 96-well plate for 24 hours at the density of 10^5 cells/well. Cells were added with 100 µl BrdU labeling solution and reincubated for additional 24 hours at 37°C. The culture medium was removed by tapping. 200 µl/well of FixDenat solution was added into the cells and re-incubated for 30 minutes at room temperature. FixDenat solution was removed by tapping then 100 µl/well of anti-BrdU-POD working solution was added and incubated for approximately 90 minutes at room temperature. Anti-BrdU-POD was removed by flicking off and rinsed wells 3 times with 200 µl – 300 µl/well of washing solution (1X PBS). The washing solution was removed by tapping and 100 µl/well of substrate solution TMB was added. The substrate was incubated at room temperature for 20 minutes. 25 µl of 1M H_2SO_4 was added to each well and incubated for approximately 1 minute then measured the absorbance of samples in ELISA reader (Human Egypt, Egypt) at 450 nm.

CHAPTER V

RESULTS

Specification of the inserted genes

The inserted genes of the provided plasmids were confirmed before the experiments began.

pCI-NEO-E6

pCI-Neo containing HPV16 E6 were extracted from transformed *E. coli* DH5α and confirmed for the specification of inserted genes by using PCR method. The specific band of E6 gene could be detected at 397 bp. (Figure 20)

pCEP4-ΔNp63 isotypes

pCEP4 containing ΔNp63 alpha, beta, and gamma were confirmed for the presence of inserted genes by using PCR method. The specific bands of alpha, beta, gamma isotypes and ΔNp63 could be detected at 189, 206, 214, and 440 bp, respectively. (Figure 21)

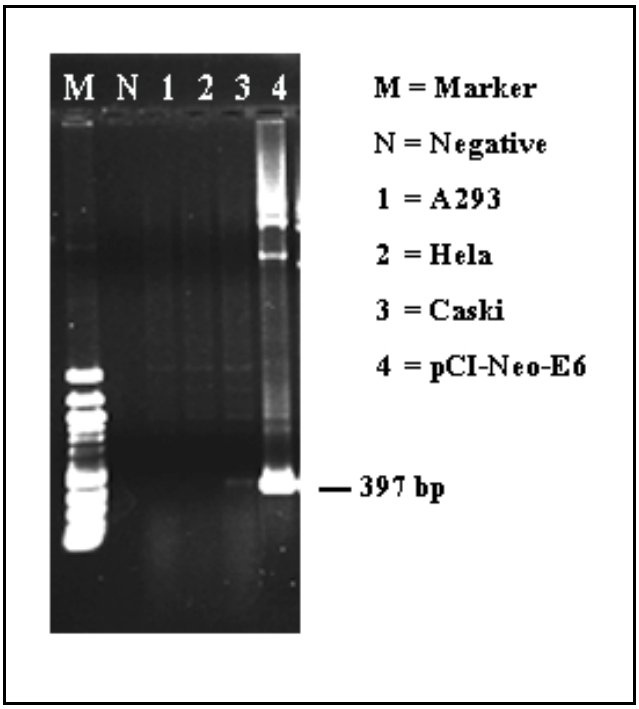

Figure 20 The PCR product from primer specific to HPV16E6 gene. M represented 100 bp DNA ladder (Fermentas, Canada). N represented the negative control derived from using distilled water instead of a template. The specific band of HPV16E6 gene was detected in CaSki, the cell lines containing HPV16 genome (lane 3) and pCI-Neo-E6 (lane 4) at 397 bp size while A293 cell lines without HPV (lane 1) and Hela cell lines containing HPV 18 (lane 2) cound not be found.

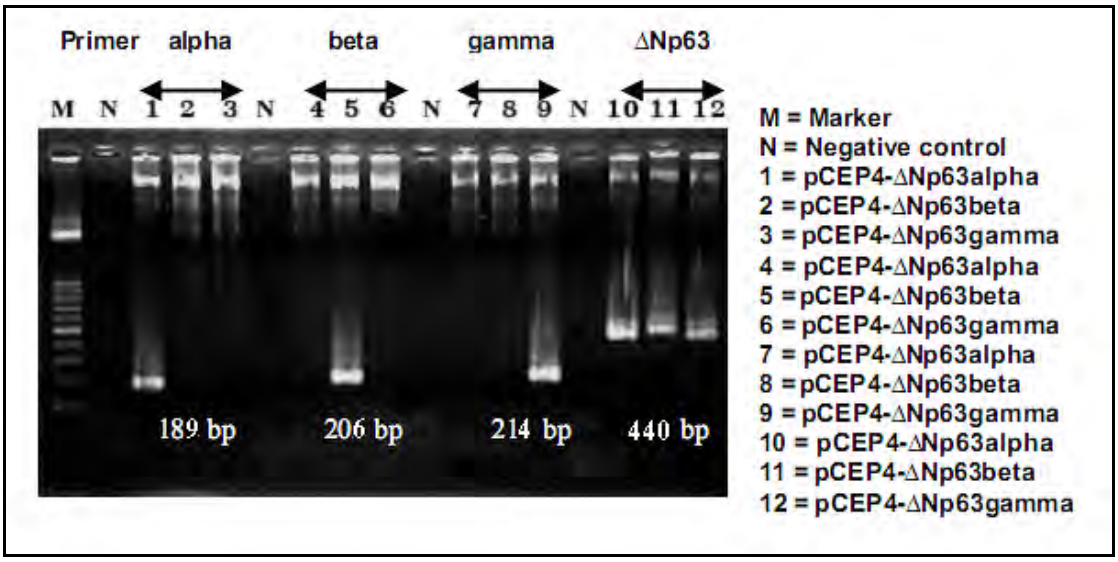

Figure 21 The PCR products of pCEP4-ΔNp63 alpha, beta, and gamma. M represented 100 bp DNA ladder (Fermentas, Canada). N represented the negative control derived from using distilled water instead of a template. The specification of three plasmid constructs, pCep4-ΔNp63 alpha, beta, and gamma was detected in lane 1, 5, 9, 10, 11 and 12 by the primers specific to alpha tail (lane 1, 4 and 7), beta tail (lane 2, 5 and 8), gamma tail (lane 3, 6 and 9), and ΔN (lane 10, 11, and 12), respectively.

Drug susceptibility test

To study the suitable dose of the drugs for transfected cells selection, neomycin and hygromycin susceptibility tests were performed in RKO cells with various concentrations of drugs.

Neomycin susceptibility test

In order to test the suitable concentration of neomycin for pCI-Neo-E6 transfected cells selection, neomycin susceptibility test against RKO cells was performed by using 20% MEM containing neomycin at the concentration of 0, 500, 1,000, 2,000 and 2,500 µg/ml for 12 days. The number of viable cells was analysed every 2 days.

Viable cell count

By observing under inverted microscopy, cells grew normally in every concentrations of neomycin until 6 days after adding neomycin. At 8 days after adding neomycin, the cells reached the stable stage and started dying at day 12. (Figure 22, Table 5)

Crystal violet assay

The reduction of OD630 of RKO cells treated with neomycin when compared with non-treated cells could be detected at 6 days after adding neomycin. However the curve still rose until 8 days and quite stable until 10 days. Finally, the curve fell at 12 days after adding neomycin. (Figure 23, Table 6)

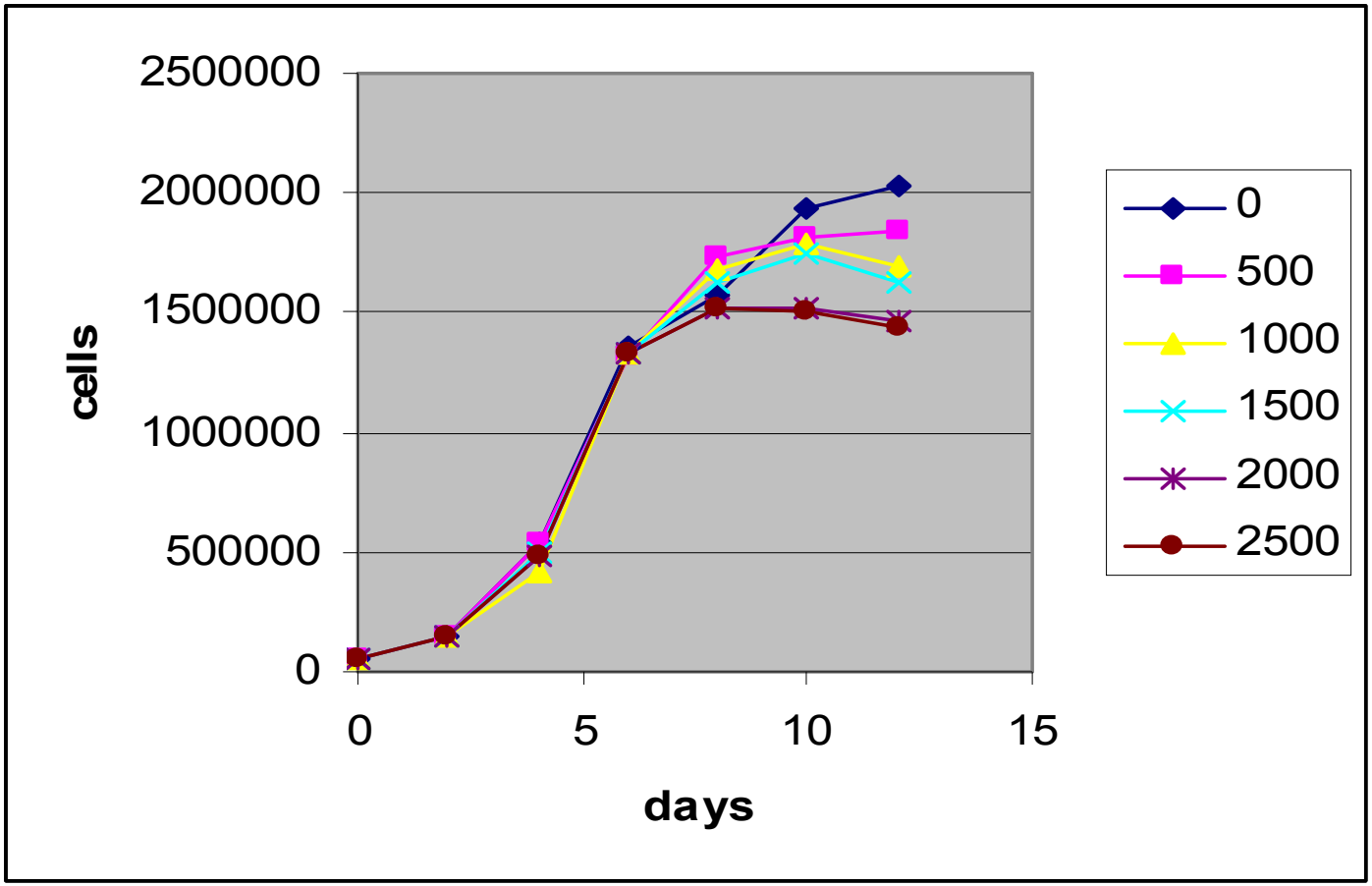

Figure 22 The viable cells count in neomycin susceptibility test for 12 days. Various concentrations of 0, 500, 1,000, 1,500, 2,000, and 2,500 µg/ml neomycin were added.

