Uterine cervical neoplasia

Aspects of biology and pathology

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PREFACE

This study was carried out at the Gade Institute, University of Bergen between 1993 and 2005. While starting as a post graduate fellow supported by the Norwegian Cancer Society, from 1996 I have held a position as consultant at the Department of Pathology, Haukeland University Hospital. The first part of this work was carried out in collaboration with Professor Flora Hartveit, who is hereby thanked for introducing me to research. During these years, diagnostic pathology has advanced from a strict morphological fundament to a more versatile existence in the sphere that lies between morphology and cell biology. This is particularly true since the introduction of immunological and molecular biological techniques. Professor Ole Didrik Lærum urged me to enter this exciting field between basic research and clinical pathology. The inspiration I gained from working in the "Lærum group", with its assorted approaches to cancer research and noble achievements, cannot be overestimated. I am deeply grateful to Professor Lærum for his skillful supervision, optimism and encouragement.

Professor Anders Molven introduced me to quantitative real-time PCR and guided me through many difficulties. He has also been an important discussion partner, and is sincerely thanked for his significant contribution.

Thanks are also extended to all my coworkers and technicians in the laboratory. Prosector Nils-Petter Aardal, Professor Einar Svendsen, Professor Inge Morild and Professor Karl Henning Kalland have in turn been chairmen of the Department and thanked for the excellent working facilities.

Finally, my thanks to my wife, Anne, and daughter, Hanne, who have borne with me during this long and somewhat winding road.

Bjørn I. Bertelsen

ABBREVIATIONS USED

bp:	base pairs
CIN:	cervical intraepithelial neoplasia
CGH:	comparative genomic hybridization
CDK:	cyclin dependent kinase
C_T:	threshold cycle
DNA:	deoxyribonucleic acid
GCK:	glucokinase gene
E6-AP:	E6-associated protein
ECM:	extracellular matrix
EGF:	epidermal growth factor
EMT:	epithelial-mesenchymal transition
ERK:	extracellular signal-regulated kinase
eNOS:	nitric oxide synthase
FHIT:	fragile histidine triad tumor suppressor
FIGO:	Fédération International de Gynécologie et Obstétrique /International Federation of Gynecology
FOXO:	the FOXO family of transcription factors
HGF:	hepatocyte growth factor
HIF1α:	hypoxia-inducible factor alpha
HPV:	human papillomavirus
HRG:	histidine-rich glycoprotein
IL-8:	interleukin 8
IKK:	IκB kinase
ILK:	integrin-linked kinase
JOE:	6-carboxy-dichlorodimethoxyfluorescein
LSIL:	low-grade squamous intraepithelial lesion
LEEP:	loop electrosurgical excision procedures
HSIL:	high-grade squamous intraepithelial lesion
HSV-2:	Herpes Simplex Virus type 2
IGF-1R:	insulin-like growth factor 1 receptor

ISH:	*in situ* hybridization
NF-κB :	nuclear factor κB
PDGF:	platelet derived growth factor
PDK-1:	3-phosphoinositide-dependent protein kinase-1
PDK-2:	3-phosphoinositide-dependent protein kinase-2
PH:	pleckstrin homology
PI3K:	phosphatidylinositol 3-kinase
PIP_3:	phosphatidylinositol 3,4,5 trisphosphate
PCR:	polymerase chain reaction
PTEN:	phosphatase and tensin homolog deleted on chromosome 10
Rb:	retinoblastoma tumor suppressor protein
RFLP:	restriction fragment length polymorphism
RTK:	receptor tyrosine kinase
RT-PCR:	reverse transcription-polymerase chain reaction
SNOP:	systematized nomenclature of pathology
SNOMED:	systematized nomenclature of medicine
SIL:	squamous intraepithelial lesion
TAMRA:	6-carboxy-tetramethylrhodamine
Taq:	*Thermus aquaticus*
T_m:	melting temperature
TOR:	target of rapamycin
TSC2:	tuberous sclerosis complex 2
VEGF:	vascular endothelial growth factor
VEGFR-1:	vascular endothelial growth factor receptor 1
VEGFR-2:	vascular endothelial growth factor receptor 2

BACKGROUND

During the course of my studies of cervical neoplasia, new insights have radically changed the understanding of this cancer type. The general introduction of this thesis will therefore start with an overview of the current knowledge of the disease, including aspects of its fundamental biology, practical clinicopathological considerations, and screening. Finally, I will give a survey of biobanks and how old, archival pathology material can yield new insights into the biology of cervical neoplasia.

The cervical cancer load

Uterine cervical cancer accounted for an estimated 274,000 deaths world-wide in 2002[1]. With nearly 500,000 new cases per year, it is the second most common cancer in women world-wide[1]. It is one of the leading causes of cancer-related deaths in young women[2]. The disease incidence shows marked geographical variation. An estimated 83% of new cases now occur in the developing countries, where it represents 15% of new cancers in women[1]. In contrast, cervical neoplasia accounts for only 3.6% of new female cancers in the developed world[1]. The low incidence rate in developed countries is a rather new phenomenon, as the incidence in most of Europe, North America, Australia and New Zealand before the introduction of cytological screening in the 1960s and '70s, was similar to that in the developing countries today[3,4]. In Norway, 270 women were diagnosed with cervical cancer in 2004 and there are about 100 deaths every year[5]. The age-specific incidence maximum was 21 per 100,000 women at age 45-49 years[5].

Morphology and classification

Cervical cancers arise in the transformation zone, i.e. the zone between the original and current squamocolumnar junctions (Figure 1)[6]. Squamous cell carcinomas make up about 80% of cervical cancers, adenocarcinomas make up about 15%,

while the rest include some rare carcinoma types and neuroendocrine tumors[7,8].

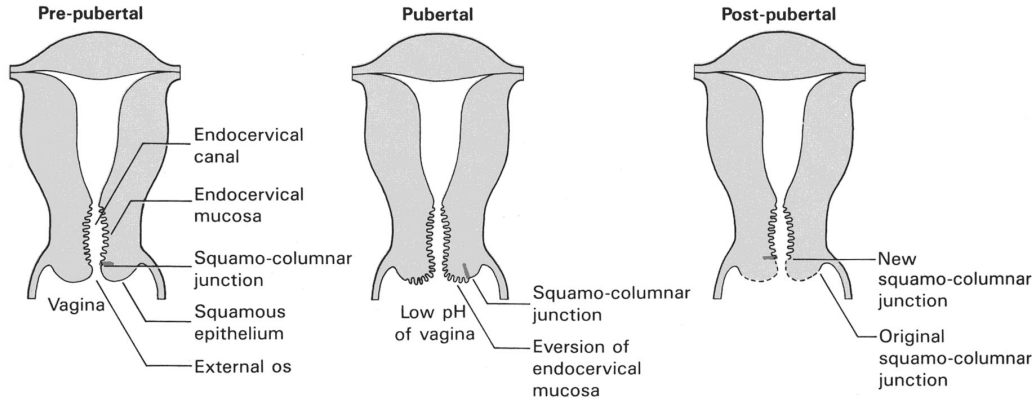

Figure 1. The cervical transformation zone at different physiological stages. From Wells [9].

Cervical squamous cell carcinomas originate from intraepithelial precursor lesions, cervical intraepithelial neoplasia (CIN) (Figure 2), characterized by a disordered morphology of the squamous epithelium with proliferation of primitive cells beginning in the basal portions of the epithelium[6,10]. These so-called undifferentiated neoplastic cells have enlarged, pleomorphic, hyperchromatic nuclei and high mitotic counts[6,11]. Abnormal mitotic figures are sometimes found in the undifferentiated neoplastic cells and are indicative of an aneuploid cell population[12]. CIN is a flat lesion located in the transformation zone, and seldom visible to the naked eye. Colposcopic examination may reveal areas of abnormal epithelium. A more specific diagnosis can be reached following microscopy of a cytological smear or a biopsy. The division into CIN grades 1-3 was meant to reflect a biological continuum of risk for the development of carcinoma[6]. In cervical intraepithelial neoplasia grade 1 (CIN 1) the undifferentiated neoplastic cells are found in the basal one-third of the epithelium, in CIN 2 they are present in the basal two-thirds of the epithelium, while in CIN 3 the upper one-third of the epithelium is also affected[6,11] (Figure 2). In CIN 1 and 2, and sometimes in CIN 3, differentiating pre-neoplastic cells are present in the epithelium superficially to the undifferentiated neoplastic cells[8]. The increased nucleo-cytoplasmic ratio in the exfoliated neoplastic cells is the clue to the cytological diagnosis of CIN. In CIN 1 the nucleus occupies up to one half of the total area of the cytoplasm, in CIN 2 it occupies between one half and two thirds of the cytoplasmic area, and in CIN 3 it

occupies more than two thirds of the cytoplasmic area[11]. In all three lesions, the nuclei are pleomorphic, hyperchromatic and irregular .

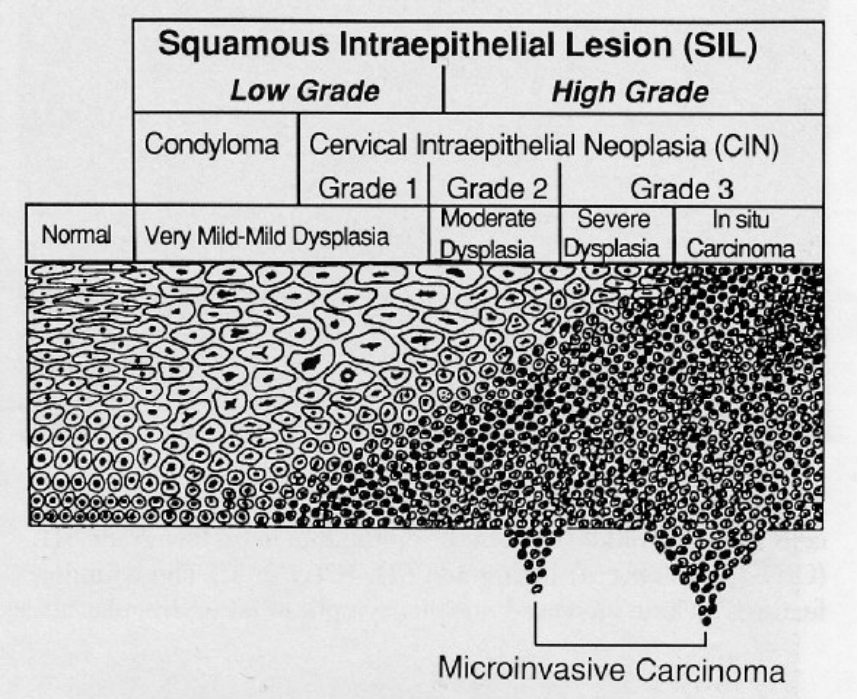

Figure 2. Pre-invasive squamous lesions in the uterine cervix. From Wright et al.[13].

The concept of a stepwise development of cervical squamous cell carcinoma was introduced nearly a century ago[14]. A variety of terms have been used for the pre-invasive lesions, i.e. atypical squamous epithelium, incipient carcinoma, carcinoma *in situ*, atypical hyperplasia, dysplasia, CIN and squamous intraepithelial lesion (SIL)[15-21]. When attention was drawn to human papillomavirus and its possible relation to cervical neoplasia, a variety of terms describing the association between viral changes and pre-invasive lesions were suggested[22-24]. Later, the Bethesda system intended for use in cytology, introduced terms for undetermined specimens, i.e., 'atypical squamous cells of undetermined significance' (ASCUS) for abnormal squamous cells that may or may not be pre-malignant or malignant and 'atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion' (ASC-H) for those cases where a high-grade lesion is difficult to exclude. Furthermore, the Bethesda system lumped human papilloma virus infection and CIN 1 together as a low-grade squamous intraepithelial lesion

(LSIL) and merged CIN 2 and 3 as high-grade squamous intraepithelial lesions (HSIL)[20,21]. However, the CIN-classification is still regarded as the system best suited for histopathology[25,26]. The suggested modified terms "low-grade-" and "high-grade CIN" for CIN 1 and CIN 2-3 respectively, would bring it closer to the Bethesda system[27,28]. In Norway the CIN classification prevails in histopathology, while the Bethesda system recently has been taken up in cytology[29].

