Hypoxia and Cell Cycle deregulation in Endometrial Carcinogenesis

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Hypoxia and Cell Cycle deregulation in Endometrial Carcinogenesis

De rol van hypoxie en ontregeling van de cel cyclus bij het ontstaan van het endometriumcarcinoom
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. W.H. Gispen, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 13 september 2007 des middags te 4.15 uur.

door

Nicole Horrée

geboren op 10 april 1980 te Arnhem

Promotores

Prof. dr. A.P.M. Heintz
Prof. dr. P.J. van Diest

Contents

Abbreviations

AKT	protein kinase B
AMPK	5′-adenosine monophosphate-activated protein kinase
ARNT	aryl hydrocarbon receptor nuclear translocator
BLAST	basic local alignment search tool
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
CAIX	carbonic anhydrase IX
CBP	CREB binding protein
CCNE	cyclin E gene
CDK	cyclin-dependent kinase
CKI	cyclin-dependent kinase inhibitor
CSA	catalyzed signal amplification
CTNNB1	beta-catenin
DAB	3,3′diaminobenzidine tetrahydrochloride
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide-triphosphate
EC	endometrial cancer
EDTA	ethylenediamine tetraacetic acid
EGLN	egg-laying defective nine
EH	endometrial hyperplasia
EIN	endometrial intraepithelial neoplasia
EPAS-1	endothelial per-arnt-sim domain protein-11
EPO	erytropoietin
ER	estrogen receptor
ERBB2	HER2/neu; named after avian erythroblastosis oncogene B,
FACS	fluorescent activated cell sorting
FIH	factor inhibiting HIF
FIGO	International Federation of Gynecology and Obstetrics
Glut-1	glucose transporter 1
HEC1B	human endometrial carcinoma cell line 1B
HIF	Hypoxia-Inducible Factor
HNPCC	hereditary nonpolyposis colorectal cancer
HNSCC	head and neck squamous cell carcinoma
HPV	human papilloma virus
HRE	hypoxia-responsive element
IE	inactive endometrium
LhS28	ciliated cell marker
LKB1	serine/threonine-protein kinase 11
Mab	monoclonal antibody
MDM2	mouse double minute 2
MMAC1	mutated in multiple advanced cancers 1
mRNA	messenger ribonucleic acid
MVD	microvascular density
NCBI	National Center for Biotechnology Information
N-TAD	N-terminal transactivation domain

ODDD	oxygen dependent degradation domain
Pab	polyclonal antibody
PAS	PER-aryl hydrocarbon nuclear translocator-SIM
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHD	prolyl 4-hydroxylase
PI(3)K	phosphatidylinositol-3-OH-kinase
PIP	phosphatidylinositol 3,4,5-trisphosphate
PR	progesteron receptor
PTEN	phosphatase and tensin homolog
PV	powervision
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Siah	seven in absentia homolog
Skp2	s-phase kinase-associated protein 2
SNP	single nucleotide polymorphism
SPSS	statistical products and service solution
TM	tubal metaplasia
VEGF	vascular endothelial growth factor
VHL	Von Hippel Lindau tumor suppressor gene
WHO	World Health Organization

chapter 1

General introduction

Because uterine endometrial carcinoma is the most common malignancy of the female genital tract and 1 of every 5 patients dies of this disease, understanding the mechanisms of carcinogenesis and progression of endometrial carcinoma is important. These insights might lead to improved diagnostic tools for the pathologist, improved prediction of prognosis and response to therapy, and eventually better disease-based management of this disease in the individual patient. In this introduction, some key clinicopathological features of endometrial cancer will be outlined first, followed by a discussion of some of this cancer's basic tumor biologic features and, finally, a formulation of the aims of this thesis.

Clinical Aspects of Endometrial Cancer

Epidemiology

Endometrial cancer represents a major health issue. Estimated incidence of cancer in the uterine corpus in the United States was 39,080 cases in 2007 (6% of all new cancers), with an estimated probability of developing uterine cancer of 1 in 40.[1] Uterine cancer rates are highest in North America, intermediate in Europe, temperate in South America, and low in southern and eastern Asia (including Japan) and in most of Africa (except southern Africa).[2] Currently, endometrial adenocarcinoma ranks fourth among the most common cancers in women behind breast, lung, and colon cancer. In the Netherlands, incidence is 1400 per year, with a yearly death rate of 300 women.[3] This relatively low mortality, compared to other cancers is attributed to early presentation with vaginal bleeding. Endometrial carcinoma is mainly a disease of postmenopausal women, although 25% of the cases occur in premenopausal patients with 5% in patients younger than 40 years of age.[4,5]

Etiology

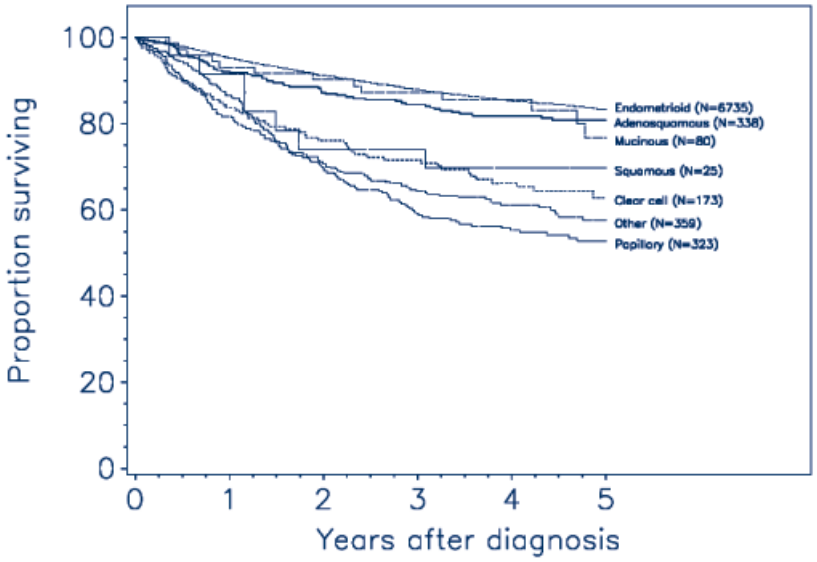
Endometrial cancers are generally classified into two major types: type 1 cancers comprise those with an endometrioid or mucinous histology, and type 2 cancers are usually papillary serous or clear cell cancers. In type 1 cancers, elevated estrogen levels are recognized as a risk factor, which can be due to prolonged unopposed estrogen intake, obesity, early menarche, late menopause, nulliparity, or polycystic ovary syndrome. Furthermore, the antiestrogen tamoxifen, given as adjuvant therapy in many breast cancer patients, also increases the risk of endometrial cancer. Besides these mainly environmental factors, genetic factors can play a major role too in uterine cancer. Hereditary nonpolyposis colorectal cancer (HNPCC), or Lynch syndrome, is an autosomal dominant cancer susceptibility syndrome, in which endometrial cancer is the second most common cancer after colonic carcinoma. For type 2 cancers, hormonal risk factors have not been identified.

Prognosis

Endometrial cancers usually arise in postmenopausal women and cause abnormal (postmenopausal) bleeding. This permits early detection and cure at an early stage. Early detection in combination with effective treatment results in a low mortality for stage 1 (confined to the uterus) disease (5-year survival, 86% to 90%;

see Table 3 in the Endometrioid Endometrial Carcinoma section). However, survival drops in higher stages of disease, resulting in an overall mortality of 20% to 25%.[6] Prognostic factors are used to decide the need for adjuvant treatment and to identify new treatment strategies. They can be clinical, hormonal, cellular, and molecular genetic. The most important prognostic factors are clinicopathologic: stage, tumor grade, histologic subtype, depth of myometrial invasion, presence of lymphovascular invasion, a primary tumor diameter of more than 2 cm, and invasion of the cervical stroma. Several factors have also been associated with prognosis: overexpression of growth factors and receptors, steroid receptor status, and angiogenetic factors. However, only the clinicopathologic data (histologic subtype [see Figure 1], age, grade of disease, and stage) are currently used to determine therapy.[6,7] The inaccuracy of these clinicopathological factors is illustrated by the fact that 1 of every 3 women who die of endometrial cancer presents with presumed localized disease. This results from the failure to recognize sites of occult extrauterine dissemination at the time of primary treatment and underlines the need for more detailed biological information concerning the behavior of this tumor.

Figure 1. *Kaplan-Meier curves showing differences in survival between histologic subtypes of endometrial cancer. (Figure adapted from annual report of the International Federation of Gynecology and Obstetrics [FIGO] 2006, with permission).*[6]



Treatment

Endometrial cancer confined to the uterus is treated by surgical removal of the tumor and, if necessary, followed by radiotherapy in high-risk patients.[7] In higher-stage tumors, lymphadenectomy is deemed necessary by most,[8] although there is still discussion on this matter. On occasion, treatment may be limited to progestogens in women who want to maintain fertility and have well-differentiated and very early–stage disease. Progestogens can also be used as adjuvant therapy in recurrent endometrial cancer, but response rates and progression-free survival intervals vary.[8,9] Chemotherapy is used as a treatment modality in higher stages.[8-10]

Histopathology of the Endometrium

Normal Cyclic Endometrium

The normal premenopausal endometrium undergoes a cyclic pattern of proliferative and secretory phases, controlled by ovarian steroid hormones. Estrogens induce mitosis in the normal endometrium. Proliferation is necessary for outgrowth of glands, supporting stroma and vascular endothelial cells. Cellular proliferation during the phases of the menstrual cycle is a tightly controlled process.[11] Estrogen receptors are upregulated during the follicular phase and decrease after ovulation because of progesterone influences. The progesterone receptors are induced by estrogen and are at maximum number around ovulation. At the end of the follicular phase, the endometrium is composed of long, coiled glands, covered by mucus-producing epithelium (proliferation phase). After ovulation, growth of the endometrium is inhibited by progesterone. In case of failing implantation of an embryo, the lamina functionalis of the endometrium is finally rejected as a result of declining estrogen and progesterone production of the corpus luteum. This phase is the menstruation. The lamina basalis sustains and functions as a base for regrowth of the endometrium during the next cycle.The endometrium is saturated with blood by two types of vessels from the stratum vasculature in the middle of the myometrium. The arteries of the basal layer of the endometrium are not influenced by hormones; however, the coiled arteries in the lamina functionalis develop under the influence of estrogens and progesterones.[12] After menopause, proliferation of the endometrium stops and the endometrium then turns to an inactive, resting state.