Table 5 The viable cells in neomycin susceptible test for 12 days.

	Number of viable cells x 10^5																	
	Neomycin concentrations																	
Exp. / Day	0			500			1,000			1,500			2,000			2,500		
	I	II	Mean	I	II	Mean	I	II	Mean	I	II	Mean	I	II	Mean	I	II	Mean
0	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
2	1.50	1.40	1.45	1.53	1.52	1.53	1.55	1.52	1.54	1.525	1.550	1.516	1.510	1.550	1.516	1.525	1.550	1.537
4	5.64	5.45	5.35	5.30	5.40	5.35	5.20	4.98	4.13	4.95	5.05	5.00	4.95	4.85	4.90	4.823	4.90	4.86
6	13.50	13.70	13.60	13.30	13.10	13.20	13.10	13.40	13.25	13.60	12.90	13.25	13.25	13.30	13.28	13.30	13.20	13.25
8	15.60	15.75	15.68	17.80	17.00	17.40	17.00	16.70	16.85	16.50	16.08	16.29	15.18	15.30	15.24	15.38	15.05	15.21
10	19.20	19.40	19.30	18.70	17.50	18.10	17.33	18.40	17.86	17.80	17.20	17.50	15.10	15.25	15.18	15.13	14.90	15.01
12	19.80	20.70	20.25	18.10	18.80	18.45	16.85	16.93	16.89	16.10	16.40	16.25	14.80	14.45	14.63	14.50	14.18	14.34

* The data was collected from 2 independent experiments. i.e. Exp I and Exp II. Each experiment was done in duplication.

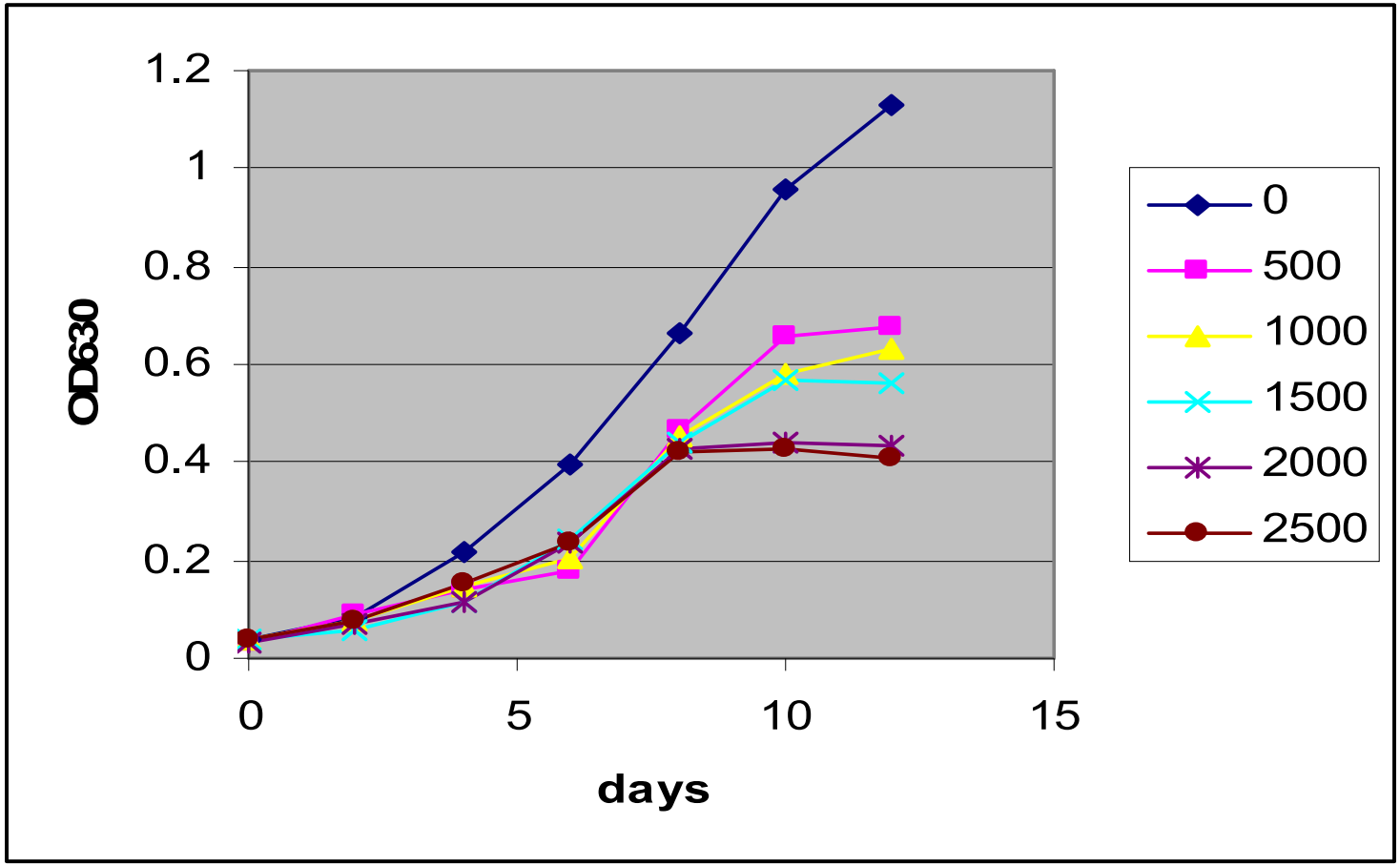

Figure 23 The absorbance at OD 630 nm of crystal violet assay in neomycin susceptibility test for 12 days. Various concentrations of 0, 500, 1,000, 1,500, 2,000, and 2,500 µg/ml neomycin were added.

Table 6 The absorbance value at OD 630 nm of crystal violet assay in neomycin susceptibility test.

	Neomycin concentrations								
Exp. / Day	0			500			1,000		
	I	II	Mean	I	II	Mean	I	II	Mean
0	0.038	0.034	0.036	0.036	0.032	0.034	0.034	0.038	0.036
2	0.089	0.083	0.086	0.087	0.092	0.089	0.078	0.074	0.076
4	0.226	0.214	0.220	0.172	0.104	0.138	0.164	0.125	0.145
6	0.402	0.384	0.393	0.144	0.214	0.179	0.185	0.221	0.203
8	0.658	0.672	0.665	0.432	0.494	0.463	0.459	0.453	0.456
10	0.942	0.967	0.955	0.665	0.652	0.659	0.588	0.576	0.582
12	1.125	1.136	1.131	0.654	0698	0.676	0.583	0.682	0.633
Exp. / Day	1,500			2,000			2,500		
	I	II	Mean	I	II	Mean	I	II	Mean
0	0.037	0.034	0.036	0.035	0.033	0.034	0.038	0.035	0.037
2	0.079	0.063	0.071	0.086	0.057	0.072	0.092	0.061	0.077
4	0.152	0.080	0.116	0.145	0.081	0.113	0.193	0.114	0.154
6	0.318	0.167	0.243	0.316	0.153	0.235	0.306	0.163	0.235
8	0.535	0.346	0.440	0.522	0.336	0.429	0.531	0.308	0.420
10	0.588	0.548	0.568	0.467	0.415	0.441	0.315	0.279	0.43
12	0.584	0.542	0.563	0.458	0.403	0.431	0.311	0.272	0.41

* The data was collected from 2 independent experiments. i.e. Exp I and Exp II. Each experiment was done in duplication.

The correlated results obtained from viable cell count and crystal violet assay showed that the concentration of neomycin 2,000 and 2500 µg/ml could be used for transfected cells selection. Thus, 2,000 µg/ml neomycin was selected in this study.

Hygromycin susceptibility test

Hygromycin susceptibility test was also performed against RKO cells by using 20% MEM containing hygromycin at a concentration of 25, 50, 100, 200 and 400 µg/ml. The minimum concentration that affected cell growth would be selected for pCEP4-ΔNp63 isotypes transfected cell selection. The number of viable cells was analysed every 3 days for 9 days.

