Monoclonal antibodies against human papillomavirus E7 oncoprotein for diagnosis of cervical neoplasia and cancer

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Gothenburg 2012

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ISBN 978-91-628-8511-3

Printed in Gothenburg, Sweden 2012
Ineko AB, Gothenburg

For my family

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Abstract

Cervical cancer is the second most common cancer among women worldwide with half a million of new cases every year. Cervical cancer is caused by oncogenic human papillomaviruses (HPVs), with HPV16 and 18 being the most frequently detected types. Genital HPV infections are common in the general population although most infections are cleared before causing malignancy. However a small proportion of the infections evade the immune system, become persistent and may cause cervical lesions and even invasive disease. Being the causative agent for cervical malignancy, HPV is an obvious target for cervical cancer diagnosis and prevention. Current screening programs, primarily based on cervical cytology, produce millions of suspicious samples every year. Specific tools to identify high-grade disease in these samples are needed to increase specificity for malignancy and thereby reduce referral rates and overtreatment.

In the current study, monoclonal antibodies were raised against the HPV E7 oncoprotein. E7 is an absolute prerequisite for malignant transformation and the protein is expressed at increasing levels during cancer development. E7 is therefore a suitable marker for HPV-induced malignancy. Antibodies specific for the E7 protein of oncogenic HPV types were selected using immunological methods such as ELISA, Western blot, Immunocytochemistry and flow cytometry. Phage display was used to identify antibody epitopes thereby predicting and verifying antibody specificity. Two of the antibodies, recognizing HPV16 and 18 E7 respectively, demonstrated strong staining of dysplastic cells in HPV-positive specimens in immunocytochemistry and may thus have the potential to be used in a clinical setting. Since the antibodies detect the protein in Liquid-based cytology, which normally leaves residual sample after standard cytology, E7 testing can easily be performed without recalling the patient for additional sampling.

Immunological detection of the E7 oncoprotein is an attractive alternative for triage of suspicious and borderline cytology to highlight and identify the often rare dysplastic cells present in a cell scrape. E7 detection can further reduce subjectivity and be performed with only standard equipment and thereby make HPV-testing available also in less developed regions.

Keywords: Human papillomavirus (HPV), Cervical cancer, Cervical neoplasia,
E7 oncoprotein, Monoclonal antibodies, Cytology, Immunocytochemistry, Phage display

ISBN: 978-91-628-8511-3
http://hdl.handle.net/2077/29710

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Maria Lidqvist, Olle Nilsson, Jan Holmgren, Christina Hall, Christian Fermér. **Phage display for site-specific immunization and characterization of high-risk human papillomavirus specific E7 monoclonal antibodies.**
Journal of Immunological Methods, 337 (2008) 88-96.

II. Maria Lidqvist, Olle Nilsson, Jan Holmgren, Sebastian Hölters, Eva Röijer, Matthias Dürst, Christian Fermér. **Detection of human papillomavirus oncoprotein E7 in liquid-based cytology**.
Journal of General Virology, 93 (2012) 356–363.

III. Michael Lebens, Susanne Källgård, Christian Fermer, Maria Lidqvist, Hubert Bernauer. **Generation of plasmid encoded protein-specific overlapping peptide libraries and the mapping of B cell epitopes in HPV E7 oncoprotein recognized by monoclonal antibodies and antibodies in the serum of immunized mice**.
Submitted manuscript.

IV. Maria Lidqvist, Olle Nilsson, Jan Holmgren, Matthias Dürst, Elin Andersson, Peter Horal, Christian Fermér. **Clinical feasibility study of anti-E7 MAb for detection of HPV-induced neoplasia and cancer in liquid-based cytology**. *Submitted manuscript.*

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Abbreviations

aa	Amino acid
Ab	Antibody
ASC-US	Atypical Squamous Cells of Undetermined Significance
ATCC	American Type Culture Collection
CIN	Cervical Intraepithelial Neoplasia
CR	Conserved region
CTB	Cholera toxin B subunit
E	Early
GST	Glutathione-S-Transferase
HPV	Human papillomavirus
HRP	Horseradish peroxidase
ICC	Immunocytochemistry
Ig	Immunoglobulin
L	Late
LCR	Long control region
mAb	Monoclonal antibody
ORF	Open reading-frame
PEG	Polyethylene glycol
pRb	Retinoblastoma protein
SCC	Squamous cell carcinoma

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1 Introduction

Cervical cancer is the second most common cancer in women worldwide, with half a million new cases every year. Cervical cancer is caused by a limited number of oncogenic human papillomaviruses (HPVs) and viral DNA can be detected in almost all cancers (99.7 %)[1]. Most women and men are infected with genital HPV at least once in their life-time and the prevalence of genital HPV is over 10 % in the world[2]. The vast majority of all infections are transient and/or asymptomatic. However, a small proportion will evade the immune system and if left untreated, progress to cancer. The major challenge in cervical cancer diagnosis and management is thus to discriminate persistent high-risk infections, that should be treated or closely monitored, from transient infections and low-grade lesions that will regress, while minimizing overtreatment and unnecessary recalling of patients.

The Pap test is the most widely used screening method, often complemented by colposcopy and histology. In the Pap test, the morphology of exfoliated cervical cells is studied to identify pre-malignant and cancerous cells. Pap screening has reduced the number of cancer cases dramatically in many areas[3, 4], but to be efficient the test has to be repeated frequently and requires trained personnel. Millions of pap tests yield inconclusive or borderline results every year and require further sampling and evaluation to identify underlying disease. In addition, many women in the world are not included in a cervical cancer prevention program and thus cervical cancer still causes 270 000 deaths every year[5].

To reduce the number of cancer cases, both in regions with and without functional screening, more cost-effective, cancer-specific and preferably objective diagnostic methods are needed. Since Harald zur Hausen's discovery of the causality between high-risk HPV infection and cervical cancer in 1980, for which he was awarded with the Nobel Prize in physiology or medicine in 2008, a lot of effort has been put into understanding the viral life cycle and its ability to induce malignancy. Based on this knowledge HPV has become an important target for diagnosis and prevention of cervical cancer.

This thesis describes the generation of monoclonal antibodies (mAbs) detecting the E7 oncoprotein of carcinogenic HPV types and the characterization of the antibodies with special reference to their ability to specifically recognize E7 of high-risk types and finally a preliminary evaluation of their usefulness for immunological detection of E7 oncoprotein

in clinical specimens. Different immunization and selection strategies were used to obtain antibodies with desired functionalities. Antibodies were identified that proved to be useful in different immunological methods for E7 detection and can hopefully be used for development of more specific tests for early diagnosis of HPV-induced malignancy.

1.1 Human papillomaviruses & Cervical cancer

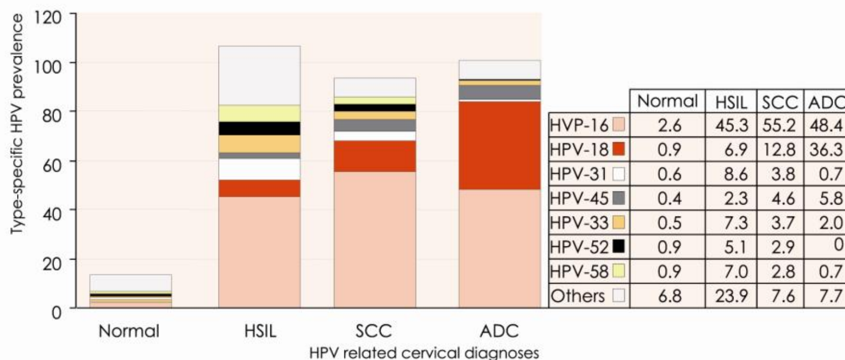
1.1.1 The Human papillomavirus

Papillomaviruses are small, non-enveloped DNA viruses infecting epithelial surfaces in most vertebrates. The viruses cause a broad range of disease in humans, such as anogenital cancer, head- and neck cancer, skin or genital warts and recurrent respiratory papillomatosis. The outcome of the infection will depend on HPV type and site of infection as well as different host factors. HPV is necessary for the development of cervical cancer and viral DNA can be found in virtually all cases[1]. The two most common types in cervical cancer are HPV16 and 18, together responsible for approximately 70 % of all cases[6].

So far, at least 120 HPV types with completely characterized genomes have been identified. The virus is classified based on the nucleotide sequence of the HPV L1 capsid gene and viruses with at least 10 % difference in L1 sequence are defined as different types[7]. The HPV genome evolves slowly, in parallel with its host and has more and less conserved regions such as L1 being well conserved and the noncoding Long control region (LCR) being more diverse[8].

Different HPV types target different epithelia and at least 40 types infect the human genital tract. Of these, 12 are frequently found in cervical cancers and therefore considered high-risk types[9]. Women, and men, are often infected with several HPV types at the same time, often transmitted together and different infections can cause independent lesions side by side in the cervix[10, 11]. HPV16 is by far the most dominant HPV type, responsible for 55 % of the cervical cancers in the world, followed by HPV18 present in at least 15 % of the invasive cancers[6]. The other high-risk types are HPV33, 45, 31, 58, 52, 35, 59, 51, 56 and 39 in descending order in the world-wide distribution though there are geographical variations[6, 12]. The other mucosal types, besides the 12 high-risk types, are classified as intermediate or low-risk and only rarely detected in cervical cancer. HPV6 and 11 are the two most common genital low-risk types together responsible for 90 % of genital warts[13].

The viruses are prone to cause cancer at transformation zones where two types of epithelia meet, especially the cervix where squamous epithelium replaces glandular[13]. Most cervical cancers are of squamous origin but approximately 15 % are adenocarcinomas. HPV18 is more common in adenocarcinoma than in Squamous cell carcinoma (SCC), detectable in 37 % of cases, compared to 13 % of the SCC[6]. Conversely, HPV16 is less frequently found in adenocarcinoma than in SCC. HPV16, 18 and 45 are often considered more aggressive since they are present in a larger proportion of SCC than in high-grade lesions compared to other HPV types[6] (Fig. 1). Invasive cancer caused by HPV16, 18 and 45 is in addition diagnosed at a younger mean age[12].



	Normal	HSIL	SCC	ADC
HVP-16	2.6	45.3	55.2	48.4
HPV-18	0.9	6.9	12.8	36.3
HPV-31	0.6	8.6	3.8	0.7
HPV-45	0.4	2.3	4.6	5.8
HPV-33	0.5	7.3	3.7	2.0
HPV-52	0.9	5.1	2.9	0
HPV-58	0.9	7.0	2.8	0.7
Others	6.8	23.9	7.6	7.7

Figure 1. Type-specific HPV prevalence in relation to cervical diagnoses. HPV16, 18 and 45 are often considered more aggressive than the other high-risk types as the prevalence of these types increase more with the severity of the disease.HPV16, 18 and 45 are further responsible for a larger portion of the adenocarcinomas (ADC) than squamous cell carcinoma (SCC). Multiple infections are counted several times. HSIL: high-grade squamous intraepithelial lesions (corresponding to Cervical intraepithelial neoplasia 2/3). Reprinted from[14] Copyright (2008), with permission from Elsevier.

