DETECTION OF HPV-DNA IN CERVICAL AND URETHRAL SCRAPINGS USING HYBRID CAPTURE 2 AND NESTED-PCR AND THEIR CORRELATION WITH CYTOMORPHOLOGY

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UNIVERSITI SAINS MALAYSIA

2010

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by

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Thesis submitted in fulfillment of the requirements
for the degree of
Master of Science

MAY 2010

ACKNOWLEDGEMENTS

In the name of Allah, the most Gracious, the most Merciful.

Praise Allah, the Almighty God for giving me the strength and patience, making this possible for me to finally complete this study.

My sincere thanks and deepest appreciation to my supervisor, Professor Dr. Nor Hayati Othman, for her willingness to accept me as her student. Her dedication, support and constant supervision have been great motivation for me to complete my research. I am also very grateful for the financial help I received during my course of study and her patience in seeing me through this project.

My greatest appreciation to all staff and friends at the Institute for Research in Molecular Medicine (INFORMM) especially to Prof Asma Ismail, for giving me a place and a chance to use the equipment there. To Dr. Tang Thean Hock, my co-supervisor – I thank him for his stimulating scientific discussion and broad knowledge in the field of molecular research. To Dr. Hamid, Kak Norhaslizai, Kak Zurinariah, Kak Fiza, Kak Siti, Kak Ina, Kak Zuriati, Foong, and all the “Informmer” that I did not mentioned their names here; thank you for your co-operation and support. The kind and friendly atmosphere which they provided enable me to enjoy doing this research.

My deepest gratitude to all staff from Department of Pathology, School of Medical Sciences, namely technologists, cytologists and pathologists specially not forgetting Tae Han Shean, Dr. Shariman, Zulkefli Sanip and Dr. Shady El-Tawel for their help throughout this study.

Special thanks to husband and wife, Prof. Syed Mohsin Sahil Jamalullail and Puan Hasnah Ishak for their help, attention and moral support to me while I was completing this study.

To my family especially my father, Othman bin Mohd and mother, Rossnah binti Mohamed who whole-heartedly give me the freedom to do whatever I deem best for my future. I am thankful for their endless love, support and understanding during my study period.

To all named and unnamed friends and peers, I again extend my thanks.

This study was funded by an IRPA grant from the Ministry of Science, Technology and Innovation (MOSTI), Malaysia Government.

Norodiyah Othman

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LIST OF ABBREVIATIONS

ACOG	American College of Obstetricians and Gynecologists
ADC	Adenocarcinoma
AGUS	Atypical grandular cells of undetermined significance
AP	Asia Pacific
ASCCP	American Society for Colposcopy and Cervical Pathology
ASCUS	Atypical squamous cells of undetermined significance
ASR	Age-standardized rates
bDNA	Branched DNA
bp	Base pair
CIN	Cervical intraepithelial neoplasia
CP	Conventional Pap smear
CR	Crude incidence rate
DNA	Deoxy ribonucleic acid
DR	Denaturation Reagent
E	Early
FN	False negative
FP	False positive
HC2	Hybrid capture 2
HGSIL	High-grade squamous intraepithelial lesion
HPV	Human papillomavirus
HR	High-Risk

HSV	Herpes simplex virus
IARC	International Agency of Research on Cancer
ICC	Invasive cervical cancer
ISH	In situ hybridization
L	Late
LBC	Liquid-based cytology
LGSIL	Low-grade squamous intraepithelial lesion
LR	Low-Risk
$MgCl_2$	Magnesium chloride
Min	Minute
NPV	Negative predictive value
OC	Oral contraceptive
ORF	Open reading frame
Pap	Papanicolaou
PC	Positive control
PCR	Polymerase chain reaction
PHR	Probable high-risk
PP	Polypropylene
PPV	Positive predictive value
RLU	Relative light unit
RLU/CO	Relative light unit/cut-off
RNA	Ribonucleic acid
RR	Relative risk
S	Second

SCC	Squamous cell carcinoma
SD	Standard deviation
spp	Species
STM	Specimen transport medium
suppl	Supplement
T_m	Annealing temperature
TP	ThinPrep® Pap smear
UR	Undetermined risk
URR	Upstream regulatory region
USFE	Unsatisfactory smear for evaluation
v/v	Volume per volume
VLP	Virus-like particle
vs	Versus
w/v	Weight per volume
WNL	Within normal limit

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ABSTRAK

Pengesanan DNA-HPV di Dalam Kikisan Servik dan Uretra Menggunakan Hybrid Capture 2 dan Nested-PCR dan Kaitannya dengan Sitomorfologi.

Jangkitan virus papilloma manusia (HPV) adalah agen penyebab kepada pertumbuhan kanser servik. Kajian ini bertujuan untuk mengesan kehadiran HPV pada pap smear wanita yang menghadiri pemeriksaan rutin di Hospital Kota Bharu, Hospital Universiti Sains Malaysia dan Hospital Kuala Terengganu. Sampel serviks dari setiap wanita dalam dua bentuk (split-sampling); Pap smear konvensional (CP) dan Pap smear ThinPrep® (TP). Wanita ini diberikan boring soal selidik untuk menentukan faktor risiko untuk pertumbuhan kanser serviks. Pasangan laki-laki dijemput untuk memberikan kikisan uretra untuk kajian HPV. Sample serviks dan uretra diuji dengan Hybrid Capture 2 (HR-HC2) dan tindak balas berantai polymerase (nested-PCR). Keputusan HPV di dalam kikisan serviks dikaitkan dengan diagnosis sitologi dari CP dan TP.

Sebanyak 705 kikisan serviks dan 99 kikisan uretra laki-laki dikumpulkan. 702 sampel serviks mempunyai keputusan penilaian sitologi untuk kedua-dua CP dan TP. Dari CP, 92.7% sampel kikisan serviks adalah normal, 2% adalah tidak normal Pap smear manakala 5.3% smear tidak memuaskan untuk penilaian sitologi. Dengan TP, 87.1% adalah normal, 1.7% adalah tidak normal dan 11.1% tidak memuaskan untuk penilaian sitologi. Keputusan diagnosis Pap smear secara konvensional dan ThinPrep® adalah sama sebanyak 88.9%. Kecukupan untuk pesampelan dengan TP (88.9%) adalah setanding dengan CP (94.7%).

635 sampel serviks adalah mencukupi untuk ujian HPV. 44 (6.9%) sampel positif untuk HPV; 27 dengan HR-HC2 dan 28 dengan nested-PCR. Yang terakhir ini lebih sensitif untuk mengesan HPV; dengan sensitiviti 72.7% dibandingkan dengan sensitiviti HR-HC2; 36.4%, sedangkan spesifisiti adalah sama, 96.8% dan 96.3%. Pengesanan HPV meningkat apabila diagnosis sitologi dari Pap smear ThinPrep® (TP) digunakan sebagai standard. Genotip dari 21/28 (75.0%) nested-PCR HPV positif sampel adalah; HPV-16 (57.1%), HPV-58 (19.0%), HPV-6 (9.5%) dan HPV-18, -33 dan -61 (4.8%).

