การเปรียบเทียบลำดับนิวคลีโอไทด์ของ gene L1 ในเชื้อ Human papillomavirus ที่แยกได้จากตัวอย่างต้อเนื้อ และตัวอย่างมะเร็งปากมดลูกชนิด Adenocarcinoma

นางสาวลักคณา ธรรมโชติรุจน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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COMPARISON OF L1 GENE OF HUMAN PAPILLOMAVIRUS FROM PTERYGIUM AND ADENOCARCINOMA CERVICAL TISSUE

Miss Luckana Thammachoteruja

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Medical Microbiology
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Thesis Title	COMPARISON OF L1 GENE OF HUMAN PAPILLOMAVIRUS FROM PTERYGIUM AND ADENOCARCINOMA CERVICAL TISSUE
By	Miss Luckana Thammachoteruja
Field of Study	Medical Microbiology
Thesis Advisor	Associate Professor Parvapan Bhattarakosol, Ph.D.
Thesis Co-advisor	Associate Professor Somchai Niruthisard, M.D.
Thesis Co-advisor	Associate Professor Ngamjit Kasetsuwan, M.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

……. Dean of the Graduate School

(Associate Professor Pornpote Piumsomboon, Ph.D.)

THESIS COMMITTEE

……. Chairman

(Associate Professor Ariya Chindamporn, Ph.D.)

……. Thesis Advisor

(Associate Professor Parvapan Bhattarakosol, Ph.D.)

……. Thesis Co-advisor

(Associate Professor Somchai Niruthisard, M.D.)

……. Thesis Co-advisor

(Associate Professor Ngamjit Kasetsuwan, M.D.)

……. External Examiner

(Associate Professor Wasan Chantratita, Ph.D.)

ลักคณา ธรรมโชติรุจน์: การเปรียบเทียบลำดับนิวคลีโอไทด์ของ gene L1 ในเชื้อ Human papillomavirus ที่แยกได้จากตัวอย่างต้อเนื้อ และตัวอย่างมะเร็งปากมดลูกชนิด Adenocarcinoma (COMPARISON OF L1 GENE OF HUMAN PAPILLOMAVIRUS FROM PTERYGIUM AND ADENOCARCINOMA CERVICAL TISSUE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร.ภาวพันธ์ ภัทรโกศล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.นพ.สมชัย นิรุตติศาสน์ และ รศ.พญ.งามจิตต์ เกษตรสุวรรณ 87 หน้า

โรคต้อเนื้อ คืออาการที่เกิดเป็นรอยโรคของเนื้อเยื่อเส้นใยของหลอดนำส่งของเหลวบริเวณลูกตา ที่มีลักษณะเป็นเนื้องอกของเยื่อตา การศึกษาเกี่ยวกับสาเหตุของโรค และกลไกในการเกิดโรคไม่เป็นที่แน่ชัด สันนิษฐานว่าน่าจะเกิดจากปัจจัยทางด้านสิ่งแวดล้อม เช่นแสง ultraviolet จากดวงอาทิตย์, ความผิดปกติในระดับโมเลกุล รวมถึงไวรัสที่ทำให้เกิดโรค ได้แก่ HPV ส่วน Adenocarcinoma คือชนิดของมะเร็งปากมดลูกที่แบ่งออกตามลักษณะพยาธิสภาพของเซลล์ ซึ่งเป็นมะเร็งที่เกิดขึ้นในเซลล์ต่อมเกิดขึ้นบริเวณ endocervix มีสาเหตุมาจากการติดเชื้อ HPV และปัจจัยเสี่ยงอื่นร่วมด้วย ได้แก่ ปัจจัยทางสิ่งแวดล้อม, ปัจจัยของโฮสต์ และ ปัจจัยของไวรัส ก่อนหน้านี้ได้มีการศึกษาอุบัติการณ์การพบและชนิดของ HPV ในตัวอย่างมะเร็งปากมดลูกชนิด adenocarcinoma พบ HPV ชนิด 18 และ 16 มากที่สุด ส่วนอุบัติการณ์การพบและชนิดของ HPV ในตัวอย่างต้อเนื้อ พบ HPV ชนิด 6 และ 11 มากที่สุด และยังพบ HPV ชนิด 16 และ 18 ด้วย วัตถุประสงค์ของการศึกษาครั้งนี้คือ ศึกษาเปรียบเทียบลำดับนิวคลีโอไทด์ของ L1 gene ในเชื้อ HPV ชนิดที่ตรวจพบได้ในตัวอย่างต้อเนื้อ เปรียบเทียบกับลำดับนิวคลีโอไทด์ของ L1 gene ในเชื้อ HPV ชนิดที่ตรวจพบได้ในตัวอย่างมะเร็งปากมดลูกชนิด adenocarcinoma

การศึกษาครั้งนี้ทำการหาชนิดของ HPV โดยวิธี reverse hybridization จากกลุ่มตัวอย่างที่เป็น DNA ที่สกัดได้จากเนื้อเยื่อของผู้ป่วยที่เป็นโรคต้อเนื้อจำนวน 25 ตัวอย่าง และกลุ่มมะเร็งปากมดลูกชนิด adenocarcinoma จำนวน 89 ตัวอย่าง จากผลการศึกษาความชุกของ HPV ในกลุ่มตัวอย่างต้อเนื้อ สามารถตรวจหาชนิดของ HPV ได้ทั้งหมด 16 ตัวอย่าง คิดเป็น 64% โดยพบ HPV-16 ในทุกตัวอย่าง รองลงมาเป็นการติดเชื้อร่วมกับ HPV-18 (2/25, 8%) และ HPV-58 (2/25, 8%) ส่วนในกลุ่มของ มะเร็งปากมดลูกชนิด adenocarcinoma สามารถตรวจหาชนิดของ HPV ได้ทั้งหมด 23 ตัวอย่าง คิดเป็น 25.8% พบว่าเป็นการติดเชื้อร่วมกันของ HPV หลายชนิด โดยพบ HPV 51 มากที่สุด (7/23, 30.43%) รองลงมาเป็น HPV 58 (5/23, 21.74%) ตามมาด้วย HPV16 (4/23, 17.39%) และ HPV 53 (4/23, 17.39%)

ผลการศึกษาเปรียบเทียบลำดับเบสของ L1 gene ของ HPV-16, 31, 52, 53, 58, 59, 66 and 70 เทียบกับลำดับเบสของ reference HPV ในแต่ละชนิด พบว่ามีตำแหน่งเบสบางตำแหน่งเปลี่ยนไป นอกจากนี้ยังพบว่าตำแหน่งเบสที่เปลี่ยนไปในตัวอย่างที่เป็น HPV-31, 53, 59 และ 70 มีผลทำให้ตำแหน่งของกรดอะมิโนของโปรตีน L1 เปลี่ยนไปเช่นกัน และจากการเปรียบเทียบลำดับเบสของ HPV-16 ในกลุ่มตัวอย่างต้อเนื้อเทียบกับกลุ่มตัวอย่างมะเร็งปากมดลูกชนิด adenocarcinoma พบว่าตำแหน่งของนิวคลีโอไทด์ มีความแตกต่างกัน 3 ตำแหน่ง

การศึกษานี้แสดงให้เห็นว่า ลำดับนิวคลีโอไทด์ของ gene L1 ในเชื้อ HPV ที่แยกได้จากตัวอย่างต้อเนื้อ เทียบกับตัวอย่างมะเร็งปากมดลูกชนิด Adenocarcinoma มีความแตกต่างกันเล็กน้อย แต่ยังอยู่ในกิ่งเดียวกันของแผนภูมิต้นไม้

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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LUCKANA THAMMACHOTERUJA: COMPARISON OF L1 GENE OF HUMAN PAPILLOMAVIRUS FROM PTERYGIUM AND ADENOCARCINOMA CERVICAL TISSUE. ADVISOR: ASSOC. PROF. PARVAPAN BHATTARAKOSOL, Ph.D., CO-ADVISOR : ASSOC. PROF. SOMCHAI NIRUTHISARD, M.D., ASSOC. PROF. NGAMJIT KASETSUWAN, M.D.87 pp.

Pterygium is a common eye disease and is a fibrovascular lesion of the ocular surface. It is unknown pathogenesis and origin. Environment factors(UV-B light), genetic factors including viral infections may be implicated. Oncogenic virus, HPV has been hypothesized to be related with pterygium progress. Adenocarcinoma is a major histological types of cervical cancer, which emerges within glands and locates in the endocervix. In previous study, HPV 18 and 16 were the most common type found in adenocarcinoma, while HPV 6, 11, 16 and 18 have been previously reported in pterygium. Aim of this study was to determine the prevalence of HPV genotypes and compared the DNA sequence of L1 gene of HPV in pterygium and adenocarcinoma of cervical tissue.

In pterygium patients, HPV genotyping by using reverse hybridization was successfully done in 16 of 25 patients (64%). HPV-16 was the most common genotype. Among those 16 samples, 4 samples had mixed infection with HPV-18 (2/25, 8%) and HPV-58 (2/25, 8%). In cervical tissue, the infection of mixed genotypes was the most frequently found (17/23, 73.91%). HPV 51 was found 30.43% (7/23), HPV 58 was 21.74% (5/23), followed by each the HPV16 (4/23, 17.39%) and HPV 53 (4/23, 17.39%)

Intragenotypic variations in the L1 regions of each HPV type (HPV-16, 31, 52, 53, 58, 59, 66 and 70) were aligned with reference HPV. Several mutations were identified. The presence of different nucleotide mutations in HPV type 31, 53, 59 and 70 led to amino acid change in the L1 protein. The comparison of L1 gene sequences of HPV type 16 in pterygium and adenocarcinoma cervical tissue showed only 3 different single nucleotide mutations. The results indicate DNA sequence of L1 gene of Human papillomavirus from pterygium and adenocarcinoma cervical tissue were very few different in genomic variant and they were clustered in the same branch of Phylogenetic tree.

Field of Study: Medical Microbiology
Academic Year: 2011

Student's Signature…….....
Advisor's Signature……..……
Co-advisor's Signature ……
Co-advisor's Signature ……

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CONTENTS

CHAPTER Page

CHAPTER Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AC	=	Adenocarcinoma
ATP	=	Adenosine triphosphate
β-globin	=	Beta globin
bp	=	Base pair
^{0}C	=	Degree Celsius
CIN	=	Cervical intraepithelial neoplasia
CSIL	=	Cervical squamous intraepithelial lesions
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleotide triphosphate
E.coli	=	*Escherichia coli*
et al	=	et alii
EV	=	Epidermodysplasia verruciformis
HCl	=	Hydrochloric acid
HPV	=	Human papillomavirus
Hr	=	Hour
HSV	=	Herpes simplex virus
ICC	=	Invasive cervical cancer
Kbp	=	Kilo base pairs
KCl	=	Potassium chloride
LB	=	Luria Bertani medium
LCR	=	Long control region
M	=	Molar
$MgCl_2$	=	Magnesium chloride
min	=	Minute
mL	=	Milliliter
mM	=	Millimolar
NCBI	=	National Center for Biotechnology Information
ng	=	Nanogram

nm	=	Nanometer
ORF	=	Open reading frame
PCR	=	Polymerase Chain Reaction
pmole	=	Picomole
pRb	=	Retinoblastoma protein
RRP	=	Recurrent respiratory papillomatosis
SCC	=	Squamous cell carcinoma
sec	=	Second
Tris	=	Tris-(hydroxymethyl)-aminoethane
μL	=	Microliter
UV	=	Ultraviolet
VLPs	=	Virus-like particles

CHAPTER I

INTRODUCTION

Human papillomavirus (HPV) is a member of the family *Papillomaviridae*. It is a small non-enveloped virus containing closed circular double stranded DNA genome. HPV particle is about 55 nm in size. The circular DNA genome, approximately 8 kb in length has 3 functional major regions: early (E: a gene coding early viral proteins), late (L: a gene coding late viral proteins), and a long control region (LCR or noncoding region [NCR]):(1). The early region encodes 6 non-structural proteins (E1, E2, E4, E5, E6 and E7). The E1, E2 and E4 contribute to the viral replication and E5 - E7 are oncogenic proteins that involve in malignant transforming of cervical cancer and associate undifferentiated or intermediately differentiated keratinocytes. E6 and E7 are two viral oncoproteins that inactivate, 2 cellular tumor suppressor proteins, p53 and retinoblastoma (pRb), respectively. The late region of all papillomavirus genomes encodes major (L1) and minor capsid (L2) proteins. The LCR regulates viral replication and gene expression(2-7).

There are different HPV genotypes more than 100 types and the genomes of more than 80 have been completely sequenced. The classification of different HPV types have been identified based on DNA sequence analysis, the genotype are considered distinct if they share less than 90% homology in the nucleotide sequence of the E6, E7 and L1 open reading frame (ORF). Subtypes have between 90% and 95% homology, and variants between 96% and 98% (3, 4, 8). Approximately 30-40 distinct HPV genotypes affect the genital tract, at least 15 genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) are characterized as 'high-risk' group as they associate with the development of cervical cancer. HPV genotypes 16 and 18 are found in 70 to 80% of all cervical cancers. HPV genotypes designated as 'low-risk' group are such as 6,11,40,42,43,44, 54,61,70,72,81 and CP6108. HPV genotypes 6 and 11 associate with 90% of genital warts (3, 9). HPV can be classified into cutaneous and mucosal types. Cutaneous types infect the squamous epithelium of the skin and produce common warts, plantar warts and flat warts. Mucosal HPVs infect the mucous membranes and can cause cervical neoplasia in adults as well as anogenital warts(10-13).

There are 2 major histological types of cervical cancer, 80 - 85% are squamous cell carcinoma (SCC) while adenocarcinoma (AC) accounts for 15 - 20%. Squamous cell carcinoma arises from the squamous epithelia on the ectocervix while adenocarcinoma emerges within glands and located in the endocervix, usually at the area where the ectocervix connects to the endocervix. The patients with cervical adenocarcinoma present with symptoms such as abnormal vaginal bleeding or discharge, or with pelvic pain. Most cervical adenocarcinomas are characterized by an abnormal mass or growth on the cervix which can be seen during a colposcopy(14-17). HPV 18 and 16 were the most common types found in adenocarcinomas (18).

Pterygium is a common eye disease and most found in tropical countries. Pterygium is a fibrovascular lesion of the ocular surface; it is unknown pathogenesis and origin. The characteristic of disease is a wing-like of the conjunctiva onto cornea with inflammation of fibroblast and blood vessel. The development of pterygia appears to be involved to environmental factors, such as exposure to ultraviolet light, genetic factors and viral infections. Besides these, inflammatory reactions, degeneration of the cornea, fibroblastic proliferation, allergic factors, and immunopathological mechanisms are probably factors to induce the disease. Oncogenic virus, HPV has been hypothesized to be related with pterygium progress(19, 20). HPV 6, 11, 16 and 18 have been previously reported in pterygium(18, 21-29). It is interesting to know whether HPV of the same genotypes that cause pterygium are the same as that cause cervical carcinoma. This knowledge may support the use of HPV vaccine in prevention pterygium disease.

In this study, the nucleotides of HPV L1 gene among HPV-DNA obtained from pterygium and adenocarcinoma cervical tissues were analyzed and compared.