1.1.2 Natural history of HPV infections

The 8 kb circular HPV DNA genome normally encodes two classes of genes, Early (E) genes, E1, E2, E4, E5, E6 and E7, involved in viral replication and cellular transformation and Late (L) genes encoding the L1 and L2 capsid proteins (Fig. 2). To be able to infect, the viral particles have to reach the proliferating basal cells and probably do so through microabrasions in the epithelia, though the exact mechanisms of viral uptake is not fully understood[15]. The life cycle of the virus is then totally dependent on the differentiation program of the keratinocytes and cellular DNA synthesis.

During a productive infection, the expression of viral proteins is tightly regulated in order to produce infectious viral particles and avoid the immune system. The early proteins are expressed soon after infection but at low levels and the viral genome is maintained as stable episomes or plasmids, replicating with the cellular DNA in the basal cells[15]. In this initial part of the viral life cycle the HPV E1 and E2 proteins are necessary for replication of the viral DNA[15]. The infected basal cells undergo asymmetric mitosis, leaving one daughter cell as an undifferentiated proliferating basal cell acting as a reservoir of infection while the other becomes a differentiating suprabasal cell. The E6 and E7 proteins associate with cell cycle regulators and drive the cells, which would otherwise exit the cell cycle as they differentiate, into S-phase[16] and are thus considered oncoproteins. As the infected cells spread throughout the epithelia, the expression of the E6 and E7 oncoproteins is upregulated and the viral DNA increases in copy number. The capsid proteins are only expressed in the upper epithelial layers, after viral genome amplification[15] and assemble into viral particles comprised of the viral genome, covered by a capsid consisting of 360 copies of the L1 protein and 12 copies of the L2 protein. Papillomaviruses are non-lytic and shed when the infected cells reach the epithelial surface[17]. This infectious cycle takes approximately three weeks, the same time as it takes for the basal cells to differentiate and move up the epithelium.

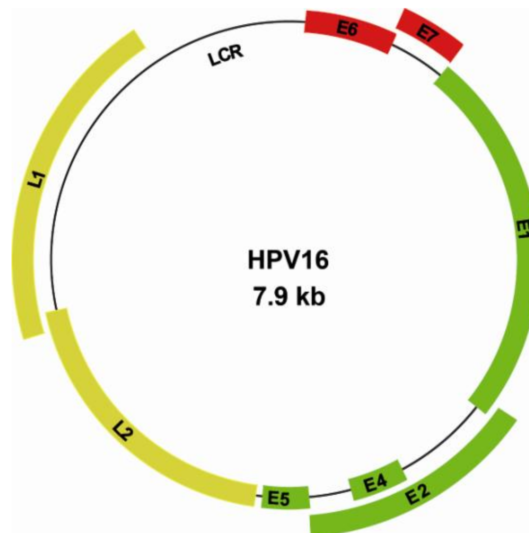


Figure 2. A schematic presentation of the HPV 16 circular DNA genome. The Early (E) and Late (L) genes and the Long control region (LCR) are shown.

The majority of the HPV infections are subclinical or cause only mild dysplasia, cleared by the immune system within two years although the mechanisms of HPV clearance are not fully understood. The immune response after HPV infection is normally low and/or delayed, most probably because of the low-level expression of the early HPV proteins, production of the capsid proteins only in the upper epithelial layers far from immune cells in the submucosa and the fact that the infection is non-lytic, thereby not inducing an inflammatory immune response[15].

1.1.3 From HPV infection to cancer

If a high-risk HPV infection is not cleared by the immune system, it becomes persistent and able to induce malignant transformation. Cervical cancer development normally follows defined stages starting with infection with a carcinogenic HPV type followed by the establishment of viral persistence, the development of pre-cancer and culminating in invasion (Fig. 3). Lesions at all stages until invasive cancer can regress, but the chance of clearance decreases with the severity of the lesion.

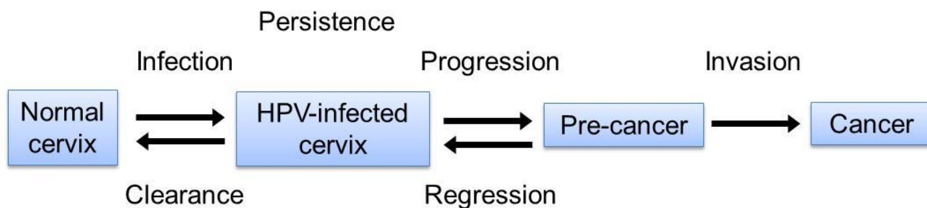


Figure 3. From HPV infection to cancer. The majority of the genital HPV infections is asymptomatic and cleared before causing visible lesions. The majority of all mild dysplasia regress spontaneously within less than a year. A proportion of the high-risk HPV infections will however become persistent and may, if left untreated, proceed to high-grade lesions and invasive cancer.

The estimated life-time risk of acquiring a genital HPV infection is over 50 %[18] and most women are infected soon after becoming sexually active. The HPV prevalence therefore peaks in young women and is as high as 40 % in some female populations[19]. Due to the long pre-cancerous phase and the fact that most infections do not cause malignancy, cancer incidence in young women is however normally low[19]. Since the virus is transmitted by skin-to-skin contact, the risk factors for acquiring HPV are linked to sexual behavior, such as the number of partners and other genital infections[20]. Approximately half of the carcinogenic HPV infections are resolved within six months of exposure and less than 10 % become persistent and are still detectable after

two years[19]. The two most important risk factors for cervical cancer are thus persistent infections with oncogenic HPV and lack of screening[13]. Consequently most cases and deaths from cervical cancer are in low-resource regions, where the possibilities to screen and treat are limited. Other minor risk factors for cervical cancer in HPV-infected women are multiparity[21], inflammation[22] and long-term use of oral contraceptives[23]. Smoking is a risk factor for SCC but not adenocarcinoma[24].

Viral persistence is necessary for carcinogenesis and as the probability of clearance decreases with time from exposure, the risk of invasion increases[25] (Fig. 4). For malignant progression however, other genetic events, induced by the virus, must take place in the host genome[15]. As discussed below, the viral oncoproteins interact with different cell cycle regulators, increase cell proliferation and induce genomic instability which leads to an accumulation of somatic mutations during the long pre-cancerous phase. The pre-cancer (Cervical intraepithelial neoplasia, CIN2/3) incidence actually peaks approximately 5-15 years after infection and the invasive cancer decades later. Approximately 1 % of all high-risk infections will finally, if left untreated, cause cervical cancer[26].

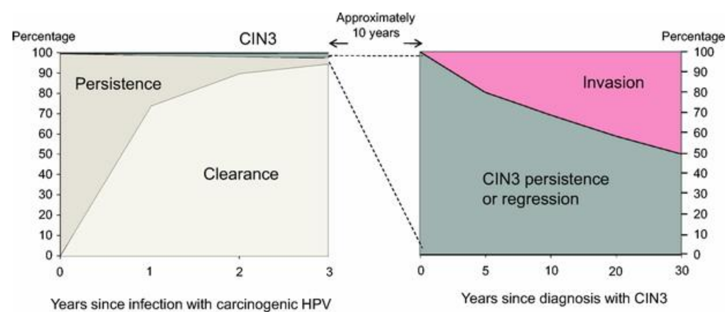


Figure 4. Risk of HPV persistence and cervical cancer progression. The majority of the HPV infections are solved within two years after infection. However, the longer the infection persists, the larger risk of developing high-grade dysplasia. Most high-grade lesions (Cervical intraepithelial neoplasia, CIN3) develop many years after primary infection. Half of the high-grade lesions will, if left untreated proceed to invasive cancer and true CIN3 should be the preferred threshold for treatment. Reprinted from[19] Copyright (2010), with permission from Wolters Kluwer Health.

Cervical cancer precursors are often histologically classified as Cervical Intraepithelial Neoplasia (CIN) grade 1-3, where CIN1 is mild, CIN2 moderate and CIN3 severe dysplasia[27], defined as the extension of abnormal, undifferentiated cells in the epithelium caused by persistent HPV infections

(Fig. 5). Low-grade lesions (CIN1) are the first manifestation of the HPV infections, caused by both high- and low-risk HPV types. CIN1 should not be considered as pre-cancer and has a relatively low risk of progression to invasive disease. The abnormal cells only occupy the deepest third of the epithelium and these mild to subclinical changes are hard to detect histologically. CIN2 is a heterogeneous state, often with a mix of acute infection and severe dysplasia, with abnormal cells in two thirds of the epithelium. In CIN3, or carcinoma *in situ*, the undifferentiated cells are spread throughout the epithelia and this is the immediate precursor of invasive cancer and should always be treated due to the high risk of progression.

A key event during carcinogenesis is integration of the viral DNA into the host genome. This is seen in most cancers as well as in many high-grade lesions and some low-grade lesions[28]. Integration is probably not necessary for malignancy since not all cancers have integrated viral genome, but leads to a more aggressive progression. Integration often leads to disruption of the E2 gene, which otherwise is a regulator of the E6/E7 expression. This loss of negative feedback control of the expression of the oncogenes results in elevated oncoprotein levels[15]. Integration cannot be considered a natural event in the viral life cycle since no viral particles are produced from this truncated integrated form.

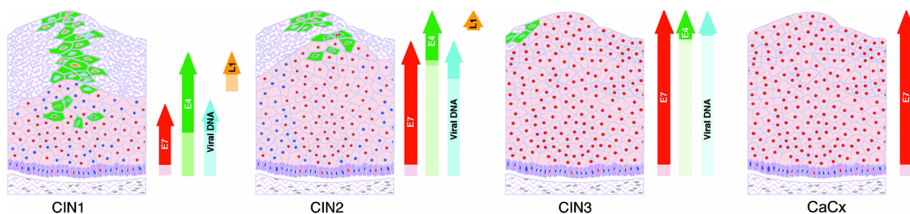


Figure 5. The expression pattern of the viral proteins changes during the progression to cervical cancer. In the productive infection, viral particles are produced in the upper epithelial layers and shed. The early viral proteins are expressed, at moderate levels, in the lower epithelium. In high-grade dysplasia and cancer, there is no or very low expression of the capsid proteins, while the expression of the oncoproteins is upregulated, especially after viral integration. CIN: Cervical intraepithelial lesion; CaCx: Cervical cancer. Reproduced with permission from John Doorbar (2006) Clinical Science (110) p 533 © the Biochemical Society.

1.1.4 HPV E6 and E7 oncoproteins

Several studies have shown that two viral proteins, E6 and E7, are necessary both for the induction of the malignant transformation and for maintaining the transformed phenotype[29, 30]. The E6 and E7 oncoproteins are expressed at increasing levels during cervical carcinogenesis[31, 32] and are accepted markers for cervical cancer progression. E6 and E7 have numerous cellular targets, with the tumour suppressor p53 and the retinoblastoma protein family (pRb) being the most studied. By interacting with important cell cycle regulators, the virus overrides cell cycle check points and induces cell proliferation. The viral oncoproteins further inhibit apoptosis, induce genomic instability and increase the telomerase expression, important steps in the transformation towards cancer phenotype (reviewed by[16]) (Fig. 6).