Viable cells count

Cells died rapidly at the beginning of the experiment. All concentrations of hygromycin could reduce of the number of viable cells when compared with non-treated cells at 3 days. At day 9, cells in 200 and 400 µg/ml could not be detected. (Figure 24, Table 7)

Crystal violet assay

The amount of viable cells was determined after crystal violet staining. The cell reduction was observed in hygromycin concentration greater than 50 µg/ml comparing to non-treated cells and cells at 25 and 50 µg/ml since day 3 after adding hygromycin. The reduction of absorbance curve could be detected at 9 days in all concentration of hygromycin except 25 µg/ml. (Figure 25, Table 8)

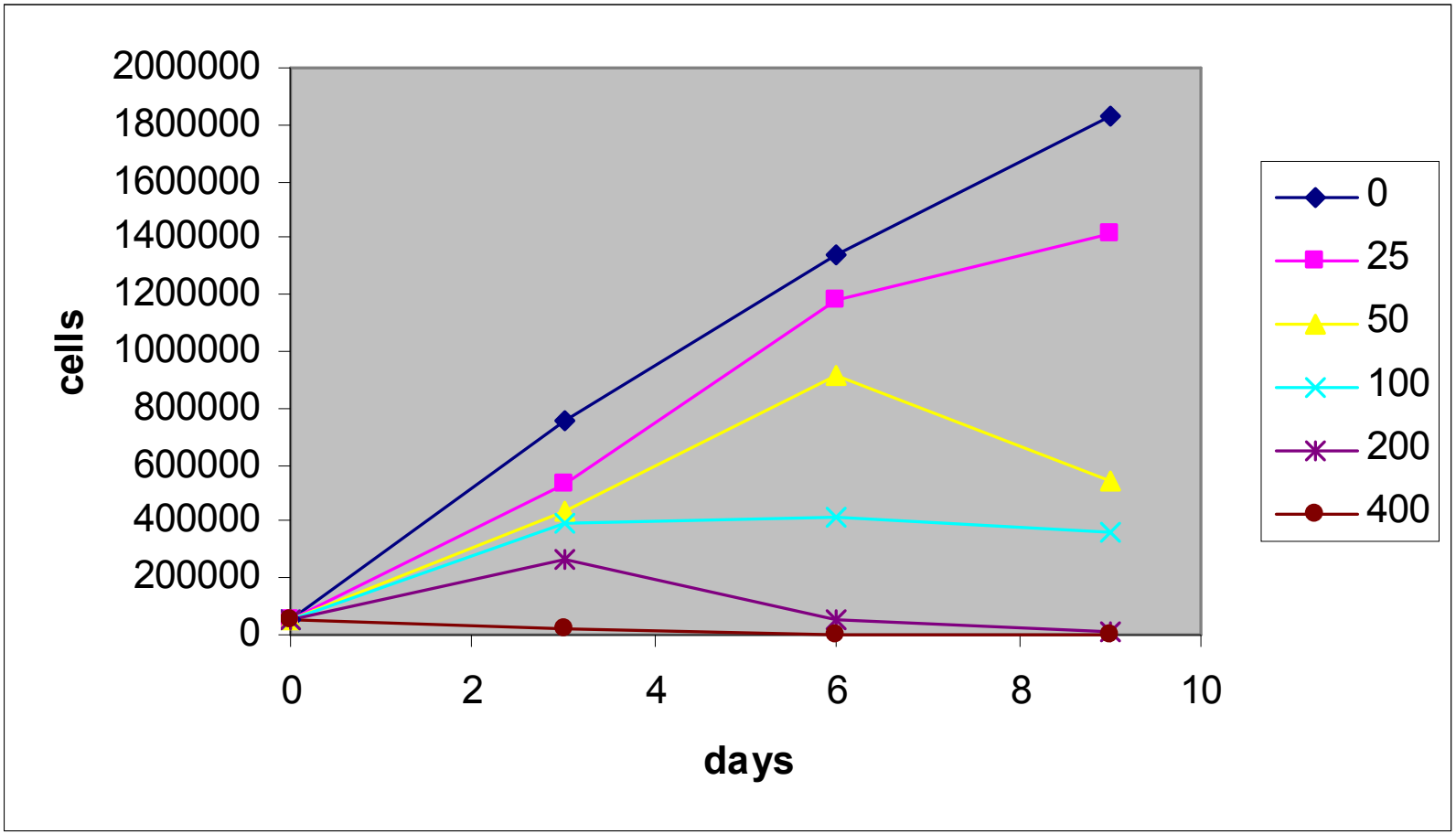

Figure 24 The viable cells count in hygromycin susceptibility test for 9 days. Various concentrations of hygromycin (0, 25, 50, 100, 200, and 400 µg/ml) were added.

Table 7 The viable cell count (1×10^5) in hygromycin susceptibility test.

	Hygromycin concentrations								
Exp. / Day	0			25			50		
	II	I	Mean	I	II	Mean	I	II	Mean
0	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
3	7.60	7.60	7.60	5.25	5.35	5.30	0.45	0.43	0.44
6	13.20	13.60	13.40	11.65	11.95	11.80	9.20	9.20	9.20
9	18.40	18.60	18.30	14.10	14.30	1420	5.30	5.50	5.40
Exp. / Day	100			200			400		
	I	II	Mean	I	II	Mean	I	II	Mean
0	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
3	3.80	4.00	3.90	2.70	2.70	2.70	0.20	0.20	0.20
6	4.10	4.10	4.10	0.50	0.50	0.50	0	0	0
9	3.60	3.60	3.60	0.10	0.10	0.10	0	0	0

* The data was collected from 2 independent experiments. i.e. Exp I and Exp II. Each experiment was done in duplication.

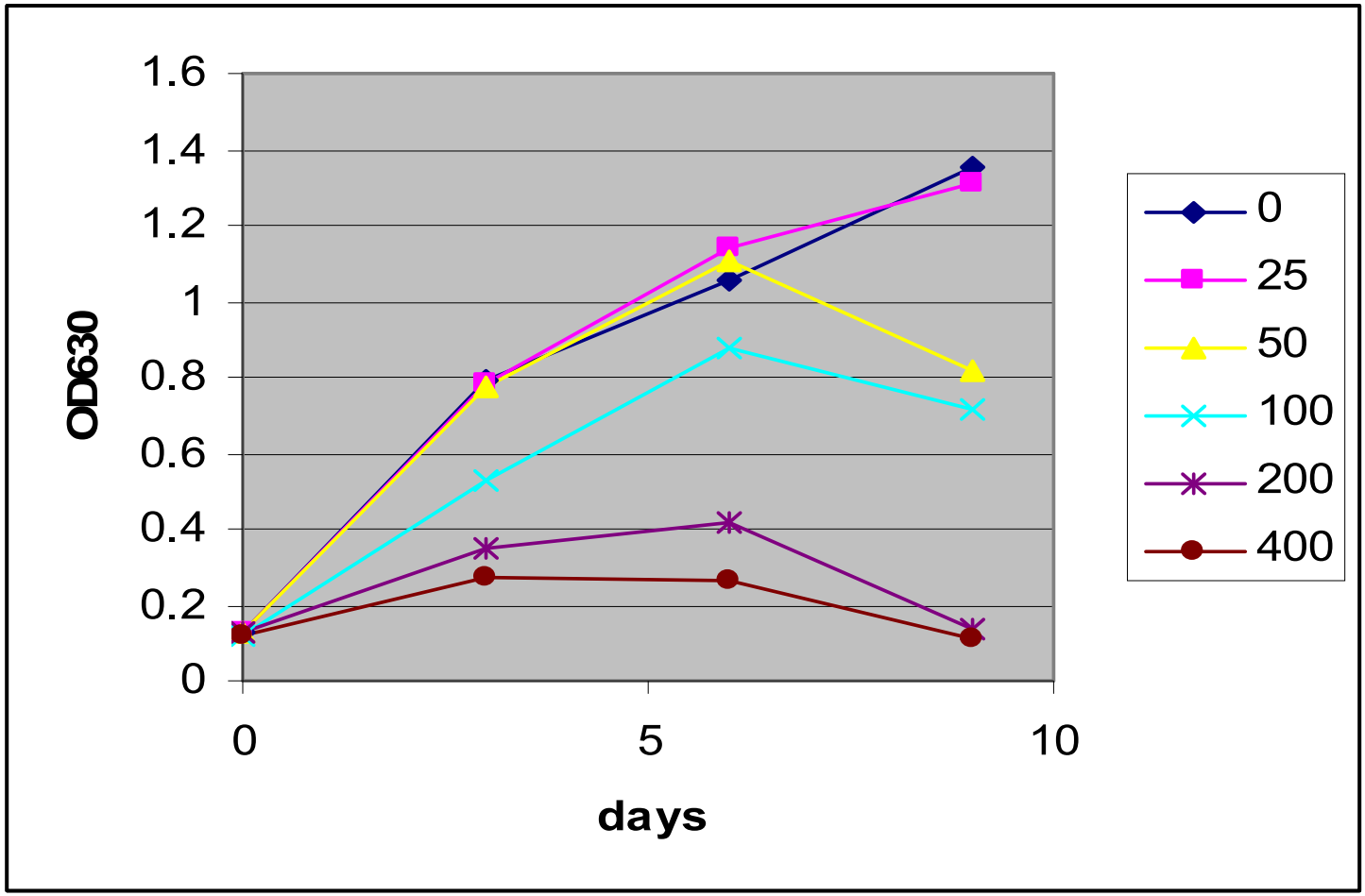

Figure 25 The absorbance at OD 630 nm of crystal violet assay in hygromycin susceptibility test for 9 days.

Table 8 The absorbance values at OD 630 nm of crystal violet assay in hygromycin susceptibility test.

	Hygromycin concentrations								
Exp. / Day	0			25			50		
	II	I	Mean	I	II	Mean	I	II	Mean
0	0.118	0.129	0.124	0.117	0.135	0.126	0.113	0.138	0.126
3	0.842	0.737	0.790	0.824	0.750	0.787	0.830	0.713	0.772
6	1.015	1.089	1.052	1.061	1.224	1.143	1.089	1.128	1.109
9	1.4	1.309	1.355	1.359	1.261	1.310	0.727	0.182	0.016
Exp. / Day	100			200			400		
	I	II	Mean	I	II	Mean	I	II	Mean
0	0.112	0.135	0.123	0.123	0.134	0.129	0.114	0.131	0.123
3	0.754	0.295	0.525	0.514	0.19	0.352	0.415	0.128	0.272
6	0.823	0.937	0.880	0.361	0.474	0.418	0.397	0.127	0.262
9	0.687	0.734	0.711	0.125	0.149	0.137	0.114	0.11	0.112

* The data was collected from 2 independent experiments. i.e. Exp I and Exp II. Each experiment was done in duplication.