Tubal Metaplasia

As a variation of the normal endometrium described previously, tubal metaplasia (increased amount of ciliated and secretory cells, normally found in the fallopian tubes) may be observed.[13] Ciliated cells are supposedly normal constituents of the human endometrium, increasing in number during age.[14] Ciliated cell changes are seen in normal endometrium, hyperplasia, and carcinomas of the endometrium[15,16] and also in the remainder of the gynecological tract. Endometrial tubal metaplasia may be extensive especially in perimenopausal and postmenopausal women. Knowledge concerning cellular biological changes in tubal metaplasia is very scarce.

Precursor Lesion of Endometrioid Endometrial Carcinoma

Endometrial hyperplasia occurs most commonly around menopause or in association with persisting anovulation in younger women. Hyperplasia is associated with endometrioid endometrial carcinoma. For more than 40 years, it has been generally accepted that hyperplasia can progress to a spectrum of atypical changes, eventually leading in some cases to endometrial carcinoma. Therefore, hyperplasia has been seen as a precursor lesion.[17] Furthermore, risk factors for hyperplasia and carcinoma are the same; for example, prolonged or excessive estrogen stimulation with diminution or absence of progestogens. However, hyperplasia comprises a heterogeneous group of lesions, some thought to be reversible and some to be truly neoplastic, so attempts have been made to subclassify these lesions in a biologically and clinically meaningful way.

Histologically, hyperplasia is defined by an abnormal increase in the volume of the endometrium and changes in the shape and arrangement of the glands. The gland–stroma ratio is increased. Based on architectural changes and cellular atypia, 4 types of hyperplasia are distinguished according to the World Health Organization (WHO) nomenclature: simple and complex, with or without atypia. Especially complex hyperplasia with atypia shows an elevated risk to develop to invasive cancer (Table 1).[18]

Table 1. *Follow-up of Simple and Complex Hyperplasia and Atypical Hyperplasia in 170 patients*[a]

		Regressed		Persisted		Progressed to Carcinoma	
Type of hyperplasia	No. of Patients	n	(%)	n	(%)	n	(%)
Simple	93	74	(80)	18	(19)	1	(1)
Complex	29	23	(80)	5	(17)	1	(3)
Simple atypical	13	9	(69)	3	(23)	1	(8)
Complex atypical	35	20	(57)	5	(14)	10	(29)

[a] *Adapted from Kurman et al.*[18]

Regrettably, studies have revealed a low reproducibility of the WHO classification.[13,19,20] An alternative classification method was therefore proposed making a distinction between endometrial hyperplasia caused by reversible estrogen effects and endometrial intraepithelial neoplasia (EIN) considered to be a real precancerous lesion,[20-23] with PTEN (phosphatase and tensin homolog (mutated in multiple advanced cancers 1) inactivation as an important event in progression.[24] The D score is used as a diagnostic tool to differentiate between the two groups.

Endometrioid Endometrial Carcinoma

Cancers with an endometrioid histology account for about 75% of endometrial carcinomas. These tumors are referred to as *endometrioid* because they resemble proliferative-phase endometrium. The nomenclature also maintains consistency with the terminology used for describing tumors with the same histologic appearance in the gynecologic tract. Remaining types are serous, mucinous, clear cell or mixed cancers. Endometrial carcinomas are divided into 3 groups based on the extent of solid growth (Table 2) and excluding squamous parts. Conspicuous nuclear atypia of glandular cells raises the grade by one. Serous papillary and clear cell cancers are considered grade 3 by definition. Furthermore, tumors are staged based on invasion and metastases in the surroundings and rest of the body (Table 3). The most important prognostic features in endometrial cancer are the differentiation grade, myometrial invasion, histologic type and surgical FIGO stage (see Table 3 and Figure 2).

Table 2. Differentiation Grade of Endometrial Cancer.[a]

Grade	Characteristics
1	5% or less of a nonsquamous or nonmorular solid growth pattern[b]
2	6%-50% of a nonsquamous or nonmorular solid growth pattern[b]
3	More than 50% of a nonsquamous or nonmorular solid growth pattern

[a] *Annual Report FIGO 2006.*[6]

[b] *Upgrade 1 grade in case of conspicuous nuclear atypia.*

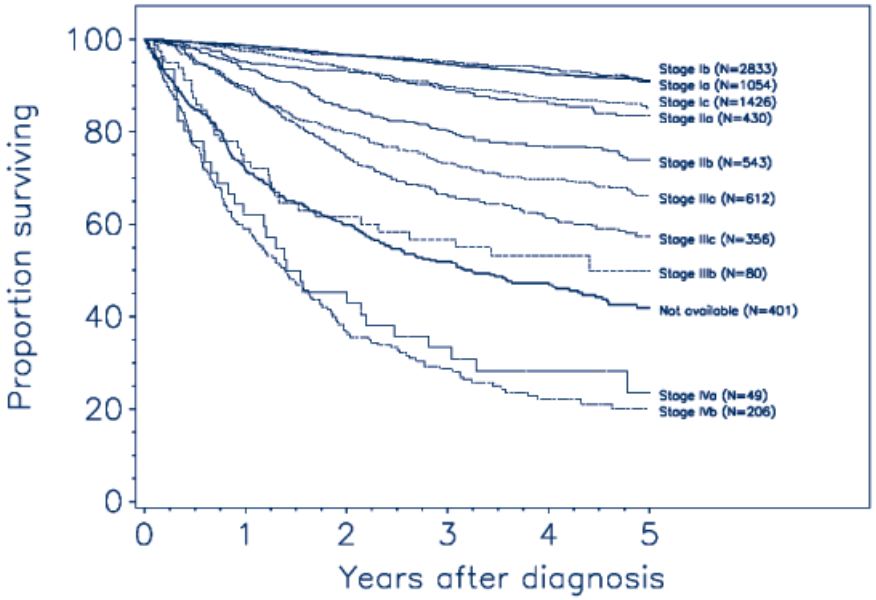
Table 3. Surgical Staging of Endometrial Cancer with 5-year survival.[a]

Stage	Characteristics	5-year survival 'b'	
Ia	Tumor limited to endometrium	96%	86%-90 %
Ib	Invasion to less than one-half of the myometrium	95%	
Ic	Invasion to more than one-half of the myometrium	82%	
IIa	Endocervical glandular involvement only	66%-83%	
IIb	Cervical stromal invasion		
IIIa	Tumor invasion of serosa and/or adnexa and/or positive	43%-66%	
IIIb	Vaginal metastases		
IIIc	Metastases to pelvic and/or paraaortic lymph nodes		
IVa	Tumor invasion of bladder and/or bowel mucosa	16%-25%	
IVb	Distant metastases including intraabdominal and/or		

[a] *Annual Report FIGO 2006.*[6]

[b] *Summarized data of Baak et al,*[25] *Wolfssen et al,*[26] *Maneschi et al,*[27] *Annual Report FIGO 2006.*[6]

Figure 2. Kaplan-Meier curves showing proportion of survival of endometrial cancer by stage defined by FIGO staging system. (Adapted from the Annual Report FIGO 2006, with permission.[6])



Molecular studies have shown that endometrioid tumors differ from nonendometrioid tumors in their genetic profile.[28] Endometrioid cancers are characterized by mutations of the PTEN, K-ras and β-catenin genes (sporadic cancers), microsatellite instability, and defects in DNA mismatch repair (hereditary and sporadic cases, mechanism differs). Serous cancers show mutations in p53 and ERBB-2 (HER-2/*neu*) and are often nondiploid. Estrogen receptor (ER) and progesterone receptor (PR) typically occur together and vary by histologic type: endometrioid tumors are mostly ER and PR positive, whereas serous cancers rarely express these receptors.

Tumor Biology

Several phenomena are known to contribute to carcinogenesis and progression of most malignancies, of which the following are perhaps the most essential: uncontrolled proliferation outweighing cell death, cellular dedifferentiation, and the ability to invade the environment and metastasize through the lymphatics or blood vessels. Intratumoral hypoxia induces cellular dedifferentiation and promotes angiogenesis and metastases. Angiogenesis is essential for tumor growth and spread of metastases. After activation of the endothelial cells of an existing mature blood vessel, the surrounding basement membrane is degraded, followed by movement of adjacent endothelial cells into the connective tissue, proliferation of these endothelial cells, formation of tubular structures and a network. Ultimately, this will lead to functioning blood vessels.

In tumors, however, often leaky, dilated, or nonfunctioning vessels are generated, and vessels may derive from differentiating fibroblasts or bone marrow–derived precursor cells.

Hypoxia

A developing solid tumor will outgrow its own vasculature beyond the size of several cubic millimeters,[29] resulting in hypoxia (defined as an oxygen tension below the physiologic level, far below 20%). *In vivo*, the tissue oxygen tension decreases with increasing distance of a tumor cell from a vessel. Because the oxygen diffusion distance is maximally 200 micrometer, tumor cells that lie beyond this distance from a vessel become anoxic.[30] Two kinds of hypoxia are known: acute and chronic. Acute hypoxia results from a sudden closure of the vasculature or from an acute decrease in blood flow. This form of hypoxia has been shown to be present in experimental tumors.[31,32] In general, acute responses are thought to be modulated by posttranslational modification of proteins that can occur in seconds to minutes. Responses to chronic hypoxia represent changes in gene transcription and take place in minutes to hours. Both chronic and acute hypoxia may coexist within the same tumor.[33] Direct measurement of hypoxia via oxygen electrodes has been shown in several cancers[34] with a wide range of oxygen levels within tumors. Not all tumors showed hypoxia, and almost all tumors harbored normoxic tumor parts.