Kehadiran HPV-DNA dikesan di semua lesion termasuk 'Dalam Batas Normal' (5.4%) dan 'Tidak memuaskan untuk Evaluasi' didiagnosis (11.7%) oleh CP dan 5.1% dan 10.8% masing-masing oleh TP. HPV dikesan dalam semua kes didiagnosis sitologi sebagai HGSIL (1 kes) dan kanser (3 kes). Untuk LGSIL didiagnosis oleh CP, 50.0% mempunyai HPV dan 57.1% di dalam TP. Kajian ini menunjukkan ujian HPV lebih sensitif berbanding sitologi untuk mengesan HPV.

99 pasangan laki-laki memberikan kikisan uretra secara sukarela untuk ujian HPV. 10 sampel mempunyai tahap DNA yang mencukupi untuk analisis, tidak ada yang positif untuk HPV.

Kaji selidik analisis tidak menunjukkan sebarang faktor risiko yang signifikan dalam semua subjek.

ABSTRACT

Detection of HPV-DNA in Cervical and Urethral Scrapings using Hybrid Capture 2 and nested-PCR and Their Correlation with Cytomorphology

Human papillomavirus (HPV) infection is a causative agent for development of cervical cancer. This study aims to detect the presence of HPV in routine pap smears of women who attended Hospital Kota Bharu, Hospital Universiti Sains Malaysia and Hospital Kuala Terengganu. The cervical sample from each woman was in two forms (split sampling); conventional smear (CP) and ThinPrep® smear (TP). These women were given a questionnaire to determine the risk factors for cervical cancer development. The male spouses were invited to provide urethral scrapings for HPV studies. The cervical and urethral samples were subjected to Hybrid Capture 2 and nested-PCR. HPV results of the cervical scrapings were correlated with cytological diagnosis from CP and TP.

A total of 705 cervical scrapings and 99 male urethral scrapings were collected. 702 cervical samples were available for both CP and TP cytological evaluation. By CP, 92.7% of the collected cervical scrapings had normal smear, 2.0% had abnormal smear and 5.3% smears were unsatisfactory for evaluation. By TP, 87.1% was normal, 1.7% was abnormal and 11.1% was unsatisfactory for evaluation. The diagnoses of the conventional Pap smears and ThinPrep® Pap smears were 88.9% in agreement. The adequacy of sampling by TP (88.9%) is comparable to CP (94.7%).

635 cervical samples were sufficient for HPV testing. 44 (6.9%) samples were positive for HPV; 27 by HR-HC2 and 28 by nested-PCR. The latter is more sensitive to detect HPV; 72.7% of sensitivity compared to HR-HC2; 36.4% sensitivity while the specificity is fairly similar; 96.8% and 96.3%. HPV detection improved when cytological diagnosis from ThinPrep® smear (TP) was used as gold standard. The genotypes of the 21/28 (75.0%) nested-PCR HPV positive samples were; HPV-16 (57.1%), HPV-58 (19.0%), HPV-6 (9.5%) and HPV-18, -33 and -61 (4.8%).

HPV-DNA was seen in all lesions including 'Within Normal Limit' (5.4%) and 'Unsatisfactory for Evaluation' (11.7%) diagnosed by CP and 5.1% and 10.8% respectively by TP. HPV was detected in all cases diagnosed cytologically as HGSIL (1 case) and cancer (3 cases). For LGSIL diagnosed by CP, 50.0% had HPV and by TP 57.1%. This study shows HPV testing is more sensitive than cytology in detecting HPVs.

99 male spouses volunteered the urethral scrapings for HPV testing. 10 samples had adequate level of DNA for analysis, none was found to be HPV positive.

Questionnaire survey analysis did not show any significant risk factor in all subjects.

CHAPTER ONE

INTRODUCTION AND REVIEW OF LITERATURE

1.0 Introduction

Cervix is an organ of the female reproductive system. It is located at the lower part of the uterus (womb) and is lined by a protective stratified squamous epithelium. Endocervix is part of the cervix that is closest to the body of uterus whereas ectocervix is part of the cervix next to the vagina. Cervical cancer commonly starts at where these 2 parts meet and occurs when the normal cells keep dividing. These cells also infiltrate and proliferate to become malignant abnormal cells. They reproduce in the epithelium and spread to adjacent stromal tissues and supporting muscles in the cervix. Cervical cancer does not form suddenly but gradually develop from a precancerous stage that turn into cancer at a later time.

Several terms had been used to describe the precancerous changes that occur in the cervix. These include cervical intraepithelial neoplasia (CIN), squamous intraepithelial lesion (SIL) and dysplasia. The Bethesda system has been introduced in order to uniform the nomenclature for abnormal cytology report. There are two types of cervical cancer, namely squamous cell carcinoma (SCC) and adenocarcinoma (ADC). SCC is the most common and develops from the squamous metaplastic cells on the surface of the cervix. ADC is uncommon and they develop from the glands in the endocervical canal. Cervical cancer is nearly always fatal if it is not detected and treated early.

It is commonly accepted that the development of almost all cervical cancers are associated with human papillomavirus (HPV) (Malik, 2005, Steenbergen *et al.*, 2005). Their association was first postulated by Harald zur Hausen in 1970s. It was suggested that HPV-16 and HPV-18 could play a role in cervical cancer based on specimens that these authors were studying (zur Hausen, 2002). HPV is a sexually transmitted infection (STI) that is very common among young men and women in many parts of the world. It is asymptomatic in nature and had a long incubation period. They become highly prevalent and infection could be easily and unknowingly transmitted (Tovar *et al.*, 2008). Women are at an increased risk of developing severe dysplasia and cervical cancer if infected with certain oncogenic type of the virus. The most common (and benign) symptomatic presentation of an HPV infection is genital warts (*Condyloma acuminatum*) – a wart that occurs on the genitalia. Other visible HPV infections are the plantar warts (*Verruca pedis*) – usually only found on pressure points on the soles of the feet and the common warts (*Verruca vulgaris*) – most common on hands but can grow anywhere on the body. All are curable, but evidence suggests that the virus persists in the tissue for a long period of time and can develop into cancer (Schiffman and Castle, 2003).

The incidence and mortality rates of cervical cancer have been drastically reduced since the introduction of cervical cancer screening programs. Over the past 5 decades, cervical cytology becomes the major screening program worldwide. The conventional Papanicolaou test and the newer liquid-based cytology (LBC) techniques had allowed an early detection of cervical abnormalities prior to the development of invasive cervical cancer.