The results from both viable cells count and crystal violet assay showed that the doses of hygromycin that affected cell growth effectively were 200 and 400 µg/ml. In this experiment, 200 µg/ml hygromycin was chosen for transfected cell selection.

Optimization of transfection ratio

In order to get the high efficiency of transfection, the proper ratio between transfection reagent and amount of DNA should be optimized (Figure 26). The ratio for transfection was observed by transfecting pCI-Neo-E6 at the ratio of 3:2, 4:2, 5:2, 6:2 and 7:2 (µl transfection reagent:µg DNA). The efficiency of transfections at ~10-30% was observed after IFA staining at 1 week after transfection (Figure 26). Since the transfected cells were found in all ratios, and the ratio of 4:2 was recommended by the manufacturer's instruction, it was also used throughout this study.

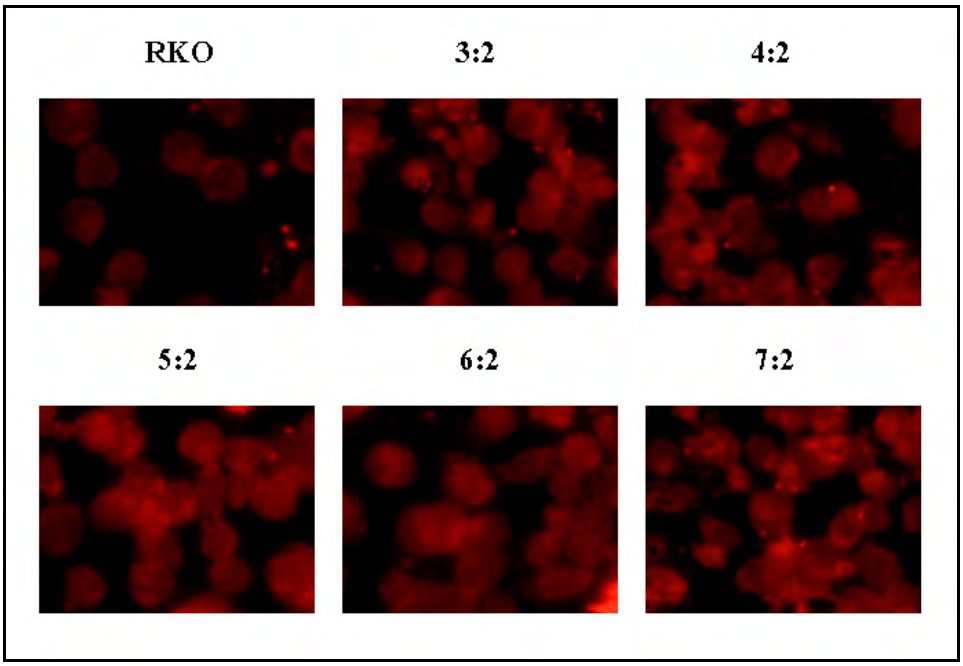

Figure 26 pCI-Neo-E6 transfected RKO cells in any ratios of µl transfection reagent:µg DNA, 3:2, 4:2, 5:2, 6:2 and 7:2. Non-transfected RKO cells were served as a negative control.

Protein expression detection

Western blot

In order to detect the expression of the protein of interest in transfected cells, the cells after transfection for 1 week were grown in tissue culture flask for 3 days. 10^7 cells of transfected cells were used for detecting the protein expression by Western blot analysis. The expected E6 protein was appeared at 18 kDa. At first, the protein expression was detected by Western blot analysis however this method needed the large amount of cells, taken long times for detection and had non-specific binding protein (Figure 27). Therefore the indirect immunofluorescense assay was used for detecting the expression.

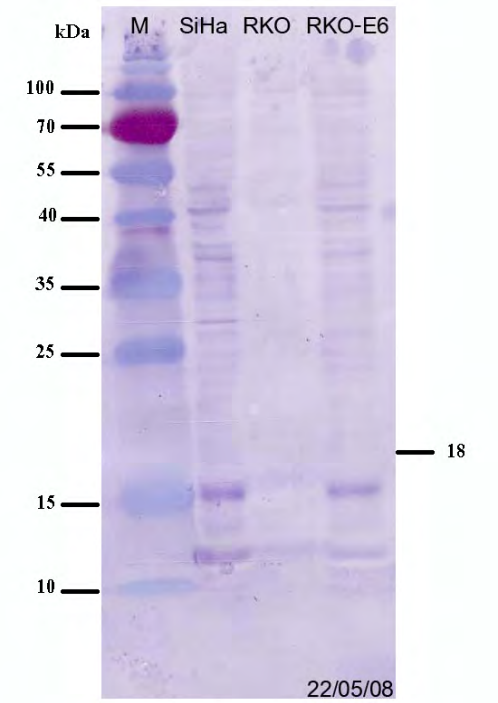

Figure 27 Western blot assay of pCI-Neo E6 transfected RKO cells. M represented prestain protein marker. SiHa served as a positive control of cells expressed HPV16E6 protein. RKO cells were a negative control of the experiment. RKO-E6 represented the transfected cells.

Indirect immunofluorescence assay

Transient transfected cells

At one week after transfection, RKO cells transfected with HPV16E6 and deltaNp63 isotypes showed the expression of the protein of interest about 10-30% in cytoplasm (Figure 28).

Stable transfected cells

At 6 months after transfected cells selection, all transfected cells (100%) had the expression of the protein of interest and could be claimed as the stable expressed cells as demonstrated in Figure 29.

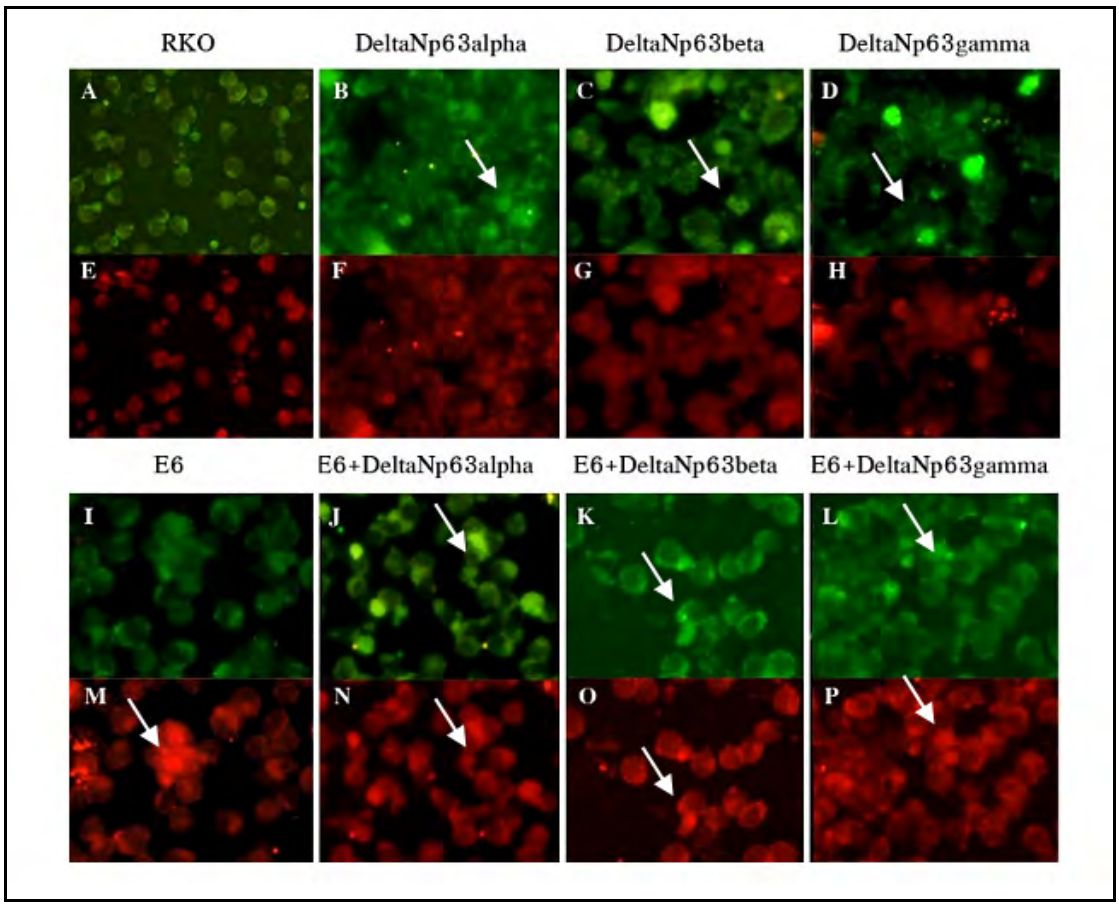

Figure 28 Cells after transfection for 1 week. The illuminate cells (green) indicated cells expressed Np63 isotypes. In the upper row of each group (A, B, C, D, I, J, K, and L) showed the cells stained with the primary antibody specific to ΔNp63 and the secondary antibody tagged with FITC (green). In the lower row of each group (E, F, G, H, M, N, O, and P) showed the cells stained with the primary antibody specific to HPV16E6 and the secondary antibody tagged with PE (red). White arrows pointed to the transfected cells that expressed the protein of interest.

