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Adenocarcinoma of the cervix uteri. Methods to improve diagnostics: biological markers and HPV testing

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ADENOCARCINOMA OF THE CERVIX UTERI. METHODS TO IMPROVE DIAGNOSTICS:BIOLOGICAL MARKERS AND HPV TESTING

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ABSTRACT

Cervical adenocarcinoma (ADC) represents about 20% of invasive cervical cancers. Implementation of screening programs that have led to a decrease in squamous cell carcinoma (SCC) has only had a limited preventive effect on adenocarcinoma as the incidence of ADC, especially among younger women, has steadily increased in recent decades. Human papillomavirus (HPV) is considered the most important single factor for development of SCC. Adenocarcinomas of the cervix are also related to HPV of high risk types (HR-HPV), but the correlation is less pronounced. HPV infection alone is insufficient and other factors are required for oncogenic transformation to ADC.

The aim of this thesis is to investigate molecular markers such as tumor suppressor $p16^{INK4a}$, proliferation marker Ki-67, possible tumor suppressor proteins in the LRIG family, the TP53 target WIG-1, and E6/E7 mRNA in cervical adenocarcinoma in order to provide a deeper understanding of the etiology of the disease and to develop more efficient screening strategies for adenocarcinoma of the cervix.

Formalin-fixed/paraffin-embedded tumor tissue samples of cervical ADC were investigated by immunohistochemistry (IHC) for the occurrence of $p16^{INK4a}$ using the CINtec® kit, of Ki-67 with the MIB-1 antibody, and of LRIG1, LRIG2 and LRIG3 using polyclonal rabbit antibodies. In 38 samples of cervical cancer (ADC or SCC), Wig-1 was examined by IHC using a monoclonal antibody. In cervical cancer cell lines the WIG-1 gene locus was investigated by SKY, CGH, Southern and FISH; mRNA expression by Northern and RT-PCR; and protein expression by Western analysis. Testing for E6/E7 mRNA was carried out using the PreTect HPV Proofer; testing for HR-HPV DNA was done by consensus PCR and subsequent typing by SSCP, direct sequencing and Linear Array®. The examined markers were evaluated regarding prognostic impact and association with HPV infection.

We found that poorly differentiated tumors stain with less intensity and in a lower fraction than do well differentiated tumors. All HPV-positive tumors showed $p16^{INK4a}$ staining, but 60% of HPV-negative tumors also stained for $p16^{INK4a}$. We found a significant correlation between staining for Ki-67 and histological grade ($p=0.031$) as well as worse outcome ($p=0.004$).

High staining intensity for LRIG1 and a high fraction of LRIG3-positive cells were significantly associated with improved patient survival ($p=0.03$ and $p=0.04$). LRIG1 and LRIG3 expression correlated with HPV infection, since higher staining intensity was observed in HR-HPV-positive cases.

mRNA extraction from paraffin-embedded tissue samples was successful, as shown by positive results in the GAPDH mRNA integrity control in all cases. HR-HPV infection was detected by mRNA in 64% of the tumors, compared with detection by DNA in 62% of the tumors. There was an 87% agreement in results between the two methods regarding HPV positivity and 84% agreement regarding HPV type.

WIG-1 is not the primary target for genomic alteration on chromosome 3, even though analysis revealed chromosome 3 gains in all lines. WIG-1 mRNA expression was

higher in the two HPV-negative cervical cell lines (C33-A, HT-3) than in the HPV-positive lines. Wig-1 expression in tumor tissue, as assessed by IHC, showed significantly higher nuclear Wig-1 levels in ADC than in SCC ($p<0.0001$). We observed higher nuclear Wig-1 expression in the HPV-negative tumors than in HPV-positive tumors ($p = 0.002$). Patients with tumors that demonstrated moderate nuclear and positive cytoplasmic Wig-1 expression had a better prognosis ($p = 0.042$) than those with high nuclear and negative cytoplasmic Wig-1 expression.

According to our findings the tested markers appear to be potential diagnostic supplements for cervical adenocarcinoma as expression of Wig-1 as well as LRIG1 and LRIG3 could serve as prognostic markers, $p16^{INK4a}$ and Ki-67 might be helpful markers for grading endocervical malignancies and mRNA testing has been shown to be as sensitive as DNA testing. The combination of improved detection of precursors through integration of molecular markers into screening programs with vaccination against HPV 16 and 18 will help cervical cancer including adenocarcinoma to become one of the most preventable cancers.

LIST OF PUBLICATIONS

I. SUSANNE MÜLLER, Carmen Flores-Staino, Barbro Skyldberg, Ann-Cathrin Hellstrom, Bo Johansson, Bjorn Hagmar, Keng-Ling Wallin and Sonia Andersson
Expression of p16^{INK4a} and MIB-1 in relation to histopathology and HPV types in cervical adenocarcinoma.
International Journal of Oncology 2008 Feb; 32(2):333-40

II. Hovland S, SUSANNE MÜLLER , Skomedal H, Mints M, Bergström J, Wallin KL, Karlsen F, Johansson B, Andersson S
E6/E7 mRNA expression analysis: a test for the objective assessment of cervical adenocarcinoma in clinical prognostic procedure.
International Journal of Oncology 2010 Jun; 36(6):1533-9

III. SUSANNE MÜLLER, Lindquist D, Kanter L, Flores-Staino C, Henriksson R, Hedman H, Andersson S
Expression of LRIG1 and LRIG3 correlates with human papillomavirus status and patient survival in cervical adenocarcinoma.
International Journal of Oncology 2013 Jan; 42(1):247-52

IV. Lidi Xu, SUSANNE MÜLLER, Srinivasan R. Thoppe, Fredrik Hellborg, Mikael Lerner, Biying Zheng, Nohamin Tamyalew, Svetlana Lagercrantz, Dan Grandér, Keng Ling Wallin, Klas G. Wiman, Catharina Larsson and Sonia Andersson
Expression of the p53 target Wig-1 is associated with HPV status and patient survival in cervical carcinoma
To be submitted to International Journal of Cancer in November 2013

CONTENTS

LIST OF ABBREVIATIONS

ADC	Adenocarcinoma
AIS	Adenocarcinoma in situ
ASC-US	Atypical squamous cells- uncertain significance
ASR	Age standardized ratio
CC	Cervical cancer
CIN	Cervical intraepithelial neoplasia
E6AP	E6 associated protein
FFPE	Formalin fixed paraffin embedded
HC	Hybrid Capture
HPV	Human papillomavirus
HR-HPV	High risk HPV
HSIL	High grade squamous intraepithelial lesion
IARC	International Agency for Research on Cancer
ICC	Invasive cervical cancer
ICESCC	The International Collaboration of Epidemiological Studies of Cervical Cancer
IHC	Immunohistochemistry
LR-HPV	Low risk HPV
LRIG	Leucine-rich repeats and immunoglobulin-like domains
LSIL	Low grade squamous intraepithelial lesion
OR	Odds ratio
ORF	Open reading frame
PV	Papilloma virus
RCT	Randomized Controlled Trial
SCC	Squamous cell cancer
SCJ	Squamocolumnar junction
TERC	Human telomerase gene
TZ	Transformation zone
WIG-1	Wild type p53-induced gene 1

1 POPULÄRVETENSKAPLIG SAMMANFATTNING

Livmoderhalscancer är den tredje vanligaste tumörtypen hos kvinnor i hela världen. Det finns två olika former av livmoderhalscancer beroende på vilka celler som omvandlas till cancer. I Sverige, liksom i många andra utvecklade länder, har förekomsten av skivepitelcancer i livmoderhalsen minskat dramatiskt de sista årtiondena, till följd av gynekologisk screening med cellprover sedan 60-talet. Trots detta drabbas ca 450, till övervägande del unga kvinnor, av livmoderhalscancer och 150 kvinnor dör årligen av sin sjukdom. När det gäller adenocarcinom i livmoderhalsen (mindre vanlig form, ca 20 % av alla tumörer i livmoderhalsen, som utgår från körtelepitelceller) har däremot inte någon minskad frekvens observerats utan snarare en stegring, främst hos yngre kvinnor. Adenocarcinom har inte samma tydliga förstadium som skivepitelcancer, och det ger sig inte lika ofta till känna vid screeningen. Diagnostiken som bedrivs inom dagens rutinsjukvård, cytologprovet, är ett otillräckligt screeninginstrument för att upptäcka adenocarcinom i tidigt skede, och ger tyvärr ofta otillräcklig information. Idag vet vi att livmoderhalscancer orsakas av ett virus, humant papillomvirus (HPV), som sprids sexuellt. Vidare vet vi att HPV är en av de vanligaste, om inte den vanligaste, sexuellt överförbara sjukdomen bland unga individer i världen idag. Detta gör livmoderhalscancer till en av de vanligaste cancerformar bland kvinnor i världen. Humant papillomvirus (HPV) av s.k. "högrisk typ" är en förutsättning för utveckling av livmoderhalscancer och nästan 100 % av skivepitelcancer har visats innehålla "högrisk" HPV, men ytterligare faktorer bidrar till cancerutvecklingen. Adenocarcinom innehåller HR-HPV i 70-80% av alla fall. Det visade sig att det fanns HPV-16 och HPV-18 i normala cytologiska prover 14 år innan diagnosen av adenocarcinom ställdes. HPV typning som komplement skulle kunna öka möjligheten att upptäcka adenocarcinom i tidigt skede, främst hos unga kvinnor, en grupp som oftast har HPV-positiva tumörer.

Målet med denna avhandling var att undersöka molekylära markörer i fall av adenocarcinom och deras relation till HPV samt prognosen av cancersjukdomen. Nya känsliga och specifika tester som skulle kunna förbättra den gynekologiska hälsokontrollen är av största värde.

I det första delarbetet analyserade vi 101 arkivprover av tumörvävnad från adenocarcinom i livmodertappen, med avseende på expression av $p16^{INK4a}$ (en tumörhämmare) och Ki-67 (en tillväxt markör), och resultat relaterades till HPV-infektion och stadie av cancer och den mikroskopiska tumörbilden. Vi fann att tumörer som var lågt differentierade uttryckte cancer markören $p16^{INK4a}$ i mindre utsträckningen och med lägre intensitet än de högt differentierade tumörer. Alla tumörer som innehåll HPV, och även 60 % av tumörer utan HPV infektion uttryckte $p16^{INK4a}$. Dessutom fann vi ett starkt samband mellan Ki-67 och den mikroskopiska tumörbilden. Sämre prognos var förknippat med högre Ki-67 uttryck.

Den andra studien undersökte värdet av olika metoder för detektion av HPV infektion. Mätning av HPV mRNA av de fem vanligaste typerna som tecken på expression av tumörbefrämjande gener jämfördes med resultat från HPV DNA mätningen hos kvinnor med adenocarcinom. Vi visade att denna metod hade ungefär samma

känslighet som HPV DNA metoden, E6/E7 mRNA av de 5 HR-HPV typer uppvisades i 64 % av tumörerna jämfört med HPV DNA som vi fann bara i 62 % av alla tumörer. Resultat mellan metoderna överenstämde i 87 %.

Den tredje delarbete testade hypotesen om att påvisande av LRIG1, 2 och 3 i tumörvävnader skulle kunna användas som en prognostisk markör hos patienter med adenocarcinom i livmodershalsen. Vi fann att hög immunfärgning för LRIG1 och LRIG3 var förknippat med förbättrad överlevnad och det fanns ett samband mellan uttrycket av LRIG1 och LRIG3 och HPV infektion.

I den fjärde studien undersökte vi WIG-1. Det är en av de gener som kan uppregleras av TP53 (en s.k. tumörhämmare och ha en central roll för att skydda oss mot cancer), men WIG-1 kan också själv reglera TP53. Vi undersökte därför uttryck av Wig-1 protein i livmoderhalscancer och fann kraftigare uttryck av proteinet i körtelcellcancrar än i skivepitelcancrar. Vi hittade högre uttryck i cellkärnor hos HPV-negativa tumörer jämfört med HPV-positiva tumörer.

Adenocarcinom i livmoderhalsen är en svår diagnostiserad sjukdom. Att påvisa korrelation mellan förändringar i cancercellen och kliniskt förlopp av tumörsjukdomen är önskvärt. Socialstyrelsen har förordat införande av vaccin för flickor mellan 10-12 års ålder redan hösten 2008. Vaccination mot HPV är ett nytt effektivt verktyg som kommer att utgöra första steget i den kedjan av åtgärder som ingår i prevention av livmoderhalscancer. För första gången ges möjlighet till effektiv primärprevention av livmoderhalscancer, men vaccinet kommer inte att skydda mot alla virustyper. Därför är det desto viktigare att fånga upp kvinnor som löper risk för adenocarcinom, genom att hitta tidiga markörer för snabb och inriktad prevention, vilket är hela detta projekts utgångspunkt.

2 BACKGROUND

2.1 THE CERVIX

The uterine cervix is the lower part of the uterus (Figure 2-1) and is composed of dense fibro muscular tissue. The outer part of the cervix protrudes into the vagina. It mainly functions as a sphincter (especially during pregnancy), a barrier to prevent ascending infections and a source of lubricating mucous.

The outside of the cervix is called the ectocervix and the inner portion is called the endocervix. The ectocervix is covered by nonkeratinized stratified squamous epithelium (Figure 2-2a), while the endocervix is covered by a single layer of mucin-secreting columnar epithelium (Figure 2-2b). The cervical canal runs through the cervix. The internal os is where the canal opens into the uterus and the external os is where it opens into the vagina.

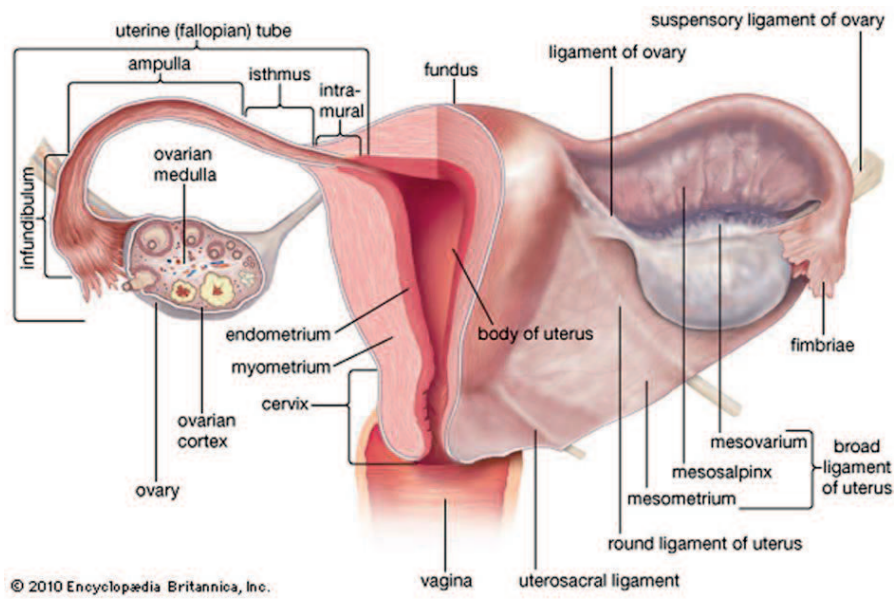

Figure 2-1 Anatomy of the human uterus.
(taken from http://global.britannica.com/EBchecked/topic/620603/uterus)

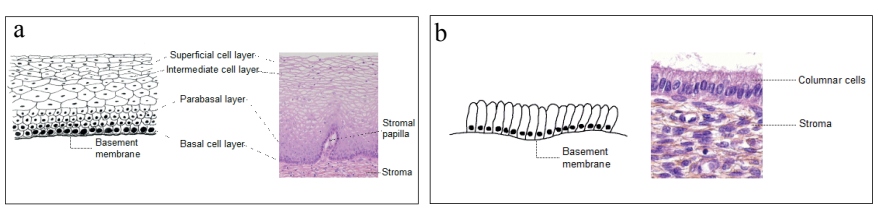

Figure 2-2 Stratified squamous epithelium (a); columnar epithelium (b); taken from IARC (http://screening.iarc.fr/colpochap.php?chap=1).

2.1.1 Transformation zone (TZ)

The original squamocolumnar junction (SCJ) between squamous and columnar epithelium, called the congenital junction, remains unchanged until puberty. Post puberty, in addition to the congenital junction, the adult or functional junction forms and is termed the new SCJ. The columnar epithelium is exposed to the acid, non-sterile environment of the vagina and will gradually be replaced by squamous epithelium through a process known as metaplasia (Schiffman *et al.* 2007), which occurs in the transformation zone (TZ). The TZ occupies the area between the original and the new SCJ and becomes larger with age as it expands toward the cervical opening (Figure 2-3).

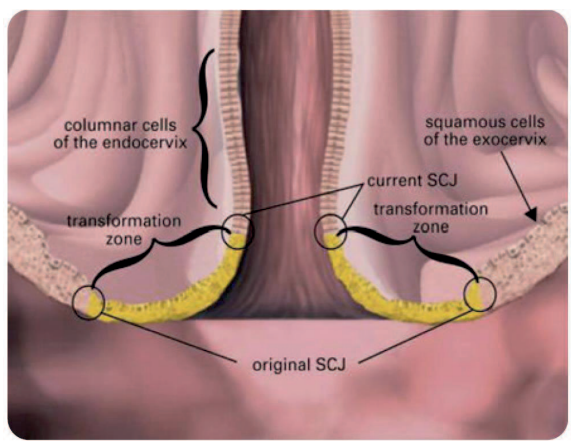

Figure 2-3 Transformation zone with original and current squamocolumnar junction in an adult woman; taken from: Herzog *et al.* 2007.

The TZ plays a crucial role in the pathogenesis of cervical cancer (CC). It is assumed that oncogenic HPV infect reserve cells of the TZ that are committed to glandular differentiation, which eventually leads to the proliferation of glandular lesions and AIS (Herzog *et al.* 2007; Doorbar *et al.* 2012). A special target cell in the squamocolumnar junction with embryonic characteristics has been described. This cell is highly susceptible to oncogenic HPV DNA incorporation and is speculated to be strongly related with cervical carcinogenesis (Herfs *et al.* 2012).

2.2 CERVICAL CANCER

2.2.1 Histology, clinical features and diagnosis

There are two major histological types of cervical cancer, one derived from each of the two different types of cervical epithelium: squamous cell carcinoma (SCC) and adenocarcinoma from glandular cells (ADC). The highest incidence of cervical cancer, 80-95%, is attributable to SCC. ADC accounts for 5–20% of all cervical cancers (Jemal *et al.* 2011). ADC may be categorized into further subgroups such as mucinous and non-mucinous adenocarcinoma. The most common histological type of invasive adenocarcinoma is mucinous adenocarcinoma. There are also carcinomas composed of a mixture of malignant glandular and squamous cells, known as adenosquamous

carcinoma, and in very rare cases, cancer may even arise from other types of cervical cells (IARC publications chapter 5).

The cardinal symptom of invasive cervical cancer is abnormal bleeding. In addition, vaginal discharge and in advanced cases, pelvic or lower back pain and/or sciatic nerve root pain radiating into the lower extremities may occur. The presence of bowel or urinary symptoms would indicate advanced late-stage disease. Clinical suspicion of cervical cancer requires work-up that includes biopsy or diagnostic conization for a definitive histological diagnosis. Once the diagnosis is confirmed, the stage of the disease needs to be ascertained to determine a suitable treatment plan. The International Federation of Gynecology and Obstetrics (FIGO) has formulated a widely used and recommended system as described in
Table 2-1 (Pecorelli *et al*. 2009; Quinn *et al.* 2006).

FIGO stage	**Extension of tumor**
Stage I	*Carcinoma confined to the cervix.*
IA	Microinvasive carcinoma, not clinically visible. Can only be diagnosed by microscopy.
IA1	Stromal invasion <3mm in depth and <7 mm horizontal spread
IA2	Stromal invasion ≥3≤5 mm in depth and < 7 mm horizontal spread
IB	Carcinoma clinically visible; or a microscopic lesion greater than IA2
IB1	≤4 cm in greatest dimension
IB2	≥4 cm
Stage II	*Carcinoma spread beyond the cervix, but not as far as the lower third of the vagina or the pelvic wall*
IIA1▪	Tumor size <4 cm with involvement of less than the upper two-thirds of the vagina, but not extending to tissues surrounding the uterus (parametria).
IIA2▪	Tumor size ≥4 cm with involvement of less than the upper two-thirds of the vagina and without invasion of parametria.
IIB	Parametrial invasion, but not as far as the pelvic wall or the lower third of the vagina.
Stage III	*Tumor extends to pelvic wall or involves the lower third of the vagina, or causes hydronephrosis or non-functioning kidney*
IIIA	Extension to the lower third of the vagina, with no extension to the pelvic wall, and no hydronephrosis or non-functioning kidney
IIIB	Extension to the pelvic wall, with hydronephrosis or non-functioning kidney.
Stage IV	*Tumor has spread.*
IVA	Spread to involve the mucosa of the bladder or rectum.
IVB	Spread to distant organs, such as extrapelvic lymph nodes, kidneys, skeleton, lungs, liver and brain.

Table 2-1 Summary of the FIGO stages (WHO, 2006) ▪ Subdivision of Stage IIA according to Pecorelli et al, 2009.

2.2.2 Epidemiology

Currently, cervical cancer ranks third among the most common female malignancies (after breast and colorectal cancer) with an estimated 530,000 new cases and 275,000 cancer deaths in 2008 (de Martel *et al.* 2012). Incidence varies immensely between different regions, with the highest risk seen in Eastern and Western Africa where age-standardized rates (ASR) are >30/100,000, while the lowest risk is seen in Western Asia, North America and Australia where ASRs are <6/100,000. More than 85% (450,000 cases) of the global burden of cervical cancer occurs in developing countries, where the majority of women have little or no access to preventive screening and adequate treatment nor, more recently, to HPV vaccination (GLOBOCAN 2008, Figure 2-4).