Cervical carcinogenesis

A modern definition of a neoplastic cell is one that has clonally expanded as a result of somatic mutations[30]. Neoplastic development may be viewed as an evolutionary process where the cells acquire genetic lesions selectively advantageous to their survival and proliferation[31]. CIN 3 and invasive cervical carcinomas are monoclonal, and it seems that the pre-invasive lesions develop into a monoclonal lesion during the progression from CIN 1 to CIN 3[32-35]. Self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis have been postulated as hallmark alterations in cell physiology shared by most human cancers[36]. Many of these changes may be incited by viruses that have evolved mechanisms to modulate cellular signaling pathways to reprogram host cells to support the viral life cycles or modulate host defense responses.

Human papillomavirus infection

A role for human papillomavirus (HPV) in cervical neoplasia was suggested by Harald zur Hausen in the early 1970's[37]. Work in his laboratory resulted in the identification of HPV DNA in condyloma accuminatum and cervical cancer tissues[38-40]. It is now generally accepted that human papillomavirus (HPV) play a necessary, causative role in cervical carcinogenesis[41-44]. Besides, HPV is a probable causative factor in a proportion of anal, perianal, vulvar, penile, oropharyngeal and skin cancers[45].

HPV is a small non-enveloped virus that contains a double-stranded circular DNA genome of about 8,000 basepairs[46]. More than 200 different types of HPV have been identified on the basis of genomic differences[46]. Specific subtypes of HPV that carry a considerable risk of causing malignant progression in the uterine cervix are termed high-risk HPV (Table 1)[2,43]. HPV 16 is the most commonly occurring subtype in cervical neoplasia[43], however, HPV 18 is associated with more advanced cervical neoplasia than HPV 16[47]. The virus infects the basal cells of the epithelium, probably binding to the α6β4-integrin[48]. HPV DNA is maintained at a low copy number in the nuclei of the differentiating epithelial cells as they move toward the epithelial surface, while replication to a high copy number with production of progeny virus occurs in terminally differentiating cells[46].

Viral feature	HPV types
Carcinogenic	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82.
Probably carcinogenic	26, 53, 66.

Table 1. HPV types considered carcinogenic or probably carcinogenic. After Munoz et al.[43].

The virus is transmitted through sexual contact[49-51]. Genital HPV infections are abundant, and infection with more than one HPV type is not uncommon[52-58]. Sexual activity at early age, large number of sexual contacts, poor hygiene, uncircumcised male partners and compromized immune defense are factors that increase the risk for genital HPV infection in women[26,59-65]. The prevalence of high-risk HPV has been found to peak in the youngest women, decline in middle aged women, and then increase slightly in older women[66]. HPV DNA has been detected by Southern blot in 25-40% of adolescents in a single screening visit[67]. Estimates of life-time risk based on cytological screening showed that up to 79% of Finnish females would contract at least one HPV infection between ages 20 and 79 years[52]. Even if most infections clear without sequelae in 1-2 years, persistence of the infection may eventually result in integration of HPV DNA into the host cell genome, an event considered a prerequisite for malignant development[46,68-71].

The morphological changes of HPV-infection are commonly found in association with CIN and have been described in detail[11,23,72-75]. The most

consistent of the so-called epithelial cytopathic effects are koilocytosis, dyskeratosis and multinucleation[11]. Histologically, a flat lesion is usually seen (flat condyloma/condyloma planae), but it may be elevated (condyloma accuminatum) or endophytic (inverted)[11,24].

HPV oncoproteins

HPV integration sites are randomly distributed over the whole human genome with a clear predilection for fragile genomic sites[76]. While most of the open reading frames of the integrated viral DNA are lost or interrupted, the high-risk HPV proteins E6 and E7 continue to be expressed, accounting for the transforming ability of the virus[45,68,77]. E6 binds and degrades the tumor suppressor p53[78,79], while E7 disrupts the function of the retinoblastoma (Rb) family of cell cycle regulators[80]. Even if the functions of each viral protein have been thoroughly examined in isolation, these analyses do not accurately represent the *in vivo* scenario in cases of cervical cancer, where both E6 and E7 are always present[45]. E6 and E7 interact with numerous cellular proteins resulting in a diversity of biological effects[45,46,81]. In many cases the relevance of these interactions still remains to be determined[46]. However, it is clear that high-risk HPV E6 and E7 are both individually able to immortalize many different human cells, even if their cooperative action results in a considerably increased immortalization efficiency[81-83]. The E6 and E7 genes of non-carcinogenic HPV have only weak immortalizing activity in human epithelial cells[84].

E6 has mutagenic and antiapoptotic effects that are key elements in tumor progression[45]. It binds the cellular tumor suppressor protein p53, indirectly through a complex formed with the E6-associated protein (E6-AP)[46]. This results in the degradation of p53 by ubiquitin-dependent proteolysis[79,85]. Because of the p53 degradation, E6 overrides the G_1/S checkpoint control in DNA-damaged cells and exerts a mutagenic effect[45]. E6 also has antiapoptotic effects through the elimination of p53 and degradation of the proapoptotic protein Bak, a member of the Bcl-2 family[45]. Besides, it has recently been demonstrated that E6-mediated inactivation of the Bax-dependent proapoptotic pathway is crucial for protection of HPV-positive cancer cells from apoptotic cell death[86]. Upregulation of c-IAP2 gene expression by both E6 and E7 has also been reported and seems to confer

resistance to apoptosis contributing to sustained growth in high-risk HPV positive cancer cells[87]. Shortening of the chromosomal telomeric termini with each round of DNA replication autonomously restricts the number of cell divisions in a normal cell, while in many malignant tumors this effect is prevented by the expression of telomerase[46]. In high-risk HPV induced lesions, E6 stimulates expression of host cell telomerase, contributing further to cell immortalization[88,89].

E7 has cell cycle driving effects. It binds the retinoblastoma-susceptibility gene product pRb as well as some of its family members, including p107 and p130, regulators of the progression of cells from the G_1-phase into the S-phase of the cell cycle[80,90,91]. This results in phosphorylation and degradation of pRb and its family members and to a release of the E2F transcription factor that activates expression of several growth promoting genes[45]. This ability of E7 to destabilize pRb and related proteins with a resultant truncation of an important mechanism in the control of cell proliferation seems to be critical for cellular transformation[46]. Another essential growth stimulatory effect of E7 is the inactivation of the cyclin-dependent kinase inhibitors $p21^{CIP1}$ and $p27^{KIP1}$, thus bypassing cell cycle arrest during keratinocyte differentiation[46]. The newly identified cellular protein retinoblastoma protein-associated factor, p600, that regulates cellular pathways contributing to anchorage-independent growth, binds to E7 and seems to contribute to the transforming properties of E7[92].

The viral load and E6/E7 expression level appear to be important determinants for neoplastic development. Recently, a dose-response relationship between increasing high-risk HPV viral load and risk of pre-invasive lesions was suggested[93]. Likewise, higher HPV DNA copy numbers per cell are associated with increasing risk of development of adenocarcinoma of the cervix[94]. Further, the HPV E6/E7 expression level has been found to play a key role in progression of invasive carcinoma of the cervix[95]. A suggested scheme of high-risk HPV-mediated carcinogenesis is shown in Figure 3.

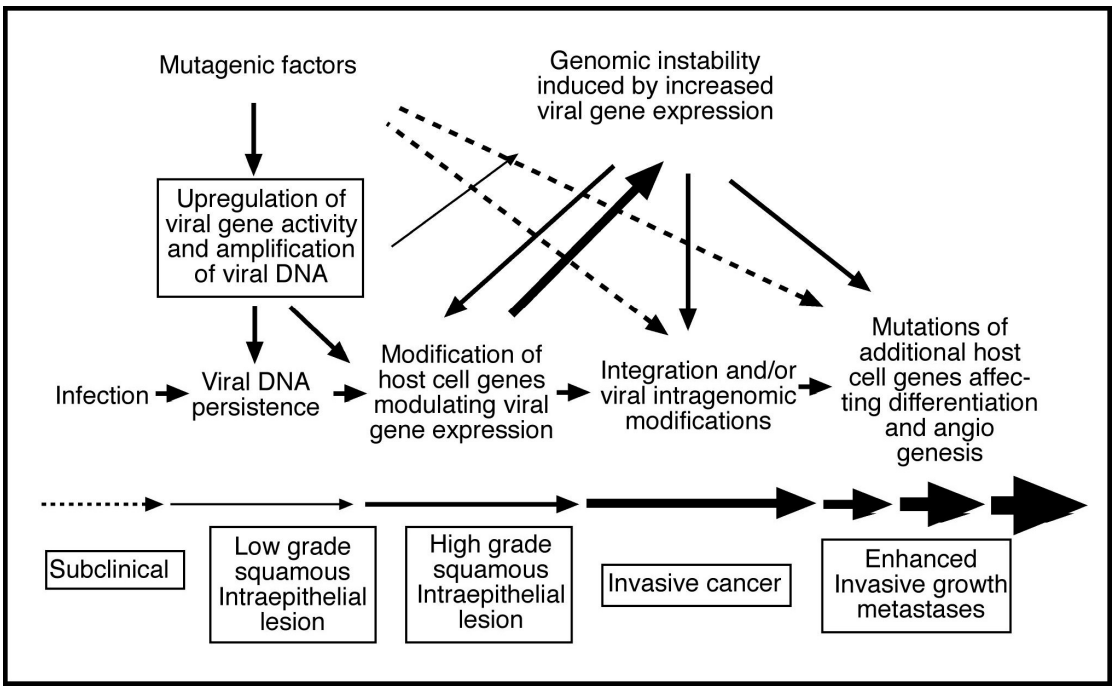

Figure 3. A possible scheme of high-risk HPV-mediated carcinogenesis. After zur Hausen[45].

Genetic aberrations

Chromosomal instability is an early event in cervical carcinogenesis[96]. Collectively, the expression of E6 and E7 gives rise to mitotic defects and numerical and structural chromosome instability through the induction of centrosome abnormalities, thus enabling the cells to acquire genetic alterations necessary for survival and clonal expansion[97-102]. However, the alternative view that chromosomal instability and aneuploidization precede integration of high-risk HPV genomes are supported by one study of biopsies from CIN and carcinomas[103].

Cervical carcinomas show a recurrent pattern of cytogenetic changes where chromosomal gains generally are more common than losses[104,105]. Gains of chromosomal regions 1q, 3q, 5p and 8q and loss of 2q, 3p, 4p, 4q, 6q, 11q, 13q and 18q are the most frequent changes[104,105]. The most common deletion is that of chromosome 3p with loss of 3p14.2 encoding the tumor suppressor fragile histidine triad (FHIT) identified in 40% of cervical cancers[104,105]. Gain of the long arm of chromosome 3 is the single most frequent change overall[97,105-109]. The same aberration has been demonstrated in HPV-transfected keratinocytes, pre-malignant

cervical precursor lesions and cervical cancer cell lines[97,106,110-112]. By comparative genomic hybridization (CGH), the areas of gain have been refined to parts of chromosomal bands 3q24-29[98,107-109]. *PIK3CA* at 3q26.3 is a candidate oncogene in this region[113,114].

The PI3K-AKT pathway

One of the major intracellular signaling pathways leading to cell growth involves phosphatidylinositol 3-kinase (PI3K)(Figure 4). This kinase principally phosphorylates inositol phospholipids. It can be activated by receptor tyrosine kinases, as well as by many other types of cell surface receptors. PI3K generates inositol phospholipids that trigger phosphorylation of AKT, an effector of potentially tumor-driving signals[113]. PTEN reverses the action of PI3K[115]. Deregulation of PI3K-AKT signaling occurs in many types of cancers, and may result from PTEN inactivation or overexpression of PI3K[113,115-118].

PI3K is a heterodimer with a regulatory subunit, p85α, and a catalytic subunit, p110α[118]. Several isoforms and subgroups of PI3K exist, however, only class IA PI3K is involved in carcinogenesis[113,118], and the one dealt with here. p110α is encoded by the above-mentioned gene *PIK3CA* located at 3q26.3[113].

