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IMPROVED HUMAN PAPILLOMAVIRUS DNA TYPING METHODS AND BIOLOGY OF CERVICAL CANCER

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To my family

ABSTRACT

Cervical cancer is the second most common cancer among women worldwide. Persistent type-specific HPV has been identified as the main risk factor for cervical cancer development. The oncoproteins E6 and E7 of high-risk HPVs interact with host cellular proteins (biomarkers), disrupting cell cycle checkpoints and induce carcinogenesis. However, only a small proportion of HPV infected women will develop cervical cancer. Cofactors can contribute to carcinogenesis, such as high parity, smoking, other sexually transmitted infections (STIs), genetic predisposition and impaired immune response. Early diagnosis and treatment of cervical precancerous lesions can prevent disease progression. Widespread use of the Pap smear has decreased the incidence of cervical cancer by 70% in developed countries. HPV DNA testing may further improve the efficiency of screening and treatment for cervical neoplasia. In addition, biomarkers may be used as diagnostic markers in predicting disease progression. HPV vaccines, currently in development, may eventually prevent HPV infection and further decrease the incidence of cervical cancer.

Accurate detection and genotyping of HPV is important for the clinics. Using the multiple sequencing primers method, we were able to genotype a panel of 65 cervical and 17 oropharyngeal samples for the most clinically important HPV types (HPV 6, 11, 16, 18, 31, 33 and 45) with Pyrosequencing technology. By introducing sequence pattern recognition, we identified multiple co-infections of HPV. We also showed that this method is suitable for samples that generated non-specific amplification products or low amplification yield (**Paper I**).

Furthermore, we developed a rapid assay utilizing multiple sequencing primers for the twelve most common oncogenic HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59). This technique identifies a single base that acts as a sentinel for the particular HPV genotypes in the specimen. We genotyped 244 HPV positive cervical specimens amplified by nested PCR with primers MY09/11 and GP5+/6+. Approximately 25% of the samples demonstrated multiple co-infections of HPV. Thus this sentinel-base method is capable of rapidly genotyping samples containing single or multiple HPV co-infections (**Paper II**).

Other STIs may act as cofactors for cervical carcinogenesis. Co-infection of AAV and HPV can occur in the cervix. The AAV-2 Rep78 gene inhibits HPV-induced cell transformation *in vitro*. To investigate the role of AAV in cervical cancer development, we analyzed the presence of AAV-2 DNA in a retrospective, population-based nested case-control study. One hundred and four cases and an equal number of control individuals were included and they were matched by age and time of sampling. Our data showed an association between AAV and cervical cancer. A larger population-based study will be required to confirm this finding (**Paper III**).

Immunological factors play an important role in cervical cancer development. Two genetic variations in the chemokine receptors CCR2 and CCR5 were investigated for their association with HPV infection in cervical neoplasia. We analyzed 50 CIS or CIN cases and 50 healthy controls, and 100 cervical cancer and 100 control individuals. All

the cases and their corresponding controls were matched by age and date of sampling. We found that those with the Δ32/Δ32 genotype of the CCR5 gene have a risk of 4.58 (CI 0.40-52.64, p-value=0.045) for HPV infection, whereas the CCR2-64I polymorphism did not confer any increased risk for HPV infection. However, there is no evidence of association between these two chemokine receptor genes polymorphisms and the development of cervical neoplasia (**Paper IV**).

During carcinogenesis, oncoproteins E6 and E7 of high-risk HPVs interact with host cellular proteins, particularly through the $p16^{INK4A}$-cyclin D1-CDK4/6-pRb-E2F and $p14^{ARF}$-MDM2-p53 pathways. This results in host cell over-proliferation and genomic instability, further leading to tumorigenesis. We employed immunohistochemistry to investigate the expression of host cellular proteins $p16^{INK4A}$, $p14^{ARF}$, p53 and PCNA on paraffin sections of serial consecutive biopsies from cervical cancer patients. Our results suggested that increased $p16^{INK4A}$ and $p14^{ARF}$, and decreased or stable p53 expression, are associated with disease progression. The disease developed more rapidly among patients whose samples stained positive for $p16^{INK4A}$ or $p14^{ARF}$, and those with altered p53 expression. Thus, these biomarkers can act as prediction markers for cervical cancer progression (**Paper V**).

Key words: cervical cancer, human papillomavirus (HPV), Pyrosequencing, chemokine receptors, adeno-associated virus (AAV), biomarkers, cell cycle checkpoint.

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LIST OF PUBLICATIONS

This doctoral thesis is based on the following five publications, referred to in the text by their Roman numerals (I-V).

I. Gharizadeh B, Oggionni M, **Zheng BY**, Akom E, Pourmand N, Ahmadian A, Wallin KL, Nyrén P. Type-specific multiple sequencing primers: A novel strategy for reliable and rapid genotyping of human papillomaviruses by Pyrosequencing technology. J Mol Diagn. 2005 May; 7(2): 198-205.

II. Gharizadeh B*, **Zheng BY***, Akhras M, Ghaderi M, Jejelowo O, Strander B, Nyrén P, Wallin KL, and Pourmand N. Sentinel-base DNA genotyping using multiple sequencing primers for high-risk human papillomaviruses. Mol Cell Probes. 2006 Mar 1; [Epub ahead of print] (* Shared first authorship)

III. **Zheng BY***, Li XD*, Wiklund F, Chowdhry S, Ångström T, Hallmans G, Dillner J, Wallin KL. Detection of adeno-associated virus type 2 genome in cervical carcinoma. Br J Cancer. Accepted (* Shared first authorship).

IV. **Zheng BY,** Wiklund F, Gharizadeh B, Mehdi S, Gambelunghe G, Hallmans G, Dillner J, Wallin KL, Ghaderi M. Genetic polymorphism of chemokine receptors CCR2 and CCR5 in cervical neoplasia. Submitted.

V. Wang JL, **Zheng BY**, Li XD, Ångström T, Lindström MS, Wallin KL. Predictive significance of the alterations of p16INK4A, p14ARF, p53, and proliferating cell nuclear antigen expression in the progression of cervical cancer. Clin Cancer Res. 2004 Apr 1; 10(7): 2407-2414.

OTHER RELATED PAPERS

Zhang A, Wang J, **Zheng B**, Fang X, Ångström T, Liu C, Li X, Erlandsson F, Björkholm M, Nordenskjöld M, Gruber A, Wallin KL, Xu D. Telomere attrition predominantly occurs in precursor lesions during in vivo carcinogenic process of the uterine cervix. Oncogene. 2004 Sep 23; 23(44): 7441-7447.

Kaller M, Hultin E, **Zheng B**, Gharizadeh B, Wallin KL, Lundeberg J, Ahmadian A. Tag-array based HPV genotyping by competitive hybridization and extension. J Virol Methods. 2005 Nov; 129(2): 102-112.

Wang JL, **Zheng BY**, Li XD, Nokelainen K, Ångström T, Lindström MS, Wallin KL. p16INK4A and p14ARF expression pattern by immunohistochemistry in human papillomavirus-related cervical neoplasia. Mod Pathol. 2005 May; 18(5): 629-637.

Strander B, Ryd W, Wallin KL, Wärleby B, **Zheng BY**, Milsom I, Gharizadeh B, Nader P, Andersson-Ellström A. Does HPV-status after treatment of high grade dysplasia in the uterine cervix predict long time recurrence? Submitted.

CONTENTS

LIST OF ABBREVIATIONS

aa	amino acid
AAV	adeno-associated virus
ASC-US	atypical squamous cell of undetermined significance
ALTS	ASCUS/LSIL Triage Study
APC	antigen presenting cell
APS	adenosine phosphosulfate
ASO	allele-specific oligonucleotide
bp	base pair
CIS	carcinoma *in situ*
CIN	cervical intraepithelial neoplasia
CDK	cyclin-dependent kinase
DC	dendritic cell
DNA	deoxyribonucleic acid
HC	Hybrid Capture
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPV	human papillomavirus
HSIL	high-grade squamous intraepithelial lesion
HSV	herpes simplex virus
Ig	immunoglobulin
LCR	long control region
LSIL	low- grade squamous intraepithelial lesion
MCP	monocyte chemoattractant protein
MCM	minichromosome maintenance proteins
MIP	macrophage inflammatory protein
NK cell	natural killer cell
ORF	open reading frame
OC	oral contraceptive
Pap Smear	Papanicolaou smear
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PPi	pyrophosphate
RANTES	regulated on activation, normal T cell expressed and secreted
Rb	retinoblastoma
RFLP	restriction fragment length polymorphism
RLB	reverse line blotting
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
STI	sexually transmitted infection
SNP	single nucleotide polymorphism
SSCP	single strand conformation polymorphism
SCC	squamous cell carcinoma
SIL	squamous intraepithelial lesion
TSG	tumor suppressor gene
URR	upper regulatory region
VLP	viral-like particle

1 INTRODUCTION

Cervical cancer is the second most common cancer among women worldwide (Parkin et al., 2005), and persistent infection with type-specific human papillomavirus (HPV) has been identified as a main risk factor for its development (Bosch et al., 2002; Wallin et al., 1999; van der Graaf et al., 2002; zur Hausen, 1999b). The oncoproteins E6 and E7 of high-risk HPVs can bind to and degrade the tumor suppressor gene (TSG) products p53 and pRb that interact with the cell cycle checkpoint, particularly through the pathways $p16^{INK4A}$-cyclin D1-CDK4/6-pRb-E2F and $p14^{ARF}$-MDM2-p53. This will in turn contribute to tumorigenesis through host cell over-proliferation and genome instability. However, since only a subset of HPV infected patients will eventually develop cervical cancer, additional factors are needed for carcinogenesis. Several cofactors have been proposed such as high parity, smoking, long-term use of oral contraceptives, genetic predisposition, hormonal levels, and impaired immune response. These cofactors may influence the patient's ability to clear HPV infection thus contributing to cervical cancer development. Once cervical cancer has been diagnosed, clinical staging is important for prognosis. Accurate and early diagnosis as well as treatment of precancerous cervical lesions is important to prevent the disease progression. In developed countries, the widespread use of Papanicolaou (Pap) smear has dramatically reduced cervical cancer incidence and mortality. The application of HPV DNA testing on Pap smears may improve the outcome of screening programs. HPV-induced cellular proteins may be used as biomarkers for the prediction of disease progression. The ongoing development of vaccines against HPV infection represents a promising approach for the future prevention of cervical cancer.

1.1 CERVICAL CANCER

Cervical cancer is the cancer of uterine cervix. It is a common cause of death among middle–aged women (40-60 years old), with an estimated 493,000 new cases and 274,000 deaths worldwide in 2002. Today majority of the cases (83%) occur in developing countries, which is primarily related to the lack of intensive screening programs (Parkin et al., 2005).

1.1.1 Cervical cancer pathology

Most cervical tumors arise from the metaplastic area – a transformation zone where the squamous epithelium of the ectocervix meets with the columnar epithelium of the endocervix.

There are two main types of cervical cancers: squamous cell carcinoma and adenocarcinoma. About 80% of cervical cancers are squamous cell carcinomas (SCC), while the remaining 20% are adenocarcinomas and cervical cancers of other origins (DiSaia PJ, 1997).

1.1.2 Pathogenesis of cervical cancer

Cervical cancer development is a multi-step process. The major steps are HPV infection and HPV persistence for >1 year, followed by slow progression to precancerous lesions, and eventually to invasive cancer. Most HPV infections resolve spontaneously in 6-12 months and the majority of precancerous lesions regress due to immune response (Figure 1). Since only a small proportion of HPV infections will eventually lead to cervical cancer, other cofactors are needed for cervical cancer development (Schiffman & Kjaer, 2003).