HPV testing has now been incorporated (as primary or secondary test) into screening programs that previously relied only on cytology (Burd, 2003). Some HPV tests such as Hybrid Capture and a variety of Polymerase Chain Reaction protocols (using consensus or specific primer) have been implemented in developed countries. Both assays are suitable for high-throughput testing and automated execution of the result. The identification of specific HPV genotypes can be achieved (at high degree of resolution) by a variety of methods, which may be more or less comprehensive in their number of detectable HPV types, including Southern and Northern blot, dot blot or DNA sequencing (Brestovac et al., 2005).

HPV tests allowed an early identification of populations at different risk levels for this neoplasia since HPV infection is a "necessary cause" of cervical cancer (de Lang and Wilander, 2005, Fontaine *et al.*, 2007). It is also more sensitive for the detection of high-risk group of HPV in cervical lesions than cytology (De Francesco *et al.*, 2008). Furthermore, it shows encouraging results when used in conjunction with cytological analysis in women 30 years of age or older. It is also recommended for most women with equivocal findings on cervical cytological analysis (atypical squamous cells of undetermined significance, or ASCUS) (Schiffman and Solomon, 2003).

There are some factors that had been identified (based on experience in developed countries) which can significantly reduce the number of new cases of cervical cancer and the mortality rate associated with it. It has been shown that high coverage, well planned and organized screening programs, general awareness about cervical cancer and the improvement of existing health care services can reduce the

burden of cervical cancer for women and for the health care system. As detailed by Cuzick *et al.* (2008), cost, performance (accuracy and reproducibility), coverage and acceptability, infrastructural requirements, and complexity of technology and implementation (i.e., number of visits required to effect treatment) are very important factors in reducing the incidence of disease.

The introduction of currently licensed HPV vaccines - Gardasil® (Merck & Co., Inc., Whitehouse Station, NJ USA); quadrivalent (HPV types 6, 11, 16, and 18) and Cervarix™ (GlaxoSmithKline Biologicals, Rixensart, Belgium); bivalent (HPV types 16, and 18) may prevent the transmission of this virus (Zonfrillo and Hackley, 2008, Ronco *et al.*, 2006). However, it does not replace routine cervical cancer screening because vaccines do not protect against all HPV types. Furthermore, it cannot be assured that the women are automatically protected against the virus. They are also not cost effective for developing countries (Zimet *et al.*, 2008). The probability of getting HPV infection remains high and population are prone to develop cervical cancer. The secondary prevention by screening and treatment will continue to be crucially important in cervical cancer prevention program especially through the detection, treatment and follow-up of its precursors (Denny, 2005).

1.0.1 Justification of the study

This study is expected to provide information on the cervical cancer screening program that compares conventional cytology and liquid-based cytology using split-sampling technique and HPV testing. Liquid-based cytology had been used worldwide as a replacement of conventional Pap smear in order improve quality of smear and getting better cytomorphological diagnosis.

The association of HPV and development of cervical intraepithelial changes had been widely studied. HPV testing had been incorporated in cervical cancer screening as adjunctive to Pap smear or standalone test in developed countries. Due to this reason, this study is expected to give the statistically significant difference between HPV testing and cytology result. HPV testing either using HC2 or PCR is expected to sensitively detect HPV infection in women. Thus, HPV testing should also be included as early preventive method of cervical cancer instead of cytology in Malaysia.

Besides, limited data on epidemiological distribution of HPV infection among Malaysian women should get an attention. Furthermore, no study regarding HPV infection in men had been reported. The present results are expected to provide a database of HPV infection distribution in the study area. Early detection of HPV makes it possible to avoid development of pre-cancerous stage and invasive cancer in the future.

1.0.2 Rationale of doing the study

Study on HPV infection in Malaysia is very limited. The use of HC2 (the only FDA approved HPV test) in detecting HPV infection in Malaysia still not widely practised. There are only 6 HC2 DML units installed in Malaysia; 2 are for research purposes only (in HUSM and HUKM) while another 4 are in the private sector (all located at Federal territory and Selangor).

So far, HPV testing has not yet been provided at Malaysian government hospitals. Very few clinicians know and are aware about HPV testing and their role in helping early detection of the development of cervical cancer. There is no record of studies conducted in Malaysia describing the significant usage of HPV testing and cytology (current screening tool of cervical cancer). Therefore a robust study needs to be done to determine the performances of these HPV tests and to determine the differences between it and cytological diagnosis. Furthermore, data epidemiological distribution of HPV infection is needed in order to implement the usage of HPV vaccine among Malaysian in the future.

1.1 Cervical Cancer Burden

Cervical cancer is the most common cause of cancer death among women in developing countries and remains second only to breast cancer worldwide (Figure 1.1). An estimated 2329.08 million women ages 15 years and older are at risk of developing cervical cancer with 493,243 new cases being diagnosed with invasive cervical cancer worldwide each year, 80% of which were from developing countries (Castlellsagué *et al.*, 2007). It is responsible for over 250,000 deaths representing nearly 10% of all cancers in women in 2005 (WHO, 2006). Because the disease progresses over many years, it is estimated that up to 7 million women worldwide may have precancerous conditions that need to be identified and treated (Baseman and Koutsky, 2005).