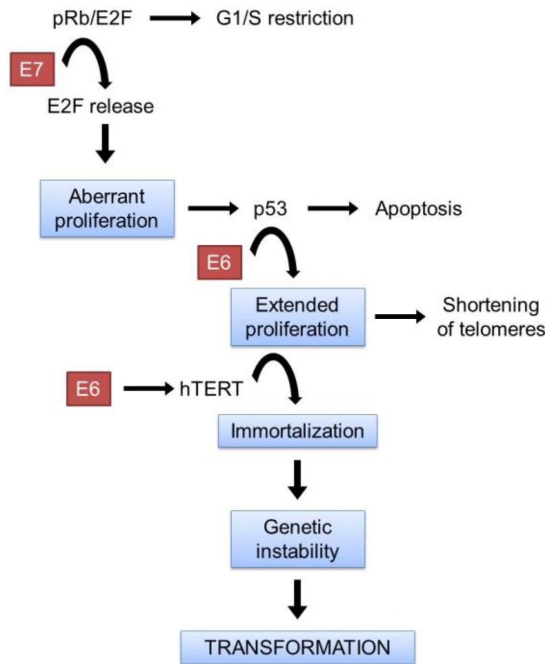


Figure 6. Illustration of key steps in HPV induced malignancy. In the normal cell, cell cycle progression is controlled by the retinoblastoma protein (pRb) and the E2F transcription factor. The E7 protein can, by degrading the tumour suppressor pRb, release E2F and induce S-phase entry in HPV-infected epithelial cells. Due to loss of feed-back, the levels of tumour suppressor p53 increase causing growth inhibition and apoptosis. When E6 is present however, p53 is degraded which leads to extended proliferation, loss of p53-mediated DNA damage control and accumulation of mutations. E6 can further activate the catalytic subunit of telomerase (hTERT) which is normally inactive in parabasal cells, causing telomere extension and immortalization[16].

E6 and E7 of high- and low-risk HPVs have large sequence similarities and share some interaction partners (e.g. pRb). However, the low-risk HPV proteins has lower affinity to the cellular proteins and the low- (and intermediate) risk HPV types are not able to induce malignant transformation[33, 34] and are seldom detected in cervical cancer.

The E7 proteins are small acidic proteins, of approximately 100 amino acids (aa), with more and less conserved regions (Fig. 7). The N-terminal part of the protein is unfolded and flexible[35]. This region contains two conserved regions, CR1 and CR2. The conserved Leu-X-Cys-X-Glu (LXCXE) motive in CR2 is necessary for pRb-binding and the CR2 domain shares sequence similarity and transforming activity with proteins of other DNA tumour viruses as well (e.g. Adenovirus E1A, simian virus 40 large tumour antigen)[36]. The C-terminal end contains a third conserved region, CR3, with two Cys-X-X-Cys (CXXC) motifs separated by 29 or 30 residues, necessary for the formation of a zinc binding fold and involved in protein stabilization and dimerization although dimerization has not yet been shown *in vivo*[35].

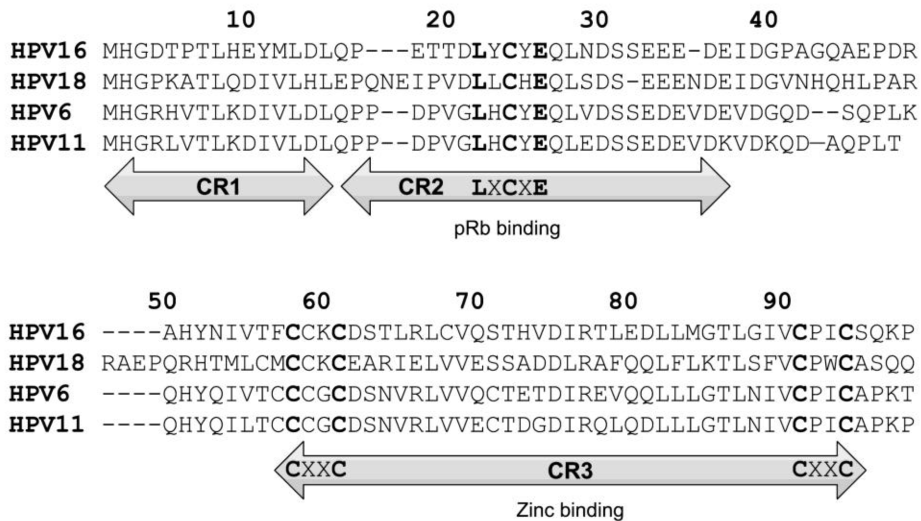


Figure 7. Alignment of HPV16, 18, 6 and 11 E7 amino acid sequences.
The conserved regions (CR) one to three are indicated with respect to the HPV16 E7 amino acid sequence[37].

1.2 Cervical cancer prevention

1.2.1 Screening and diagnosis

The long pre-invasive phase, together with the fact the cervix is easily accessible for sampling and treatment, makes cervical cancer ideal for screening. Large parts of the world are covered by screening programs and this has led to a dramatic reduction in cervical cancer incidence with over 70 % reduction in some populations[4] and women diagnosed by screening have a significantly higher chance of being cured than those diagnosed by detectable symptoms[38]. However, many women are not yet covered by such programs and due to limitations of the available tests, a not insignificant portion of the screened women still gets cancer.

Screening prevention programs normally include primary screening, triage of equivocal results and colposcopy-guided biopsy in patients identified with abnormal screening results, followed by treatment and follow-up. Detection methods, start-points and screening intervals, as well as when and how to treat vary between settings. For primary screening, most programs use cervical cytology. In the widely used Pap test (Pap smear), first described by George Papanicolau in the 1940s, the morphology of the exfoliated cervical cells is examined under magnification[39]. The sensitivity of the test is limited (around 50 % for CIN2/3) and the test needs to be repeated frequently, typically every 2-3 years, to be efficient[26]. The evaluation identifies a spectrum of abnormalities and is subjective. Studies have shown that even experienced cytologists differ in their evaluation[40].

A refinement of the Pap smear, where the cervical cells are spread directly onto a microscope slide, is Liquid-based Cytology (LBC). In LBC the exfoliated cells are first transferred into a fixative liquid and then used to prepare monolayer slides. LBC has been shown to increase neither sensitivity nor specificity compared to the conventional pap smear[41], however this technique has two major advantages; it creates more homogenously spread cell preparations and it leaves residual material for further analysis such as HPV testing, without requiring an extra sample. Furthermore, LBC samples can be processed using automated systems.

1.2.2 Cervical cancer screening in a vaccinated population

Two HPV vaccines are approved today, Cervarix (GlaxoSmithKline) and Gardasil (Merck). Both vaccines protect against HPV16 and 18 and Gardasil also targets HPV6 and 11 that cause genital warts. The vaccines are HPV L1 Virus-like-particle (VLP) vaccines. The vaccines have shown almost 100 %

protection against the targeted HPV types and offer cross-protection against closely related high-risk HPV types as well[42]. Vaccines for protection against additional carcinogenic types are under evaluation.

Since most women are infected at first intercourse, women should optimally be vaccinated before sexual debut. The vaccines have no therapeutic effect and vaccination of older, already exposed women is not protective[42]. For these generations of unvaccinated women screening will still be necessary. It is also important that vaccinated women continue to undergo regular cervical screening since the vaccines do not cover all high-risk types. There will also always be women who are not, for different reasons, covered by the vaccination program and need regular examinations.

1.2.3 Challenges in cervical cancer prevention

Histological evaluation has long been the gold standard for diagnosis of cervical malignancy and is used to verify abnormal cytology results and solve inconclusive cytology. In most screening programs, the threshold for treatment is histological CIN2 or worse, despite the fact that 40 % of the CIN2 lesions regress spontaneously within two years[43]. Most guidelines recommend treatment of the entire transformation zone at confirmed high-grade dysplasia since the entire zone is at risk. Fertility sparing methods, such as cryotherapy, cone-shaped excision and loop electrosurgical excision procedure (LEEP) are to be preferred, but in case of cancer, surgery and even radical hysterectomy, in combination with radiotherapy and chemotherapy, might be the best choice[13]. Most protocols recommend treating CIN2, rather than waiting and observing, due to the risk of missing underlying cancers[44, 45]. This leads to a lot of overtreatment of lesions that would otherwise regress and which is extra problematic in women in childbearing age, due to the increased risk of preterm delivery following treatment[46].

The highest grade of CIN identified in a biopsy sample decides the management of the patient and since one patent can have several lesions of different severity and caused by different HPV types, the site of sampling becomes crucial[10, 47]. This, in combination with large inter-observer variations in the histological evaluation[40], contributes to the complexity of diagnosing cervical pre-cancer histologically. Multiple biopsies and immunohistochemical staining have been suggested to increase sensitivity and specificity for malignancy[19].

A particular challenge in cervical cancer prevention is the management of low-grade and mildly atypical cervical abnormalities. The most common abnormality in cytology is Atypical Squamous Cells of Undetermined

Significance (ASC-US)[48]. Millions of women are actually diagnosed with minor or equivocal abnormalities every year[49] and though most of these lesions will regress approximately 5% have underlying high-grade disease (CIN3+) that needs to be identified and treated[50]. The high proportion of inconclusive cytology results, poor reproducibility in grading of lesions and large number of recalled and over-treated women highlights the need for alternative, preferably more objective approaches, such as biomarkers to solve some of the problems with today's screening methods.

1.2.4 Biomarkers in cervical screening

To increase the interobserver reproducibility, reduce subjectivity and/or increase specificity/sensitivity several biomarkers have been suggested in the diagnosis of cervical malignancy. Safer diagnosis has the potential of reducing the overtreatment of lesions that would otherwise regress, decrease the number of missed cases and hopefully make screening available for a larger part of the world.

HPV DNA testing is now widely used for triage of equivocal cytology findings and as co-testing to cytology, but has also been for suggested as a more sensitive test for high-grade dysplasia compared to cytology in primary screening[51]. The primary benefits of HPV DNA testing are the high sensitivity and the high negative predictive value and consequently, the screening interval after a negative HPV DNA test can safely be lengthened to six years due to the low risk of high-grade dysplasia[52]. However, a single DNA tests cannot discriminate transient infections from persistent and due to the high prevalence of transient HPV infections in the population, especially in young women, the utility of HPV testing in primary screening is somewhat limited. Another approach claimed to be more specific for malignancy than DNA testing is to measure HPV mRNA. mRNA testing is generally more specific and less sensitive than DNA at detecting high-grade dysplasia, depending the detection method used[53].

Women with ASC-US and borderline cytology should always be further managed in order to identify those women at risk due to the risk of underlying high-grade disease (10-15 %)[54, 55]. High-risk HPV DNA and mRNA tests normally perform better in ASC-US populations than borderline[49, 56]. Since most low-grade lesions are HPV positive HPV testing is not selective for high-grade dysplasia in this group and especially not in women under 50[48]. Thus, most guidelines recommend HPV DNA testing (in case of LBC), repetition of the smear and/or colposcopy for triage of ASC-US, while today only cytology and colposcopy is recommended for triage of borderline

samples[57]. Other complementary tests are therefore needed for the large group of borderline diagnoses in cytology.