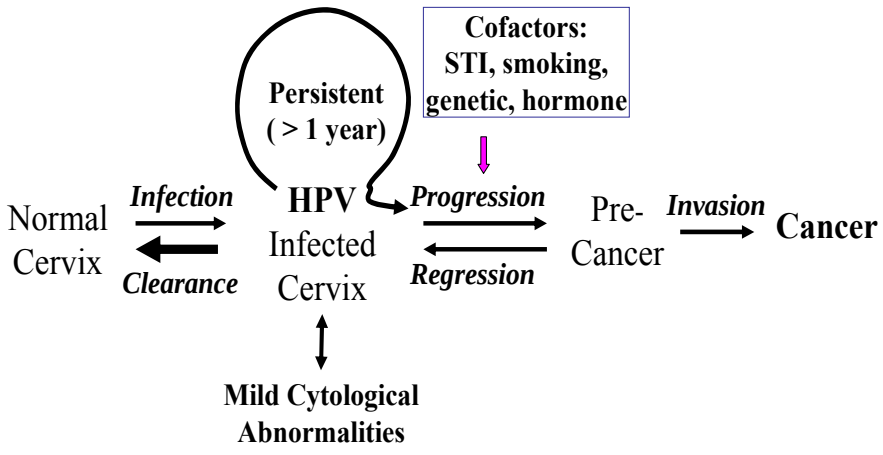

Figure 1. Natural history of cervical cancer development.
Courtesy of Dr. Wang Jianliu.

During carcinogenesis the oncoproteins E6 and E7 of high-risk HPVs interact with host cellular proteins through different pathways (zur Hausen, 2002), particularly the $p16^{INK4A}$-cyclin D1-CDK4/6-pRb-E2F and $p14^{ARF}$-MDM2-p53 pathway. This interaction subverts cell cycle checkpoints, resulting in cell over-proliferation, genome instability, and carcinogenesis (Kim & Zhao, 2005) (Figure 2). $p14^{ARF}$ and $p16^{INK4A}$ arise from the same gene and are strongly associated with HPV-positive cervical cancer (Kanao et al., 2004). High-risk HPV E6 binds to p53 and degrades it through the ubiquitin pathway. $p14^{ARF}$ act as a cell cycle regulator, inhibiting MDM2 proteins and blocking the formation of MDM2-p53. This prevents the degradation of p53 by MDM2 (Zhang et al., 1998). High-risk HPV E7 binds to and degrades pRb, and releases the cellular transcription factor E2F1, leading to increased expression of cyclin A, cyclin E and $p16^{INK4A}$ (Giarre et al., 2001).

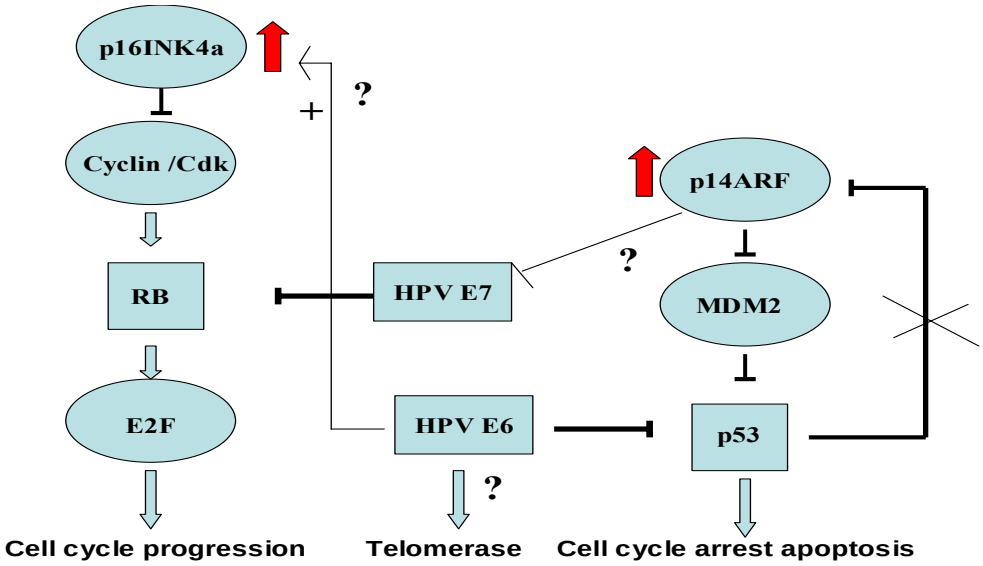

Figure 2. Interactions between HPV oncoproteins E6, E7 and host cellular proteins. Courtesy of Dr. Mikael S Lindström.

1.1.3 Cervical precancerous lesions

Cervical precancerous lesions are defined by cytological abnormalities within the cervical epithelium and are usually classified in three- and two-tier systems (Figure 3). The cervical intraepithelial neoplasia (CIN) system was introduced in 1973 and is based on tissue architecture (Richart, 1973). CIN1 refers to abnormal cells occupying the lower third of the cervical epithelial stratum, CIN2 indicates that two thirds are occupied, and in CIN3 the entire epithelial layer are occupied. CIN1, CIN2 and CIN3 describe different processes - CIN1 indicates a self-limiting HPV infection, and CIN2 or CIN3 are actual cervical cancer precursors (Kiviat & Koutsky, 1993). The other reporting system is the Bethesda system that was introduced in 1988. This system classifies cytological abnormalities as either low-grade (LSIL) or high-grade (HSIL)

(Solomon et al., 2002). LSIL corresponds to CIN1 and HPV infection; and HSIL corresponds to CIN2, CIN3 and carcinoma *in situ* (CIS). In addition, the abnormal cells with "borderline" changes are classified as atypical squamous cell of undetermined significance (ASC-US) or atypical squamous cells, which are difficult to distinguish from HSIL (ASC-H).

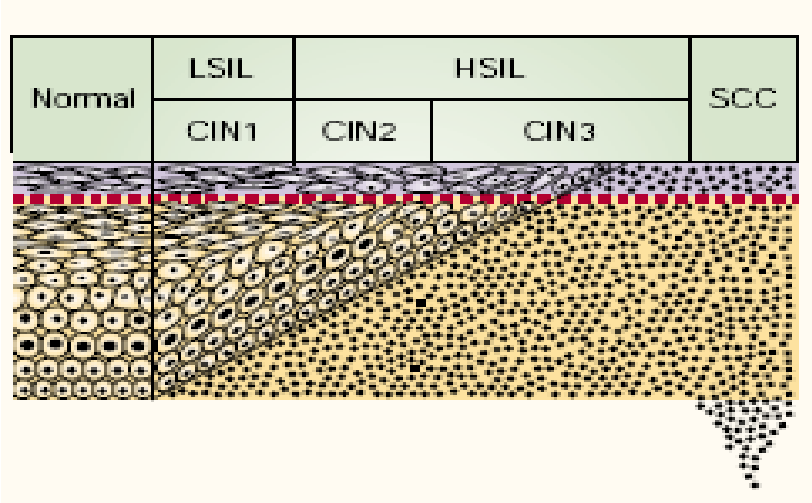

Figure 3. Premalignant and malignant diseases in cervical squamous epithelium identified by morphological abnormalities. Modified from Baldwin et al., 2003.

Table1. The 2001 Bethesda classification system of cervical squamous cell dysplasia

ASC	ASC-US (Atypical squamous cells of undetermined significance)
	ASC-H (Atypical squamous cells, cannot exclude HSIL)
LSIL	Low-grade squamous intraepithelial lesion
	- HPV-related changes
	- Mild dysplasia
	- Mild CIN (CIN1)
HSIL	High-grade squamous intraepithelial lesion
	- Moderate and severe dysplasia
	- Moderate and severe CIN (CIN2, 3)
	- Carcinoma *in situ*
SCC	squamous cell carcinoma

Modified from Solomon et al., 2002.

1.1.4 Risk factors, cofactors

1.1.4.1 HPV risk factors

HPV is the most prevalent sexually transmitted viral infection among both men and women. It is estimated that 80% of sexually active adults have been infected with at least one HPV type (Baseman & Koutsky, 2005). It is well established that high-risk HPV infection causes cervical cancer. Other factors pertaining HPV infection such as variants, viral load, multiple co-infections and viral integration may modify viral-host biological interaction and play a role in the development of cervical cancer.

1.1.4.1.1 High-risk HPV types

More than 200 different HPV types exist in the current database and not all of them share the same properties (zur Hausen, 1999a). The different HPVs are generally grouped according to their association with cervical cancer and precancerous lesions and their genomic sequence such as: oncogenic high-risk types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82), probable oncogenic high-risk types (HPV 26, 53 and 66), and non-oncogenic low-risk types (HPV 6, 11, 42, 43, 44, 54, 61, 70, 72, 81 and 89). Low-risk HPVs are primarily found in genital warts, while high-risk HPVs are found in cervical cancer and precancerous lesions (Munoz et al., 2003). Furthermore, it has been shown that high-risk HPV can transform human cells in tissue culture (Hawley-Nelson et al., 1989; Munger et al., 1989). Oncoproteins E6 and E7 of high-risk HPV have a high affinity for binding and degrading p53 and pRb, resulting in essentially immortal cells. However, E6 proteins of low-risk HPVs do not bind p53 at detectable levels and have no effect on p53 stability *in vitro.* Also, the E7 proteins of low-risk HPV types binds pRb with decreased affinity (Burd, 2003).

1.1.4.1.2 Viral variants

There are over 200 known closely related HPV types (zur Hausen, 1999a). In order for an HPV type to be classified as a new HPV type, it must exhibit less than 90% sequence similarity to known HPV types in the E6, E7 and L1 open reading frames (ORFs). If the type shows 90% or higher sequence similarity to the prototype in these regions it can either be classified as a subtype (90-98% similarity) or as a variant (>98 % similarity).

HPV variants have different biological and biochemical properties important to cancer risk (Burd, 2003). These variants often appear to have disparate geographical and

ethnic origins. Most studies of HPV variants focus on high-risk HPV 16. HPV 16 has 5 known variants: European (E), Asian (As), Asian-American (AA), African-1 (Af1) and African–2 (Af2). Asian-American variants have apparently higher oncogenic potential than European variants (Veress et al., 1999).

1.1.4.1.3 Viral load

The term viral load refers to the HPV virus copy number in the infected cells. It has been suggested that viral load correlates to cervical disease severity (Swan et al., 1999; Zerbini et al., 2001). However, some studies suggested that a high viral load alone is insufficient to induce progression from HPV infection to CIN2, 3 and cancer (Lorincz et al., 2002; Ylitalo et al., 2000). Moreover, low levels of all types of high-risk HPVs are able to induce tumorigenesis (zur Hausen, 1996).

1.1.4.1.4 Viral integration

Viral integration has been reported to be associated with carcinogenesis. Integrated HPV is more frequently found in HSIL and cervical cancer than in LSIL (Klaes et al., 1999). During carcinogenesis part of HPV E2 is deleted, and the loss of full length E2 expression leads to increased expression of E6 and E7 oncoproteins (Yoshinouchi et al., 1999). High-risk HPV E6 and E7 impairs p53 and pRb functions and causes the cell to escape cell cycle check point surveillance, subsequently leading to genome instability and cell immortalization (Ferenczy & Franco, 2002). Persisting HPV infection may be a consequence of viral integration.