Hypoxia Inducible Factor-1α

Although it is strenuous for a cell to survive in a hypoxic environment, there are factors that help to accomplish this task. The key survival factor for a cell in a hypoxic environment is hypoxia inducible factor-1 (HIF-1). As HIF-1 regulates cellular oxygen homeostasis, it is implicated in hypoxic conditions that occur during embryogenesis, cardiovascular disease, and tumor development, such as endometrial and breast cancer.[35,36] HIF-1 is a transcription factor consisting of the subunits HIF-1α and HIF-1β, which both contain basic helix-loop-helix and PAS (PER-aryl hydrocarbon nuclear translocator-SIM) domains to bind to DNA.[37,38] Besides HIF-1α, HIF-2α (EPAS-1) and HIF-3α have also been described.[39] They have been discovered on basis of their cDNA sequence similarity. Both HIF-2α and HIF-3α dimerize with HIF-1β in hypoxic cells to form DNA-binding complexes. HIF-2α is considered to be an important transcription factor promoting tumor growth associated with loss of Von Hippel Lindau protein. It has been shown that HIF-2α is thought to be responsible for proliferation and neovascularization, although function is dependent on cell type, which is nicely reviewed by Gruber and Simon.[40] In an earlier study on endometrial cancer, HIF-2α was expressed less than HIF-1α and not associated with prognosis,[35] although it is possible that stromal presence of HIF-2α is more important than epithelial expression.[41] HIF-3α is induced by hypoxia and is a negative regulator of HIF-1α function and therefore serves as a negative feedback loop.[42]

As outlined in Figure 3, in the presence of oxygen HIF-1α is hydroxylated at the proline residues 402 and 564[43] of the oxygen-dependent degradation domain by specific HIF-1α-proline hydroxylases.[44,45] Iron as a cofactor and dioxygen and 2-oxoglutarate as cosubstrates are required for the prolyl hydroxylation, which facilitates the specific interaction of pVHL to HIF-1α, resulting in proteasomal degradation. The factor-

inhibiting HIF (FIH) hydroxylates the asparagine residue in position 803 and inhibits HIF-1α's interaction with p300/CBP. Additionally, a number of studies have shown that HIF-1 activity can be increased through phosphorylation.[46]

Either a mutation of pVHL or lack of (part of) the ODD domain in the HIF-1α protein will lead to normoxic overexpression of HIF-1 and HIF-1 activation. In addition to hypoxia and aberrations in the VHL-HIF-1α pathway, increased expression of HIF-1α in tumors might be caused by various genetic alterations such as changes in the protooncogene ERBB2 (HER-2/*neu*)[47], activation of the PI-3K/Akt pathway,[48-52] or loss of p53.[53]

A nuclear localization signal at the C-terminal end of HIF-1α allows its transport from the cytoplasm to the nucleus, where it forms an active HIF-1 complex by binding to HIF-1β. The amount of HIF-1α protein in the nucleus is rate limiting and determines the functional activity of the HIF-1 complex. The HIF-1 complex binds the consensus sequence 5'-RCGTG-3' in the hypoxia-response elements (HREs) of target genes,[54] such as glucose transporters, glycolytic enzymes, and genes involved in gluconeogenesis, high-energy phosphate metabolism, growth factors, erythropoiesis, haem metabolism, iron transport, vasomotor regulation and nitric oxide synthesis, to activate their transcription.[55-57] Protein products of these downstream genes help the cell to survive the hypoxic stress by increasing oxygen delivery, activating alternate metabolic pathways that do not require oxygen, or stimulating proapoptotic proteins.

Cell Cycle

Deregulation of the normal constraints on cell proliferation lies at the heart of malignant transformation. A tumor may increase in size through any one of three mechanisms involving alterations pertaining to the cell cycle: shortening of the time of transit of cells through the cycle, decrease in the rate of cell death, or reentry of quiescent cells into the cycle. In most human cancers, all three mechanisms appear to be important in regulating tumor growth rate, a critical parameter in determining the biological aggressiveness of a tumor. The cell cycle consists of a DNA synthesis (S) phase, a mitosis (M) phase, and two gap (G1 and G2) phases (Figure 4). These phases are controlled by cyclins A, B, D1 and E, cyclin-dependent kinases (CDKs) like cdk2 and cdk4, cyclin-dependent kinase inhibitors (CKIs) like $p16^{INK4A}$, $p21^{WAF-1/CIP-1}$, and $p27^{KIP-1}$ and several other tumor suppressor gene products like p53.[58] Cyclins form a complex with CDKs, enabling transition to the next phase of cell cycle.

Figure 3. *Oxygen-dependent regulation of HIF-1α.*

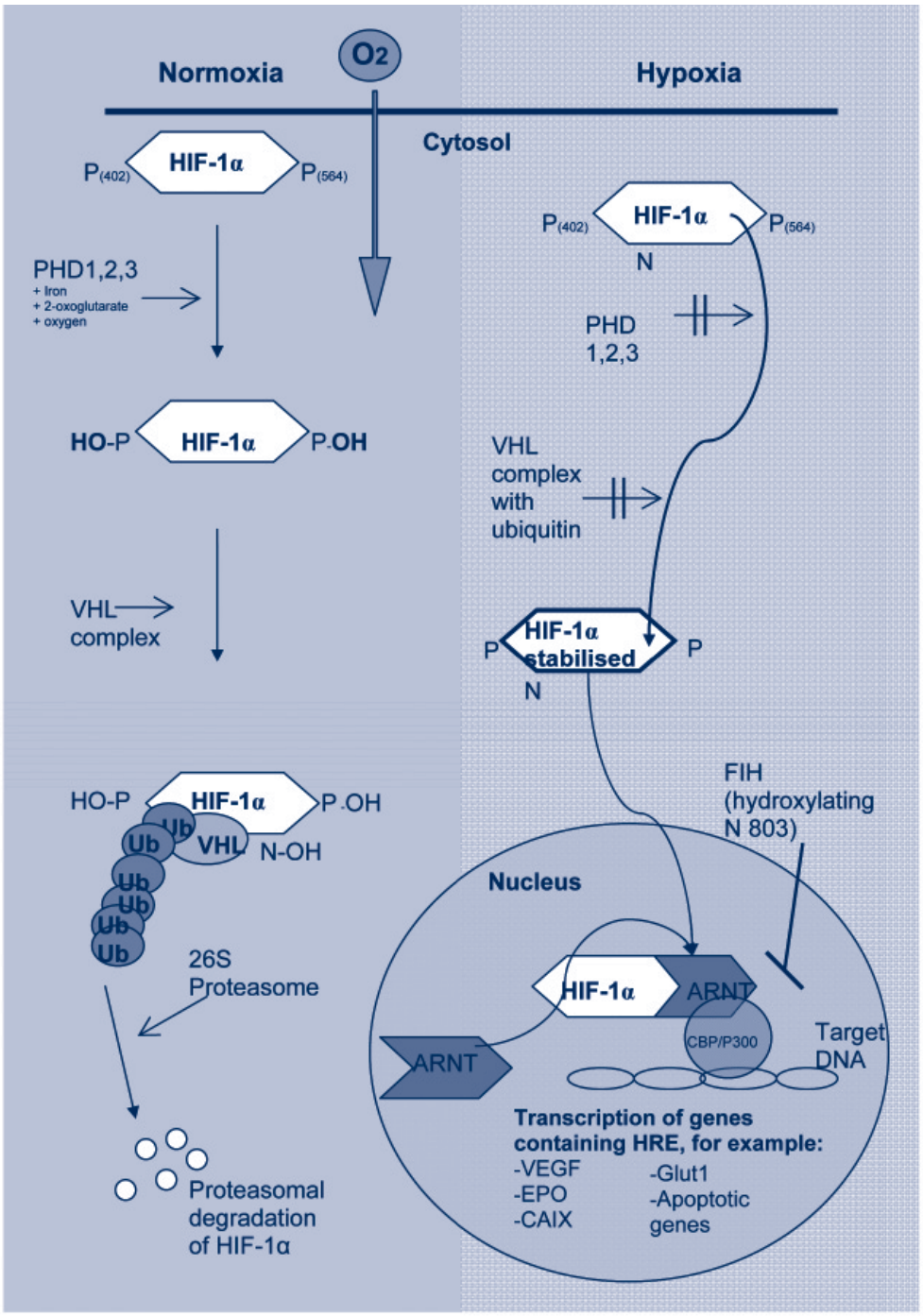
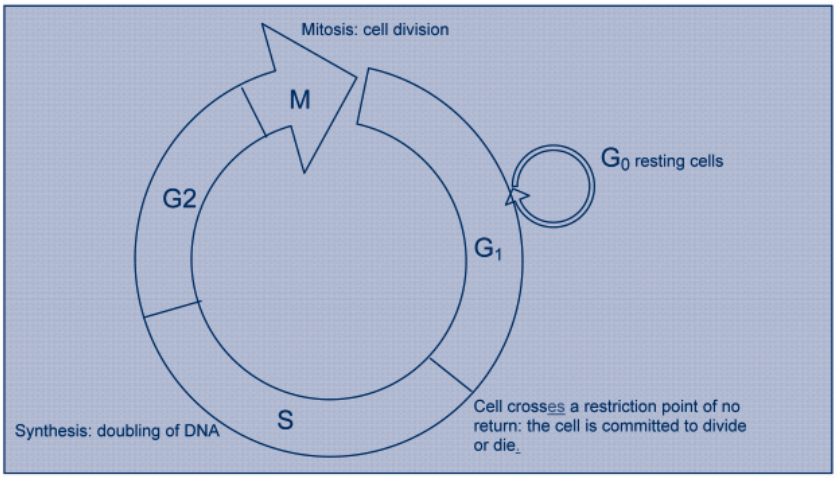
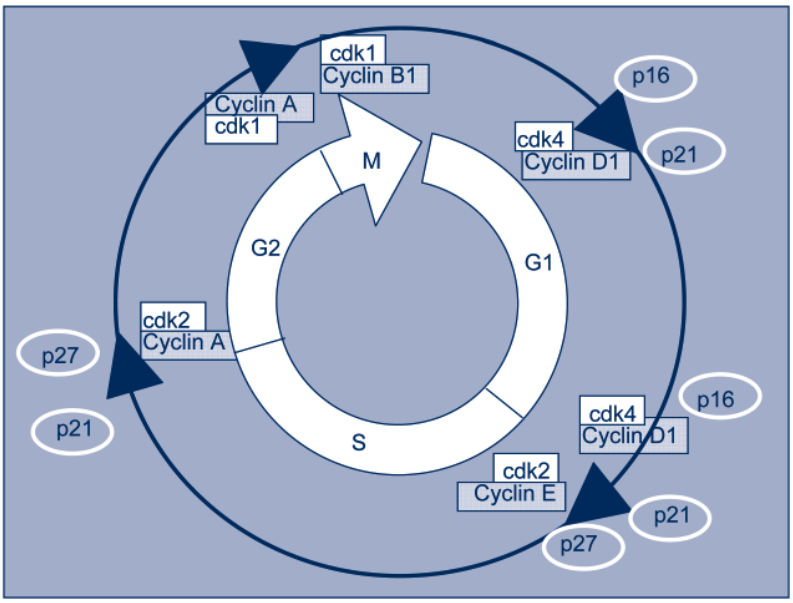