Some cellular markers have been suggested for more specific detection of cervical neoplasia. So far, the tumour suppressor gene p16 ($p16^{ink4a}$) is the most widely evaluated and promising cellular marker. p16 is overexpressed in high-grade dysplasia and cervical cancer due to E7-induced pRb degradation and loss of negative feedback[58]. p16 cytology has been shown to have higher specificity but lower sensitivity for CIN2+ than HPV DNA testing[59]. To further improve the test performance and facilitate the interpretation of the staining results, the combination of p16 and the proliferation marker Ki-67 has been introduced (mtm laboratories)[60]. This dual staining protocol, judging a sample as positive based on the detection of at least one double stained cell irrespective of cellular morphology, has been shown to be more specific for detection of CIN2+ in the ASC-US and low-grade lesions compared to both HPV DNA testing and p16 single-stain cytology[61].

Although the importance of the viral proteins for the malignant transformation is well established, no HPV protein test has yet been FDA approved, which is probably due to the lack of sensitive reagents. A few groups have managed to detect viral protein markers in clinical material e.g. E7[31, 62], E6[63] and L1[64], but larger studies are needed to prove the clinical utility of these reagents.

2 Aim

The overall objective of this thesis was to develop immunological reagents against biomarkers associated with malignant transformation of cervical epithelium for improved diagnosis of cervical cancer.

The specific aims were to

- Generate useful monoclonal antibodies against HPV E7 oncoproteins. The antibodies should have high affinity to oncogenic HPV types without cross-reactivity to cellular proteins.
- Develop sensitive protocols for Immunocytochemistry, Immunohistochemistry and other immunoassays to assist in early detection of cervical cancer.
- Evaluate the diagnostic potential of the established methods using representative clinical specimens.

3 Key methodologies

3.1 Cervical cancer cell lines

Three cervical cancer cell lines were obtained from the American Type Culture Collection (ATCC) and used as positive and negative controls throughout the studies; CaSki (ATCC: CRL-1550) expressing the HPV16 E7 protein, HeLa (ATCC: CCL-2) expressing the HPV18 E7 protein and C-33A (ATCC: HTB-31) negative for HPV DNA. The cell lines have the major advantage of being monoclonal, compared to the mixed nature of patient samples and are therefore trustable controls.

3.2 Generation, identification and production of monoclonal antibodies

Two different immunization strategies were employed in this study; Site-specific immunization with peptides displayed on phage particles and immunization with full-length recombinant HPV E7 protein. Female Balb/c mice (B&K Universal) were used and kept at the Experimental Biomedicine facility, Sahlgrenska Academy at the University of Gothenburg. All experiments were approved by the Ethical Committee for Animal experimentation in Gothenburg, Sweden.

The mice were intraperitoneally injected with either 5 x 10^{11} phage particles displaying E7 peptides, or first 25 µg and then 10 µg recombinant HPV16 or 18 E7 protein, in Sigma Adjuvant System. The serum reactivity against the full-length E7 protein was studied by ELISA and spleen cells from high-titer mice were collected for the establishment of antibody-producing hybridomas. Newly isolated B-lymphocytes were fused with the mouse myeloma cell line P3X63Ag8.653 (ATCC: CRL-1580), by treating the cells with Polyethylene glycol (PEG) in a strictly controlled manner[65]. The newly fused cells were grown in 96-well plates and selected through the addition of hypoxanthine, aminopterin and thymine (HAT) to the growth media (Fig. 8). The resulting hybridomas were then selected for reactivity to full-length HPV16 or 18 E7 in ELISA.

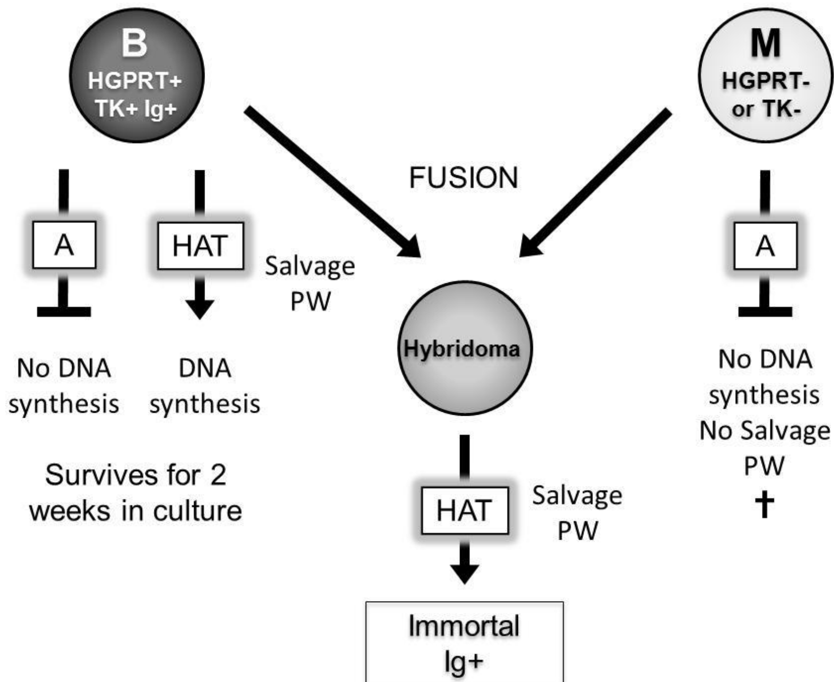


Figure 8. Fusion and in vitro selection of in HAT medium. When aminopterin (A) is added to the growth media normal DNA synthesis is inhibited and the cell has to use a salvage pathway (PW) which requires hypoxanthine (H) and thymine (T) for DNA synthesis. The mutant mouse myeloma cell line (M) is defective in one of the genes necessary for the salvage pathway and will die in the presence of aminopterin. Myeloma cells fused with B-cells (B), however, are provided with the necessary genes and become immortal antibody-producing hybridomas. Non-fused B-lymphocytes only survive for up to two weeks in culture. Ig: Immunoglobulin.

Different immunological methods, primary ELISA, but also Western blot and Immunocytochemistry, were used to identify antibodies with the desired properties. Antibodies with reactivity to the low-risk HPV types were discarded and the high-risk specific ones were further evaluated. Promising hybridomas were expanded and cloned by limiting dilution. Antibody (Ab) isotypes were determined by ELISA using isotype-specific antibodies.

The mAbs used in all studies, were produced by *in vitro* cultivation in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich) with 2.5 % FBS and 1 % DMEM supplement in roller bottles. The antibodies were purified on Protein A and eluted at pH 4. Purity and concentration were determined by gel chromatography and by measuring the absorbance at 280 nm.

3.3 Phage display

Phage display, display of peptides on the surface of the bacteriophage, has become a widely used technique to study protein interactions and produce large amounts of peptides attached to the phage carrier. Wild-type filamentous phage particles are rod-shaped and composed of a single-stranded phage genome, covered by approximately 2700 copies of the major pVIII protein and five copies each of the minor coat proteins pIII and pVI. By cloning a chosen nucleotide sequence in fusion with one of the phage coat proteins, either directly into a modified phage genome or into a separate phagemid, phage particles displaying the corresponding peptide on its surface are created[66]. Phagemids are plasmids carrying a modified gene of one of the phage coat proteins together with antibiotic resistance and both a plasmid and a phage replication origin. A wild-type so called helper phage is then needed to support phage production. The resulting phage preparation is be a mixture of phage particles, carrying either the helper phage genome or the phagemid, all displaying the peptide encoded by the phagemid on a portion of its coat proteins. In this study, phage display was used for the construction of overlapping E7 peptide libraries for epitope determination, immunization of mice and mimotope mapping with commercial random peptide libraries.

3.3.1 Site-specific immunization

Phage particles are immunogenic and can thus be used as immunogenic carriers of peptides[67]. In paper I, phage display was used for Site-specific immunization, to direct the immune system to a specific pre-defined region of the E7 protein. A high-risk specific region of the HPV16 E7 sequence, corresponding to aa 33-60 of the E7 protein, was identified and cloned in phage vector f88-4, kindly provided by professor G.P. Smith (University of Missouri, Columbia, US), in fusion with the major coat protein pVIII. Phage particles were produced in *E. coli*, purified by double PEG precipitation and used to immunize mice. Spleen cells from mice with high HPV16 E7 titer were used to establish antibody-producing hybridomas, as described above. Serum reactivity as well as the reactivity of the resulting antibodies was measured by ELISA against full-length E7 protein.

3.3.2 Epitope determination using overlapping E7 peptides

Since phage particles carry the sequence encoding the peptide it displays it is suitable for the construction of peptide libraries. Phage clones binding to a specific target, such as a mAb can be selected from the library in a panning

process. The sequences of the identified phage clones reveal the region, epitope or mimotope involved in binding.

In paper I and II, overlapping HPV E7 peptides, 16 to 37 aa long, were displayed on phage and antibody binding to the different clones was studied by ELISA. The antibodies to be examined were immobilized in microtiter wells and different phage clones were added each wells. After washing off the non-binding phage particles, phage binding was detected by incubation with a rabbit-anti-M13 antibody (raised against M13 phage particles at Fujirebio Diagnostics AB), a Horseradish peroxidase (HRP)-conjugated swine-anti-rabbit Immunoglobulin (Ig, Dako) and the HRP substrate Enhance K-blue (Neogen). The binding pattern to the different phage clones thereby directly revealed the E7 regions involved in binding.

3.3.3 Mimotope determination using random peptide libraries

In paper I and III, a commercially available random 7-mer peptide library displayed on phage (New England Biolabs) was used for mimotope identification. The library was panned against the mAbs in four rounds and binding clones were identified and sequenced. The consensus aa sequences were compared with the E7 aa sequence and aa essential for binding, the mimotope, were identified.

3.4 Construction of a vector system for the display of peptide libraries

In paper III, three vectors for the display of peptides on either bacteriophage, in fusion with Glutathione-S-Transferase (GST), or in fusion with the cholera toxin B subunit (CTB) were generated. The vectors and inserts were designed so that the synthesized DNA could be cloned into any of the three vectors, depending on application. By arranging the peptide-encoding fragments, interspersed with linkers carrying restrictions sites for cloning, on the same DNA construct cloning of the entire library could easily be performed in a single reaction mix (Fig. 9). Here, the vector system was used to create libraries of HPV16 E7 peptides, 12 aa long, covering the entire E7 open reading-frame (ORF) and overlapping by 8 aa.