1.1.4.1.5 Multiple HPV co-infections

Multiple HPV co-infections have been reported in the literature (Jacobs et al., 1997; Kleter et al., 1999; Quint et al., 2001). Most of these are double co-infections, but triple, quadruple and even quintuple HPV co-infections have also been detected (Gharizadeh et al., 2006; Jacobs et al., 1997; Quint et al., 2001). Multiple HPV genotypes, usually with at least one high-risk type, were found in 11.8% of patients with normal cytology and ASC-US, in 34.5% of patients at CIN1 or 2, and in 4.5% of cervical cancer tissue samples (Kleter et al., 1999).

1.1.4.2 Non-HPV risk factors

HPV infection alone is not sufficient for cervical cancer development. Some endogenous and exogenous factors can act as cofactors by influencing the risk of HPV persistence and cancer progression. Exogenous cofactors include the use of oral contraceptives (OCs), high parity, diet, life style, tobacco smoking, cervical trauma, and co-infection with other sexually transmitted agents such as human immunodeficiency virus (HIV). Endogenous cofactors include hormonal levels, and genetic factors associated with human leukocyte antigen (HLA) (Castellsague & Munoz, 2003).

1.1.4.2.1 Long-term use of oral contraceptives (OCs)

Some studies have shown that long-term use of OCs increases the risk of high grade cervical neoplasia, however, other studies do not support this association (Adam et al., 2000; Brisson et al., 1994). The International Agency for Research on Cancer (IARC) have reported that long-term use of OCs is associated with a moderate increase in cancer risk (OR=1.4), and that there is a strong dose-response relationship with increased periods of use (Moreno et al., 2002). The upstream regulatory region of HPV contains sequences similar to the glucocorticoid responsive elements that are induced by steroid hormones such as progesterone (the active component of OCs) (Burd, 2003). OC hormones may therefore promote HPV DNA integration into the human genome, deregulating E6 and E7 expression and influencing the progression of carcinogenesis (IARC, 1995).

1.1.4.2.2 High parity

High parity has been consistently found to increase the risk of squamous cell cervical carcinoma among HPV positive women. High parity leads to direct exposure of the transformation zone in the cervix to HPV infection, hence increasing the risk of cervical cancer (Autier et al., 1996; Munoz et al., 2002). Increased levels of estrogen and progesterone during pregnancy may also modulate the immune response to HPV and influence the risk of persistence or progression (Munoz et al., 2002).

1.1.4.2.3 Smoking

Smoking shows a moderate and statistically significant association with cervical cancer in case-control studies, even after adjusting for HPV infection (OR=2) (Castellsague et al., 2002). Nicotine- and tobacco-specific carcinogens have been detected in the cervical mucus of cigarette smokers (Prokopczyk et al., 1997). Smoking

increases a woman's susceptibility to tumorigenesis by lowering the immune surveillance in the mucosa (Daling et al., 1996; Parazzini et al., 1998).

1.1.4.2.4 Other sexually transmitted infections (STIs)

It has been suggested that other STIs may be cofactors of HPV in cervical cancer progression (Schmauz et al., 1989). Herpes simplex virus 2 (HSV2) has been suggested as a cofactor in cervical cancer development in a multi-center case-control study (Smith et al., 2002). Antibodies to *Chlamydia trachomatis* in high-risk HPV-infected women increases the risk of cervical cancer two-fold (Anttila et al., 2001). Furthermore, HIV infected women have significantly higher recurrence rates (87%) of HPV-associated cervical lesions than women without HIV infection (18%) (Calore et al., 2001). Adeno-associated virus (AAV) is a helper-dependent parvovirus. For its replication in a host cell, co-infection with other viruses (such as adenovirus, HPV, vaccinia virus or HSV) is necessary. An inverse association between seropositivity for AAV-2 and cervical cancer has been reported (Georg-Fries et al., 1984). However, studies on the detection of AAV DNA in cervical smears or biopsies do not fully support the serological findings (Ahn et al., 2003). Several studies detected AAV DNA in cervical samples, while others failed to do so (Odunsi et al., 2000; Strickler et al., 1999). Whether or not the presence of AAV DNA is associated with cervical cancer risk has not been assessed in an epidemiologically controlled format.

1.1.4.2.5 Life style

In 1842, Rignoni-Stern reported that sexual activity is associated with cervical cancer (Rigoni-Stern, 1842). An individual has an increased risk of cervical cancer with an early onset of sexual activity (<16 years), multiple sexual partners (more than four), or having partners with multiple sexual partners (Herrero et al., 1990). These behaviors increase the risk of HPV infection as well as other sexually transmitted diseases.

1.1.4.2.6 Immunosuppression

HIV infection is associated with a higher prevalence of HPV-related diseases. Higher persistence rates of HPV 16 and HPV 18 among HIV patients have been reported (Sun et al., 1995). Furthermore, HIV infected patients and patients receiving immunosuppressive medication are at increased risk of developing cervical cancer (Palefsky & Holly, 2003).

1.1.4.2.7 Genetic predisposition

Biological daughters of women with cervical cancer had an increased risk of the disease as compared to adopted daughters, with an approximately 50% reduced risk for half-sisters (Magnusson et al., 1999). These findings suggested that genetic predisposition may contribute to the risk of cervical cancer. Gene polymorphisms or genetic variations in immune-related genes might be related to HPV persistence and progression to cancer. Human leukocyte antigens (HLAs) are the most extensively studied immune-related genes. HLA molecules play an important role in immune function, through the presentation of peptides for recognition by T lymphocytes. For example, the HLA type DRB1*1301 is consistently negative associated with cervical cancer (Hildesheim & Wang, 2002). Other immune-related genes such as chemokines and their receptors are also under investigation. CCR2 and CCR5 are two chemokine receptors that act as co-receptors for HIV-1. Chemokines are small chemotactic cytokines that direct the migration of leukocytes during inflammation. Chemokines also regulate angiogenesis and may contribute to tumor growth (Rossi & Zlotnik, 2000). Chemokine receptors are mainly expressed on immune and inflammatory cells, such as B- and T-lymphocytes and antigen-presenting cells. Genetic variations within the chemokine receptor genes are associated with inflammatory-mediated and autoimmune disorders (Garred et al., 1998; Zapico et al., 2000). CCR5-Δ32 and CCR2-64I are two genetic polymorphisms associated with slower progression from HIV infection to AIDS (Berger et al., 1999; Smith et al., 1997). These data suggest that the CCR5-Δ32 and CCR2-64I alleles may play a role in immunity-mediated functions.

1.1.5 Cancer treatment and prognosis

Once cervical cancer is diagnosed, clinical staging is important for treatment planning and estimation of prognosis. Early-stage tumors can be treated with cone biopsy and hysterectomy, and the five-year survival rate is over 95% for this group of patients. Late-stage cervical cancer needs to be treated with radical surgery, radiotherapy, and/or concurrent chemotherapy. Less than 40% of these patients survive over five years (Table 2) (DiSaia PJ, 1997).

Table 2. Treatment and 5-year survival in cervical cancer stages.

Stage	Treatment	5-year survival
Stage 0	LEEP, laser therapy, conization, cryotherapy	100%
Stage Ia	Simple hysterectomy or cone biopsy	> 95%
Stage Ib or IIa	Radical hysterectomy and radiotherapy	80-90%
Stage IIb	Pelvic radiotherapy plus chemotherapy	65%
Stage III	Pelvic radiotherapy plus chemotherapy	40%
Stage IVa	Pelvic radiotherapy plus chemotherapy	<20%
Stage IVb	Radiation therapy plus chemotherapy	7%

1.1.6 Cervical cancer prevention

1.1.6.1 Pap smear screening

Since its discovery in 1949 by the pathologist George Papanicolaou, the Pap smear screening has helped to decrease cervical cancer incidence and mortality rates by 70% (Kurman et al., 1994). The Pap smear identifies cytological abnormalities of the transformation zone. However, this method has several limitations: it is subjective with wide variations in sensitivity (30-87%); single Pap smear tests have relatively low specificity (86-100%) (Nanda et al., 2000); and false negative rates as high as 20-45% have been reported, probably due to sample preparation or misinterpretation of Pap smear results (Woolf et al., 1996). Techniques for improving the sensitivity and specificity of detection are highly desirable, such as liquid-based cytology.

1.1.6.2 HPV screening

High-risk HPV can be found in almost every cervical cancer case, and persistent infection is likely to lead to cervical cancer development (Walboomers et al., 1999). HPV DNA testing by polymerase chain reaction (PCR) is highly sensitive for detection of HPV infection(s). HPV DNA testing has great potential as a screening tool for cervical cancer, especially for women older than 35 years of age, those with persisting high-risk HPV infections and those with ASC-US. However, since most HPV infections are transient, particularly in young women, the specificity of this method is reduced. HPV testing alone cannot be used as a screening tool, but it should be combined with Pap smear analysis.

1.1.6.3 Biomarkers

During carcinogenesis high-risk HPV oncoproteins interact with host cellular proteins, particularly cell cycle proteins, resulting in genome instability and cell immortality. These host cellular proteins can act as biomarkers to indirectly predict HPV infection, allowing unambiguous identification of HPV-related disease in epithelial cells (von

Knebel Doeberitz, 2002) (Table 3). Detection of these biomarkers could be an objective, reproducible, and reliable method for cervical cancer screening. It also has the potential to predict the outcome of the disease through identification of individuals at risk of progression from HPV infection to cancer (Baldwin et al., 2003). For example, high-risk HPV E7 binds to and degrades the pRb protein, leading to upregulated $p16^{INK4A}$ expression (Klaes et al., 2001). The over-expression of $p16^{INK4A}$ is related to HPV infection and high-grade squamous neoplasia (Guo et al., 2004). $p16^{INK4A}$ has been used as a biomarker in cervical squamous neoplasia and is a good candidate for an additional screening tool to be used in cervical cancer screening (Murphy et al., 2004; Murphy et al., 2003; von Knebel Doeberitz, 2001).

Table 3. Candidate biomarkers of cervical neoplasia.

p16 (INK4A)
Ki 67
Cyclins (A, E, D1)
p14 (ARF)
Cyclin-dependent kinase (CDK)
Proliferating cell nuclear antigen (PCNA)
p53
MDM-2
pRb/E2F
p21(WAF1/CIP1)
p27(Kip1)
Minichromosome maintenance proteins (MCMs)

1.1.6.4 HPV vaccines

HPV infection is the major etiological factor in cervical cancer. Vaccines against HPV infection are designed for use in preventing or treating cervical cancer and other HPV-related diseases. Two types of vaccines have been developed.

Prophylactic vaccines target HPV L1 or both HPV L1 and L2 and prevent HPV infections by inducing neutralizing antibodies against the virus (Christensen et al., 1994; Roden et al., 1996; White et al., 1998). Natural HPV infections are inefficient in eliciting such an immune response (Heim et al., 1995; Le Cann et al., 1995a; Le Cann et al., 1995b). Vaccination with viral-like particles (VLPs) for animal papillomavirus vaccines has shown excellent potential for immunogenicity and protection against papillomavirus-related diseases (Breitburd et al., 1995; Jansen et al., 1995; Jarrett et al., 1990a; Jarrett et al., 1990b; Suzich et al., 1995). Such VLP vaccines produce type-specific antibodies and high titers against infection (Breitburd et al., 1995; Jansen et al., 1995; Jarrett et al., 1990a; Jarrett et al., 1990b; Suzich et al., 1995; White et al., 1998).

Developing a cocktail of VLPs for the most common high-risk HPVs is optimal against different HPV infections (Munoz et al., 2004). Phases I and II prophylactic vaccine trials have demonstrated their immunogenicity and safety (Koutsky et al., 2002; Villa et al., 2005). Two vaccines (HPV 16 and/or HPV 18) are currently in phase III of clinical trials (Mahdavi & Monk, 2005).