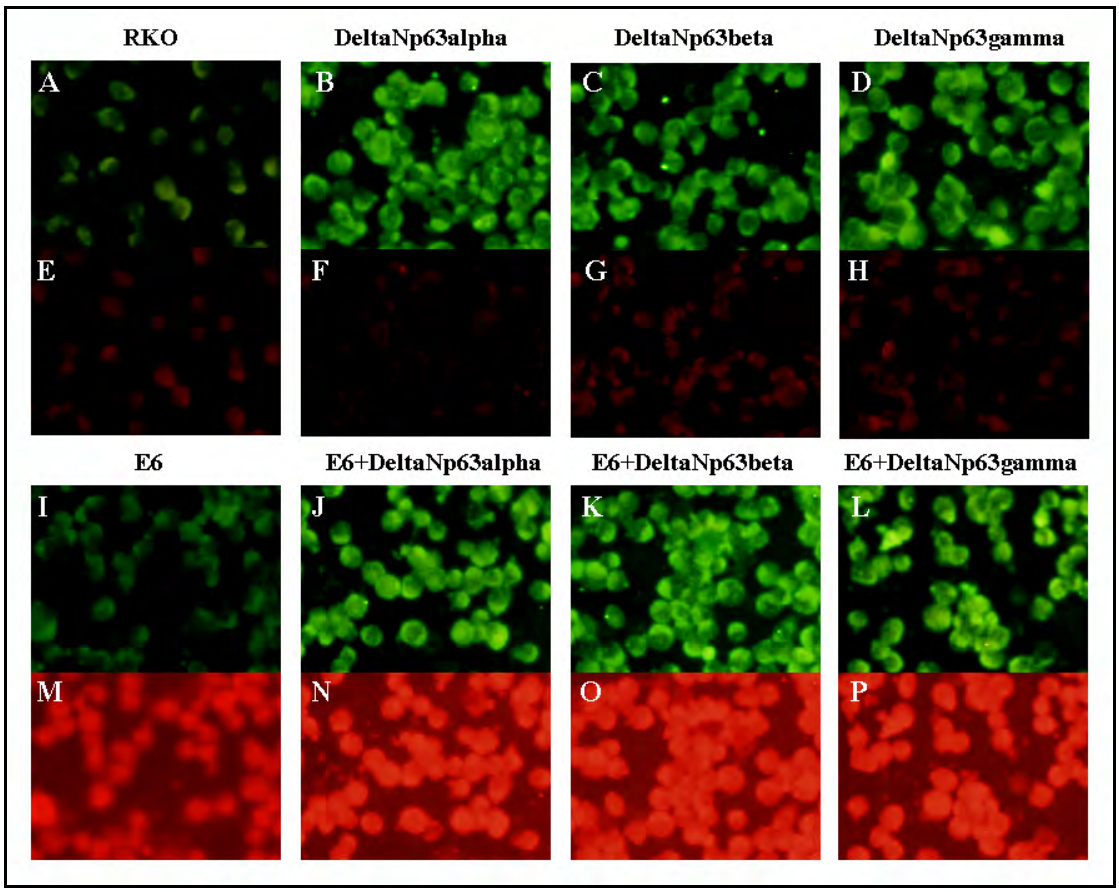

Figure 29 Cells after transfection for 1 week followed by transfected cells selection and grew for 6 months. The illuminate cells (green) indicated cells expressed ΔNp63 isotypes. In the upper row of each group (A, B, C, D, I, J, K, and L) showed the cells stained with the primary antibody specific to ΔNp63 and the secondary antibody tagged with FITC (green). In the lower row of each group (E, F, G, H, M, N, O, and P) showed the cells stained with the primary antibody specific to HPV16E6 and the secondary antibody tagged with PE (red).

Determination of transfected cell properties

Studying about the properties of ΔNp63 isotypes and the correlation between ΔNp63 isotypes and HPV16E6 proteins disclosed the roles of ΔNp63 isotypes in cervical cancer caused by human papillomavirus infection. To study the properties of ΔNp63 isotypes and HPV16E6 protiens, cell proliferation assay and cell death assay were performed.

Condition for cell death assay

To find out the suitable condition for cell death assay, the number of RKO cells (10^4 and 10^5 cells/well), the suitable camptothecin concentration (1, 2 and 4 µg/ml), and the incubation time were optimized.

-Determination of the suitable concentration of camptothecin.

In table 9 the suitable number of cells and camptothecin concentration were varied while the incubation time was fixed at 4 hours. The positive control was derived from the internal positive control of cell death detection kit. The absorbance values at OD_{405} of cell number:camptothecin concentration conditions which were comparable to the positive controls could be observed in 10^5:2 and 10^5:4 (cells/well):camptothecin (µg/ml)). Therefore the chosen condition for this study was 10^5 cells/well and the camptothecin concentration was 2 µg/ml.

-Determination of the suitable incubation period for cell death detection assay.

In table 10, the incubation time was varied whereas the number of cells at 10^5 cells/well and the camptothecin concentration at 2 µg/ml were used. The positive control was derived from the internal positive control of cell death detection kit. The absorbance values at OD_{405} of cell number:camptothecin concentration conditions which were comparable to the positive controls could be observed at 4 hours. The result

indicated that the suitable condition for cell death assay was cells 10^5 cells/well in 2 µg/ml camptothecin for 4 hours.

Table 9 OD_{405} at various Cell numbers and camptothecin concentrations.

	Cells (cells/well):camptothecin (µg/ml)				
	10^4:N	10^4:1	10^4:2	10^4:4	Positive control
I	0.102	0.215	0.517	0.616	2.884
II	0.113	0.207	0.532	0.628	2.882
mean	0.108	0.211	0.525	0.622	2.883
	10^5:N	10^5:1	10^5:2	10^5:4	Positive control
I	1.247	2.456	2.871	2.834	2.860
II	1.252	2.463	2.834	2.802	2.884
mean	1.250	2.460	2.853	2.818	2.872

* N represented cells without camptothecin.

** The data was collected from 2 independent experiments. i.e. Exp I and Exp II. Each experiment was done in duplication.

Table 10 OD_{405} of RKO cells (10^5 cells/well) treated with camptothecin 2 µg/ml at various incubation times.

	Incubation times (hours)			
	1		2	
	N	camptothecin	N	camptothecin
I	0.369	0.270	0.444	0.965
II	0.382	0.273	0.374	0.937
Mean	0.376	0.272	0.409	0.951
	3		4	
	N	camptothecin	N	camptothecin
I	0.371	1.761	0.358	2.799
II	0.382	1.754	0.367	2.750
Mean	0.377	1.758	0.363	2.775
Positive control = 2.884				

* N represented cells without camptothecin.

* The data was collected from 2 independent experiments. i.e. Exp I and Exp II. Each experiment was done in duplication.

Cell death detection assay

Cell death detection assay was performed in all transfected cell types. The absorbance values were calculated to get the enrichment factors. These factors indicated the enrichment of mono- and oligonucleosomes released into cytoplasm which represented an apoptosis. Enrichment factors were calculated from the formula described in materials and methods.

Transient transfected cells

The enrichment factors which were calculated from the absorbance value in table 11 were plotted (figure 30). The result showed that ΔNp63 isotype proteins and HPV16E6 protein had the ability to reduce apoptosis however HPV16E6 protein stronger inhibited cell death than ΔNp63 isotype proteins. Co-expressed ΔNp63 isotypes and HPV16E6 cells promoted the superior results in the reduction of apoptosis.

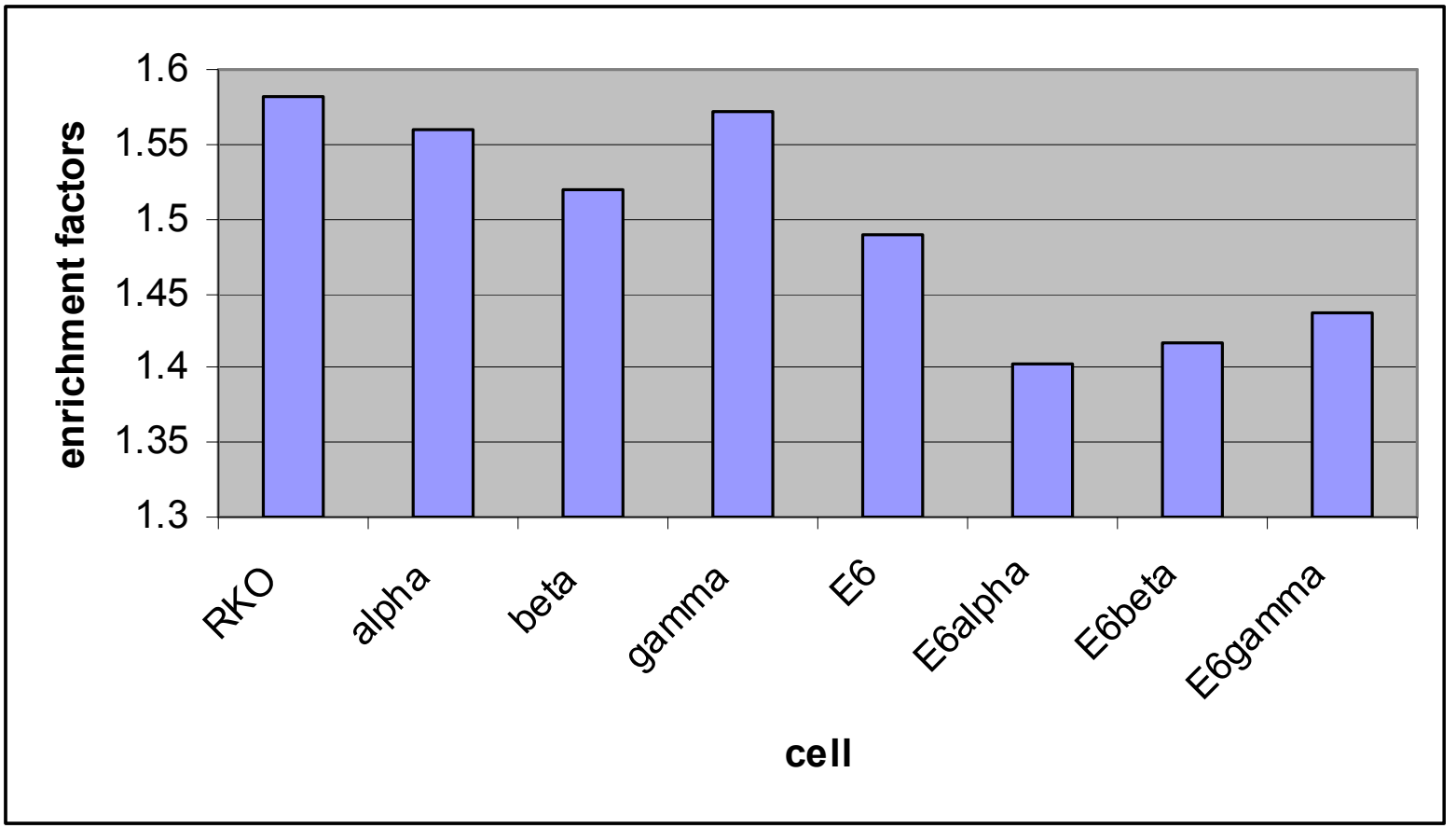

Figure 30 The enrichment factors of transient transfected cells.