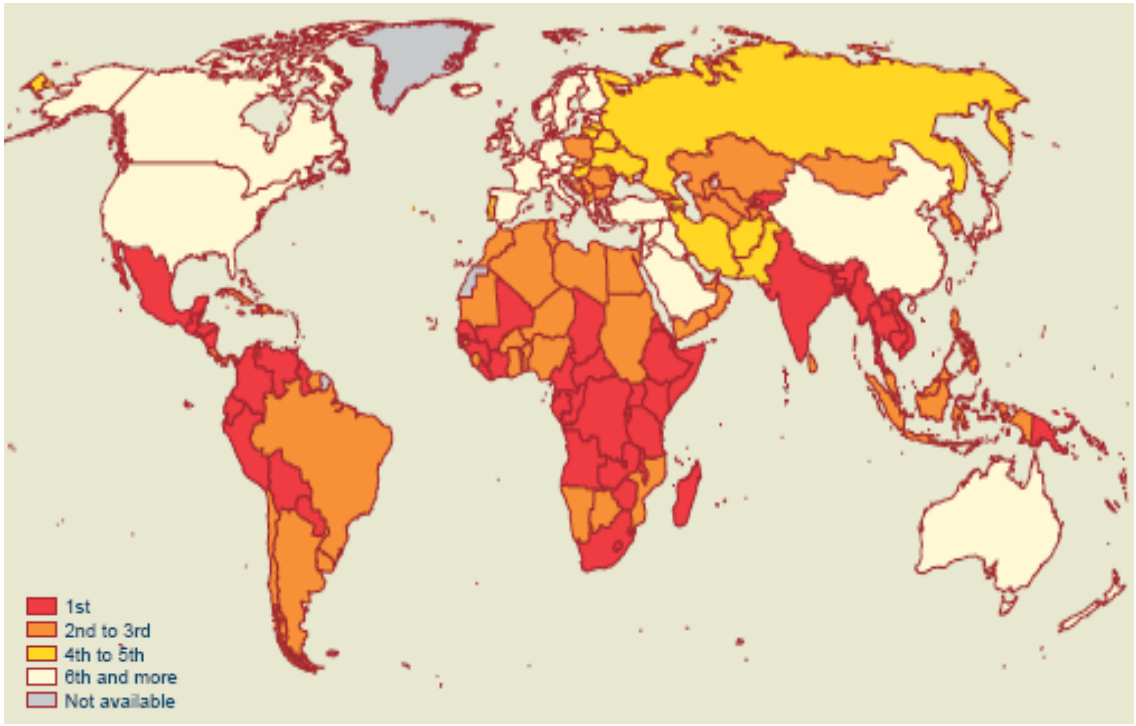

Figure 1.1 Ranking of incidence of cervical cancer in comparison to other cancers in women with all ages by country.
Adapted from Castlellsagué *et al.* (2007).
Key represent ranking of incidence.

1.1.1 Incidence and mortality rates

Mortality rates are substantially lower than incidence rates. Worldwide, the age-standardized incidence rates (ASR) for cervical cancer is 16.2 per 100,000 and the mortality rate is 9.0 per 100,000 populations (Parkin *et al.*, 2008). In South-Eastern Asia (the region where Malaysia belongs to), incidence and mortality rates are 18.7 and 10.2, respectively. According to the National Cancer Registry (NCR) report (2003), the ASR cancer incidence and mortality rates in Malaysia for the year 2002 were 15.7 and 8.4 per 100,000 populations, respectively. It occurrence slightly increases in the following year (2003) which represents ASR cancer incidence and mortality rate of 19.2 and 15.9 per 100,000 populations (NCR, 2004). Table 1.1 and 1.2 shows the estimated incidence and mortality rates of cervical cancer in Malaysia, South-Eastern Asia and the world (WHO, 2007).

Malaysia has a population of 8.49 million women aged 15 years and older who are at risk of developing cervical cancer (Castlellsagué *et al.*, 2007). Current data indicated that 1,492 women were diagnosed with cervical cancer and 766 die from the disease every year (WHO, 2007). Its remains the second most frequently reported cancers among women after breast cancer, where it stands at 12.0% of total cancer cases recorded in 2002 (NCR, 2003) and 12.9% the following year (NCR, 2004). As reported by Malaysian Medical Association (MMA) in 2002, nearly 10.5% of death among women in government hospitals is due to cancer of the cervix.

Table 1.1 Incidence of cervical cancer in Malaysia, South-Eastern Asia and the World.

Indicator	Malaysia	South-Eastern Asia	World
Crude incidence rate	13.1	15.9	16
Age-standardized incidence rate	15.7	18.7	16.2
Cumulative risk (%)	1.2	1.5	1.3
Age period 0-64 years			
Standardized incidence ratio (SIR)	95	112	100
Annual number of new cancer cases	1,492	42,538	493,243

Rates are per 100,000 women.
Standardized rates are estimated using the direct method and the World population as the reference.

Table 1.2 Mortality of cervical cancer in Malaysia, South-Eastern Asia and the World.

Indicator	Malaysia	South-Eastern Asia	World
Crude mortality rate	6.8	8.4	8.9
Age-standardized mortality rate	8.4	10.2	9
Cumulative risk (%)	0.6	0.8	0.7
Age period 0-64 years			
Standardized mortality ratio (SMR)	92	112	100
Annual number of deaths	766	22,594	273,505

Rates are per 100,000 women.
Standardized rates are estimated using the direct method and the World population as the reference.

The incidence and mortality rates vary between different age groups. The schematic diagram for the age-specific incidence and mortality rates of cervical cancer in Malaysia as compared to estimates for South-Eastern Asia and the World are shown in Figure 1.2 and 1.3 (WHO, 2007). The graph showed that the incidence and mortality rates of women in Malaysia increased at age above 55, contrary to the rates in South-Eastern Asia and the World. The reasons for this difference may due to the late screening for the cervical cancer or ineffective screening program among Malaysian women.

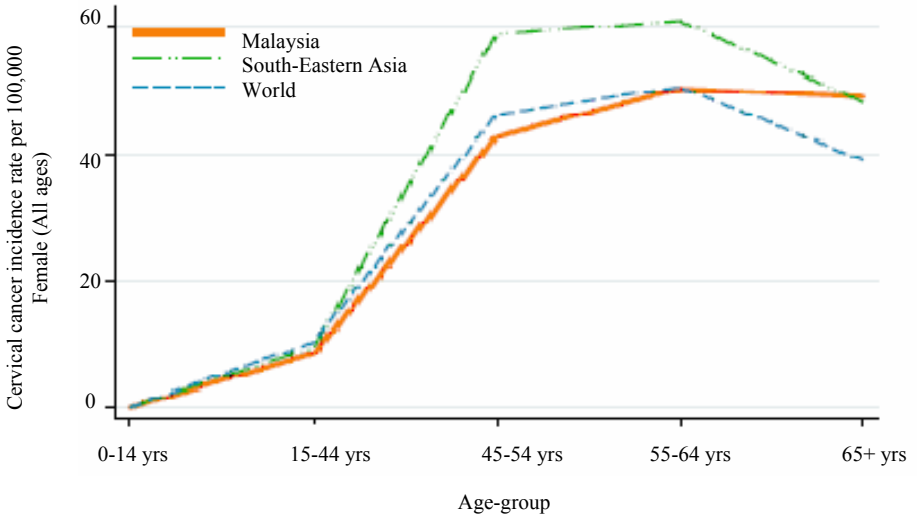

Figure 1.2 Age-specific incidence rates of cervical cancer in Malaysia as compared to estimates for South-Eastern Asia and the World.