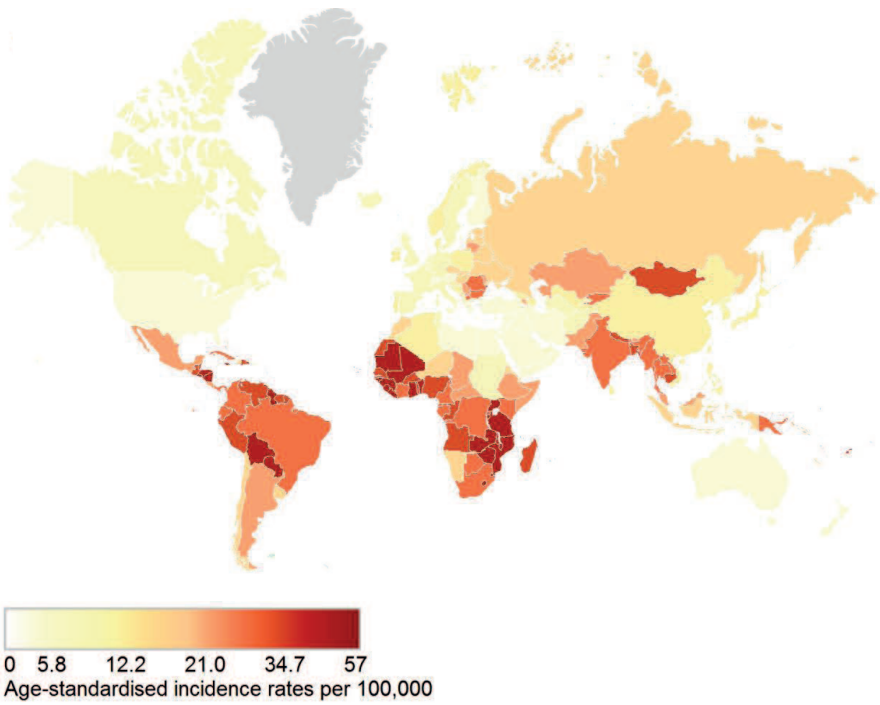

Figure 2-4 Global map showing the estimated age- standardized cervical cancer incidence rate per 100,000 worldwide in 2008; taken from GLOBOCAN 2008, IARC (http://globocan.iarc.fr/factsheet.asp).

Incidence and mortality rates of CC in countries with well-organized screening programs have decreased significantly since the introduction of screening. In Sweden the incidence of CC has declined from 20 cases per 100,000 women in 1965 and seems to have leveled off with a rate of approximately 7 cases per 100,000 since the late 1990s (The National Board of Health and Welfare 2012). In the 35-39 year age group, the National Swedish Cancer Registry shows a drop in age-specific rate from 46.0 per 100,000 women in 1958 to 15.5 in 2004 – a 66% reduction. In the 70-74 year age group the rate dropped from 27.0 to 11.4 – a 42% reduction. The Swedish Cause of Death

Register shows an age standardized mortality rate of 7.7 per 100,000 women for CC in 1959 and 2.8 in 2004 (Pettersson *et al*. 2011).

In contrast to rates for squamous cell cervical carcinoma, the incidence rates of cervical adenocarcinoma have steadily increased in developed countries in women born in 1930 and in successive cohorts thereafter through the mid-1960s (Gustafsson *et al*. 1997, Vizcaino *et al*. 2000). The age-adjusted incidence rates for ADC increased throughout Europe and ranged from ~0.5 to >3% per year (Vizcaino *et al.* 1998; Bray *et al.* 2005). This upward trend, noted particularly among women under age 40, has occurred despite extensive Pap smear screening (Vizcaino *et al.* 1998; Visioli *et al.* 2004; Bray *et al*. 2005, Bergström *et al.* 1999; Wang *et al.* 2004).

The Swedish National Cancer Registry shows an increase in incidence of ADC in the 25-29 year age group from 0.7 per 100,000 for women born between 1935 and 1939, to 1.2 per 100,000 for women born between 1950 and 1954. In the 30-34 year age group a similar increase was seen from 1.0 for women born between 1930 and 1934 to 1.9 for women born between 1945 and 1949 (Pettersson 1998). A subsequent study also showed a twofold increase in incidence of ADC during a 35 year period (Bergström *et al.*1999).

Studies have shown that SCC and ADC of the cervix seem to share most risk factors. However, our understanding of the epidemiology and natural history of CC may still be incomplete.

The strong link between infection with HPV 16 or 18 and malignant transformation of the glandular epithelium of the cervix suggests that screening for these two HPV types might be a useful tool for improving prevention and/or early detection of cervical adenocarcinoma, which has proven to be difficult through regular cytological screening (Sasieni *et al*. 2009). It was recently estimated that Swedish women who did not undergo regular cytological screening had a modest 60% increased risk of being diagnosed with cervical adenocarcinoma, but a 200% increased risk of squamous cell carcinoma, compared with women who participated in screening. Thus cytological screening provides substantially lower protection against adenocarcinoma than squamous cell cancer of the cervix (Andrae *et al.* 2008).

An increase of in-situ forms has preceded the decrease of invasive cancer for squamous lesions. Paradoxically, the rising incidence of AIS over the past decades has corresponded with an increase in ADC rates (Hemminki *et al.* 2002; Wang *et al*. 2004; Sherman *et al.* 2005).

2.2.3 Risk factors

As early as the nineteenth century, the association between cervical cancer and sexual activity was suggested by the absence of cervical cancer among nuns (Rigoni 1987). Research to demonstrate the possible role of HPV in cervical cancer began in 1974-1976 (z. Hausen 2009) and eventually garnered the Nobel Prize in 2008. The first papilloma virus types were isolated 35 years ago (Orth *et al.*1977) and in 1983 a new type was isolated from cervical cancer tissue and called human papillomavirus type 16 according to order of discovery (Dürst *et al.* 1983). Sexually transmitted HPV is now

recognized as playing a causative role in the development of cervical cancer (Munoz 2000). By the last decade, HPV had been detected in almost all (99.7%) cervical cancers (Walboomers *et al.* 1999).

Although HPV infection is necessary for development of cervical cancer, that alone is insufficient, because the majority of HPV-infected women do not develop cervical cancer. Since the establishment of the causative role of HPV infection, previous risk factors for cervical cancer have now been redefined as surrogate markers for risk of HPV or as important cofactors (Bosch *et al.* 2002).

Lifetime number of sexual partners is a surrogate marker, highly correlated with HPV exposure and thereby cervical cancer. The International Collaboration of Epidemiological Studies of Cervical Cancer (ICESCC) conducted a reanalysis showing that risk of cervical cancer increases with total number of sexual partners. A lifetime history of >6 partners doubles risk, while >11 partners triple risk, compared with women who had only one partner. However, risk of becoming infected with HPV depends on many factors, including the sexual behavior of the partner (ICESCC 2009). First intercourse at an early age also increases risk of cervical cancer, which is subject to a variety of interpretations. Early first intercourse may be a marker of high-risk behavior and also of more HPV infections. According to another interpretation, early first intercourse may increase duration of HPV infections, which could be more harmful. A third interpretation may be that the cervix is more susceptible to HPV infection in young women (ICESCC 2009).

Medium (5-9 years) or long-term use (>10 years) of combined estrogen-progestin contraceptives is considered to be a co-factor. Combined oral contraceptives are classified by the IARC as carcinogenic factors because they increase the risk of cervical cancer, as well as breast and hepatocellular cancer (Cogliano et al. 2005). A large 2007 reanalysis of 24 studies, showed a twofold increase for developing cervical cancer among current medium-term and long-term users which declines after use ceases (ICESCC 2007). Use of combined oral contraceptives is not believed to increase susceptibility to HPV infection, for which reason the increase in risk appears to be associated with established HPV infection (Cogliano *et al*. 2005). However, no significant difference between women with and without HR-HPV infection was found in the reanalysis study (ICESCC 2007).

The IARC also classifies smoking as a carcinogenic factor for cervical cancer (Secretan *et al.* 2009). According to the ICESCC, smoking increased the risk of invasive cervical cancer (ICC) or carcinoma in situ (CIS)/ cervical intraepithelial neoplasia 3 (CIN 3), where relative risk (RR) of 1.60 was found compared with never-smokers. Risk was also increased for former smokers, though less so than among current smokers (RR: 1.12). However, the increase in risk for current and former smokers applied only to SCC and was not demonstrated for ADC (ICESCC 2006).

Another co-factor for development of cervical cancer analogous to exogenous hormonal use is high parity. The risk of cervical cancer increases with the number of full-term pregnancies. An IARC study of HPV-positive women demonstrated a fourfold risk among women with more than 7 full-term pregnancies, compared with

nulliparous women, while risk among women who had only one or two full-term pregnancies was doubled (Bosch *et al.* 2002).

Herpes simplex type 2 (HSV-2) infection was found to double risk of SCC and triple risk of ADC in an IARC study of HPV-positive women (Smith *et al.* 2002a, de Sanjose *et al.* 1994). One Swedish cohort study showed that women with prior history of Chlamydia trachomatis infection were at increased risk (RR: 2.09) of HPV persistence, but no such correlation was demonstrated for HSV-2 (Silins *et al*. 2005). Chlamydia trachomatis infection also doubled risk of SCC, but no association was found with ADC (Smith *et al.* 2002b).

A case-control study showed that prevalence of cervical cancer in HPV was higher among HIV-infected women. The explanation for the increased prevalence may relate to immunosuppression causing persistent infection, as well as to riskier sexual behavior among HIV-positive women, which in turn may be associated with greater risk of other STIs such as HPV (Odida *et al.* 2011). However, subjecting the data concerning cancer incidence among HIV-positive women and patients receiving immunosuppressive treatment, such as transplant recipients, to meta-analysis showed that both these groups were more likely to develop infectious disease-related cancers, including HPV-related cancers. Similar patterns among patients with HIV infection and patients with iatrogenic immunosuppression suggest that immunosuppression, rather than other risk factors may be responsible for the increased risk of cancer (Grulich *et al.* 2007; Frisch *et al.* 2000). An inverse association between degree of neoplasia and interleukin-2-production in response to HPV-16 E6 and E7 proteins has been observed (Tsukui *et al.* 1996). As well as an impaired T-helper cell response been discussed to predispose for the progression of cervical disease (Hildesheim *et al.* 1997).

Genetic predisposition is another proposed co-factor for cervical cancer. In 1999, data from a Swedish register study could supply first evidence for a significant familial clustering of cervical cancer which is likely to result from genetic rather than environmental factors. The results were not specified for histological type (Magnusson *et al.* 1999). Subsequent studies supported the hypothesis of hereditary co-factors (Czene *et al.* 2002; Zelmanovic Ade *et al.* 2005; Hemminki *et al*. 2006; Couto *et al*.2006).

Differences in Co-factors between SCC and ADC

Some heterogeneity in the co-factors may be associated with adenocarcinoma. According to Castellsague et al., cofactors having a statistically significant positive association with ADC and HPV-positive cases included long-term use of OCs and very high parity. IUD use was shown to have a protective effect (Castellsague *et al.* 2006).

In contrast to SCC, several studies found no association between smoking and incidence of cervical ADC (Green *et al.* 2003**;** Plummer *et al.* 2003**;** ICESCC 2006**;** Berrington de Gonzalez *et al.* 2004). Nor was any correlation confirmed between age at first birth and ADC (Berrington de Gonzalez *et al*. 2004). In a small sample Lacey noted that unopposed estrogens used in HRT were independently associated with ADC (Lacey *et al.* 2000). Obesity was also found to have a stronger association with ADC than SCC (Lacey *et al*. 2003).

2.2.4 Precancerous lesions of the cervix

Precursor lesions of SCC are divided into CIN stages 1, 2 and 3 based on histological appearance, according to the classification system developed by Richart. This classification is based on the proportion of atypical cells and disruption of architecture, which reflects the severity of the lesion. CIN 1 represents mild dysplasia with atypical cells in the lower third of the epithelium and intact stratification, CIN 2 is characterized by moderate dysplasia with atypical cells in two thirds of the epithelium, while CIN 3 is associated with severe dysplasia or the equivalent of CIS, with atypical cells throughout the entire epithelium. ICC is defined as penetration of the epithelial basement membrane by atypical cells (Richart 1973).

In the 1990s a supplementary classification system, the Bethesda system, was introduced by the National Cancer Institute in the United States. This system is based on cytology, where low-grade squamous intraepithelial lesions (LSIL) correspond to CIN 1 and high-grade squamous intraepithelial lesions (HSIL) correspond to CIN 2 and 3, because of the difficulty in distinguishing between them using cytology. Moreover, the Bethesda system includes nomenclature for cells that cannot be designated as LSIL, yet still demonstrate atypical appearance. It differentiates between atypical squamous cells – uncertain significance (ASC-US) and atypical squamous cells – cannot exclude HSIL (ASC-H) (Solomon *et al.* 2002).

2.2.4.1 Adenocarcinoma in situ (AIS)

Since the glandular epithelium of the cervix is a monolayer, there is no such stepwise division of potentially precancerous lesions. The Bethesda system discriminates between atypical glandular cells, atypical glandular cells- favour neoplastic and endocervical adenocarcinoma in situ (AIS).

AIS is a relatively rare condition that has not drawn as much attention as precursor lesions originating in the squamous epithelium of the cervix. AIS is known to occur beneath the transformation zone and may thus be covered by normal metaplastic or dysplastic epithelium.

An abundance of evidence supports cervical adenocarcinoma in situ as a precursor of invasive cervical adenocarcinoma (Zaino 2002). AIS lesions are frequently found immediately adjacent to invasive adenocarcinoma (Boon *et al.* 1981). Cases of untreated AIS preceding invasive adenocarcinoma have been reported (Boon *et al.* 1981; Poynor *et al.* 1995) and the duration of progression from AIS to ADC has been estimated at 5-13 years (Lee *et al.* 2000; Plaxe *et a*l. 1999). In fact, with a mean age at diagnosis of about 35-39 years, AIS is diagnosed in an age group 10-20 years younger than invasive ADC (Ostör *et al.* 2000, Azodi *et al*. 1999, Boon *et al*. 1981).

Similar HPV types dominate in both AIS and invasive ADC. A recent investigation by Andersson et al. found that HPV 18/45 were clearly predominant, followed by HPV16 in (Andersson *et al.* 2013). Data regarding multifocal lesions are controversial. True multifocality has been described in 13-15% of cases (Ostör *et al.* 2000; Jaworski 1990). Coexistence with squamous cell lesions is a frequently observed phenomenon in glandular lesions. Zaino described concurrent squamous lesions with AIS in approximately 50% of cases (Zaino 2002), while more recent studies reported even

higher rates of up to 64-90% (Andersson *et al*. 2013; Ault *et al.* 2011). Furthermore, AIS multicentricity has been reported in ~15% of patients (Jaworski 1990). In about 50% of cases, AIS is diagnosed only because of detection of a squamous lesion (Ostör *et al.* 2000; Ault *et al*. 2011; Andersson *et al.* 2013). A study reported the same HPV types in AIS and in the adjacent squamous mucosa (Tase *et al.* 1989).

2.2.5 Natural history of precancerous lesions

The risk of developing CIS or ICC is cumulative in relation to severity of dysplasia. A review based on follow-up studies of women with abnormal cytology between 1950-1990 presented the natural history of CIN and showed that CIN 3, diagnosed by biopsy in women who were left untreated and only followed up by cytology, turned into invasive cancer in an estimated 30%-50% of cases (Ostör 1993; McCredie *et al.* 2008). A more recent large Canadian retrospective cohort study based on cytology samples collected between the 1960s and 1980s, during which time CIN management was relatively conservative, showed that 10% of mild dysplasia progressed to severe dysplasia or worse within 10 years, compared with 32% for moderate dysplasia. The study also showed that the majority of all mild dysplasia would spontaneously regress. The regression ratio (first normal Pap smear) for mild dysplasia was 44% within 2 years and 88% within 10 years. The ratio for spontaneous regression of moderate dysplasia was lower, but 83% still regressed within 10 years (Holowaty *et al.* 1999).

2.2.6 Treatment and prognosis

Treatment protocols for SCC and ADC are similar and the therapy is based on the FIGO clinical staging system. High-grade preinvasive lesions and FIGO stage IA cancer are treated by conization or radical hysterectomy with excellent results. Tumors diagnosed in more advanced stages are treated with radiation therapy alone or concomitantly with chemotherapy using cisplatin-based regimens. A Cochrane review of 19 randomized controlled trials (RCTs) involving locally-advanced cervical cancer confirmed benefit on overall and progression-free survival with use of platinum-containing chemotherapy regimens together with radiation therapy (Stern *et al.* 2012; Green *et al.* 2005).

Data on prognosis of ADC compared with SCC remain equivocal. For carcinoma diagnosed with lymph node metastasis, Nakanishi et al. found that prognosis was worse for ADC than for SCC. However, in the absence of lymph node metastases, no difference was seen (Nakanishi *et al.* 2000). A similar conclusion was drawn from a literature review in which survival rates for patients with small tumors in the absence of lymphovascular involvement were comparable for ADC and SCC. With advancing tumor stage, survival rates for ADC were significantly lower than for SCC (Gien *et al*. 2010). In contrast, a population-based analysis from the US found lower survival rates for ADC in both early and advanced stages (Galic *et al.*2012). The incidence of distant metastasis is higher for ADC than for SCC (Eifel *et al.*1995), as is the uncommon finding of ovarian metastasis (5.3% vs. 0.8%) (Shimada *et al.* 2006).

Andrae et al. found a significantly better prognosis among women diagnosed through screening compared with those diagnosed because of symptoms, which is largely attributable to the earlier stage at which cancer is detected by screening. The authors investigated all cases of cervical cancer occurring in Sweden over a 3-year period with

follow-up at 8.5 years and found no significant difference in prognosis for SCC or ADC (Andrae *et al.* 2012). Analyses of HPV type though indicate a significantly poorer prognosis for HPV-18 associated tumors, particularly adenocarcinomas (Im *et al.* 2003), which the authors attribute to deeper cervical invasion and greater pelvic lymph node involvement.

2.2.6.1 Discriminating primary cervical and endometrial adenocarcinoma

Due to topographical proximity it can sometimes be challenging for the pathologist to reliably discriminate primary endocervical adenocarcinoma from endometrial adenocarcinoma, which originates from the mucosa of the corpus uteri. However, the confident distinction between the two is of substantial clinical and prognostic importance. In cases of unequivocal histological assessment a panel of markers is applied which includes staining for estrogen receptor (ER), vimentin, monoclonal carcinoembryonic antigene (CEA) and $p16^{INK4a}$. The panel may be completed with HPV testing and p53 staining.

In most cases primary cervical adenocarcinoma is CEA positive and vimentin negative as well as negative or focally weak positive for ER. HR-HPV is present in a high percentage and thereby diffuse $p16^{INK4a}$ staining is common. 'Wild-type' p53 is more likely to be found in cancers originating from the cervix (McCluggage 2013).

2.2.7 Screening

The goal of well-organized screening programs, including treatment of detected precancerous lesions, is to prevent invasive cancer. Since cervical cancer develops slowly over 10-15 years, screening programs are essential for early detection and treatment of neoplasia (Sankaranarayanan *et al.* 2006).

2.2.7.1 Screening methods

2.2.7.1.1 Exfoliative cytology

Pap smear

Pap smear cytology, named for developer, the Greek pathologist Dr. George Papanicolaou, has long been in use and is still the screening method recommended by the WHO. Exfoliating cells taken from the ectocervix with a spatula and from the endocervix with a brush are directly smeared onto a slide, fixed in ethanol and air dried. After multichromatic staining the slide is examined under a microscope.

Although Pap smear screening greatly reduces the incidence of cervical cancer, the sensitivity of the test is low, with an overall detection rate of 50% to 60% (Schiffman *et al.* 2011). A systematic review of 94 studies showed that sensitivity ranged between 30% and 87% and specificity between 86% and 100% (Nanda *et al.* 2000). Due to the low accuracy of the Pap smear and the many false-negative results, alternative methods are currently under evaluation.

Liquid-based cytology (LBC)

Liquid-based cytology (LBC) is a new method, introduced in the 1990s, in which similarly sampled exfoliating cervical cells are preserved in liquid and then smeared

onto a slide and stained with the Papanicolaou stain. LBC produces more uniform samples, thereby facilitating exact morphological interpretation. Unsatisfactory sample quality with the need for repeat sampling is substantially decreased when using LBC compared to conventional smear (Ronco *et al.* 2007; Strander *et al*. 2007).

The outstanding advantage of the method is the possibility for supplementary analysis such as HPV testing or immunocytochemical methods on the same sample. The so called 'reflex test' refers to the subsequent HPV testing on the same sample if the cytology is assessed as abnormal.

The method has been adopted in some countries, though only in high-income settings due to high cost. Studies comparing efficacy of LBC with conventional cytology show conflicting results. A meta-analysis evaluated the sensitivity and specificity in detecting CIN 2+ and found comparable results for LBC and conventional cytology (Arbyn *et al.* 2008) as did other studies (Ronco *et al.* 2007; Siebers *et al.* 2009).

According to WHO, LBC provides higher sensitivity than conventional cytology (WHO 2006), and there are other studies that have confirmed its superiority Schiffman *et al.* 2011; Strander *et al.* 2007; Davey *et al.* 2007).

2.2.7.1.2 HPV testing

HPV testing is method to detect viral DNA as the precondition for development of cervical cancer. Some studies show that HPV testing is more sensitive for detection of CIN 3 than conventional cytology. The greatest advantage of HPV testing, however, is the high negative predictive value for development of cervical cancer among HPV DNA-negative patients. This advantage is important for risk stratification of patients and it is also cost-effective because it allows the screening interval to be prolonged in HPV-negative women (Schiffman *et al.* 2011). HPV testing for primary screening is still controversial, mainly due to its lower specificity, especially in young women (Ronco *et al.* 2010). In a large review of routine screening data from more than 60,000 North American and European women, the sensitivity of HPV testing for detection of CIN2+ was unambiguously higher than for cytology (96.1% vs. 53.0%). As expected, specificity was lower for HPV testing than for cytology (90.7% vs. 96.3%), although this parameter improved with increasing age (Cuzick *et al.* 2006). Primary HPV testing with cytology triage was more sensitive in detecting CIN 3+ than primary cytology alone (Anttila *et al.* 2010). A multinational cohort study by Dillner et al. found that a negative baseline HPV test had a significantly higher negative predictive value for developing CIN3 or cancer than a negative baseline cytology (Dillner *et al.* 2008).