Different mechanisms of PI3K activation exist. The regulatory subunit p85α is a direct substrate of receptor tyrosine kinases and G-protein-coupled receptors, and triggers activation of the catalytic subunit p110α[113,114]. Activation of p110α can also occur through the Ras protooncogene, possibly acting in concert with p85α[114,119]. Upon activation, class IA PI3K is recruited to the inner surface of the plasma membrane, where it catalyzes the transfer of phosphate from ATP to the D-3 position of the inositol ring of membrane-localized phosphoinositides, thereby generating 3'-phosphorylated phosphoinositides, most notably phosphatidylinositol 3,4,5 trisphosphate (PIP_3)[114,119]. PIP_3 interacts with the pleckstrin homology domain of AKT, and as a consequence AKT is translocated from the cytoplasm to the inner leaflet of the plasma membrane[119].

AKT (or protein kinase B) is a Ser/Thr kinase that has three isoforms, AKT1, AKT2 and AKT3. It is activated by ligand-stimulated growth-factor-receptor signaling in a PI3K-dependent manner. AKT is thereby phosphorylated at threonine 308 and serine 473 by two kinases localized at the inner surface of the

plasma membrane, 3-phosphoinositide-dependent protein kinase-1 (PDK-1) and the still elusive 3-phosphoinositide-dependent protein kinase-2 (PDK-2)[113]. Thr308 phosporylation by PDK-1 is sufficient for AKT activation[120], however, maximal activation of AKT requires additional Ser473 phosphorylation[121]. The lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) negatively regulates AKT by dephosphorylating PIP_3 at the 3-position[122]. PTEN, traditionally considered a strictly cytoplasmic protein, also shuttles to the nuclear compartment, where its role is still elusive[123].

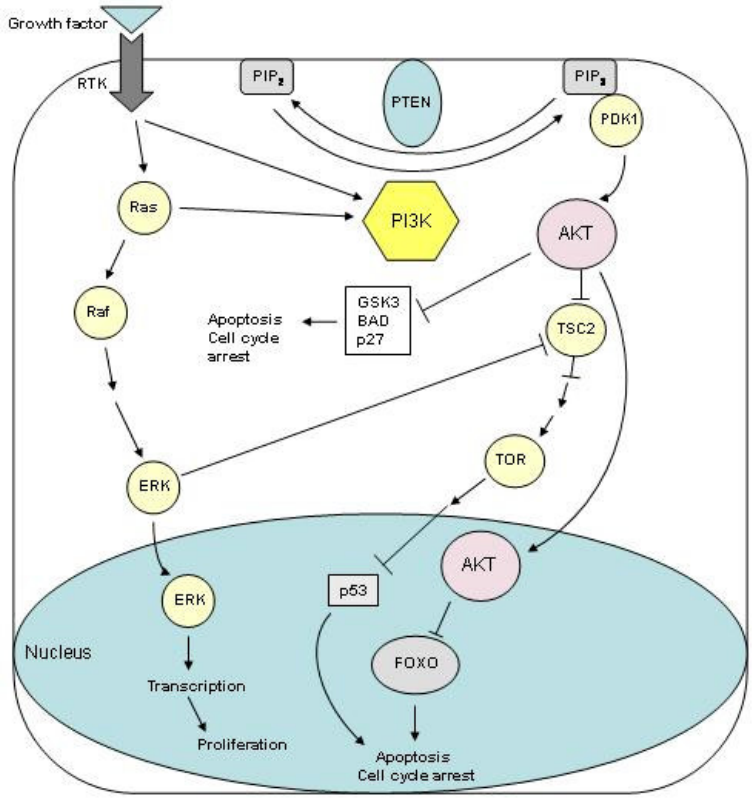

Figure 4. A core scheme of the PI3K-AKT signaling network. After Cully et al.[114].

PI3K-AKT deregulation in cancer

Deregulation of the PI3K-AKT pathway from one of a variety of causes has been reported in large number of cancers[124,125]. Constitutive activation of PI3K resulting from *PIK3CA* mutations has been found in a proportion of cancers of the colon, brain, stomach, breast and lung[117,126] and from mutation in the gene coding for p85α in some colonic and ovarian carcinomas[127]. Further, *PIK3CA* amplification

has been reported in cancers of the ovaries, uterine cervix, head and neck, stomach and brain[128-132].

Germline mutations in *PTEN* are present in two rare autosomal dominant syndromes with the common feature of the development of hamartomas in various organs and increased cancer risk, namely Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome[122]. Somatic point mutations in *PTEN* occur in many sporadic tumors in which the other copy of the gene is mostly inactivated by deletions, for instance glioblastoma, melanoma and carcinomas of the breast, prostate, endometrium, kidney and colon[133-140]. The germline mutations associated with the syndromes and the somatic mutations in tumors are clustered in exon 5 encoding the catalytic domain of PTEN[139]. *PTEN* point mutations usually result in inactivation of the PTEN-protein[141]. PTEN down-regulation may also result from promoter hypermethylation, as reported for endometrial and colorectal cancers[142-144].

Some cancers and cancer cell lines of the stomach, brain, ovaries, pancreas and breast display *AKT* amplification[132,145-147]. Furthermore, overexpression of AKT has been reported in colorectal and hepatocellular carcinomas[148,149].

Upstream changes that activate PI3K occur in some tumors. One example is overexpression of the receptor tyrosine kinase gene *ERBB2* (*HER2/NEU*) in breast cancer, which results in constitutive activation of PI3K-AKT and may contribute to tumor aggressiveness by enhancing cell survival[150,151].

Important substrates of the AKT kinase are $p27^{KIP1}$, the forkhead box transcription factors of the FOXO class, glycogen synthase kinase 3, serum-and glucocorticoid-induced kinase 1 and tuberous sclerosis complex 2 (TSC2)[114]. Crosstalk between the PI3K-AKT pathway and other signaling pathways can contribute to tumorigenesis through synergistic effects, in particular the Ras pathway and the p53 pathway[114] (Figure 4). Phosphorylation of TSC2 by AKT results in the activation of TOR (target of rapamycin)[114]. The carcinogenesis-driving effects of TOR remain elusive[114]. TOR can form two different complexes, one of which, the rapamycin-insensitive TOR complex, can also phosphorylate and activate AKT[114].

Recent reports have introduced additional mechanisms of PI3K-AKT activation which need further elucidation. Several viral proteins can interfere with

the PI3K-AKT pathway, and it is of special note that HPV E7 have been shown to amplify AKT signaling by inhibition of AKT dephosphorylation[152,153].

Effects of PI3K-AKT deregulation

Deregulation of the PI3K-AKT pathway may stimulate several alterations in cell physiology that are hallmarks of cancer development, i.e. evasion of apoptosis, limitless cell proliferation, sustained angiogenesis, invasion and metastasis[36]. PI3K-AKT also affects other cellular changes that may promote malignant development like epithelial-mesenchymal transition, cell matrix interactions and anoikis resistance.

Activation of PI3K-AKT protects cells from apoptosis by coordinating programs that directly inhibit apoptotic effectors and suppress transcription of pro-apoptotic genes, as recently reviewed[154]. AKT is the principal mediator by preventing the release of cytochrome c from the mitochondria, and by inactivation of the pro-apoptotic factor BAD and the cell death effector Caspase 9. Also, by repressing the activity of the FOXO class transcription factors and activating IκB kinase, AKT blocks activation of several pro-apoptotic proteins and brings about nuclear translocation of the pro-survival transcription factor NF-κB,[118]. AKT can affect the pro-apoptotic tumor suppressor p53 by phosphorylation of MDM-2, which speeds up translocation of MDM-2 to the nucleus, thus increasing p53 degradation[154].

Epithelial cells die in an apoptotic process called anoikis if they lose contact with the matrix[155]. Anoikis resistance is fundamental in malignant transformation of epithelial cells[156], and has been demonstrated to be brought about by PI3K-AKT mediated caspase suppression induced by expression of the TrkB tyrosine kinase receptor[157].

AKT stimulates cell cycle progression at the G1/S transition by aiding in activation of cyclin dependent kinases (CDKs). Firstly, it blocks the degradation of Cyclin D1 through inactivation of its regulating kinase, glycogen synthase kinase-3β[158]. Furthermore, AKT indirectly restrains expression of CDK inhibitors, such as $p27^{KIP1}$ and $p21^{CIP1}$ [125,159].

Development of new vascular channels is necessary to nourish a growing number of tumor cells. The PI3K-AKT pathway plays an essential role in the induction of angiogenic cytokine release (most notably vascular endothelial growth factor and interleukin 8) as a response to hypoxia and growth factor stimulation[160-164]. Furthermore, the PI3K-AKT pathway is central in the endothelial cell response as it mediates endothelial cell survival and migration through several effectors[165-167]. It may be of relevance in this context that colposcopic evaluation of the cervix is based partly on the visualization of vascular changes in the mucosa.

Activating mutations of PI3K have been shown to boost cancer cell invasivenes[117]. PI3K-AKT has a central role in the interaction between neoplastic cells and extracellular matrix (ECM) during tumor invasion. It regulates both the urokinase plasminogen activator-1 which degrades plasminogen in the ECM and matrix metalloproteinase 9 that degrades collagen IV[168-170], and through mTOR it plays a central role in upregulation and activation of matrix metalloproteinase 2[171].

Invasion and metastasis seem to be dependent on the acquisition by the neoplastic cell of mesenchymal, fibroblast-like properties with reduced intercellular adhesion and increased motility, a process called epithelial-mesenchymal transition (EMT)[172]. PI3K-AKT has been demonstrated to induce EMT in various cancer cell types, including HPV-infected cervical cancer cell lines[173,174].

Integrins are the major family of cell surface receptors that mediate attachment to the ECM, an essential process for cancer cell motility. PI3K-AKT regulates integrin-dependent cell motility by modulating integrin responses[175]. In addition PI3K may regulate cell movement through modulation of a variety of other effectors controlling formation and extension of cellular actin protrusions (i.e. lamellipodia and filopodia)[176,177] .

Even if deregulation of the PI3K-AKT pathway usually seems to promote cancer development, paradoxical effects of AKT have been reported. It appears that hyperactivation of AKT in some instances may suppress cell motility and invasion. Thus, in one report breast cancer cell migration and invasion was shown to be blocked by AKT activation[178]. In addition, overexpression of activated myr-Akt1 in human breast cancer cells has recently been found to reduce motility and invasion[179] . Furthermore, the three AKT isoforms do not always seem to share the same cellular functions, as evidence for AKT isoform-specificity in regulating

growth-factor stimulated phenotypes in breast epithelial cells has been provided[180]. In that study AKT1 downregulation seemed to promote EMT and cell migration, while AKT2 downregulation reversed hyperproliferation and anti-apoptotic activities[180].

Host factors

As high-risk HPV infections give rise to cervical carcinoma only in a small fraction of women and usually after a latency period of 10-15 years, additional host factors are probably necessary for development of the malignant phenotype[45,181,182]. Experimental studies show that neither the individual HPV oncogenes nor their cooperative action are sufficient to convert normal cells into a malignant state[45].

A genetic predisposition in cervical neoplasia was first demonstrated by a study utilizing data from the Swedish Cancer Registry[183]. The host-cell gene alterations responsible for this predisposition are at present under investigation. Single nucleotide polymorphisms in *p53* may play a role, but have not been proved important in the development of human papillomavirus-associated cancer[184-188]. Claims that polymorphisms in the Fas promoter, Fas gene, Fas ligand and the genes coding for matrix metalloproteinase-1 (MMP1) and TNF-α are relevant for genetic predisposition for cervical cancer, need further exploration [105].

Changes in host genes involved in control of HPV oncogene expression would be of special importance[45]. While the existence of such genes is likely, the molecular mechanisms of a host-mediated antagonism of the viral oncoproteins is poorly understood[45]. A negative interference of cyclin-dependent kinase inhibitors with the HPV oncoproteins has been suspected[2]. Blocking of HPV DNA transcription through modifications of the transcription factor AP1 has been demonstrated, and interruption of this effect has been shown to occur during malignant transformation[189]. Host factors may also directly affect the persisting viral DNA, for instance by upregulation of viral gene transcription, modification of the viral promoter region or by amplification of the viral genomes[2].