Therapeutic vaccines target high-risk HPV E6 and E7 and are designed for treatment for existing HPV infection and HPV-related diseases. Various forms of therapeutic HPV vaccines are presently undergoing testing in clinical trials, including those based on DNA, dendritic cell (DC), protein, peptide, and bacterial vector. However, these therapeutic vaccines are in an earlier stage of development than prophylactic vaccines.

1.2 HUMAN PAPILLOMAVIRUS (HPV)

HPVs are a group of tumor DNA viruses in the family *Papillomaviridea* that can infect the basal epithelia of skin and mucosa and are categorized as cutaneous types and mucosal types. HPVs are found in cutaneous warts on the hands and feet, plantar warts (HPV types 1, 2), common warts (HPV types 1, 2, 7), and flat warts (HPV types 3, 10). About 90% of genital warts are caused by HPV 6 or 11 (Greer et al., 1995; Meisels & Fortin, 1976) and about 70% of cervical cancer cases are caused by HPV 16 or 18 (Munoz et al., 2003).

1.2.1 Viral structure

HPVs are small circular double-stranded DNA viruses, with approximately 8000 base pair (bp) genomes encased in a naked icosahedral capsid about 55 nm in diameter (Munger et al., 2004; Sinal & Woods, 2005). Most HPVs have eight ORFs that are located on one strand. Some of these are overlapping, encoding a total of eight to ten proteins. The HPV genome is functionally divided into three regions. The first 400 to 1,000 bp is a non-coding region called the long control region (LCR) or upper regulatory region (URR), which regulates viral DNA replication. It contains the p97 core promoter together with an enhancer and a silencer. The second region, known as the early region, encodes 6 early proteins (E1, E2, E4, E5, E6, E7), involved in viral replication and oncogenesis through interactions with the host cell. The third, so called late region, encodes the major (L1) and minor (L2) structural proteins for the viral capsid (Schneider, 1993) (Figure 3).

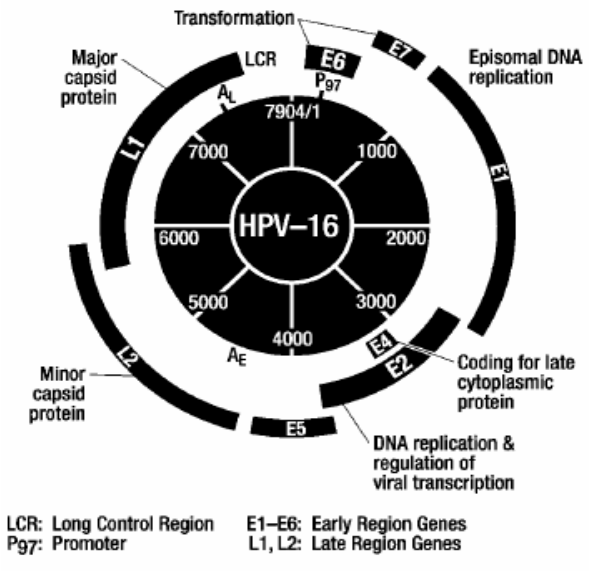

Figure 3. Viral structure and gene functions of HPV 16.
Adapted from Hubbard et al., 2003.

1.2.2 Viral proteins and their functions

The HPV genome encodes 8 to 10 proteins (6-8 early proteins and 2 late proteins) (Table 4).

•**E1** contains about 650 amino acids (aa), encoded by the largest conserved ORF. It exhibits both ATPase and 3´-5´ helicase activities (Chen et al., 1994; Hughes & Romanos, 1993; Wilson et al., 2002). E1 weakly binds to origin of replication sequences, helping to form heterodimers with E2 (Dixon et al., 2000; Frattini & Laimins, 1994a; Frattini & Laimins, 1994b; Lu et al., 1993), and initiating viral replication (Sverdrup & Khan, 1995).

•**E2** is a DNA binding protein that contains approximately 380 aa. Its main functions are viral DNA replication and viral transcription regulation. E2 also inhibits over expression of E6 and E7 (Thierry & Howley, 1991) and permits E1 to initiate viral replication.

•**E4** protein is approximately 100 aa long and plays a role in the maturation and release of viral particles. It is considered a late protein. After translation from spliced transcripts, E4 is fused with E1 to generate E1^E4 fusion proteins (Brown et al., 1995).

E1^E4 proteins from high-risk HPV types associate with keratin networks in cells and can induce their collapse by over-expression in transient transfection assays (Doorbar et al., 1991)(Wang et al., 2004; Wilson et al., 2005).

•**E5** is approximately 90 aa long and consists of small hydrophobic proteins located in the endosomes, the Golgi apparatus, and cellular membranes (Conrad et al., 1993). It causes transformation by interacting with growth factor receptors and activating the MAPK pathway, resulting in cell growth stimulation (Crusius et al., 1997; Crusius et al., 2000; Straight et al., 1993).

•**E6** is about 150 aa in size and contains two zinc-binding domains with the motif Cys-X-X-Cys. It is localized in both the nucleus and the cytoplasm, and it has been shown to bind to more than 12 different proteins (zur Hausen, 2002). The high-risk HPV E6 proteins interact with certain cellular proteins and involve the $p14^{ARF}$-MDM2-p53 pathway (Zhang et al., 1998). E6 transforms cells by targeting p53 for degradation via ubiquitin pathway (Thomas et al., 1999). In addition, E6 activates telomerase activity (Veldman et al., 2001) and inhibits SRC-family degradation (Oda et al., 1999), causing over proliferation of infected cells.

•**E7** comprises around 100 aa. High-risk HPV E7 binds to pRb and degrades it, releasing the transcription factor E2F which subsequently up-regulates INK4A through the $p16^{INK4A}$-cyclin D1-CDK4/6-pRb-E2F pathway (Dyson et al., 1989; Kiyono et al., 1998). E7 also stimulates the S-phase proteins cyclin A and cyclin E (Zerfass et al., 1995) and blocks CDK inhibitors e.g. p27 (WAF1) and p21 (KIP1) (Funk et al., 1997; Jones et al., 1997; Ruesch & Laimins, 1998).

E6 and E7 can independently immortalize human cells, but at reduced efficiency (Halbert et al., 1991). Their joint function leads to a marked increase in the transformation ability of the virus.

Late genes: **L1** and **L2** are major and minor capsid proteins, respectively. They are not expressed in precancerous or tumor cells, but they are important for vaccine development.

Table 4. HPV viral proteins and functions.

Protein	Cellular location	Function
L1	Nuclear	major capsid protein
L2	Nuclear	minor capsid protein
E1	Nuclear	episomal replication
E2	Nuclear	DNA replication and regulation of viral transcription
E4	Cytoplasmic	disrupt cytokeratin networks
E5	Cytoplasmic	activate EGF receptor, stimulate cell growth
E6	Nuclear	oncoprotein, target p53 for degradation, anti-apoptosis
E7	Nuclear	oncoprotein, bind to pRb and degrade pRb, disrupting cell cycle regulation

1.2.3 Pathogenesis of HPV

In productive HPV infection, HPVs exploit host cell factors to regulate viral transcription and replication. After infection by HPV in skin or mucosal epithelium (basal cell layer) through microabrasion, specific "early" viral genes (such as E5, E6 and E7) are first expressed, resulting in low-level viral replication. Following entry into the suprabasal layers, "late" viral gene expression is initiated, high copy numbers of the circular viral genome are amplified and capsid proteins are synthesized. In the upper layers of the epidermis or mucosa, complete viral particles are assembled and released.

In non-productive HPV infection, high-risk HPV is integrated randomly into the host genome and part of the E2 ORF is deleted, resulting in loss of full length E2 gene expression, leading to increased E6 and E7 genes expression (Yoshinouchi et al., 1999). The E6 gene product binds to the tumor suppressor protein p53 and rapidly degrades it through the cellular ubiquitin pathway (Thomas et al., 1999). This subverts the $p14^{ARF}$-MDM2-p53 pathway and leads to deregulation of G_1 arrest, apoptosis and cell cycle checkpoints. The HPV E7 gene product binds to the tumor suppressor gene pRb and degrades it, releasing the transcription factor E2F-1 and upregulating $p16^{INK4A}$ (Kiyono et al., 1998). Host cells increase their proliferation rate and genomic instability, finally leading to tumorigenesis.

1.2.4 HPV detection and typing

Cervical infection by certain HPV types is a precursor event to cervical cancer. HPV prevalence differs with respect to geographical region, age, and social economic groups. Thus, accurate typing and diagnosis of HPV infection is important for identifying patients at risk. Furthermore, genotyping contributes to epidemiological studies, vaccine development and research on HPV-related diseases. HPV infection is mainly diagnosed by molecular biology methods, because culturing and *in vitro*

propagation of virus is impractical, and serological methods are not sensitive enough (Dillner, 1999).

HPV DNA can be detected in cervical smears and biopsy samples by several methods. These include:

1) Direct nucleic acid hybridization methods, such as Southern blot or dot blot hybridization and *in situ* hybridization (ISH). ISH can localize HPV infection in cytological or biopsy samples and enable co-localization with other markers (Sato et al., 1998). Southern blot or dot blot hybridization can detect and type the DNA genome directly, but they are time-consuming and are limited by low sensitivity and the requirement for large amounts of highly purified DNA.

2) Signal amplification systems such as Digene Hybrid Capture II system (HC2, Digene Corp., USA) is a non-radioactive immunoassay based on the hybridization of target HPV DNA to labeled RNA probes. RNA-DNA hybrids are then detected using a specific antibody and a chemiluminescence detection system (Bozzetti et al., 2000; Lorincz, 1996). A cocktail of two different probes is used, one targeted against 5 low-risk HPV genotypes (HPV 6, 11, 42, 43 and 44) and the other against 13 high-risk HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). This method is widely used to identify patients susceptible to high- or low-risk HPV infection in clinics in many countries and is approved by the Food and Drug administration (FDA). However, this technique is somewhat limited because it cannot determine the specific HPV type present in the sample.

3) Target amplification systems: PCR is the most commonly used method to detect HPV DNA because of its simplicity and high degree of sensitivity. Two approaches are used: a) Type-specific PCR using type-specific primers which only allow amplification of a single HPV genotype. This method is comparatively labor-intensive and expensive and is used primarily in research. b) Broad-spectrum PCR employing general PCR primers and consensus PCR primers to amplify a broad spectrum of HPV genotypes. The most common primers used are MY09/11 (Hildesheim et al., 1994), PGMY (Gravitt et al., 1998), GP5+/6+ (Jacobs et al., 1997), and SPF_{10} (Kleter et al., 1999), which target conserved and variable regions in the HPV L1 ORF. In order to identify at-risk patients with persistent HPV infections (the same HPV type is present >1 year), we need to know the HPV genotype. HPV genotypes can be detected by reverse line blotting (RLB) (van den Brule et al., 2002), restriction fragment length polymorphism (RFLP) analysis (Grce et al., 2000), and sequencing (Maki et al., 1991). RLB detects HPV genotypes through the hybridization of PCR products to different HPV probes immobilized onto nitrocellulose membrane. This method is adequately sensitive to

detect specific HPV types and multiple genotypes in a single sample, but there is the possibility of false hybridization and non-specific discrimination of closely related types. RFLP is based on HPV genotype-specific restriction patterns. It is a time-consuming procedure, and sometimes HPV RFLP data is difficult to interpret. DNA sequencing is the gold-standard method for HPV genotyping. It provides sequence information for uncharacterized HPV genotypes as well as mutation information. But conventional DNA sequencing technique is often not effective for the detection of multiple HPV co-infections in a single sample, because the sequence signals generated are mixed and the results are difficult to interpret. In contrast to traditional Sanger sequencing, Pyrosequencing is a real-time DNA sequencing method. Integrating the multiple sequencing primers method with Pyrosequencing allows for accurate detection of multiple HPV co-infections in one sample (Gharizadeh et al., 2003a; Gharizadeh et al., 2005).