2.2.7.1.3 Visual screening methods

Visual screening methods in colposcopy include visual inspection with acetic acid (VIA) and Lugol's Iodine (VILI). Application of acetic acid to the cervix turns abnormal cells white, while iodine turns them dark-yellow. Average sensitivity is 77% and specificity 86%. Instant results and low cost make VIA a promising screening option in low-income settings. However, further research, including RCTs, is needed to compare VIA, which is currently recommended only for pilot projects with conventional cytology (WHO 2006).

2.2.7.2 Organized screening

The Swedish national screening program notifies all women aged 23 to 49 to come for a Pap smear every third year and women aged 50 to 60 to come every five years, which complies with WHO recommendations with the exception of age at initiation (Stockholm County Counsil 2011, Vårdprogramm).

In Sweden, organized screening for cervical cancer has been introduced in 1964 and fully established in 1974. The introduction of organized screening showed great impact on the incidence of cervical SCC whereas the incidence of ADC was rather uninfluenced (Bergström *et al.*1999). Women who belong to the screening generation were diagnosed at an earlier stage than those of the prescreening generation (55% compared to 17% at stage I). Likewise, the proportion of advanced cases, stages III and IV, dropped from 29.3% to 21.2% (Pettersson *et al.* 2011).

The effectiveness in reducing mortality (>60%) was demonstrated in a Swedish population-based study. The study concluded that non-adherence to screening programs was the major cause of mortality in cervical cancer. Thus screening programs are presumably the key to reducing the incidence of cervical cancer (Jemal *et al.* 2011; Andrae *et al.* 2008).

A rather new approach to increase coverage of cervical cancer screening is self-sampling. The woman collects a vaginal sample herself with a simple device and sends it to the laboratory for HPV testing. As self-sampled material does not reliably contain cells representing the transformation zone it is not suitable for cytological assessment. The approach of self-sampling increases participation in organized screening (Sanner *et al.* 2009) and shows reliable results in detecting precancerous lesion (Brink *et al.* 2006; Sanner *et al.* 2009).

2.2.7.3 Screening for adenocarcinoma

Detection and treatment of in situ cancers are more effective at preventing SCC than invasive ADC, as confirmed by the observation that the vast majority of cervical cancer cases in poorly screened populations are SCC. In contrast, regions with good cervical cancer screening programs have a higher proportion of ADC (Schiffman *et al* 2007). However, a Swedish study from 2008 found that screening also has an effect on the incidence of ADC. The authors concluded that the increasing incidence of adenocarcinoma is the net result of increases in background risks, such as acquisition of HPV infection, and the counteracting effect of screening programs is unable to keep pace improving outcomes (Andrae *et al.* 2008).

There is overwhelming evidence that a remarkable percentage of AIS fails to be detected by cytological screening, or may progress rapidly to invasion. Adenocarcinomas frequently occur among women who underwent screening within 5 years of diagnosis (Hildesheim *et al.* 1999; Andersson *et al*. 2003). A negative cytology report affords less assurance against development of adenocarcinoma than squamous carcinoma (Mitchell *et al.* 1995).

Inadequate screening results may be due to compromised sample quality secondary to the less-than-adequate devices and techniques used to obtain the sample (Herzog *et al.*

2007). The introduction of cytobrush techniques, which allow sampling in the cervical canal, and of liquid-based cytology, which provides a monolayer of cells for assessment, were the first steps toward more accurate sampling and diagnoses of glandular lesions (Ashfag *et al*. 1999).

The location of glandular lesions and thereby the limited access for colposcopic assessment amplifies the challenges in diagnosing precursors of ADC. Irregular distribution of abnormalities within glands, the small size of some lesions and multifocality can further hamper assessment.

Inadequately validated cytological criteria for neoplasm precursors have been suggested to be a possible reason for screening deficiencies concerning glandular lesions, thereby subjecting results to a wide variation of inter-observer interpretation. A minor improvement in specificity could be made through subtype characterization, given the increasing possibility of identifying histological subtype over time (Gunnel *et al.* 2007).

2.3 HUMAN PAPILLOMAVIRUS

2.3.1 Classification of papillomaviruses

Papillomaviruses (PV) belong to the Papillomaviridae family of DNA viruses. They display high diversity and are able to infect all kinds of mammals, as well as birds and reptiles. To date well over 200 types of PV are known. The L1 gene encodes for the major viral L1 capsid protein, which together with the L2 protein constitute the complete capsid. Overall, L1 is the most conserved open reading frame (ORF) of the PV genome. Sequence comparisons of whole genomes lead to a similar distribution of interrelated HPV types as found when comparing the L1 ORFs (de Villier *et al.* 2004). Papillomaviruses are classified in taxonomic levels including **genus**, **species** (due to close phylogenetic relationship between certain types and because the concept species typically lumps PV types with common biological and pathological properties), **types** (a newly discovered papillomavirus type is defined as a complete papillomavirus genome, whose L1 gene nucleotide sequence is at least 10% different from that of other known papillomavirus types), **subtypes** (the L1 gene sequence differs by 2-10%) and **variants** (the L1 gene sequence is no more than 2% dissimilar) (Bernard 2013).

Human papillomaviruses are PV with the capacity to infect humans. Members of this multifaceted group have different epithelial tropisms and life-cycle strategies. Most types are ubiquitous with global distribution where different types dominate in different areas (Doorbar *et al.* 2012). Isolation and characterization of new HPV types is an ongoing process; currently 170 HPV types have been identified (deVilliers 2013; Bernard 2013; Figure 2-5).

HPV are members of 5 PV genera: the alpha-, beta-, gamma-, mu-, and nu-Papillomaviruses. Alpha-PV is divided into cutaneous and mucosal types according to epithelial tropism. A total of 40 sexually transmitted types have been detected in the

anogenital mucosal epithelium. These are clinically further divided into high-risk and low-risk types according to their potential to mediate human carcinogenesis (Wise Draper *et al*. 2008; Doorbar *et al.*2012). The WHO classifies the following 12 types as high risk: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59. Several other types, such as types 68 and 73, belong to the high-risk clade and are classified as "possibly cancer-causing" because of their similarity to cancer-causing types, even if they have not been found in cancer. Even low-risk types may occasionally be associated with cancer (Doorbar *et al*. 2012). There are also putative high-risk types, such as HPV 26, 53, and 66 (IARC Monographs 2007; Munoz *et al.* 2003). Although the life-cycle organization of high-risk types shares many similarities with low-risk HPV, the two groups differ significantly in their ability to drive cell cycle entry and cell proliferation.

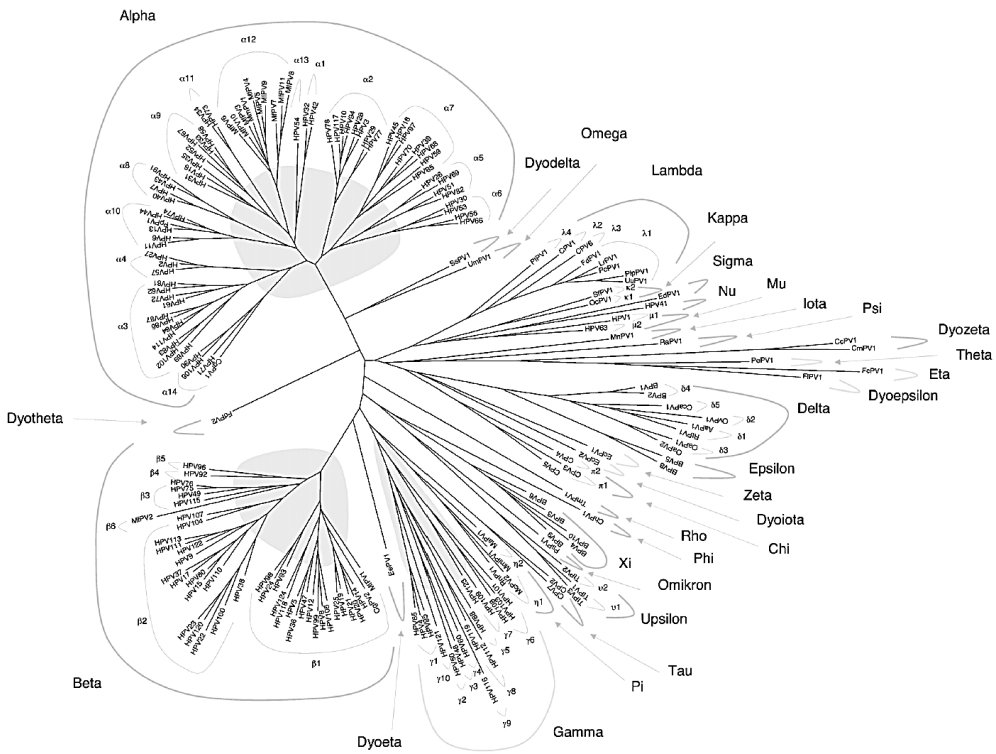

Figure 2-5 Phylogeny of papillomavirus types based on the nucleotide sequence of the L1 gene; taken from Bernard 2013.

2.3.2 Genome structure and viral particle

HPV is a small non-enveloped DNA virus containing almost 8000 base pairs. Circular double-stranded DNA is surrounded by a protein shell composed of two structural proteins, the major capsid protein L1 and the minor L2.

The genome is functionally divided into 3 domains: the long control region (LCR) and eight genes that are divided into early and late regions according to the order of the action of these genes in the viral life cycle (Figure 2-6). The LCR is a non-coding upstream regulatory region of 400 to 1000 base pairs. It contains promoter, enhancer and silencer sequences that regulate replication by controlling the transcription of the open reading frames (ORF). This region contains the highest degree of variation in the viral genome. The early region consists of six ORFs: E1, E2, E4, E5, E6, and E7. These genes are involved in regulation of replication and transcription, cell growth and maturation and virus release, as well as malignant transformation. The late region encodes the structural proteins L1 and L2 for the viral capsid, which is exclusively produced in the fully differentiated cells in the top layers of the epithelium (Burd 2003; Bernard 2013).

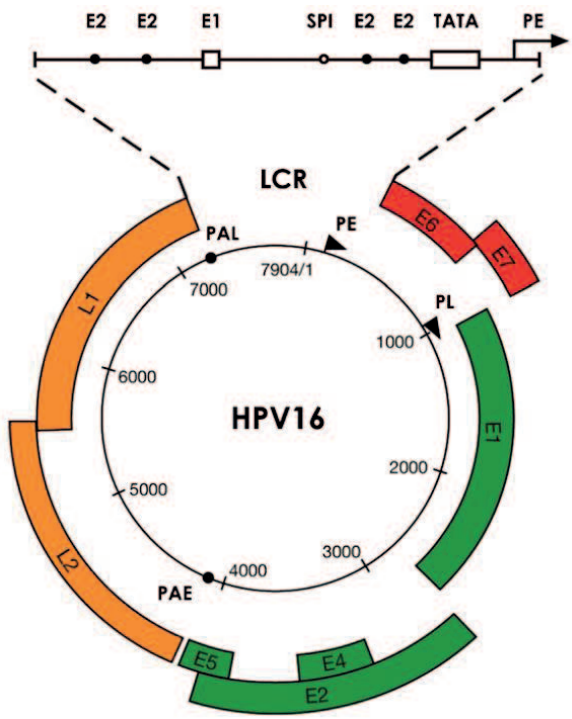

Figure 2-6 Schematic depiction of the genome organization of human papillomavirus type 16; taken from Doorbar J *et al*. 2012.

E1: E1 protein is the initiator of viral DNA replication. It acts as an ATPase and DNA helicase and binds to DNA polymerase.

E2: E2 protein is the main regulator of viral gene transcription. It modulates viral gene expression, has multiple binding sites in the viral LCR, binds the viral transcriptional promoter, and interacts with E1 to locate the viral origin of replication. E2 mediates down-regulation of E6/E7 transcription, which results in undisturbed

function of p53 and pRb proteins and allows the life cycle of the host cell to progress normally. Integration of HPV DNA into the host cell genome disrupts E2, which leads to reduced down-regulation of E6/E7 and thereby to increased expression of E6/E7 (McBride 2008).

E4: E4 protein acts late in the viral life cycle and accumulates at very high levels in cells that support virus synthesis. It facilitates assembly, maturation and release of virus particles, interacts with the keratin cytoskeleton and intermediate filaments, and induces arrest in the G2 phase of the cell cycle.

The **E5**, **E6**, and **E7** gene products have proliferative properties and mediate progression to malignancy. HPV oncoproteins cause DNA damage as well as both numerical and structural chromosome instability during HPV-associated carcinogenesis (Duensing *et al.* 2002). They do not possess enzymatic activities themselves, but instead interact directly or indirectly with a number of cellular target proteins (Wise Draper *et al.* 2008). Deregulation of E6 and E7 expression is the critical event in neoplastic dedifferentiation; their action is more efficient when the two act in concert, but even high levels of E7 alone are adequate (Burd 2003).

E5: E5 induces aberrant cell proliferation by stimulating cell growth through stabilization of growth factor receptors (EGFR, PDGFR beta and CSF1R), by enhancing EGF signaling and by inhibiting apoptosis (Genther *et al*. 2003). E5 interferes with intracellular trafficking of endocytotic vesicles (IARC Monographs 2007). The E5 gene sequence is deleted when viral DNA is integrated into host cells (Schwarz *et al.* 1985). E5 inhibits transport of major histocompatibility complexes to the cell surface and thereby helps the virus to evade immune response.

E6: E6 codes for a small protein of approximately 150 amino acids that contains two zinc finger motifs. HR-HPV E6 protein is found both in the nucleus and cytoplasm (Moody *et al*. 2010; Howie *et al.* 2009). HR-HPV E6 has been shown to block apoptosis, promote proliferation, activate telomerase, and to disrupt cell differentiation, adhesion and polarity, as well as to reduce immune recognition (Howie *et al.* 2009).

The E6 protein disrupts both the extrinsic and intrinsic pathways of p53 dependent apoptosis as well as p53 independent apoptosis (Howie *et al*. 2009). It interacts with tumor suppressors, especially p53, abrogating its normal function including cell cycle arrest in G1, activation of DNA repair and induction of apoptosis. E6 induces degradation of p53 through complex formation and induction of proteolysis via ubiquitin ligase (Wise Draper *et al.* 2008), blocks the specific binding site of p53 and can relocate p53 to the cytoplasm where it cannot carry out transcription (Howie *et al.* 2009). Degradation of p53 is specific to the HR-HPV E6 protein (Wise Draper *et al*. 2008).

E6 exerts its proliferative effect by interacting with transcriptional activators and inducing DNA synthesis; it prevents cell differentiation and interacts with factors involved in cell polarity and motility.

Activation of telomerase contributes to immortalization of malignant cells. Telomerase is activated in many cancer cells, but remains quiescent in most normal somatic cells. E6 can activate telomerase in epithelial cells independent of p53 (Klingelhutz *et al.* 1996; Wise Draper *et al.* 2008; Howie *et al.* 2009)

All these mechanisms in concert result in genomic instability, accumulation of mutated cells and development of malignant phenotypes. Moreover, E6 has been shown to help the virus to avoid the immune defenses by interacting with two proteins that are part of the innate immune response to viral infection: Interferon regulatory factor-3 (IFR-3) and Toll-like receptor 9 (TLR9) (Ronco *et al.* 1998; Hasan *et al*. 2007; Howie *et al*. 2009).

E7: E7 encodes a protein of approximately 100 amino acids mainly localized to the nucleus, although some studies have suggested that E7 also has a cytoplasmic component (Moody *et al*. 2010; Mc Laughlin-Drubin *et al.* 2009). E7 is the major transforming protein needed for maintenance of a transformed phenotype. E7 induces abnormal cell proliferation, interacts with histone acetyl transferases and interacts with negative regulators of the cell cycle and tumor suppressors, primarily the retinoblastoma (Rb) family.

The tumor suppressor proteins of the Rb family are major targets of E7. So-called pocket proteins are phosphor proteins that usually repress E2F transcription factors, which are key regulators of S-phase genes (Wise Draper *et al.* 2008; Moody *et al*. 2010). Binding of E7 inactivates Rb by disrupting the complex between pRb and E2F, which leads to liberation of E2F. E2F action on the methyltransferase gene exposes a gene sequence, allowing transcription of genes required for the S-phase of the cell cycle (Burd 2003), resulting in stimulation of cell cycle progression and proliferation. Both HR-HPV and LR-HPV E7 proteins can interact with Rb, but the binding affinity of HR-HPV E7 is many times greater than that of LR-HPV E7 (Münger *et al*. 1992). See Figure 2-8 on page 36.

E7 proteins also alter cell cycle control through interactions with cyclins and the cyclin-dependent kinase inhibitors p21 and p27, which are critical for cell cycle arrest in response to DNA damage (Moody *et al.* 2010).

L1: L1 is the major viral structural protein component in the assembly of capsomeres and capsids. It plays a key role in viral entry into the host cell and interacts both with cell receptors and L2. L1 is expressed exclusively in non-transformed cells and is highly immunogenic.

L2: L2 is a large protein, albeit a minor component of the viral capsid. It facilitates cell entry, genome encapsidation and intracellular endosomal trafficking of virions to the nucleus, and plays a role in capsid stabilization.

(reviewed in IARC Monographs 2007)

2.3.3 Viral life cycle

Human papilloma virus is an obligatory intracellular parasite. Since HPV genomes do not encode enzymes necessary for viral replication they are dependent on the

replication capacity of a mitotically active cell (Moody *et al*. 2010; Pyeon *et al.* 2009). The human papilloma virus must deliver its genome and accessory proteins into the host cell and use the biosynthetic cellular machinery for viral replication. Consequently, HPV's productive life cycle is restricted to terminally differentiating epithelium (Schiller *et al.* 2010).

Infection with HPV requires epidermal or mucosal epithelial cells that are able to proliferate (z. Hausen 1996). In multilayer epithelium, those cells are located in the basal layer, which is accessible through tiny tears in the epithelium (Schiffman *et al*. 2007). One area especially prone to infection and severe lesions is the previously mentioned transformation zone.

This area is characterized by increased accessibility and proliferation of the basal cell layers, especially during puberty and onset of sexual activity (Doorbar *et al*. 2012). Cells close to the squamous columnar junction, such as epithelial reserve cells or columnar epithelial cells, may be associated with development of adenocarcinoma (Doorbar *et al.* 2012).

Papillomaviruses are the only viruses known to initiate the infectious process at an extracellular site (Schiller *et al.* 2010). The virus gains access through micro wounds to the basement membrane of the disrupted epithelium, where it initially binds. Only after it binding to the basal keratinocyte cell surface can the virus gain subsequent entry (Scheelhaas *et al.* 2008).

Virus entry is achieved through the following steps:

Via its major capsid L1 protein, HPV interacts with heparan sulfate proteoglycans (Giroglou *et al.* 2001) on the epithelial surface or the basement membrane (Raff *et al*. 2013). Heparan sulfate proteoglycans (HSPG) are considered the primary attachment receptors (Joyce *et al.* 1999; Sapp *et al*. 2009). In addition to cell surfaces, PV capsids bind to the ECM via HS and Laminin-5 (Culp *et al.* 2006). This primary attachment process is solely dependent on L1 and does not require L2 (Horvath *et al.* 2010). Binding of HPV triggers structural changes in the virion capsid that affect both L1 and L2; specifically, the binding exposes the N-terminus of L2 to proteolytic cleavage. These changes expose the binding site of a secondary uptake receptor on the surface of basal epithelial cells. This interaction is necessary for virus internalization and subsequent transfer of the viral genome to the nucleus (Richards *et al*. 2006; Selinka *et al.* 2007; Day *et al*. 2008; Kines *et al.* 2009).

At the same time HPV binds to alpha 6 integrins, which initiates further intracellular signaling events. HPV 16 has been shown to bind to a newly identified L2-specific receptor, the Annexin A2 heterotetramer, which triggers endocytosis (Woodham *et al.* 2012; Raff *et al.* 2013).

Following receptor interaction, the virus is internalized by endocytosis. At this point, the various viral types use different pathways. Regardless of pathway, the process occurs slowly over a period of several hours (Schiller *et al*. 2010, Raff *et al.* 2013).

Once internalized, virions probably undergo endosomal transport and uncoating in endocytic vesicles. L1 is shed from the viral genome and ultimately subjected to lysosomal degradation. Prior to transport from endosome to nucleus along microtubules, disassembly of the viral particle occurs (Schelhaas *et al.* 2012; Florin *et al.* 2006).

The L2 protein DNA complex ensures correct nuclear entry of the viral genomes. Cell division is needed to establish and express the viral genome in the nucleus (Pyeon *et al.* 2009). Entry of the viral genome into the nucleus may follow from nuclear membrane breakdown during mitosis, rather than through active transport (Darshan *et al*. 2004).

Initially, inside the proliferative host cell, the HPV genome is replicated at low, non-productive levels using the host cell DNA replication machinery. During this phase both virus and host cell replicate together. Viral copy number is maintained at low levels of about 50-200 copies per cell (Pyeon *et al.* 2009, McBride 2008).

The infected basal cells divide and their daughter cells migrate toward the epithelial surface. As long as cells continue to divide, expression of viral proteins is tightly controlled. E6 and E7 are expressed at very low levels. (z Hausen 2002; Stanley *et al.* 2012). As cells reach the upper epithelial layer, they stop dividing and instead differentiate into mature epithelial cells. The virus switches to DNA amplification mode; all viral genes (including "late genes") are activated and the circular viral genome is replicated in high copy numbers, while structural proteins are also synthesized. The genomes are encapsidated and the assembled virions are released along with the most superficial dying and detaching epithelial cells (z Hausen 2002). E4 is thought to control virion release and infectivity in the upper epithelial layer (Doorbar *et al.* 2012).

The full viral cycle takes a long time, and even in the optimal scenario the time from cell entry to virus release takes about 3 weeks, since this is the time required for basal keratinocytes to move up through the epithelium, where they are sloughed off. Eventually, large numbers of infectious virus are shed for transmission to naive individuals (Stanley *et al.* 2012).