The immune system is a key player in host control of HPV infections, and a large proportion of infected women clear the infection by immunological mechanisms[2]. Still, low copy number high-risk HPV infections can persist in some women for decades without causing clinically overt lesions[46], as recently also

demonstrated for elderly women[190]. Spontaneous regression of HPV lesions appears to depend on either naturally acquired or iatrogenically related stimulation of HPV type-specific immunity[191]. While the humoral antibody response to HPV particles may be important in preventing infection, the local events surrounding regression of HPV lesions are primarily associated with specific cell-mediated immunity[191]. Malfunction of the cellular immune response following for instance HIV-induced depletion or iatrogenic inhibition of CD4-lymphocyte activity, enhances the progression of HPV-induced cervical lesions to malignancy[192]. T-cells with specificity against E7 seem to participate in the control of HPV, and may be associated with resolution of pre-invasive lesions, while carcinomas do not seem to be eliminated[193,194].

HLA class II-mediated immune response to HPV may control the viral load[195]. It may therefore not be surprising that carriers of certain HLA class II alleles seem to be more prone to HPV infection and cervical carcinoma, while other HLA class II alleles appear to have a protective effect[196-202]. In a recent study patients with HLA*201 who had CIN 2 or 3 caused by HPV types other than 16 had a significantly lower regression rate than other patients[203]. A meta–analysis indicates that a HLA-restricted HPV-specific immune response plays a key role in controlling disease outcome[202].

Cigarette smoking has been associated with an increased risk for cervical neoplasia[204-207]. A recent meta-analysis of 23 epidemiological studies with stratification for age, number of sexual partners, age at first intercourse, oral contraceptive use and parity concluded that current smokers have a relative risk of 1.60 for cervical squamous cell carcinoma as compared to never smokers, while no association between smoking and adenocarcinoma was found[208]. Although high levels of nitroso-compounds derived from tobacco smoke have been detected in the cervical mucus of smokers[209,210], it remains to be settled whether these mutagenic components take part in the development of cervical malignancy[211]. Loss of FHIT has recently been shown to be significantly more common in smokers than non-smokers with cervical cancer, and may represent a molecular target in cigarette smoking-associated cervical carcinogenesis.[212].

Glucocorticoids and progesterone may hypothetically predispose to cervical neoplastic development by activating HPV expression[211]. Thus, indications of a role for oral contraceptives in the causation of cervical neoplasia have been

reported[54,213-216]. A multicentric case-control study concluded that long-term use of oral contraceptives could increase the risk of cervical carcinoma by up to four-fold in women who are positive for cervical HPV DNA[217]. However, findings on the effect of oral contraceptive use are inconsistent between studies of different designs[207] and elevations of the physiologic levels of sex hormones do not seem to contribute to the development of cervical neoplasia[218]. Oral contraceptive use and parity were not associated with increased risk of CIN 3 or cervical cancer in a long-term prospective study[219].

It has been speculated that poor nutritional status and low body weight may be risk factors of cervical cancer, but this could not be confirmed in studies where HPV status was also taken into account[220]. However, as women with high levels of folate are less likely to acquire genital infection with HPV, improved folate status may to some extent protect against cervical cancer[221]. An increased risk of CIN in women with glomerulonephritis, independent of immunosuppressive treatment, has also been reported[222].

Infections other than HPV may be relevant for malignant development. *Chlamydia trachomatis* serotype G has been associated with increased risk of subsequent development of cervical squamous cell carcinoma[223]. Furthermore, Herpes Simplex Virus type 2 (HSV-2) infection may act in conjunction with HPV infection to increase the risk of cervical carcinoma[224].

Cervical intraepithelial neoplasia

Occurrence

The prevalence of the causative viral factor is the key determinant for the prevalence of cervical intraepithelial neoplasia. Accordingly, sexual habits and host factors rendering women susceptible to genital HPV infections, will increase the risk of developing CIN[26,54,60,61,225-229]. In a screened population, the risk of CIN has been demonstrated to increase with time since the previous cervical smear[230,231].

CIN has been reported in 1.4% to 4% of cervical smears in different screening projects[232-237]. The detection rate of CIN 3 has been reported from 0.2% to 2.7%[237,238]. An annual age-adjusted incidence rate of CIN of 233 per 100,000

women has been estimated in a district with a high cervical cancer mortality[239]. Results from screening projects indicate that CIN has the highest prevalence in women between 20 and 40 years of age[237,240,241], and not uncommon in teenagers[236,242]. In Sweden, the estimated age-specific incidence of carcinoma *in situ* reached a maximum of 650 per 100,000 woman-years at the age of 30 years[243]. The prevalence rates for CIN 1 and CIN 2 have been reported to fall with age[236,237,244-247], while higher rates of CIN 3 have been reported in older women[236,237,247].

Course

An established CIN lesion may regress, persist unchanged or progress to a more severe lesion, i.e. a higher grade of CIN or invasive carcinoma. Theoretically, the natural course of CIN may be influenced by removal of tissue for histological examination or treatment. Moreover, given the substantial interobserver variation and low reproducibility of cervical cytologic and histologic interpretation, the course of CIN is problematic to document[248]. The conclusions from a major review of studies dealing with the course of CIN[10] are summarised in table 2. A large cohort study concluded that the majority of untreated CIN 1 regress to normal within 2 years[249]. Both CIN 1 and CIN 2 are more likely to regress than to progress, while CIN 3 seems to regress in about one-third of cases[10,249]. Recently, regression of 28% of biopsy-confirmed CIN 2-3 was reported from a study were 100 women were followed prospectively for 15 weeks[203]. Others have reported regression of 32 and 50% of biopsy-proven CIN 2-3 during follow up for 12 weeks and 12 months, respectively[250,251]. Still, two other studies that followed women with biopsy-proven CIN 3 for about 6 months found regression of 19 and 31% of the lesions, respectively[252,253]. Regression of CIN 3 may be age-dependent, as data from some screening programs indicated that it occurred in 70-80% of younger women and in 40-50% of older women[254,255]. Regression of CIN 2 has been reported as 19% or 27% during 6 months of observation[252,253].

The progression rate from CIN 1 to CIN 3 or worse was in one large study found to be only 1% per year, 5% within 5 years, 10% within 10 years[249], while Östör concluded that it eventually occurred in about 12% of the cases[10]. However, it is debated whether CIN 1 really is a precursor to CIN 2-3[6]. The Bethesda

classification reflects the alternative view that CIN 1 should be regarded as the manifestation of a productive HPV infection, and thus included in LSIL[21]. Holowaty and co-workers found progression of CIN 2 to CIN 3 or worse in 16% within 2 years, in 25% within 5 years and in 32% within 10 years[249]. Östör found that a total of 27% of all CIN 2 progressed to CIN 3 or worse[10].

Lesion grade	*Regress*	*Persist*	*Progress to CIN 3*	*Progress to invasion*
CIN 1	57%	32%	11%	1%
CIN 2	43%	35%	22%	5%
CIN 3	32%	<56%	-	>12%

Table 2. The course of CIN. Data from Östör[10].

The above mentioned host factors that increase the risk for cervical cancer must be expected to also enhance the risk for progression of CIN. The use of biomarkers to predict CIN progression could have an important potential[256]. CIN 1 lesions with diffuse immunohistochemical staining for $p16^{INK4a}$ have been reported to be more likely to progress to CIN 3 than those that were $p16^{INK4a}$ negative[257,258]. Quantitation of increased Ki67 immunostaining has strong prognostic value for progression in early CIN lesions, and are better at predicting CIN 3 in follow-up than are routine and review CIN grades[259-261]. Combined quantitation of Ki67, Rb, CK13, and CK14 gave accurate information about the progression risk of early CIN lesions[262]. Furthermore, Baak and co-workers reported that HSILs with combined negativity or low positivity for p53 and pRb protein in small histologic biopsies were highly likely to persist, contrasting those in which one of these cell cycle regulators was strongly positive[263]. Finally, the

size of the viral load may be relevant for prediction of progression, as a large load of high-risk HPV has been reported to represent an increased risk for progression to CIN 3[264-266].

Screening, diagnosis and treatment

As cervical cancer develops through a recognizable and treatable pre-invasive stage, it should be amenable to screening in agreement with the Wilson Criteria[267]. The conventional approach to cervical cancer screening has been the identification of cells from the cancer or its potential precursors.

The conventional cytological smear

Use of a cytological smear for the diagnosis of cervical pre-invasive and invasive lesions was demonstrated by Babès and extensively documented by Papanicolaou[268-271]. Cytological screening of asymptomatic women was initiated on an experimental basis from the late 1940's, and in Norway in Østfold county in 1959[272-276]. Apart from some population-based test projects, up to 1995 the cytological screening in Norway was based on the initiative of the individual woman or medical practitioner, a practice termed opportunistic screening. A nationwide organized screening program with women being invited for smear tests every third year, was finally introduced in 1995[277,278]. Norwegian and international guidelines for the follow-up of abnormal cervical smears have been presented[279-281]. Sampling is carried out with a wooden spatula in combination with a brush to ensure sampling of the ecto- and endocervix. The sampled cells are smeared onto a glass slide and immediately fixed by spraying with an alcohol-based fixative. The average cervical cytological smear contains between 50,000 and 300,000 cells, and a varying proportion of these may be inflammatory cells and/or erythrocytes[6]. Staining is usually done by the Papanicolaou method.

Limitations and alternative methods

The standard cervical cytological smear test has some known limitations[267]. The sensitivity is low, somewhere in the range of 30-87% , resulting from cell

clumping, admixed blood, frequent inadequate fixation and the fact that only a limited proportion of the collected cells, as little as 5%, are transferred to the slide[6,248,255,282-290]. Discordance between cytological and histological reports may result[291]. Because the specificity of the test varies from 86 to 100% for the same reasons, some overtreatment must be expected[286,287,290,292,293]. Furthermore, interobserver variability is substantial[248].

Laboratory programs for quality assurance and certification of cytotechnicians as well as pathologists, may reduce the false-negative rate[294,295]. In our laboratory, cytotechnicians must pass a national exam or the International Academy of Cytology exam.

In order to increase the sensitivity and specificity of cervical screening, alternative procedures have been advocated[267]. Liquid-based cytology is a new method of preparing cervical samples for cytological examination. The collected cervical cells are suspended in a liquid medium, from where a representative aliquot is deposited as a thin layer of cells on a glass slide. Debris, blood and suboptimal fixation of the cells are avoided, thus reducing the proportion of specimens classified as technically unsatisfactory[267,296,297]. A large meta-analysis of studies comparing conventional cervical smears with liquid-based cytology concluded that use of the latter reduces the number of false-negative test results and the number of unsatisfactory specimens and also that it may decrease the time needed for examination of specimens[298,299]. However, the efficiency of liquid-based cytology is still debated, and a recent review reported no evidence that it reduces the proportion of unsatisfactory slides, or detects more high-grade lesions than conventional cytology[300].

As cytological screening for cervical cancer is limited by moderate sensitivity and low reproducibility, the merit of such programs relies on many rounds of screening throughout adulthood[301]. Testing for high-risk HPV has a better sensitivity and reproducibility, and would theoretically serve as a better primary screening test[301-307]. Such screening should begin at about age 30, 10-15 years after the average age of sexual debut, avoiding the ages of frequent transient HPV infections[301]. To increase the specificity of HPV screening, women with positive tests could be examined by cervical cytological smears, and if cytologically negative, they should be tested for HPV a year or two later to identify persistent infection[301,308]. However, infection with multiple HPV types is

common[58], and type specific persistent high-risk HPV infection as monitored by genotyping has been shown to identify women at increased risk of cervical neoplasia more accurately than a single or repeated presence/absence HPV test[309].

Primary screening by HPV testing has also been suggested as more cost-efficient and easier to implement in developing countries[297]. HPV DNA testing by self-obtained samples has been demonstrated as a feasible approach for cervical cancer screening with sensitivity at about the same level as traditional cytology, and a good alternative for women who refuse to participate in conventional screening[297,310].

While HPV testing is still not widely used in primary screening, it has gained an established role as an alternative or supplement to a repeat smear in the triage of women with ASCUS or LSIL[280,281]. Such adjunctive HPV-testing is scientifically backed up by several studies[266,311-313]. However, even if this practice results in a reduction in the rate of repeat smears, it raises the rates of referral to colposcopy[314]. Nevertheless, it has recently been reported to be cost-effective[313].