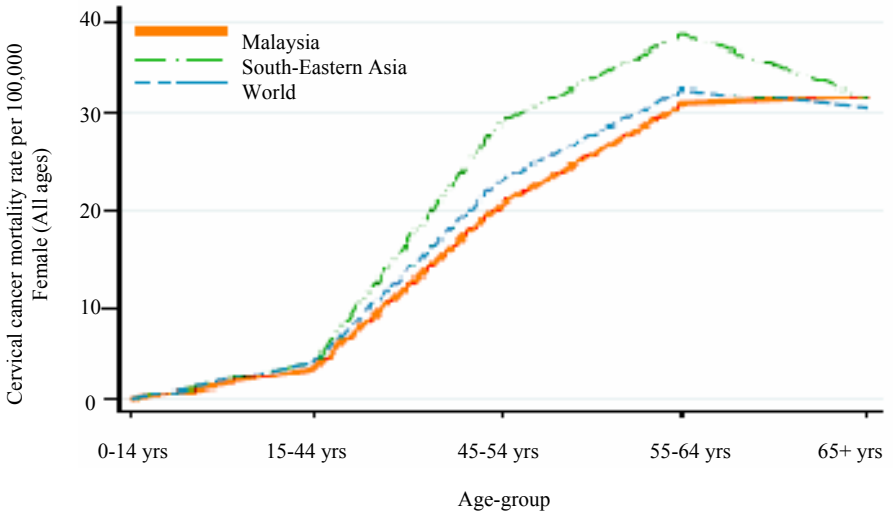

Figure 1.3 Age-specific mortality rates of cervical cancer in Malaysia as compared to estimates for South-Eastern Asia and the World.

There are some variations of ASR between the major ethnics groups in Malaysia. As reported in the first (2003) and second (2004) report of NCR, for the year 2002, Chinese women have the highest ASR with 33.6 per 100,000, followed by Indian women with 27.7 per 100,000 population and Malay women with 12.6 per 100,000. In 2003, Chinese women still lead with ASR of 28.8 per 100,000, followed by Indians and Malay women with 22.4 and 10.5 per 100,000 women respectively.

In term of economical status, there is a big difference in the incidence and mortality rates of cervical cancer between developed and developing countries. According to Parkin and Bray (2006), the hardest-hit regions are among the worlds poorest. Central and South America, the Caribbean, sub-Saharan Africa, and parts of Oceania and Asia have the highest incidence rates of over 30 per 100,000 women. These rates are lower compared with the figures for North America and Europe which is no more than 10 per 100,000 women. In 2008, Parkin and coworkers reported that the highest estimated ASR incidence rates (per 100,000 populations) for the year 2002 were observed in the Melanesian populations. These countries include Solomon Islands (42.8), Papua New Guinea (40.4) and Fiji (33.4). Lowest ASR incidence rates were observed in China (6.8), Australia (6.9) and Japan (8.0).

1.1.2 HPV Burden in Women

HPV prevalence among countries may vary considerably across studies. This is due to the insufficient degree of geographical coverage and sample size, diversity in the techniques used for HPV detection and variety methods used for estimating HPV type-specific prevalence (Giuliano *et al.*, 2008, Bosch *et al.*, 2008). Worldwide and regional estimates of HPV type-specific prevalence in women with and without cervical lesions have been estimated from highly standardised multicentric studies. For example, International Agency for Research on Cancer (IARC) of cervical cancer series (Franceschi, 2005), IARC HPV prevalence surveys (Maucort-Boulch *et al.*, 2008) as well as from the wider meta-analyses of all published data (Bae *et al.*, 2008, Bao *et al.*, 2008a;b, Bhatla *et al.*, 2008).

The adjusted global prevalence of HPV infection was 10.41% based on a meta-analysis of 78 studies of women with normal cytology around the world (Burchell *et al.*, 2006). In the same year, Clifford and co-workers reported that the overall HPV-DNA prevalence in invasive cervical cancer was 96%, based on pooled analysis of 12 studies conducted in 25 countries (3,085 cases). The prevalence of HPV types that cause cervical cancer; HPV-16 (53.5%), -18 (17.2%), -45 (6.7%), -31 (2.9%), -33 (2.6%), and other high-risk types; -52, -58, -35, -59, -56, -39, -51, -73, -68 and -66 (15-20%) (Clifford *et al.*, 2006).

HPV prevalence in women with different cytological diagnosis in Malaysia, South-Eastern Asia and the world are shown in Table 1.3 (WHO, 2007). Complete data is not yet available on the HPV burden in the general population of Malaysia. However, according to the South-Eastern Asia HPV prevalence estimate, about 6.2% of women in the general population are estimated to harbour cervical HPV infection at any given time (Castlellsagué *et al.*, 2007).

Table 1.3 Burden of HPV in women with and without cervical disease.

	Malaysia		South-Eastern Asia		World	
	No. tested	HPV prev.[a] % (95% CI[b])	No. tested	HPV prev.[a] % (95% CI[b])	No. tested	HPV prev.[a] % (95% CI[b])
Normal	-	-	4,194	6.2 (5.5-6.9)	157,879	10.0 (9.8-10.1)
LGSIL	-	-	27	33.3 (16.5-54.0)	8,640	71.6 (70.6-72.5)
HGSIL	-	-	207	61.8 (54.8-68.5)	7,094	84.9 (84.1-85.7)
ICC	23	95.7 (78.1-99.9)	1,090	92.1 (90.3-93.6)	14,595	87.2 (86.7-87.8)

The samples for HPV testing come from cervical specimens (fresh / fixed biopsies or exfoliated cells).
[a]Prev. = prevalence; [b]CI = confidence interval

Domingo and coworkers (2008) reported that, HPV-16 and -18 are the two most common HPV types in Malaysia (excluding East Malaysia), which contribute 73.9% and 65.2% of total HPV prevalence, respectively. HPV-31 contributes 13% followed by HPV-33, 4.3% in a total of 23 cases of cervical cancer.

1.2 HPV and Cervical Cancer

HPV infection is now a well-establish cause for the development of a variety of epithelial lesions, which range in severity from benign warts to invasive cervical cancer (Tristram and Fiander, 2007). More than 90% of invasive cervical cancer specimens contain HPV DNA, however, approximately 5% of cervical carcinomas may be unrelated to HPV infection (Janicek and Averette, 2001, Bhatla *et al.*, 2008).

Persistent infection with one of 15 high-risk HPV types is considered a necessary cause of cervical cancer and are found in the vast majority of cervical and anogenital cancers, including vaginal, vulvar, penile and anal cancers (Winters *et al.*, 2006, Wu *et al.*, 2006). Nearly all occurrences of cervical cancer are linked to HPV-16 and -18 (Gilson and Mindel, 2001). HPV-16 is the most prevalence of all of the high-risk types and it is detectable in more than 50% of all cervical cancer cases (Peitsaro *et al.*, 2002, Middleton *et al.*, 2003, Harper, 2004). On the other hand, according to Remmerbach *et al.*, (2004) HPV-18 is found in almost 15% of the total cases of cervical cancer. Almost 10% of women with HPV-16 infection and 5% with HPV-18 infection developed CIN 3 within 36 month. According to Khan and coworkers (2005), women who were HPV-DNA negative at enrolment developed a 1% cumulative incidence for CIN 3 or cervical cancer at 3 years of follow up. As continuation, they said at 10 years follow up, 17% of the women who were infected with HPV-16 at enrolment and 14% of those infected with HPV-18 at enrolment had developed CIN 3 or higher (Khan *et al.*, 2005).