CHAPTER II

HYPOTHESIS AND OBJECTIVES

Hypothesis

DNA sequence of L1 gene of Human papillomavirus from pterygium and adenocarcinoma cervical tissue are different

Objectives

1. To determine the genotypes of HPV in pterygium
2. To determine the genotypes of HPV in adenocarcinoma cervical tissue
3. To compare the genotypes and DNA sequence of L1 gene of HPV from pterygium and adenocarcinoma cervical tissue

CHAPTER II

REVIEW AND RELATED LITERATURES

HPV:Structure and Genome

Human papillomavirus (HPV) is a member of the family *Papillomaviridae*. It is a small non-enveloped virus with a diameter of 40-55 nm in size. The particle of HPV has icosahedral symmetry, isometric capsid consists of 72 capsomers. HPV genome is closed circular double stranded DNA approximately 8 kbp. The genome includes of 8 – 10 open reading frames (ORFs), all locates on the same strand(1). The genome can be divided into 3 functional major regions: early (E: a gene coding early viral proteins), late (L: a gene coding late viral proteins), and a long control region (LCR or noncoding region [NCR]: a region between E and L)(Fig.1). Early genes encode proteins which play roles in HPV genome replication, transcriptional regulation and transformation. The late genes, L1 and L2, encode for major and minor capsid proteins (2-5, 7, 8, 13). E1 protein is a regulator of viral replication and transcription. E2, which is responsible for recognition and binding of origin of replication, forms a protein complex with E1 and regulates transcription of viral genome. The E4 protein involves in maturation and release of viral particles. E5 which involves in cell transformation binds with host membrane proteins such as growth factor receptors. The E6 and E7 proteins are proteins associated with malignant transformation activies. These proteins activate cell proliferation by interacting and suppressing the functions of tumour suppressor proteins p53 and pRb that are important in controlling cell proliferation. E6 encoded by high risk HPV is able to bind with p53 tumour suppressor protein, this interaction encourages the degradation of p53. The high risk HPV E7 protein binds to pRb tumor suppressor protein with a higher affinity than the low risk HPV E7 proteins. pRb plays an important role in cellular proliferation. The pRb releases E2F that stimulates cell cycle progression in hypophosphorylation form. The binding association of the E7 protein with pRb is in hypophosphorylation. Late region L1 ORF is the most conserved region, responsible for initial binding to the cell surface. L2 is the minor capsid protein, coexpressed with L1 to incorporate of

the viral DNA into viral like-particles (VLPs) (3, 8, 11, 30-35). These functions of HPV genes are shown in Table 1.

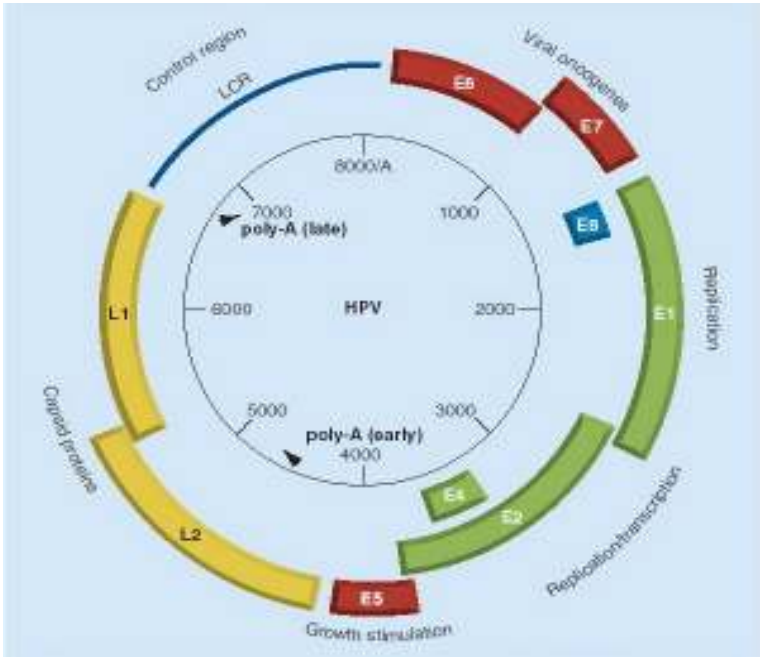

Figure 1 Genetic map of human papillomavirus: The 3 major regions of the genome contain the long control region (LCR), the early (E) gene region and the late (L) gene region(36)

Table 1 A summary of the functions of HPV open reading frames (ORFs)(37)

Viral Protein	Functions and Properties
E1	ATP-dependent DNA helicase. Role in segregation of genomes during cell division, genome replication
E2	Regulatory role in viral transcription and replication (binds to regulatory region on viral genome and forms initiation complex with E1 protein). Regulation of cellular gene expression; cell cycle and apoptosis regulation
E3	Unknown functions
E4	Remodels cytokeratin network; cell cycle arrest; virion assembly
E5	Control of cell growth and differentiation by complexed with epidermal-growth-factor receptor
E6	Binds to and degrades p53, inhibits apoptosis and differentiation
E7	Binds to the hypophosphorylation form and degrades of the retinoblastoma (pRb) protein promoting E2F release and S-phase entry of cell cycle
L1	Major capsid protein
L2	Minor capsid protein; virus assembly

Classification

The classification of different HPV types has been identified based on DNA sequence similarity. The genotype is considered distinct if they share less than 90% homology in the nucleotide sequence of the E6, E7 and L1 open reading frame (ORF). Subtypes have between 90% and 95% homology, and variants have between 96% and 98% homology (3, 4, 7, 38). At present, there are more than 100 different HPV genotypes and the genomes of more than 80 have been identified and completely sequenced. There are 40 distinct HPV genotypes that affect the genital tract. The genital type of HPV are further classified into 2 groups, high-risk and low-risk group. High-risk group, at least 15 genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82) is associated with the development of cervical cancer. HPV genotypes 16 and 18 are the causative agents in 70 to 80% of all cervical cancers. HPV genotypes designated 'low-risk' group such as 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP6108 (3, 9). HPV genotypes 6 and 11 associate with 90% of genital warts and 100% of recurrent respiratory papillomatosis (RRP)(39).

Phylogenetic analysis, a comparison of HPV genomic variations, is based on DNA sequences of different regions. Classification of viruses base on species of origin and relationship between viral genomes. The different HPV type is classified by comparison the nucleotide sequences among their viral genomes. HPV genotypes are grouped into subtypes and variants depending on the similarity of sequence in the L1 region (Fig.2)(3, 40, 41).

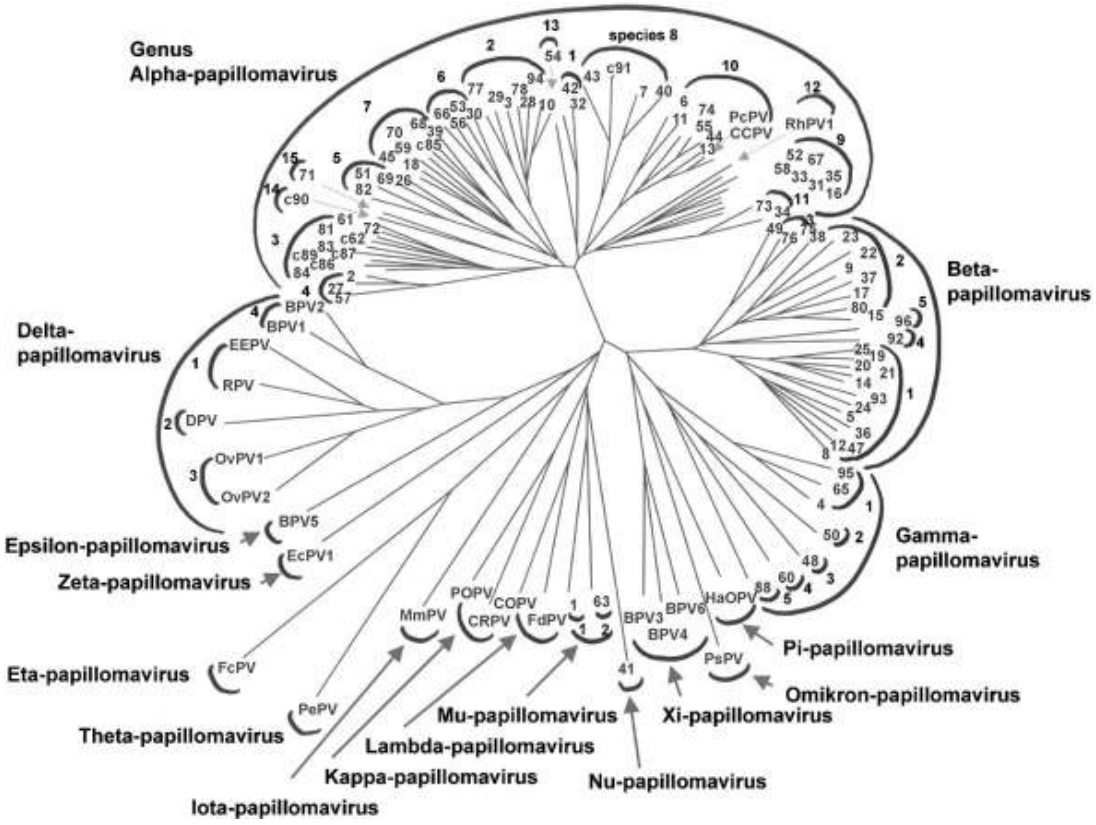

Figure 2 Phylogenetic tree containing the sequences of 118 papillomavirus types. The numbers at the ends of each of the branches identify HPV type; c-numbers refer to candidate HPV types. All other abbreviations refer to animal papillomavirus types. The outermost semicircular symbols identify papillomavirus genera, e.g. the genus alpha-papillomavirus. The number at the inner semicircular symbol refers to papillomavirus species (3).

HPV Life cycle

HPV virion infects epithelial tissues through micro-abrasions within the squamous epithelium and enters the basal epithelial cells. The replication of HPV has two modes: stable and vegetative. The stable replicative form occurs as a circular episomal genome in the cells of the basal layers of low-risk HPV infected squamous epithelial cells while the vegetative form occurs in highly differentiated cells at the epithelial surface. Viral genome is replicated to multicopy episome and maintained the viral DNA as a low copy number in dividing basal epithelial cells. A round of viral DNA replication is independent of the cell cycle. Viral assembly occurs in the mature squamous epithelium and assembled virions are released in the terminally differentiated outer epithelial layer (Fig.3)(10, 37, 41-49).

Pathogenesis

HPV infection stimulates cellular proliferation in stratified squamous epithelial cells. Infection initiates with the viral entry via the proliferating of the epithelium cells, then the early proteins are expressed and viral DNA replicates from episomal DNA. Finally, the coat proteins L1 and L2 are expressed and assembly of infectious virus is in upper layer epithelium. HPV DNA persists in an episomal form in normal infected cells and can be integrated in the host cell chromosome in malignant cells(2, 37, 50, 51). The viral DNA integration, the breakage always occurs in the E1 region leading to the loss of functions of the E1/E2 genes cause of negatively control the expression of the E6 and E7 genes resulting in cellular transformation. HPV early genes E6 and E7 associated with malignant transformation that are produced in lower and upper layers of the epithelium. E6 protein is a protein that binds and forms complex with tumour suppressor proteins p53 and the enzyme ubiquitin ligase, producing its degradation. The HPV E7 is a protein that binds to the pRb resulting in interruption of complex between pRb and the E2F cellular transcription factor at the G1 cell cycle stage. The inhibition of genes expression relates to the replication of DNA and cell proliferation(37, 44, 51-53).

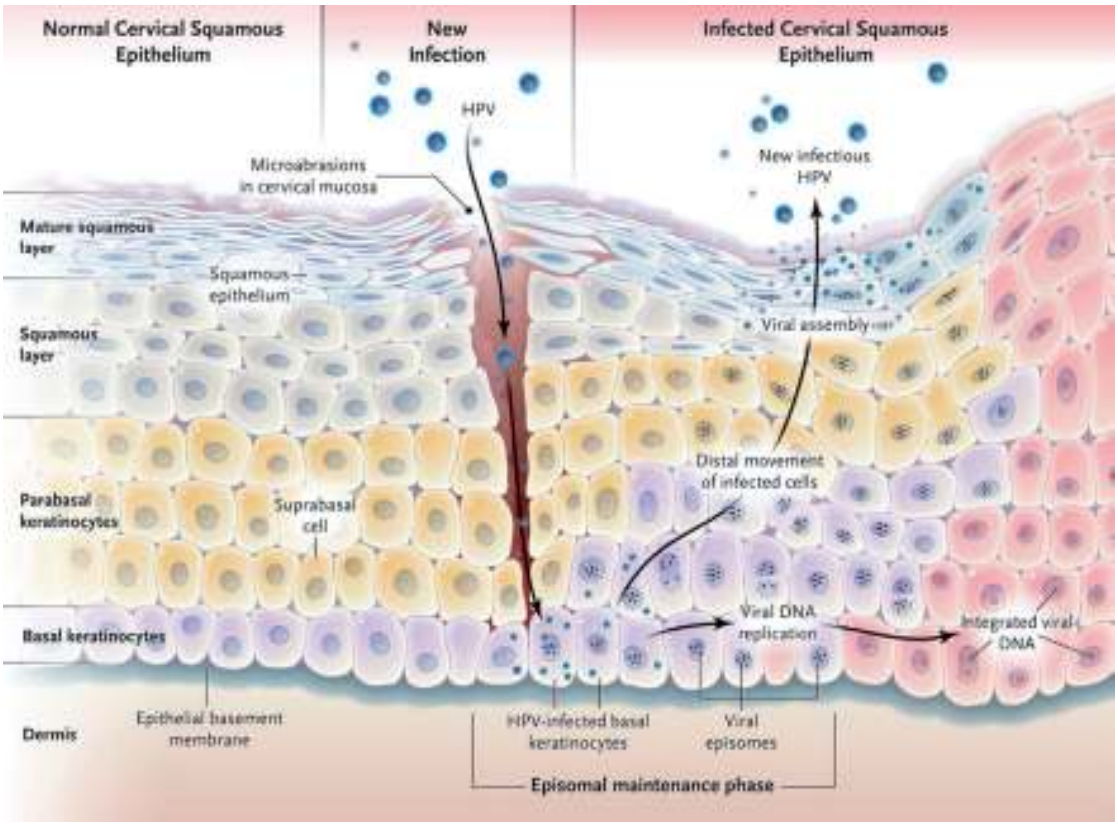

Figure 3 Life Cycle of human papillomavirus in the squamous epithelium. HPV infects basal keratinocytes through microabrasions in the skin or mucosa. In the replication of viral DNA, the virus is amplified as a low copy number. The initial genome amplification is followed by an episomal maintenance phase in the basal cells then enters the suprabasal compartment. Early and late genes are expressed and productive genome amplification to high copy numbers. Viral assembly, L1 and L2 encapsidate the viral genome to form virion occurs in the upper layer of the squamous epithelium. Virions are released and can initiate a new infection(54).

Transmission

HPV transmission typically occurs through direct skin-to-skin contact. The sexual intercourse is primary route of genital HPV infection including both vaginal and anal. Thereby, the infection of HPV can cause warts (common warts, plantar warts and flat warts) of cutaneous epithelium. Transmission of skin warts are by direct contact with an infected tissue or indirectly by contact with virus contaminated objects. The factors of modulating progression of HPV infection period are dependent on pathogen and host factors, such as HPV exposure, molecular variant of HPV genotype, HPV viral load, intensity or rate of exposure and immune status of the host(55-57).

Epidemiology

HPV is estimated to be the most common cause of sexually transmitted disease in women worldwide. The prevalence of HPV has found highest in young persons within the first few years after sexual beginning. HPV infects the genital tract through sexual contact, in primarily the cervix, vagina, vulva, penis and anus. Genital HPV types are divided into high and low-risk groups, according to the association with genital tract cancer. Cervical cancer is the second most common cancer in women worldwide. High-risk HPV types are detected in 99.7% of cervical cancers, HPV type 16 was the most found in invasive cervical cancer (ICC) (55%), followed by HPV 18 (12.8%), HPV types 33, 45, and 31 are the next most frequently. In the adenocarcinoma of the cervix, HPV type 16 was the most prevalence (48%), followed by HPV 18 (36%) and HPV 45 (6%). The low-risk HPV types, the most common of which are HPV 6 and 11, can cause genital warts and was found approximately 90%(4, 53, 55, 56, 58).

Clinical Manifestations

HPV is a virus that presents in epithelial cells, causing infections of the skin and mucous membranes. HPV is divided by considering tissue tropism of stratified squamous epithelium and depending on the type of epithelium infected. There are 2 groups of HPV; cutaneous and mucosal types. Cutaneous HPV types infect the keratinizing epithelium causing warts (plantar warts, common warts and flat warts). Mucosal HPV types infect nonkeratinizing epithelium, and also be found in the oral mucosa, conjunctiva and respiratory tract(4, 59). HPV causes a wide range of disease processes, depending on HPV types (Table 2).