HPV can be detected by PCR, often using a general primer-pair[315]. Several commercial systems for HPV testing are also in use. The Hybrid Capture II® Assay (Digene, Gaithersburg, MD, USA) and AMPLICOR® Microwell Plate Detection (Roche Diagnostics, Indianapolis, IN, USA) are based on detection of HPV DNA, while the Pre Tect® HPV-Proofer (NorChip AS, Klokkarstua, Norway) is based on HPV mRNA identification[316]. The Hybrid Capture II® Assay has won wide acceptance[304,317]. Theoretically, detection of E6/E7 mRNA could be a more reliable predictor of cancer risk than the more traditional HPV DNA detection technologies, and several studies have evaluated these systems[318-320]. The Pre Tect® HPV-Proofer has been found more appropriate for cancer risk-evaluation than Hybrid Capture II, however, population based studies are needed for solid evaluation of the predictive values[318].

A variety of different screening systems are still under evaluation. Since conventional cervical screening is labor-intensive and entirely dependent on human diagnostic skills, automated, computerized screening devices have been developed, but they have still not been shown to reduce screening cost, nor to substitute for manual microscopy by a cytotechnician[267,321-325]. Furthermore, screening approaches utilizing spectroscopic quantification of various physical characteristics

or measurements of responses to voltage or light are being tested both on cell samples or directly on the cervix[267].

The impact of screening

Well-organized screening programs have been highly effective in reducing the incidence of cervical squamous cell carcinoma[4,278,326]. In the Nordic countries organized screening programs have had a major impact on the reduction in incidence and mortality of cervical cancer[4,327]. The mass screening program in Østfold county in Norway resulted in a reduction in the incidence and mortality of cervical cancer of 22% and 24%, respectively[276]. Besides, mass cytological screening has shifted the presentation of cervical neoplasia from the clinical to the preclinical stage, with increased detection of pre-invasive lesions[241,328,329]. Whereas in the pre-screening era invasive carcinoma accounted for around 80% of the diagnosed cervical neoplastic lesions, it was reduced to 16-35% after screening [328,330]. An increased cure rate in the screened population and prolonged survival times in those with invasive lesions followed[330-332]. The incidence of cervical squamous cell carcinoma and its mortality in Norway have decreased since the 1970's in parallel with a substantial increase in the use of cervical smears over the period (Figure 5)[333,334] (see also Table 3). Nevertheless, such opportunistic screening tended to result in over-examination of young women and insufficient examination of women over 50 years of age[335,336], a fact that could explain the higher cervical cancer incidence in Norway compared to countries with centrally organized screening[327,337]. Recently, an increase in cervical cancer incidence in younger women in Europe has been reported, and has been explained by changing risk from sexual conduct[4].

As cytological screening is labor- and resource-intensive, and it reportedly results in treatment of many women not destined to develop invasive cancer, the value of screening has been questioned[293,338].

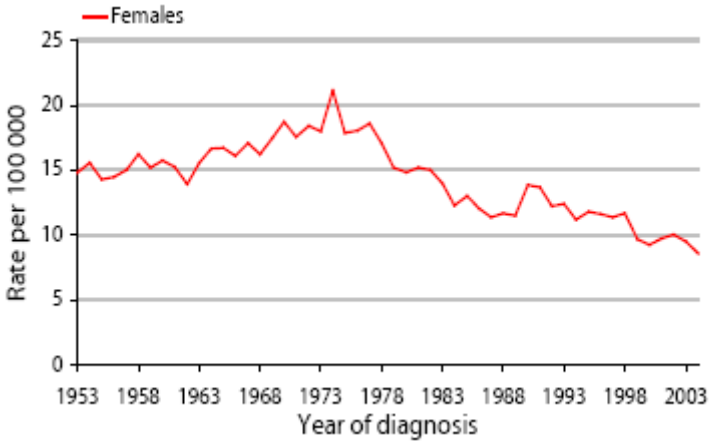

Figure 5. Age-adjusted incidence rates of cervical cancer from 1953 to 2004. From the Cancer Registry of Norway[5].

Year	1962	1972	1982	1992	2002
Smears	115	20600	35900	42000	47000

Table 3. The development in the yearly number of cervical cytological smears analyzed in our laboratory.

Diagnosis and treatment

Colposcopy supplemented with histology is advised following a smear report indicating HSIL or ASC-H and also after persisting LSIL or ASCUS in combination with a positive test for high-risk HPV[29]. The usual approach is to take colposcopically directed punch biopsies, combined with an endocervical curettage when the transformation zone is not visualized[6,13]. However, significant observer variability in colposcopic skill and biopsy placement have been reported, in consequence the biopsy may not in every case represent the true severity of the lesion[248]. Therefore, in cases where cytology suggests a high-grade lesion and the biopsy is less than high-grade, conization may be required to solve the discrepancy[6].

Cervical intraepithelial neoplasia is treated by a cone-shaped excision of the cervix encompassing the transformation zone. Conizations are usually carried out using a laser beam, and may vary in size and extent depending on the anatomy and the woman's desire for fertility[6]. Loop electrosurgical excision procedures may alternatively be used. The transformation zone should be excised with adequate margins of normal endo- and ectocervix to allow pathological examination of the entire area of the cervix at risk[6]. Treatment of CIN by administration of chemopreventive agents such as oral aliretinoin, 4-hydroxyphenylretinamide or beta-carotene, or locally applied all-trans-retinoic acid has not been succesful[250-253]. Cervical cancer is usually treated by radical hysterectomy with pelvic lymph node dissection, sometimes with adjuvant radiotherapy and/or chemotherapy according to the stage of disease, as advised by the International Federation of Gynecology and Obstetrics (FIGO)[339].

Archival pathology

With the development of modern cell and molecular biology, clues to individual physical dispositions and disease can increasingly be detected from nucleic acid molecules in cell and tissue specimens[340]. Huge quantities of specimens are already stored in various types of biobanks, and large, population-based biobanks are in progress[341-344].

Biobanks

Biobanks are systematized collections of human biological material[341,345]. Tissues, cells or other constituents of the human organism from large numbers of persons are stored for a variety of purposes[341]. The biobanks may be classified accordingly. *Diagnostic* biobanks consist of material used diagnostically, for example the tissue – and cell archives of pathology laboratories. *Therapeutic* biobanks are collections of biologic material intended for use in medical treatment, for instance frozen, fertilized ova and donor blood banks. Human biological material used for scientific or developmental studies are kept in *research* biobanks.

Commercial biobanks now also flourish, for the most part being utilized by the pharmaceutical industry and medical diagnostic companies[346].

The pathology archives

The new preparation techniques, including fixation, paraffin embedding and microtome sectioning, together with improved light microscopy were important prerequisites for the cellular pathology that revolutionized medicine in the second part of the 19th century[347]. As formalin-fixed and paraffin-embedded tissue was found to be lasting, diagnostic specimens have routinely been kept in Norwegian pathology archives from around 1930. Specimens from people born in the middle of the 19th century are thus kept in the archives, and assuming short generation gaps, tissue material from up to 5 or 6 generations of a family may thus exist.

On a national basis, the number of specimens in Norwegian pathology archives are formidable. In the year 2000, 340,000 biopsies and 560,000 cytological specimens were examined[348]. Every specimen may consist of several tissue blocks, in some cases more than 50. In the same year, 5000 autopsies were carried out[348]. A recent calculation showed that a total of 20 million paraffin embedded tissue specimens were kept in the 26 Norwegian pathology laboratories[341]. These represent nearly 5.5 million patients over a time span of more than 70 years. When cytological specimens are included, the total number reaches 10 million patients. All age groups are represented, and most women 18 years or older are included. In addition, corresponding files containing the clinical and pathological diagnoses are usually available.

New methods and applications

During the last decade we have seen the application of various molecular biological techniques to the examination of archival specimens. DNA can be retrieved from formalin-fixed, paraffin-embedded tissue blocks, as well as from stained sections and cellular smears. Identification of genes and genetic alterations such as mutation in cancer cells, can be carried out on the basis of the retrieved DNA[349]. Amplification of the DNA segment in focus using polymerase chain reaction (PCR) is the usual approach, yielding substrate for further analyses.

Archival specimens can be examined by standard molecular biologic techniques for genotyping and mutation analyses, such as DNA sequencing, restriction fragment length polymorphism (RFLP) determination, allele-specific amplification, and detection of mRNA using the reverse transcription-polymerase chain reaction (RT-PCR). Quantitation of gene expression or gene copy number can be done using real-time PCR[350,351]. Furthermore, human telomere lengths at the individual cell level may be assessed in sections from formalin-fixed paraffin-embedded tissues[352]. *In situ* hybridization and *in situ* PCR techniques allow the demonstration of specific nucleic acid sequences in their cellular environment[353,354]. The specificity of the molecular analyses can also be enhanced using laser-assisted microdissection of cell groups or single cells[355,356].

On the protein level, the continuous development of new antibodies in combination with modern antigen retrieval techniques have produced a broad selection of antibodies well suited for qualitative immunohistochemical detection of microorganisms and gene products in formalin-fixed, paraffin-embedded tissue[357-360]. Tissue array systems allow for parallel analysis of hundreds of specimens[361].

Using electronic image analysis systems, microanatomic measurements may be carried out on paraffin sections[362]. By deparaffination and proteinase treatment, cells from archival tissue are rendered suitable for suspension and staining with fluorescing dye permitting flow cytometric analyses[363]. Ploidity in malignant tumors may be assessed cytometrically or through image analysis[364-366]. Ultra-structural studies using electron microscopy are well established[367], while high-resolution three-dimensional, computerized pictures may be examined using confocal laser scanning microscopy[368,369].

Genes, proteins, microbiological agents and other substances can now be examined and correlated to morphological changes in cells and tissues from a vast array of diseases. Characterization of microbiological agents is now feasible, as exemplified by *Helicobacter pylori*, polio virus and Spanish influenza virus[370-372].

Little material has been wasted from Norwegian pathology archives. Thus, biomedical examinations of archival specimens from a large part of a stable population with population data at hand, may constitute a potent research tool. However, sensitive data may be revealed, for instance information on venereal disease, deviating parentage or information about inherent disease. Also, revised

pathological diagnoses after renewed examination or special analyses, may be inconvenient to the pathologist. Thus, the use of biobanks for research raises relevant questions about medical, legal and ethical implications for confidentiality, autonomy and community benefits[373-375]. Regulatory provisions attending these issues are now generally being implemented[341,376,377]. As future scientific developments must be expected to increase the value of the archival material, the biobanks should be well kept and not emptied.

AIMS OF THE STUDY

As our understanding of the pathogenetic mechanisms of cervical cancer is rapidly developing and new technology is available, established diagnostic principles and practices need confirmation. The main approaches in this thesis were to examine the characteristics of the traditional principles and practices of cytological diagnostics of cervical neoplasia in Western Norway during the last 20 years, to establish whether the viral factor in cervical neoplasia changed during a time span of three quarters of a century, and to examine the PI3K-AKT pathway in cervical neoplasia.

On this basis the following objectives were formulated:

1. To characterize the compiled pathology database smear histories in women with cervical intraepithelial neoplasia or squamous carcinoma and to assess its relevance to the present diagnosis.
2. To determine the effect of resection margin status, HPV status and p53 expression on lesion-free survival after laser conization for CIN 3.
3. To compare the level of papillomavirus changes in cervical lesions that progressed to CIN 3 to that in lesions that did not progress.
4. To explore the practicability of sequencing DNA from up to 75 years old formalin-fixed, paraffin-embedded tissue and to examine HPV subtype distribution in cervical neoplasia between 1931 and 2004.
5. To determine the status of the PI3K-AKT pathway in cervical neoplastic tissue in order to elucidate some of the biological mechanisms in cervical carcinogenesis.

LIST OF PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

Paper I

The cervical smear record: Its relevance to the subsequent development of cervical neoplasia.

Bertelsen B, Hartveit F.

Acta Obstetricia et Gynecologica Scandinavica 1995; **74**: 729-733.

Paper II

Laser conization of cervical intraepithelial neoplasia grade 3: Free resection margins indicative of lesion-free survival.