Figure 4. *Schematic illustration outlining the phases of the cell cycle.*



Cyclin D1 with its catalytic partner cdk4 functions to make cells leave the G0 phase and progress through G1. The transition of G1 to S is controlled by cyclin E/cdk2 and cyclin D1/cdk4 complexes, whereas p27 and p21 inhibit cyclin E, and p16 and p21 inhibit cyclin D1. The p21 protein is a p53 and cyclin D1 regulated potent inhibitor of CDKs and can thereby inhibit the phosphorylation of Rb (retinoblastoma protein). In the transition of S to G2, cyclin A/cdk2 plays a role with the CKIs p27 and p21 exerting effects here as well. In the switch from G2 to M, cyclin B/cdk1 and cyclin A/cdk1 are functional (Figure 5).

Figure 5. *Schematic diagram outlining the cell-cycle proteins regulating the cell cycle.*



Overexpression of cell-cycle stimulating factors such as the CDKs and cyclins and aberrant expression of inhibiting factors such as the CKIs are frequently found in tumors. This is often associated with a more malignant phenotype, a higher proliferation rate, recurrence, and worse survival in different tumors, including endometrial carcinoma.[59-68] Deletions, mutations, or promoter methylation of the cyclins, CDKs, or CKIs are all underlying anomalies causing malignant potential, for example in the *p16* or *CCNE* (cyclin E) gene.[68-77]

In contrast to these results is the overexpression of p21 and p16, both CKIs, which is paradoxically often seen in cancers. Presumably it reflects a failing feedback attempt of a normal protein rather than an alteration in the genes. Transcriptional control by another gene is also a possible cause, similarly to the association of β-catenin regulation with cyclin D1 and p16. Additionally, proteins are posttranslationally regulated by ubiquitin-proteasome–dependent degradation mechanisms like p27.[63,78]

The Invasive Front

The ability of cancer cells to invade the surrounding stroma and vessels is linked to advanced local invasive tumor growth and distant spread, affecting patient prognosis. Tumor invasion is a complex process that involves cell attachment, proteolysis of matrix components, migration of cells through the disrupted matrix, proliferation, and angiogenesis. During cancer progression, some endometrial cancers grow as polyps, but most invade downwards into the myometrium. Conceptually, the deepest parts of the tumors invading the myometrium must be the most active and aggressive ones, showing highest proliferation, invasive behavior, and angiogenesis. However, this theory has not been studied in endometrial cancers.

Aims of Thesis

More research is necessary to improve current understanding of the molecular basics of endometrial cancer. Such research will provide tools to use in prognostic and diagnostic studies and ultimately in randomized controlled trials to evaluate novel disease-based therapeutic strategies.

In general, this thesis can be summarized as a study of the role of cell-cycle proteins and the HIF-1α pathway in progression from normal endometrium to endometrioid endometrial carcinoma.

In **Chapter 2**, the role played by HIF-1α in carcinogenesis of endometrial carcinoma is elucidated. Expression patterns in normal, precancerous, and cancerous tissue are studied. Furthermore, to ensure that a possible expression of HIF-1α is functional, we searched for correlations with the downstream proteins VEGF, Glut-1, and CAIX and with microvessel density as a measure of angiogenesis.

In Chapter 2, which details the study on HIF-1α expression in endometrial carcinomas, different staining patterns were observed throughout the tumor, including a diffuse pattern. In addition to hypoxia, derailments in the HIF-1α pathway (loss of pVHL,

prolyl hydroxylases, or mutations in the HIF-1α gene) and oncogenic activation of HIF-1α by proteins such as phospho-AKT are possible contributors to elevated levels of HIF-1α. Our finding of a relatively high amount of diffusely expressed HIF-1α tumors was the rationale for searching for other factors than purely hypoxia to influence HIF-1α in **Chapter 3** and **Chapter 4**.

Expression of cell-cycle proteins in normal, premalignant, and malignant endometrial lesions, representing the morphologically well-defined stepwise model of human endometrial carcinogenesis, is explored in **Chapter 5**. Expression of cell-cycle regulators has been studied before in endometrial carcinoma, but a comprehensive analysis of expression of cyclins, CDKs, and CKIs in a progression model of hyperplasia to endometrioid endometrial carcinoma has not been performed, especially not with regard to EIN. We therefore studied the expression of cyclin A, cyclin B1, cyclin D1, cdk2, p16, p21, p27, p53, and the proliferation marker Ki67(MIB-1) in normal endometrium, hyperplasia (subclassified as EIN/non-EIN), and endometrioid adenocarcinoma of the endometrium, and correlated expression with some relevant clinicopathologic features.

Chapter 6 describes the differential expression of hypoxia and cell-cycle–related proteins between the tumor center and invasive front of endometrial cancers because differences were expected in proliferation (and thus expression of cell-cycle proteins) and expression of proteins in the hypoxia pathway.

Chapter 7 reconsiders the tissue type tubal metaplasia (TM). It is generally thought that endometrial TM is a benign disease, although molecular studies are scarce. For example, in previous studies aberrant expression of p16 has been noticed, but this observation has never been placed in a broader context. Aberrant expression of cell-cycle regulatory proteins or proteins in the HIF-1α pathway, both known to be more extensively expressed in cancer, might necessitate changing the common view that this is a purely benign entity. Therefore we studied these proteins in TM compared to normal tissue.

While we scored immunohistochemical staining for p27 in endometrial carcinomas in Chapter 5, we observed a staining pattern that we describe as "perinecrotic" or central within the tumor glands. Such a pattern has been described earlier for HIF-1α stainings in breast cancer, and we observed the pattern in endometrial cancer. This prompted us to search for interactions, direct or indirect, between hypoxia, HIF-1α, and p27 in endometrial carcinoma *in vitro* and *in vivo*. The results are described in **Chapter 8**.

In **Chapter 9**, the results of this thesis are summarized and placed in a broader context.

chapter 2

Hypoxia and angiogenesis in endometrioid endometrial carcinogenesis

Cell Oncol. 2007;29:219-227

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Abstract

Background

Methods Expression of HIF-1α and proteins in the HIF-1α pathway (Glut-1, CAIX, VEGF) in paraffin-embedded specimens of normal (n=17), premalignant (n=23) and endometrioid endometrial carcinoma (n=39) was explored by immunohistochemistry, in relation to microvessel density (MVD).

Results

HIF-1α overexpression was absent in inactive endometrium but present in hyperplasia (61%) and carcinoma (87%), with increasing expression in a perinecrotic fashion pointing to underlying hypoxia. No membranous expression of Glut-1 and CAIX was noticed in inactive endometrium, in contrast with expression in hyperplasia (Glut-1 0%, CAIX 61%, only focal and diffuse) and carcinoma (Glut-1 94.6%, CAIX 92%, both mostly perinecrotically). Diffuse HIF-1α was accompanied by activation of downstream targets. VEGF was significantly higher expressed in hyperplasias and carcinomas compared to inactive endometrium. MVD was higher in hyperplasias and carcinomas than in normal endometrium ($p<0.001$).

Conclusion

HIF-1α and its downstream genes are increasingly expressed from normal through premalignant to endometrioid adenocarcinoma of the endometrium, paralleled by activation of its downstream genes and increased angiogenesis. This underlines the potential importance of hypoxia and its key regulator HIF-1α in endometrial carcinogenesis.

Introduction

Solid tumors will outgrow their own vasculature beyond the size of several mm^3, resulting in hypoxia. Hypoxia is an important issue in carcinogenesis because it renders a more aggressive phenotype with increased invasiveness and proliferation, formation of metastases and poorer survival[79,80]. Besides, hypoxic malignant cells are more resistant to radiotherapy and chemotherapy[81,82]. In reaction to hypoxia, cells will alter their metabolism and activate certain survival genes. Hypoxia-inducible factor 1 (HIF-1) plays an essential role in the adaptive cellular response to hypoxia[83]. HIF-1 is a transcription factor consisting of 2 subunits, HIF-1α and HIF-1β, which both contain basic helix-loop-helix and PAS (PER-aryl hydrocarbon nuclear translocator-SIM) domains to bind to DNA[37,38]. HIF-1α and HIF-1β together form the active HIF-1 complex which binds the consensus sequence 5'-RCGTG-3' in the hypoxia-response elements of various target genes[54]. Genes involved control glucose transporters, glycolytic enzymes, gluconeogenesis, high-energy phosphate metabolism, growth factors, erythropoiesis, haem metabolism, iron transport, vasomotor regulation and nitric oxide synthesis[54-56]. Under normoxia, HIF-1α protein has a very short half-life due to its continuous ubiquination and proteasome mediated degradation, while in hypoxia this process is inhibited[84]. As HIF-1β is constitutively expressed, HIF-1α protein levels determine HIF-1 activity. HIF-1α is overexpressed in many cancers[85]. Endometrial cancer is the most common malignant tumor of the female genital tract. Estimated incidence of cancer in the uterine corpus in the US was 40,880 for 2006 (6% of all cancers), with an estimated probability of developing uterine cancer of 1 in 38[86]. HIF-1α was associated with poor prognosis in stage 1 endometrial cancer in one study[35]. Carbonic anhydrase IX (CAIX) is a membrane-associated carbonic anhydrase, that plays a role in pH regulation[87]. Its gene contains a hypoxia response element in the promoter region and is activated by HIFs[88]. A role for this enzyme in the adaptation of tumor cells to hypoxic conditions and in tumor cell progression is suggested by a significant overlap between CAIX expression and regions of hypoxia in solid tumors[88,89]. Elevated CAIX levels are predictive of hypoxia in various types of cancer and are related to poor prognosis[90,91] although no studies were found in which CAIX expression is determined in endometrial cancer. In endometrial hyperplasia, expression of Glut-1, a facilitative glucose transporter upregulated by HIF-1α, appeared to be a useful indicator of high risk for development of endometrial carcinoma[92]. Increased angiogenesis, one of the effects of HIF-1α through upregulation of vascular endothelial growth factor (VEGF), was a statistically significant predictor of decreased survival in endometrial cancer[93-97]. These results point to an important potential role of hypoxia and its key regulator HIF-1α in endometrial carcinogenesis and progression. However, present data are fragmentary. The aim of this study was therefore to comprehensively explore the role of HIF-1α and its downstream genes in normal, premalignant and malignant endometrial lesions representing the morphologically well defined stepwise model of human endometrioid carcinogenesis, the most frequent carcinogenetic pathway in the endometrium.