HPV-16 and HPV-18 account for 70% of cervical cancer cases, 41–67% of high-grade squamous intraepithelial lesion (HGSIL), 16–32% of low-grade squamous intraepithelial lesion (LGSIL) and 6–27% of atypical squamous cells of undetermined significance (ASCUS), based on the meta-analysis study worldwide. These proportions are broadly similar for all global regions, and highlight the increasing importance of HPV-16/-18 with increasing lesion severity (Parkin *et al.*, 2008).

HPV can produce infection that may be latent and progressive to malignancy. It means once cervical cells are infected, abnormalities can progress or regress along a spectrum of disease. Through microabrasions, virus is introduced into the basal layer of the stratified squamous epithelium. It takes 1 to 10 years for HPV to change normal cell into becoming severe dysplasia and invasive carcinoma through viral proliferation, genomic integration, and oncogenic transformation. Although the majority of HPV infections regress following initial infection, persistent HPV infection remains a serious concern because it may lead to cervical cancer (Baseman and Koutsky, 2005). Expression of viral non-structural proteins stimulates cellular proliferation, resulting in latent and subclinical infection, condyloma, or dysplasia. The cytological hallmark of HPV infection is when infected cells display koilocytosis. The characteristics of koilocytosis is when ‘halo’ cells appear, nuclei enlarged, irregular chromatin, perinuclear clearing, and a cytoplasmic border that varies from thick to thin. Each koilocyte contained approximately 50-100 virions (Paavonen, 2007). The schematic of the progression from HPV infection to invasive cancer are shown in Figure 1.4.

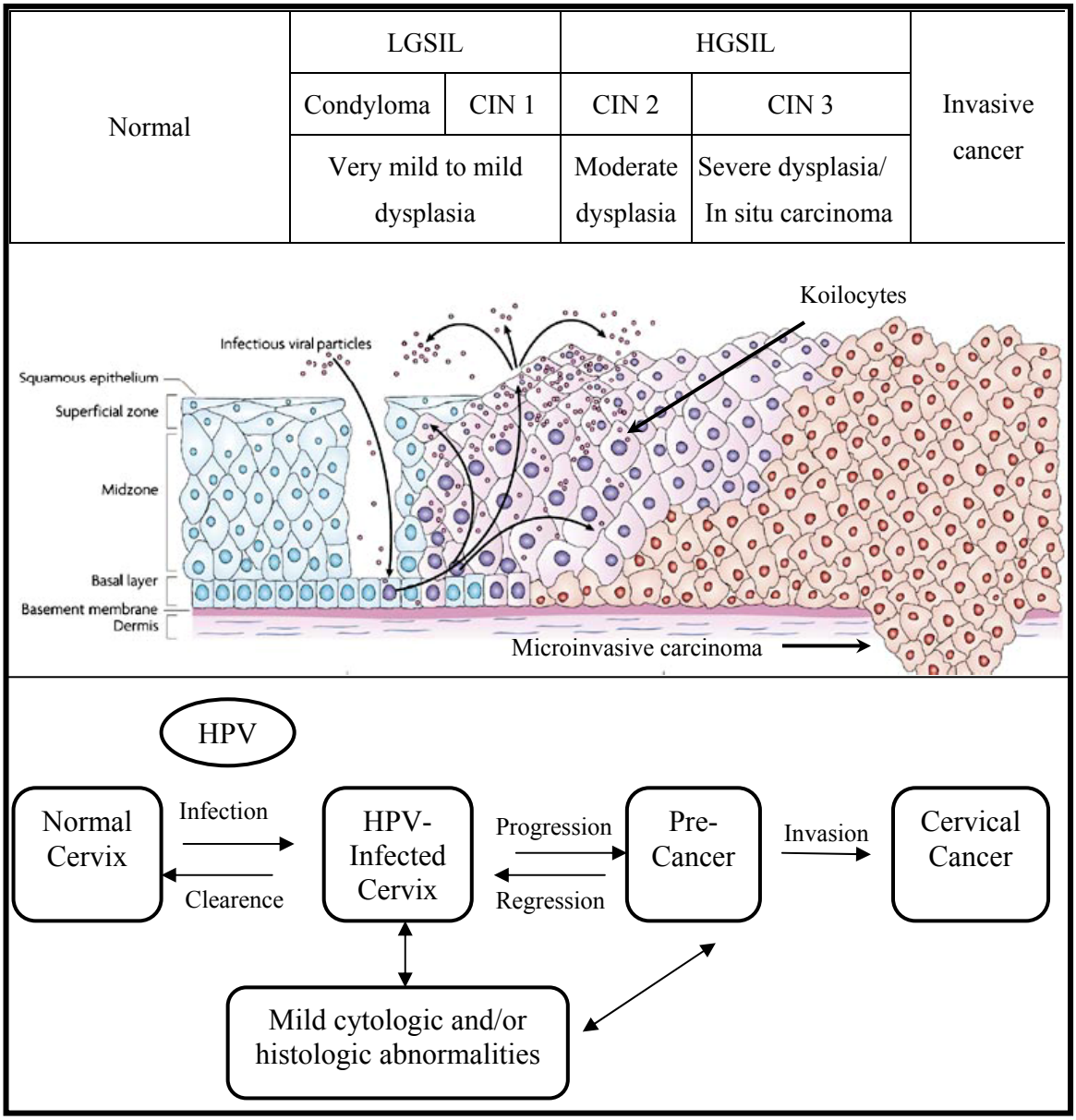

Figure 1.4 Schematic of the progression from HPV infection to invasive cancer. Modified from Park (2008), Moscicki *et al.* (2006) and Palefsky (2001).

**HPV, Human papillomavirus; LGSIL, Low-Grade Squamous Intraepithelial Lesion; HGSIL, High-Grade Squamous Intraepithelial Lesion; and CIN, Cervical Intraepithelial Neoplasia.*

1.2.1 HPV Genome

HPV belongs to the Papillomaviridae family, a relatively small with 55 nm in diameter, non-enveloped virus with circular double stranded DNA of approximately 8 kb in size (Tristram and Fiander, 2007). The genome can be divided into three regions: an upstream regulatory region (URR), an early (E) and a late (L) region (Villa, 2006). HPV has eight genes, known as open reading frames (ORFs), which encode for functional viral proteins (Figure 1.5). The upstream regulatory region (URR) contains the p97 core promoter along with enhancer and silencer sequences. It regulates DNA replication by controlling the transcription of the ORFs and contains the highest degree of variation in the viral genome (Braaten and Laufer, 2008).