Table 11 The absorbance values at OD_{405} and the enrichment factors of cell death detection.

	Absorbance values of cells treated with camptothecin 2 µg/ml							
Exp	RKO	alpha	beta	gamma	E6	E6alpha	E6beta	E6gamma
Ia	2.953	2.899	2.740	1.836	2.774	2.837	2.538	2.687
Ib	2.878	2.829	2.862	2.588	2.819	2.936	2.751	2.295
N	1.843	1.837	1.843	1.408	1.877	2.058	1.867	1.733
	Enrichment factor							
	1.582	1.559	1.52	1.571	1.49	1.402	1.416	1.437
Positive control = 2.882								

* N represented cells without camptothecin.

However, the efficiency of transient transfection was only 10-30%.The stable cell lines were generated in order to get the accurate results.

Stable cells

The enrichment factors which were calculated from the absorbance value in table 12 were plotted (figure 31). To analyze the effects of HPV16E6 and ΔNp63 isotypes proteins in apoptosis, the statistical analysis (ANOVA with Post Hoc test) of the enrichment factors of 8 cell types were tested. The result showed that there was statistically significant difference among 8 cell types (*p*-value = 1.38×10^{-06}) in apoptosis. All HPV16E6 transfected RKO cells (with and without ΔNp63 isotypes) showed the statistically significant difference in apoptosis when compared to RKO cells without HPV16E6. Besides HPV16E6, ΔNp63α and ΔNp63β play the role in the inhibition of apoptosis induced by camptothecin while the effect was not found in ΔNp63γ. However, there was no statistically significant difference between ΔNp63α and ΔNp63β transfected RKO cells [*p*-value = 1.00 (without HPV16E6) and 0.999 (with HPV16E6)]. The compared data was showed in table 13.

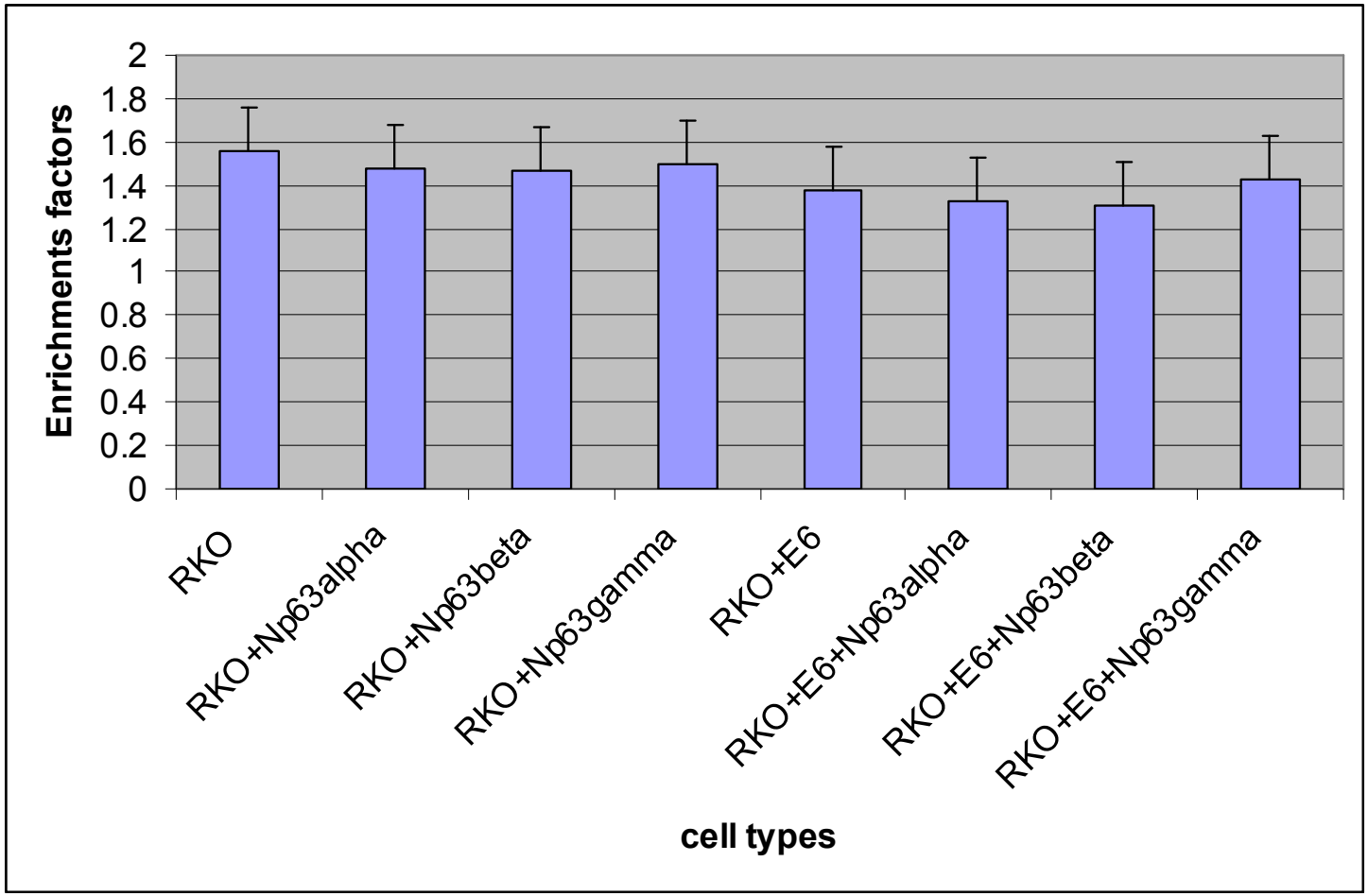

Figure 31 The enrichment factors of stable transfected cells.

Table 12 The enrichment factors of stable transfected cells.

	Absorbance							
Exp	RKO	Alpha	beta	gamma	E6	E6alpha	E6beta	E6gamma
Ia	2.974	2.719	2.768	2.751	2.617	2.516	2.506	2.713
Ib	2.977	2.706	2.678	2.807	2.393	2.433	2.356	2.620
N	1.848	1.836	1.853	1.862	1.813	1.845	1.867	1.832
IIa	2.811	2.873	2.815	2.841	2.621	2.427	2.529	2.727
IIb	2.727	2.597	2.663	2.708	2.398	2.430	2.372	2.472
N	1.829	1.875	1.864	1.856	1.826	1.853	1.872	1.854
	Enrichment factors							
Ia	1.609	1.481	1.494	1.477	1.443	1.364	1.342	1.491
Ib	1.611	1.473	1.445	1.508	1.320	1.319	1.262	1.430
IIa	1.536	1.532	1.510	1.531	1.435	1.309	1.351	1.470
IIb	1.491	1.438	1.429	1.459	1.313	1.311	1.267	1.333
mean±s.d.	1.561±0.059	1.481±0.039	1.470±0.019	1.494±0.032	1.378±0.071	1.326±0.026	1.306±0.048	1.430±0.070

Table 13 The *p*-value of the enrichment factors of stable transfected cells analyzed by ANOVA with Post Hoc test.

	alpha	beta	gamma	E6	E6alpha	E6beta	E6gamma
RKO	0.351	0.208	0.559	0.001*	0.000*	0.000*	0.023*
alpha		1.000	1.000	0.118	0.004*	0.001*	0.847
beta			0.997	0.214	0.010*	0.002*	0.955
gamma				0.057	0.002*	0.000*	0.650
E6					0.821	0.486	0.803
E6alpha						0.999	0.105
E6beta							0.032*
E6gamma							

* The mean difference is significant at 0.05 level.

Cell proliferation assay

The proliferation assays were performed by using cell proliferation ELISA. This method quantitated cell proliferation based on the measurement of BrdU incorporation during DNA synthesis in proliferating cells. The absorbance values at OD_{450} were collected. The results from 2 independent experiments analyzed by ANOVA with Post Hoc test showed that the proliferation of RKO cells transfected with HPV16E6 and HPV16E6+ΔNp63γ HPV was better than ΔNp63γ alone. The proliferation of RKO cells transfected with ΔNp63α was significant difference when compared to RKO cells transfected with ΔNp63γ, HPV16E6 and HPV16E6+ΔNp63γ (*p*-value = 0.048, 0.005 and 0.00, respectively). The proliferation of RKO cells transfected with ΔNp63α was lower than ΔNp63γ with the *p*-value = 0.0.036. The results could be concluded that HPV16E6 and ΔNp63γ had the ability to support cell proliferation while ΔNp63α did not support cell proliferation. The compared data was showed in table 14 and 15.