Another method that may be useful in HPV genotyping is mRNA detection by reverse transcription (RT) PCR and real-time PCR to identify high-risk HPV E6 and E7 expression (Lamarcq et al., 2002; Wang-Johanning et al., 2002). This assay can actually determine whether these transforming genes are present and active, which could be more clinically valuable to identify at-risk patients as compared to available methods (Lie et al., 2005).

1.2.5 HPV immunology

1.2.5.1 Innate immunity

The first line of defense against HPV infection is the innate immune system, which provides non-specific protection and also enhances the adaptive immune response. However, HPV often escape innate immune recognition and elimination (Woodworth, 2002). They are resistant to lysis by natural killer (NK) cells but are sensitive to cytokine-activated NK cells. Activated macrophages also kill HPV-infected cells and prevent invasive cancer development (Woodworth, 2002).

1.2.5.2 Humoral immunity

Epidemiological studies have revealed that HPV seropositive women are at risk for cervical neoplasia (De Sanjose et al., 1996; Lehtinen et al., 1996). HPV antibody is a marker of current and/or past exposure to HPV (De Sanjose et al., 1996; Lehtinen et al., 1996). HPV antibody production is important in preventing viral transmission and reinfection. Most HPV antibodies are type-specific and appear six months to a year after HPV infection (DNA) has been detected. HPV-specific immunoglobulin A (IgA) is correlated with virus clearance, however, immunoglobulin G (IgG) is associated with persistence of HPV infection (Burd, 2003).

1.2.5.3 Cellular immunity

The primary immune response for HPV clearance is cell–mediated (Stanley, 2001). This response involves interactions with professional antigen presenting cells (APCs), helper T-cells, cytolytic T-cells, and co-stimulatory cytokines. In immuno-suppressed women, the incidence of HPV infection and progression to invasive cancer is increased (Arends et al., 1997). The T-cell proliferative responses to HPV 16 E7 are stronger in women with persistent HPV infection and progressive disease (99% reactive) than those who cleared the infection (41% reactive) (de Gruijl et al., 1996). These data indicates that the CD4 Th1 cell response is important for preventing HPV-induced diseases.

1.3 PYROSEQUENCING TECHNOLOGY

Pyrosequencing is a DNA sequencing technique to determine nucleic acid sequences (Ronaghi et al., 1996). This method is a sequencing-by-synthesis technique that employs a series of enzymes to accurately detect short nucleic acid sequences during DNA synthesis (Hyman, 1988; Melamede & Wallace, 1985). In principle, after one of the nucleotides has been incorporated into the primed DNA template by DNA polymerase, an inorganic pyrophosphate (PPi) molecule is released. The released PPi is converted to ATP by ATP sulfurylase using adenosine phosphosulfate (APS) as substrate and this reaction provides energy for luciferase to oxidase luciferin, consequently a light is produced that is presented as a peak in the pyrogram. Unincorporated nucleotides are removed by apyrase before the addition of the next nucleotide, permitting *de novo* sequencing (Figure 4). The advantages of Pyrosequencing are accuracy, flexibility, parallel processing and it can be easily automated. In addition, this technique eliminates the need for labeled primers, labeled nucleotides and gel-electrophoresis.

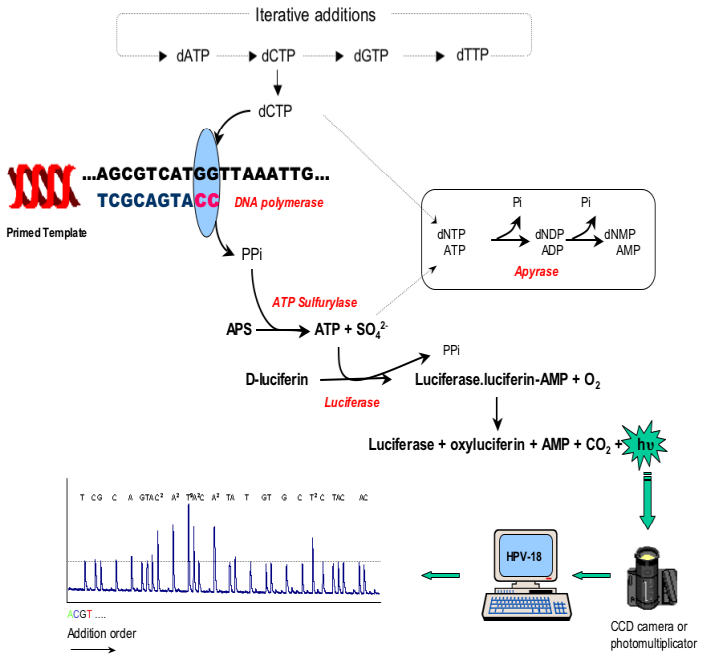

Figure 4. The principle of Pyrosequencing technology.
Adapt from Gharizadeh et al., 2001.

1.3.1 Applications

Pyrosequencing is primarily used for analyses of single nucleotide polymorphism (SNP) and mutations (Ahmadian et al., 2000), but it can also be used in microbial and viral genotyping (Gharizadeh et al., 2001; Gharizadeh et al., 2004; Gharizadeh et al., 2005; Gharizadeh et al., 2003b), methylation analyses (Colella et al., 2003), and SNP discovery (Ahmadian et al., 2000; Ronaghi, 2003).

1.3.1.1 Single nucleotide polymorphism (SNP)

SNPs are frequently occurring nucleotide variations in the human genome (Collins, 2000) with a random distribution of approximately 1 SNP per 200-300 bp (Judson & Stephens, 2001; Kruglyak & Nickerson, 2001). SNPs can contribute directly to disease predisposition by changing the function of a gene. Furthermore, SNPs can be assembled in haplotypes (a set of polymorphism alleles co-occur on a single chromosome) to investigate genetic variations (Ahmadian et al., 2000; Odeberg et al., 2002). SNPs are commonly used as markers to locate disease causing genes through association studies or family-based linkages studies (Craig & Stephan, 2005; Engle et al., 2006). A feature of SNP typing by Pyrosequencing technology is that each allele combination (homozygous, heterozygous) will give a specific pattern of pyrogram readouts due to non-synchronized extension (Ahmadian et al., 2000) (Figure 5).

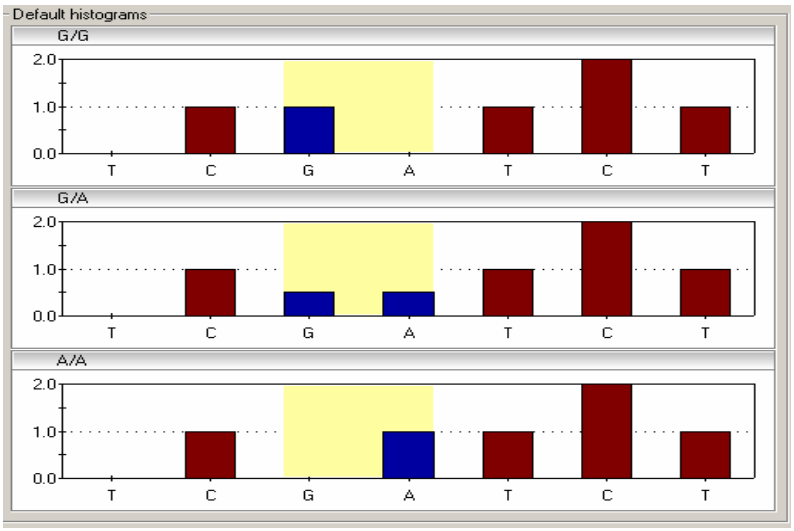

Figure 5. Default histograms of SNP typing using Pyrosequencing. G/G homozygous, A/G heterozygous, A/A homozygous.

1.3.1.2 DNA sequencing

DNA sequence determination techniques are crucial to the study of biological systems. At present, the state-of-the-art method for DNA sequencing is the Sanger dideoxy chain termination reaction, introduced in 1977 (Sanger et al., 1977). The application of this method has had significant impact on the biological and biomedical sciences, and the method has been successfully used in such diverse areas as molecular biology, genetics, biotechnology, pharmacology, forensics, archaeology and anthropology. However, the technique faces limitations in both throughput and cost for many future applications. Many alternative DNA sequencing technologies have been investigated. Among them, Pyrosequencing has been successful for confirmatory and *de novo* sequencing (Hyman, 1988; Ronaghi et al., 1996; Ronaghi et al., 1998). Pyrosequencing is being used for whole-genome sequencing, which is able to sequence 25 million bases at 99% or better accuracy in one four-hour run (Margulies et al., 2005).

1.3.1.3 Viral typing

Microbial and viral infections have a substantial impact on human health. Accurate detection and typing are critical to clinical diagnosis. Treatment and vaccine development requires detailed information about pathogen types. Most viral genomes contain both conserved and variable regions which are targeted for genotyping. Pyrosequencing technology has been employed in genotyping of microorganisms with heterogeneous sequences. Analysis of up to 25 bases in the highly heterogeneous HPV L1 is sufficient to classify many genotypes (Gharizadeh et al., 2001). Furthermore, it has been successfully used to genotype multiple HPV co-infections in the same sample (Gharizadeh et al., 2005; Gharizadeh et al., 2003b; Gharizadeh et al., 2006).

1.3.2 Multiplex Pyrosequencing

1.3.2.1 The principle of the new method

Multiplex Pyrosequencing employs several primers hybridized to one or more target DNA templates (Gharizadeh et al., 2003a; Gharizadeh et al., 2005). DNA sequencing is a useful technique for detection and typing of many different organisms. However, if several species/genotypes are present in a sample, the general sequencing primer will bind to all types present in the sample, producing mixed signals and making sequence information difficult or impossible to interpret. Figure 6 and 7a show a sample containing multiple co-infections and non-specific amplification products genotyped with the general primer that binds to all the HPV DNA present in the samples. As evident from Figure 6, the sequencing results are not possible to interpret. However, the problem will be overcome using a set of target-specific sequencing primers and Pyrosequencing. Using this method, only the primers that have a specific complementary nucleotide sequence in the sample will hybridize, and thus, only the genotype related to the specific primer will result in sequence signals (Figure 8b). Consequently, this approach is highly advantageous for analysis of a sample with multiple HPV co-infections or a sample with non-specific amplification products.

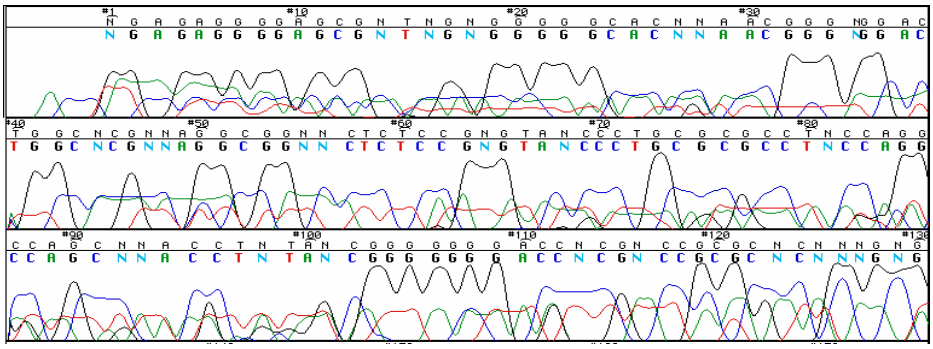

Figure 6. Electropherograms of a sample with multiple HPV infections (HPV 16/72/6) genotyped using general primer GP5+ and Sanger dideoxy chain termination method.