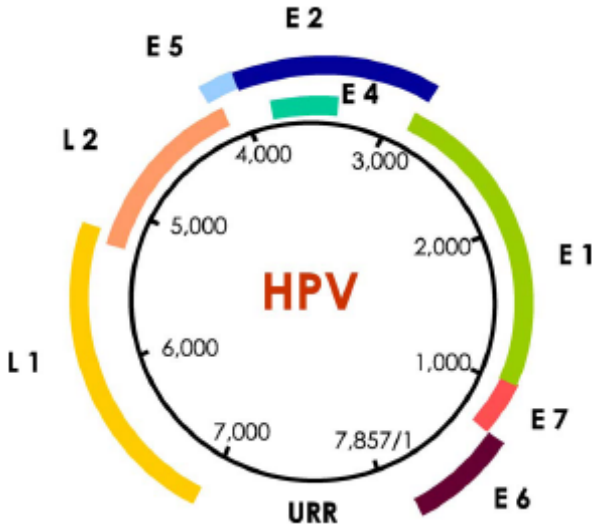

Figure 1.5 Schematic presentation of the HPV genome showing the arrangement of the early protein (E1-E7), late proteins (L1-L2) and the upstream regulatory region (URR).
Adapted from Munoz et al., (2006).

1.2.1.1 Function of viral proteins

The viral proteins are named after their encoding open reading frame (ORF)s; DNA sequence that does not contain a stop codon in a given reading frame. For over the past decades, the L1 ORF has been used for genotyping and identification of new HPV genotypes because it contained the most conserved gene within the HPV genome. A summary of the key functions of each protein is given in Table 1.4 (Burd, 2003). The early (E) region codes for regulatory functions engaged in genome persistence, DNA replication and activation of the lytic life cycle (Burd, 2003). E2 is a transcriptional repressor of E6 and E7. The loss of this ORF during viral integration in anogenital neoplasia results in an upregulation of E6 and E7 expression. The two viral capsid proteins L1 and L2 are encoded by ORFs within the late region (Vetter and Geller, 2007).

Table 1.4 Summary of the key function of proteins encoded by HPV.

ORFs	Protein function
L1	Major viral capsid protein; viral conformation eg. Viral-like particles (VLPs)
L2	Minor viral capsid protein; viral genome encapsidation
E1	Viral replication; maintain episome
E2	Regulates viral transcription and replication
E4	Interacts with cytoskeletal proteins
E5	Down regulation of MHC class 1 molecules
E6	Oncoproteins; binds to tumour suppressor protein p53
E7	Oncoproteins; binds to tumour suppressor protein retinoblastoma (Rb)

1.2.1.2 Types

HPV was first recognized as the cause of cutaneous warts (plantar warts, common warts, and flat warts) and it depends on the type of HPV infection (Burd, 2003). One hundred and eighteen types of HPV have been recognized based on nucleotide sequence homology (Burd, 2007). More than 40 HPV-types have been isolated from genital lesions (Tristram and Fiander, 2007). Munoz *et al.,* (2003) had classified genital HPV into 4 groups based on their association with cervical cancer and precursor lesions from multiple countries; 15 HPV types have been classified as high-risk (HR) for development of cervical cancer, 3 have been classified as probable high-risk (PHR), 12 have been classified as low risk (LR) and 3 are considered to have undetermined risk (UR) (Table 1.5).

Table 1.5 Classification of HPV types by cervical oncogenecity.

Risk classification	HPV types
High-risk	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82
Probable high-risk	26, 53, 66
Low risk	6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, CP6108
Undetermined risk	34, 57, 83

Multiple HPV types can be found co-existing within the same lesion and may be though as multiple infections. Types 6 and 11 are the most common LR types found in approximately 90% of warts (Bernard, 2005). High-risk (HR) HPV types are associated with anogenital neoplasia and have been reported in 99.7% of cervical cancers worldwide (Tristram and Fiander, 2007).

1.2.2 Molecular basis of oncogenesis of HPV-induced cancer

HPV contributes to neoplastic progression predominantly through the action of two viral oncoproteins; E6 and E7 under the regulation of the E2 gene product (Wise-Draper and Wells, 2008). These two oncoproteins have been shown to posses transforming ability when transfected into cell lines (Smotkin and Wettstein, 1987). The mechanism by which some viral infection remain low risk and some are high risk for developing high grade CIN or invasive cancer are different. In HPV infected lesions, the low risk viral DNA remains structurally as episomes. In the invasive cancers, the viral genome is usually found integrated into chromatin material of the host nucleus (Munoz *et al.*, 2003). In brief, many of current research shows that HPV interacts with host proteins to deregulate the cell cycle (Cordano *et al.*, 2008).

High-risk HPV E6 and E7 products interfere with critical cell cycle pathways which are governed by the tumour suppressor proteins (p53) and retinoblastoma protein (pRB), resulting in the accumulation of DNA damage and the development of cervical cancer (Kaufmann *et al.*, 2002).

The HPV E6 gene product binds to p53 tumor suppressor gene and induces p53 degradation via a cellular enzyme ubiquitin ligase. As a consequence, the normal activities of p53 which govern G1 process, apoptosis, and DNA repair can be suppressed. LR-HPV E6 proteins do not bind to p53 at detectable levels and have no effect on p53 stability *in vitro* (Burd, 2003).

The HPV E7 gene product binds to the retinoblastoma gene product (pRB) and altering its phosphorylation state and inactivates the protein. This binding disrupts the complex between pRB and the cellular transcription factor E2F-1, resulting in the liberation of E2F-1. Specifically, pRB normally binds with the transcription factor E2F, which functions in cell cycle progression from G1 to the S phase following interaction with cyclin and cyclin-dependent kinases. The E7 gene product can also associate with other mitotically interactive cellular proteins such as cyclin E resulting in stimulation of cellular DNA synthesis and cell proliferation. The E7 protein from LR-HPV types binds pRB with decreased affinity (Janicek and Averette, 2001). The illustration of the oncogenesis of HPV E6 and E7 genes at cellular event and their mechanisms is shown in Figure 1.6.

The ability of binding and inactivation activity against p53 and pRB can also be found in gene products of other DNA tumor viruses such as SV40 (large T antigen; involved in viral genome replication and regulation of host cell cycle) and Adenovirus (E1A and E1B; independent transcription units in E1 region) (Janicek and Averette, 2001).