2.3.4 Methods of detection

2.3.4.1 Polymerase chain reaction (PCR)

The two HPV detection methods currently in large- scale- use are PCR and the Hybrid Capture (HC) test. PCR has high analytical sensitivity and is able to detect as few as 10 copies of HPV genomic DNA in a few microliters of specimen. This method selectively targets a DNA sequence through a set of consensus (GP5+/6+, PGMY09/11) primers directed at a conserved region within the L1 gene, and is capable of detecting virtually all mucosal HPV types. Commercially available PCR-based detection systems include Amplicor and Linear Array by Roche, CH. Amplicor allows detection of 13 HPV types in a cocktail (similar to HC), although it is not able to perform genotyping. The Amplicor test has 93-95% sensitivity for detecting HSIL/CIN2+, which is equal to that of HC2 (Mo *et al*. 2008; Monsonego *et al*. 2005).

Linear Array allows detection and typing of 37 HPV types using a nylon strip covered with immobilized type-specific oligonucleotides. The method is faster than other genotyping methods, but is not yet suitable for high-throughput.

Luminex-based HPV genotyping is a novel technique that combines PCR amplification with hybridization to fluorescence-labeled polystyrene bead microarrays. The detection limit for the various HPV types is above 500 plasmids and the results from this technique are in excellent agreement with an established microarray chip (Oh *et al*. 2007).

2.3.4.2 Hybrid capture (HC)

HC is a high-throughput, semi-automated test approved by the U.S. Food and Drug Administration (FDA). HCII is a refinement of HCI, and permits detection of 13 HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) and 5 LR-HPV types (6, 11, 42, 43, 44) with a cut-off limit of 5000 HPV genomes per reaction. The LR-HPV and HR-HPV probe cocktails are used in separate reactions.

2.3.4.3 Other methods

After PCR amplification, HPV DNA can also be detected by Southern blot, in which the PCR products are separated by electrophoresis on an agarose gel and transferred onto a nylon membrane, which is hybridized with type-specific probes. This method has been considered the gold standard for evaluation of HPV genomes, since it is specific, able to determine the physical status of the genome (episomal or integrated), and semi quantitative. A simplified version without gel separation is dot blot or slot blot hybridization, in which PCR product (single-stranded DNA) is directly applied to a nylon membrane for hybridization with type-specific probes.

Another method for relative HPV typing is single-strand conformational polymorphism. PCR is performed with radioactive consensus primers and PCR products are separated on a non-denaturing polyacrylamide gel, where migration band patterns of the amplicons are compared with HPV controls. The most accurate technique for HPV genotyping is sequencing, which can be done directly following the PCR reaction or after cloning of amplified fragments (reviewed in IARC Monographs 2007).

2.3.5 Prevalence of genital HPV infection

Even though HPV is a very common sexually transmitted infection, most individuals eliminate the infection without leaving any detectable evidence (Baseman *et al.* 2005). The virus is transmitted via intimate genital contact or contact with other mucosal surfaces (Moscicki *et al.* 2006). Acquisition of the virus peaks soon after onset of sexual activity. Cumulative incidence of HPV infection among young women aged 15-19 was found to be as high as 60% within 5 years and risk of infection increased with number of sexual partners (Woodman *et al*. 2001; Koutsky 1997; Peto *et al*. 2004).

HPV is highly infectious. The incubation period ranges from 3-4 weeks to months or years, probably depending on dose of virus acquired (Stanley *et al.* 2012). The majority of infected individuals eventually clears the infection and becomes DNA-negative (Stanley *et al*. 2006).

The median duration of infections is about 8-13 months for HR-HPV, compared with 4.8-8 months for LR-HPV (Bosch *et al*. 2002). Up to 50% of infections are cleared within 6 months, 70% within 1 year and 90% clear within 2-5 years of acquisition (Rodriguez *et al.* 2010; Winer *et al.* 2011; Moscicki *et al.* 2012; Plummer *et al.* 2007). A minority of about 10% does not effectively clear the virus, but instead remains DNA-positive as a sign of persistent active viral infection. Persistent infection is defined as infection with the same viral type detected after a number of years, varying between 4 and 12 years after initial infection. HPV-16 tends to persist longer than the average for other high-risk types (Franco *et al.* 1999). The risk for persistence increases if the infection has been present for >18 months, compared with newly diagnosed infections (Plummer *et al*. 2007). Persistent HR-HPV infection is requisite for development and maintenance, as well as progression to high-grade intraepithelial lesions (Bosch *et al.* 2002; Moscicki *et al* .2006). Apart from persistence, the type of infection (with HPV-16 linked to the highest risk) is an important determinant of risk for severe lesions (Moscicki *et al.* 2012). However, average persistence of HPV 16 is longer compared with most other HR-HPV types, which in turn may contribute to its higher cancer risk (Richardson *et al*. 2003; Franco *et al.*1999).

Globally, HR-HPV is found in 10-12% of the female population (de Sanjose *et al.* 2007; Bruni *et al.* 2010; Guan *et al*. 2012), with considerable variation among continents ranging from 8% in Asia and Europe to 24% in Africa. Prevalence is highest among younger women (<25 years), followed by a continuous decline that plateaus at 30-35 years, with the exception of Mexico, Colombia and Chile, where a slight second increase is seen in women over the age of 45-50 (Franceschi *et al*. 2006; Moscicki *et al.* 2012). The rate of newly detected carcinogenic HPV infections declines with increasing age (Rodriguez *et al.* 2010). Dunne et al. found the overall prevalence of HPV infection (including both low-risk and high-risk types) to be 26.8% among US women aged 14 to 59 years (Dunne *et al.* 2007).

Each HPV genotype acts as an independent infection (Schiffman *et al.* 2007), where individual HR types differ enormously in their relative carcinogenic potential (Bouvard *et al.* 2009). In benign and low grade lesions due to HPV, the viral DNA is located extra chromosomally within the nucleus, whereas in cancer, the DNA is often found to be integrated in the host cell genome. To a lesser extent, viral DNA integration is also seen with high-grade intraepithelial lesions (IARC Monographs 2007).

In low grade lesions, an overall HPV prevalence of 70-84% has been observed, depending on the investigated region. The predominant types include HPV 16 in 26.3% of samples, followed by HPV 31 in 11.5%, HPV 51 in 10.6% and HPV 53 in 10.2% (Clifford *et al*. 2005; Insinga *et al.* 2008).

Prevalence of HR-HPV increases with severity of lesion, ranging from 12% in normal cytology to about 90% in CIN 3 and invasive cancer. HPV 16, 18, 45 are the only types that are found more frequently in invasive cervical cancer (ICC) than in normal cytology (Guan P *et al.* 2012). When retesting samples under optimal conditions that were initially classified as HPV-negative, Walboomers reported a worldwide prevalence of HPV in cervical carcinoma of 99.7% (Walboomers *et al.* 1999).

In a meta-analysis of 30,848 cases of ICC, the prevalence of all carcinogenic HPV types was 89.9%, a figure that reflects an increase in prevalence from 85.9% in the 1990-1999 interval to 92.9% in the 2006-2010 interval (Li *et al*. 2011). Differently designed studies have unequivocally shown that, assuming adequate specimens, HPV DNA can be detected in up to 90%-100% of cases, compared with 5%-20% of cervical specimens from women identified as suitable epidemiological controls (Bosch *et al.* 2002).

Prevalence of HPV16 and 18 has been most widely studied since they are known to be the two most commonly found HPV types in cancerous lesions and are the two HPV types targeted by currently available vaccines. Joint prevalence worldwide of HPV types 16 and 18 in invasive cervical cancer is reported to be 70%-76% (de Sanjose *et al.* 2010), except for a slightly higher prevalence of 82% in Western/Central Asia and a slightly lower prevalence of 68% in East Asia (Li *et al.* 2011). However, HPV types 16 and 18 were detected in only 52% of severe intraepithelial lesions (Smith *et al.* 2007).

A Swedish study of cervical cancer cases before the introduction of the vaccine confirms these figures. Overall HPV prevalence for all types of cervical cancer was 92.9%. In SCC, HPV16 and 18 were seen in 69.7% of cases (Du *et al.* 2011).

Overall prevalence of carcinogenic HPV in different studies ranges from 62%-91.4% for ADC, compared with 87.3%-95.9% for SCC (Andersson *et al.* 2003; Clifford *et al.* 2003; Insinga *et al*. 2008; deSanjose *et al*. 2010, Du *et al.* 2011; Li *et al.* 2011, Chan *et al.* 2012). The meta-analysis mentioned above also showed an increase in the prevalence of HPV for adenocarcinoma from 77.8% (1990-1999) to 86.0% (2006-2010) (Li *et al.* 2011).

In 1995, Bosch et al. reported a worldwide HPV 18 prevalence of 56% in adenocarcinoma, clearly making it the predominant type in these cancers (Bosch *et al.* 1995). It has further been reported that HPV-18 and phylogenetically related types in the Alpha-7 species, such as types 39, 45 and 59, are strongly associated with cervical glandular intraepithelial and invasive lesions, whereas SCC is closely linked with HPV 16 and its phylogenetically related family (HPV types 31, 35, and 52) (Bosch *et al.* 2002; Clifford *et al.* 2008). Possibly, oncogenic potential relates to differences in tropism exhibited by various HPV types in different tissues.

Recent studies indicate a tendency towards similar contributions from HPV-16 (36.3-43.6%) and HPV-18 (36.8-41.8%) in ADC. The explanation may be related to the increase in joint detection of ADC and SIL, or to an increase in the percentage of adenosquamous carcinoma, which in turn seems to be related to HPV 16 more often than is ADC (Li *et al.* 2011; Quint *et al.* 2010). HPV-18 is still more commonly found in ADC (36.8%) than in SCC (13.2%) (Li *et al.* 2011).

When comparing HPV positive women with HPV negative women with normal cytology, Dahlström et al. found an odds ratio (OR) of 11 for future adenocarcinoma in situ (AIS) and an OR of 16 for future ADC. Comparing HPV-18 positive women with HPV negative samples, the OR for AIS was 26.0 and for ADC was 28.0 (Dahlström *et al.* 2010).

Epidemiological studies suggest that the genomic variants of HPV-16 that prevail in different regions of the world exhibit different carcinogenic potential (Schiffman *et al.* 2010), with the European variant E being most common in CIN 3 (83.8%), SCC (71.4%) and AIS (73.9%), while the Latin American variant AA is most common in ADC (41.7%), which seems to be preferentially associated with this histological type (Quint *et al.* 2010).

With improved detection methods the observed prevalence of multiple infection (mostly 2, but up to 5 types) has increased from the previously reported 4%-7% to the current 12%-16% in invasive cervical cancer, a concurrent change which applies to the different histological types (Burd 2003; Bosch *et al.* 2002; Li *et al*. 2011; Guan *et al.* 2012). There is no evidence for interaction between multiple HPV types or for an increase in risk of malignancy due to presence of multiple infections (Plummer *et al*. 2007; Bosch *et al.* 2002).

2.3.6 The role of HPV in malignant transformation

In uninfected epithelium, controlled cell division occurs in the para/basal layer. Regulated stimulation of cell cycle entry is controlled by growth factors such as Cyclin D and CDK that phosphorylate Rb. Rb/E2F are separated and the S-phase is entered. Stimulated $p16^{INK4a}$ suppresses cyclin D/CDK through negative feedback and prevents overstimulation (Doorbar *et al.* 2012)

Expression of the viral genome can sometimes be suppressed by epigenetic mechanisms such as methylation, a process that leads to persistent, but silent, infection where the viral genome is retained in the basal layer, but causes no apparent disease. Only 20% of HR-HPV infections cause morphological changes in the epithelium.

In low-grade lesions, HPV leads to an ordered pattern of viral gene expression with virus life cycle entry and release of virus particles from the superficial layer. Basal cell proliferation is mostly regulated by growth factors, as in uninfected lesions. Viral genes E6/E7 promote cell cycle entry, but not proliferation, and also stimulate HPV genome amplification in the upper epithelial layer. E7 binds to Rb p170 and separates it from E2F, while E2F stimulates host gene expression, which is necessary for DNA replication and cell cycle progression. Most low-grade lesions will regress spontaneously within 18 months (reviewed in Doorbar *et al.* 2012).

In high-grade lesions, a transforming infection is characterized by deregulated expression of viral oncogenes E6 and E7 with varying vertical expansion up to the full thickness of the epithelium in CIN 3. E6 and E7 stimulate additional cell cycle entry and proliferation. Inactivated inhibition of cell cycle progression by $p16^{INK4a}$ causes accumulation of Ki-67, MDM and finally p53 (due to decreased degradation by MDM). However, increased p53 cannot exert its function because it is eventually degraded by E6 associated with E6AP. It is notable that certain non-carcinogenic HPV types are capable of producing lesions diagnosed as CIN2, which demonstrates the etiologic heterogeneity of these lesions (reviewed in Doorbar *et al.* 2012.

Deregulated expression of the HPV oncogenes is responsible for chromosomal instability and accumulation of genetic errors in the infected cell, as well as the

eventual integration of the viral episomes into the host cell chromosome and progression to cancer (Wentzensen *et al*. 2007; Doorbar *et al.* 2012). Elevated E6 and E7 levels are directly related to increasing severity of neoplasia (Moscicki *et al* .2012).

About 70% of HPV 16-associated cervical cancers contain integrated HPV-16 sequences and in lesions due to HPV-18, the genome is almost always integrated (Cullen *et al.*1991; Badarocco *et al.* 2002). Cancer progression is facilitated when integration preserves the integrity of the long control region and the E6, E7, 5'portion of the E1 gene, but disrupts the regulatory E1 and E2 genes (Doorbar *et al.* 2012). The majority of cervical cancers contain one or many copies of HPV more or less randomly integrated (but often at fragile sites) into the host chromosome with the viral integration site lying within the regulatory E1 or E2 gene (Yu *et al.* 2005).

The period between acquisition of infection and detection of lesions is highly variable and can range from weeks to months. CIN 2 may occur in young women soon after infection (Paavonen *et al.* 2007; Paavonen *et al.* 2009). The timeframe between onset of sexual activity and therefore the most likely time of acquisition of HPV and detectable CIN 3+ is 7-10 years (Winer *et al.* 2005). The average time between infection and establishment of a high-grade lesion is shorter than the time between the first small CIN 3 lesion and cancer (Schiffman *et al.* 2007). However, estimates are somewhat imprecise since we neither know the exact moment of HPV acquisition, nor the exact time that lesions arise due to the relatively long intervals between screenings.

Compared with the estimated 80% lifetime risk of acquiring genital HPV infection, the 0.03% incidence of cervical cancer in the absence of screening is very low (Parkin *et al.* 2005). About 20% of CIN I progresses to CIN 2 if left untreated (Doorbar 2006). CIN 2 regression rates for women <25 years are 70% for lesions induced by any HPV type and 50% for HPV-16 (Moscicki *et al.* 2010).

2.3.7 HPV related cancer in sites other than the uterine cervix

An estimated 12.7 million cancer cases occurred worldwide in 2008, of which 610,000 cases (4.8%) could be attributed to HPV infection. The overwhelmingly most frequent of HPV related cancers was cervical cancer, accounting for 530,000 cases (86.9%) (Forman *et al*.2012). While cervical cancer is by far the most common malignant consequence of the carcinogenic potential of HPV infection, HPV is known to play an important etiological role in various other cancer entities, especially in the anogenital region. These include vulvar cancer, where HPV has been detected in 40%-50% of cases, with HPV-16 clearly being the dominant type, occurring in 80% of cases (Smith *et al.* 2009; Bosch *et al.* 2002). The same applies to vaginal, anal and penile cancers, which are HPV-positive in 65.5%, 72% and 47% of cases respectively, again with HPV-16 invariably being the predominant type (Smith *et al.* 2009; Hoots *et al.* 2009; Miralles- Guri *et al*. 2009).

In recent years a causal relationship has been found between HPV and head and neck cancer, especially tonsil cancer. Between 13% and 56% of head and neck cancers can be attributed to HPV infection, with considerable variation depending on histological origin of lesions and global region (Forman *et al*. 2012, Ramqvist 2011). In HPV

positive oropharyngeal squamous cell cancers HPV 16 is the by far dominating type being detected in 90% (Ramqvist 2011).

2.3.8 Immune response and HPV

Host response to HPV infection mobilizes both cell-mediated and humoral immunity (Stanley 2010). Clearance of incident infection is mainly mediated by cellular immunity, while neutralizing antibodies against the L1 capsid protein protect against infection by preventing initial entry of the virus into the basal cell layer (Carter *et al*. 1996; Stanley 2010).

Primary immune response to HPV infection is cell-mediated and targeted against the early proteins, specifically E2 and E6 (Woo *et al*. 2010). Failure of effective cell-mediated immune response is linked to an increased risk of progression. Some studies report a reduced T cell response to antigens of the respective HPV types among cancer patients (Welters *et al*. 2003; de Jong *et al*. 2004).

The cell-mediated immune response is followed or accompanied by seroconversion and production of antibodies to the major L1 capsid protein. Whether or not serology is specific to HPV type and protective for a second infection is not fully clear. Transient infections are cleared by the innate cell-mediated immune response without creating memory immunity (Farhat *et al.* 2009).

Seroconversion in natural infection occurs slowly, over an average of 8-9 months after HPV DNA is first detected and results in neutralizing antibodies to the L1 capsid protein. However, results are variable and antibody concentrations are low; only 50-70% of women with incident HPV infection show type-specific seroconversion (Viscidi *et al*. 1997; Carter *et al.* 2000). Nevertheless, these low antibody titers seem to provide adequate protection against disease (Olsson *et al.* 2009).

HPV successfully evades recognition by the immune system for many weeks or months. Due to the exclusively intraepithelial pathogenicity of HPV, the viral life cycle does not require a viremic phase, so viral access to vascular and lymphatic vessels and lymph nodes is limited. Infected cells shed virus from the epithelial surface, well removed from circulating immune cells. HPV is not cytolytic, but the virus replicates and assembles within cells that are destined for death anyway. Consequently, no inflammatory process occurs to alert the immune system (Stanley *et al.* 2012).

A major innate antiviral mechanism, the interferon response (Pett *et al*. 2006), is down-regulated by E6 and E7 in high-risk HPV through inhibition of the interferon receptor signaling pathway in keratinocytes (Kanodia *et al*. 2007).

Despite all these mechanisms to evade host defenses, at least 80% to 90% of genital HPV infections resolve with time due to the cell-mediated immune response (Stanley *et al.* 2012)

2.3.9 HPV Vaccines

After a long period of development, two licensed prophylactic HPV vaccines are now available on the market. Gardasil®, a quadrivalent HPV 16, 18, 6 and 11 vaccine from

Sanofi Pasteur MSD, has been approved in the US and Europe since 2006. Cervarix®, a bivalent HPV 16 and 18 vaccine from GlaxoSmithKline (GSK), was approved in Australia and Europe in 2007 and by the FDA in 2009.

The vaccines are based on virus-like particle (VLP) technology. VLPs are empty non-infectious viral shells consisting of recombinant L1 capsid protein; they are able to evoke an immune response without containing any viral DNA (Schiffman *et al.* 2007). These VLP vaccines are highly immunogenic, producing 50 times higher titers of type-specific neutralizing antibodies to L1 than those produced by natural infections (Schiffman *et al.* 2007; Harper *et al.* 2004; Villa *et al*. 2006).

This enhanced immune reaction to the VLP immunization is likely related to the route of administration, since VLP vaccines are given by intramuscular injection, providing direct access to the vascular and lymphatic system.

Randomized controlled trials have shown that both vaccines are well-tolerated and safe (Lu *et al.* 2011). They are highly efficacious against HPV-16 and HPV-18 related high-grade intraepithelial lesions among women aged 15 to 26, who test negative for these two HPV types at 5-year follow-up (Kjaer *et al*. 2009; Munoz *et al*. 2010; Lehtinen *et al*. 2012) (Paavonen *et al.* 2007, Paavonen *et al.* 2009). Even among women who tested seropositive but DNA-negative, as a sign of prior exposure, vaccination has proven to be protective in individuals up to age 25 (Munoz *et al.* 2010; Lehtinen *et al*. 2012). Among women with ongoing infection (seropositive and DNA positive), no benefit could be observed (Haupt *et al.* 2011). The effect on cervical cancer has not yet been assessed due to the long incubation period of the disease.

Although these vaccines target the two most prevalent HR-HPV types (16 and 18), a significant cross-protective effect against related types HPV 31, 33, 45 and 51 has been demonstrated for both vaccines (Wheeler *et al.* 2012; Paavonen *et al.* 2009; Brown *et al.* 2009; Einstein *et al*. 2011). One concern that has occasionally been expressed is that by removing HPV 16 and 18, an empty ecological niche may be created for other oncological types to fill. Although no evidence supporting this concern has been found to date, long-term follow up is needed.

In Sweden, HPV vaccination of girls aged 10-12 has been included in the national vaccination program since 2008. A free catch-up vaccination program is currently available for girls aged 13-18. Subsidized vaccination is available to women up to age 26.

Since HPV-16 and 18 account for about 70% of all invasive cervical cancers and the remaining 30% are caused by other types, the available vaccines cannot guarantee complete protection, despite the cross-protection phenomenon (Smith *et al.* 2007).

Serrano et al. have estimated that a nine-valent vaccine targeting HPV types 31, 33, 45, 52, and 58, as well as the types currently included in available vaccines, could prevent almost 90% of ICC cases worldwide (Serrano *et al.* 2012). A nine-valent vaccine is currently being tested in phase III trials.

A conceivable future alternative may be broad spectrum protection achieved by vaccination targeting L2, the minor capsid protein, which has been shown to evoke cross-neutralizing protection (Schiffman *et al.* 2007; Karanan *et al*. 2009). Another approach currently under investigation is to induce regression of established disease by therapeutic vaccination. Current strategies include improving access and acceptance of vaccines, as well as long-term follow-up to assess the effects of vaccination on the incidence of cancer.

2.4 MOLECULAR MARKERS OF CARCINOGENESIS

2.4.1 Viral markers of carcinogenic activity

2.4.1.1 Viral load

Viral load, the amount of viral DNA copies in a cell, is a quantitative test based on real-time PCR technology that reflects the content of human DNA in the sample. Viral load is a surrogate for persistence (Munoz *et al.* 2009) and correlates with severity of disease (Burd 2003; Moberg *et al.* 2005).