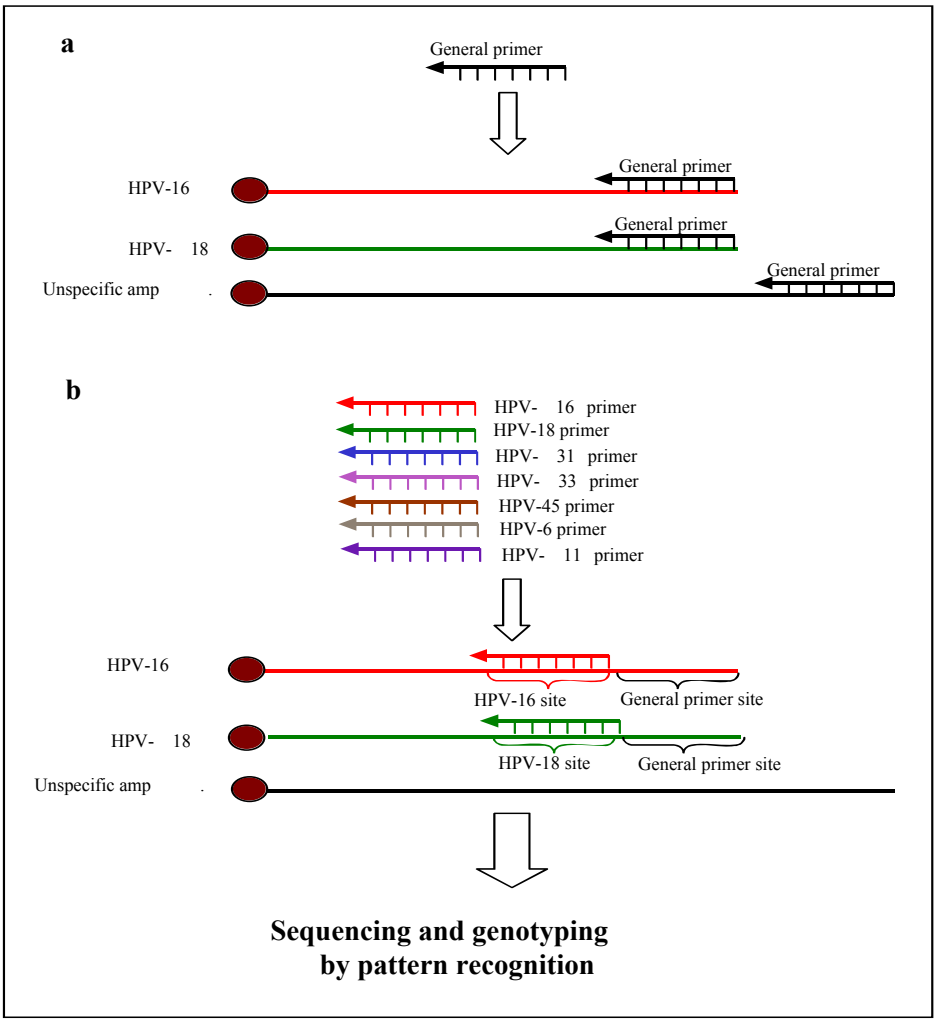

Figure 7. An amplicon containing HPV 16, HPV 18 and non-specific amplification product genotyped by Pyrosequencing. (a) Genotyped with general primer GP5+, the primer bind to HPV 16, HPV 18 and non-specific amplification products. (b) Genotyped with type-specific primers, HPV 16 primer only bind to the specific amplicon that contains HPV 16 DNA and HPV 18 primer only bind to the specific amplicon that contains HPV 18 DNA.

1.3.2.2 Pattern recognition

Pattern recognition refers to the comparison-by-alignment of at least two sequence-pattern results. The pattern recognition approach of pyrograms is applicable to typing of multiple types in one sample (Gharizadeh et al., 2005). Pyrosequencing provides sequence signals in real-time and it is possible to align the different sequence signals in a pyrogram. Pattern recognition can reveal the dominance or subdominance of multiple infections in the sample. However, conventional gel-based sequencing generally does not distinguish multiple infections (Figure 6). Figure 8 demonstrates three different clinical samples containing double infections of HPV 16 and HPV 18. These samples

were genotyped by comparing characteristic sequence signals specific for each type. The common and specific bases for characterizing each type facilitate genotyping. Sequence peaks on positions 1, 2, 5, 6, and 9 represent HPV 16 and sequence peaks on positions 1, 2, 7 and 10 represent HPV 18. The dominant type could be easily observed by comparison of single bases shown by arrows. Figure 8a shows HPV 16 and HPV 18 in almost equal proportions, while Figure 8b shows a dominant HPV 18 infection and Figure 8c shows a dominant HPV 16 infection.

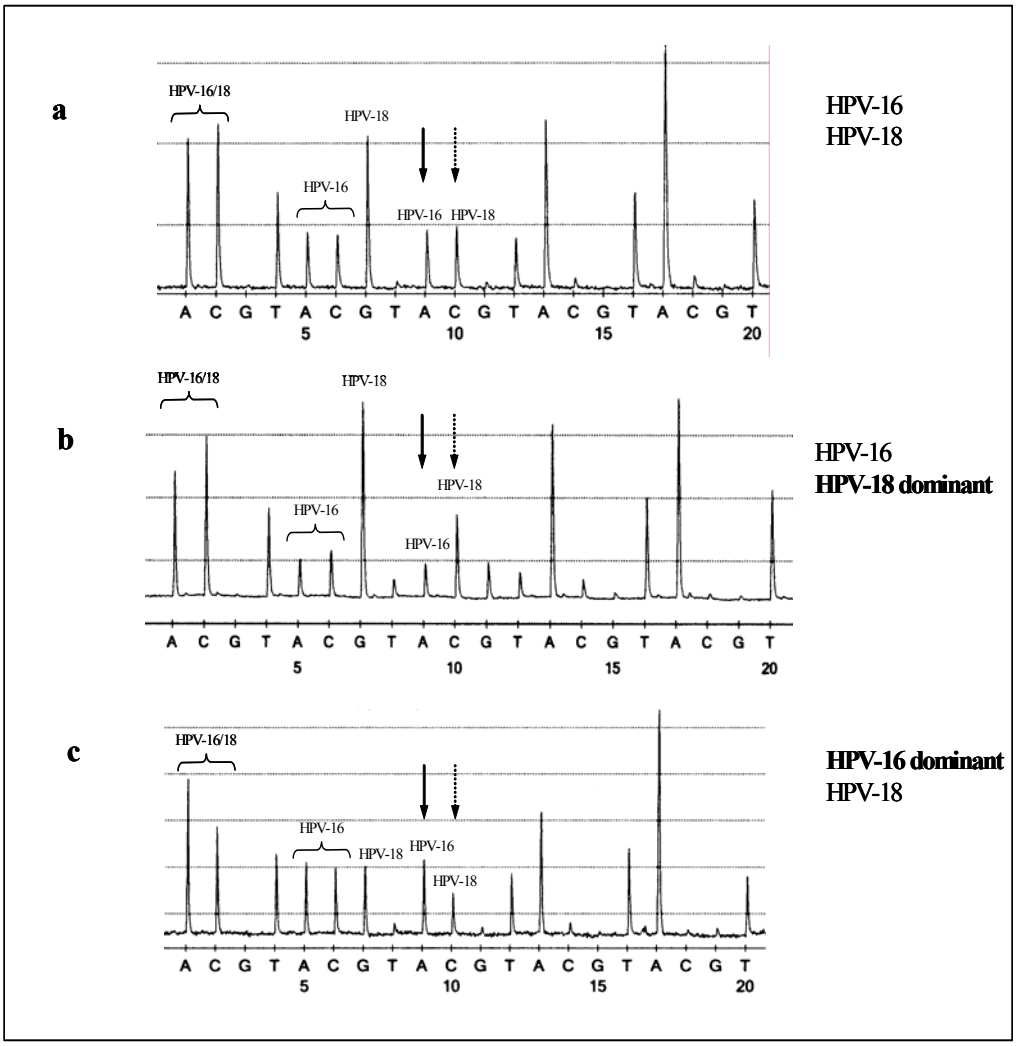

Figure 8. Pyrograms of co-infections of HPV 16 and HPV 18 in three different clinical samples sequenced by the seven multiple sequencing primers and genotyped by pattern recognition. The dominant type could be easily observed by comparison of single bases shown by arrows. a: HPV 16 and HPV 18 almost equal in dominance; b: HPV 18-dominant; and c: HPV 16-dominant.

1.3.2.3 Sentinel base sequencing

Multiple co-infections of HPVs are a common phenomenon. Studies show that multiple HPV infections can range from a few percent to almost 39% of all cases (Kleter et al.,

1999). This assay focuses on identifying just one characteristic base, referred to as a "sentinel", that indicates the presence of a single or multiple HPV infections. The type-specific multiple sequencing primers are designed for the most prevalent high-risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59). In brief, HPV-positive PCR products are genotyped by Pyrosequencing in four sequencing reactions using the GP5+ PCR primer and three pools consisting of four primers each. Each primer in each pool results in specific sequence pattern that can identify a specific HPV type. Figure 9 highlights the sentinel base that is unique for each genotype. This assay has the potential to genotype all of these HPVs in a clinical sample in less than 10 minutes, a significant improvement over the more time-consuming *de novo* methods.

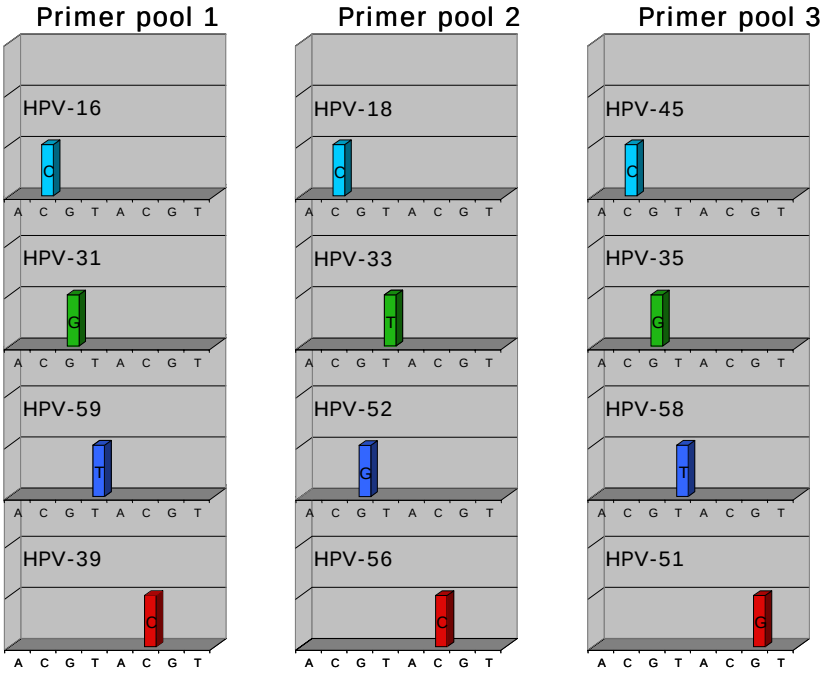

Figure 9. Highlights of sentinel-base for each genotype.

2. AIMS OF STUDY

The primary purpose of this thesis was to develop methods for improved HPV genotyping using multiple sequencing primers integrated with Pyrosequencing technology. We also aimed to determine the role of HPV cofactors and evaluate the clinical significance of biomarkers in cervical cancer development. Specifically, we proposed to:

- Evaluate HPV genotyping methods using type-specific multiple sequencing primers with Pyrosequencing technology (Paper I).