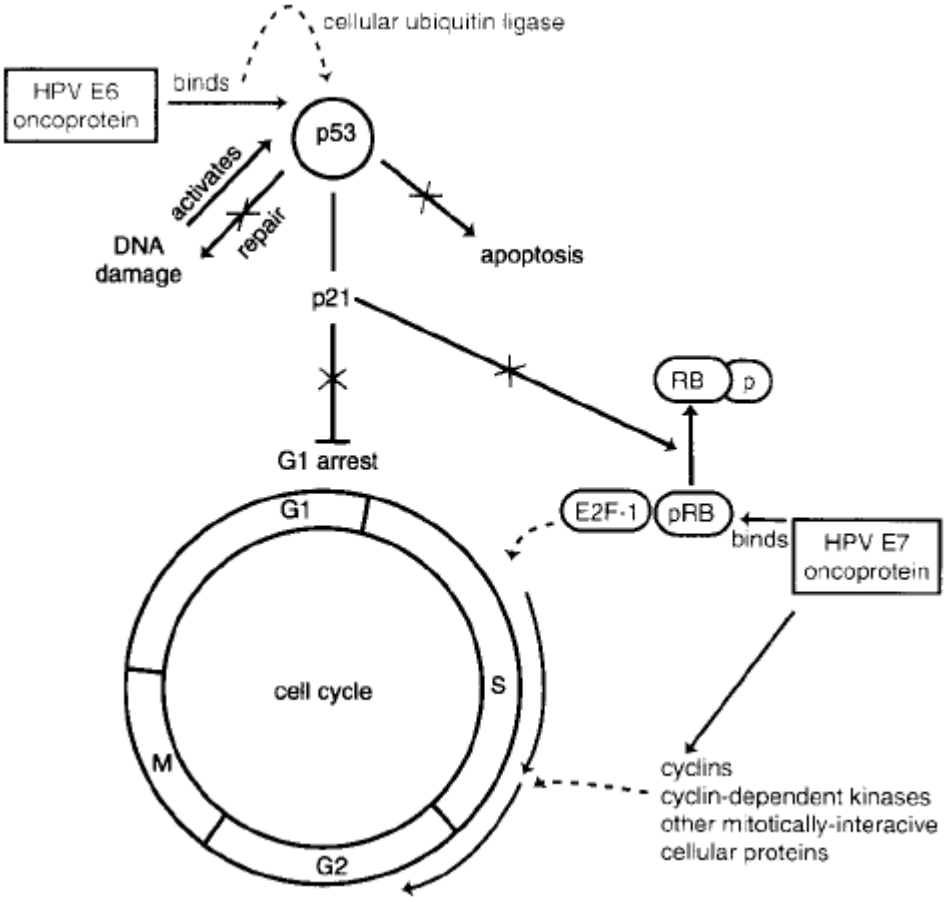

Figure 1.6 The illustration of the oncogenesis of HPV E6 and E7 genes at cellular event.
Adapted from Burd (2003).

1.2.3 Epidemiology of HPVs

Transmission of HPV occurs predominantly by sexual transmission (penetrative genital or anal contact). HPV is very resistant to heat, desiccation and nonsexual transmission via fomites can also occur, such as by prolonged exposure to shared contaminated clothing (Baseman and Koutsky, 2005). However, there is little or no evidence to suggest that HPV can be transmitted by nonsexual routes.

An individual is at a greater risk of becoming infected with HPV if he or she has had multiple sexual partners at any time or is the partner of someone who has had multiple sexual partners (Schiffman and Castle, 2003, Burd, 2003).

HPV infection is most common in sexually active young women, 18 to 30 years of age (Syrjanen *et al.*, 2008a, de Sanjose *et al.*, 2008). There is a sharp decrease in prevalence after 30 years of age. However, cervical cancer is more common in women older than 35 years, suggesting infection at a younger age and slow progression to cancer (Domingo *et al.*, 2008). Meta-analysis of data on cervical infection demonstrated an overall prevalence of 10-12% in adult women; highest in younger women (<30 years) (Franceschi, 2005, Syrjanen, 2008, Syrjanen *et al.*, 2008b).

1.2.3.1 Risk factors

Two factors enhancing the oncogenic power of HPV have already been identified: smoking and oral contraceptives. It has been proven that women with oncogenic HPV and minimally abnormal Papanicolaou smears who smoke were up to three times more likely to be diagnosed with CIN 3 than nonsmokers (McIntyre-Seltman *et al.*, 2005). A recently published meta-analysis and a large cohort study showed independently that use of oral contraceptives (OC) leads to an increased relative risk (RR) of cervical cancer. The RR increased with duration of OC use and is 1.90 after 5 years or more (95% CI: 1.69-2.13) and decreased upon cessation of OC use and was normal again 10 years later. Longstanding OC use enhances human papillomavirus (HPV) transcription and decreases HPV clearance, resulting in more frequent persistence of HPV, an increase of cervical intraepithelial neoplasia, and an increased RR of cervical cancer (Schmeink *et al.*, 2008).

Other determinants of the progression of HPV infection to cervical cancer relate to a woman's immune status. Those with an immune system compromised as a result of malnutrition are also at increased risks from cervical dysplasia. Women in the lowest serum retinol quartile were at an increased risk of CIN I compared with women in the highest quartile (Yeo *et al.*, 2000). Risk of type-specific, persistent HPV infection was lower among women reporting intake values of vitamin C in the upper quartile compared with those reporting intake in the lowest quartile (Giuliano *et al.*, 2003). A statistically significantly lower level of alpha-tocopherol was also observed in the blood serum of HPV-positive patients with cervical intraepithelial neoplasia. The risk of dysplasia was four times higher for an alpha-tocopherol level

<7.95 mmol/l (Kwaśniewska *et al.*, 1997). Studies by Piyathilake *et al.*, (2004) have shown that CIN development can be prevented by improving folate status in subjects. Meanwhile, women who are co-infected with the human immunodeficiency virus (HIV) are at increased risk for malignant progression in the cervix due to the low CD4+ T cell counts in the body (Yamada *et al.*, 2008, Minkoff *et al.*, 2008).

Others factors such as hormonal influences (pregnancy) (Selleret and Mathevet, 2008), other STDs (e.g., *Chlamydia trachomatis*, HSV-2) (Sasagawa *et al.*, 2000, Hara *et al.*, 1997, Hawthorne *et al.*, 2005), or genetic predisposition and immunosuppressive chemotherapy (Venturoli *et al.*, 2008, Ab Hamid and Wastie, 2008), appear to be at an increased risk of HPV progression and can lead to cervical cancer. Some studies had related that high parity, race, alcohol consumption, circumcision, and low socioeconomic status as significant risk factors for cervical cancer (Safaeian *et al.*, 2008, Mariani *et al.*, 2008, Madsen *et al.*, 2008, Morris, 2007, Svare *et al.*, 2002). However, none of these have consistently been shown to be significant independent risk factors. The number of studies that follow women longitudinally is still insufficient to provide solid data on the relative importance of these risk factors. Persistence of infection is more common with the high-risk oncogenic HPV types and is an important determinant in the development of cervical cancer (Moscicki *et al.*, 2006).