Materials and Methods

Patients and Tissues

Paraffin-embedded clinical specimens from inactive endometrium (n=17), hyperplasia (n=23) and endometrioid adenocarcinoma (n=39) were selected from the archives of the Department of Pathology of the University Medical Center, Utrecht, The Netherlands. These tissues were derived from patients operated between 1991 and 2004. None of the carcinoma patients received preoperative radio- or chemotherapy. The ages ranged from 31 to 85 years; all women with inactive endometrium were postmenopausal. Table 1 gives an overview of the patient demographics and main pathological features. We included relatively more high stage patients to better balance the groups and to evaluate possible difference between stages.

Table 1. Patient demographics and main pathological features (n=79)

		N	(%)
Age	Mean	59.05	
	Median	58.46	
	Minimum	31	
	Maximum	85	
Histologic diagnosis	Inactive endometrium	17	(21.5%)
	Hyperplasia	23	(29.1%)
	Carcinoma	39	(49.4%)
Hyperplasia	Simple without atypia	7	(30.4%)
	Simple with atypia	0	(0.0%)
	Complex without atypia	9	(39.1%)
	Complex with atypia	7	(30.4%)
Endometrial Intraepithelial Neoplasia	Non-EIN/hyperplasia	13	(56.5%)
	EIN	10	(43.5%)
Grade of carcinoma	Grade 1	6	(15.4%)
	Grade 2	21	(53.8%)
	Grade 3	12	(30.8%)
Stage of carcinoma	Stage I	13	(33.3%)
	Stage II	10	(25.6%)
	Stage III	11	(28.2%)
	Stage IV	5	(12.8%)

Haematoxylin and eosin-stained sections were revised and histologically typed and graded by 2 experienced gynecopathologists (PvD, DSG). Hyperplasias were categorized according to the World Health Organization (WHO) nomenclature and reclassified as endometrial intraepithelial neoplasia (EIN) or non-EIN[21,98]. For carcinomas, the tumor stage was defined by the

International Federation of Gynecologists and Obstetricians (FIGO) system. One of the endometrial carcinomas showed a mucinous carcinoma part and one a serous carcinoma, but we analyzed in this study only the endometrioid part. Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our hospital[99].

Immunohistochemistry

HIF-1α, Glut-1, CAIX, VEGF and CD31 were immunohistochemically stained on serial 5 µm thick paraffin slides as extensively described before[100]. Table 2 presents all antibodies, dilutions, incubation times and antigen-retrieval methods used. For all stainings, slides were deparaffinized with xylene and serial ethanol dilutions, and endogenous peroxidase activity was blocked followed by antigen retrieval.

Table 2. *Overview of the antibodies used and tissue processing details*

Primary Antibody	Type Ab	Source	Dilution	Antigen Retrieval	Second step	Positive control	Incubation time / temp (primary antibody)	Procedure
HIF-1α	MoAb, Mouse	BD Pharmingen	1:50	TRS, DAKO, 45 min, 97°C	CSA	Mamma-carcinoma	60 minutes / room temp	By hand
Glut-1	PoAb, Rabbit	DAKO	1:200	Citrate, pH 6.0, 20 minutes, 93°C	G-aR IgG + Strep1	Red blood cells in slide	60 minutes / room temp	Autostainer
CAIX	PoAb, Rabbit	Novus-Biological	1:1000	Citrate, pH 6.0, 20 minutes, 93°C	Powervision	Grawitz tumour	60 minutes / room temp	By hand
VEGF	PoAb, Goat	R&D Systems	1:50	Citrate, pH 6.0, 20 minutes, 93°C	R-aG IgG + Strep2	Endothelium (internal)	60 minutes / room temp	By hand
CD31	MoAb	DAKO	1:40	Citrate, pH 6.0, 20 minutes, 93°C	Powervision	Endothelium (internal)	60 minutes / room temp	By hand

HIF-1α = hypoxia-inducible factor -1α; Glut-1 = glucose transporter-1; CAIX = carbonic anhydrase IX; VEGF = vascular endothelial growth factor, MoAb = monoclonal anitbody; PoAb = polyclonal antibody; DAKO = DAKOCytomation, Glostrup, Denmark; TRS = Target Retrieval Solution, DAKO S1700; CSA = catalyzed amplification kit, DAKO; G-aR IgG = biotinylated Goat-anti Rabbit IgG (BA-1000, Vector laboratories, CA, diluted 1:500) + Strep1 = Streptavidin peroxidase labeling (Streptavidin HRP, IM0309, Beckman Coulter, diluted 1:1000); R-aG IgG = biotinylated Rabbit-anti Goat IgG (E0466, DAKOCytomation, Glostrup Denmark) + Strep2 = Streptavidin peroxidase labeling (K0377, DAKOCytomation, Glostrup, Denmark); Powervision = Powervision ready to use (Poly-HRP-anti Ms/Rb/RtIgG biotin free, ImmunoLogic, ImmunoVision Technologies, Brisbane, CA, USA).

For HIF-1α, endogenous peroxidase was blocked by hydrogen peroxide (Dako CSA kit), after which antigen retrieval followed. A cooling off period of 30 minutes preceded blocking of the avidin by biotin block (Dako; 10 min) and protein block (Dako; 5 min). Then, the primary antibody was applied followed by the catalyzed signal amplification system (DAKO, Glostrup, Denmark). For CAIX, VEGF, Glut-1 and CD31 endogenous peroxidase activity was blocked for 30 minutes in methanol containing 0.3% hydrogen peroxide.

Finally, peroxidase activity was developed with DAB and counterstained with hematoxylin. In between steps, slides were washed in PBS. Positive controls were used throughout, see table 2 for types of tissue. Negative controls were obtained by omission of the primary antibodies from the staining procedure.

Evaluation of Staining

Two authors (PvD, NH) scored all slides blinded to clinicopathologic data and results of other stainings. For HIF-1α, the percentage of dark, homogenously stained nuclei was estimated as before[101], ignoring cytoplasmic staining. Glut-1 and CAIX were considered positive when membrane staining was seen. VEGF staining was semiquantitatively scored as negative, +, ++, or +++. For nuclear HIF-1α, cytoplasmic VEGF and for membranous Glut-1 and CAIX expression, the pattern of staining was noted as diffuse (throughout the tumor without emphasis on areas with necrosis, thought to be due to non-hypoxic stimuli), perinecrotic (only positive staining around a necrotic area, thought to be hypoxia induced) or a combination of these two. No double staining was performed as this was done previously in breast cancer[101], and no further topographic analysis of staining was performed. In the inactive endometrium, areas of tubal metaplasia were skipped. To assess the microvessel density (MVD), the most hypervascular areas ("hot spots") were selected under low magnification in the CD31 stained slides. Herein, microvessels were counted in the 4 most hypervascular adjacent fields at a magnification of 20x, the 'hot-spot'- method. The total area counted was 3.80 mm2, and MVD values were expressed per mm^2. If there was not enough tissue for 4 fields of vision, counts were extrapolated. All slides were counted twice, and the mean was taken. MVD was not assessable in 5 cases of inactive endometrium because of fragmentation.

Statistical Analysis

Frequencies of expression of CAIX, Glut-1 and VEGF for inactive, hyperplastic and carcinomatous tissue were compared with Fisher's exact test. The Kruskal-Wallis test was used to assess differences in MVD and HIF-1α expression between the three different lesion categories, and for differences between two groups the Mann-Whitney test was used.

Fisher's exact test was also used to search for differences in expression between EIN (n=10) vs non-EIN (n=13), and between the WHO subgroups of hyperplasia: simple without atypia (n=7), simple with atypia (n=0), complex without atypia (n=9) and complex with atypia (n=7). When HIF-1α was entered in Fisher's exact test, the usual 5% threshold was used for positivity. As there were no significant differences in expression of any of the proteins and MVD between EIN and non-EIN, or between any of the WHO categories of hyperplasia, the results are only shown for the hyperplasias as a group.

Two sided p-values <0.05 were considered significant. All statistical analysis were performed by using SPSS for Windows version 12.0.1., 2003 (SPSS Inc., Chicago, IL).

Results

Table 3 shows a summary of the expression of the different hypoxia related proteins in inactive endometrium, hyperplasia (as a group) and endometrioid carcinoma

of the endometrium. Normal (inactive) endometrium completely lacked HIF-1α expression. Endometrial hyperplasia as a group showed HIF-1α expression in 14/23 (60.9%) cases, 13 showing diffuse and 1 only perinecrotic expression (an EIN/complex atypical case). In endometrioid carcinoma, HIF-1α expression was seen in 34/39 cases (87.2%). In 9 cases (26.5%) the expression was only diffuse (*Figure 1A*), in 20 cases (58.8%) the expression was mixed diffuse/perinecrotic, and in 5 cases (14.7%) expression was exclusively perinecrotic (*Figure 1B*). The median percentages of HIF-1α positive nuclei in inactive endometrium, endometrial hyperplasia (as a group) and endometrioid carcinoma were 0%, 5%, 20%, respectively ($p<0.001$), and differences between hyperplasia and carcinoma were significant as well ($p = 0.025$).