Bertelsen B, Tande T, Sandvei R, Hartveit F.

Acta Obstetricia et Gynecologica Scandinavica 1999; **78**: 54-59.

Paper III

Human papillomavirus infection in progressive and non-progressive cervical intraepithelial neoplasia.

Bertelsen B, Kalvenes MB, Hartveit F.

APMIS 1996; **104**: 900-906.

Paper IV

Subtypes of HPV in cervical cancer biopsies between 1930 and 2004: Detection using general primer pair PCR with sequencing of the products.

Bertelsen B, Kugarajh K, Skar R, Laerum OD.

Virchows Archiv 2006; **449**: 141-147.

Paper V

Molecular analysis of the PI3K-AKT pathway in uterine cervical neoplasia: Frequent *PIK3CA* amplification and AKT phosphorylation.

Bertelsen B, Steine S, Sandvei R, Molven A, Laerum OD.

International Journal of Cancer 2006; **118**: 1877-1883.

SUMMARY OF THE PAPERS

Paper I

The previous smear history (1981-88) and short term outcome (up to and including 1992) in all 850 women with a cytological diagnosis of cervical neoplasia in 1989 were assessed. In every case, the cytological and histological diagnoses recorded were read out from the hospital's Norsk Data database and entered manually into a relational database program. The data comprised 6314 cytological specimens and 1824 histological specimens. One half of the women had a previous history of only negative smears, irrespective of the type of lesion present in 1989. About every third woman had a previous diagnosis of CIN, while about every fifth woman lacked previous smears. In 1989, those without previous examination more often than the previously screened women had a diagnosis of invasive cervical squamous cell carcinoma. Moreover, we found that the women's previous smear histories were similar, independent of the current grade of CIN in 1989.

Paper II

Lesion-free survival was assessed in 598 women who had undergone laser conization for cervical intraepithelial neoplasia grade 3. The state of the resection margins was recorded and related to the findings on follow-up, up to 15 years post-operatively. Lesion-free survival times were analyzed by the Kaplan-Meier method. Furthermore, every fifth case was examined for human papillomavirus infection using DNA *in-situ* hybridization with probe cocktails against HPV types 6/11, 16/ 18 and 31/ 33/ 35, respectively . Also, every fifth case was examined for *p53* expression using immunohistochemistry with the monoclonal antibody DO-7 which recognizes epitopes of both the wild-type and mutant human *p53*. Lesion-free survival was significantly more common after complete than incomplete excision of cervical intraepithelial neoplasia. In the latter, lesions tended to appear shortly after surgery, indicating the presence of residual disease. The few lesions appearing later during follow-up were evenly divided between those with and those without complete excision. No association could be demonstrated between lesion-free survival and HPV DNA status or p53 immunoreactivity. We concluded that

the presence of CIN in the cone margin gave strong indication of potential treatment failure. In its absence, laser conization was a highly effective treatment of CIN 3.

Paper III

The presence of HPV was examined in archival material from two groups of women with progressive and non-progressive CIN, respectively. Using morphological criteria for HPV on cervical smears and *in situ* hybridization on formalin-fixed paraffin-embedded tissue no significant difference was found between the two groups. PCR with a general HPV primer pair was carried out on the cases that were negative on *in situ* hybridization. Combined evaluation of the *in situ* hybridization and PCR results showed that HPV was found significantly more often in the cases with lesions progressing to histologically proven CIN 3.

Paper IV

In this paper we studied the practicability of sequencing DNA from formalin-fixed, paraffin-embedded tissue stored for up to 75 years and investigated human papillomavirus subtype distribution in cervical neoplasias from the period 1931 - 2004. Three protocols for DNA retrieval were examined using sequencing of *p53* exons 7 and 8 as test system for cellular DNA quality. Magnetic bead DNA extraction proved advantageous as it gave superior specimen purity and effortless sequencing. Successful sequencing was achieved in more than 70% of the specimens from 1931-60. Mutations in *p53* exon 7 and 8 were uncommon. Next, magnetic bead DNA retrieval was utilized to study viral subtypes using general primer pair PCR with sequencing of the products in a series of 97 cases of neoplastic and non-neoplastic cervical specimens from 1931-1960 and 73 similar cases from 1992-2004. HPV was detected in 61% of neoplastic specimens from 1931-60, and in 89% of those from 1992-2004. In specimens from 1931-34 only HPV type 16 was detected, whereas in the specimens from 1940 and later other HPV subtypes were identified in one third of the cases. The difference was statistically significant and suggests an increase in papillomavirus subtype heterogeneity in Western Norway after 1930. The results demonstrated the utility

of studying cancer genotypes in up to 75 years old formalin-fixed, paraffin-embedded specimens.

Paper V

This work was a molecular analysis of the PI3K-AKT pathway in uterine cervical neoplasia. We studied 46 specimens of formalin-fixed, paraffin-embedded cervical neoplastic tissue. The activation state of the PI3K-AKT pathway was assessed immunohistochemically using an antibody with specificity towards serine-473 phosphorylated AKT. AKT phosphorylation was found in 39 out of 46 examined specimens. In order to examine the possible molecular basis for this activation, we searched for *PIK3CA* amplification using quantitative real-time PCR. *PIK3CA* gene copy number was estimated to be 3 or more in 28 out of 40 successfully examined cases. In addition, a *PTEN* mutation analysis of all nine *PTEN* exons was carried out, but except for one metastasis with a heterozygous mutation in exon 9 (V369I), all cases showed normal *PTEN* sequence. Immunohistochemical staining for PTEN was strong in all lesions. We concluded that an increased activation state of AKT kinase appeared to be present in cervical carcinogenesis, and could be accounted for by *PIK3CA* amplification, whereas *PTEN* mutation seemed to be of little importance.

DISCUSSION

Study material

This work is based on the routine material from Department of Pathology, the Gade Institute, Haukeland University Hospital. The specimens came from general practitioners, gynecologists and hospital gynecological departments. The cytological specimens in paper I, II and III, resulted from the standard opportunistic screening of asymptomatic women (including the follow-up specimens generated), the examinations of symptomatic women and the follow-up of women that had been treated for CIN 3. The histological specimens either resulted from follow-up of cytological findings or the clinical work-up in symptomatic patients. The study material must be expected to be representative of the cervical, diagnostic specimens from this region.

The records of this department have been computerized since 1981. Information on specimens diagnosed before that year was not included in the study. In the Norsk Data system (Norsk Data, Oslo, Norway) used in this department up to 1997, data on specimens and diagnoses were manually entered from the typed specimen reports, while the DocuLive Patologisystem (Siemens Norge, Oslo, Norway) used later included full computerization of reports and records. In the Norsk Data system the cytological diagnoses were recorded using the SNOP (Systematized Nomenclature of Pathology, the College of American Pathologists) code up to 1992, later the SNOMED (Systematized Nomenclature of Medicine, by SNOMED®International, the College of American Pathologists) codes were used. The diagnoses used in paper I-III were retrieved from printouts of the computerized archives where typing or coding errors may have occurred. However, in every case where a recording error was suspected, the original report was checked.

As the specimens have been collected by many different medical practitioners over an extensive time period, the indications for the cyto- and histological examinations may not have been totally consistent throughout the study, and the representativity of the cells or tissues retrieved may have varied.

Likewise, inconstant quality of the pre-laboratory preparational techniques, including fixation, must be expected. Further, as the specimens were processed in a large, routine pathology laboratory, suboptimal laboratory processing (i.e. fixation, embedding, microtome cutting, or staining) of some specimens cannot be excluded, and may have influenced the representativity of the material.

All diagnoses were translated from the dysplasia terminology to the CIN terminology, otherwise all diagnoses of pre-invasive lesions in paper I and III were the original ones set by the pathologists at this laboratory. The value of light-microscopy in diagnostic cytology and histology rests on the observer's interpretation of complex images. Interobserver variation in the analyses of cervical cytological and histological specimens is a common phenomenon[248,378-382]. Thus, a review of all 6314 cytological smears and 1824 histological specimens could have increased the diagnostic consistency of the study material. However, as one of the aims of this study was to characterize the compiled pathology database smear histories in women with CIN, such an approach was not considered to be practical. Also, it may be noted that a similar approach has later been used in the large Canadian cohort study of progression and regression of cervical dysplasia during 1962-1980[249].

Besides, discordance between the cytological and histological evaluation of CIN has been reported[291]. Use of MIB-1 and/or p16 immunohistochemistry can reduce false-negative and false-positive biopsy interpretation and thereby significantly improve cervical pre-cancer diagnosis[256,259,382,383]. Such immunostaining was, however, not in routine use in the diagnostic laboratory in this department during the study period of paper I-III.

In papers I-III, specimens diagnosed as inflammatory or undetermined (i.e. M4000/40000 or M6900/M69000) were recorded as negative for neoplasia. However, it was at that time in such cases standard procedure to advice the clinician to take a repeat specimen. The diagnosis of HPV changes was not in general use during the time period covered by papers I-III. Some cases with HPV may have been interpreted as negative, while others may have been labelled CIN 1, as reported[24].

About 18% of cervical cytological specimens from the region covered were analyzed outside this laboratory in 1989 (personal communication from former head of cytopathology, dr. Elsa Skaarland). In some cases of interest, cervical

specimens may have been sent to other pathology laboratories. Accordingly, there is a possibility that relevant cytological or histological diagnoses in some case may not have been known to us.

Methodological aspects

Immunohistochemistry

Immunohistochemistry is the use of labeled antibodies for detection of antigens in tissue sections. The commonly used indirect method involves an unlabeled primary antibody reacting with tissue antigen and a labeled secondary antibody reacting with several sites on the primary antibody, resulting in signal amplification and increased sensitivity. The peroxidase anti-peroxidase method or the avidin-biotin complex method are commonly used for labeling of the secondary antibody[384,385]. Antigen retrieval by pre-treatment of the tissue with proteolytic enzymes or heating is commonly used to increase the sensitivity of the assay. Suboptimal antibody concentration, antibody denaturation or low antigen density, as well as post-fixation loss of antigen, are common causes of negative results[386]. Low specificity may in some cases result from antibody cross-reactivity, non-specific binding, tissues with endogenous peroxidases or avidity for the avidin-biotin complex, or, notably, entrapment of normal tissues or tissue proteins by tumor cells[386]. Positive controls to test the protocol and negative controls to test for the specificity of the antibody were applied in this study.

A major cause of variation in the reproducibility of immunohistochemical staining is induced by tissue fixation and, to a lesser degree, tissue processing[387]. As we worked with routine, diagnostic biopsies and surgical specimens covering a long time span, some variation in tissue fixation and tissue processing must be expected. Also, unbuffered formalin was used before 1970. The application of laboratory robots, for instance the DakoTechMate™ (Dako, Denmark) that was used in paper V, should increase standardization and reproducibility of the immunohistochemical staining. Quantitative, objective methods may be used to

reduce interpretational errors and improve the reproducibility of immunohistochemical assessments[256].

Immunohistochemistry with the monoclonal mouse antibody DO-7 (Dako) with application of microwave antigen retrieval and the avidin-biotin-peroxidase complex was manually carried out in paper II for examination of p53 expression. DO-7 is a widely used antibody that binds epitopes of wild-type and mutant p53[388-390]. In paper V, immunohistochemistry was applied for assessment of the phosphorylation status of AKT using the polyclonal goat anti-phospho-Akt antibody #9277 (Cell Signaling Technology, USA), applying the DakoChemMate™ avidin-biotin kit with peroxidase/DAB (Dako). Phosphorylation state-specific antibodies have been reported to raise some interpretive problems as the staining patterns may be complex[391], and, accordingly, there are no standard systems for scoring of P-AKT staining[392]. Therefore, we developed a system based on recent publications[392-394]. Also, due to a report of rapid dephosphorylation of phosphoproteins in human tumor specimens, it has been emphazised that caution should be applied in the interpretation of phosphorylation state[395].