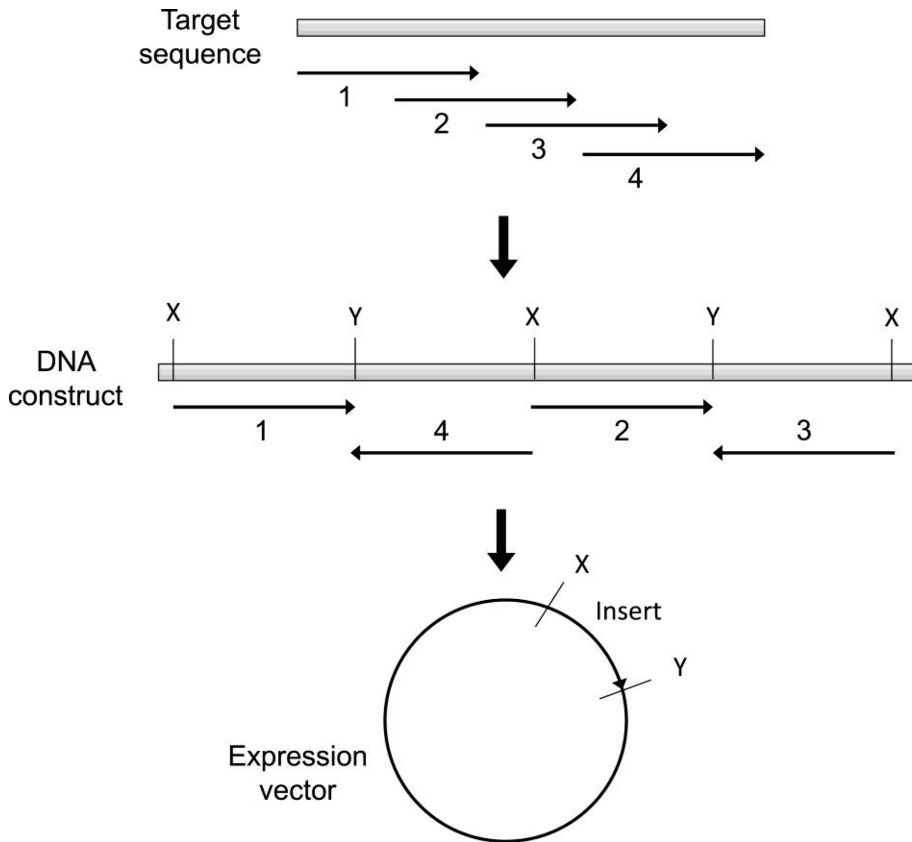


Figure 9. Arrangement of overlapping sequences for cloning into expression vectors. Alternating inserts were inverted to minimize the number of bases to synthesize and shuffled to reduce secondary structure of overlapping sequences. The restriction sites (represented by X and Y) on the inserts and expression vectors were placed so that the vectors could be used interchangeably. The digested inserts were dephosphorylated to prevent them from ligating to each other.

Four HPV16 E7 mAbs were epitope mapped by colony blot, using the E7 peptide library fused to GST. 135 random clones from the library were grown as single colonies in a grid pattern on LB plates and then transferred to sterile nitrocellulose filters by placing them on top of the colonies. The filters were then placed on fresh LB agar plates supplemented by IPTG to induce the expression of the GST fusions. The cells on the filters were lysed and the filters were incubated with the HPV16 E7 mAbs. MAb-binding was detected using HRP-conjugated goat anti-mouse Ig and visualized with HRP substrate 2-chloronaphtol. Plasmids of the positive clones on the filter were prepared from the same position on the corresponding LB-plate and sequenced.

The same overlapping HPV16 E7 peptides, displayed on phage particles, were used to map dominant B-cell epitopes in crude serum from mice immunized with full-length HPV16 E7 protein. The serum to be mapped was immobilized in microtiter wells. A mixture of 10^8 phage particles, corresponding to nearly 5 x 10^7 copies of each phage clone, was added to each well and 20 binding clones were sequenced after one round of panning. The peptide sequences were compared to the HPV16 E7 sequence to identify linear B-cell epitopes.

3.5 ELISA

ELISA (Enzyme-linked immunosorbent assay) offers the opportunity to study antibody interaction to the native antigen in solution. In this study, ELISA was used for different applications such as specificity studies with recombinant antigen, epitope mapping with phage particles and to monitor immune response in immunized mice. Although variables such as incubation times and Ab concentrations varied between applications, the key steps are common to all.

The catching antibody was immobilized in Maxisorp wells (Nunc A/S), either directly or, for unpurified Ab caught by an immobilized polyclonal goat-anti-mouse Ig antibody (Jackson Immunoresearch Laboratories). After a wash step, the antigen, diluted in blocking buffer was added. Antigen binding was detected using antibodies directed directly against the E7 protein or against a protein tag such as the phage particle or GST and an HRP-conjugated secondary antibody. Two different HRP substrates were used, either the *o*-Phenylenediamine (OPD) substrate (Sigma-Aldrich) or the more sensitive Enhance K-Blue (Neogen) substrate. Absorbance was measured at 450 or 620 nm in a microplate spectrophotometer (V_{max}, Molecular Corporation), depending on the substrate used. An automatic plate washer, in strip mode with overflow, with 5mM Tris-HCl pH 7.8, 150 mM NaCl and 0.005 % Tween20. All incubations were at room temperature, either in a humid chamber or on a microplate shaker (900-1100 oscillations/min). When measuring serum reactivity against the E7 protein, GST-tagged E7 protein was directly coated in glutathione-coated wells (Pierce). Serial dilutions of serum were added to the E7-coated wells and anti-E7 reactivity was detected with HRP-conjugated rabbit anti-mouse (Dako).

3.6 Western blot

The specificity of antibodies was also studied by Western blot. A panel of high- and low-risk (HPV1, 6, 11, 16, 18, 31, 33, 35 and 45) E7 proteins were separated by SDS-PAGE (NuPAGE) and blotted to PVDF membranes. The membranes were incubated with the E7 antibodies in 5 % non-fat dry milk and binding was detected with HRP-conjugated rabbit anti-mouse Ig (Dako) and chemiluminescent detection (ECL+, GE Healthcare).

Western blot was also used to ensure that the antibodies did not cross-react with other proteins present in cervical cells. Cell lysates were prepared from cervical cancer cell lines CaSki (expressing HPV16 E7 protein), HeLa (expressing HPV18 E7 protein) and C-33A (negative for HPV DNA). The cells were lysed by freeze-thawing and cell debris was removed by centrifugation. The total protein concentration was determined with the Bio-Rad protein assay kit and 50 μg of protein was loaded to each lane. The analysis was then done as described above.

3.7 Immunocytochemistry

All high-risk HPV-specific antibodies were evaluated using Immunocytochemistry (ICC). The newly harvested cervical cancer cell lines were either directly mounted on polysine slides and fixed in 10 % neutral buffered formalin or first fixed in the LBC preservative fluid (Thinprep Preservcyt, Hologic or Surepath, BD) and then mounted on slides. Different methods and buffers for antigen retrieval were evaluated such as heat-induced antigen retrieval in citrate or Tris buffer, to try to find the optimal protocol for each antibody. Other parameters, such as antibody concentrations, incubation times and incubation temperatures were also optimized in parallel.

3.8 Staining of clinical LBC samples

The optimized protocols with the anti-HPV E7 mAbs E716-41 and E718-79 were, as presented in paper IV, evaluated on 49 patient cytology samples collected in Thinprep LBC fluid (Hologic). All samples were HPV DNA genotyped. 19 samples were judged as normal in the cytological evaluation. The 20 dysplastic samples, all HPV16 or 18 positive, were from a referral population and cytology samples and biopsies for histological evaluation were taken at the same visit. All women provided informed consent.

The samples were either mRNA genotyped or evaluated in p16/Ki-67 cytology (CINtech PLUS, mtm laboratories AG). All mRNA genotype results were in agreement with the DNA genotyping. For p16/Ki-67 evaluation only double stained cells, with red nucleus and brown cytoplasm, were considered as positive. E7 cytology was interpreted as positive if cells abnormal cells were stained. All slides were evaluated by trained cytologists. The cytologists knew the distribution of the specimens, but were blinded to the specific cytology diagnosis and HPV genotype result. The E7 cytology results were then correlated to HPV mRNA status, p16/Ki-67 cytology and histology results.

4 Results & Discussion

Using two different immunization strategies, over 100 hybridomas producing anti-HPV16 or HPV18 E7 antibodies were established. From these, high-risk HPV-specific antibodies with different properties were selected, with focus on finding those that detected the antigen by ICC, especially in LBC samples. The identified antibodies had different specificities and properties depending on the immunogen and selection method used. (Fig. 10)

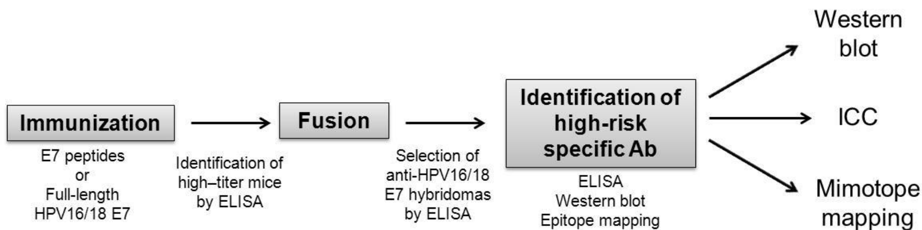


Figure 10. Schematic presentation of the workflow. Two different immunization strategies were used; Site-specific immunization with a high-risk specific HPV16 E7 peptide displayed on phage particles and immunization of recombinant full length E7 protein. Spleen cells from mice with high serum titers against full-length recombinant E7 were fused with a mouse myeloma cell line to get antibody-producing immortal hybridomas. The primary screening of the hybridomas was done by ELISA against full-length HPV16 and 18 E7 proteins. The reactivity of the selected hybridomas were further evaluated against a panel of high- and low risk HPV types in ELISA and Western blot and the epitopes were roughly mapped using overlapping E7 peptides displayed on phage. High-risk HPV-specific antibodies (Ab) were cloned and carefully evaluated against endogenous E7 in Immunocytochemistry (ICC) and Western blot. Mimotope analysis was performed for a subset of the high-risk Abs.

The HPV E7 protein is an antigen with more and less conserved regions. Antibodies, especially those reacting with the highly conserved regions, will most probably detect the E7 protein of several HPV types. Whether type-specific antibodies or antibodies with broader specificity is preferred, will depend on the clinical application. Throughout these studies, high-risk HPV-specific antibodies were saved while the ones cross-reacting with the common, but not carcinogenic HPV-types were discarded. The reason for this was to avoid antibodies giving false positive results. This was done by immunizing with high-risk specific regions of the protein (site-specific immunization) and by selecting for high-risk specificity.

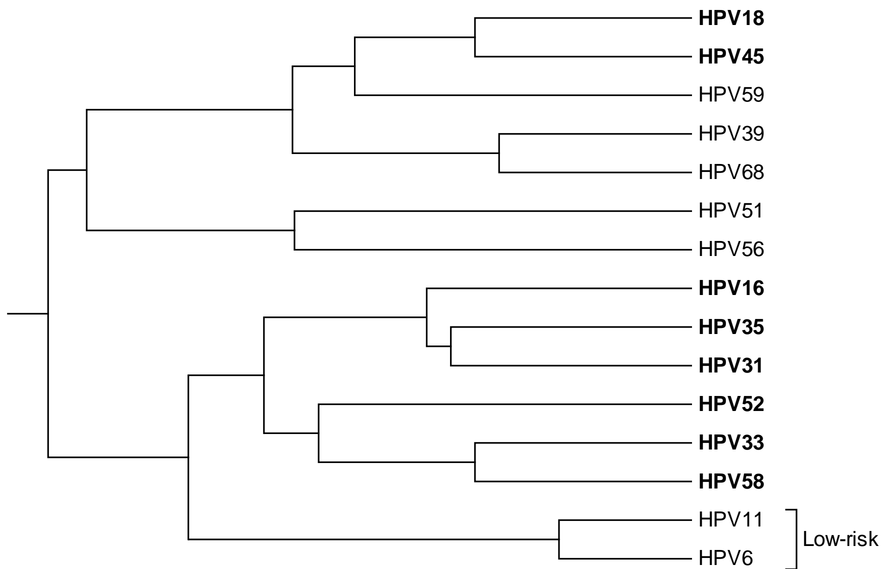


Figure 11. Phylogenetic tree based on the E7 amino acid sequences of 12 high-risk HPV types, two low-risk HPV and the probably carcinogenic HPV type 68. Due to the high sequence similarity between types, E7 antibodies often detect several E7 proteins, especially the more closely related such as HPV16, 31 and 35 or HPV18 and 45. The types in bold are the eight most common types, together found in approximately 90 % of all cervical cancers. The sequence differences between high- and low-risk HPV E7 made the establishment of high-risk specific E7 antibodies possible. The phylogenetic analysis is based on the multiple alignments of the E7 amino acid sequences in Clustal W. The evolutionary analyses were conducted in MEGA5.