Table 3. *Expression of hypoxia related proteins in different endometrial lesions representing the endometrial endometrioid carcinogenetic spectrum*

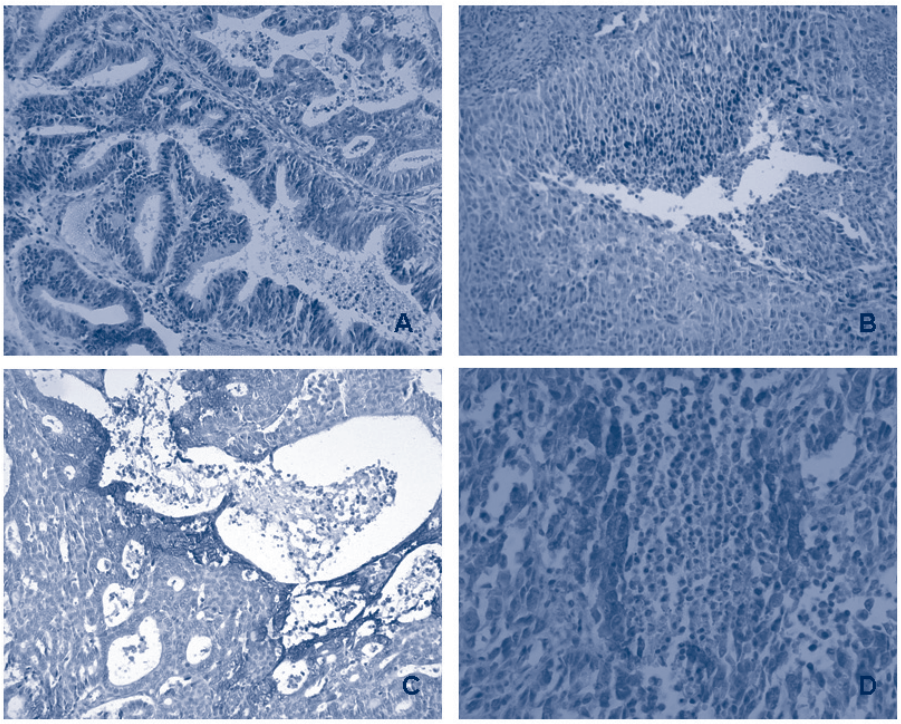
		Inactive		Hyperplasia		Carcinoma		P-value for difference between IE, EH, EC	P-value for difference between EH and EC
CAIX	Negative	17	(100%)	9	(39.1%)	3	(7.7%)	p < 0.001*	p = 0.006*
	Positive	0	(0%)	14	(60.9%)	36	(92.3%)		
Glut-1	Negative	17	(100%)	13	(56.5%)	1	(2.6%)		
	Cytoplasm positive, Membrane negative	0	(0%)	10	(43.5%)	1	(2.6%)		
	Membrane positive	0	(0%)	0	(0%)	37	(94.9%)	p < 0.001*	p < 0.001*
VEGF	-	7	(41.2%)	1	(4.3%)	1	(2.6%)		
	+	10	(58.8%)	12	(52.2%)	16	(41.0%)	p < 0.001*	p = 0.768*
	++	0	(0%)	8	(34.8%)	16	(41.0%)		
	+++	0	(0%)	2	(8.7%)	6	(15.4%)		
MVD (n/mm2)	Mean	18.1		43.4		30.3			
	Median	15.8		40.4		27.6		p < 0.001†	p = 0.009‡
	Range	5.3 – 47.9		12.1 – 84.5		9.7 – 72.6			
HIF-1α	<5% positive	17	(100%)	9	(39.1%)	5	(12.8%)	p < 0.001*	P = 0.027*
	≥ 5% positive	0	(0%)	14	(60.9%)	34	(87.2%)		
	Median	0		5		20		p < 0.001†	p = 0.025‡
	Range	0 – 0		0 – 75		0 – 90			

** = Fisher exact; † = Kruskal Wallis; ‡ = Mann-Whitney-test*

There was no expression of CAIX in inactive endometrium. In hyperplasia, CAIX was expressed in 14/23 cases (60.9%) in a focal and diffuse way, in contrast to 36/39 (92.3%) of carcinomas ($p = 0.006$). In the carcinomas, the CAIX (*Figure 1C*) staining pattern was just diffuse in only 3 (8.3%) cases, perinecrotic in 23 (63.9%) cases, and mixed in 10 (27.8%) cases. There was no membranous expression of Glut-1 in inactive endometrium and hyperplasia in contrast to 37/39 (94.9%) of carcinomas. The pattern of expression

was diffuse in 3 (7.7%) cases, perinecrotic (*Figure 1D*) in 27 (69.2%) and mixed in 3 (7.7%) cases.
VEGF was significantly less expressed in inactive endometrium compared to hyperplasia and carcinoma ($p<0.001$). 3 out of 39 carcinomas (7.7%) showed a pure perinecrotic VEGF expression, 20 cases (51.3%) showed a diffuse pattern and 16 (41.0%) showed a mixed patttern of these. MVD was higher in hyperplasias and carcinomas compared to normal endometrium ($p<0.001$).
In the carcinomas, there were no significant correlations between expression of any of the proteins and grade or stage.

Figure 1. *Expression of HIF-1α, Glut-1 and CAIX in endometrial carcinomas. A) Diffuse nuclear expression of HIF-1α (10x magnification). B) perinecrotic nuclear expression of HIF-1α (20x magnification). C) Perinecrotic membrane CAIX expression (10x magnification). D) Perinecrotic membrane Glut-1 expression (20x magnification).*

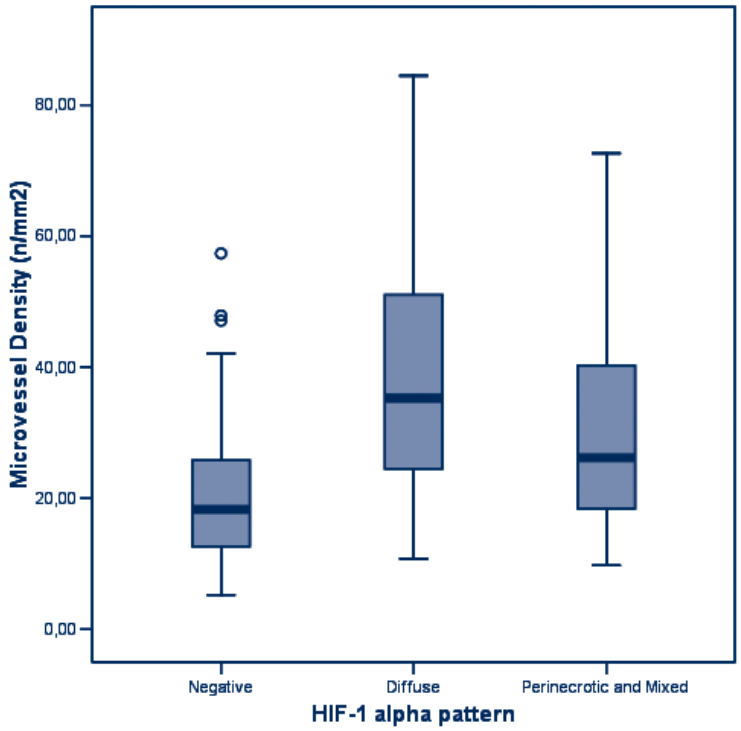


Correlation between HIF-1α and its downstream proteins and microvessel density

In the hyperplasias (non-EIN and EIN), diffuse HIF-1α expression was associated with CAIX expression in 13/19 (68.4%) cases, with Glut-1 expression in 0/19. In the carcinomas, the 9 cases with only diffuse HIF-1α expression showed CAIX expression in all cases (1 diffuse, 5 perinecrotic and 3 mixed), Glut-1 expression in 8 (88.9%) cases (2 diffuse, 4 perinecrotic, 1 mixed, 1 focal) and VEGF expression in all cases (6 diffuse, 3 mixed). The 5 carcinoma cases with pure perinecrotic HIF-1α all showed perinecrotic CAIX expression and perinecrotic Glut-1, without diffuse staining. In these perinecrotic HIF-1α stained carcinoma cases, VEGF was positive in all

(2 perinecrotic, 2 diffuse and 2 mixed). The 20 tumors with a mixed expression of HIF-1α showed CAIX expression in 19 cases (12 perinecrotic, 2 diffuse and 5 mixed), Glut-1 expression in all cases (15 perinecrotic, 1 diffuse and 1 mixed, 3 focal) and in all cases VEGF expression (1 perinecrotic, 10 diffuse and 9 mixed). Diffuse HIF-1α in carcinoma (n=9) was accompanied by CAIX expression (1 diffuse, 5 perinecrotic, 3 mixed), Glut-1 expression (2 diffuse, 4 perinecrotic, 1 mixed) and VEGF expression (6 diffuse, 3 mixed). Low HIF-1α expression was associated with negative/low VEGF staining in the total group (Fisher exact, p=0.001). Figure 2 shows that diffuse expression of HIF-1α was associated with highest MVD; perinecrotic and mixed patterns were associated with an intermediate MVD ($p<0.05$).

Figure 2. *Microvessel Density (MVD) for different expression patterns of HIF-1α. Diffuse expression of HIF-1α was associated with highest MVD; perinecrotic and mixed patterns were associated with an intermediate MVD (Kruskal-Wallis, p<0.05).*



Discussion

The purpose of this study was to investigate the expression of HIF-1α, its downstream genes Glut-1, VEGF and CAIX, and angiogenesis in the endometrioid carcinogenetic spectrum represented by inactive endometrium, endometrial hyperplasia and endometrioid endometrial carcinoma. This is the first study in which CAIX in human endometrial cancer is assessed. It is also the first publication on the expression of HIF-1α in endometrial hyperplasia.