One nested case-control study found consistently higher viral loads of HPV-16 among cancer cases than in controls. The high viral loads detected up to 13 years before the diagnosis of cervical cancer reflected a 30-times higher risk of developing cervical cancer and a 60-times higher risk of developing carcinoma in situ, compared with HPV-negative women (Ylitalo *et al*. 2000; Josefsson *et al*. 2000). However, these results were contradicted by Lorincz et al., who found no predictive value of viral load for CIN3+ (Lorincz *et al*. 2002). Other clinically relevant findings include the correlation between reduction or clearance of viral load and regression of CIN lesions to normalcy (van Duin *et al.* 2002) and the predictive value of such clearance or reduction for harmless lesions (Snijders *et al.* 2006).

So far, this method has been of limited clinical use since high viral load has been associated with the productive phase of infection and low viral load has been associated with cancer (Schiffman *et al*. 2007; Gravitt *et al.* 2007).

2.4.1.2 E6/E7 mRNA

Messenger RNA is the surrogate for transcription. Evaluation of mRNA from the viral oncogenes E6 and E7 thereby provides indirect functional information about the transcriptional activity of the virus. The presence of mRNA associated with viral oncogenes E6 and E7 is closely linked to a transforming infection and the oncogenic potential of the particular virus (Hudson *et al.* 1990; Cuschieri *et al.* 2008; Gravitt *et al.* 2008). In 2007, Castle et al. described the correlation between detection of HPV E6 and E7 mRNA and severity of cervical dysplasia. They evaluated 531 liquid cytology samples for E6 and E7 mRNA for 14 oncogenic HPV genotypes. They found that 94% of women with CIN3 and 100% (5 of 5) of women with cancer tested positive for high-risk HPV E6 and E7 mRNA activity (Castle *et al*. 2007).

A prospective study from Norway compared the effectiveness of mRNA assessment with DNA detection to predict the risk of CIN2+ in women with atypical squamous

cells of uncertain significance (ASC-US) or low-grade squamous intraepithelial lesion (LSIL) cytology. Subsequent cytology and biopsy sampling were performed two years after initial HPV DNA and mRNA testing. Women with a positive high-risk mRNA test were about 70 times more likely to be diagnosed with CIN2+ within two years than women with comparable cytologic findings but a negative high-risk HPV mRNA test. Compared with mRNA testing, detection of HPV DNA in the same group of women had a 10-fold lower predictive value for CIN2+within two years than detection of mRNA (Molden *et al.* 2005).

A meta-analysis published in 2011 showed higher specificity for detecting precursor lesions using RNA testing compared with DNA testing (Burger *et al*. 2011). RNA tests have even been shown to detect double-stranded (ds) DNA, albeit with substantially lower sensitivity (Getman *et al*. 2009; Boulet *et al*. 2010). A recent meta-analysis concluded that HPV assays to detect the mRNA of 5 HR-HPV types may reduce overdiagnosis of women with minor cytologic abnormalities. However, given the lower sensitivity, the authors concluded that women with negative mRNA test results cannot be considered free of CIN2+ and therefore require further surveillance (Verdoodt *et al*. 2013).

2.4.1.3 HPV integration status

In contrast to precancerous lesions where HPV genomes are mainly found in an episomal state, high-grade lesions often present with the viral genome integrated into the host cell chromosome (Moody *et al.* 2010). Integration of viral DNA occurs at random sites, but fragile sites and transcriptionally active regions are preferential sites for integration (Ziegert *et al.* 2003; Wentzensen *et al*. 2004). Integrated viral DNA is found in 70% of cancers related to HPV-16, while 30% contain viral episomes. In HPV 18-positive tumors, the viral genome is predominantly integrated (Badarocco *et al*. 2002; Woodman *et al.* 2003). Recent studies have suggested that coexistence of HPV episomes with integrated copies could be crucial for carcinogenesis (Kadaja *et al*. 2009).

Whether viral integration is an early or late event—in other words, whether it is the cause of chromosomal instability or the result—is still open to debate. However, chromosomal abnormalities have been detected in cells containing exclusively episomal DNA, an argument supporting integration as a rather late event in carcinogenesis (Wentzensen *et al.* 2004).

The initial step in the integration process is disruption of the viral genome, typically at the E1/E2 ORF site, causing loss of important host regulatory function in these proteins, while the ORF of oncogenes E6 and E7 remains intact. Disruption of E2 interferes with viral transcriptional control, leading to deregulated expression of early viral genes, including accelerated expression of E6 and E7 (Schwarz *et al.* 1985). This in turn leads to increased proliferative capacity, a crucial step in progression to cancer (Hannahan and Weinberg 2000). E6 and E7 mRNAs expressed from integrated copies show increased stability and integration is associated with growth advantage over cells containing episomal viral DNA (Jeon *et al*. 1995).

Several methods can be used to detect the integrated HPV genome. In situ hybridization can detect integrated viral sequences in morphological structures such as chromosomes or episomes (Cooper *et al.* 1991). PCR-based methods offer an alternative approach where integrated sequences are amplified together with cellular sequences.

2.4.2 Host cell markers

2.4.2.1 Genomic alterations

Genomic instability is mandatory for malignant transformation. E6 and E7 achieve genomic instability through various mechanisms, such as induction of DNA damage or centrosome abnormalities. HPV-associated tumors harbor numerous chromosomal alterations, including gains or losses of whole chromosomes, or chromosomal rearrangements (z. Hausen 1999). Induction of genetic instability is thought to be an early event in the malignant process induced by HPV (Dünsing *et al.* 2001).

2.4.2.2 Chromosomal aberrations

Chromosomal copy number alteration may lead to overexpression of oncogenes or decreased expression of tumor suppressors.

A recent systematic meta-analysis by Thomas et al. investigated chromosomal copy number alterations in HPV-positive vs. HPV-negative cancer and precancerous lesions in the lower genital tract, in which they compared SCC and ADC. The most frequent alteration in SCC was a gain of 3q, especially between 3q25 and 3q29. The frequency of 3q gain increased with the severity of the lesion up to 55% in cancers and was especially common in the presence of HPV-16. Other frequent copy number alterations in SCC were loss of 3p and 11q, as well as gain of 1q and 5p. The rate of alteration was generally lower in adenocarcinoma. Here, gain of 3q was the second most common alteration, exceeded only by gain of 17q and with accumulation in HPV 18-positive ADC (Thomas *et al*. 2013; Yang *et al*. 2001). One gene localized on 17q is ErbB2, which encodes the oncogenic growth factor receptor HER2/neu, which is overexpressed in many other adenocarcinomas. HER-2/neu has been expressed in cervical glandular lesions, especially in those positive for HPV-16 (Roland *et al*. 1997).

An interesting gene in the frequently gained 3q is TERC (human telomerase gene, or telomerase RNA component), which maps to 3q26. Increased telomerase activity is characteristic of many cancers, including cervical cancer. Heselmeyer et al. found 3q gain in 90% of SCC samples, while no gain was found among LSIL samples or those with normal histology. The authors therefore suspected that gain of 3q plays a key role in the transition from severe dysplasia to invasive cancer (Heselmeyer *et al.* 1996). A prospective study by Andersson et al. found a correlation between increasing TERC detection in cytology and higher grade of dysplasia (Andersson *et al.* 2009). TERC amplification in normal smears has also been shown to predict development of cervical SCC since 3q gain was detected in 33% of normal slides among women subsequently diagnosed with cervical cancer within the following 3-year period (Heselmeyer-Haddad *et al.* 2005). The same study showed that CIN1/CIN2 lesions showing a diploid pattern for TERC were more likely to undergo spontaneous regression, while CIN 1/CIN2 lesions with extra copies of TERC were more likely to progress (Heselmeyer-Haddad

et al. 2005). Andersson et al. examined 12 cases of ADC and found TERC gain in all cases (Andersson *et al.* 2006a). Identification of TERC amplification has the potential to become a useful marker for cervical cancer, including ADC.

2.4.2.3 LRIG1,2,3

The LRIG proteins are a family of integral surface proteins that all show a similar domain organization, an extracellular signal peptide composed of leucin-rich repeats and immunoglobulin-like domains, a transmembrane glycoprotein, and a cytoplasmic tail (Holmlund *et al.* 2004).

The LRIG family has three members. LRIG, initially cloned in 2001 and named Lig-1, is located on 3p14. That region is often deleted in human cancers, including cervical cancer (see the chapter on chromosomal copy number alterations). LRIG1 protein shows similarities to Kekkon-1, an epidermal growth factor receptor antagonist in the Drosophila melanogaster (Nilsson *et al.* 2001). Expression of mRNA was found in the normal tissue of most organs, albeit in varying concentrations with the highest in brain and lowest in spleen (Nilsson *et al.* 2001). Interestingly, the protein was not expressed in the healthy uterine cervix, despite normal mRNA expression in this tissue (Nilsson *et al.* 2003).

LRIG2, which is located on 1p13, was identified in 2003. It was found to have the same domain organization as LRIG1 with 47% homology in amino acid sequences. Similarly, LRIG2 was found to be expressed in all human tissues (Holmlund *et al.* 2004). Soon thereafter LRIG3, located on 12q13, was also identified (Guo *et al.* 2004).

Because of the significant structural similarities between LRIG and Kekkon-1, it was anticipated that LRIG would exert a tumor suppressive function through inhibition of epidermal growth factor receptors (EGFR) (Hedman *et al.* 2007). LRIG up-regulation is followed by ubiquitylation and degradation of growth factor receptors belonging to the Erb family, thereby inhibiting proliferation (Gur *et al.* 2004; Laederich *et al.* 2004). LRIG1 has recently been identified as an intestinal stem cell marker with tumor suppressor capacity (Powell *et al.* 2012).

LRIG1 is down-regulated in carcinomas of the skin, cervix, bladder and lung, and overexpressed in astrocytoma, prostate cancer and lung carcinoid tumors (Hedman *et al.* 2007). LRIG genes are not universally down-regulated in human cancers and the question is whether LRIG proteins always exert a tumor-suppressive effect, or whether they may act as tumor promoters under certain circumstances (Hedman *et al.* 2007).

In breast cancer, LRIG1 was found to be poorly expressed in ErbB2-positive breast cancers (Miller *et al.* 2008), while showing significantly higher expression in estrogen receptor-positive cancers compared with estrogen receptor-negative cancers. The results suggest that LRIG1 has an estrogen-regulated growth inhibitor function (Krig *et al*. 2001). Recent results show that both LRIG1 and LRIG3 interact with the ErbB2 receptor, albeit with opposite results. LRIG3 increases receptor expression, thereby counteracting the effect of LRIG1. In turn, LRIG1 destabilizes LRIG3, thereby identifying it as a target for LRIG1 (Abreira *et al*. 2010; Rafidi *et al.* 2013).

LRIG 1 has been investigated as a prognostic marker in skin cancers: higher levels were associated with better survival and higher tissue differentiation, while being inversely related to the rate of metastasis (Tanemura *et al.* 2005). LRIG1 is expressed in various subcellular locations, such as within the nuclear, perinuclear and cytoplasmic compartments. In glioma, perinuclear staining was associated with lower stage, better survival, and lower proliferation index, and was also assessed to be an independent prognostic factor (Guo *et al.* 2006).

As mentioned above, in contrast to mRNA, LRIG protein was not expressed in normal cervical tissue (Nilsson *et al.* 2003). Lindström et al. investigated LRIG1 expression together with 9 other tumor markers in 128 patients with invasive squamous cervical cancer. LRIG1 levels decreased with increasing stage of disease and the level significantly correlated with better prognosis in early stage (IB-IIA) disease. In higher stages, LRIG level had no predictive value (Lindström *et al.* 2008). A subsequent study by Hedman et al. on 165 cervical cancer patients (including 36 patients with adenocarcinoma) found an association between expression of LRIG2 and poorer survival in early stage (IB-IIB) squamous cell cervical cancer. No correlation could be established for the relatively small sample of ADC. The authors concluded that a combination of high LRIG2 and low LRIG1 expression could identify women with very poor prognosis (Hedman *et al*. 2010). In the wake of these results, Lindström et al. investigated LRIG1 and LRIG2 together with 11 tumor markers in 171 cervical tissue samples (normal and CIN). Expression of both glycoproteins showed a concordant increase with increasing tumor grade and was associated with the tumor suppressor protein FHIT (Lindström *et al*. 2011).

2.4.3 Expression of cellular proteins

2.4.3.1 TP53 and WIG-1

2.4.3.1.1 TP53

The tumor suppressor protein that has been most extensively examined in a broad variety of malignancies is p53.TP53 is activated by cellular stress and responds with multifaceted actions such as cell cycle arrest, DNA repair and apoptosis to avert irreparable damage. Its function is mediated through transcriptional regulation of target proteins (Vousden *et al.* 2009). Clearly, TP53 plays an important role as tumor suppressor since more than 50% of human cancers contain mutation or deletion of the TP53 gene (Hollstein *et al.* 1991; Vogelstein *et al.* 2000; Soussi *et al.* 2007).

The frequent presence of mutant TP53 seen in cervical cancer and its dependence on HPV infection and histological type remains controversial. Early cell line studies identified TP53 mutations exclusively in HPV-negative cervical cancer cells (Scheffner *et al.*1991) and this finding was confirmed by a separate study of cervical cancer tissue (Crook *et al*. 1992). Several other studies reached the conclusion that TP53 mutation is generally uncommon in cervical cancer, regardless of HPV status (Kessis *et al.* 1993; Busby-Earle *et al.* 1994).

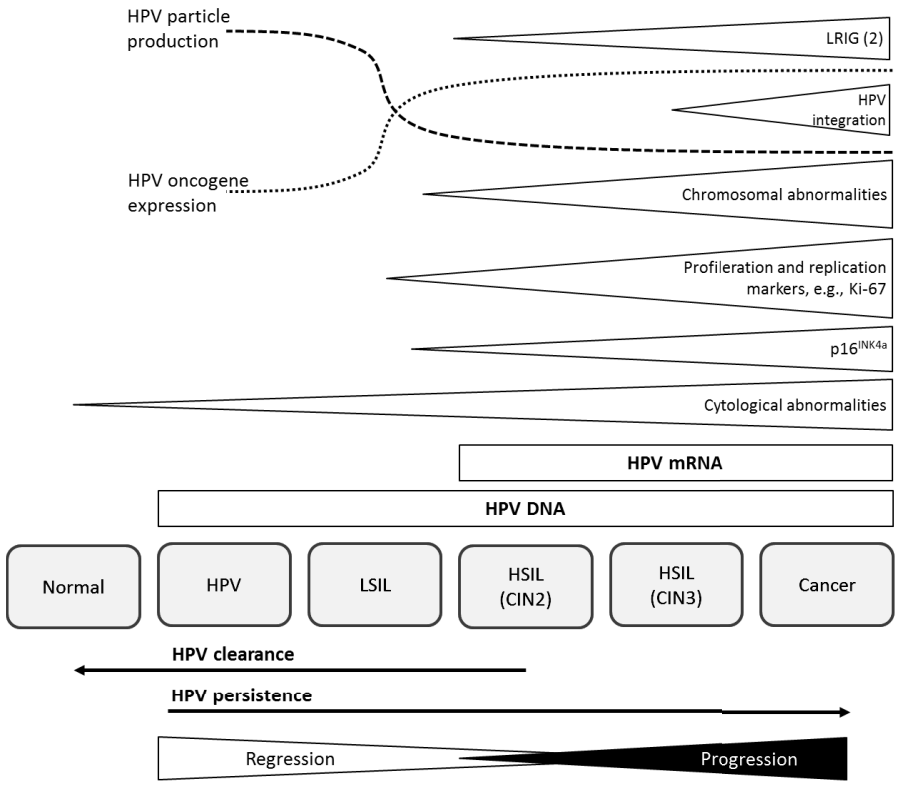

Figure 2-7 Progression model of cervical carcinogenesis and biological marker levels (adapted from Wentzensen *et al.* 2007)

In 2006, Andersson found mutant TP53 in 20% of cervical adenocarcinomas in a Swedish sample with no difference between HPV-positive and HPV-negative specimens (Andersson *et al.* 2006b). A recently published review described a higher rate of mutant TP53 in cervical ADC than in SCC, with remarkable geographic differences, where the highest frequency of mutant TP53 in ADC was found in Asia (19%) and Europe (12.2%) (Tornesello *et al.* 2013).

In HPV-induced cervical cancer in the presence of wild-type TP53 (wt TP53), transformation is provoked by the interaction of the viral oncogene HPV-E6 with TP53. E6 forms a complex with E6-associated protein (E6AP) and together they block the TP53 DNA binding site and target TP53 for degradation. Inactivation of TP53 by mutation, deletion, or degradation leads to proliferation and immortalization of the affected cell (Werness *et al.* 1990; Scheffner *et al.* 1990; Scheffner *et al.*1991; Münger et al. 1992).

2.4.3.1.2 WIG-1

The human WIG-1 gene (wild type p53-induced gene 1; also named PAG608 and ZMAT3) is a direct transcriptional target gene of p53 (Varmeh-Ziaie *et al.* 2001). It

maps to chromosome 3q26.32 and encodes the Wig-1 protein that exists in two isoforms with either 289 or 290 amino acids (Vilborg *et al.* 2011). The protein contains 3 Cys2His2-type zinc fingers, a structure shared with a few other RNA-binding proteins (Reuter *et al.* 1990).

WIG-1 is expressed in all tissues, with the highest levels found in the brain (Vilborg *et al.* 2011). Low levels of WIG-1 mRNA and protein are found in normal human tissues, whereas levels may rise when wt TP53 expression is induced following activation (Hellborg *et al.* 2001).

The fact that Wig-1 protein is mainly localized to the nucleus, but may also extend to cytoplasm, indicates that it can shuttle between cytoplasm and nucleus. Wig-1 preferentially binds to dsRNA with high affinity compared with dsDNA or ssRNA (Mendez-Vidal *et al.* 2002).

The WIG-1 promoter contains a TP53 binding site, but its expression is not exclusively regulated by TP53. Transcription factors other than TP53 can also regulate WIG-1, since WIG-1 has been detected in cells lacking TP53. Some of these transcription factors even promote proliferation, indicating that WIG-1 expression might be induced under diverse circumstances (Vilborg *et al.* 2011). WIG-1 can in return positively regulate TP53 mRNA through stabilization via a U-rich region in the p53 3'UTR (Vilborg *et al*. 2011).

Ectopically expressed WIG-1 inhibits colony formation of tumor cells by up to 25%-30h% (Hellborg *et al.* 2001), but the exact cellular mechanism of WIG-1 remains unknown. On the other hand, WIG-1 has been found to be amplified and overexpressed in squamous cell carcinoma of the lung (Varmeh-Ziaie *et al.* 2001) indicating a possible oncogenic effect. Recently, WIG-1 was identified as a positive regulator of the N-Myc mRNA oncogene; knockout of WIG-1 significantly delayed N-Myc-driven tumor growth (Vilborg *et al*. 2012).

2.4.3.2 $p16^{INK4a}$

One promising marker is the cyclin-dependent kinase inhibitor $p16^{INK4a}$. This tumor suppressor protein inhibits E2F-dependent transcription and cell-cycle progression. Usually, E2F-mediated cell-cycle progression is blocked by binding of phosphorylated retinoblastoma (pRb) to E2F. The phosphorylation of pRb is mediated by the cyclin-dependent kinases CDK4 and CDK6. $p16^{INK4a}$ exerts its effect by blocking the CDK4- and CDK6-mediated phosphorylation of pRb. pRb has in turn been shown to negatively regulate $p16^{INK4a}$ transcription via suppression of E2F activity (Li *et al*. 1994; Khleif *et al.*1996; Zhang *et al.* 1999), see Figure 2-8.

In cells with transforming HPV infections and uncontrolled expression of E7, regulation of the Rb-E2F pathway is disrupted by aberrant E7 and activation of $p16^{INK4a}$ is unable to exert its downstream effect (Khleif *et al.*1996). The functional inactivation of Rb results in release of transcription factor E2F and cell cycle progression. Following the negative feedback loop between Rb and $p16^{INK4a}$, deregulation of Rb results in hypomethylation of the $p16^{INK4a}$ promoter and thereby strong overexpression of $p16^{INK4a}$, which accumulates in the cells (Sano *et al.* 1998;

Dehn *et al*. 2007). Continuous expression of E7 is necessary to maintain a malignant phenotype in HPV-associated cancer and overexpression of $p16^{INK4a}$ is a direct consequence of this process; therefore $p16^{INK4a}$ becomes a surrogate marker for deregulated HPV oncogene expression (Khleif *et al.*1996). Expression of $p16^{INK4a}$ is independent of underlying HPV type, obviating the need for type specification. In contrast to many other tumor markers, such as Ki67, $p16^{INK4a}$ is not a proliferation marker; rather, it is associated with senescence and cell cycle arrest and is therefore not expressed in cells with benign proliferative capacity (Beausejour *et al.* 2003).

However, when cells are exposed to physiological genomic stress such as aging, $p16^{INK4a}$ is expressed and induces immediate and irreversible cell cycle arrest that may ultimately lead to apoptosis (Beausejour *et al.* 2003). Thus, independent of HPV status, $p16^{INK4a}$ expression is sometimes observed in single cells that undergo modifications of their normal differentiation program due to aging or genomic stress (Wentzensen *et al.* 2007; Tringler *et al.* 2004).

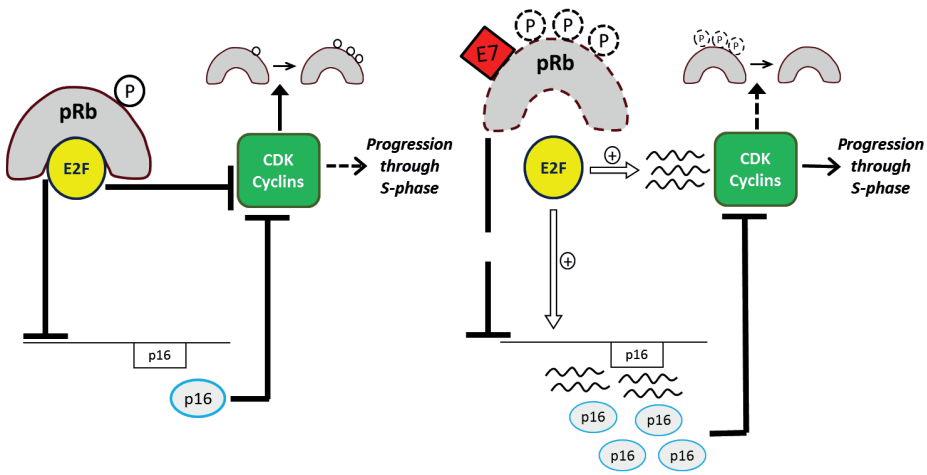

Figure 2-8 Schematic diagram of the cellular proteins involved in G1-S phase transition and the interference of HPVE7.