In situ hybridization

In situ hybridization (ISH) is a technique for visualization/localization of specific nucleic acids in tissue sections by the use of a labeled nucleic acid probe with base sequence complementary to the sought DNA or RNA, thus enabling hybridization between probe and target after a denaturating step[396,397]. The tissue sections may be treated with detergent or proteinases such as proteinase K in order to permeabilize the membranes. The probe must have sufficient access into the cells so that it can bind to the target. The degree to which the cells are permeabilized affects the degree of specificity of ISH. Cloned RNA or DNA, or synthetic oligonucleotides are used as probes[386]. They can be labeled directly, or indirectly, with a specific antibody or a labeled binding protein used to detect another molecule that is attached to the probe sequence. Labeling can be done with radioactive substances, but peroxidase, biotin or digoxigenin is more commonly used today[386,398,399]. Digoxigenin labelled probes have been found more sensitive than biotinylated probes[400]. The probe design and the temperature, pH, and salt

concentration of the buffer solution will control the degree of specificity of probe to target hybridization. "High stringency" conditions will only allow hybridization of probes with very similar homology to the target sequence, while "low stringency" conditions will allow a probe to bind with less specificity. ISH has been widely used to identify HPV DNA in tissue sections, and signal amplification systems have been developed[401-403].

In situ hybridization was used in paper II and III for studying the presence of HPV DNA in tissue sections. In paper II, we applied three commercial cDNA HPV probe sets reacting with HPV types 6/11, 16/18 and 31, 33, 35, respectively, and hybridization kit (ViraType® and Digene Tissue Hybridization Kit, both Digene, USA), while in paper III, a commercial pan cDNA HPV probe and hybridization kit (Omniprobe™ and Digene Tissue Hybridization Kit, both from Digene) covering HPV types 6, 11, 16, 18, 31, 33, 35, 42, 43, 44, 45, 51, 52 and 56. Thus, in paper III, the non-carcinogenic types 6, 11, 42, 43 and 44 were covered, while some of the types now considered carcinogenic, i.e. 39, 58, 59, 68, 73, 82 and probably carcinogenic, i.e. 26, 53 and 66, were missing[43]. The exact stringency of these commercial systems have not been given by the manufacturer, but has been reported to be low[404,405]. The sensitivity has been found similar to radioactive *in situ* hybridization, filter-, dot blot-, and Southern blot hybridization[404,406]. A detection limit of about 20 viral DNA copies per cell has been reported[407].

DNA retrieval from formalin fixed tissue

Effective retrieval of genomic DNA is needed for gene analysis of formalin fixed, paraffin embedded tissue samples[408,409]. Formaldehyde reacts with DNA through interaction with hydrogen bonds, induces protein crossbindings, and hydroxymethylation of the nucleic acids.[410]. Removal of paraffin wax using organic solvent, followed by enzymatic protein digestion, with or without purification by phenol/chloroform extraction has been the conventional approach[411-413]. In this study, genomic DNA was extracted from formalin-fixed paraffin-embedded tissue samples in paper III, IV and V. Several methods for DNA retrieval were used. In paper III, it was done by an easy and fast method

applying xylene dewaxing, followed by tissue- and macromolecule decomposition by boiling for 7 minutes[414]. This method yielded DNA of a quality sufficient for successful PCR using the GP5+/6+ primer pair. In paper IV, we tested three different procedures for DNA isolation from old formalin-fixed, paraffin-embedded tissue. Two of these procedures included conventional xylene deparaffinization, followed by proteinase K digestion, overnight in one procedure, while for six days in combination with a chelating agent and detergent in the other[411,412]. Purification by phenol/chloroform extraction was not included, as inhibition of the *Taq* polymerase by carry-over phenol has been reported[415,416]. Both procedures yielded low purity DNA. However, in both cases it could be used for DNA sequencing. In the third procedure, the GenoPrep™ DNA from Tissue Kit was run on a GenoM™-48 robotic workstation (both from QIAGEN, Germany). This system, which was also used in paper V, is based on heat pre-treatment for dewaxing, thus avoiding the use of organic solvent, followed by proteinase K digestion and DNA extraction using magnetic beads in the presence of chaotropic salts. The positively charged magnetic beads will attract the negatively charged DNA. This procedure rendered high-purity genomic DNA. The time-effectiveness of the procedure makes up, at least in part, for the expensive equipment and reagents. It therefore became the selected method when the isolated DNA was to be used for sequencing or quantitative real-time PCR (papers IV and V).

PCR

PCR is a powerful technique used to make a large number of copies of a target DNA sequence, thereby producing substrate for further analyses[417]. The principle is automated heating and cooling for 30-45 cycles of denaturation of the nucleic acids (at about 94°C), annealing between templates and primers (at about 50-55°C), and extension (at 72°C) by the activity of a heat-stable DNA polymerase, providing an exponential increase of the number of copies of the target DNA sequence. Primer annealing is the rate limiting process in PCR[418]. When working with template DNA from formalin-fixed, paraffin-embedded tissue, where DNA is degraded into shorter segments, PCR products should be optimized to a length less than 200 basepairs, even if successful amplification of longer products have been

reported[410,419]. PCR was used in paper III for studying the presence of HPV DNA using the general primer pair GP5+/6+. Detection of a 150 basepair long PCR product on agarose gel electrophoresis served as identification of HPV. This primer set has been reported to amplify HPV 6, 11, 13, 16, 18, 30, 31, 32, 33, 35, 39, 40, 43, 45, 51, 52, 54, 55,56, 58, 59, 66, thus missing the carcinogenic types 68, 73 and 82, and the probably carcinogenic types 26, 53 and 66[43,315]. Use of multiple PCR primer sets would, of course, have optimized the sensitivity of viral detection[420]. The primer pair GP5+/6+ was also used in paper IV. However, here a biotinylated GP5+ primer was applied for easy purification of the PCR product using streptavidin-coated magnetic beads, followed by sequencing of the PCR products to identify HPV subtypes. Even if the GP5+/6+ primer pair is widely used in HPV research and diagnostics, it may not be optimal, as it recently has been reported that PCR primers that target the L1 region, like GP5+/6+, may miss some cases of advanced disease. The reason is that the LI regions sometimes are lost during integration of viral DNA into the host genome[421]. Also, in paper IV PCR was used in the sequence analysis of *p53* exons 7 and 8 with product sizes of 171 and 230 basepairs, respectively. In paper V, analysis of *PTEN* was done using 11 PCR product to cover the 9 exons, with PCR product lengths between 147 and 265 basepairs.

The great sensitivity of the PCR method renders the process of sample collection, sample processing and amplification susceptible to contamination by plasmids, DNA from other samples, and PCR products (carry-over). Paraffin blocks were cut using gloves, with clean instruments and fresh microtome-blades. The preparation of the PCR reaction mix, the addition of specimen to the mix, the amplification, and the analysis of the PCR products were carried out in separate rooms using dedicated pipettes with filter plugged tips. Negative controls were run with each amplification, and were at no time positive during this study.

Sequencing of DNA

The dideoxy method is the commonly used method for determination of the nucleotide sequence of a DNA fragment[422]. This method was used in paper IV for the *p53* analysis and HPV subtype identification, and in paper V for the *PTEN* mutation analysis. Even if DNA sequencing is a standard method for mutation

analysis, it may be hampered by some problems. False positive results may occur as a result of PCR-induced changes in the base sequence. Error rates of thermostable DNA polymerases have been reported between 2.1 x 10^{-4} to 1.6 x 10^{-6} misincorporations per nucleotide per extension[423,424]. However, to overcome the possibility of PCR-induced sequence alterations, all mutations detected in this study were verified by repeated PCR amplification and sequencing from tumor DNA. False negative findings may result if DNA is extracted from specimens where the neoplastic cells make up only a minor fraction of the cells, with non-neoplastic, i.e. stromal and inflammatory, cells dominating. To overcome this problem, in paper V, tumor tissue was dissected manually from the specimens under microscopic guidance. Use of laser-assisted microdissection of tumor tissue could probably further have increased the cellular specificity of this assay[355,356].

Quantitative real-time PCR

Quantitation of gene dose or gene expression can be done using real-time analysis of the polymerase chain reaction[425]. The principle is that a fluorescing signal is released from the products of every PCR cycle, with the signal intensity being proportional to the quantity of accumulated PCR product[426,427]. The cycle number at which the reporter dye emission intensity rises significantly above background noise is called the threshold cycle (C_T) (Figure 6). The higher the template concentration, the lower the measured C_T. The C_T is determined at the exponential phase of the reaction, where none of the reaction components are limiting[355]. Real-time measurement is therefore more robust and dependable than conventional quantitative PCR methods using end-point measurements, where only a slight variation in limiting reaction component concentrations may markedly affect the quantity of PCR end product, thus resulting in poor precision. The method has successfully been applied to studies of nucleic acids from formalin-fixed, paraffin-embedded material[356]. Quantitation of gene copy number is often done using a standard curve[428]. However, if amplification of target and reference are equally efficient, the comparative C_T method, which does not depend on astandard curve, can be used[429].

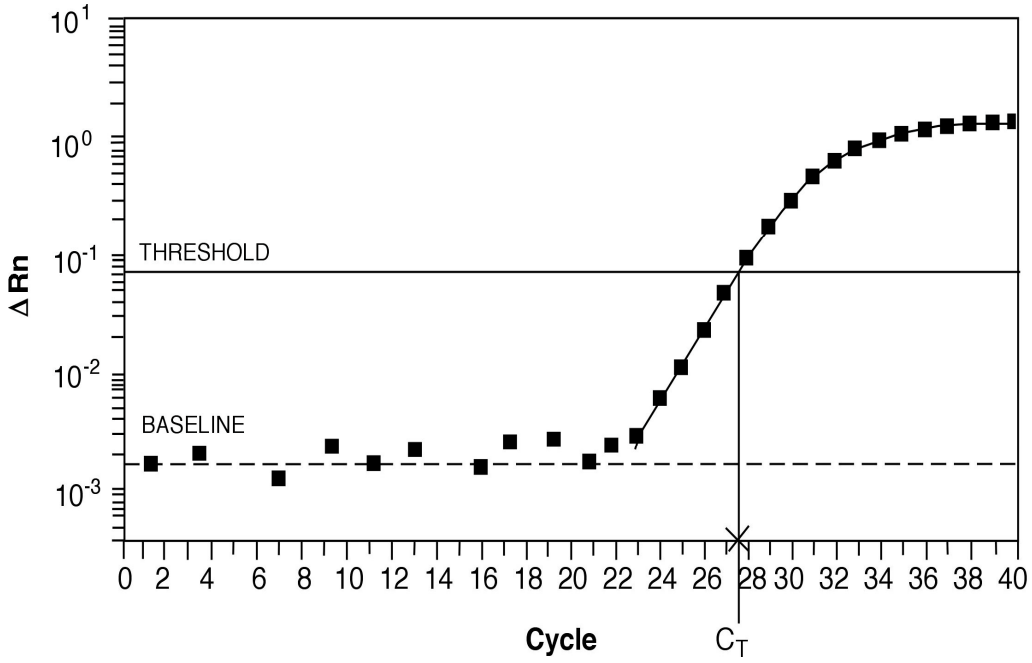

Figure 6. Real-time PCR. C_T is the threshold cycle number at which the reporter dye emission intensity rises significantly above the baseline background noise. The higher the template concentration, the lower the C_T measured. The relative fluorescence, ΔRn, will be proportional to the quantity of PCR-product.

Quantitative real-time PCR was used in paper V for assessment of relative *PIK3CA* gene copy number using the glucokinase gene (*GCK*) as reference. As we did not succeed in designing primer pairs for target and reference gene that worked together in a multiplex reaction, three separate parallels were run for both target and reference gene in every C_T determination assay, with the mean C_T value used in the calculations of gene copy number. A multiplex assay with both PCR reactions run in the same tube would probably have been beneficial, as it would have excluded well-to-well variability, reduced reagent costs, time consumption, saved sample DNA and increased throughput. In particular, when examining slight changes in gene copy number, it is crucial to avoid well-to-well variation in reagent volumes.