HIF-1α showed increasing overexpression from inactive endometrium through

hyperplasia to endometrioid carcinoma. Perinecrotic, hypoxia associated HIF-1α overexpression was absent in inactive endometrium, rare in endometrial hyperplasia and frequent in endometrioid carcinoma. This largely confirms previous studies on HIF-1α in endometrial carcinogenesis. Acs et al.[102] found 74% of carcinoma cases to be HIF-1α positive with significantly more expression in tumor samples containing areas of necrosis, and only negative benign endometrium cases. Sivridis et al.[35] found 49% of carcinoma cases to be HIF-1α positive.

For CAIX and Glut-1 we noticed an increasing overexpression from normal to malignant endometrium too. Glut-1 was often (94.9%) and exclusively expressed in carcinomas, in line with previous studies[92,103].

Interestingly, diffuse HIF-1α expression (thought to be especially due to non-hypoxic stimuli such as HIF-1α mutations and amplifications, mutations in p53, PTEN, and VHL, and HER-2/neu amplifications, although mild hypoxia cannot be excluded) was often accompanied by activation of the downstream genes CAIX, Glut-1 and VEGF. This is in contrast with previous findings in breast cancer, where only perinecrotic HIF-1α (that is thought to be hypoxia driven) was in general associated with Glut-1 and CAIX expression[101]. The activation of CAIX and Glut-1 in diffuse and perinecrotic HIF-1α expressing endometrial carcinomas may point to diffuse HIF-1α expression being functional too. This is further evidenced by the fact that highest MVD was seen in cases with diffuse HIF-1α expression compared to cases with perinecrotic/mixed expression. Although mild hypoxia cannot be excluded as reason for diffuse HIF-1α expression, especially non-hypoxic stimuli are thought to be involved. These include HIF-1α mutations and amplifications, and mutations in p53, PTEN, and VHL, Her/2neu amplifications, etc.

We noticed a significant difference in MVD in the three types of tissue where inactive endometrium showed the lowest and hyperplasia and carcinoma significantly higher MVD, in line with previous studies[104,105]. The angiogenic switch during endometrioid endometrial carcinogenesis therefore seems to lie between inactive and hyperplastic endometrium.

We found no global association between VEGF expression and MVD. Earlier reports are not consistent on this issue. Fujisawa et al.[106] found no correlation either, although others[96,105,107] concluded that VEGF was associated with higher MVD. The lack of correlation between VEGF and MVD might be due to the complex system of proangiogenic and antiangiogenic factors that regulates angiogenesis. Obviously, in endometrial carcinoma VEGF is not the only angiogenic factor. On the other hand, we observed VEGF in perinecrotic areas where also HIF-1α is preferentially expressed in 3 out of 39 carcinomas, and a mixed pattern with diffuse and perinecrotic expression in 16 out of 39 tumors. This points to a biological relation between hypoxia, HIF-1α and VEGF expression. In our study, HIF-1α was associated with VEGF expression, which underlines this idea.

The group of hyperplasias is rather heterogeneous, and various proposed systems have attempted to arrive at a biologically and clinically useful subclassification. Atypical complex hyperplasia, one of the types of hyperplasia defined by the WHO

nomenclature, in particular is considered the precursor lesion for endometrial carcinoma, although diagnostic agreement between pathologists is rather low[19,108]. The EIN classification was introduced as a potentially better reproducible alternative system and is also used in diagnosis[21,98]. We could not find any differences within the hyperplasia subgroups for both these classification systems. This can to a certain extent be explained by the small size of the subgroups. We would further like to note that no stage Ia cancers were included in this study, which would likely overlap in expression patterns with complex atypical hyperplasia/EIN.

PTEN inactivation is seen as one of the major events resulting in carcinogenesis of EIN and as a result endometrial carcinoma[109]. This is underlined by the outcome of a recent study in which it is shown that lack of PTEN expression in EIN is correlated with cancer progression[24]. Functional inactivation of the PTEN gene is associated with stabilization of HIF-1α[48]. Therefore it might be interesting in future studies to evaluate whether in addition to hypoxia, this might be one of the causes of (diffuse) HIF-1α upregulation in endometrial tissues.

In conclusion, HIF-1α is increasingly expressed over the endometrioid carcinogenetic spectrum of the endometrium and is associated with activation of its downstream targets and increased angiogenesis. This underlines the potential importance of hypoxia and the subsequent stabilisation of HIF-1α in endometrial carcinogenesis. Besides, detecting HIF-1α may identify subgroups of patients that could benefit from hypoxia targeting therapeutic strategies and may be resistant to radiotherapy.

chapter 3

Expression of prolyl hydroxylases in relation to non-hypoxic expression of HIF-1α in endometrial cancer

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Abstract

Aims

Hypoxia inducible factor 1 alpha (HIF-1α) is the key survival protein that is stabilised in response to hypoxia. Besides hypoxia induced perinecrotic expression, HIF-1α may be regulated by several other factors leading to a more diffuse expression pattern that is often seen in endometrial carcinomas. Here we evaluated several proteins for their putative role in diffuse HIF-1α expression in endometrial carcinoma.

Methods and Results

In 61 endometrioid endometrial cancers, expression of PTEN, p-AKT, PHD1, PHD2, PHD3 and pVHL was studied by immunohistochemistry and correlated to HIF-1α expression patterns. Expression of the prolyl hydroxylases, especially that of PHD1, was less in diffusely HIF-1α expression tumors than in perinecrotically stained tumors. In one case PHD2 expression was less in cancer cells than in the adjacent normal glands. HIF-1α expression did not correlate to PTEN, and p-AKT. For VHL, no clear low expression was seen, but membranous staining, previously reported to be related to missense mutations, was observed in 2 tumors around necrosis, pointing to possible hypoxic upregulation. Additionally, mosaic pVHL staining was observed in 2 tumors, analogous to p16 in the atrophic endometrium with tubal metaplasia. Since pVHL has been shown to be important for ciliogenesis, this pattern might be related to ciliated cell change.

Conclusion

Loss of expression of prolyl hydroxylases may be related to diffuse, non-hypoxia related expression of HIF-1α in endometrial cancer.

Introduction

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor complex which plays a key role in the hypoxic response and is therefore associated with an aggressive phenotype of many cancers including endometrial cancer[35,36,101]. HIF-1α and HIF-1β together form the active HIF-1 complex which binds the hypoxia-response elements of various target genes[110,111] (e.g., glucose transporters, glycolytic enzymes, growth factors, genes involved in apoptosis, erythropoiesis, vasomotor regulation and nitric oxide synthesis) to induce their expression. Whereas HIF-1β is constitutively expressed, HIF-1α is regulated by oxygen tension. Prolyl hydroxylases (PHDs) hydroxylate HIF-1α protein on proline residues 402 and 564 within the oxygen dependent degradation domain (ODDD), allowing binding of the Von Hippel Lindau (VHL) E3 ligase complex, making HIF-1α a target for ubiquitination and subsequent proteosomal degradation[44,112,113]. Since the PHDs require oxygen, this process is suppressed in hypoxia allowing HIF-1α to escape degradation, and subsequently induce target gene expression. However, PHDs are important in targeting HIF-α for degradation under normoxia and under hypoxia[114], and loss of PHD function[114,115] may cause non-hypoxia mediated HIF-1α expression. Biallelic inactivation of *VHL* or lack of wild-type pVHL leads to HIF-1α stabilization and upregulation of HIF-1 target genes, resulting in an inappropriate triggering of the hypoxic response under normal oxygen tension[116]. This especially plays a role in renal cell carcinoma. Restoration of pVHL function by stable transfection reversed normoxic HIF-α protein stability and the aberrant increase of target gene expression[116]. Therefore, one mechanism by which mutations in pVHL might cause tumor formation is by permitting the stability and activity of HIF-1α under normal oxygen tensions, resulting in the subsequent expression of genes and angiogenetic factors even before cells are exposed to hypoxic stress. In addition to pVHL, a number of other proteins have been reported to affect HIF-1α ubiquitination and stability. For example, MDM2 has been suggested to bring about ubiquitination of HIF-1α in a p53-dependent fashion[53]. Further, non-hypoxia mediated HIF-1α expression might be caused by various genetic alterations. These include mutations in the ODDD of HIF-1α, changes in oncogenes such as HER-2/*neu*[47], activation of the PI-3K/AKT pathway[48-52], and loss of the PTEN tumour suppressor gene (also known as MMAC1) which is often seen in endometrial carcinogenesis[117-120].

In a previous study we demonstrated that HIF-1α is associated with endometrial carcinogenesis[121]. A perinecrotic, hypoxia-induced expression of HIF-1α was seen in cancer samples, as well as a more diffuse expression pattern (also in areas adjacent to blood vessels), thought to be due to hypoxia-independent factors[101,122].Little is however known about the role of non-hypoxia related factors in endometrial cancer. The aim of this study was therefore to assess various proteins potentially related with diffuse, non-hypoxia-mediated HIF-1α overexpression in endometrial carcinomas: PTEN, p-AKT, pVHL and PHD1, PHD2, and PHD3 by immunohistochemistry.

Materials and Methods

Patient and tissue selection

Paraffin-embedded clinical specimens from endometrioid endometrial carcinomas (n=61) were selected from the archives of the Department of Pathology of the University Medical Center, Utrecht, The Netherlands. These tissues were derived from patients operated between 1992 and 2005. None of the carcinoma patients received preoperative radio- or chemotherapy. Haematoxylin and eosin-stained sections were revised and histologically typed and graded (see table 2). The tumor stage was defined by the International Federation of Gynecologists and Obstetricians (FIGO) system. Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our hospital[99].