2.4.3.3 Ki-67

The proliferation marker Ki-67 is a nuclear protein associated with cell proliferation. The Ki-67 antigen is visualized using monoclonal antibody MIB-1. Ki-67 is expressed during the active phases of the cell cycle G1, S, G2 and M, but absent among resting cells in G0. In normal cervical squamous epithelium the proliferation marker Ki-67 is confined to the parabasal cell layer. In intraepithelial lesions extended expression correlates with disordered maturation. As a pure proliferation marker, Ki-67 alone cannot discriminate HPV-mediated dysplasia from reactive cell proliferation in response to benign processes (Hwang *et al.* 2012), but it is used as an adjunct to morphological assessment in grading cervical dysplasia (Kruse *et al.* 2001; Baak *et al.* 2005). Figure 2-7 illustrates different marker levels during progression of carcinogenesis as discussed above.

3 AIMS

Because there has been an increase in the incidence of adenocarcinoma of the uterine cervix, especially among young women, and cytological screening for detection of this type of cancer is inadequate, other screening techniques are needed. The general aim of this thesis is to investigate the potential of different biomarkers as tools to reliably diagnose and predict prognosis for cervical adenocarcinoma.

3.1 SPECIFIC AIMS

3.1.1 Paper I

The aim of this study was to evaluate the relationship between expression of the $p16^{INK4a}$ tumor suppressor and the Ki-67 proliferation marker (detected through MIB-1) in cervical adenocarcinoma and to correlate staining patterns with clinical parameters.

3.1.2 Paper II

The aim of this study was to evaluate whether the real-time NASBA-based PreTect HPV-Proofer assay (NorChip AS, Klokkarstua, Norway) can be used to detect the full-length mRNA E6/E7-region of five HR-HPV types in paraffin-embedded tissue samples of cervical adenocarcinoma. We wanted to analyze whether the PreTect HPV-Proofer is a more sensitive method for detection of adenocarcinoma of the cervix due to transforming HPV infection, than using the DNA test in paraffin-embedded material.

3.1.3 Paper III

The aim of this study was to evaluate immunohistochemical expression of LRIG proteins in cervical adenocarcinomas, and to investigate possible correlations between LRIG expression, patient survival and HPV status.

3.1.4 Paper IV

The aim of this study was to examine whether WIG-1 (p53 target gene) is a molecular marker for cervical carcinogenesis and to evaluate whether Wig-1 expression differs between HPV-positive and HPV-negative cervical cancers. We also investigated the correlation between WIG-1 expression and clinical outcome.

4 MATERIAL AND METHODS

4.1 TUMOR MATERIAL

All tumor cases were identified from the Swedish National Cancer Registry maintained by the National Board of Health and Welfare. This registry includes all cases of malignant tumors diagnosed histopathologically since 1959, with each tumor identified by a topographical and a histopathological code. All studies were approved by the Regional Ethics Review Board in Stockholm and all women provided written informed consent to participate in the studies,

4.1.1 Paper I

The study includes formaldehyde-fixed paraffin-embedded tumor tissue specimens from 101 primary cervical adenocarcinomas diagnosed and surgically treated at Karolinska University Hospital-Huddinge between 1992 and 2000. All tumor samples were collected, with informed consent and approval from the local ethics committees. The histopathological diagnoses were based on WHO criteria. In all, 101 tumors were classified as cervical adenocarcinomas and all lacked any squamous cell component. Eight adenocarcinomas showed only superficial proliferation in the mucosa with no obvious signs of infiltration into surrounding stroma. These tumors were regarded as "in situ adenocarcinomas" and the remaining 93 tumors as "infiltrating cervical adenocarcinomas". Information concerning stage of differentiation was available for 89 of the tumors, while information on clinical stage was available for 76 cases. All patients were retrospectively followed-up from time of diagnosis until January 2007 and disease recurrence and survival data were recorded.

4.1.2 Paper II

The study includes formaldehyde-fixed paraffin-embedded tumor tissue specimens from 98 primary cervical adenocarcinomas taken from the previous material that were diagnosed and surgically treated at Karolinska University Hospital-Huddinge between 1992 and 2000. Eight adenocarcinomas showed only superficial proliferation of the mucosa with no obvious signs of infiltration into surrounding stroma. These tumors were regarded as "in situ adenocarcinomas," while the remaining 90 tumors were considered to be "infiltrating cervical adenocarcinomas." All patients were retrospectively followed-up from time of diagnosis until January 2007 and disease recurrence and survival data were recorded.

4.1.3 Paper III

This study looked at a cohort of 86 patients with primary cervical adenocarcinoma treated at Karolinska University Hospital, Huddinge, between 1992 and 2000. The previous study included 101 patients, while the current study conducted immunohistochemical analyses for the expression of LRIG proteins in 86 patients, after 15 patients were excluded due to an insufficient amount of material.

All tumors were cervical adenocarcinomas and lacked any squamous cell component; 79 were invasive, 4 were in situ and histopathological diagnosis could not be ascertained for the remaining 3 tumors. All patients were retrospectively followed up from time of diagnosis until January 2011 and survival data were recorded.

4.1.4 Paper IV

For the first part of the study eight human cervical cancer cell lines Ca Ski, SiHa, C-4 I, C-33A, ME-180, HT-3, MS751 and SW756 and a osteosarcoma cell line Saos2 were purchased from the American Type Culture Collection, ATCC (http://www.lgcstandards-atcc.org).

For the immunohistochemical portion of the study, biopsies were collected from 38 patients who were diagnosed between 1989 and 2010 at the Department of Obstetrics and Gynecology, Karolinska University Hospital. Included were 13 cases of squamous cell carcinoma samples and 25 cases of invasive adenocarcinoma samples that had also been included in studies I-III. The biopsies were grouped according to morphological diagnosis and the histopathological diagnosis was based on WHO criteria. All patients were retrospectively followed up from time of diagnosis until January 2012. Clinical information on patients, including age at diagnosis, stages and survival data, was recorded.

4.2 METHODS

4.2.1 Sample preparation

The tumor tissue was formalin-fixed and paraffin-embedded. Serial sections of 10µm were from each block. One section was stained with hematoxylin-eosin for histological evaluation, while the remainder was used for further investigation, depending on the study. Figure 4-1 gives an overview of material and methods applied in study I-III.

4.2.2 HPV DNA testing

Analyses were performed on DNA extracted from a 10-µm-thick section of paraffin blocks; the preceding section had been used for morphological diagnosis. A fragment of 150 bp was amplified from the L1 region using GP5+/GP6+ primers, PCR followed by SSCP typing, or HPV-typed by direct DNA sequencing and comparison with known HPV sequences using the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST).

In paper II for cases previously tested as HPV-negative (28), the Linear Array HPV Genotyping Test (LA) was carried out.

4.2.2.1 HPV DNA extraction and detection with Linear Array

Paraffin-embedded archival biopsies were de-waxed with xylene-ethanol and DNA was extracted using a MagNA Pure LC Robot in accordance with the procedure outlined in the manufacturer's manual (Roche Diagnostics, Basel, Switzerland). HPV-DNA detection and genotyping were carried out using the Linear Array (LA) HPV Genotyping Test outlined in the manufacturer's manual (Roche Diagnostics, Basel, Switzerland), which is capable of detecting 37 HPV types divided into three "risk categories" according to Muñoz and co-workers (Muñoz *et al.* 2003).

4.2.3 E6/E7 mRNA analysis (Paper II)

Paraffin-embedded tissue blocks were sliced with a microtome blade into five 10-µm sections and transferred to 1.5 ml RNase-free vials and stored at 4ºC. The microtome and forceps were cleaned with 70% ethanol twice between each sampling to prevent contamination between blocks. Sections for RNA analysis were incubated in two

consecutive baths of xylene for 10 minutes each before centrifugation for 5 minutes at 2500 rpm, followed by two consecutive baths in 100% ethanol, 10 minutes each. After final centrifugation for 5 minutes at 2500 rpm, the pellets were dried at 55ºC for 2-4 hours and treated for at least 3 hours with 100 µl 0.5 mg/ml proteinase K (Invitrogen, Carlsbad, USA, 20 mg/ml) in RNase-free 1x Tris-HCl (Sigma Aldrich, St. Louis, USA) at 55˚C until digestion was complete. Finally, 900 µl lysis buffer (Nuclisens, bioMérieux, Marcy l'Etoile, France) was added to each tube before extraction using the NucliSens manual extraction kit (bioMerieux, Marcy l'Etoile, France). Extracts were stored at -80ºC.

Full-length mRNA from HPV types 16, 18, 31, 33, and 45 were detected using the real-time NASBA-based assay (PreTect HPV-Proofer, NorChip AS, Klokkarstua, Norway), according to manufacturer instructions.

To avoid false negatives due to degradation of mRNA, primers and probes against human U1A mRNA are included in the HPV-Proofer kit for performance and integrity control. However, the U1A control was found to be suboptimal for integrity control in paraffin-embedded tissue due to modified or fragmented nucleic acids, and was therefore replaced by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The amplicon defined by the GAPDH primers was adjusted in size to better match the HPV amplicons.

4.2.4 Immunohistochemistry (Paper I; III; IV)

4.2.4.1 Immunohistochemistry for $p16^{INK4a}$ and Ki-67

Immunohistochemical staining was performed with the CINtec®. Histology Kit (Code No. K5336, Dako Cytomation, Glostrup, Denmark) following the manufacturer's recommendations and using the Dako Autostainer. This kit contains Tris EDTA buffer (x10) pH 9.0 intended for epitope retrieval. A Coplin jar filled with the diluted epitope retrieval buffer (1:10 distilled water) was placed in a water bath and heated to 95-99˚C. Deparaffinized sections were then incubated for 10 minutes while maintaining a temperature of 95-99˚C. Jars with slides were removed from the water bath and left to cool at room temperature for 20 minutes, followed by washing for 5 minutes in Wash Buffer (Code No. S3006, Dako Cytomation) diluted 1:10 with distilled water. The automated procedure began with 1x rinse (1x rinse equals 4 min) in wash buffer diluted.

1:10 with distilled water [Wash Buffer (x10), Code No. S3006, Dako Cytomation]. Endogenous peroxidase activity was blocked by incubating slides for 5 minutes in Peroxidase-Blocking Solution (Dako REAL™, Code No. S2023, Dako Cytomation). Slides were rinsed 1x, after which 200 µl of the primary antibody to the $p16^{INK4a}$ protein clone E6H4 was dropped onto each slide, followed by incubation for 30 minutes. Slides were rinsed once. Reaction products were visualized by incubating slides for 30 minutes with the Visualization Reagent (a horseradish peroxidase/goat anti-mouse immunoglobulin-labeled dextran polymer) and, after 2 rinses, incubating for 10 minutes in a 1:40 solution of DAB chromogen (3.3'-diaminobenzidine) in DAB Buffered Substrate, also from the CINtec® Histology Kit. Slides were then washed in distilled water for 1 minute and counterstained for 2 minutes in Harris Hematoxylin solution diluted 1:2 with distilled water. After 2 minutes of washing in water, slides

were then dehydrated in ethanol to xylene and mounted in a water-free permanent mounting medium with mounting glass. Tissue sections containing cervical cancer were used as positive controls for $p16^{INK4a}$, while negative controls consisted of incubated doublet slides in the Negative Control Reagent contained in the kit, instead of primary antibodies. Immune staining was considered positive for $p16^{INK4a}$ when the nuclei were clearly stained. In addition, cells with a distinct cytoplasmic immunoreaction were scored as positive. Scoring of the immunohistochemistry results was performed on the basis of both staining intensity and percentage of immunoreactive epithelial cells. Scoring criteria for $p16^{INK4a}$, expressed as percentages (%) were: negative =no expression; <20 =weak staining (±); 20-30 =weak to moderate staining (+); 31-50 =moderate to strong staining (++); >50 =strong staining (+++). Scores of +, ++ and +++ were considered positive for $p16^{INK4a}$.

To detect the Ki-67 antigen we used monoclonal mouse antibody (clone MIB-1), (Code No. M7240, Dako). The sections were deparaffinized, rehydrated, and microwaved in target retrieval solution diluted 1:10 with distilled water (Dako REAL Target Retrieval Solution (x10) Code No. S2023, Dako Cytomation) for 2x5 minutes at 500 W. Thereafter slides were loaded on the Autostainer according to procedure and run together with the $p16^{INK4a}$ slides as described above. Ki-67 positivity was scored on a 1-3 scale in a manner similar to p16, but only with respect to both nuclear staining and attention to heterogeneity in distribution.

4.2.4.2 Immunohistochemistry for the LRIG proteins

Immunohistochemical staining of LRIG1, LRIG2 and LRIG3 was carried out using polyclonal rabbit antibodies against the cytosolic tails of the proteins. Tissue sections containing squamous cervical cancer were used as positive controls, and appropriate negative controls were always included. A senior pathologist blinded to all clinical data, evaluated the immune staining. Immunohistochemistry results were scored based on both staining intensity and percentage of immunoreactive epithelial cells. The percentage of positive cells was based on a 5-grade semi-quantitative scale: 0= no expression; 1= >0 and <24% positive cells, 2= 25-49% positive cells, 3= 50-74% positive cells, 4= >75% positive cells. Intensity of staining was evaluated on a four-grade semi-quantitative scale: 0= no staining; 1= weak, 2= moderate, and 3= intense.

4.2.4.3 Immunohistochemistry for Wig-1

For assessment of Wig-1 a polyclonal rabbit antibody anti- Wig-1 from Genetex was used after deparaffinization and the common pretreatment, including incubation with normal goat serum (DAKO) to block nonspecific staining. Sections were first incubated with primary antibody against Wig-1 (1:400 in 1% BSA) at 4°C overnight, and subsequently with secondary antibody (Goat anti-rabbit from DAKO) and finally with ABComplex HRP (DAKO) to visualize reaction products. Slides were mounted after counterstaining with hematoxylin. Scoring of IHC results was performed on the basis of staining intensity and intracellular distribution (nuclear vs. cytoplasmic). Staining intensity was evaluated on a four-grade semi-quantitative scale: 0 (no staining), 1 (weak), 2 (moderate), and 3 (strong).

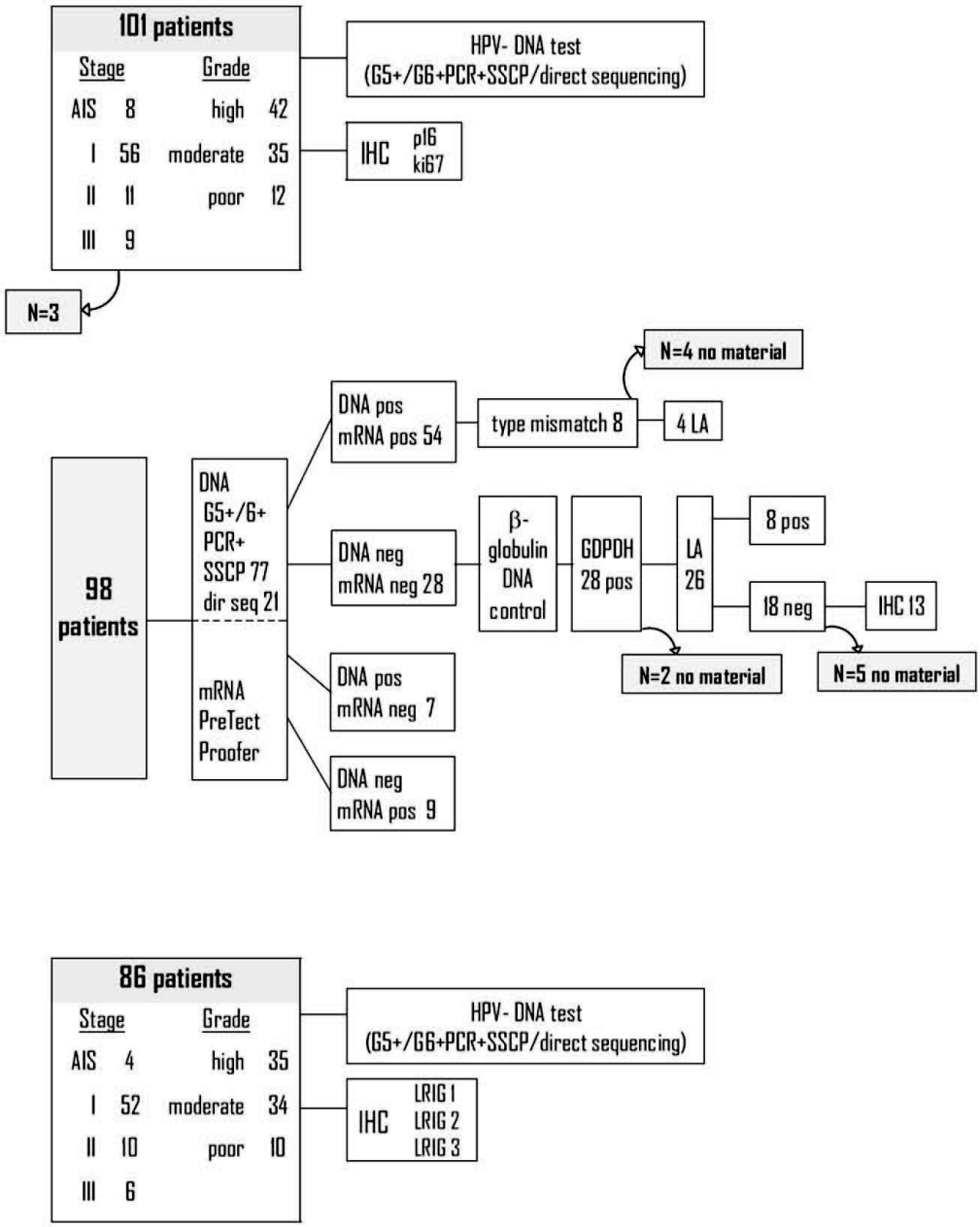

Figure 4-1 Flowchart illustrating the design of study I- III

4.2.5 Cell lines

The eight cervical cancer lines Ca Ski, ME-180, MS751, SiHa, SW 756, C-4I, C-33A, and HT-3 were examined for structural and numerical alteration in the 3q region by SKY and CGH, respectively. Possible copy number alterations of the WIG-1 gene were assessed by Southern blot and dual color FISH. mRNA expression was examined by

Northern (qualitative) and RT-PCR (quantitative). Finally, Wig-1 protein expression was evaluated by Western Blot.

4.2.6 Statistical analysis

The Statistical Program for Social Sciences (SPSS) was used for statistical analysis and a significance level of $p< 0,05$ was applied. In Paper I the impact of biomarkers on survival was evaluated by Cox regression analysis and the relation between two variables was examined using ANOVA. In paper II Cox regression analysis was applied to evaluate the difference in survival between HPV mRNA positive and negative cases. Chi-2 or Fisher's exact test were used for evaluation of ordinal variables. The impact of HPV status and expression of the LRIG proteins on survival was described in a Kaplan-Meier graph and survival in different groups was compared using the log rank test. Cox regression was applied for multivariate analysis of different molecular markers. In paper IV ANOVA was performed to examine the correlation between WIG-staining and clinical parameters, as well as HPV status. Survival distribution in different groups was tested with the Log rank test and described in a Kaplan Meier graph.

5 RESULTS AND DISCUSSION

5.1 PAPER I

The objective of this study was to evaluate expression of the $p16^{INK4a}$ tumor suppressor gene and the proliferation antigen Ki-67 in cervical adenocarcinoma. We sought to evaluate the relationship between the two markers and clinical parameters such as age, stage, grade and survival.

$P16^{INK4a}$ expression in relation to histology: 86% of tumors stained for $p16^{INK4a}$. Surprisingly, the proportion of $p16^{INK4a}$ staining decreased with lower tumor grade (95% in well-differentiated tumors, 91% in moderate and 41% in poorly differentiated tumors). Similarly, a significant negative correlation was found between $p16^{INK4a}$ staining intensity and histological grade (p=0.001); we found that highly differentiated tumors stain more strongly than poorly differentiated tumors. No correlation was found between $p16^{INK4a}$ staining intensity or proportion of stained samples and tumor stage. No correlation was found between $p16^{INK4a}$ staining and either age or survival**.**

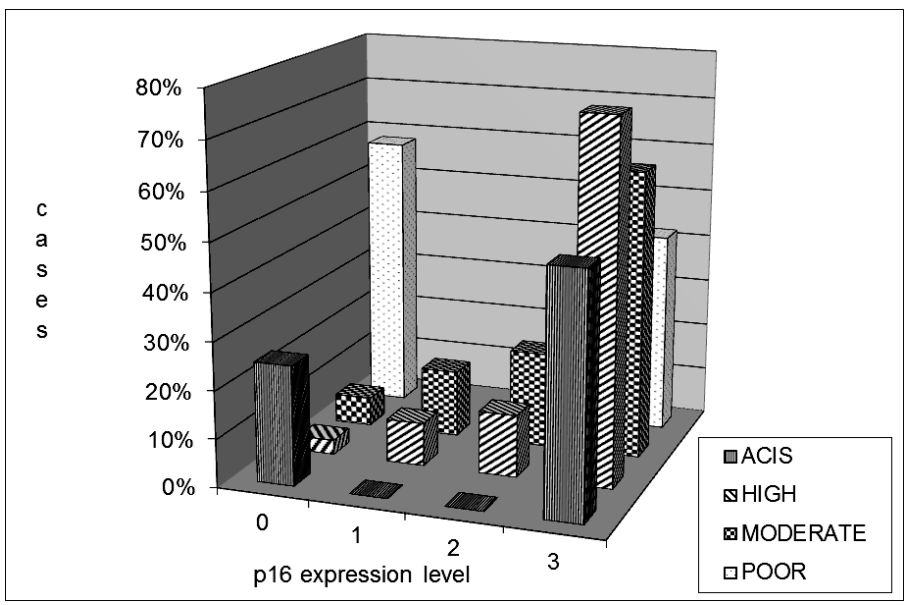

Figure 5-1 Different scores of p16 expression in samples with various grade of differentiation of cervical adenocarcinoma. Frequency of different scores of p16 antigen expression in all samples, distributed over the four grades, as shown in 97 cases. Vertical hatched bars, AIS (6 cases); crosshatched bars, highly differentiated tumors (41 cases); chess hatched bars, moderately differentiated tumors (32 cases); and punctuated bars, 5 cases where histology showed poor differentiation.