Cutaneous warts

Warts are benign proliferations of skin and the most common of HPV infection include common warts, plantar warts and flat warts. Common warts (verruca) are hyperkeratotic papulonodules most often seen on the hands, arms and legs. Flat warts are less than common warts, flat, smooth or slightly rough surface and color or pigment on skin which is slightly yellow to brownish.The locations, face and back of hands are the most common found. Plantar warts are warts that occur on the plantar region, these lesions lie deep and present in the skin of the hands and feet(4).

Epidermodysplasia verruciformis

Epidermodysplasia verruciformis (EV) is a rare disease causing from genetic disorder especially defective in cellular immunity. The disease is characterized by the appearance of flat warts liked skin lesions on the face and neck, reddish-brown pigmented plaques. Patients with epidermodysplasia verruciformis are usually infected with multiple types of HPV. More than 30 HPV types, such as 4, 5, 8, 9, 12, 14, 15, 17, 19-25, 36-38, 47, and 50 have been identified in epidermodysplasia verruciformis lesions(4, 60-62)

Conjunctival papillomas

Conjunctival papillomas are benign and common tumour of the stratified squamous epithelium of the conjunctiva(63). The clinical signs associated with squamous cell papilloma. Most lesions are asymptomatic without associated conjunctivitis or folliculitis and locate in the inferior fornix, but it also may arise in the limbus, caruncle, and palpebral regions(64). HPV types 6 and 11 are the most commonly found in conjunctival papillomas(65).

Condylomata acuminata

Condylomata acuminate, the most common manifestations of HPV infection in the genital area are anogenital warts. The lesions of disease present as papules, nodules soft, filiform, pinkish, sessile or pedunculated growths and may present exophytic or cauliflower type(4). HPV types 6 and 11, are the most commonly isolated viruses and highrisk HPV 16 and 18 can also be isolated(66, 67).

Table 2 Human papillomavirus types and clinical manifestations(4)

Manifestation	HPV types
Nongenital	
Common warts(verrucae vulgaris)	**1, 2**, 4, 26, 27, 29, 41, 57, 65
Plantar warts	**1, 2**, 4, 63
Flat warts	**3, 10**, 27, 28, 38, 41, 49
Respiratory papillomatosis	**6, 11**
Squamous cell carcinoma of the lung	**6, 11**, 16, 18
Laryngeal papilloma	**6, 11**, 30
Laryngeal carcinoma	**16, 18**
Conjunctival papillomas	**6, 11**
Conjunctival carcinoma	**16**
Epidermodysplasia verruciformis	**2, 3, 10**, **12, 15,** 19, 36, 46, 47, 50
Anogenital	
Condylomata acuminata	**6, 11**, 30, 42, 43, 44, 45, 51, 52, 54
Unspecified intraepithelial neoplasia	30, 34, 39, 40, 53, 57, 59, 61, 62, 64, 66, 67, 68, 69
Low-grade intraepithelial neoplasia	**6, 11**, 43
High-grade intraepithelial neoplasia	**16, 18**, 56, 58
Carcinoma of cervix	**16, 18**, 31
Carcinoma of vagina	**16**
Carcinoma of anus	**16**, 31, 32, 33

Bold type indicates most frequent association

Cervical cancer

Cervical cancer is the one most common cancer in women worldwide and is the second only to breast cancer as a cancer cause of death(68). HPV-16 and 18 are the most prevalent among the cervical cancers attributable of high-risk HPV infection(41, 53, 69, 70). High-risk HPV types have been related with other genital cancers, such as carcinoma of vagina, penis and anus. The clinical symptoms, abnormal Papanicolaou test results and patients with a indelicate lesion of the cervix are estimated by colposcopy and biopsy.

Cervical intraepithelial neoplasia (CIN) is a premalignant cervical disease that is also called cervical dysplasia or cervical squamous intraepithelial lesions (CSIL). CIN is divided into grades 1, 2 and 3. CIN 1 corresponds to mild dysplasia, CIN 2 to moderate dysplasia, and CIN 3 corresponds to both severe dysplasia and carcinoma *in situ*(71).

The cofactors of cervical cancer are necessary by stimulation of the persistence of HPV infection or by stimulation of progression to an invasive cancer(72). Cofactors can be divided into 3 groups: (1) environmental factors; (2) host-related factors; and (3) viral factors. Environmental factors include smoking, parity, oral contraceptives and co-infection with sexually transmitted diseases. Host-related factors include hormone factor such as oestrogen, genetic factor and immune system factor. Viral factors are such as infections with high-risk types of HPV, infections with one type or multiple types and amount of virus (viral load) present in the cervix(14, 73).

Adenocarcinoma

Invasive cervical cancer can be divided into 2 major histological types: squamous cell carcinoma (SCC) and adenocarcinoma. In cases, 80–85% are squamous cell carcinoma, 10% are adenocarcinoma, and 3% are adenosquamous carcinoma and other rare tumours(15, 74). Squamous cell carcinoma arises from the squamous epithelia on the ectocervix, while adenocarcinoma arises from the glandular epithelia from the endocervix(14). Patients with cervical adenocarcinoma present with symptoms such as abnormal vaginal bleeding or discharge, or with pelvic pain. Most cervical adenocarcinomas are characterized by an abnormal mass or growth on the cervix which can be seen during a colposcopy(14-17). The cause of the cervical adenocarcinoma is unclear, probable risk factors include history of uterine disease, certain hormone use, sexual history and HPV infection. In adenocarcinoma, HPV 18 is predominantly while in SCC, HPV 16 is more frequently(18, 75, 76).

Pterygium

Pterygium is a common eye disease and found in tropical countries. Pterygium is a fibrovascular lesion of the ocular surface originating in the limbal conjunctiva within the palpebral fissure with progressive involvement of the cornea. It is unknown pathogenesis and origin. The characteristic of disease is a wing-like of the conjunctiva onto cornea with inflammation of fibroblast and blood vessel. The development of pterygia appears to be involved to environmental factors, such as exposure to ultraviolet light, genetic factors and viral infections may be associated with the disease. Besides these, inflammatory reactions, degeneration of the cornea , fibroblastic proliferation, allergic factors, and immunopathological mechanisms are probably factors to induce the disease. Oncogenic virus, HPV has been hypothesized to be related with pterygium progress(19, 20). HPV 6, 11, 16 and 18 have been previously reported in pterygium(18, 21-29).

Detection of HPV: Molecular Diagnostics

HPV can not grow on conventional culture media and serological diagnostic methods have limited(77). In a laboratory, molecular techniques are the only method available to detect HPV DNA. Molecular diagnostic method for detection HPV DNA can be divided into 2 techniques, amplified techniques and non- amplified techniques(78). Polymerase chain reaction (PCR) is the most commonly used in the detection of HPV DNA and the most sensitive method. PCR is a target amplification technique which duplicates fragments of DNA from a targeted gene sequence. The reaction consists of the following 3 basic steps: denaturation, annealing and extension. The PCR procedures for HPV DNA detection use consensus primers targeting to the conserved viral capsid L1 regions of HPV genome, which can detect numerous HPV types. L1 consensus primer sets, MY09/11, GP5+/6+, PGMY and SPF10 were used to detect HPV(79-82). Type specific PCR, amplified a single genotype of HPV by targeting a type specific DNA sequence are based on the E6 or E7 gene of HPV subtypes(83). After amplification of viral DNA by PCR, the techniques often used for typing of the DNA amplified are Southern blot, dot blot, reverse hybridization, restriction enzyme digestion, and sequencing analysis. The signal amplification technique uses branched DNA technology or hybrid capture to increase the DNA proportional signal to detectable level(4, 78). Real-time PCR methods can detect HPV-DNA by using type specific PCR primers combined with fluorescent probes for real-time detection. Moreover Real-time PCR can be multiplexing several type specific primers within one reaction which be detected the different HPV genotypes(84, 85).

Reverse hybridization is a method for hybridization of a PCR product to multiple oligonucleotide probes which are immobilized on a solid phase and added of the amplified PCR product in the liquid phase. The solid phase is a membrane strip containing multiple probes immobilized as parallel lines. Amplified PCR product are constructed, by using biotinylated primers and hybrids probe line can be detected after hybridization and washing. This method can detect multiple HPV genotypes in a single step and type specific infections(78, 86).

Genome variation of HPV genotypes

HPV are defined as types based on their nucleotide sequences of L1 gene. A newly discovered HPV genotype is at lease 10% difference to known HPV types, while HPV subtype is defined as HPV genomes with L1 gene that differs by 2 to 10% from that of the original HPV type. Variants are defined as the nucleotide sequence of their L1 genes that were different about 2% in most genes and 5% in less conserved region from the original isolated of the reference prototypes (3, 87). The variants of the same HPV type can result in amino acid change leading to distinct biologically and make a difference in the pathogenic risk. In L1 region, the change of one or more amino acid could lead to a conformational change in the capsid protein may affect the efficacy of viral infection or change of viral antigenicity (88, 89). Variations in LCR region may affect the viral replication rates and activity of early gene transcription. The change of amino acid may affect the activity of transformation in the E6 and E7 oncoproteins, the variations of E6 gene results to the p53 protein degradation involved in host immune recognition(90, 91). Previous study reported the distinct of HPV benefit to study the genetic variability of HPV variants for understanding of the molecular mechanisms, disease progression and transformation. In the L1 region, several studies were identified mutations in HPV protein regions. Cento *et al* presented 22 different nucleotide mutations led to amino acid change in the L1 protein in Italian women with abnormal cervical smear of variations HPV-31 and 58 (92). Pande *et al* (93) showed 13 nucleotide variations were detected in 11 cervical cancer patients of India, the variants led to mutations in amino acid. In E6 oncogenes, Chagas *et al* (94) reported 3 nucleotide changes in E6 which resulted in the characterization of E6 oncogenes genetic variability of HPV-31 isolated from cervical scraping samples of Northeastern Brazilian women.

CHAPTER III

MATERIALS AND METHODS

1. Study samples

1.1 Pterygium specimen

25 DNA samples extracted from pterygium specimens and confirmed the presence of HPV DNA were obtained from the previous study entitled, Incidence of Herpes simplex virus (HSV) and Human papilloma virus (HPV) whose Dr. Ngamjit Kasetsuwan was the principle investigator. In all cases, the location of pterygium was corneal limbus. 20 samples (CU) were from King Chulalongkorn Memorial Hospital, 4 samples were from Chophaya Abhaibhubejhr Hospital (AP) and one sample was from Queen Sawangwattana Memorial Hospital (SS).

1.2 Cervical specimen

89 formalin-fixed paraffin-embedded biopsy specimens were from the Department of Obstetrics and Gynecology, King Chulalongkorn Memorial Hospital, Bangkok, Thailand between 2003 to 2009. All of them showed pathology of adenocarcinoma and approved by pathologist.

1.3 Ethical approved

This study protocol was approved by the ethical committee, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. COA No. 1085/2009 IRB No. 417/52

1.3 HeLa cells

HeLa cells used as control in this study were obtained from Virology Unit, Department of Microbiology, Chulalongkorn University. HeLa cells containing HPV-18 genome were derived from cervical cancer patients. The cells were grown in MEM media (GIBCO BRL, USA) supplemented with 10% fetal bovine serum

(FBS), 50 unit/ml of penicillin and 50 ug/ml of streptomycin. The cells were incubated at 37°C with 5% CO_2 in atmosphere.

2.Extraction of DNA

2.1 Formalin-fixed paraffin- embedded tissues

A Formalin-fixed, paraffin-embedded tissues was cut by a microtome with a disposable blade. At least 10 pieces of 5 µm thick of tissues were placed in 1.5 mL microcentrifuge tube and cut in to small pieces. The pieces of tissues were then deparafinized by adding 1 mL of xylene and incubated at room temperature until the paraffin was completely dissolved. After that, the tube was centrifuged for 2 min at 11,000 x *g* and the supernatant was discarded by pipetting. 1 mL of ethanol (96 – 100 %) was added to the pellet and mixed with vortex followed by centrifugation for 2 min at 11,000 x *g* and the supernatant was discarded by pipetting. The cells pellet was dried in open tube at 60 °C for 3 to 10 min.

The cells pellet was further extracted by using AmpliLute Liquid Media Extraction Kit (Roche Diagnostics, USA). Procedures followed the manufacturer's instructions. In brief, 80 µL Buffer ATL and 20 µL Proteinase K were added into the tube, and the cells pellet was incubated at 56°C for 30 min. After that, 250 µL of AL buffer was added, and the tube was incubated at 70°C for 15 min. After the incubation, 300 µL of absolute ethanol was added and incubated at room temperature for 5 min. The suspension was applied to QIAamp MinElute Column on the vacuum manifold and turned on the vacuum pump to remove all lysate. The QIAamp MinElute Column on the vacuum manifold was washed with 750 µL of wash buffer, turned on the vacuum pump and washed again with 750 µL of absolute ethanol. The QIAamp MinElute Column was placed into collection tube and centrifuged at 16,000 x *g* for 3 min. After that, the QIAamp MinElute Column was placed into another elution tube to remove alcohol in column by open the lid and incubated at room temperature for 15 min. The sample DNA was eluted from the QIAamp MinElute Column by adding 120 µL of elution buffer (AVE), incubated at room

temperature for 5 min and centrifuged at 16,000 x *g* for 1 min. The eluted DNA was stored at -80 °C until used.

2.2 HeLa DNA

The HeLa DNA was extracted by using NucleoSpin Tissue kit (MACHEREY-NAGEL, Germany). The procedure of DNA purification was performed according to the manufacturer's instruction. In brief, the 10^7 cells was resuspended in Buffer T1 and 25 µL Proteinase K solution. The cells suspension was mixed by vortex and incubated at 56°C until the cells complete lysis. 200 µL of Buffer B3 was added and the lysed cells was mixed by vortex and incubated at 70°C for 10 min. After that, 210 µL of absolute ethanol was added and the suspension was mixed by vortex. The supernatant was applied to NucleoSpin Tissue column in a collection tube and centrifuged for 1 min at 11,000 x *g*. DNA on silica membrane was washed by 500 µL Buffer BW and the tube was centrifuged for 1 min at 11,000 x *g*. The DNA was washed twice by 600 µL Buffer B5 and the tube was centrifuged for 1 min at 11,000 x *g*. The silica membrane was dried by centrifugation for 1 min at 11,000 x *g* and DNA was eluted from column by adding 100 µL of prewarmed Buffer BE (70°C), incubated at room temperature for 1 min and centrifuged at 11,000 x *g* for 1 min. The eluted DNA was kept at -80°C until used.

3. HPV DNA detection

The extracted DNA samples were first amplified by β-globin primers (GH20/PC04) to determine the adequate of DNA in samples, as a control for DNA amplification. The amplified product of 268 bp was expected (Table 3). In brief, a total volume of 50 µL reaction mixture contained 100 mM KCl, 20 mM Tris HCl (pH 8.0), 3 mM $MgCl_2$, 200 µM dNTPs, 25 pmole of primers GH20/PC04, 1.25 unit of Taq polymerase (Fermentas, EU) and 0.5 µL template DNA. The reaction was initially preheated at 94°C for 5 min, followed by 35 cycles

of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min.

All the β-globin positive samples were further assayed for the presence of HPV-DNA by nested PCR. The consensus primers MY09/MY11 as outer and Gp5/Gp6 as inner oligonucleotides were used to amplify the HPV L1 conserved region giving the product size of 450 bp and 150 bp (Table 3). In the first round of PCR, the PCR mixture of 25 µL contained 100 mM KCl, 20 mM Tris HCl (pH 8.0), 2.5 mM $MgCl_2$, 200 µM dNTPs, 25 pmole of primers MY09/MY11, 0.25 unit of proof reading Taq polymerase (Finnzymes, USA) and 1 µL template DNA. The reaction started by preheating at 92°C for 4 min, followed by 30 cycles of denaturation at 92°C for 30 sec, annealing at 53°C for 30 sec, and extension at 72°C for 30 sec, and a final extension at 72°C for 15 min. The 0.5 µL of MY amplified product was added to the second PCR reaction mixture containing 100 mM KCl, 20 mM Tris HCl (pH 8.0), 2.5 mM $MgCl_2$, 100 µM dNTPs, 25 pmole of Gp5/Gp6 primers and 0.25 unit of proof reading Taq polymerase. The reaction initiated by preheating at 92°C for 4 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 45°C for 30 sec, and extension at 72°C for 30 sec, and a final extension at 72°C for 15 min.