4.1 Site-specific immunization

Phage display is an effective method to produce large amounts of peptides attached to an immunogenic carrier. Phage particles displaying pathogen-derived peptides induce protective immunity to that specific pathogen[68, 69] and phage clones displaying mimotopes mimicking specific antibody epitopes and selected from random libraries, can activate immune responses against the original antigen[70, 71]. Site-specific immunization with phage particles is an attractive approach when antibodies to a specific part of a protein are wanted for example in sandwich pairing, or to avoid highly immunogenic regions. Further, as phage particles are immunogenic and persist in the circulation or longer than free peptides that are rapidly cleared, site-specific immunization is a preferable alternative when antibodies to less immunogenic regions are needed.

In paper I, the immunogenicity of the phage particles was used to direct the immune response to a specific region of the HPV16 E7 oncoprotein thereby raising antibodies recognizing a pre-defined region of the endogenous E7 protein. A high-risk specific region, aa 33-60 of the HPV16 E7 protein was identified by sequence analysis. This fragment of HPV16 E7 has large sequence homology to the E7 protein of the closely related HPV types 31, 33 and 35, but not to the low-risk types or less related high-risk types, such as HPV18. The region was further chosen not to include the conserved regions, CR1-3. The corresponding nucleotide sequence was cloned into phage display vector f88-4, resulting mosaic phage particles with approximately 5 % of the pVIII proteins fused with the E7 peptide.

After seven immunizations with the phage preparations during the period of ten month, two out of three mice had high serum reactivity towards full-length HPV16 E7 and were sacrificed for hybridoma production. The fusion procedure resulted in three hybridomas, with reactivity to the HPV16 E7 protein, which were cloned by limiting dilution and evaluated using different immunological methods, in both paper I and II.

The three antibodies, named E716-1, E716-2 and E716-9 recognized full-length recombinant E7 of the three closely related high-risk types HPV16, 31 and 35, but not HPV 1, 6, 11, 18, 33 or 45, in ELISA and Western blot. The reactivity to the denatured protein in Western blot indicated that the antibodies recognize linear epitopes. The antibodies further detected the endogenous HPV16 E7 protein in lysates of the HPV16-positive cervical cancer cell line CaSki in Western blot, without cross-reactivity to other cellular proteins present in the lysates or to HPV18 E7 in HPV18-positive

HeLa cells. The mAbs specifically stained the HPV16 E7 protein in formalin fixed CaSki cells in ICC, indicating that the antibodies can be used to detect HPV16-induced malignancy in formalin fixed clinical samples.

Since the mice were immunized with an HPV16 E7 peptide corresponding to aa 33-60 of the HPV16 E7 fragment, the resulting antibodies recognized this region. To further characterize the epitopes fragment phage display was used. Three peptides together covering the peptide used for immunization were displayed on phage particles. The reactivity of the antibodies to the different fragments was studied in ELISA and from the binding pattern, the epitope was narrowed down further. E716-1 and E716-9 recognized the two fragments aa 28-47 and 37-53, mapping the epitope to the overlapping region aa 37-47. Antibody E716-2 only bound one fragment aa 37-53, mapping the epitope of E716-2 to the junction between the other two fragments (aa 28-47 and 47-62) (Fig. 12).

To further confirm the mapping data and support the specificity results, mimope analysis was done with a commercially available random peptide library displayed on phage. The peptides of the library used here was only 7 aa long and therefore ideal for mapping of linear B-cell epitopes, though conformation dependent epitopes cannot be identified with such short peptides. The phage library, displaying the random peptides in fusion with the pIII coat protein, was panned against the antibodies in four rounds. Nine binding clones were sequenced for each mAb and the consensus sequence revealed the aa residues necessary for antigen binding, the mimotope. E716-1 and E716-9 had the exact same mimotope, Q A/S Q/R PD, corresponding to aa 44-48 on the HPV16 E7 sequence. This sequence is conserved between the three closely related types HPV16, 31 and 35 in agreement with the specificity study with recombinant protein and cannot be found in the other HPV E7 proteins. The mimotope of E716-2 was one position longer, Q A/S R PDR, corresponding to aa 44-49. This sequence can only be found in HPV16 E7, but since E716-2 also detects HPV31 and 35, the last position of the mimotope, the Arginine (-R-) is not crucial for binding. Altogether, the study shows how different methods can be used to complement and confirm results in the establishment of highly specific and well characterized antibodies for clinical applications.

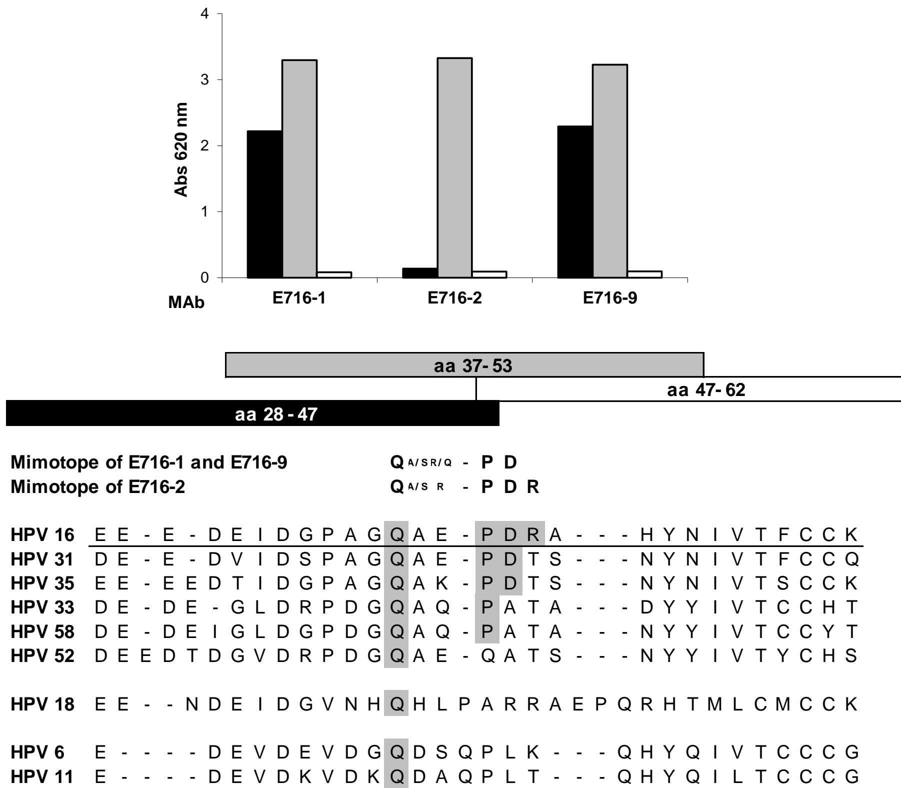


Figure 12. Mapping results for the three HPV16 E7 antibodies raised by site-specific immunization. The antibodies were raised against amino acid (aa) 33-60 of the HPV16 E7 protein (underlined sequence). This region was chosen due to the sequence homology with a group of high-risk viruses (e.g. 31 and 35). The resulting antibodies detected mimotopes corresponding to aa 44-48 and 44-49 and recognize recombinant HPV16, 31 and 35 E7 but not HPV18 E7 or low-risk HPV6 or 11 E7 protein.

4.2 Immunization with full-length E7 and the identification of high-risk specific E7 antibodies

A more widely used strategy for the establishment of monoclonal antibodies is to immunize the animals with full-length protein. In paper II, recombinant HPV16 and 18 E7 was used for immunization. The primary hybridoma selection was against recombinant HPV16 or 18 E7 proteins in ELISA and only hybridomas detecting either of the proteins were saved. This resulted in 57 hybridomas. The specificity was further evaluated in ELISA against a panel of high- and low-risk E7 proteins and those that cross-reacted with low-risk types were not further evaluated, resulting in 35 high-risk HPV-specific hybridomas. The majority of the antibodies recognized the E7 protein from more than one HPV type, which is not at all surprising, given the large sequence similarity throughout the viral genome. Most of the high-risk specific antibodies recognized a few, closely related HPV types, such as HPV18 and 45 or HPV16, 31 and 35.

The same panel of recombinant E7 proteins was used to study antibody specificity in Western blot. All the anti-E7 antibodies, selected for high-risk specificity against recombinant E7 in ELISA and functionality in ICC, had linear epitopes and recognized the E7 protein using Western blot. Overall, the specificity results from the Western blot analysis agreed with the ELISA analysis. The minor discrepancy seen between the two techniques is probably due to differences in affinities and how the specific epitopes are exposed.

As in paper I, overlapping peptides displayed on phage particles were used for epitope determination (Fig. 13). However, since the antibodies in paper II were raised against full-length protein fragments covering the entire HPV16 and HPV18 E7 ORFs were used. The peptides were of different length, from 16 to 37 aa long and displayed on phage particles in fusion with either coat protein pVIII or pIII. Antibody binding to the different peptides was then studied in ELISA to map the antibody epitopes. Epitope mapping with long peptides is a fast and straightforward technique to roughly identify the epitopes of a large number of antibodies targeting the same antigen. A separate set of clones has to be constructed for each antigen, but when this has been done, a large number of antibodies can easily be mapped in parallel. The mapping data can be used to for example find possible ELISA pairs, predict antibody specificities or identify immunogenic regions on an antigen.