HPV infection was strongly correlated with higher $p16^{INK4a}$ staining (p=0.00). None of the HPV-positive tumors was $p16^{INK4a}$-negative and 40% of the HPV-negative tumors were also negative for $p16^{INK4a}$ expression. The remaining 60% stained with varying

intensity. No single HPV type was found to be more strongly associated with $p16^{INK4a}$ expression than any other type.

The $p16^{INK4a}$ tumor suppressor gene is inactivated in many carcinomas, but in cervical carcinoma it is often overexpressed, both in the nucleus and the cytoplasm (Saqi *et al.* 2002). The majority of cervical cancer is caused by HR-HPV infection. Overexpression of $p16^{INK4a}$ in these tumors is thought to be the result of functional inactivation of the Rb tumor suppressor protein by the E6 and E7 viral oncoproteins (Klaes *et al.* 2001). $p16^{INK4a}$ transcription is induced by transcription factor E2F, which is released from Rb after Rb binding to the HR-HPV E7 protein (Khleif *et al.*1996). Release of E2F activates transcription of $p16^{INK4a}$, which accumulates to high levels in the cell. It has been shown that evaluation of $p16^{INK4a}$ immunostaining allows identification of preinvasive and invasive cervical squamous lesions in biopsies, while reducing both false negative and false positive readings (Klaes *et al.* 2001). Expression of $p16^{INK4a}$ has been discussed as a potential predictor of rapid progression, as seen in a longitudinal study (Wang *et al.* 2004) and its expression may suggest the presence of transformed cells, even if the lesions appear morphologically normal. However, the specificity of the method has been debated since it has previously been shown to have limited application in cervical glandular lesions (Murphy *et al.* 2004). Positively staining $p16^{INK4a}$ samples in the setting of relatively few Ki-67 expressing cells has been seen in squamous metaplasia (Nielsen *et al.* 1999). Our earlier study showed a positive correlation between grade of CIN and expression of $p16^{INK4a}$ in squamous cell lesions (Andersson *et al.* 2006c). $p16^{INK4a}$ has also been tested as a marker for persistence of HPV infection, a predictor of progression to cancer, and has been found to correlate with histological grade (Branca *et al.* 2004; Negri *et al*. 2004). The majority of the studies did show elevated $p16^{INK4a}$ levels in HR-HPV positive cervical cancer, although the studies mainly addressed squamous cell cancer.

In our material, $p16^{INK4a}$ staining correlated with degree of histological differentiation, showing a lower proportion of staining in poorly differentiated tumors. Some studies evaluated expression of $p16^{INK4a}$ in adenocarcinoma. Missaoui et al. found $p16^{INK4a}$ expression in all such cases, although the authors did not specify grade of differentiation (Missaoui *et al.* 2006). Ishikawa et al. found no correlation between $p16^{INK4a}$ expression and histological subtype of adenocarcinoma, age, stage or recurrence (Ishikawa *et al.* 2003).

We suggest that the reason for lower $p16^{INK4a}$ expression in less differentiated tumors is that highly differentiated tumors may still have the capacity to express the $p16^{INK4a}$ tumor suppressor owing to the oncogenic activity of HR-HPV E7, whereas poorly differentiated tumors may have lost this capacity.

Our finding that all HPV-positive adenocarcinomas stained for $p16^{INK4a}$ is consistent with results from other groups (Missaoui *et al.* 2006). The etiological role of HR-HPV in cervical adenocarcinoma is not completely understood. In our material, we found that 64% of tumors were HR-HPV positive. Impaired gene expression probably contributes to carcinogenesis in some adenocarcinomas, especially in HPV-negative cases. In our material, no detectable $p16^{INK4a}$ overexpression was found in 40% of

HPV-negative tumors. Thus loss of functional $p16^{INK4a}$ may cause uncontrolled proliferation, or even possibly result from it.

The fact that 60% of HPV-negative tumors in our sample showed overexpression of $p16^{INK4a}$ signals that HPV independent mechanisms can also lead to overexpression of $p16^{INK4a}$. Alternatively, this finding could be due to undetected past or current HPV infection.

MIB-1 levels were used to reflect Ki-67 expression and, as expected, histological grade correlated strongly with Ki-67 expression, showing both increased intensity and higher fraction of Ki-67 staining with higher tumor grade. All poorly differentiated tumors stained for Ki-67. The correlation between staining intensity and tumor grade was significant (p=0.004). Furthermore, higher MIB-1 levels were significantly associated with higher tumor stage at diagnosis (p=0.038). Higher MIB-1 levels as a marker for Ki-67 expression showed a significant correlation with poorer outcome (p=0.004) after adjusting for age.

HPV infection correlated significantly with better survival, but after adjusting for age this correlation was no longer significant.

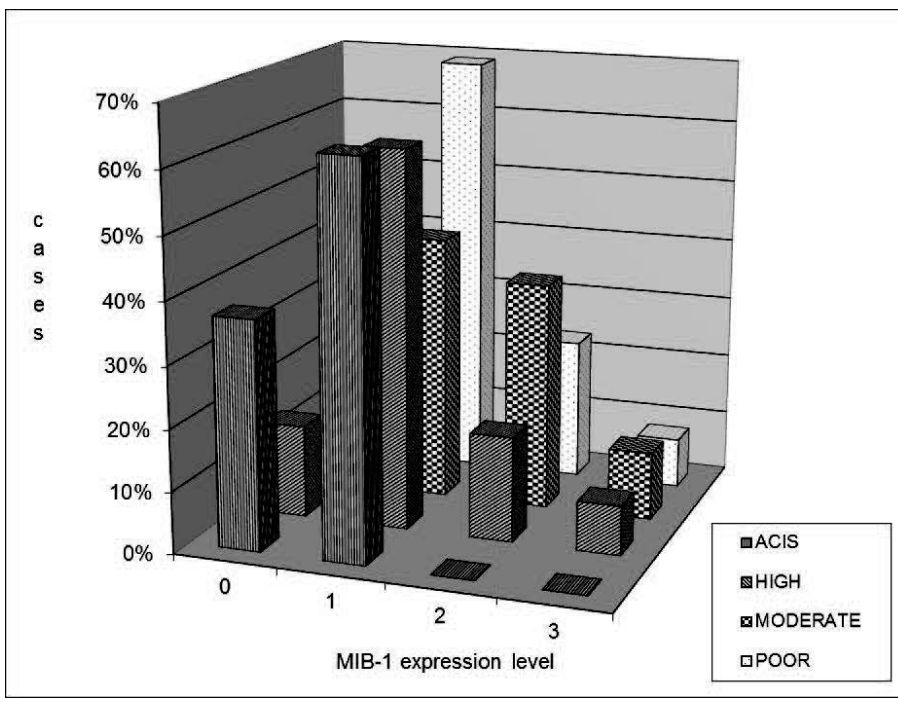

Figure 5-2 Different scores of MIB-1 expression in samples with various grade of differentiation of cervical adenocarcinoma. Ninety-nine cervical adenocarcinomas were evaluated for the expression of MIB-1 antigen. Vertical hatched bars, AIS (5 cases); crosshatched bars, highly differentiated tumors (40 cases); chess hatched bars, moderately (35 cases); and punctuated bars, 13 cases with poor differentiation histology.

Some studies have indicated the potential value of immunostaining for Ki-67 to distinguish endocervical neoplasia from benign endocervical lesions. Cina et al. reported that Ki-67 staining was negligible or low in normal endocervical tissue (Cina *et al*. 1999). In our study we found that the MIB-1-positive proportion of samples and labeling intensity both correlated with cancer stage and grade; higher levels of MIB-1 were associated with higher stage at diagnosis and more poorly differentiated tumors. Higher levels of MIB-1, representing the Ki-67 proliferation antigen, were significantly associated with poorer outcomes in our patients. This result is consistent with previous studies of squamous cell carcinomas, suggesting that higher MIB-1 levels are powerful predictors of shorter survival (Ho *et al.* 2000). However, other investigations found that MIB-1 expression had no clinical utility for predicting long-term disease-free survival (Avall-Lundqvist *et al*. 1997; Graflund *et al.* 2002).

Our study found a significant positive correlation between $p16^{INK4a}$ and Ki-67 staining. The interaction between $p16^{INK4a}$ and Ki-67, the so-called dual-stain approach, has been increasingly investigated in recent years. Under physiological conditions, simultaneous expression of the $p16^{INK4a}$ tumor suppressor and the Ki-67 proliferation marker should exclude each other. Thus, the combined detection of both markers should represent deregulation of cell cycle control, resulting from infection with oncogenic HPV. Simultaneous $p16^{INK4a}$/Ki-67 expression has been reported to increase with severity of lesion (Wentzensen *et al.* 2012). Its diagnostic potential has been shown with respect to effective triage of ASCUS and LSIL (Schmidt *et al.* 2011; Petry *et al.* 2011) and its sensitivity to detect CIN2+**.**

In summary, we found that a lower proportion of poorly differentiated tumors stain for $p16^{INK4a}$ and do so with less intensity than do well-differentiated lesions; all HPV-positive tumors demonstrated $p16^{INK4a}$ staining, but 60% of HPV-negative tumors also stained for $p16^{INK4a}$. Moreover, we found a significant correlation between staining for Ki-67 and histological grade, in regard to both staining intensity and proportion of samples that stain. Poorer outcome was significantly related to higher Ki-67 staining.

5.2 PAPER II

The aim of our study was to compare DNA-based and mRNA-based methods with respect to detection of HR-HPV in paraffin-embedded samples of cervical adenocarcinoma. Since the PreTect HPV-Proofer always detects full-length E6/E7 mRNA, a positive finding should strongly correlate with integrated HPV, loss of HPV replication, and stabilized E6/E7 full-length mRNA expression. Stabilized E6/E7 full-length mRNA resulting from integration of HPV correlates very well with high, stable expression of full-length E6 proteins. HPV mRNA testing demonstrated HPV in 64% of samples, while HR-HPV DNA testing with GP5+/GP6+ PCR and SSCP or direct sequencing revealed HPV in 62% of samples. Another eight samples turned HR-HPV positive after retesting with the reverse line blot method using Linear Array (Roche).

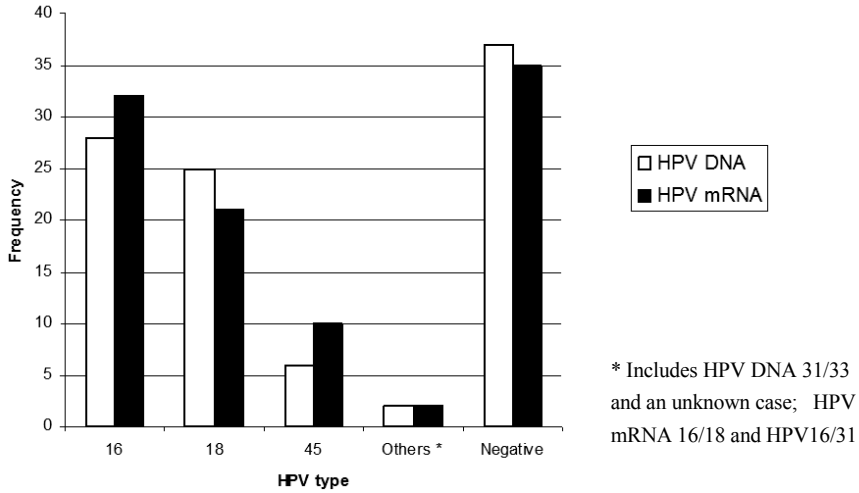

Figure 5-3 Detection of different HPV types with DNA and mRNA methods.

Previous studies using PCR techniques to detect HPV DNA on formalin-fixed, paraffin-embedded biopsy specimens of cervical adenocarcinomas, reported HPV positivity at rates ranging from 32% to 85% (Duggan *et al.* 1995; Clifford *et al.* 2006). Prevalence of HR-HPV may be higher when examining fresh-frozen tumor tissues because of alterations in the DNA sequence caused by formalin fixation and storage in paraffin (Karlsen *et al*. 1994; Williams *et al.* 1999; Tang *et al.* 2006). Successful extraction of intact mRNA from paraffin-embedded samples was confirmed using GAPDH control.

Differences in sensitivity may be due to use of different DNA/RNA extraction methods, primers, and consensus and/or HPV type-specific primers, as well as to differences in preparation, fixation, and processing of sample tissue. However, the main reason for reduced sensitivity may be the lack of control for presence of cancer tissue in all collected sections. In this study, it was not possible to include histological evaluation of tissue samples before and after the tissue was used for RNA/DNA extraction. Another factor that may reduce efficiency of HPV detection is loss of portions of the viral genome during integration. Integration of HPV DNA may result in deletion of the viral genome containing the sequences targeted by the PCR reaction. A higher proportion of DNA integration in glandular lesions compared with squamous lesions may result in a lower proportion of detectable episomal HPV genome and thereby lead to significant underestimation of HPV DNA in cervical adenocarcinomas (Park *et al.* 1997; Chung *et al.* 2008).

In 8 (8%) cases, the results of the mRNA-based and DNA-based methods differed regarding HPV type. An earlier study on cervical squamous cell cancer that compares the mRNA and DNA methods describes type mismatch in only 2% of cases (Kraus *et al.* 2006). We retested 4 of these cases using Linear Array and found type consistency with mRNA in all 4 cases. Results regarding HR-HPV presence or absence obtained through DNA and mRNA testing were consistent in 87% of samples.

Both assays revealed ~70% positive findings for HPV when targeting HPV 16 and 18 alone. HPV types 16, 18, 31, and 45 accounted for 99% of all HPV types within our group of 77 HPV-positive cases.

HPV type in cocktail	# Types	#TP	#FN	FP±	#TN	Sensitivity
0	0	0	78*	0	20	0%
HPV 16	1	28	50	0	20	35.9%
HPV 16-18	2	53	25	0	20	67.9%
HPV 16-18-45	3	59	19	0	20	75.6%
HPV 16-18-45-31	4	60	18	0	20	76.9%
HPV 16-18-45-31-33	5	60	18	0	20	76.9%

*One case with unknown HPV-type, thereby giving total positives 60 (TP60) instead of 61 (TP61)

± Specificity was overall 100%, because we included only cases with adenocarcinoma.

Table 5-1 Number of TP, FN, TN detected in HPV positives (by any method) in cervical adenocarcinoma using SSCP and direct sequencing

HPV type in cocktail	# Types	#TP	#FN	#FP ±	#TN	Sensitivity
0	0	0	78	0	20	0.0%
RNA16	1	32	46	0	20	41.0%
RNA 16-18	2	53	25	0	20	67.9%
RNA 16-18-45	3	63	15	0	20	80.8%
RNA 16-18-45-31	4	63	15	0	20	80.8%
RNA 16-18-45-31-33	5	63	15	0	20	80.8%

± Specificity was overall 100%, because we included only cases with adenocarcinoma.

Table 5-2 Number of TP, FN, TN detected in HPV positives (by any method) in cervical adenocarcinoma using PreTect HPV-Proofer

In summary, our data indicate that mRNA HPV testing is at least as sensitive as HPV DNA testing to identify women with cervical adenocarcinoma and that mRNA extracted from paraffin-embedded tissue sections could be used for further investigations. Since most adenocarcinomas are HPV-positive, it is possible that mRNA testing will have a stronger impact on prevention of adenocarcinoma than cytological screening, especially in young females. Thus, E6/E7 oncogene expression analysis of precursor lesions (i.e., adenocarcinoma *in situ* and glandular dysplasia) will be the next step to verify the potential of E6/E7 expression as a molecular marker for early detection and progression to invasive disease. In contrast to cytology, detection of E6/E7 HPV mRNA is an objective molecular marker, and may allow diagnosis of cervical adenocarcinoma at an earlier stage in the natural history of the disease. The ability to detect almost all HPV-related adenocarcinomas, including the limited HPV types covered by the PreTect HPV-Proofer assay, is a new discovery that underscores the potential of this test for adenocarcinoma screening.

5.3 PAPER III

Our main finding was a significant correlation between survival and expression of LRIG1 and LRIG3. Survival was significantly better among patients with tumor samples that exhibited moderate or intense staining for LRIG1 than among those with

tumors showing no or mild intensity of LRIG1 staining (p=0.003). Significantly better survival was also observed in patients with tumors where 50% or more of the cells stained for LRIG3 compared with patients whose tumors showed a lower fraction of LRIG3 staining (p=0.004). Multivariate regression analysis showed that the LRIG3-positive fraction was the only independent parameter exerting a statistically significant influence on survival, apart from age.

IHC analysis of LRIG proteins showed a balanced distribution of staining intensity for LRIG1 and LRIG2, but not for LRIG3 where 89.4% of tumor samples showed weak or no staining. The majority of tumor samples showed staining in >50% of cells: 64% for LRIG1, 74.4% for LRIG2, 54% for LRIG3. Intracellular distribution varies for LRIG proteins: LRIG1 was only found in cytoplasm, LRIG2 was seen in the cytoplasm and nucleus, while LRIG3 was mainly detected in the nucleus.

A	**LRIG staining intensity**			
	No staining	Weak	Moderate	Intense
	0	1+	2+	3+
LRIG1	11 (12.9%)	18 (21.2%)	26 (30.6%)	30 (35.3%)
LRIG2	5 (5.9%)	19 (22.4%)	34 (40.0%)	28 (31.8%)
LRIG3	17 (20.0%)	59 (69.4%)	8 (9.4%)	1 (1.2%)

B	**Fraction of LRIG positive cells**				
	0	>0 - 24%	25 - 49%	50 - 75%	>75%
		1+	2+	3+	4+
LRIG1	11 (12.8%)	4 (4.7%)	16 (18.6%)	25 (29.1%)	30 (34.9%)
LRIG2	5 (5.8%)	3 (3.5%)	14 (16.3%)	8 (9.3%)	56 (65.1%)
LRIG3	17 (20.0%)	11 (12.9%)	11 (12.9%)	11 (12.9%)	35 (41.1%)

Table 5-3 Staining intensity (A) and fraction of positive cells (B) of the LRIG immunohistochemical analysis.

In this study, both higher intensity of LRIG1 staining and higher proportion of stained cells in the tumor were associated with improved patient survival. A correlation has been reported between higher expression in the tumor and better patient prognosis in breast cancer (Krig *et al*. 2011), cutaneous squamous cell carcinoma (Tanemura *et al.* 2005) and early stage invasive squamous cervical cancer (Lindström *et al.* 2008). Moreover, increasing grade of CIN is associated with increasing LRIG1 expression (Lindström *et al.* 2011). These data suggest that LRIG1 is involved in both precancerous and invasive squamous cervical lesions. Our material showed that LRIG1 also functions as a prognostic marker for cervical adenocarcinoma. An earlier study evaluated the prognostic impact of LRIG1 on cervical cancers, including both squamous cell and adenocarcinoma, in which no correlation with survival was found in the adenocarcinoma portion, possibly due to the limited sample size of 36 patients (Hedman *et al.* 2010). The finding that prognosis is improved by the presence of higher LRIG1 expression in both adenocarcinoma and squamous cell carcinoma of the cervix suggests an important role for this marker in the disease.

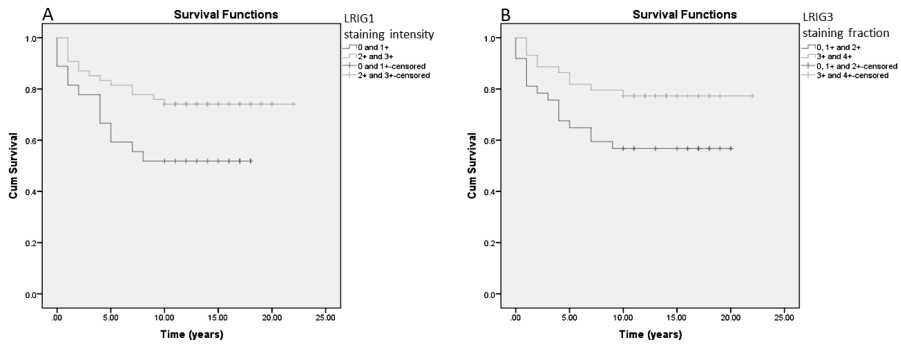

Figure 5-4 Correlation between LRIG1 staining intensity (A) and fraction of LRIG3 staining cells (B) and survival. The 4 (A) or 5 (B) groups were dichotomized using the median as a cut-off to generate two groups of equal size.

In cervical SCC, LRIG2 expression has been reported to correlate with worse survival (Hedman *et al.* 2010). In our material no such correlation between LRIG2 expression and survival or other clinical parameters was found for cervical adenocarcinoma. Thus, LRIG2 might be more important as a possible marker of prognosis in cervical SCC and other cancer types.

Staining for LRIG3 has been evaluated in oligodendroglioma and in astrocytic tumors. Perinuclear staining of LRIG3 in astrocytic tumors correlated with better survival (Guo *et al.* 2006). This is consistent with the findings in the present study where LRIG3 is linked to better survival. LRIG3 may have a tumor suppressive function similar to that of LRIG1. However, recent experimental results have shown that LRIG1 and LRIG 3 interact with ErbB2, where the latter exerts an opposite effect on the receptor (Abreira *et al.* 2010, Rafidi *et al.* 2013).

A significant correlation was seen between HPV status and staining intensity for LRIG1 ($p=0.001$) and LRIG3 ($p=0.004$), with higher staining intensity in HPV-positive cases. No significant correlation was found between LRIG expression and stage or histological findings. HPV positivity was also identified as a significant prognostic factor ($p<0.001$).