Amplified products were analysed by electrophoresis in 1.5% agarose gel stained with ethidium bromide and photographed under ultraviolet (UV) transillumination. In every PCR assay, we used HPV-DNA from HeLa cells as a positive control and distilled water as a negative control.

Table 3 Sequence of oligonucleotide primers

Primers	Sequence (5' -3')	Target	PCR Product (bp)	Reference
MY09 MY11	CGT CCM ARR GGA WAC TGA TC GCM CAG GGW CAT AAY AAT GG	L1	450	*Manos et al*., 1989
Gp5 Gp6	TTT GTT ACT GTG GTA GAT AC GAA AAA TAA ACT GTA AAT CA	L1	142	*Snijders et al*., 1990
GH20 PC04	GAA GAG CCA AGG ACA GGT AC CAA CTT CAT CCA CGT TCA CC	β – globin	268	*Bell et al*., 1993

Note: R = G/A

W= T/A

M= C/A

Y = T/C

4. HPV genotyping

DNA extracted from HPV-DNA positive pterygium specimens and cervical specimens were determined for HPV genotypes by using LINEAR ARRAY HPV Genotyping Test Kit (Roche Diagnostics, USA) which can detect 37 HPV genotypes including 13 high risk genotypes (16,18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68), 24 low risk genotypes (6,11, 26, 40, 42, 53, 54, 55, 61, 62, 64, 66, 67, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39 and CP6108) and β-globin gene. This test kit is based on the reverse hybridization principle. The assay used biotinylated primers to define a sequence of nucleotides within the polymorphic L1 region of the HPV genome (approximately 450 bp). The denatured biotin-labelled amplicons were hybridized with oligonucleotide probes that coated on membrane strip and detection of the probe-bound amplified products by colorimetric determination. The procedure of genotyping HPV was performed according to the manufacturer's instructions. Briefly, each strip with the probe line was placed into hybridization buffer in tray and 75 µL of denatured amplicon was added. The mixture was incubated at 53°C ± 2°C in shaking water bath for 30

min. After that the strip was washed with 4 mL of ambient wash buffer and washed again with stringent wash buffer and incubated at 53°C ± 2°C in shaking water bath for 15 min. After 4 mL of working conjugate was added into each strip and incubated at room temperature (20-25°C) on orbital shaker for 30 min, the strip was washed 3 times with 4 mL of ambient wash buffer at room temperature for 10 min on orbital shaker. After that 4 mL of citrate buffer was added to each well containing a strip and incubated at room temperature on orbital shaker for 5 min. Finally, 4 mL of substrate buffer was added into each strip and incubated at room temperature for 5 min on orbital shaker and rinsed with 4 mL of distilled or deionized water.

5. Sequencing analysis

5.1 Direct sequencing of HPV

HPV-DNA positive specimens were purified and sequenced. The sequence was analyzed and determined by NCBI Blast.

5.1.1 Purification of PCR product

Amplified products were analysed by electrophoresis in 1.5% agarose gel stained with ethidium bromide and cut-out gel band for purification by NucleoSpin Gel and PCR Clean-up kit (MACHEREY-NAGEL, Germany). The procedures followed the manufacturer's instructions. In brief, the cut-out gel band was mixed with 200 µL of binding Buffer NTI and dissolved the agarose by heat at 50°C for 5 – 10 min. After that, the suspension was applied to NucleoSpin Gel and PCR Clean-up column. The column was centrifuged for 30 sec at 11,000 x *g*. DNA on silica membrane was washed by adding 700 µL Buffer NT3 into column and centrifuged for 30 sec at 11,000 x *g*. Finally, silica membrane was dried by centrifugation for 1 min at 11,000 x *g* and DNA was eluted from column by adding 15–30 µL of distilled water PCR grade, incubated at room temperature for 1 min and centrifuged at 11,000 x *g* for 1 min.

5.1.2 Analysis of DNA Sequencing

The purified PCR product was submitted to Macrogen Inc. (Korea) for sequencing. The consensus primers MY09/MY11 and Gp5/Gp6 were used separately in sequencing in order to sequence both sense and antisense strands. The sequence was analyzed and determined by NCBI Blast. The sites of HPV DNA nucleotides on each genotype are numbered according to the HPV sequence published in GenBank.

5.2 Molecular cloning and nucleotide sequencing of HPV genome

The specimens that showed mixed HPV infections were further DNA cloning. The PCR products were cloned (CloneJET PCR Cloning kit, Fermentas) according to the instructions of the manufacturer, followed by DNA sequencing. The identification of the obtained sequence was verified by using alignment search tool (blast) analysis (www.ncbi.nlm.nih.gov). This test kit features the novel positive selection cloning vector pJET 1.2/blunt, blunt-end PCR products generated by proofreading DNA polymerases can be directly ligated with the pJET 1.2/blunt cloning vector and transformed to common laboratory *E.coli* strains.

5.2.1 Ligation mixture

The blunt-end PCR products generated by proofreading DNA polymerase was directly ligated with the pJET 1.2/blunt cloning vector. The ligation reaction was set up on ice according to the instructions of the manufacturer. The ligation mixture was incubated at room temperature for 5 min. The reaction conditions were shown in table 4.

Table 4 Conditions of Ligation mixture

Component	Volume
2X Reaction Buffer	10 µL
Purified PCR product	1 µL
pJET 1.2/blunt cloning vector (50 ng/ µL)	1 µL
Water, nuclease-free	Up to 19 µL
T4 DNA Ligase	1µL
Total volume	20 µL

5.2.2 Transformation

This study used *E. coli* DH5α as competent cells and transformed bacteria by using heat shock. Briefly, the competent *E. coli* DH5α cells was thawed from -80°C on ice and aliquoted 50 µL of cells into pre-chilled microcentrifuge tube. Then, 5 µL of ligation mixture was added into cells and gently stirred. The tube was incubated on ice for 30 min. After that, the cells were heated shock for 45 sec at 42°C in water bath and the tube was placed immediately on ice for 2 min to reduce damage of the cells. Then 1 mL of warmed LB (Luria Bertani) (without antibiotic) was added and the tube was incubated at 37°C for 1 hr. After incubation, 100 µL of the culture was spread on LB agar plate containing the appropriate antibiotic (50ng/mL Ampicillin) and the plate was incubated at 37°C for overnight.

5.2.3 Colony selection

After incubating the plate at 37°C for overnight, the presence of DNA inserted colonies were analysed. An individual colony was resuspended in PCR master mix, according to table 5.

Table 5 Condition of colony PCR master mix

Component	Volume
10X *Taq* buffer	2 µL
dNTP mix, 2 mM each	2 µL
25 mM $MgCl_2$	1.2 µL
pJET 1.2 Forward Sequencing Primer, 10 µM	0.4 µL
pJET 1.2 Reverse Sequencing Primer, 10 µM	0.4 µL
Water, nuclease-free	13.9 µL
Taq DNA Polymerase 5 u/ µL	0.1 µL
Total volume	20 µL

The reaction was initially preheated at 95°C for 3 min, followed by 25 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. After that, the product was analysed by electrophoresis in 1.5% agarose gel for the presence of DNA insert.

5.2.4 Preparation of plasmid DNA

The plasmids were isolated by using NucleoSpin Plasmid DNA Purification kit (MACHEREY-NAGEL, Germany). The procedure of purification was performed according to the manufacturer's instruction. In brief, the colony with DNA insertion was placed in 2 mL of LB (with antibiotic) and incubated at 37°C on shaker for 12 – 16 hr. Then, saturated *E.coli* LB culture was centrifuged at 11,000xg for 30 sec and the medium was discarded. 200 µL of Buffer A1 was added to resuspend the cell pellet and the tube was mixed by vortex. After that, 250 µL of Buffer A2 was added and the tube was mixed gently by inverting and incubated at room temperature for 5 min. The cell pellet was lysed by 300 µL of Buffer A3 and mixed by inverting again. The lysate cells were clarified by centrifugation at 11,000xg for 5 min.

The supernatant of 750 µL was applied to NucleoSpin Plasmid/ Plasmid column in a collection tube and centrifuged for 1 min at 11,000 x *g*. Plasmid DNA on silica membrane was washed by 600 µL Buffer A4 and the tube was centrifuged for 1 min at 11,000 x *g*. The silica membrane was dried by centrifugation for 2 min at 11,000 x *g* and plasmid DNA was eluted from column by adding 50 µL of distilled water PCR grade, incubated at room temperature for 1 min and centrifuged at 11,000 x *g* for 1 min.

5.2.5 Sequencing

The purified plasmid DNA was submitted to Macrogen Inc. (Korea) for sequencing by using the consensus primers MY09/MY11 and Gp5/Gp6. The sequences were analysed by using alignment search tool (blast) analysis (www.ncbi.nlm.nih.gov). The sites of HPV DNA nucleotides on each genotype are numbered according to the reference HPV sequence published in GenBank were shown in table 6.

Table 6 Reference of each genotype from GenBank

Reference each Genotype	Accession No.	Country
HPV 16	DQ469930	Germany
	AY177679	South Africa
	AF534061	East Asian type
	FJ797754	Thailand
HPV 31	U37410	USA
	JN041176	Italy
	HQ537666	Zambia
	HQ537675	Thailand
HPV 52	X74481	Germany
	HQ537739	Costa Rica
	HQ537736	Zambia
	FJ797780	Thailand
HPV 53	X397046	Germany
	EU779756	Serbia
	DQ241374	Portugal
HPV 58	EU918765	China
	HQ537754	Costa Rica
	HQ537763	Zambia
	FJ797803	Thailand
HPV 59	U45930	USA
	AB437933	Korea
	DQ486471	Brazil
	FJ797809	Thailand
HPV 66	EF177191	USA
	U31794	Germany
	DQ486474	Brazil
HPV 70	U21941	Sweden
	EF626587	Brazil
	FJ797812	Thailand

CHAPTER IV

RESULTS

1. Detection of HPV DNA

1.1 Pterygium specimens

25 HPV-DNA positive pterygium samples were obtained from the previous study entitled "Incidence of Herpes simplex virus and Human papilloma virus". 20 samples were from King Chulalongkorn Memorial Hospital (CU), 4 samples were from Chophaya Abhaibhubejhr Hospital (AP) and one sample was from Queen Sawangwattana Memorial Hospital (SS). HPV DNA was detected by nested PCR using primer MY09/MY11 and Gp5/Gp6.

1.2 Cervical specimens

In group of cervical specimens, β-globin was detected in 82 of 89 samples (92.13%). The presence of HPV was detected in 23 samples (28.05%) by nested PCR using primer MY09/MY11 and followed by Gp5/Gp6 (Fig. 4).

2. HPV Genotyping

A total of 25 HPV positive pterygium tissues were genotyped using reverse hybridization test. HPV genotype was identified in 16 of 25 patients (64%) and HPV 16 was the most common genotype (16/25, 64%). Among those 16 samples, 4 samples had mixed infection with HPV 18 (2/25, 8%) and HPV 58 (2/25, 8%). There were 9 untyped samples detected in this study (9/25, 36%) (Fig. 5). The distribution of the HPV genotypes are shown in table 7.

In adenocarcinoma tissues, HPV genotyping was successfully done in all 23 specimens. The infection of mixed genotypes was the most frequently found (17/23, 73.91%)(Fig. 6). HPV 51 was the most found (7/23, 30.43%), followed by HPV 58 (5/23, 21.74%), HPV16 (4/23, 17.39%) and HPV 53 (4/23, 17.39%). The prevalence of HPV

infection and the distribution of the different HPV genotypes were shown in tables 8**.** The maximum number of HPV genotype in mix-infection was 6 genotypes**.**

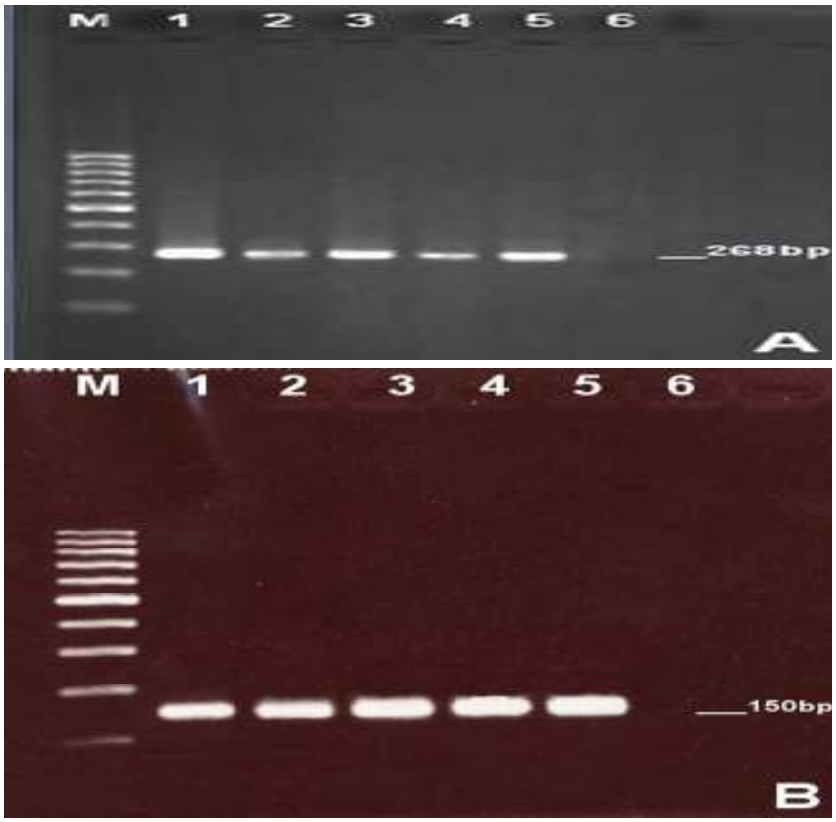

Figure 4 Electrophoresis of PCR products on 1.5% agarose gel: cervical specimens group; Lane M contains the DNA band marker. Lane 1 contains HeLa-DNA positive control and lane 6 is distilled water as negative control. Lane 2-5 are specimens. (A) β-globin PCR products (268 bp). (B) HPV amplified product of nested PCR (150 bp)

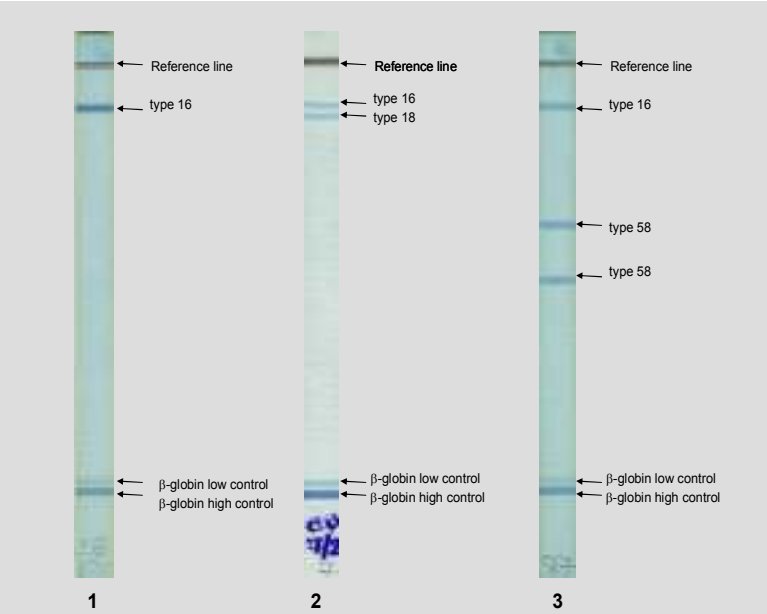

Figure 5 Patterns of genotype band of HPV positive pterygium tissue cases were determined by Reverse hybridization. Strip 1 represents HPV type 16, strip 2 represents mix-infections of HPV type 16 and 18 and strip 3 represents mix-infections of HPV type 16 and 58.