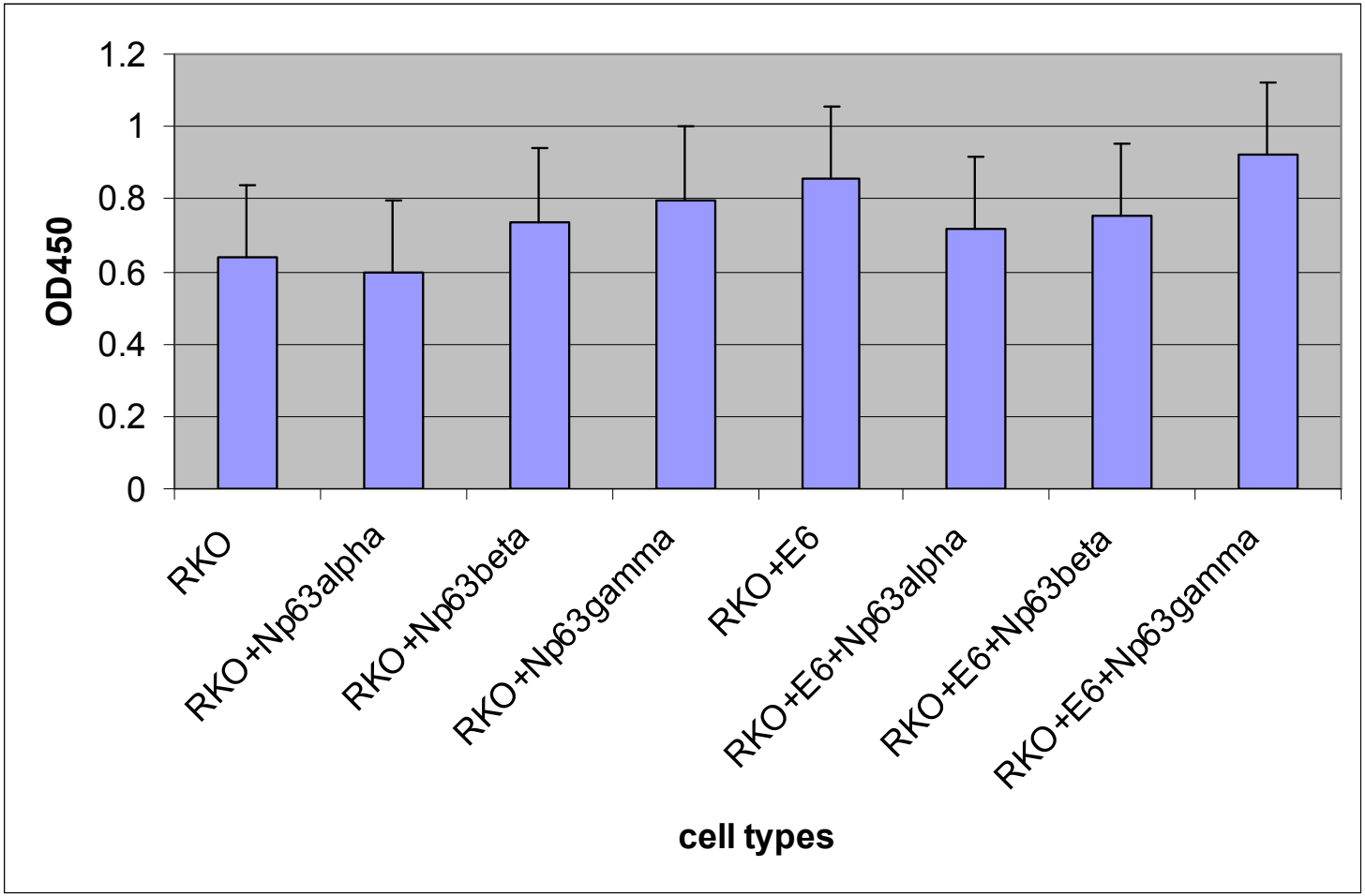

Figure 32 The absorbance values at 450 nm of cell proliferation assay.

Table 14 The absorbance values at 450 nm of cell proliferation assay.

	Absorbance							
Exp	RKO	alpha	beta	gamma	E6	E6alpha	E6beta	E6gamma
Ia	0.591	0.598	0.682	0.771	0.918	0.797	0.627	0.932
Ib	0.520	0.491	0.728	0.624	0.964	0.710	0.786	0.936
IIa	0.738	0.666	0.775	0.908	0.741	0.644	0.795	0.935
IIb	0.709	0.637	0.767	0.888	0.795	0.713	0.809	0.892
Mean± S.D.	0.640±0.102	0.598±0.077	0.738±0.043	0.798±0.131	0.855±0.104	0.716±0.063	0.754±0.179	0.924±0.021

Table 15 The *p*-value of the absorbance value at 450 nm of stable transfected cells in the proliferation assay analyzed by ANOVA with Post Hoc test.

	alpha	beta	gamma	E6	E6alpha	E6beta	E6gamma
RKO	0.996	0.722	0.191	0.027*	0.899	0.556	0.002*
alpha		0.317	0.048*	0.005*	0.523	0.203	0.000*
beta			0.971	0.538	1.000	1.000	0.079
gamma				0.978	0.865	0.995	0.443
E6					0.330	0.705	0.937
E6alpha						0.998	0.036*
E6beta							0.135
E6gamma							

* The mean difference is significant at 0.05 level.

CHAPTER VI

DISCUSSION

Cancer of uterine cervix was found to be the second most common incidence among female in developing countries. Infection with high risk human papillomaviruses (HPV) was the commonest incidence [1]. High risk HPV E6 protein plays the critical roles in tumor development by interacting with many cellular proteins that regulate cell cycle and interferes both extrinsic and intrinsic pathways of apoptosis. In the function about p53, HPV E6 protein down-regulates p53 activity by inducing p53 to the ubiquitin degradation pathway by the helping of other cellular proteins. P63 family is the group of proteins which share the similarity of protein structure and amino acid sequences with p53 proteins. P63 proteins located on chromosome 3q27 [9, 10]. ΔNp63 is the p63 proteins without NH_2-terminal transactivating domain. The alternative splicing of p63 generates three isoforms with the different COOH-oligomerization domain termed α, β, and γ. The studies about biochemical activities of p63 in transient transfection or adenoviral infection suggested that TAp63 variants could induce cell cycle arrest and apoptosis while ΔNp63 variants show the opposite function [13-15]. The studies in normal cervix showed the expression of ΔNp63 in basal and parabasal cells whereas the expression of ΔNp63 could be detected in superficial cells of cervical intraepithelial neoplasia (CIN) [19]. To study the correlation between HPV16 E6 protein and the expression of ΔNp63 family, the cell line expressing deltaNp63 and HPV E6 proteins were generated. The cell death detection assay and cell proliferation assay were performed.

Transient transfected cells were generated at the beginning of the study. The proper ratio between transfection reagent and amount of DNA was optimized however the transfection efficiency was always about 10-30%. In order to get the accurate result, stable transfected cells were generated by drug selection. Neomycin and hygromycin susceptibility of RKO cells were also optimized in order to get the suitable concentrations for transfected cells selection. In this study, the suitable

concentrations were different from the recommended concentration from the manufacturer's instruction. Thus, the concentration to be used depends on each cell type. After continuous selection, all cells completely transfected and showed protein expression.

In cell death detection assay, preliminary study using transient RKO transfected cells showed that all transfected cell types except ΔNp63γ transfected cell reduced cell death ability after stimulating with campothecin (table 11, figure 30). These results were confirmed when stable RKO transfected cells were used. Both HPV16E6 and ΔNp63 isotypes had the ability to reduce apoptosis of RKO cells (table 12). The reductions of apoptosis were statistically significant difference when compared the stable ΔNp63 isotypes transfected and stable HPV16E6 transfected RKO cells to non-transfected RKO cells. All HPV16E6 transfected RKO cells (with and without ΔNp63 isotypes) showed the statistically significant difference in apoptosis when compared to RKO cells without HPV16E6. This observation strongly supported the function of E6 protein of human papillomavirus that could inhibit apoptosis through the interaction of E6 with many cellular proteins that regulate cell cycle [29]. Beside HPV16E6, ΔNp63α and ΔNp63β also played the role in the inhibition of apoptosis induced by camptothecin. Our finding corresponding to a previous report performed in baby hamster kidney cells (BHK cells) transfected with mammalian expression vectors encoding epitope-tagged p63 isotypes [15]. The noticeable level of apoptosis was detected in BHK cells expressing ΔNp63γ while apoptosis was virtually absent in BHK cells expressing high levels of ΔNp63α. This could be explained by the structures of ΔNp63 isotypes which lack the N terminus and act in a dominant-negative manner toward p53. The assays about transactivation of p53 reporter genes by p63 isotypes in Saos-2 cells transfected with constant amount wild-type p53 and varying concentration of either ΔNp63α or ΔNp63γ indicated that ΔNp63 isotypes act in a dominant-negative manner toward p53 [15]. In this study, the reduction of apoptosis was enhanced in RKO cells expressed both HPV16E6 and each ΔNp63 isotypes. The results could be explained by the function of HPV E6 which targeted p53 into the protein

degradation pathway [3] while p53 associated with and targeted ΔNp63 into a protein degradation pathway [15, 51]. The reduction of p53 in RKO cells expressing both HPV16E6 and each ΔNp63 isotypes sustained ΔNp63 isotypes which play the roles as anti-apoptotic proteins.

In cell proliferation assay, the proliferation of RKO cells transfected with HPV16E6 and HPV16E6+ΔNp63γ were noticeable. The results were better than RKO cells transfected with ΔNp63γ alone and contrast to RKO cells transfected with ΔNp63α or ΔNp63β which played the roles in apoptosis. Our results suggested that the function of ΔNp63γ may regulate or work with the molecules which took a part in different pathway such as cox-2 [52]. The correlation between the function of HPV16E6 and ΔNp63γ in cell proliferation remains unclear because the previous reports about the function of HPVE6 were pointed to the regulation of apoptosis [53].

This study suggested that ΔNp63 isotypes played role in the ability of either apoptosis or proliferation of cells. The functions were probably related to the variation of COOH end which came from the alternative splicing at transcription step. In the presence of HPV E6 protein, the splicing of such proteins might be affected resulting to the change of cell growth properties.