Whether or not the positive correlation between HPV status and staining intensity of LRIG1 and LRIG3 is relevant to prognosis remains to be elucidated. Nevertheless, LRIG3 has been shown to be an independent prognostic factor after multivariate analysis. For other HPV-related cancers, such as cancer of the tonsils and base of the tongue, the existence of two different tumor types with different prognosis, depending on HPV status, has been suggested (Attner *et al.* 2011, Hammarstedt *et al.* 2006). Our results suggest differences regarding prognosis between HPV-positive and HPV-negative cervical adenocarcinomas, at least insofar as LRIG expression relates to patient survival.

Thus, analysis of LRIG expression in precursor lesions (i.e., adenocarcinoma in situ and glandular dysplasia) will be important to determine the potential of LRIG proteins

as molecular markers for early detection and progression to invasive disease. LRIG immunoreactivity is of prognostic importance in cervical adenocarcinoma and correlates with HPV status. Therefore, LRIG proteins may be important determinants of cervical adenocarcinoma progression, which justifies further study of their diagnostic and prognostic potential in this disease.

5.4 PAPER IV

WIG-1 is amplified and/or overexpressed in many human tumors, indicating an oncogenic function (Varmeh-Ziaie *et al*. 2001). WIG-1 has been mapped to chromosome 3q26.3, a region that shows frequent copy number gain in various human tumors (Hellborg *et al.* 2001). Gain of chromosome 3q occurs during progression from cervical carcinoma in situ to advanced invasive cancer (Heselmeyer *et al.* 1996). These findings suggest that in cervical cancer WIG-1 is associated with gain of 3q. We therefore examined Wig-1 expression in cervical carcinoma cell lines and assessed Wig-1 expression in biopsies containing samples of cervical carcinoma.

5.4.1 Cell line studies

We first assessed structural and numerical alterations of the WIG-1 locus in cervical carcinoma cell lines by SKY and CGH analyses, which showed that WIG-1 is not a primary target for chromosome 3 alterations in these cells. We found single structural chromosome 3 abnormalities by SKY in six cells, but in no case did the breakpoint involve the 3q26.32 region where WIG-1 is located. All eight lines studied by CGH exhibited copy number variations of chromosome 3.

We then studied the WIG-1 locus for possible copy number variations. Using Southern analysis, WIG-1 was compared with a control marker from the short arm of the same chromosome (3p23), which showed modest gain of WIG-1 in MS751 and ME-180 cells. High copy-number amplification of WIG-1 was excluded by dual color FISH using WIG-1 and D3Z1 probes.

WIG-1 is located telomeric to the commonly amplified 3q23-24 interval in Me180 and SiHa cells, and centromeric to the amplified 3q27-ter interval in ME-180 cells. Moreover, the 3q amplifications detected by CGH were not accompanied by WIG-1 locus amplifications as determined by Southern blotting or FISH. Thus, we conclude that WIG-1 is not the critical gene that drives 3q gain in cervical cancer.

We assessed WIG-1 mRNA and protein expression in all cell lines by Northern and Western blotting. HT-3, C-33A and Saos-2 cells showed strong WIG-1 mRNA expression, while Ca Ski, ME-180, SiHa and SW756 showed weaker but clearly detectable expression. Western blotting showed Wig-1 protein expression in all cell lines. However, no associations were found between WIG-1 expression and copy number variations at the DNA level. WIG-1 mRNA expression, as assessed by quantitative RT-PCR, was higher in the two HPV-negative cervical carcinoma lines (C33-A, HT-3) than in the HPV-positive cell lines. We found no association between WIG-1 protein expression and HPV infection in cell lines.

Cell line	HPV	Chromosome		Chromosome 3 aberrations	
Name	Infection	content		by SKY	by CGH****
Ca Ski	HPV-16	(3n)	64-74	der(3)t(3;12)(p22;q24)	+3p12-qter(**q23-q26**)
ME-180	HPV-68**	(2n)	54-57	i(3)(q10)	+3q(**q27-qter**)
MS751	HPV positive***	(2n-3n)	37-75	der(3)t(3;8)(p11;q11)	-3pter-p11, +3q24-q26
SiHa	HPV-16	(3n)	65-71	der(X)t(X;?3)(p11;?)	+3p25-q26(**q23-q24**)
SW756	HPV-18	(2n)	37-45	None	+3q26
C-4I	HPV-18	(2n)	35-45	None	+3q24-q26
C-33A	Negative	(2n)	42-47	None	+3p21, +3q26
HT-3	Negative	(3n)	57-59	der(3)t(3;12)(p11;q11)	-3p, +3q26-q27
Saos2*	Negative	(2n-4n)	52-99	dup(3)(q12q29); dic(3;19)(?;q13.3); der(X)t(X;1;3)(X?->cen->X?::1?->1?::3?->3?)	+3q

*Saos2 is a osteosarcoma cell line

**According to ATCC it contains HPV-39

*** HPV type could not be determined by direct DNA sequencing of PCR products

**** Bold in parenthesis indicates amplification within the gained interval

Table 5-4 HPV status and chromosome 3 aberrations in the cervical cancer cell lines.

	WIG-1 copy number by		*WIG-1* / Wig-1 expression			*TP53* / p53 status**	
Cell	FISH	Southern		*WIG-1* by	Wig-1		
Line	*WIG-1* vs. *D3Z1*	*WIG-1* vs. *3p23*	Northern	qRT-PCR*	by Western	*TP53* sequence	p53 expression
HPV positive							
Ca Ski	no amplification	n. d.	+	3.07	positive	wild-type	very low / absent
ME-180	no amplification	gain	+	4.91	positive	wild-type	very low / absent
MS751	no amplification	gain	-	5.95	positive	wild-type	absent
SiHa	no amplification	no gain	+	2.75	positive	wild-type	very low / absent
SW756	no amplification	no gain	+	3.13	positive	n. a.	n. a.
C-4I	no amplification	no gain	n. d.	4.67	positive	wild-type	very low / absent
HPV negative							
C-33A	no amplification	no gain	++	5.79	positive	mutated (p.Arg273Cys)	high but inactive
HT-3	no amplification	n. d.	++	5.47	positive	mutated (p.Gly245Val)	high but inactive
Saos2	n. d.	no gain	++	11.74	positive	homozygously deleted	absent

- = negative; + = detectable to moderate expression; ++ = strong expression; n.d. = not determined; n.a. = not available;
* Given in arbitrary units as compared to fibroblasts (1.0)
** Reiss et al., 1992; Yaginuma et al., 1991; Srivastava et al., 1992; Scheffner et al., 1991; Masuda et al., 1987

Table 5-5 Copy number and expression data of WIG-1 / Wig-1 data in relation to HPV infection and TP53 / p53 status.

5.4.2 Cervical carcinoma tissue studies

The next step was to evaluate Wig-1 protein expression by immunohistochemistry in tissue samples of cervical carcinoma. All 38 cervical cancer cases in this study stained positive for Wig-1, mainly in cell nuclei. None of the 38 cases were Wig-1 negative; 40% showed moderate and 55% showed strong staining. Cytoplasmic Wig-1 staining intensity was relatively low compared with nuclear staining in most tumors; 58% of tumors showed negative cytoplasmic Wig-1 staining, while 42% showed weak staining. We observed two distinct Wig-1 immunostaining patterns: A) high nuclear staining with negative cytoplasmic staining and B) low nuclear staining with positive cytoplasmic staining. Of the 38 tumors, 8 showed pattern A and 14 showed pattern B. Normal tissue adjacent to the tumors showed moderate nuclear Wig-1 staining. Interestingly, these staining patterns showed a significant correlation with HPV status. Moreover, nuclear Wig-1 staining intensity was significantly associated with tumor type ($p < 0.0001$); ADC samples showed higher nuclear Wig-1 expression than did SCC samples.

We observed a possible correlation between Wig-1 expression and HPV status in these cervical carcinomas. HPV DNA is known to be almost invariably present in SCC (Walboomers *et al.*1999, Bosch *et al*. 2002}; however, our tumor material included 4 HPV-negative SCC cases. We first investigated whether Wig-1 expression differed between HPV-positive and HPV-negative adenocarcinomas (ADC). We found a statistically significant difference in nuclear Wig-1 staining intensity between HPV-positive and HPV-negative ADC cases ($p = 0.049$). After including all SCC cases, nuclear Wig-1 staining intensity remained significantly different between the HPV-positive and HPV-negative cervical tumors ($p = 0.002$).

We previously found that 70% of ADC cases were HPV DNA positive by PCR and direct HPV DNA sequencing (Andersson *et al*.2003). Thus, factors other than HPV are likely to play a role in the development of cervical ADC. The higher nuclear Wig-1 expression in the HPV-negative tumors suggests that elevated expression of Wig-1 might play a role in cervical carcinogenesis in the absence of HPV infection.

Furthermore, we found a trend indicating that moderate nuclear Wig-1 expression and positive cytoplasmic Wig-1 expression are associated with better overall survival ($p= 0.126$ and $p= 0.110$, respectively). In agreement with previous studies, patients with HPV-positive tumors had better survival ($p= 0.066$) than those with HPV negative tumors (though this relationship was not adjusted for age).

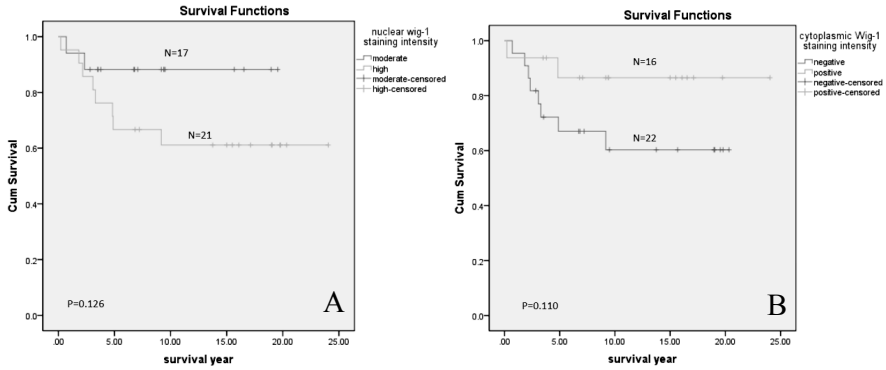

Figure 5-5 Survival curve to compare overall survival rates of patients between different nuclear (A) and cytoplasmatic (B) WIG-1 staining intensities of tumors by Kaplan-Meier analysis. Cases no. and Log Rank P values are indicated.

We then examined survival for the subgroup of patients who had moderate nuclear and positive cytoplasmic Wig-1 expression in their tumors. We found that this subgroup of patients had a significantly better prognosis than patients with moderate nuclear Wig-1 staining and negative cytoplasmic staining ($p = 0.042$). Since we observed higher nuclear Wig-1 expression in HPV-negative cell lines and tumors ($p = 0.002$), we compared the prognosis for this pattern with the prognosis associated with HPV-positive tumors characterized by moderate nuclear Wig-1 expression and found the prognosis ($p = 0.049$) to be worse for the former group (HPV-negative cell lines with high nuclear Wig-1 expression). Moreover, we found that HPV-negative tumors characterized by negative cytoplasmic Wig-1 staining were associated with worse prognosis than HPV-positive tumors characterized by positive cytoplasmic Wig-1 staining ($p = 0.033$).

How does high nuclear WIG-1 expression contribute to progression of cervical cancer? Our previous results indicate that WIG-1 has a pro-survival function. WIG-1 may stimulate cell proliferation and antagonize cell death through up regulation of putative mRNA targets such as N-Myc, c-Fos, c-Jun and Cyclin D1, and down regulation of the pro-apoptotic Fas (Vilborg *et al.*2009; Vilborg *et al.* 2012; Bersani *et al.,* manuscript in preparation). Thus, it is plausible that high nuclear Wig-1 expression in tumor cells drives cell proliferation through stabilization of pro-growth mRNA targets and destabilization of pro-apoptotic mRNA targets. However, the exact roles of nuclear and cytoplasmic Wig-1 remain to be elucidated.

Since WIG-1 is a p53 target gene, the question arises whether WIG-1 expression correlates with TP53 status in tumors and/or with presence of HPV, which encodes the E6 protein that targets p53 for degradation. The TP53 gene is more frequently mutated in HPV-negative than in HPV-positive cervical cancer cell lines and tumors (Tommasino *et al.* 2003). Among the 8 cervical cancer cell lines that we studied, the only two HPV-negative cancer cell lines, C-33A and HT-3, both carry the TP53 mutation. The TP53 status of the cervical carcinomas in our study has not been determined. Further studies to determine TP53 status in all tumor samples and to examine the correlation between WIG-1 expression and TP53 status are required. Yet,

it should be noted that TP53 is not the only transcription factor that might regulate WIG-1. Indeed, we detected WIG-1 protein in all cervical cell lines irrespective of TP53 status or genomic gain of the WIG-1 gene. According to Pscan (http://159.149.109.9/pscan), several other potential transcription factors, such as SOX, may induce WIG-1 expression (Vilborg *et al.* 2011). The finding that elevated WIG-1 expression does not correlate with functional TP53 indicates that WIG-1 expression also depends on other mechanisms, in addition to direct TP53-mediated transcriptional transactivation.

In summary, moderate to high Wig-1 protein expression is common in cervical cancer tissue, especially in the nucleus. Nuclear Wig-1 expression was significantly higher in samples of ADC compared with SCC. HPV-negative cervical tumors showed significantly higher nuclear Wig-1 expression than HPV-positive tumors. High nuclear expression in combination with negative cytoplasmic Wig-1 expression is associated with poor prognosis, whereas moderate nuclear expression in combination with positive cytoplasmic expression is associated with better prognosis. Amplifications in 3q occur in cervical cancer cells, but WIG-1 is not the primary target for driving 3q gains according to our SKY, CGH, FISH and Southern blotting analyses.

In conclusion, from the clinical standpoint it is very important to identify women who remain at risk of developing cervical cancer by finding early markers that permit rapid and focused preventive action, and to elucidate the factors that influence development of cancer in both HPV-infected and HPV-negative women. Understanding the molecular mechanisms underlying carcinogenesis in cervical cancer to improve early diagnosis, differential diagnosis, and evaluation of tumor aggressiveness, as well as to improve molecular markers for screening is crucial. Molecular markers will help differentiate between benign disease and malignancy, and thereby prove useful in clinical practice. The finding that Wig-1 protein expression is higher in HPV-negative cervical carcinoma suggests a possible role for WIG-1 in HPV-negative cervical carcinogenesis. Moderate nuclear Wig-1 expression and positive cytoplasmic Wig-1 expression are associated with better survival rates, indicating that balanced Wig-1 expression may be able to counter tumor growth. These findings may well contribute to optimization of current tumor diagnostics and pave the way for development of new treatments.

6 CONCLUSIONS

6.1 PAPER I

We evaluated the relationship between $p16^{INK4a}$ and Ki-67 expression in cervical adenocarcinomas in a series of 101 histological sections and correlated the findings with presence of HPV infection and clinical course. We found lower levels of $p16^{INK4a}$ tumor suppressor protein in poorly differentiated tumors than in higher differentiated tumors and higher levels in HPV-positive tumors than in HPV-negative tumors. Some HPV-negative tumors express $p16^{INK4a}$, indicating HPV-independent mechanisms that lead to $p16^{INK4a}$ overexpression in these tumors. The finding that 40% of HPV-negative cases lacked detectable $p16^{INK4a}$ suggests that impaired gene expression may contribute to carcinogenesis in these cases. Loss of functional $p16I^{NK4a}$ could be either the cause or the effect of uncontrolled proliferation. Higher levels of the MIB-1 proliferation marker were associated with worse outcome. Higher MIB-1 levels are seen in tumors with lower histological grade and higher tumor stage at diagnosis.

6.2 PAPER II

Detection of E6/E7 mRNA expression using the real-time nucleic acid sequence-based amplification assay (NASBA) PreTect HPV-Proofer was compared with results of HPV DNA detection in 98 paraffin-embedded samples from patients with cervical adenocarcinoma. We discovered that E6/E7 oncogene expression analysis may provide a more objective test for assessment of neoplastic glandular cells. E6/E7 mRNA testing is as feasible as HPV DNA testing to detect HR-HPV in paraffin-embedded samples of cervical adenocarcinoma. Most cervical adenocarcinomas are HPV-positive, therefore E6/E7mRNA as an objective marker has the potential to identify women with the disease. Despite the limited number of HPV types included, the assay has the ability to detect almost all HPV-related cervical adenocarcinomas. Further studies may reveal whether the clinical performance of the E6/E7 mRNA assay will be of prognostic value in management of cervical adenocarcinoma.

6.3 PAPER III

In this study, LRIG protein immunoreactivity was evaluated in 86 cervical adenocarcinomas. Both high staining intensity of LRIG1-positive cells and a high proportion of LRIG3-positive cells were significantly associated with patient survival, and positive correlations were found between both LRIG1 and LRIG3 staining intensity and HPV status. Since low expression of LRIG1 correlates with worse survival in cervical adenocarcinoma, LRIG1 may be of interest as a potential target for treatment. However, whether LRIG1 can inhibit tumor growth remains to be determined.

6.4 PAPER IV

The p53 target gene WIG-1 (PAG608, ZMAT3) is located on human chromosome 3q26.32, a region that is frequently amplified in human tumors, including cervical cancer. We have examined the status of WIG-1 in cervical carcinoma cell lines and in 38 cervical tumor samples, including both squamous cell carcinoma or adenocarcinoma. We detected that Wig-1 expression was positively associated with age at diagnosis and histological grade. ADC samples had significantly higher nuclear Wig-1 levels (than SCC samples. We observed higher nuclear Wig-1 expression in

HPV-negative ADC samples. Patients with moderate nuclear Wig-1 expression levels and positive cytoplasmic Wig-1 expression in their tumors had a better prognosis. Moreover, patients with HPV-positive tumors and positive cytoplasmic Wig-1 expression had better survival rate. These results are consistent with a growth-promoting and/or anti-cell death function of nuclear Wig-1 and suggest that Wig-1 expression can serve as a prognostic marker in cervical carcinoma.

7 FUTURE PERSPECTIVE

In recent years evidence has accumulated that combined detection of $p16^{INK4a}$ and Ki-67, using a single immunocytochemistry kit (CINtec PLUS), can help to predict high-grade or invasive lesions and this methodology is nearing clinical application. Most of the studies were carried out on squamous lesions (Petry *et al.* 2011; Schmidt *et al.* 2011; Wentzensen *et al.*2012). The studies approaching the predictive value of dual staining in glandular lesions are small in scope and few in number. However, the limited evidence shows a close association between a positive test result and subsequent diagnosis of invasive or pre-invasive cervical glandular lesions (Samarawardana *et al.* 2011; Ravarino *et al.*2012) and the potential to increase specificity of HPV testing when used to supplement regular testing (Singh *et al*.2012). An interesting next step would be to analyze the potential of this combination test to serve as a marker for cervical adenocarcinoma and its precursor lesions in a larger cohort and to address both cytological and histological considerations.

mRNA cytology testing to triage women with minor cytological abnormalities such as ASCUS, or positive DNA testing to detect CIN2+ have been shown to be more specific but less sensitive for evaluating squamous lesions (Verdoot *et al*. 2013; Stoler *et al.* 2013; Perez Castro *et al.* 2013; Waldström *et al.* 2012) and less reliable for follow-up (Persson *et al*. 2012). Most studies that use mRNA testing for screening or follow-up were not specifically concerned with glandular lesions, which presents an opportunity to investigate these methods as a potential tool for triage and follow-up of glandular lesions.

LRIG proteins have been evaluated in squamous cell cancer and precursor lesions, showing a correlation between an increase in LRIG1 and LRIG2 expression and increasing grade of CIN (Lindström *et al.* 2011). LRIG1 and LRIG2 have also been identified as prognostic markers for early stage SCC (Lindström *et al.* 2008; Hedman *et al.* 2010). Our next step would be to examine expression of the three LRIG proteins in precursor lesion tissue sections and carry out cytological evaluation.

Our studies of WIG-1 in cervical cancer open the door to questions that can be answered by follow-up experimental studies. Previous results indicate that WIG-1 may stimulate cell proliferation and antagonize cell death through up regulation of putative mRNA targets such as N-My, c-Fos, c-Jun and Cyclin D1, and down regulation of the pro-apoptotic Fas (Vilborg *et al*. 2009; Vilborg *et al.* 2012; Bersani et al., manuscript in preparation). Thus, it is plausible that high nuclear Wig-1 expression in tumor cells drives cell proliferation through stabilization of pro-growth mRNA targets and destabilization of pro-apoptotic mRNA targets. The exact roles of nuclear and cytoplasmic Wig-1 and their correlation with survival, independent of HPV status, need to be further elucidated.

Because WIG-1 is a p53 target gene, the question arises whether Wig-1 expression correlates with TP53 status and presence of HPV in tumors, since HR-HPV E6 protein targets p53 for degradation. The TP53 gene is more frequently mutated in HPV-negative than in HPV-positive cervical cancer cell lines and tumors (Tommasino *et al.* 2003). Among the 8 cervical cancer cell lines that we studied, the only two HPV-negative cancers cell lines, C-33A and HT-3, both carry the TP53 mutation. The TP53

status of the cervical carcinomas in our study has not been determined. Further studies are required to examine TP53 status in all tumor samples so that Wig-1 expression can be correlate with TP53 status. The natural antisense transcript Wrap53 is another interesting potential marker in this context. It is encoded by the Wrap gene located on chromosome 17 and directly overlapping the first exon of TP53. The WRAP53 gene gives rise to an antisense transcript that regulates the actions of the tumor suppressor TP53 and encodes for a protein with its own oncogenic properties.

More effective and reliable markers to predict women at risk for developing cervical cancer, especially adenocarcinoma, are clearly needed. Knowledge in this field has grown immensely in recent decades and initial success was achieved through the introduction and improvement of cytological screening, supplemented by HPV testing, and by the introduction of new biomarkers. Studies indicate that the first generation of HPV vaccines targeting HPV 16 and 18 could prevent at least two-thirds of cervical cancers and precursor lesions. Through a combination of vaccination against HPV and further improvements in detecting precursor lesions of cervical cancer using molecular markers in screening programs, this cancer may become the most preventable on a global scale.

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