Table 7 HPV genotypes prevalence in pterygium specimens

Code of sample	Genotype
CU-011	16
CU-018	untyped
CU-025	16, 58
CU-031	16, 18
CU-038	untyped
CU-049	16
CU-050	16
CU-070	16
CU-087	16, 58
CU2-001	16
CU2-007	16, 18
CU2-008	untyped
CU2-019	16
CU2-027	untyped
CU2-029	16
CU2-031	16
CU2-036	16
CU2-042	16
CU2-044	16
CU2-057	16
SS-053	untyped
AP-037	untyped
AP-068	untyped
AP-084	untyped
AP-121	untyped

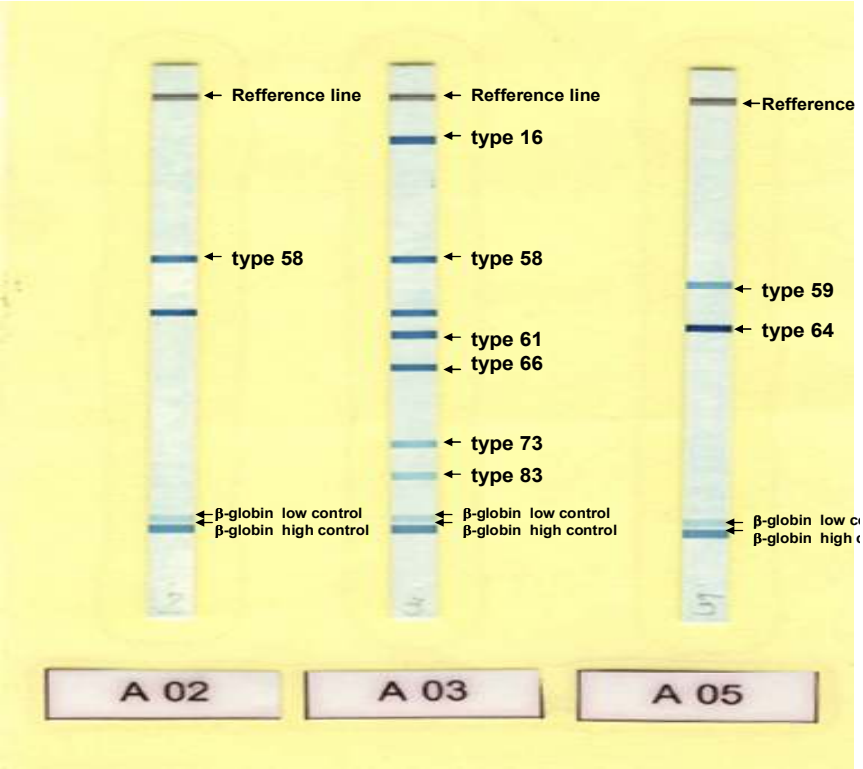

Figure 6 Patterns of genotype band of HPV positive cervical tissue cases were determined by Reverse hybridization. Strip 1 represents HPV type 58; strip 2 represents mix-infection of HPV type 16, 58, 61, 66, 73 and 83; and strip 3 represents mix-infection of HPV type 59 and 64.

Table 8 HPV genotypes in cervical specimens

Code of sample	Genotype
ADC-002	58
ADC-003	16,58,61,66,73,83
ADC-004	31
ADC-005	59,64
ADC-006	55
ADC-007	6,54,62
ADC-008	31,59
ADC-012	52
ADC-015	51,56
ADC-019	16,53,62,81,84
ADC-020	52,70
ADC-022	51,52
ADC-028	51,53,70
ADC-036	71,81
ADC-044	42,53,54
ADC-048	51,58,59,42,73
ADC-052	39,84,IS39
ADC-060	16
ADC-061	51,66,72
ADC-064	31,58,53
ADC-072	16,51
ADC-078	45,58
ADC-079	51

3. DNA sequencing analysis

The amplified fragment of the L1 gene was used in a sequencing reaction to determine the HPV type. HPV genotype was assigned based on ≥98% sequence similarity over 400 nucleotides (http//www.ncbi.nlm.nih.gov/BLAST).

3.1 Pterygium specimens

Among 12 specimens of HPV 16 monotype infection, 11 samples were amplified. The 9 PCR products amplified using the MY09/MY11 (450 bp) and 2 PCR products were amplified using the MY09/MY11 followed by Gp5/Gp6 primer (150 bp) (CU2-019 and CU2-029). All of 11 amplified fragments of the L1 gene were HPV-16 by subjected to DNA sequence analysis and subsequent BLAST (Table 9).

3.2 Cervical specimens

Among 6 monotype infected samples, only 3 samples could be amplified for direct sequencing by using the MY09/MY11 (450 bp). 11 samples of mixed HPV infections were cloned in pJET 1.2/blunt cloning vector with PCR products amplified by MY09/MY11 primers. At least 10 clones from each sample were further amplified and sequenced. Only 1 sample (ADC-064) could be cloned for all mixed HPV types. The rest 10 samples were found only one HPV type. Sequence identity of all amplified fragments of the L1 gene was determined using BLAST (Table 10).

Table 9 HPV genotypes prevalence in pterygium specimens by Direct sequencing analysis

Code of sample	**Genotype** (by Reverse hybridization**)**	**Genotype** (by Direct sequencing**)**
CU-049	16	16*
CU-050	16	16*
CU-070	16	16*
CU2-001	16	16*
CU2-031	16	16*
CU2-036	16	16*
CU2-042	16	16*
CU2-044	16	16*
CU2-057	16	16*
CU2-019	16	16**
CU2-029	16	16**

* 450 bp

** 150 bp

Table 10 HPV genotypes prevalence in adenocarcinoma specimens by sequencing analysis

Code of sample	**Genotype** (by Reverse hybridization)	**Genotype** (by sequencing)
ADC-003	16,58,61,66,73,83	
Clone 1 - 5		16
ADC-004	31	31*
ADC-005	59,64	
Clone 1 - 9		59
ADC-008	31,59	
Clone 1		31
ADC-012	52	52*
ADC-019	16,53,62,81,84	
Clone 1 - 3		53
ADC-020	52,70	
Clone 1 - 10		52
ADC-022	51,52	
Clone 1 - 2		52
ADC-028	51,53,70	
Clone 1 - 10		70
ADC-044	42,53,54	
Clone 1 - 9		53
ADC-060	16	16*
ADC-061	51,66,72	
Clone 1 - 2		66
ADC-064	31,58,53	
Clone 1 – 3, clone 6		31
Clone 4		53
Clone 5, clone 7		58
ADC-072	16,51	16
Clone 1 - 10		

* By direct sequencing

4. Genetic Variability

4.1 HPV 16 L1 Sequence Variations

In pterygium group, 11 out of 12 single HPV 16 infection samples were able to performed L1 gene sequencing. The nucleotide sequence of 9 samples (9/11, 81.82%) were similar to all HPV 16 L1 reference sequences. The different nucleotide mutations in 139 bp of the L1 gene of two samples (CU2-019 and CU2-029) were observed in 2 positions; T1078G and T1081A.

In adenocarcinoma cervical cancer group, ADC-060 and 10 clones of ADC-072 were identical to all of HPV-16 L1 gene reference sequences, while 5 clones of ADC-003 showed mutations at position A1390G.

The nucleotide sequences of HPV-16 L1 gene (420 bp) had 0.2% difference. Table 11 indicates the abbreviation of amino acids. Sequence variations observed among HPV 16 amplified products were summarized in table 12 and nucleotide mutations were shown in Fig. 7.

Table 11 Abbreviation of amino acids

Amino acid name	**Abbreviation**
Alanine	Ala, A
Arginine	Arg, R
Asparagine	Asn, N
Aspartic acid	Asp, D
Cysteine	Cys, C
Glutamine	Gln, Q
Glutamic acid	Glu, E
Glycine	Gly, G
Histidine	His, H
Isoleucine	Ile, I
Leucine	Leu, L
Lysine	Lys, K
Methionine	Met, M
Phenylalanine	Phe, F
Proline	Pro, P
Serine	Ser, S
Threonine	Thr, T
Tryptophan	Trp, W
Tyrosine	Tyr, Y
Valine	Val, V

Table 12 Mutation positions of HPV 16 comparing to reference HPV strains

HPV-16	Nucleotide Position (L1)		
	1078	**1081**	**1390**
Reference HPV-16 DQ469930, Germany	T	T	A
Reference HPV-16 HPV-16 AY177679, South Africa	T	T	A
Reference HPV-16 HPV-16 AF534061, East Asian type	T	T	A
Reference HPV-16 HPV-16 FJ797754,Thailand	T	T	A
ADC-003*	T	T	**G**
ADC-072**	T	T	A
ADC-060	T	T	A
CU-049	T	T	A
CU-050	T	T	A
CU-070	T	T	A
CU2-001	T	T	A
CU2-031	T	T	A
CU2-036	T	T	A
CU2-042	T	T	A
CU2-044	T	T	A
CU2-057	T	T	A
CU2-019	**G**	**A**	A
CU2-029	**G**	**A**	A

* ADC-003 (5 clones)

** ADC-072 (10 clones)

Figure 7 Nucleotide sequences alignment of HPV 16

4.2 HPV 31 L1 Sequence Variations

All 3 specimens of cervical cancer group presented 4 patterns with 5 different single nucleotides (ADC-004, ADC-008 and 4 clones (2 patterns) of ADC-064). The nucleotide sequence of ADC-004 was similar to all of HPV-31 L1 gene reference sequences. The variations of 5 nucleotide positions of ADC-008 were T951G, A957C, C960T, T1113C and G1192A. The nucleotide mutation position of G1192A led to amino acid change in L1 protein. The non-synonymous A398T was identified. ADC-064 had at least 2 different HPV 31 patterns. 2 nucleotides mutations of ADC-064/pattern 1 (A957C and C960T), and mutations of ADC-064/pattern 2 in 3 positions (T951G, A957C and C960T) were observed when compared with the reference sequences.

The difference among nucleotide sequences of HPV-31 L1 gene (435 bp) was 0.5-1.1%. The nucleotide mutations and amino acid of HPV-31 isolates are summarized in table 13 and the nucleotide mutations and amino acid positions were shown in Fig. 8 and Fig 9.

Table 13 Mutation positions of HPV 31 comparing to references HPV strains

HPV-31	Nucleotide Position (L1)					
	951	**957**	**960**	**1035**	**1113**	**1192**
Reference HPV-31 U37410, USA	T	A	C	T	T	G
Reference HPV-31 JN041176, Italy	-	-	-	**G**	T	G
Reference HPV-31 HQ537666, Zambia	T	A	C	T	T	G
Reference HPV-31 HQ537675, Thailand	-	A	C	T	T	G
ADC-004	-	-	-	T	T	G
ADC-008	**G**	**C**	**T**	T	**C**	**A**
ADC-064/pattern 1*	T	**C**	**T**	T	T	G
ADC-064/pattern 2**	**G**	**C**	**T**	T	T	G
HPV-31	**Amino acid Position (L1)**					
	317	**319**	**320**	**345**	**371**	**398**
Reference HPV-31 U37410, USA	A	G	H	V	F	A
Reference HPV-31 JN041176, Italy	A	G	H	V	F	A
Reference HPV-31 HQ537666, Zambia	A	G	H	V	F	A
Reference HPV-31 HQ537675, Thailand	A	G	H	V	F	A
ADC-004	A	G	H	V	F	A
ADC-008	A	G	H	V	F	**T**
ADC-064/pattern 1*	A	G	H	V	F	A
ADC-064/pattern 2**	A	G	H	V	F	A

* ADC-064/pattern 1 (clone 1, clone 2and clone 6)