- Develop an assay that can accurately genotype the twelve oncogenic high-risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59) based on a single base by Pyrosequencing (Paper II).

- Evaluate the role of AAV infection in relation to invasive cervical cancer by performing a nested case-control study within a population-based cohort (Paper III).

- Investigate the association between HPV infection and cervical cancer, and DNA polymorphisms of the chemokine receptors CCR5-Δ32 and CCR2-64I (Paper IV).

- Investigate the clinical significance of host cellular biomarkers $p16^{INK4A}$, $p14^{ARF}$, p53, and proliferating cell nuclear antigen (PCNA) expression during cervical cancer development (Paper V).

3. MATERIALS AND METHODS

Papers I and **II** are methodological studies on HPV genotyping methods employing multiple sequencing primers in Pyrosequencing.
Papers III and **IV** are nested case-control epidemiological studies, with patients diagnosed with cervical cancer or with CIN and their matched controls from within the same population-based cohort.
Paper V is a study on the role of cell cycle markers during the natural history of cervical cancer development.

Paper I: Whole genomic plasmids of HPV types 6, 11, 16, 18, 33, 40, 72 and 73 were used to evaluate the specificity of these multiple sequencing primers. HeLa, CaSki and SiHa cell lines were employed as HPV-positive controls. 65 HPV-positive cervical cancer specimens from Sweden, and 17 HPV-positive oropharyngeal samples from Italy were amplified by nested PCR with the consensus primers MY09/11 (outer primers) and GP5+/6+ (inner primers). All samples were genotyped using Pyrosequencing with seven type-specific sequencing primers specifically targeting the clinically most prevalent HPV types (HPV 6, 11, 16, 18, 31, 33, and 45).

Paper II: Two hundred forty-four cervical samples from Sweden were identified as HPV-positive amplified by nested PCR with MY09/11 outer primers and GP5+/6+ inner primers. In order to rapidly genotype HPV in clinical settings, we designed a method that was based on a single "sentinel base'' to identify different single HPV genotypes and simultaneously recognize multiple HPV co-infections in the same sample. We designed multiple sequencing primers targeting the 12 most common high-risk HPV types (HPV 16, 18, 31, 33, 35, 45, 51, 52, 56, 58 and 59). These were pooled into three sets of four sequencing primers each, and Pyrosequencing was performed with these combinations as well as with GP5+.

Paper III: The patients studied here consisted of women residing in the Västerbotten county in northern Sweden, who took part in the national cervical cancer screening program introduced since 1969. The program invites women (aged 25 to 59 years) to participate every 3 to 5 years for Pap smear screening, and it has an attendance rate of over 80%. All cases were identified by linking the cytological registry to the Swedish cancer registry during the period between 1969 and 1995. Eligible cases were women with invasive cervical cancer who had at least one normal smear before cancer diagnosis. As controls cytologically normal women were matched with cervical cancer cases according to age and date of sampling. One hundred and four cases (85 squamous cell carcinomas, 19 adenocarcinomas) and their matched controls were available for the

study (Figure 10). HPV DNA was detected by nested PCR in a single tube (MY09/11 and then GP5+/6+), and genotyped by Sanger sequencing. AAV DNA was detected using three sets of AAV primers: the first set included pan-AAV primers targeting AAV-2, -3, and -5; the second set contained non-nested AAV-2 PCR primers; and the third set consisted of nested AAV-2 PCR primers. Both the second and the third primer sets target the AAV-2 *rep* gene.

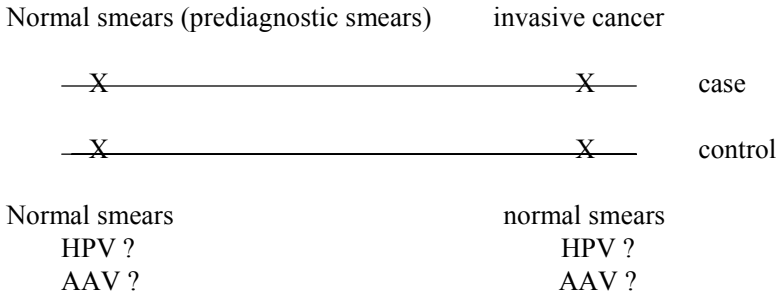

Figure 10. A schematic illustration of the study design for investigating the role of HPV and its cofactors during cervical cancer development.

Paper IV: All samples used in this study were obtained from the population-based cervical cancer screening program of Västerbotten county in northern Sweden. The samples included 50 CIS or CIN patients with 50 matched controls, and 100 invasive cancer patients with 100 matched controls. The controls were selected to correspond to cases according to age and date of sampling. Linkage between the cytology registry and the Swedish Cancer Registry identified cases that developed CIN between 1987 and 1993, as well as cases developing invasive cancer between 1969 and 1995. Controls were women from the same cohort who had normal cytology results at the time of selection. DNA extracted from smears and biopsies was used for the detection of HPV and the CCR2 and CCR5 gene polymorphisms. CCR5-Δ32 was genotyped by conventional PCR fragment analysis. Pyrosequencing technology was used to genotype CCR2-64I.

Paper V: The study includes seventeen women from northern Sweden who developed cervical cancer between 1982 and 2000. All patients were treated in Umeå University Hospital. From the pathological archive, 2 to 3 consecutive biopsy samples from each woman were retrieved that were histologically diagnosed as normal squamous epithelium, cervical inflammation, CIN or invasive squamous cell carcinoma. In total, there were 40 biopsy samples taken from the 17 patients. All specimens were formalin-fixed and paraffin-embedded. Serial sections were cut, and the end section was stained with hematoxylin and eosin to confirm that the lesion was still present in the paraffin block. Immunohistochemistry was used to evaluate the expression of biomarkers $p16^{INK4A}$, $p14^{ARF}$, p53, and proliferating cell nuclear antigen (PCNA). HPV DNA was

detected by nested PCR with MY09/11 and GP5+/6+ and HPV was typed by DNA sequencing. Immunohistochemistry data were scored according to staining intensity, as follows: "-", no expression; "+", weak expression; "++", moderate expression; "+++", strong expression. Nuclear staining with or without cytoplasmic staining was positive for $p16^{INK4A}$ and $p14^{ARF}$. Over 20% of cells staining was considered to be a positive result for $p16^{INK4A}$, PCNA and $p14^{ARF}$ and over 5% of staining was considered positive for p53, according to published protocols (Herbsleb et al., 2001; Klussmann et al., 2003; Tjalma et al., 2001).

4. RESULTS AND DISCUSSION

4.1 Paper I Type-specific multiple sequencing primers, a novel strategy for reliable and rapid genotyping of human papillomaviruses by Pyrosequencing technology

HPVs are highly diverse - there are more than 200 known genotypes, classified based on DNA sequence homology. Among these, approximately 30 types infect the anogenital tract. According to their association with cervical cancer and precancerous lesions, HPVs can be grouped into high-risk, probable high-risk, and low-risk categories. Low-risk HPV– primarily types 6 and 11– cause genital warts (Bosch et al., 1995), while high-risk HPV has been found in 99.7% of cervical cancer (Bosch et al., 1995; Walboomers et al., 1999). The most common types are HPV 16 and 18. HPV types 16, 18, 31, 33, 45 together comprise over 80% of cervical cancer cases (Bosch et al., 1995). Other high-risk HPVs include types 31, 33, 35, 39, 45, 51, 52, 56, 58, 66 and 68. Up to 39% of cases show multiple co-infection with several HPV strains (Kleter et al., 1999). Because of the range of symptoms and patient outcomes associated with different HPV types, accurate detection and genotyping is important for clinical management of cervical lesions and cancer prevention (Giovannelli et al., 2004).

Despite their diversity in certain regions, HPVs are closely related. Most of HPV DNA can be detected by consensus primers, which target both conserved and variable L1 region. There are many different HPV genotyping methods, but conventional DNA sequencing is considered the most reliable as it provides actual sequence data. However, DNA sequencing is limited in its ability to type multiple HPV co-infections in one sample, because multiple sequence signals arise and overlap with one another, making sequence results difficult to interpret. Pyrosequencing is a sequencing-by-synthesis DNA sequencing method and provides sequence signals in real-time and is possible to align the different sequence signals in a pyrogram. However, conventional gel-based sequencing generally does not distinguish multiple infections

In order to accurately genotype HPVs and multiple HPV co-infections, we first evaluated the efficacy of Pyrosequencing by using Single-stranded DNA-Binding Protein (SSB) along with the standard reactants. We found that the sequencing signals were greatly improved by using SSB.

To compare HPV genotyping methods, we sequenced all the amplicons from GP5+/6+ and MY09/11 (82 samples in total) with Pyrosequencing. The primers MY11 and GP5+, as well as seven type-specific sequencing primers that target the most prevalent HPV types (HPV 6, 11, 16, 18, 31, 33 and 45) were used in this assay. With primer

MY11, 72 of 78 samples that were amplified yielded poor-quality sequence data due to the presence of non-specific amplicons (confirmed also by ethidium bromide gel electrophoresis) or multiple HPV co-infections; with primer GP5+, 22 of 82 samples could not be typed correctly due to either non-specific amplification products or HPV co-infections or both; for the seven-type specific sequencing primers, all samples were genotyped accurately.

In order to correctly genotype multiple HPV co-infection, we employed sequence pattern recognition with Pyrosequencing. This technique is based on comparing characteristic sequences specific to each type in the samples. Pattern recognition is not applicable when examining electropherograms obtained by conventional DNA sequencing. Through this method, we could detect different proportions of multiple HPV co-infections. Genotype dominance cannot be detected by conventional DNA sequencing.

Our results suggested that the multiple sequencing primers methods can used to 1) specifically detect HPV and multiple HPV co-infections in a sample, 2) target HPV DNA in a PCR product containing non-specific amplicons, 3) detect dominant and subdominant HPV types, and 4) provide reliable sequence data even for amplicons with low PCR yield.

4.2 Paper II Sentinel-base DNA genotyping using multiple sequencing primers for high-risk human papillomaviruses

Certain types of HPV act as the primary cause of cervical cancer (zur Hausen et al., 1984). Due to the high genetic and functional diversity of HPVs, an accurate and inexpensive genotyping method is necessary for detection of viral persistence (over 4-6 months) and monitoring of antiviral treatment (van Doorn et al., 2001).

In vitro culturing of HPV is difficult, and serological methods lack the required sensitivity (Dillner, 1999). Molecular methods for detecting and genotyping HPV are therefore highly desirable. After successful PCR, HPV can be genotyped by hybridization methods that can give false results. DNA sequencing is therefore the standard method for viral genotyping because it provides sequence information (Feoli-Fonseca et al., 2001; Gharizadeh et al., 2001). In the past, DNA sequencing has been limited to sequencing of single amplicons for single HPV infection.

In order to overcome the problems associated with Sanger sequencing, and to generate an accurate and cost-effective method to genotype HPV, we developed a rapid multiple sequencing primer assay that focuses on a specific single base-calling. This base acts

as a sentinel target to identify the presence of particular HPV genotypes in the specimen. This sentinel-base system targeted 12 most common high-risk HPV genotypes. We compared sequencing results obtained using this sentinel-base method and using the general primer GP5+. We found that 207/244 (85%) of samples were HPV-positive for the 12 high-risk HPV genotypes in our assay, with 147 single infections, 41 double, 10 triple, 7 quadruple and 2 quintuple HPV co-infections. The remaining 37 samples were not in the detection range.