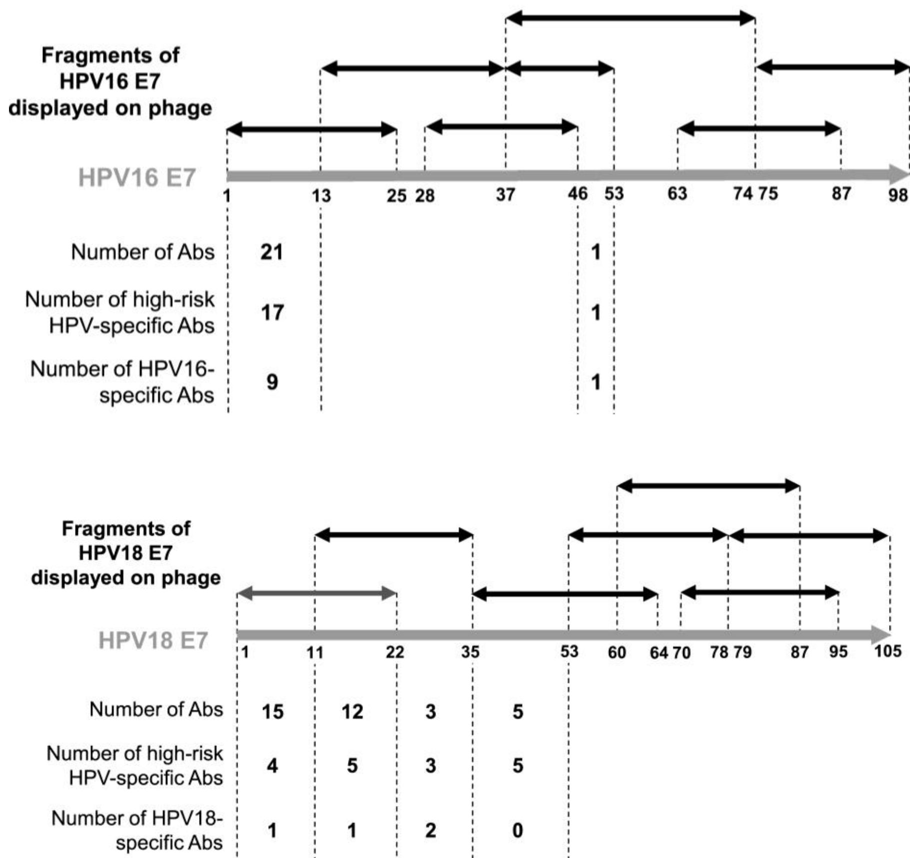


Figure 13. Epitope mapping of 57 antibodies (Ab) raised against the full-length HPV16 or 18 E7 protein. In total, 48 hybridomas recognized to the first 22 amino acids of the E7 protein, a region that includes conserved region (CR) 1 and several well conserved positions. The majority of these N-terminal antibodies recognized several HPV-types and only 14 were HPV16 or 18-specific. All antibodies that detected more central part of the protein were high-risk specific.

As expected, the 57 hybridomas targeted different regions of the E7 antigens. However, the majority of the antibodies were directed to the N-terminal of the E7 protein and all 57 antibodies actually detected epitopes within aa 1-53, a region which is known to lack secondary structure[35]. This is in agreement with earlier studies identifying the N-terminal half of the E7 protein as more immunogenic[72]. All 22 hybridomas that cross-reacted with the low-risk HPV types, detected aa 1 to 22, including CR1 which is highly conserved throughout all HPV E7 proteins. The majority of the N-terminal antibodies

did not however, detect the E7 protein in ICC, indicating that the amino terminus is immunogenic but not well accessible in ICC.

In our hands, site-specific immunization gave fewer positive hybridomas than immunization with full-length protein, but since all detected the desired region the selection process was short and no hybridomas were discarded due to cross-reactivity to low-risk types. When immunizing with full-length protein the more immunogenic regions of the protein obviously dominated but after a more laborious selection process involving a much larger number of hybridomas high-quality antibodies with different specificities and functionalities were identified. When antibodies to a protein like E7 with highly immunogenic, conserved regions are wanted site-specific immunization might be a more efficient option to obtain antibodies with the desired specificity.

4.3 Construction of a vector system for the display of peptide libraries

A vector system for the display of peptide libraries on phage particles or in fusion with GST or CTB was constructed. The inserts encoding the peptides to be displayed were arranged on long synthesized DNA constructs, with restriction sites in between, so that they could be inserted in any of the three expression vectors, depending on application. Using the described method, a 12-mer library, overlapping by 8 aa and covering the entire ORF of HPV16 E7 was constructed. The library was used to identify HPV16 E7 antibody epitopes and map antibody reactivity in HPV16 E7 immunized mice.

Four HPV16 E7 antibodies where examined with colony blotting, using the 12-mer library displayed in fusion with GST and the epitopes were mapped down to four to twelve amino acids. The mapping results were in agreement with earlier results using longer overlapping peptides and the random 7-mer peptide library (Table 1). The mimotope of two mAbs, E716-9 and E716-41 had previously been mapped with the random peptide library. The mimotope of E716-9 was identified as Q A/S Q/R PD and two 12-mer peptides in the overlapping library carry this epitope. However, only one was isolated indicating that there are some conformational constrains or that all clones were not represented among the 135 colonies in the blot. The epitope of E716-41 was mapped down to four amino acids, HEYM, corresponding to aa 9-12 on the HPV16 E7 sequence and in agreement with mimotope data identifying HE Y/F as necessary for binding.

Table 1. Epitope identification of three HPV16 E7 mAbs using three complementary techniques.

Clone	Method	Consensus sequence	Corresponding aa of the HPV 16 E7 protein
E716-9	E7 peptide display	EIDGPAGQAEPD	37-47
	12-mer peptides	EIDGPAGQAEPD	37-48
	Mimotope	QxxPD	44-48
E716-41	E7 peptide display	MHGDTPTLHEYM	1-12
	12-mer peptides	HEYM	9-12
	Mimotope	HEY	9-11
E716-74	E7 peptide display	EPDRAHYN	46-53
	12-mer peptides	RAHYNIVT	49-56
E716-81	E7 peptide display	MHGDTPTLHEYM	1-12
	12-mer peptides	TPTLHEYM	5-12

The 12-mer E7 library was also displayed on phage particles and used to map antibody responses in serum of mice immunized with full-length recombinant HPV16 E7 oncoprotein. After a single round of panning the library against serum immobilized in microtiter wells, 2.8×10^4 particles were eluted compared to 400 from naïve serum. 20 of these were sequenced which was enough to obtain conclusive results. The panning experiment showed that the serum reactivity in the E7 immunized mice was predominately directed to the N-terminal of the HPV16 E7 protein, while that of the control animals was randomly distributed. This further proved the immunogenicity of the amino terminus seen when hybridomas were raised against full-length E7 protein in paper II. The constructed library can thus be used to map dominant B-cell epitopes and predict the antibody specificities of the resulting hybridomas. In antibody production, this can be used to choose mice with the desired antibody response for hybridoma production and encourage the use of alternative immunization strategies to obtain antibodies to less immunogenic regions such as site-specific immunization. Mapping of B-cell epitopes to different antigens is also important for other applications, such as the analysis of immune response to vaccines or pathogens.

Overlapping peptide libraries are obviously an easy way of mapping several antibody epitopes to the same antigen and can even be used to map conformational dependent epitopes. Several studies have shown that peptides often better mimic antibody epitopes when attached to the hydrophobic phage

surface as compared to free peptides[70, 73, 74]. Peptide libraries can in addition be used to map antibody epitopes to a specific target in a mixture of other, irrelevant antibodies or antibodies with several specificities to the same antigen, such as crude serum and polyclonal antibodies. A new library has to be constructed for each antigen, but once this is done, is a continuous, inexpensive source of peptides, compared to e.g. Pepscan. Random peptide libraries can only be used on antibodies with single specificities, but can on the other hand be used to map antibodies with unknown specificities. The mimotope data from random libraries can be used to search sequence databases, thereby identifying possible interaction partners for a specific antibody.

4.4 Detection of E7 in LBC samples

4.4.1 Protocol optimization

All high-risk specific antibodies raised in this study were carefully evaluated in ICC using different protocols for fixation, antigen retrieval and antibody staining. As presented in paper II, ten high-risk specific E7 antibodies detected the endogenous protein in formalin-fixed cells. Seven of the antibodies also stained cervical cancer cell lines fixed in LBC fluid, with an overall higher sensitivity than in formalin fixed cells. A difference in staining between the two fixatives is expected since formalin is a crosslinking fixative while the alcohol of the LBC fluid denatures the proteins. As the epitopes are often differently exposed in different fixatives[75], ICC protocols have to be evaluated for every specific antibody/antigen pair and fixative. As seen both in this and other studies ICC staining could not be achieved with all antibodies established which is probably due to both epitope conformation and antibody properties such as affinity.

Heat-induced antigen retrieval had no positive effect on staining in any of the protocols with any of the antibodies, but incubation with Triton X-100 improved staining of LBC samples considerably and is included in the final protocol. This positive effect of Triton X-100 on E7 staining has previously been reported[62], but whether this positive effect is caused by unmasking of epitopes or permeabilization of cell membranes is not known.

The optimized protocol was the same for all antibodies and the only parameter that differed between the protocols was antibody concentration. After mounting and fixation in either formalin or LBC preservative, the cells were pretreated for 15 minutes with 0.3 % Triton X-100 followed by blocking of endogenous peroxidases for 5 min in 1 % hydrogen peroxide.

The slides were then incubated with the E7 antibodies, diluted to the optimal concentration in Dako antibody diluent, in a humid chamber. E7 antibody binding was visualized with REAL EnVision Detection system (Dako) and diaminobenzidine (DAB), staining the E7-expressing cells brown. To facilitate identification of cell morphology, the cells were counterstained blue with haematoxylin.

The final ICC protocols for detection of E7 in LBC were highly sensitive and able to detect single HPV positive cells in a background of HPV negative cells (Fig. 14). The most promising antibodies were mAb E716-41, specific for HPV16 E7 and E718-79, specific for HPV18 E7. If constructing a test based on these two HPV types it would cover approximately 70 % of all cervical cancers[6]. The cells were stored in the LBC liquid for over two years without loss of signal or adding background. This indicates that the antibodies have the potential to be used in a clinical setting to detect and highlight rare dysplastic cells in LBC samples.

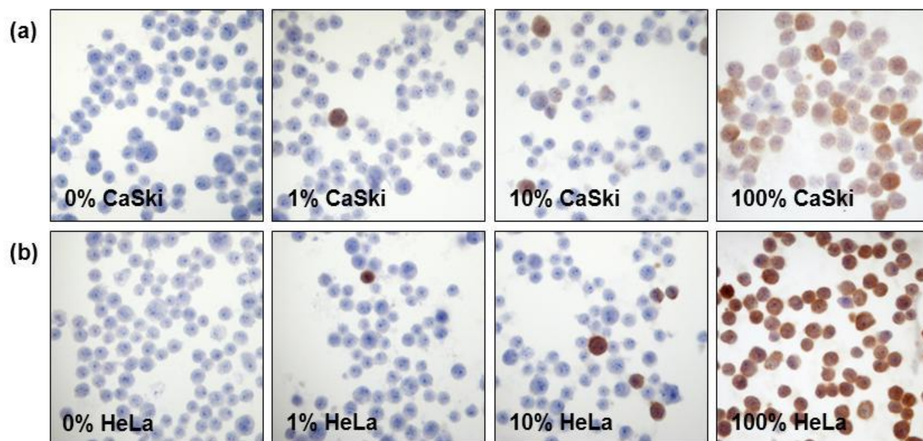


Figure 14. To mimic a clinical sample with only a proportion HPV-positive cells in a background of uninfected cells, HPV16-positive CaSki cells and HPV18-positive HeLa cells were mixed, in different proportions, with the HPV negative cervical cancer cell line C-33A and stained with a) mAb E716-41 and b) mAb E718-79. Both antibodies easily detected 1 % of HPV-positive cells without staining C-33A[76].