** ADC-064/pattern 2 (clone 3)

- No data available

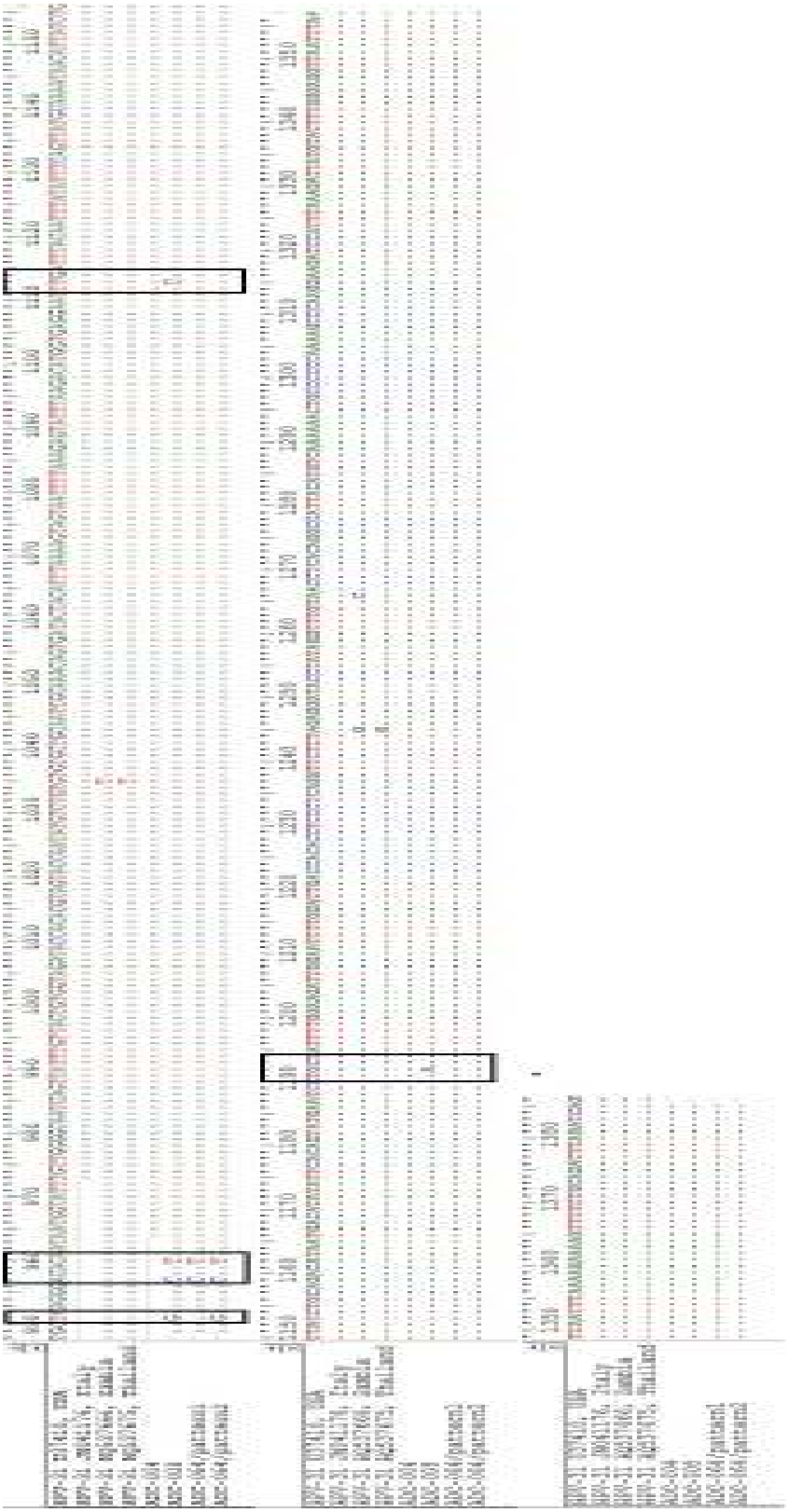

Figure 8 Nucleotide sequences alignment of HPV 31

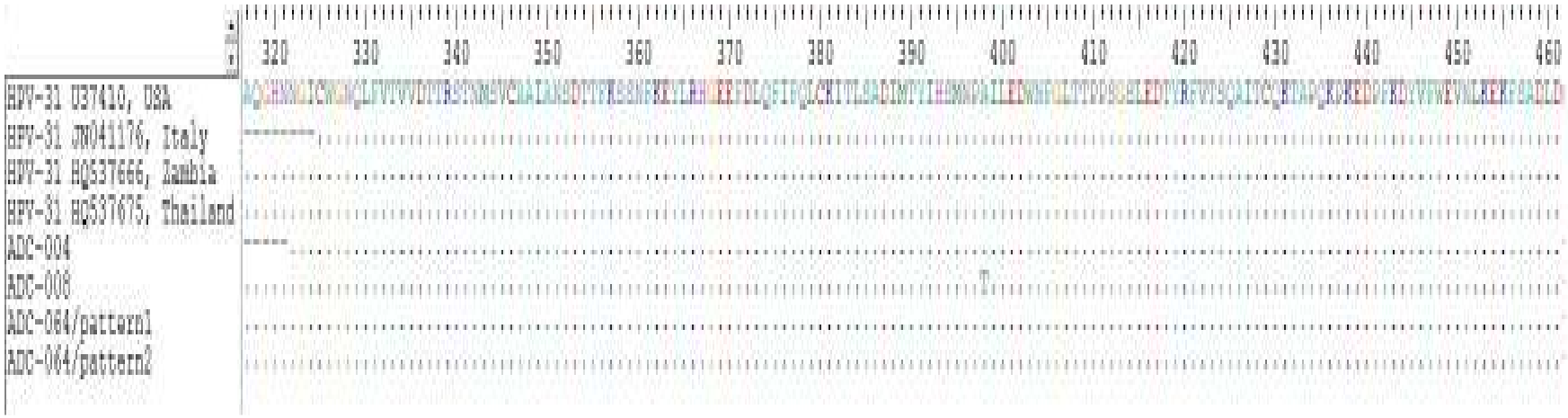

Figure 9 Amino acid alignment of HPV 31

4.3 HPV 52 L1 Sequence Variations

The variations at nucleotide position of 3 cervical cancer specimens (ADC-012, 3 clones of ADC-020 and 2 clones of ADC-022) were aligned with 4 of HPV- 52 L1 gene references. The nucleotide sequence of ADC-012 was identical to the reference HPV-52 X74481, while nucleotide mutation of 3 clones of ADC-020 were found 5 positions (T1137G, T1200C, A1230G, C1260T and C1353A). Two nucleotide mutations (G1134A and C1353A) were shown in 2 clones of ADC-022 (Fig. 10). The difference among nucleotide sequences of HPV-52 L1 gene (368 bp) was 0.5-1.4%. Sequence variations observed in HPV-52 amplified products were summarized in table 14.

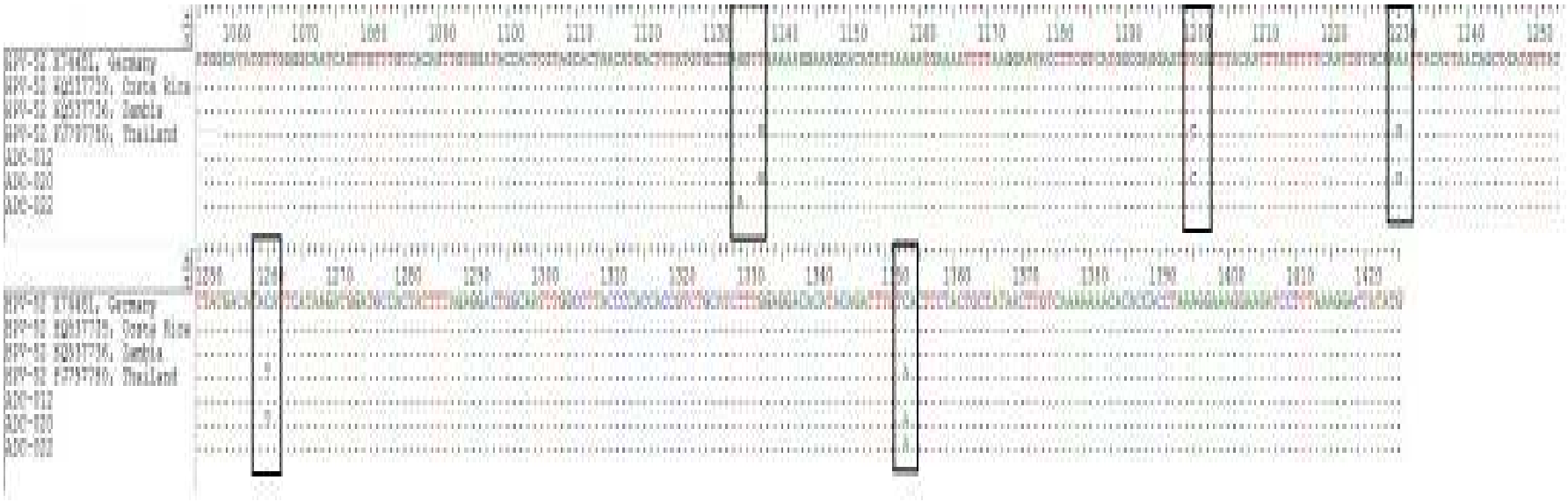

Figure 10 Nucleotide sequences alignment of HPV 52

Table 14 Mutation positions of HPV 52 comparing to references HPV strains

HPV-52	Nucleotide Position (L1)					
	1134	**1137**	**1200**	**1230**	**1260**	**1353**
Reference HPV-52 X74481, Germany	G	T	T	A	C	C
Reference HPV-52 HQ537739, Costa Rica	G	T	T	A	C	C
Reference HPV-52 HQ537736, Zambia	G	T	T	A	C	C
Reference HPV-52 FJ797780, Thailand	G	**G**	**C**	**G**	**T**	**A**
ADC - 012	G	T	T	A	C	C
ADC - 020*	G	**G**	**C**	**G**	**T**	**A**
ADC - 022**	**A**	T	T	A	C	**A**

* ADC-020 (3 clones)

** ADC-022 (2 clones)

4.4 HPV 53 L1 Sequence Variations

3 cervical cancer specimens, i.e., ADC-064, 3 clones of ADC-019 and 9 clones of ADC-044 (3 patterns) revealed 9 different single nucleotides aligned with 3 HPV-53 L1 gene references. The nucleotide sequences of ADC-019 were similar to HPV-53 X397046 reference sequence. ADC-064 had 6 different nucleotide mutations (T1054A, C1060T, T1080C, A1117G, G1285A and G1375A). There are 3 patterns of the nucleotide position mutations of 9 clones of ADC-044. Pattern 1 of ADC-044 (clone 1, 3-4, and clone 6-8), were found 6 nucleotide mutations (C1060T, T1080C, A1117G, G1285A, C1319T and G1375A). The nucleotide mutations of ADC-044 pattern 2 (clone 2 and clone 8) were identified in 7 positions (T985A, C1060T, T1080C, A1117G, G1285A, C1319T and G1375A), while the difference of nucleotide mutations of ADC-044 pattern 3 (clone 5) was 7 positions (C1060T, T1080C, A1117G, C1270A, G1285A, C1319T and G1375A). The variation at nucleotide position T985A of ADC-044 pattern 2 caused the change N to K at position 318 of the L1 protein and the nucleotide mutation position C1319T led to amino acid change in position P430S of all 3 patterns of ADC-044. The difference among nucleotide sequences of HPV-53 L1 gene (429 bp) was 1.4-1.6%.

The nucleotide mutations and amino acid positions of HPV-53 isolates were shown in Fig. 11 and Fig 12 and summarized in table 15.

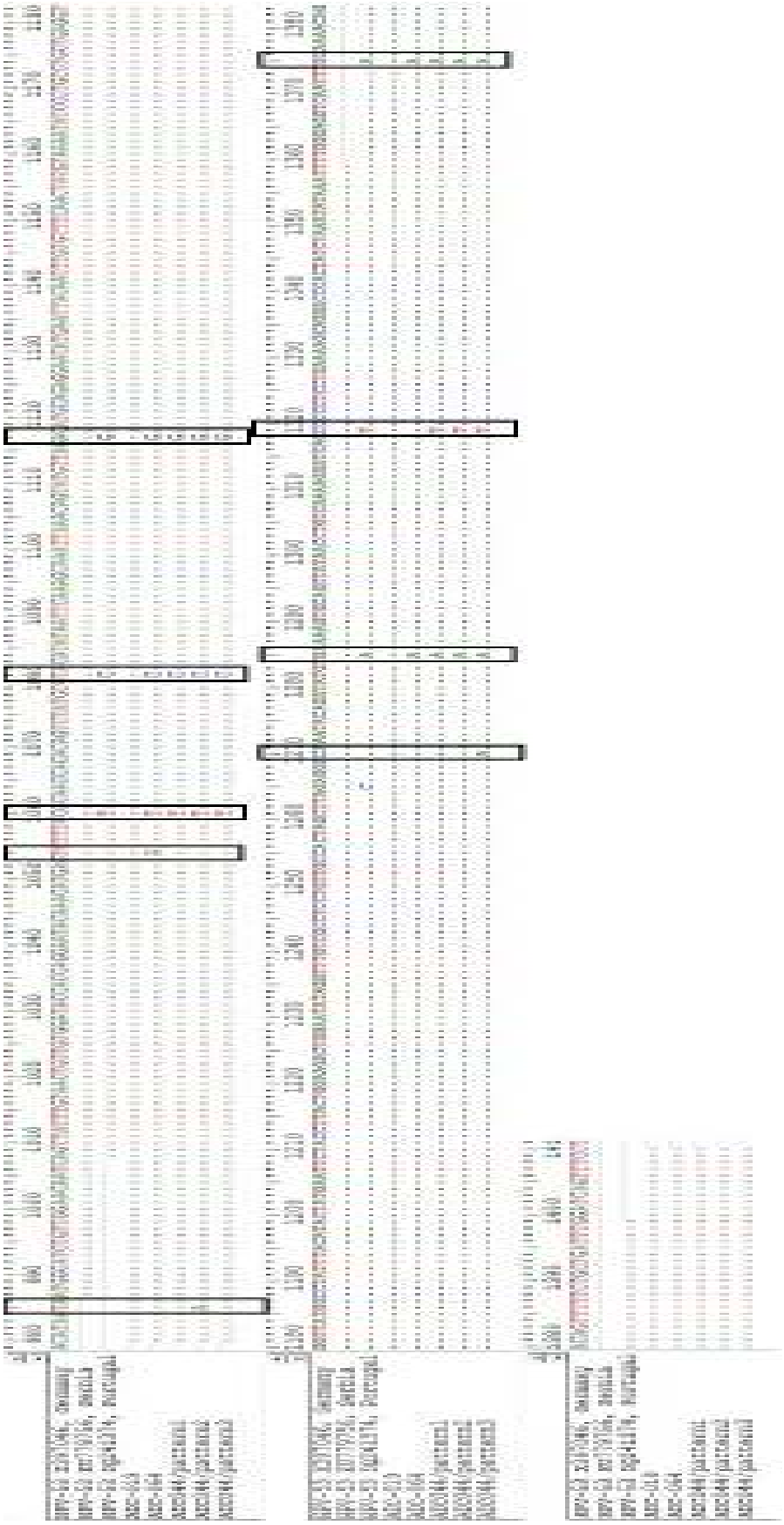

Figure 11 Nucleotide sequences alignment of HPV 53

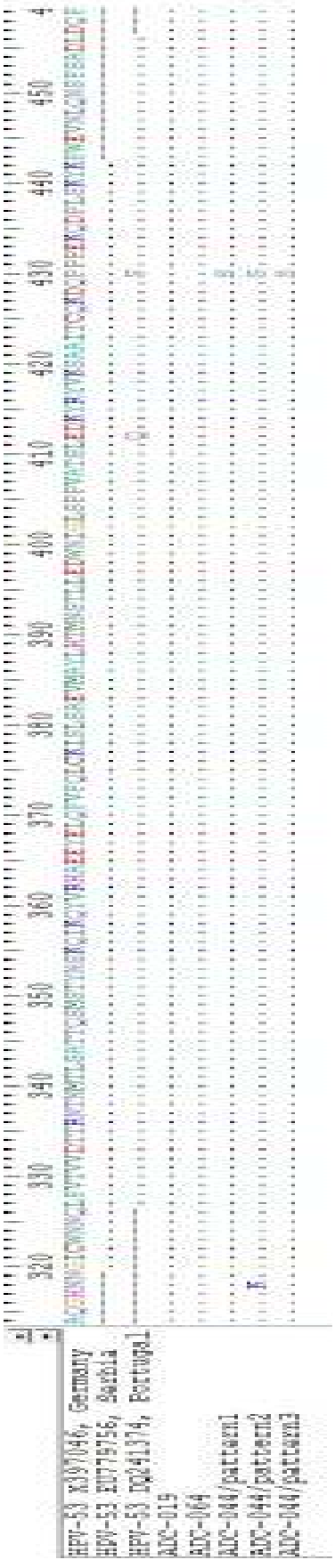

Figure 12 Amino acid alignment of HPV 53

Table 15 Mutation positions of HPV 53 comparing to references HPV strains

	Nucleotide Position (L1)								
HPV-53	**985**	**1054**	**1060**	**1080**	**1117**	**1270**	**1285**	**1319**	**1375**
Reference HPV-53 X397046, Germany	**T**	**T**	**C**	**T**	**A**	**C**	**G**	**C**	**G**
Reference HPV-53 EU779756, Serbia	_	T	C	T	A	C	G	C	-
Reference HPV-53 DQ241374, Portugal	_	T	**T**	**C**	**G**	C	**A**	**T**	**A**
ADC-019	T	T	C	T	A	C	G	C	G
ADC-064*	T	**A**	**T**	**C**	**G**	C	**A**	C	**A**
ADC-044/pattern 1[A]	T	T	**T**	**C**	**G**	C	**A**	**T**	**A**
ADC-044/pattern 2[B]	**A**	T	**T**	**C**	**G**	C	**A**	**T**	**A**
ADC-044/pattern 3[C]	T	T	**T**	**C**	**G**	**A**	**A**	**T**	**A**
	Amino acid Position (L1)								
HPV-53	**318**	**341**	**343**	**350**	**362**	**413**	**418**	**430**	**448**
Reference HPV-53 X397046, Germany	**N**	**T**	**S**	**S**	**R**	**D**	**V**	**P**	**L**
Reference HPV-53 EU779756, Serbia	N	T	S	S	R	D	V	P	L
Reference HPV-53 DQ241374, Portugal	N	T	S	S	R	D	V	**S**	L
ADC-019	N	T	S	S	R	D	V	P	L
ADC-064*	N	T	S	S	R	D	V	P	L
ADC-044/pattern 1[A]	N	T	S	S	R	D	V	**S**	L
ADC-044/pattern 2[B]	**K**	T	S	S	R	D	V	**S**	L
ADC-044/pattern 3[C]	N	T	S	S	R	D	V	**S**	L

* ADC-064 (clone 4)

A: ADC-044/pattern 1 (clone 1, 3-4, and clone 6-8)

B: ADC-044/pattern 2 (clone 2 and clone 8)

C: ADC-044/pattern 3 (clone 5)

- No data available

4.5 HPV 58 L1 Sequence Variations

2 clones of ADC-064 had 7 mutation positions (G1105A, A110G, A1112G, T1115G, C1121T, G1265A and C1346A) when compared with the HPV-58 L1 gene reference. The nucleotide sequence difference of 440 bp HPV-58 L1 gene was 1.6% (Table 16 and Fig. 13).