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APPENDICES

APPENDIX A

REAGENTS, MATHERIALS AND INSTRUMENTS

A. Media and Reagents

Absolute ethanol	(Merck, Germany)
40% AccuGel™ 29:1	(Kimberly research, U.S.A.)
Acetic acid	(Merck, Germany)
Agarose	(Research Organic, Inc., U.S.A.)
Ammonium persulfate	(Bio Basic, U.S.A.)
Amplicilin	(Bio Basic, U.S.A.)
Boric acid	(Sigma-Aldrich, U.S.A.)
Bromphenol blue	(USB, U.S.A.)
100 bp DNA ladder	(Fermentas, EU)
Calcium chloride	(Sigma-Aldrich, U.S.A.)
Cell Death Detection ELISA plus	(Roche, USA)
Cell Proliferation ELISA, BrdU (colorimetric)	(Roche, USA)
DNA extraction kit	(Qiagen, U.S.A.)
dNTPs	(Promega, U.S.A.)
DTT	(Bio Basic, U.S.A.)
Ethydium bromide	(Bio-Rad, U.S.A.)
EDTA	(Biobasic, Canada)
Fetal Bovine Serum	(PAA, Austria)
FuGENE HD® Transfection kit	(Roche, USA).
Glysine	(Biobasic, Canada)
Goat polyclonal anti-deltaNp63 IgG	(Santa cruz, USA)
Goat polyclonal anti-mouse IgG PE	(Abcam, UK)
HPV16E6 mouse monoclonal antibody	(Santa Cruz, U.S.A.)
HEPES	(Sigma-Aldrich, U.S.A.)

Hygromycin	(Sigma-Aldrich, U.S.A.)
LB broth powder	(Bio Basic, U.S.A.)
MEM medium	(GIBCO^{TM} Invitrogen, UK)
Methanol	(Merck, Germany)
Neomycin	(Sigma-Aldrich, U.S.A.)
Penicilin G	(Bio Basic, U.S.A.)
Plasmid extraction	(Qiagen, U.S.A.)
Polyethyleneglycon	(Sigma-Aldrich, U.S.A.)
Potassium chloride	(Merck, Germany)
Potassium phosphate	(Bio Basic, U.S.A.)
Prestained protein ladder	(Fermentas, EU)
Rabbit polyclonal anti-goat IgG FITC	(Abcam, UK).
SDS	(Bio Basic, U.S.A.)
Sodium bicarbonate	(Bio Basic, U.S.A.)
Sodium chloride	(Merck, Germany)
Sodium phosphate	(Bio Basic, U.S.A.)
Streptomycin	(Bio Basic, U.S.A.)
Taq DNA polymerase	(Fermentas, U.S.A.)
TEMED	(Bio Basic, U.S.A.)
Triton X-100	(Sigma-Aldrich, U.S.A.)
Tris Base	(Research Organic,U.S.A.)
Trypsin 1:250	(Bio Basic, U.S.A.)
Trypan blue	(Sigma, UK)

B.Materials

Centrifuge tube	(Labcon, Germany.)
Disposable serological pipette	(Labcon, Germany.)
ELISA plate	(Millipore, U.S.A.)
Filter Tip	(Labcon, Germany.)

Microcentrifuge tube	(Sorenson, U.S.A.)
24-well flat plate	(Costar, USA)
96-well flat plate	(Costar, USA)
Tissue culture flask	(Nunc,Denmark)

C. Instruments

Autoclave (model-SX-700)	(Tomy, Japan)
CO_2 Incubator	(Thermo Forma, U.S.A.)
Culturing shanking incubator	(Ztictene, Chaina)
DNA thermocycle system	(Takara, Japan)
Electrophoresis chamber	(CBS, U.S.A.)
ELISA plate reader	(Human Egypt, Egypt)
Fluorescence microscope	(Olympus, USA)
Incubator	(Memmert, Germany)
Microcentrifuge	(Fotodyne, U.S.A.)
Orbital shaker (TPM-2)	(Sarstedt , Germany)
pH meter	(Accumet Basic, Singapore)
Power supply (Model 1000/500)	(Bio-Rad, U.S.A.)
Refrigerator	(Sanyo, Japan)
Spectrophotometer (SmartSpect™ 3000)	(Bio-Rad, U.S.A.)
Refrigerated centrifuge	(Thermo Fisher Scientific Inc., MA)
Vertical electrophoresis chamber	(Cleaver scientific Ltd., UK)
Water bath	(Julabo, Germany)
Western blot chamber	(Cleaver scientific Ltd., UK)

APPENDIX B

REAGENTS PREPARATION

Reagents for agarose gel electrophoresis

1. **10X Tris-borate buffer (TBE)**

Tris-base	60.50	g
Boric acid	30.85	g
$Na_2EDTA{\cdot}2H_2O$	3.72	g
Distilled water	800	ml

Adjust volume to 1 liter. Sterilize the solution by autoclaving at 121°C for 15 minutes.

2. **10 mg/ml Ethidium bromide**

Ethidium bromide	1.0	g
Distilled water	100	ml

Mixed the solution and store in the dark at 4°C.

3. **Agarose gel**

Agarose	0.5	g (2.5 % gel)

(0.3 g for 1.5 % gel and 0.16 g for 0.8 % gel)

4. **1xTBE** 20 ml

The solution was dissolved by heating in microwave oven and occasional mix until no granules of agarose are present

Reagents for transformation

1. **TE buffer**

Tris-HCl, pH 7.5	10	mM
EDTA	1	mM

Dissolve in distilled water and adjust volume to 5 ml. Sterilize the solution by autoclaving at 121°C for 15 minutes.

2. **2 M $MgCl_2$ (10 ml)**

$MgCl_2$	1.9	g

Dissolve in distilled water and adjust volume to 10 ml. Sterilize the solution by autoclaving at 121°C for 15 minutes.

3. **Ampicilin stock (100 mg/ml)**

Ampicilin	1	g

Dissolve in 10 ml of distilled water. Store at -20°C.

4. **LB Medium (1Litter)**

Bacto-Tryptone	10	g
Yeast Extract	5	g
NaCl	5	g

Dissolve in distilled water and adjust volume to 1 liter. Sterilize the solution by autoclaving at 121°C for 15 minutes.

5. **LB- Ampicillin plates**

Bacto-Tryptone	10	g
Yeast Extract	5	g
NaCl	5	g
Agar	15	g

Dissolve in distilled water and adjust volume to 1 liter. Sterilize the solution by autoclaving at 121°C for 15 minutes. After autoclaved, allowed media cool down, added 1 ml ampicilin stock, poured and stored plates at 4°C in the dark.

Reagents for SDS-PAGE

1. **Running gel solution**

Deionized distilled water	3.03	ml
1.5 M Tris-HCl pH 8.8	1.90	ml
10% (w/v) SDS	75	µl
40% Acrylamide	2.47	ml
10%(w/v) APS	37.5	µl
TEMED	4.95	µl

2. **Stacking gel solution**

Deionized distilled water	1.80	ml
0.5 M Tris-HCl pH 6.8	750	µl
10% (w/v) SDS	30	µl
40% Acrylamide	402	µl
10%(w/v) APS	15	µl
TEMED	3	µl

3. **Running Buffer (1 liter)**

Tris base	15.1	g
Glycine	72	g
SDS	5.0	g

Dissolve in distilled water and adjust volume to 1000 ml. Store at room temperature.

Regents for Western blot

1. **TBS buffer**

1 M Tris pH 7.5	20	ml
NaCl	29.22	g
Distilled water to	1	L

Sterilized by autoclaving and stored at room temperature

2. **TTBS buffer**

TBS	100	ml
Tween20	100	µl

3. **Blocking buffer**

TBS	10	ml
Bovine serum albumin	0.3	g

4. **Alkaline phosphate buffer (Soak APS)**

1 M Tris pH 9.5	50	ml
NaCl	2.92	g
2 M $MgCl_2$	625	µl
Distilled water to	1	L

Sterilized by autoclaving and stored at room temperature

5. **10X Blotting buffer**

Tris base	30.3	g
Glycine	144	g
Distilled water to	1	L

Sterilized by autoclaving and stored at room temperature

Regents for IFA

1. **10 X PBS**

NaCl	80	g
KCL	2	g
$NaHPO_4$	11.5	g
KH_2HPO_4	2	g
Deionized distilled water to	900	ml

Adjusted to pH 7.4 and adjusted volume to
1000 ml with additional deionized distilled water
Sterilized by autoclaving and stored at room temperature

2. **1X PBS**

10 X PBS	100	ml
Deionized distilled water	900	ml

3. **Mounting fluid**

Glycerol	5	ml
1X PBS	5	ml

Reagents for cells cultivation

1. **Penicillin 100,000 Units/ml**

1,000,000 Unit Penicillin G	1	ampoule
Deionized Distilled water	10	ml

Sterilized by filtration and stored at -20 ^{o}C

2. **Streptomycin 100,000 µg/ml**

1 gm Streptomycin	1	ampoule
Distilled water	10	ml

Sterilized by filtration and stored at -20 ^{o}C

3. **1 M HEPES**

HEPES	23.83	g
Deionized distilled water to	100	ml

Sterilized by autoclaving and stored at 4 ^{o}C

4. **10 % $NaHCO_3$**

$NaHCO_3$	10	g
Deionized distilled water to	100	ml

Sterilized by autoclaving and stored at 4 °C

5. **Growth medium**

MEM medium	80	ml
100,000 unit/ml Penicillin G	1	ml
100,000 µg/ml Streptomycin	1	ml
Fetal Bovine Serum (FBS)	20	ml
1M HEPES	1	ml
10 % $NaHCO_3$	1	ml

Stored at 4°C

6. **5% Trypsin**

Trypsin	5	g
Deionized distilled water to	100	ml

Sterilized by filtration and stored at -20 °C

7. **1% EDTA**

EDTA	1	g
Deionized distilled water to	100	ml

Sterilized by filtration and stored at 4 °C

8. **Trypsin versine (0.025% trypsin, 0.01% EDTA)**

5% Trypsin	0.5	ml
1% EDTA	0.2	ml
1X PBS up to	100	ml

Do not autoclave

Sterilized by filtration and stored at 4 °C

9. 1X PBS

10 X PBS	100	ml
Deionized distilled water	900	ml

BIOGRAPHY

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