4.4.2 Evaluation on clinical LBC samples

Only a few studies have been able to detect the E7 protein in cervical cytology samples[62, 77] and to our knowledge no studies have shown E7 ICC staining of clinical LBC samples. In paper IV, the most promising ICC mAbs E716-41 and E718-79, were evaluated on 49 clinical samples stored in LBC fluid. Histology, HPV DNA genotyping, HPV mRNA genotyping and p16/Ki-67 staining was performed in parallel. Both antibodies were able to detect the E7 protein in dysplastic HPV-infected samples without staining normal cytology samples and can potentially be included in a test to detect the HPV16 and 18 E7 proteins in residual LBC samples.

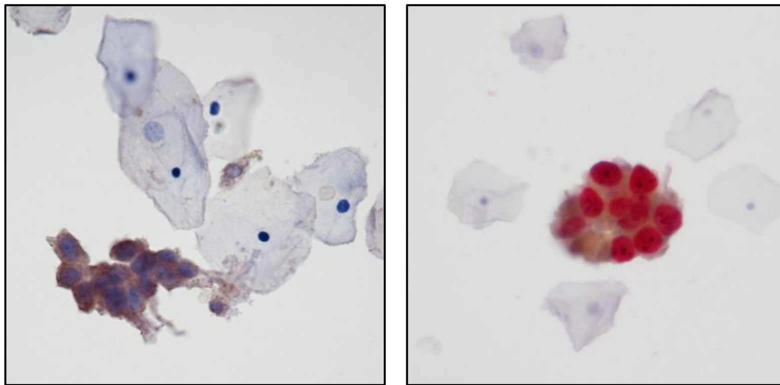


Figure 15. Evaluation of the E7 cytology protocols on clinical LBC samples. Staining of an HPV16 positive histologically verified cervical intraepithelial neoplasia (CIN) 2 sample with a) mAb E716-41 and b) p16/Ki-67 dual staining. The E7 protocols stain both nucleus and cytoplasm of dysplastic cells brown. The haematoxylin stains all cells blue.

The p16/Ki-67 staining was used to identify dysplastic cells in the LBC samples. Getting an estimation of the number of dysplastic cells actually present in the sample facilitated the evaluation of the E7 staining. In all samples, the number of E7-positive cells was the same or fewer than those detected by the p16/Ki-67 staining. This difference is most likely due to lower levels of E7 protein expressed in the cells compared with the accumulated secondary markers and not the sensitivity of E7 antibodies since both have affinity constants in the order of 10^{-10} (determined by QCM, unpublished data). False positive p16/Ki-67 staining is also a possible explanation since the secondary markers can be elevated for other reasons than in response to HPV-infection[78]. For five samples, histologically verified as high-grade lesions (CIN2-3), no cells were stained with p16/Ki-67,

indicating that no dysplastic cells were actually present in the cytology sample. The same samples were also negative for E7 staining. Deviation between cytology and histology results is normal, due to both sampling techniques and biological variations, though it can be minimized through training and method optimization. ICC protocols, such as those presented here, can however highlight the dysplastic HPV-positive cells present in the scrape, possibly irrespectively of morphology, thereby increasing specificity and sensitivity and reduce inter-observer variability.

The antibodies evaluated in paper IV, clearly stain dysplastic cells of different severity (Fig. 15). New complementary tests for borderline and ASC-US cytology are highly needed and the antibodies presented here might have the potential to be included in such tests. To prove clinical utility, however, larger studies are obviously necessary, preferably on a true referral population including multiple infections. Since LBC leaves residual sample after the cytology evaluation the E7 staining can be done on the same sample, without recalling the patient. The protocols presented here can easily be accomplished in a few hours with only basic laboratory equipment and such tests might therefore be a cheap alternative to HPV DNA/mRNA testing or genotyping, or be included in an automated test to identify true cervical malignancy.

5 Concluding remarks

Today's cervical cancer screening programs yearly produce millions of suspicious samples that require expensive retesting, recalling and follow-up. Only a small proportion of these women however, have underlying disease that needs to be detected and treated. This group should optimally be identified without causing too much anxiety and overtreatment in the large group of women who are not at increased risk of getting cervical cancer. To do this, complementary tests specific for high-grade dysplasia are needed. The antibodies presented here detect the E7 oncoprotein using different immunological techniques such as Western blot, Immunocytochemistry and flow cytometry and can also be combined in pairs for ELISA analysis. Immunological methods can offer objective and possibly more specific evaluation of HPV-induced disease as compared to cytology since elevated levels of E7 protein is a direct marker of malignant transformation.

A major focus of this study has been to generate and characterize HPV E7 antibodies in detail, regarding both specificity and usefulness in immunological detection of HPV-induced cancer. Well characterized antibodies are necessary for the construction of safe immunological tests and techniques for antibody establishment and characterization are obviously important tools in the creation of new immune reagents. The different immunization strategies, as well as the methods used for screening and epitope characterization, resulted in antibodies with different properties and specificities, highlighting the importance of choosing the most efficient strategy when generating new antibodies for a specific application.

The generated antibodies specifically detect the E7 protein from cervical cancer cell lines in Western blot and ELISA. However, in these techniques the sample is lysed prior to analysis and the E7 protein will be diluted in the lysate of an unknown number of normal cells present in the cell scrape. This makes E7 quantification problematic and future studies will need to assess whether the required sensitivity for detection of E7 in clinical samples can be achieved using ELISA or Western blot techniques.

Several of the antibodies presented here recognize the E7 protein in cervical cancer cells. A cytology sample normally contains between 500 000 and 2 000 000 cells out of which only a few, possibly less than 0.1 %, are dysplastic and express E7 oncoprotein. For obvious reasons, cytology evaluation can be tiresome and the often small numbers of abnormal cells present can be hard to identify, causing large variation in diagnosis even

between experienced cytologists[40]. The antibodies described in the present study stain single HPV-positive cells in immunocytochemistry and preliminary data indicate that the antibodies can identify down to 0.1 % HPV-positive cells in a mixture of normal cells in flow cytometry. Both ICC and flow cytometry are excellent techniques to highlight rare dysplastic cells and offer the possibility to automatize the screening process.

The two most promising antibodies for detection of E7 in LBC detect the two most common types, HPV16 and 18, together responsible for approximately 70 % of all cervical cancers[6]. The remaining types in the world-wide distribution contribute much less to the total cancer burden and antibodies detecting the nine most common types would be needed to achieve 90 % coverage[79]. E7 detection can either be done with the antibodies mixed in a high-risk specific antibody cocktail, or as separate, type-specific staining protocols. Type-specific E7 cytology can be useful to identify what HPV type/types causes lesions in for example a patient with multiple infections. This could be used to determine disease prognosis and identify patients at a higher risk since for example HPV16 and 18 are overrepresented in more aggressive disease[6]. A mixture of antibodies, possibly both to the E6 and E7 proteins or in combination with antibodies to other cellular markers, could be used to improve sensitivity further.

Some of the major questions in cervical cancer testing are; how to reduce overtreatment in screened populations, how to increase sensitivity for malignancy detection and how to make screening available for a larger part of the world. No test available today can solve all these questions and new, simple and preferably objective tests are needed to identify women at high risk of proceeding to invasive disease. Immunological methods have the potential to offer specific and objective detection of dysplasia by detecting not only infection but the protein causing the malignancy and can possibly be included in automatized systems. Methods such as ICC or flow cytometry can easily be combined with HPV DNA/mRNA testing and/or cytology to identify rare dysplastic E7-expressing cells in a cervical sample and to achieve this, sensitive and specific immunological reagents are absolutely necessary.

The antibodies presented here are specific for oncogenic HPV E7 protein and detect the target protein in tumour cells using several immunological methods. The most promising application is LBC and the optimized protocol detects single HPV16 and 18 E7 positive cells in patient material. Though clinical utility needs to be evaluated in larger studies, the antibodies hopefully have the potential to be included in a test to identify HPV-induced

dysplasia in inconclusive or borderline cytology samples, thereby distinguishing high-grade dysplasia from transient infections and reducing unnecessary recalling, anxiety and overtreatment.

Acknowledgements

Först vill jag rikta mitt stora tack till **Olle Nilsson**, **Christina Hall**, **Christian Fermér** och övriga på **Fujirebio Diagnostics** som gett mig möjligheten att genomföra det här projektet. Tack också till vänner, familj och alla som på olika sätt bidragit till den här avhandlingen och för allt stöd jag fått under de senaste åtta åren. Jag vill särskilt tacka:

Christian Fermér för ditt utmärkta handledarskap och din ständiga uppmuntran. Det har varit en ynnest att ha en så bra chef, handledare och vän tillgänglig genom hela arbetet.

Olle Nilsson som med lagom delar piska och morot samt en stor portion kunskap och erfarenhet handlett mig genom arbetet.

Jan Holmgren för att du genom dina mångåriga erfarenheter av forskarvärlden varit ett stort stöd och en kunnig handledare.

Michael Lebens for interesting collaboration, fruitful discussions and for critically reading my thesis.

Alla på **Avdelningen för Mikrobiologi & Immunologi** som på olika sätt hjälpt mig att lösa både praktiska och administrativa problem och alltid fått mig att känna mig välkommen på avdelningen. Ett extra tack till **Sukanya Raghavan** och **Louise Sjökvist Ottsjö** som hjälpt mig med FACS-analyserna.

Alla mina underbara kollegor på **Fujirebio Diagnostics** för allt stöd ni gett mig och inte minst för alla trevliga fikaraster.

Ett extra varmt tack till mina rumskamrater, **Ulrika** och **Karin**, för alla uppmuntrande kommentarer och bra förslag.

Åsa för att du och din familj finns som en viktig del i mitt liv och för att du är en så klok och ärlig vän.

Jenny för all hjälp med bilder och layout och framförallt för hjälpen med framsidan på den här avhandlingen.

Professor Matthias Dürst and colleagues at the Department of Gynecology and Obstetrics, Jena University Hospital. It was a pleasure to visit you and your experience in the field of HPV and cervical cancer has been an invaluable contribution to this thesis.

Monika Dohsé för att du delat med dig av din erfarenhet och expertis i cytologi och hjälpt mig med utvärderingen av mina antikroppsinfärgningar.

Dr Zivjena Vucetic for critically reading this thesis.

Mina ambitiösa och duktiga studenter, **Nagham Musa** och **Sara Tronarp**. Era examensarbeten har varit viktiga bidrag till den här avhandlingen och jag önskar er all lycka i era framtida karriärer.

Sofie & Johanna, mina ständiga livscoacher och kära vänner. Ett extra tack till min egen lilla HPV-doktor Johanna för all hjälp genom doktorandtiden. Det har varit så skönt att få gå i dina fotspår.

Johanna, min allra bästa vän, för att du alltid finns där med goda råd och många kramar.

Gunilla & Torgny för alla era försök att förstå er på mina krångliga virus och för att ni gjort Gotland till mitt tredje hem. Bättre svärföräldrar kan man inte önska sig.

Mamma, Pappa & Erik för att ni alltid uppmuntrar mig att göra det jag vill och er ständiga tro att jag klarar allt.

Mattias & Elvis, min fina lilla familj. Tack för all er kärlek och ert ständiga stöd. Tack för alla gånger ni kommit hem och sagt ”Nu har du jobbat färdigt, mamma”.

The studies presented in this thesis were financially supported by the Swedish Research Council, the SmartHEALTH Integrated Project and Fujirebio Diagnostics AB.

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