Table 16 Mutation positions of HPV 58 comparing to references HPV strains

HPV-58	Nucleotide Position (L1)						
	1105	**1109**	**1112**	**1115**	**1121**	**1265**	**1346**
Reference HPV-58 EU918765, China	**G**	**A**	**A**	**T**	**C**	**G**	**C**
Reference HPV-58 HQ537754, Costa Rica	**G**	**A**	**A**	**T**	**C**	**A**	**C**
Reference HPV-58 HQ537763, Zambia	**G**	**A**	**A**	**T**	**C**	**A**	**A**
Reference HPV-58 FJ797803, Thailand	-	-	-	-	-	**A**	**C**
ADC-064*	**A**	**G**	**G**	**G**	**T**	**A**	**A**

* ADC-064 (clone 5 and clone 7)

- No data available

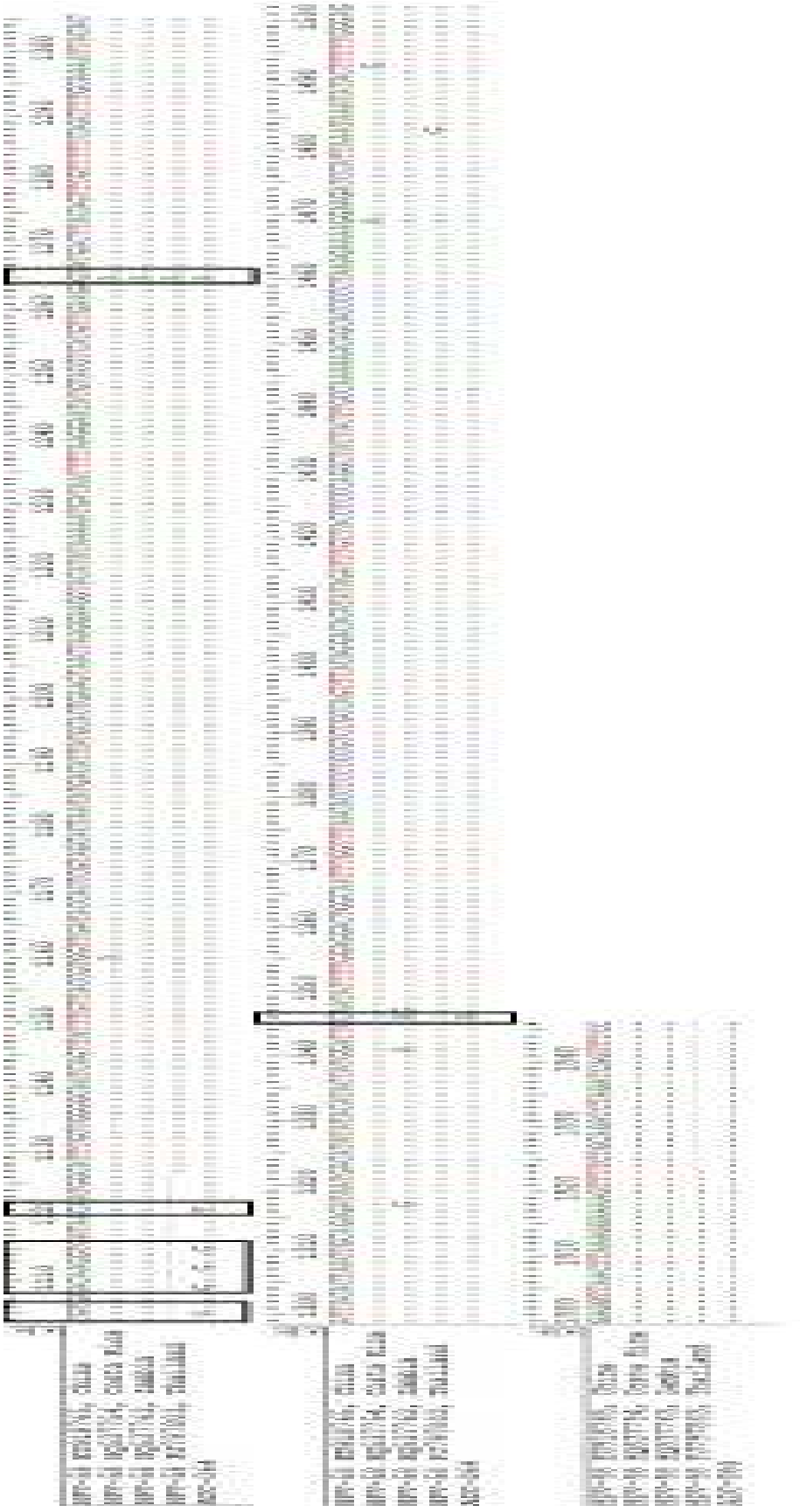

Figure 13 Nucleotide sequences alignment of HPV 58

4.6 HPV 59 L1 Sequence Variations

Only one nucleotide mutation (C1052T) was observed in 9 clones of ADC-005) when compared with 4 HPV- 59 references (Fig.14). At this position, the amino acid of L1 protein was changed P35S (Table 17 and Fig. 15). The nucleotide sequence of ADC-005 L1 gene (331 bp) in HPV-59 differed from reference strain about 0.3%.

Table 17 Mutation positions of HPV 59 comparing to references HPV strains

HPV-59	**Nucleotide Position (L1)** **1052**
Reference HPV-59 U45930, USA	C
Reference HPV-59 AB437933, Korea	T
Reference HPV-59 DQ486471, Brazil	G
Reference HPV-59 FJ797809, Thailand	T
ADC-005*	T
HPV-59	**Amino acid Position (L1)** **35**
Reference HPV-59 U45930, USA	P
Reference HPV-59 AB437933, Korea	S
Reference HPV-59 DQ486471, Brazil	A
Reference HPV-59 FJ797809, Thailand	S
ADC-005*	S

* ADC-005 (9 clones)

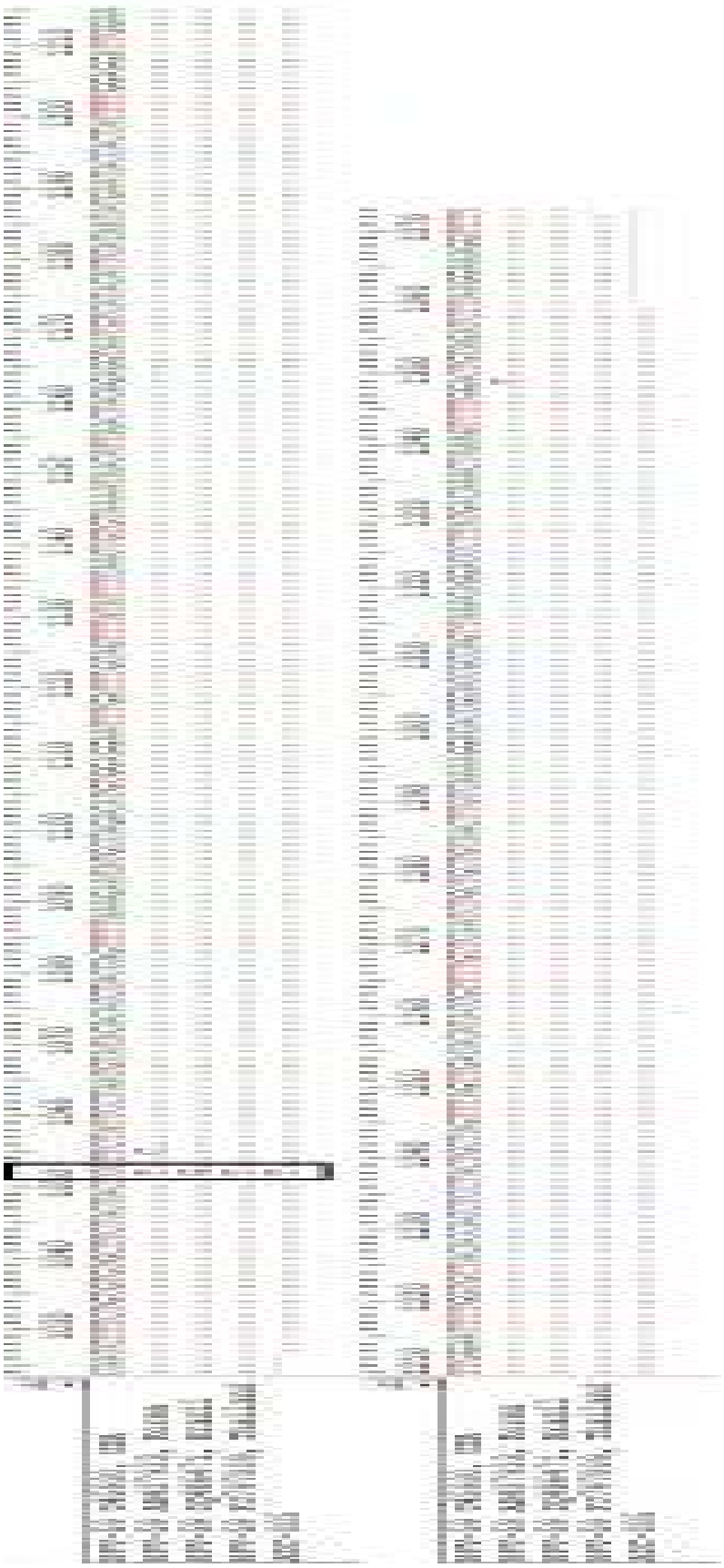

Figure 14 Nucleotide sequences alignment of HPV 59

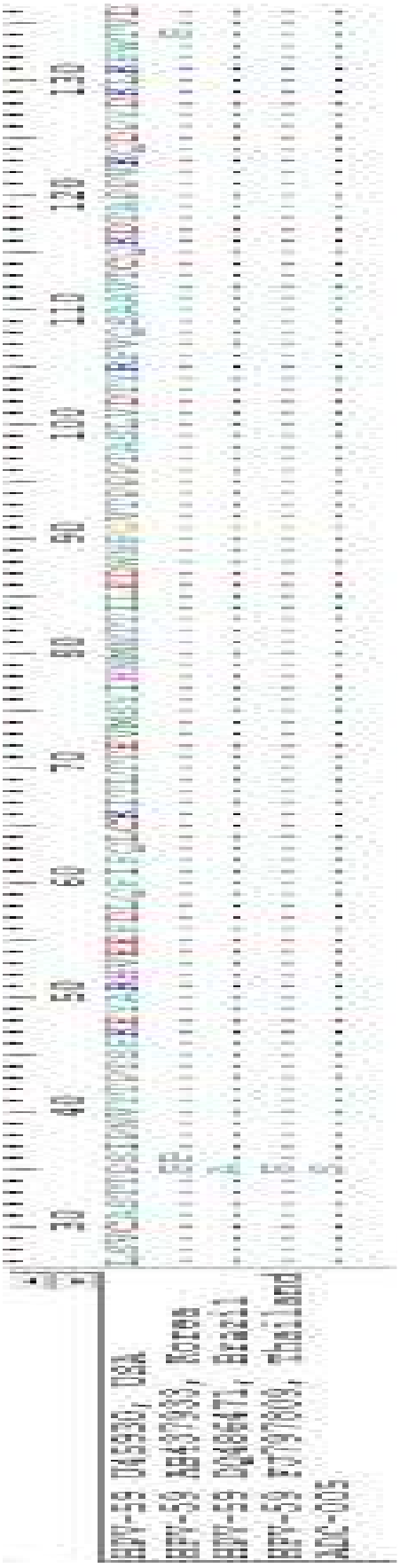

Figure 15 Amino acid alignment of HPV 59

4.7 HPV 66 L1 Sequence Variations

The variation of 2 nucleotide positions of ADC-061 (A1050G and C1056G) was demonstrated when compared with the 3 of HPV- 66 L1 gene references. The nucleotide sequence of ADC-061 HPV-66 L1 gene (424 bp) was 0.5% different (Table 18 and Fig.16).

Table 18 Mutation positions of HPV 66 comparing to references HPV strains

HPV-66	Nucleotide Position (L1)	
	1050	1056
Reference HPV-66 EF177191, USA	A	C
Reference HPV-66 U31794, Germany	A	C
Reference HPV-66 DQ486474, Brazil	A	T
ADC-061	G	G

* ADC-061 (2 clones)

Figure 16 Nucleotide sequences alignment of HPV 66

4.8 HPV 70 L1 Sequence Variations

There were 2 patterns of HPV-70 L1 mutation obtained from 10 clones of ADC- 028 which had 3 mutation positions compared with the 3 HPV-70 references. The pattern 1 has 2 nucleotide mutations (T1152A and G1324A), while mutations of ADC-028/pattern 2 were observed in 3 positions (T1152A, G1311A and G1324A). The variation at nucleotide position G1324A caused the change of D to N at position 442 of the L1 protein. The difference among nucleotide sequences of HPV-70 L1 gene (419 bp) was 0.5-0.7%.

The nucleotide mutations and amino acid of HPV-70 isolates were shown in Fig. 17 and Fig 18 and summarized in table 19.

Figure 17 Nucleotide sequences alignment of HPV 70

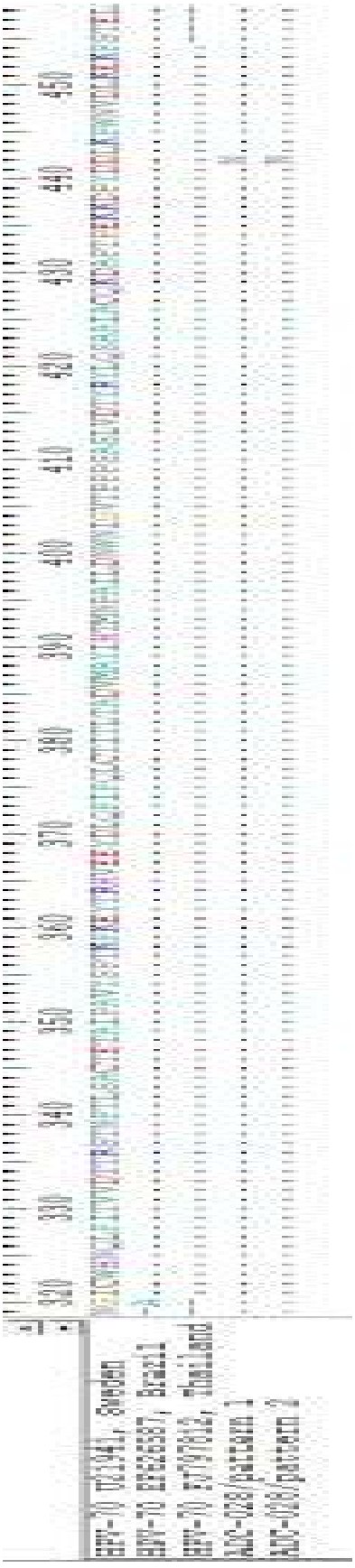

Figure 18 Amino acid alignment of HPV 70

Table 19 Mutation positions of HPV 70 comparing to references HPV strains

HPV-70	Nucleotide Position (L1)		
	1152	**1311**	**1324**
Reference HPV-70 U21941, Sweden	T	G	G
Reference HPV-70 EF626587, Brazil	A	G	G
Reference HPV-70 FJ797812, Thailand	A	G	G
ADC-028/pattern 1*	A	G	A
ADC-028/pattern 2**	A	A	A
HPV-70	**Amino acid Position (L1)**		
	384	**437**	**442**
Reference HPV-70 U21941, Sweden	A	K	D
Reference HPV-70 EF626587, Brazil	A	K	D
Reference HPV-70 FJ797812, Thailand	A	K	D
ADC-028/pattern 1*	A	K	**N**
ADC-028/pattern 2**	A	K	**N**

* ADC-028/pattern 1 (clone 1, 3-4, 6, and 8-10)

** ADC-028/pattern21 (clone 2, 5 and 7)

5. Phylogenetic Analysis

Phylogenetic analysis of the L1 gene (331 bp) of HPV-DNA from pterygium specimens and cervical specimens was shown in Figure 19. The different levels of sequence variability among the L1 regions and phenogram with different branching have been obtained. All HPV 16 isolates in this study (not included 139 bp L1 gene of CU2-019 and CU2-029) were clustered with those HPV 16 reference sequences (Europe, South Africa, East Asian and Thailand) presumably they come from the same origin (branch B6). All patterns of ADC-044 and ADC-064 were clustered with the HPV 53 reference from Portugal (branch B1). ADC-019 isolate clustered with HPV 53 reference from Germany. In branch of HPV 66, the ADC-061 isolate clustered together with the HPV reference from Germany, USA and Brazil (branch B2). The ADC-005 isolate clustered with the HPV 59 reference sequences originating from USA, Brazil, Korea and Thailand (branch B3). In branch of HPV 70, ADC-028 pattern 1 and pattern 2 isolates clustered with HPV 70 references from Brazil, Sweden and Thailand (branch B4). ADC-008, ADC-064 pattern 1 and ADC-064 pattern 2 isolates clustered with HPV 31 references from USA, Zambia, and Thailand (branch B5). In branch of HPV 52, isolates were clustered together with HPV52 references originating from 4 countries; Germany, Zambia, Costa Rica and Thailand. ADC-012 isolate clustered with the HPV references of Germany, Zambia and Costa Rica while ADC-020 closed to the HPV reference from Thailand (branch B7). Interestingly, the ADC-022 was separated to a new branch. The ADC-064 isolates (clone 5 and clone 7) clustered with the HPV 58 reference sequences originating from China, Costa Rica and Zambia (branch B8).