Diagnostics and treatment

Diagnostic and therapeutic aspects are dealt with in papers I and II. The focus is on the merits of the screening system and treatment used at the time period

examined. Paper I characterizes the compiled smear histories between 1981 and 1992 of all 850 women that were given a cytological diagnosis of CIN by this laboratory in the index year, 1989. In that year, 54% of the cases were given a diagnosis that would equal to HSIL in the Bethesda classification.
Moreover, the finding that 49% of those HSIL cases had a smear history of only negative specimens during 1981-88, may reflect the common existence of fast-developing pre-malignant lesions[430]. However, it can alternatively be seen as a demonstration of low sensitivity of the cervical cytological smear test, and as such underscoring the need for a more sensitive screening test, possibly a test for high-risk HPV. On the other hand, the finding that nearly 60% of those diagnosed with invasive carcinoma in the index year lacked earlier cervical smears during 1981-88, brings attention to the flaws of the opportunistic screening system used at the time.

Paper II examines the merits of laser conization in 598 women treated for CIN 3, with up to 15 years follow-up. As the follow-up diagnoses used in paper II were those originally given, interobserver variation must be expected. More consistent and solid follow-up status could have resulted from a re-examination of all follow-up diagnoses[248]. Soutter and co-workers recently performed a meta-analysis of the risk of invasive cervical cancer after treatment of CIN, including data from paper II[431]. In paper II, the risk of residual disease after conization was found to be significantly higher in cases where CIN was present in the resection margins, in line with other reports[432]. It is of note that the presence of CIN in the endocervical resection margin later has been reported to be a predictor of HPV persistence after conization[433]. Further, in paper II, the risk of post-conization CIN was found to fall with time, in keeping with the conclusions of Soutter et al.[431]. However, as post-conization CIN sometimes occurred late, there is reason to recommend annual cytological smears for 10 years after conization, in agreement with Soutter et al.[431]. Also, in paper II, HPV status and expression of p53 in the conization specimens were correlated to occurrence of residual or recurrent disease. Cervical carcinomas with mutations in *p53* and no or low papillomavirus load had at that time recently been reported[434]. However, in view of the later knowledge of a virtual non-existence of HPV-negative cervical neoplasia and p53 generally being abrogated by the E6 oncoprotein[43,46], the possibility that p53

expression or HPV status in the cone specimen could be potential prognostic factors after treatment for CIN seems to be of little concern.

Mechanisms of cervical neoplasia

The biological mechanisms of cervical neoplasia are the focus of papers III-V. In paper III the presence of HPV infection was examined by morphology, *in situ* hybridization and general primer PCR in CIN that progressed to grade 3 and in CIN that did not reach grade 3. As the cases studied here were not randomly picked, but selected according to a set of given criteria, the representativity of the two groups may be questioned. Reservations as to inconsistency and reproducibility of CIN grades apply, as the diagnoses used in this paper were those orginally given in the diagnostic routine. Also, it must be remembered that the histological diagnosis of CIN3 results from the fact that a histological specimen was made on the basis of a clinical decision and the smear history, and may therefore not necessarily be a valid reflection of the natural course of CIN. Besides, in the non-progressive cases, tissue for HPV examination by in situ hybridization and PCR was available in only 38 cases out of 133. There is thus reason to question the representativity of these 38 cases. Further, the examination of material scraped off from cervical smears using general primer PCR in cases lacking tissue, could have been a relevant option. Subtyping of HPV would also have been of interest here. It could have been done using the ViraType® (Digene) probe set instead of the Omniprobe™ (Digene). However, as DNA sequencing was not established in this laboratory at that time, HPV subtyping by GP5+/6+ PCR product sequencing was not easy to implement. Many cases that had progressed to CIN 3 were HPV DNA negative by *in situ* hybridization, but positive on general primer PCR. In view of the greater sensitivity of PCR, this could be seen as a demonstration of low viral load in some cases of CIN 3. HPV was detected on PCR in 69% of the CIN 3 cases tested, in line with an earlier study using type specific PCR[435].

In paper IV, archival cervical cancer specimens dating back to 1931 were examined for presence of HPV. Different methods for retrieval of genomic DNA from formalin-fixed, paraffin-embedded tissue were tested, and a commercial system utilizing magnetic beads was found more efficient than the conventional techniques. As the tissue archives of this department are vast, the establishment of an effective method for examination of old DNA opens for further studies of genetic variation in disease. The HPV findings demonstrate that, even if the distribution of genital HPV infections is closely related to the human factor, and cervical cancer incidence has been changing over time, HPV infection as a causative factor in cervical cancer has been persistent for at least 75 years. Furthermore, the indication of increasing viral subtype heterogeneity during the period studied is of interest, as geographic variation in HPV subtype distribution is well known[43]. As the increasing heterogeneity was detected in cancer specimens from 1940 and onwards, the change in HPV subtype prevalence must have occurred at a minimum of about 15 years earlier, i.e. in the mid 1920's.

The findings reported in paper V of an activated AKT kinase, probably caused by low-level amplification of the potential oncogene *PIK3CA*, add to the known inactivation of tumor suppressors p53 and pRb caused by HPV oncoproteins in cervical carcinogenesis. In general, amplification represents a major molecular pathway by which gene expression can be constitutively enhanced above the level of physiologically normal variation[436]. Therefore, amplification can confer a selective advantage to cells under stress, and is considered important in carcinogenesis, as well as in development of chemoresistance[437,438]. Amplification may be a manifestation of genetic instability, which is present in most cancers[438,439]. Genetic instability, as earlier discussed, is induced early in cervical carcinogenesis by the expression of viral oncoproteins E6 and E7[96,102]. It must be noted that most cancers show a multiplicity of genetic changes or mutations, the great majority of which probably have no significant effect on tumor growth or progression[440]. Functional analyses are needed to distinguish noncausative "passenger" mutations from those with true roles in carcinogenesis[441]. In colon cancer, experimental studies have demonstrated that the most frequently observed *PIK3CA* mutations are functionally overactive, and thus likely to be involved in carcinogenesis[117,126,442]. Amplification must be expected to yield too much of the normal gene product, which is reflected in this

study by the detection of an increased phosphorylation status of AKT. However, it would be of interest to assess the PI3K expression in cervical cancers. As the PI3K-AKT pathway serves a fundamental role in connecting growth signals from outside the cell with various parts of the cell's machinery for proliferation and anti-apoptosis, the activation of this pathway in cervical cancer must be expected to have a major impact on tumor growth and progression. It may well prove to be a necessary second hit in cervical carcinogenesis after integration of HPV DNA[45,181].

Perspectives on prevention and therapy

Effective immunization of women against HPV must be expected to reduce the incidence of cervical cancer[443-445]. However, as HPV subtype distribution varies with geographic localization[43,44] and possibly with time, as indicated in paper IV, continuous global monitoring of HPV subtype prevalence may be required in order to maintain potent vaccines.

As demonstrated by the successful use of kinase inhibitors in cases of chronic myelogenous leukemia with BCR-ABL kinase, gastroeintestinal stromal tumors displaying mutations in the *c-KIT* gene, breast cancers with *HER2* gene amplification and non-small cell lung cancers with mutated EGFR receptor tyrosine kinase, mutated kinases are ideal drug targets[446]. Targeting PI3K-AKT has been considered a rational therapeutic approach in cancers with deregulation of this pathway[118,144,447,448]. Hypothetically, restriction of tumor cell survival, proliferation, angiogenesis, invasion and metastasis would be anticipated. Besides, as the p53 apoptotic response requires downregulation of the PI3K-AKT pathway through the transcriptional activation of *PTEN*, it has been speculated that simultaneous inhibition of PI3K-AKT and activation of apoptosis downstream of p53 might have synergistic effects that could be utilized therapeutically in tumors that carry p53 mutations[114].

The presence of cancer-specific mutations and amplifications in *PIK3CA* that activate PI3K-AKT signaling make the p110α catalytic subunit of PI3K a promising drug target[118]. Several PI3K inhibitors exist, most notably wortmannin, demethoxyviridin and LY294002[449,450]. Even if wortmannin and LY294002 have

been shown to induce growth inhibition in several model cancers, they have been regarded as unsuited for clinical use due to toxicity and low specificities and potencies[144,449-454]. PI3K inhibitors used as a supplement to radiotherapy or cytotoxic agents may be a future treatment option in cervical cancer. This is emphasized by a recent study where increased expression of phosphorylated AKT in cervical cancer specimens was demonstrated to be associated with radiation resistance of the tumor[455]. Further, inhibition of PI3K by LY294002 has been shown to radiosensitize cervical cancer cell lines[456]. Interestingly, LY294002 has been demonstrated to block export of doxorubicin from drug-resistant colon cancer cells, resulting in massive cancer cell apoptosis[457]. The field is constantly developing, and selective and potent PI3K inhibitors must be expected in the future. They may show clinical utility alone or in combination with conventional chemotherapy.

Targeting PDK1, AKT or effectors downstream of AKT is also a promising therapeutic strategy[114,458]. The mTOR inhibitor rapamycin reportedly restrains metastatic tumor growth and angiogenesis in tissue culture and mouse models[144,459]. The effectiveness of various rapamycin derivatives are currently being tested in breast, renal, non-small cell lung, cervical and uterine carcinomas, as well as anaplastic astrocytomas, mesotheliomas and soft tissue sarcomas[122,458].

As activated AKT in experimental systems has been demonstrated to promote drug resistance by restraining apoptosis, targeting PI3K-AKT in AKT expressing tumors to restore cancer drug sensitivity may be a fruitful strategy[460,461]. There are already indications that mTOR inhibitors may be effective in overcoming resistance to cytotoxic agents in a variety of malignancies, including carcinomas[458,461]. However, the recently described paradoxical effects of AKT (see Background) must be taken into account when designing AKT inhibitory drugs[178-180].

Because cervical cancer growth is dependent on continuous expression of E6 and E7, anti-HPV gene therapy could have a therapeutic potential[462]. *In vitro* studies have indicated that inhibition of E6 and E7 gene expression by antisense RNA, ribozymes or siRNA, results in loss of the transformed phenotype[462]. However, in lack of an effective delivery system for anti-HPV gene therapy, clinical trials are still awaited. Nevertheless, substances specifically targeting

PI3K-AKT used in combination with anti-HPV gene therapy, may well prove to be the future adjuvant treatment modality of choice in cervical cancer.

CONCLUSIONS

Based on this work, the following conclusions may be drawn:

1. In cases with CIN diagnosed in the index year, the pathology database smear record showed no previous smear in nearly 20%, only negative smears in about half of the cases, CIN 1 in about 15%, CIN 2 in about 10%, and a diagnosis of CIN 3 or carcinoma in less than 10%. This distribution was relatively constant, irrespective of the grade of lesion present in the index year. Invasive lesions were significantly more common in the unscreened women than in those that had been screened.
2. In cervical laser conization for CIN 3, resection margin status is an important determinant of long term outcome. The presence of CIN in the resection margins is a strong indicator of potential treatment failure. Analyses for HPV status and p53 expression in the cone specimen did not add predictive information on treatment outcome.
3. The levels of papillomavirus changes cytologically, histologically and on *in situ* hybridization were the same in non-progressive and progressive pre-invasive lesions. However, detection of HPV by general primer-mediated PCR was more common in women with lesions progressing to CIN 3 than in those with non-progressive lesions.
4. DNA could be retrieved and sequenced from up to 75 years old formalin-fixed, paraffin-embedded tissues. An automated DNA extraction method based on magnetic beads was superior to the proteinase-K based techniques. HPV was present in the majority of old and new cervical cancer specimens. However, only type 16 was detected in the oldest specimens.
5. The AKT kinase is activated in cervical cancer. The activation may be accounted for by *PIK3CA* amplification, whereas *PTEN* mutations appear less important.

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Errata

The following typographical and grammatical corrections have been made after submission of the thesis:

Page 10, line 5 of the third paragraph : carcinogenesis, changed to carcinogenesis .

Page 17, line 1 of the fifth paragraph : p27/KIP1 changed to $p27^{KIP1}$.

Page 18, line 5 of the last paragraph: p27/KIP1 changed to $p27^{KIP1}$ and p21/WAF1/CIP1 changed to $p21^{CIP1}$.

Page 20, line 6 of the third paragraph: FAS promoter changed to Fas promoter.

Page 35, line 1 of the third pararaph: conisation changed to conization.

Page 35, line 4 of the fifth paragraph: in press changed to **449**: 141-147.

Page 35, last line: *2006; **118**: 1877-1883* changed to 2006; **118**: 1877-1883.