Our results suggested that the sentinel-base technique for identifying the 12 most oncogenic HPVs is capable of genotyping samples containing HPV infection, multiple HPV co-infections, non-specific amplification products and low yield of amplicons. The process is quite user-friendly, the time required for sample preparation, and sequencing has been significantly decreased.

4.3 Paper III Detection of adeno-associated virus type 2 genome in cervical carcinoma

HPV is a necessary factor for cervical cancer development. However, only a small proportion of HPV-infected women will develop cervical cancer – several other cofactors may enhance persistent infection and oncogenesis.

Other STIs may act as cofactors for cervical carcinogenesis. Co-infection of AAV and HPV can occur in the cervix. Among all AAV types, AAV-2 is the most common type found in human (Friedman-Einat et al., 1997). The Rep78 gene of AAV-2 inhibits HPV-induced cell transformation *in vitro* (Hermonat, 1994; Hermonat et al., 1997; Zhan et al., 1999), implying that AAV can impair HPV replication. However, a recent study was unable to detect any significant viral inhibition in all cervical cancer cell lines by the AAV-2 Rep 78 gene (Ahn et al., 2006). AAV seroprevalences have been reported to be lower among women with cervical cancer, but studies on detection of AAV DNA in cervical smears or biopsies do not consistently support the serological findings. Some studies detected a high prevalence of AAV DNA in cervical samples (Ahn et al., 2003; Han et al., 1996; Venturoli et al., 2001), while others failed to detect AAV DNA in cervical samples (Odunsi et al., 2000; Strickler et al., 1999). Coker et al suggested that AAV is associated with reduced risk in HSIL, but not in LSIL (Coker et al., 2001), indicating that the stage of disease may influence the nature of the interaction between AAV and HPV. However, Ahn et al found that AAV is not associated with all stages of cervical precancer and invasive cancer lesions by *in situ* hybridization and immunohistochemistry (Ahn et al., 2003).

In order to investigate the role of AAV in cervical cancer development, we analyzed for the presence of AAV-2 DNA in a population-based, nested case-control retrospective study. With the pan-AAV PCR system, no AAV DNA was detected in any samples. The non-nested AAV-2 PCR system detected AAV DNA in one cervical cancer biopsy and one matched control smear, while the nested AAV-2 PCR was positive for 2/104 (2 %) of the prediagnostic smears of cancer cases and 3/104 (3 %) of the prediagnostic smears of matched control women. At the time of cancer diagnosis, 12/104 (11.7%) of biopsies with invasive cancer and 3/104 (3 %) of matched normal control smears to the biopsies were positive for AAV DNA (RR 4.39 (95% CI: 1.11-20.29)). AAV infection did not seem to persist in any of the women in our study. Results of HPV DNA testing have been reported previously (Wallin et al., 1999): at baseline among cytological smears, HPV was detected in 29.8% (31/104) of cancer cases and 2.8% (3/104) of controls. 27 of the women with cervical cancer had the same HPV type at both the baseline smear and cancer biopsy, while only one match control had the same type of HPV in her baseline smear and her second smear. This persistence confers, therefore, a 58.7% increased risk of developing cancer (95% CI 10.2-∞) (Wallin et al., 1999).

When we correlated the presence of AAV-2 DNA with HPV DNA within the same sample for all available patients, none of the prediagnostic smears and their matched control smears had both AAV and HPV, while 10 biopsy case samples had both viruses. 97 of the control samples were negative for both HPV and AAV-2.

The low prevalence of AAV in our study group could be related to the nature of the specimen. Fixed and stained samples often contain partially degraded DNA. To overcome any technical problems, the PCR systems used were carefully optimized and validated with variable template volumes, in order to overcome the effects of inhibition. Furthermore, the PCR products produced were within the range suitable for amplification by PCR from archival DNA. However, biopsies contain more cells than Pap smears, which could have led to greater success in amplifying AAV DNA. The detection limits for the PCR systems we used were similar for both biopsy and Pap smear specimens. Despite the higher AAV DNA prevalence among the invasive cancers (11%) as compared to the matched smears from control women, the data are probably not adequate to state a conclusive association between the outcome of HPV infection and AVV. A larger sample population utilizing fresh smears or biopsies would be ideal for this purpose. Our results indicate that only a low proportion of cervical cancer biopsies contain AAV-2 genomes, and AAV DNA persistence was not detectable.

4.4 Paper IV Genetic polymorphism of chemokine receptors CCR2 and CCR5 in cervical neoplasia

HIV-infected patients have a higher risk for the development of cervical cancer. These patients are immunocompromised, therefore immunological-related factors have been suggested as a risk factor in cervical cancer development. Furthermore, natural polymorphisms or genetic variations in immune-related genes might be related to HPV persistence and progression to cancer. Immune response genes have been linked to cervical cancer pathogenesis (Ghaderi et al., 2000).

CCR5 and CCR2 are two human chemokine receptors that are mainly expressed on immune and inflammatory cells, such as such as B- and T-lymphocytes and professional antigen-presenting cells. Genetic variations within the chemokine receptor genes have been linked to inflammatory-mediated and autoimmune disorders (Garred et al., 1998; Nakajima et al., 2002; Zapico et al., 2000). CCR5-Δ32 is a modified CCR5 gene with a 32-bp deletion, which encodes a truncated, inactive receptor not expressed on the cell membrane. CCR2 has a valine to isoleucine substitution at aa 64 (a G→A mutation at nucleotide 190) in the first transmembrane region of the receptor (Smith et al., 1997). It has been suggested that CCR5-Δ32 and CCR2-64I alleles could be important for protection or susceptibility to immune-mediated disorders (Berger, 1997; Berger et al., 1999; Sica et al., 2000; Smith et al., 1997).

In order to investigate the distribution of CCR2-64I and CCR5-Δ32 polymorphisms, and their association with HPV infection among cervical neoplasia and cancer samples, we conducted a population-based case-control study. The distribution of the CCR2 and CCR5 genotypes from our study group of 150 patients (with CIN or invasive cancer) and 150 healthy controls were representative of the general population according to Hardy-Weinberg equilibrium analysis.

The CCR5 analysis showed that individuals with the Δ32/Δ32 genotype have a 4.58-fold increased risk (CI 0.40-52.64, p=0.045) for HPV positivity, but no association with cervical neoplasia; Δ-32 mutation at the CCR locus is imperceptibly associated with increased risk of HPV infection, in contrast, the CCR2-64I polymorphism did not confer any increased risk for either HPV infection or cervical neoplasia.

Our data on the frequency of the CCR5 Δ32 and CCR2 64 I genotypes is consistent with a previous study which also targeted European Caucasians (Gonzalez et al., 2001). Δ32/Δ32 of CCR5 is a rare genotype with a frequency of only about 1% in the healthy

population (Martinson et al., 1997; Zhao et al., 2003). Due to the small study group utilized here, we cannot confidently conclude the role of this genotype in HPV infection. To confirm our finding, a considerably larger population should be analyzed for CCR mutations.

4.5 Paper V Predictive significance of the alterations of p16INK4A, p14ARF, p53 and proliferating cell nuclear antigen expression in the progression of cervical cancer

During carcinogenesis, high-risk HPV oncoproteins interact with host cellular proteins, particularly cell cycle proteins, resulting in genome instability and cell immortality. These host cellular proteins can act as biomarkers to indirectly predict HPV infection and allow unambiguous identification of disease progression and HPV-related disease in epithelial cells (von Knebel Doeberitz, 2002). These biomarkers predict the biological behaviors of the disease and the need for clinical intervention or continued surveillance after a preventive or therapeutic measurement. They also have the potential to enhance the screening efficiency of the Pap smear or even HPV DNA testing (Klaes et al., 2002).

$p14^{ARF}$ and $p16^{INK4A}$ arise from same gene; both are strongly associated with HPV-positive cervical cancer (Kanao et al., 2004). High-risk HPV oncoproteins E6 and E7 bind and suppress the tumor suppressor genes p53 and pRb, respectively, resulting in cell over-proliferation and genome instability, and eventually tumorgenesis. $p14^{ARF}$ is cell cycle regulator that inhibits the MDM2 proteins, blocking the formation of MDM2-p53 and preventing p53 degradation (Zhang et al., 1998). When high-risk HPV E7 degrades pRb, it releases the cellular transcription factor E2F1, thereby increasing cyclin A, cyclin E and $p16^{INK4A}$ expression (Giarre et al., 2001). Because over-expression of $p16^{INK4A}$ is related to HPV infection and high-grade squamous neoplasia (Guo et al., 2004), $p16^{INK4A}$ has been suggested as an additional screening tool in cervical cancer screening (Murphy et al., 2004; Murphy et al., 2003; von Knebel Doeberitz, 2001).

PCNA is involved in cell proliferation, particularly those with active DNA replication. However, PCNA cannot distinguish normal and abnormal cell proliferation.

In order to understand the role of these markers in the development of cervical cancer, we employed immunohistochemistry to investigate the expression of $p14^{ARF}$, $p16^{INK4A}$, PCNA, and p53 on paraffin sections of serial consecutive biopsies from cervical cancer patients. The results showed that $p16^{INK4A}$ over-expression was significantly higher in CIN (75%) and in SCC (75%) specimens than in normal or cervical inflamed samples

(12.5%; $P < 0.01$, $P < 0.05$, respectively). $p14^{ARF}$ expression was higher in SCC samples (83%) than in normal/cervical inflamed samples (25%; $P < 0.05$). PCNA expression was negative in normal or cervical inflamed cases, but an increase in expression was seen in 63.2% of CIN and 100% of SCC specimens ($P < 0.01$, $P < 0.05$). p53 expression increased gradually with progression of lesions and was up-regulated in HPV-negative to HPV-positive status (57%), as compared with 30% of cases with persistent HPV infection.

Our results suggested that increased $p16^{INK4A}$ and $p14^{ARF}$, and decreased or stable p53 expression are associated with disease progression. The times for progression were shorter among patients whose specimens stained positive for $p16^{INK4A}$ or $p14^{ARF}$, and those with altered p53 expression.

5. CONCLUSIONS

This thesis work aimed at improving the HPV genotyping methods by Pyrosequencing, investigating HPV cofactors in cervical cancer development, and evaluating biomarkers for cervical cancer prevention. The primary conclusions of the studies in this thesis demonstrate that:

- By using the multiple type-specific sequencing primers method, we were able to genotype a panel of 65 cervical and 17 oropharyngeal samples for the most clinically important HPV types (HPV 6, 11, 16, 18, 31, 33 and 45) using Pyrosequencing technology. By introducing sequence pattern recognition we were able to identify multiple HPV co-infections in the same sample. We also showed that the application of multiple sequencing primers is suitable for PCR products consisting of non-specific amplicons and for samples with low amplification yield.

- HPV genotyping using multiple sequencing primers that target a single "sentinel" base was successfully applied to twelve high-risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59). This technique permits accurate and rapid genotyping of single HPV infection as well as multiple HPV co-infections in the same sample.

- There is an association between AAV-2 and cervical cancer. A larger population-based study will be required to confirm this finding.

- Analysis of genetic variations within the chemokine receptor CCR5 and CCR2 genes demonstrated that the Δ32 mutation on the CCR5 locus is associated with a 4.58-fold increased risk of HPV infection. CCR2-64I did not show any clear association with HPV infection. There is no evidence of association between the two chemokine receptor gene polymorphisms and cervical neoplasia.

- Increased $p16^{INK4A}$ and $p14^{ARF}$, and decreased or stable p53 expression, are associated with cervical cancer progression. The disease developed more quickly in patients whose samples stained positive for $p16^{INK4A}$ or $p14^{ARF}$, and in those with altered p53 expression. Thus, these biomarkers can act as prediction markers for cervical cancer progression

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