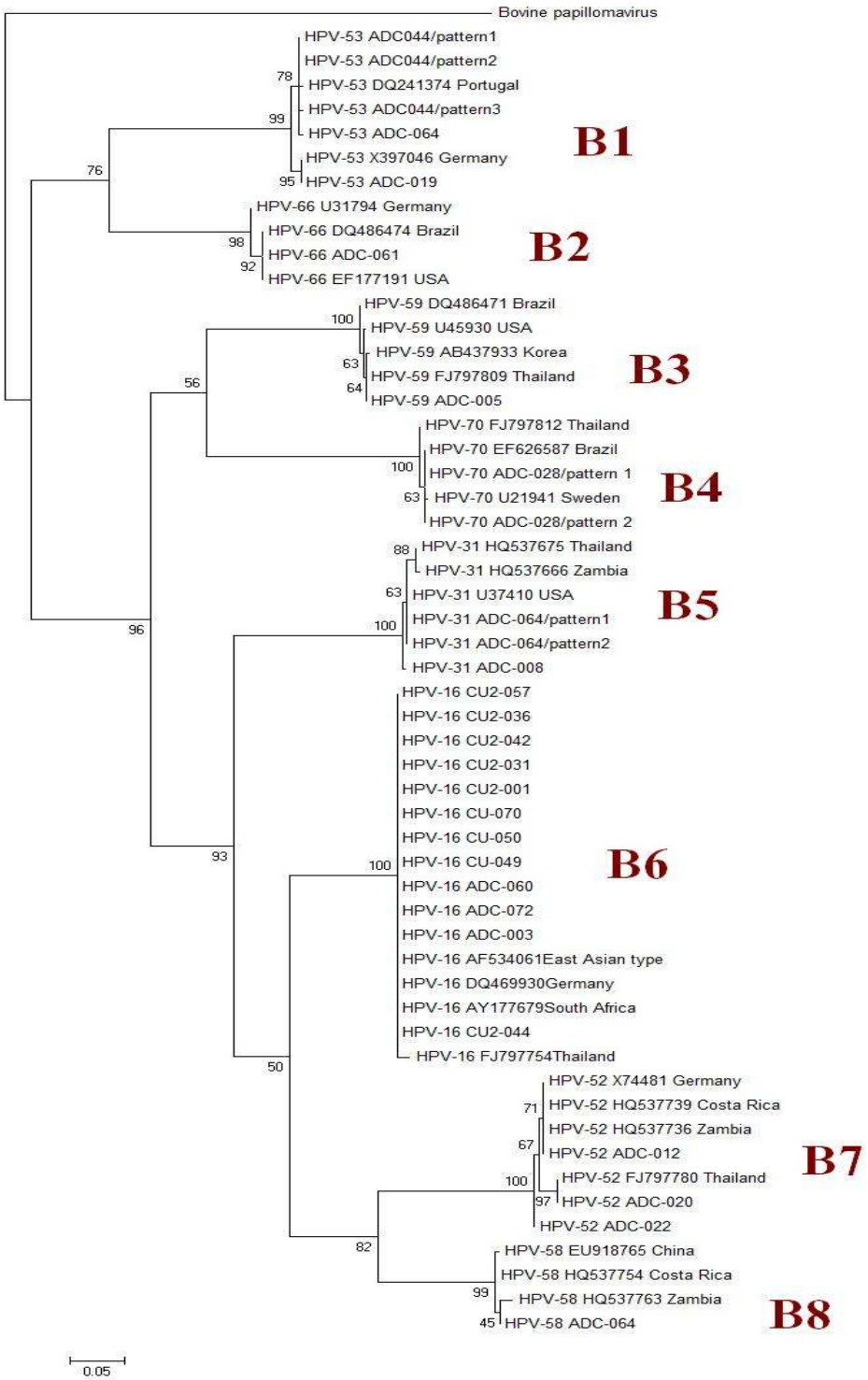

Figure 19 Maximum likelihood phylogenetic tree based on L1 (331 bp), samples labeled pterygium specimens (CU) and cervical carcinoma (ADC). Reference sequences for HPV genotypes are included in the tree. The branch leading to phylogenetic clusters B1-B8 is shown. Bootstrap values (1000 replicates) was represented in the branches.

CHAPTER V

DISCUSSION

This study describes the distribution of HPV types in pterygium tissues and adenocarcinoma cervical cancer of Thai patients. HPV infection in pterygium of Thai patients has been previously detected by nested PCR using MY09/MY11 followed by Gp5/Gp6 primers. The prevalence of HPV infection in pterygium was 6.43% (25/389, unpublished data). All 25 HPV positive samples were recruited for typing in this study. The result revealed that HPV type 16 was the most prevalence (64%). The prevalence of HPV in pterygium has been reported in many countries as shown in Table 20 (25-29). All studies were determined by PCR and the results of the prevalence of HPV infection were varied from 4.4 to 53.6% (25-29). Moreover, HPV genotypes were also different. Piecyk-Sidor *et al* (95) from Poland showed the prevalence of HPV in pterygium was 27.6% (16/58) and 56% were HPV type 16. In United Kingdom, Gallagher *et al* (28) detected HPV in pterygium and conjunctival papilloma. They showed that 5 of 10 (50%) HPV-positive pterygia which 2 were HPV type 6 (40%), 2 were HPV type 11 (40%) and one was HPV type 16 (20%) while in conjunctival papilloma, HPV type 6 was the most prevalent (40%, 4/10) followed by HPV type 11 (30%) and HPV type 16 (20%). Detorakis *et al* (29) found HPV type 18 in all HPV positive pterygium samples (24%, 12/50) in Greece. In Denmark, SjÖ *et al* (26) reported 4.4% (4/90) of HPV-positive pterygia and all of them were HPV type 6 (100%). The results reported by Piras *et al* (25) indicated the presence of HPV-DNA in 17 (100%) pterygia from Italy and 5 (21%) of 24 pterygia from Ecuador which HPV type 52 (50%) was found the most. In Asia, Hsiao *et al* (96) detected the presence of HPV type 18 (3.1%) in 2 of 65 patients in Taiwan. Altogether, HPV prevalence and HPV genotype in pterygium may be different according to the geographical regions. HPV either low risk (type 6 and 11) or high risk (type 16 and 18) involves in the pathogenesis of pterygium but in Thailand, HPV type 16 plays important role. Recently, Rootman DB, *et al* (97) in 2012 reported intraocular extension of conjunctival invasive squamous cell carcinoma after pterygium surgery. Unfortunately, they did not perform HPV-DNA detection. However, the presence of HPV-16 which is highly oncogenic type in pterygium may be a causative agent of conjunctival squamous cell carcinoma.

Table 20 Human papillomavirus (HPV) DNA in pterygium. Comparison of published data

References	Assay for Typing	HPV types
Gallagher *et al* (2001) United Kingdom	Dot-blot hybridization	HPV 6 (2/10, 40%) HPV 11 (2/10, 40%) HPV 16 (1/10, 20%)
Detorakis *et al* (2001) Greece	DNA sequence analysis	HPV 18 (12/12, 100%)
Piras *et al* (2003) Italy a Ecuador	DNA sequence analysis	HPV 52 (11/22, 50%) HPV 90 (5/22, 23%) HPV 54 (4/22, 18%)
Sjo *et al*. (2007) Denmark	DNA in situ hybridisation (ISH)	HPV 6 (4/4, 100%)
Piecyk-Sidor *et al* (2009) Poland	Reverse hybridization (INNOLiPA)	HPV 16 (9/16, 56%) HPV 6 and 16 (3/16, 18%) HPV 6 and 18 (2/16, 12.5%) HPV 16 and 31 (1/16, 6.25%) HPV 11, 16, 18, 52, 53, 59 (1/16, 6.25%)
Hsiao *et al (2010)* Taiwan	Fluorescence in situ hybridization	HPV 18 (2/65, 3.1%)

HPV DNA was detected in 23 of 89 (25.8%) adenocarcinoma cervical cancer tissues of Thai patients. The prevalence of HPV infection in this study seemed to be lower than other studies which reported the prevalence varied between 80-90% (98, 99). The reason is hardly explained. Actually the method of HPV-DNA detection in this study have higher sensitivity than those previous studies because nested PCR was used instead of conventional PCR. Moreover, beta-globin, house keeping gene, was detected in nearly all samples meaning that samples have enough DNA. Only possible explaination might rely on low HPV-DNA in tissues and possibly that HPV-DNA was very short due to fragmentation which caused false negative results.

It was clearly showed that multiple type infection was the most frequently found (17/23, 73.91%), while monotype infection was detected in 6 samples (26.09%). HPV 51 was predominantly found (7/23, 30.43%), followed by HPV 58 (5/23, 21.74%), HPV16 (4/23, 17.39%) and HPV 53 (4/23, 17.39%). A few study has previously described the HPV genotypes detected in adenocarcinoma cervical cancer. In contrast to our results, An *et al* (98) reported the prevalence of HPV infection was 90% (121/135) in Korean women with adenocarcinoma of uterine cervix. HPV type 16 (42%) was the most prevalent followed by HPV type 18 (29%), HPV type 16 and 18 (7%) and HPV 33 (5%). Li *et al* (99) showed the presence of HPV-18 more common in adenocarcinomas than HPV-16 (21.4% vs 3.1%, $p<0.02$) and 94% of them had at least one of the 2 types. Here, no any HPV-18 was detected and HPV-16 was less than 20%. Since no information of HPV detection and typing in Thai patients with adenocarcinoma has been reported, more investigation by increase number of samples should be done.

In this study, the genetic variability of L1 gene of HPV 16, 31 52, 53, 58, 59, 66 and 70 of the pterygium and adenocarcinoma cervical cancer isolates were aligned with HPV reference sequences. The sizes of the partial genome L1 sequences ranged from 139 to 440 bp. Every types showed 98.4–99.8% nucleotide similarity to the HPV L1 reference sequences obtained from different geographical worldwide. These results suggested no new variants was isolated in this study. Moreover, it may imply that HPV genotypes around the world are the same origin. Few intragenotypic variations of L1 gene is expected because this gene plays important role in attachment and penetration into host cells. In addition, HPV is a DNA virus which its replication

control by DNA polymerase that has proofreading function. Although the sequence variations of the HPV 16, 52, 58 and 66 L1 genomic region isolates were demonstrated, they did not affect the amino acid sequences. However, the nucleotide mutations of HPV 31, 53, 59 and 70 led to amino acid change. Among those amino acid changes, A398T in HPV 31 isolate, N318K in HPV 53 isolate, P430S in HPV 59 isolate and D442N in HPV 70 isolate were the position that firstly found by this study. Pande *et al* (93) showed the data of 13 nucleotide variations of HPV type 16 variants were detected in 11 cervical cancer patients of India; of four variants of L1 gene led to mutation in amino acid serine at codons 343 and 351, serine to phenylalanine at position 423 and glutamine to proline at position 429. Cento *et al* (92) reported intratypic variation of HPV-31 was identified in Italian women with abnormal cervical smear, the presented of 22 different nucleotide mutations led to amino acid change in the L1 protein (L365F, F371Y, K381Q, H393Y and E454K). The phylogenetic comparison of HPV genomic variants isolated from pterygium and adenocarcinoma cervical cancer showed that all Thais isolates were clustered with HPV reference type from other parts of the world although 2 types (HPV-31 ADC 008 and HPV-52 ADC022) were separated in a new branch. However, the difference in nucleotide sequence was less than 2%. These results suggested no new variants was isolated and no difference of HPV genotypes that cause pterygium and adenocacinoma cervical cancer. These may imply that HPV genotypes around the world were from the same origin. Intragenotypic variations of L1 gene was demonstrated ranging from one to several single nucleotide polymorphisms. It indicated that L1 gene should be conserved region and required for viral survive, which we have already known that this gene plays important role in viral attachment and penetration into host cells. In addition, proof reading viral DNA polymerase property of HPV may result in low genomic variations.

In this study, HPV type 16 was found the most prevalent in pterygium, followed by HPV 16 and 18. Recently, HPV vaccines are highly effective in preventing infections with high risk types HPV 16 and 18. Our study suggested that in Thailand, HPV vaccine can efficiency protect the infection since the L1 gene of HPV 16 was not much different from others. Moreover, the vaccine may beneficially to protect pterygium which might associate with the development of conjunctival squamous cell carcinoma.

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APPENDICES

APPENDIX A

REAGENTS, MATERIALS and INSTRUMENTS

A. Media and Reagents

Absolute ethanol	(Merck, Germany)
Agarose	(Reserch Organic, Inc., U.S.A.)
Ampicillin	(Bio Basic, U.S.A.)
100 bp DNA ladder	(Fermentas,EU)
DNA extraction kit	(Roche Diagnostics, USA), (MACHEREY-NAGEL, Germany)
dNTPs	(Fermentas,EU)
Ethidium bromide	(Bio-Rad, U.S.A.)
Genotyping Test Kit	(Roche Diagnostics, USA)
LB (Luria Bertani) broth powder	(Merck, Germany)
$Na_2EDTA.2H_2O$	(Merck, Germany)
PCR Cloning kit	(Fermentas,EU)
Plasmid extraction kit	(MACHEREY-NAGEL, Germany)
Proof reading Taq DNA polymerase	(Finnzymes, U.S.A.)
Purify PCR product kit	(MACHEREY-NAGEL, Germany)
Taq DNA polymerase	(Fermentas, U.S.A.)
Tris Base	(Reserch Organic, Inc., U.S.A.)
Xylene	(Merck, Germany)

B. Materials

Disposible serological pipette	(Labcon, Germany)
Filter tip	(Sorenson, U.S.A.)
Microcentrifuge tube	(Sorenson, U.S.A.)
Centrifuge tube	(Labcon, Germany)

C. Instruments

Autoclave	(Tomy, Japan)
Culturing shaking incubator	(Ztictene, China)
Electrophoresis chamber	(BIOER, China)
Gel Documentation	(Bio-Rad, U.S.A.)
Microcentrifuge	(Denville, U.S.A.)
PCR Thermal cycler	(Takara, Japan)
Refrigerator	(Sanyo, Japan)
Water bath	(GFL, Germany)

APPENDIX B

REAGENTS PREPARATION

1. Luria-Bertani broth

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Distilled water to	1 L

Sterilized by autoclaving 121^{0}C, 15 minutes

2. Luria-Bertani agar plate

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Distilled water to	1 L

Sterilized by autoclaving 121^{0}C 15 minutes

To pour plates, the medium was allowed to cool to 55^{0}C and added 2 mL of ampicillin (50 ng/mL) to a final concentration of 100 µg/mL

3. Ampicillin (50 mg/mL)

Ampicillin	2.5 g
Distilled water	50 mL

Sterilized by filter and stored in aliquots at 4^{0}C

4. 10X Tris-borate buffer

Tris-base	60.50 g
Boric acid	30.85 g
$Na_2EDTA.2H_2O$	3.72 g
Distilled water to	1 L

Sterilized by autoclaving 121^{0}C, 15 minutes and stored at room temperature

BIOGRAPHY

Name:	Miss Luckana Thammachoteruja
Date of birth:	20 August 1975
Place of birth:	Bangkok, Thailand
Education:	Bachelor degree of Science (Microbiology) Srinakharinwirot University, Bangkok, Thailand
Publication:	Luckana Thammachotruja, Parvapan Bhattarakosol, Ngamjit Kasetsuwan. Prevalence of Human papillomavirus genotypes in pterygium. Proceeding in The 20th Annual Scientific Meeting. The Virology Association (Thailand), Nov 2010:139-147