Table 1. Overview of the antibodies used and tissue processing details

Primary Antibody	Type Ab	Supplier	Dilution	Antigen Retrieval	Second step	Positive control	Incubation time / temp (primary antibody)
HIF-1α	MoAb	BD Pharmingen	1:50	EDTA, pH 9.0, 20 min, 97°C	Powervision	Breast cancer	Overnight / 4°C
PTEN (clone 6H2.1)	MoAb	Cascade Biosciences	1:300	10 mM TRIS/1 mM EDTA (pH 9.0), 120 °C, pressure of 1.9 bar, 2 minutes	Chemmate Envision	Normal endometrial glands	30 minutes / room temp
PHD1	PoAb	Abcam	1:100	Citrate pH 6.0, 20 min, 97°C	Powervision	Normal kidney	60 minutes / room temp
PHD2	PoAb	Abcam	1:100	EDTA, pH 9.0, 20 min, 97°C	Powervision	Normal kidney	Overnight / 4°C
PHD3*	PoAb	Novus Biologicals	1:400	Citrate pH 6.0, 20 min, 97°C	Powervision	Normal kidney	60 minutes / room temp
pVHL (Ig32)	MoAb	BD Pharmingen	1:100	Citrate pH 6.0, 20 min, 97°C	Powervision	Normal kidney	Overnight / 4°C
p-AKT (phospho-s473)	PoAb	Abcam	1:200	Citrate pH 6.0, 20 min, 97°C	Powervision	Breast cancer	60 minutes / room temp

** Before incubation with primary antibody against PHD3, slides were incubated for 60 minutes with 10% normal goat serum in sterile phosphate buffered saline.*

Abbreviations used: HIF-1α = hypoxia-inducible factor-1α; PHD1 = prolyl hydroxylase 1; PHD2 = prolyl hydroxylase 2; PHD3 = prolyl hydroxylase 3; pVHL = Von Hippel Lindau protein; p-Akt = phospho-akt, MoAb = monoclonal antibody; PoAb = polyclonal antibody; Powervision = Powervision ready to use (Poly-HRP-anti Ms/Rb/RtIgG biotin free, ImmunoLogic, ImmunoVision Technologies, Brisbane, CA, USA); CheMate Envision Detection kit, Dako.

Suppliers: BD Pharmingen, Biosciences, San Diego, CA, USA; Cascade Biosciences, Winchester, MA, USA; Abcam, Cambridge, UK; Dako, Glostrup, Denmark.

Immunohistochemistry

HIF-1α, PTEN, pVHL, PHD1, PHD2, PHD3, phospho-AKT (p-AKT) were immunohistochemically stained on serial 4 µm thick paraffin slides as extensively described before[24,49,121]. Table 2 presents all antibodies, dilutions, incubation times and antigen-retrieval methods used. Immunohistochemical staining, was carried out manually, with the exception of PTEN stains which were performed with an automated highly standardized autostainer (for details, see below). Slides were deparaffinized with xylene and serial ethanol dilutions. Then, for all stainings except for PTEN, endogenous peroxidase activity was blocked for 15 minutes in a buffer solution of pH 5.8 with hydrogen peroxide. This was followed by antigen retrieval. Next, slides were incubated with the primary antibody, followed by the secondary. Finally, peroxidase activity was developed with 3,3'-diaminobenzidine (DAB) for 10 minutes, slides were counterstained with hematoxylin and mounted. In between steps, slides were washed in PBS. For PTEN, paraffin sections of 4 micrometer thickness, adjacent to the H&E sections used for diagnosis, were mounted onto silanized slides (Dako, Glostrup, Denmark, S3002) and dried overnight at 37^{0}C followed by 1 hour at 60^{0}C. The sections were deparaffinized in xylene and rehydrated in a graded series of alcohol solutions. Antigen retrieval method and antibody dilution were optimized prior to the onset of the study. To ensure uniform handling of the samples, all sections were processed simultaneously. Antigen was retrieved with a highly stabilized retrieval system (ImmunoPrep; Instrumec, Oslo, Norway) at 120 °C and a pressure of 1.9 bar in 10 mM TRIS/1 mM EDTA (pH 9.0) for two minutes at full pressure and cooled for 15 minutes. Immunostaining was performed using an autostainer (DAKO, Glostrup, Denmark). TBS (S1968) 0.05% Tween 20 (pH 7.6) was used as rinse buffer. Endogenous peroxidase activity was blocked by peroxidase blocking reagent S2001 (DAKO, Glostrup, Denmark) for 10 minutes and incubated with the monoclonal antibody PTEN (clone 6h2.1, Cascade Biosciences, Winchester, MA, USA), 1:300, for 30 minutes. DAKO antibody diluent S0809 was used and the immune complex was visualized by Peroxidase/DAB (ChemMate Envision Kit, DAKO, Glostrup, Denmark) with incubation of Envision/HRP, Rabbit anti-mouse (ENV) for 30 minutes and DAB+chromogen for 10 minutes. The sections were counterstained with Haematoxylin, dehydrated and mounted. Controls for the immunostaining were performed using normal endometrial tissue control sections and positive normal cell compartments (i.e., stroma) within test sections. The section adjacent to the sections used for immunostaining was cut and stained with H&E to ensure the presence of the same lesion in all test sections.

Evaluation of staining

PTEN (JB, NH) and other (PvD, NH) stainings were scored by two authors blinded to clinicopathologic data and results of other stainings. For HIF-1α, the percentage of dark, homogenously stained nuclei was estimated as before[19], ignoring cytoplasmic staining. Special attention was given to pattern of staining: diffusely throughout the tumor (thought to be due to non-hypoxic stimuli), perinecrotic (only around a necrotic area, thought to be hypoxia induced) or a combination of these ("mixed"). Using stromal PTEN-signal as a positive control, immunohistochemical PTEN expression in glands was classified as PTEN-expressing (+) or PTEN-null (-). Endometrial tumors that contained any PTEN-null glands were scored as PTEN-"null", even if these co-existed with some PTEN-expressing glands in other areas. If

the intensity of staining was obviously less than other stainings (though present), tumors were scored as +/-. For pVHL, only cytoplasmic staining was scored. For PHDs, and p-AKT, nuclear and cytoplasmic staining was scored. Special attention was given to subcellular localisation of staining in the cell and staining throughout the tumor for pVHL and PHDs.

Statistical Analysis

Correlations between immunohistochemical stainings were analyzed with Kendall's tau-b correlation coefficients when the outcome was ordinal, otherwise Fisher's exact test was used. For differences of expression between groups of HIF-1α staining patterns the Anova test was used. Two sided p-values <0.05 were considered significant. All statistical analyses were performed using SPSS for Windows version 12.0.1., 2003 (SPSS Inc., Chicago, IL, USA).

Results

Immunohistochemical results

Tables 2 and 3 show a summary of the immunohistochemical staining results in endometrial carcinomas. For HIF-1α the earlier described patterns with or without association with necrosis was observed. 3/61 (4.9%) of cases were HIF-1α negative; 11/61 (18.0%) showed a purely perinecrotic staining pattern; 18/61 (29.5%) diffuse; 29/61 (47.5%) showed mixed (diffuse and perinecrotic) patterns. For pVHL, 2 tumors showed membranous perinecrotic expression (figure 1F). The 59 other tumors showed no association with necrosis, and stained diffusely positive, except for 2 cases which showed a extraordinary heterogeneous, mosaic-like pattern (figure 1G). One tumor showed pVHL negative immature squamous parts in a further pVHL-positive tumor (figure 1H). Subcellular localisation of pVHL staining differed between tumors. All tumors expressed pVHL in the cytoplasm, except for one tumor that showed only membrane staining. In summary, in 39 of 61 cases membranous staining was observed, in 19 of 61 nuclear, 5 tumors showed perinuclear staining and in 4 tumors cytoplasmic staining was emphasized at the apical side of the cell. PHD1, PHD2 and PHD3 showed no obvious expression patterns in relation with necrosis. For PHD1, the positive staining was accentuated at the apical site. In one tumor, the squamous parts stained more for PHD1 than the non-squamous parts (figure 1E). For PHD2, 3 tumors stained in their squamous parts of the tumor more nuclei positive than in the non-squamous parts (figure 1D). In one specimen normal non-cancerous glands expressed more PHD2 than the cancer cells (figures 1A-C). As shown in table 2, PHD1 was more abundantly expressed than PHD2 and 3.

Table 2. Main pathological features and pVHL and PTEN immunohistochemical staining results of 61 endometrial carcinomas

		N (%)
Grade	Grade 1	15 (24.6%)
	Grade 2	30 (49.2%)
	Grade 3	16 (26.2%)
Stage	Stage I	28 (45.9%)
	Stage II	17 (27.8%)
	Stage III	12 (19.7%)
	Stage IV	4 (6.5%)
pVHL	+	17 (27.9%)
	++	29 (47.5%)
	+++	15 (24.6%)
PTEN	-	37 (60.7%)
	-/+	5 (8.2%)
	+	19 (31.1%)

Table 3. Results of immunohistochemical staining (percentage nuclear staining) for HIF-1α, phospho-AKT and the three prolyl hydroxylases in 61 endometrial carcinomas.

	Mean	Median	(interquartile range)
HIF-1α	28.28	20	(10 – 50)
p-AKT	54.75	65	(0 – 5)
PHD1	27.89	20	(10 – 35)
PHD2	4.95	2	(0 – 5)
PHD3	5.44	2	(2 – 5)

Correlation HIF-1α and its regulators prolyl hydroxylases and pVHL

pVHL was not correlated to level of HIF-1α expression (HIF-1α cutoff 5, Fisher's exact test, p=0.493). Level of HIF-1α expression was also not correlated to nuclear or cytoplasmic PHD1, PHD2 and PHD3 expression (Kendall's tau correlation; Fisher's exact test p>0.05). Expression of each individual PHD was not different between the 3 groups of HIF-1α staining patterns (Anova test, p > 0.05, see figure 2), although a trend was observed for less PHD1 expression of PHD1 in diffusely HIF-1α expressing cancers. As PHDs show redundance in function[114,123], we also analyzed whether the sum of expression of PHD1, PHD2 and PHD3 was related with HIF-1α staining pattern. Indeed, total PHD expression was higher in HIF-1α perinecrotically stained tumors than in diffusely stained tumors, whereas cancers with a mixed HIF-1α expression pattern showed a total PHDs expression in between (see figure 2D), although this was not statistically significant.

Figure 1. *PHD en VHL immunohistochemical staining on endometrioid endometrial cancers.* ***A****. PHD2 staining. Overview of uterus with tumor (T) and normal (N) tissue, showing less staining of PHD2 in the tumor compared to the normal tissue. This is further illustrated in picture* ***B****, showing higher magnification of the tumor and in picture* ***C****, showing higher magnification of the normal endometrial glands.* ***D****. PHD2 staining showing more expression in the squamous part of the endometrial tumor than in the non-squamous parts.* ***E****. PHD1 staining showing more expression in the squamous part of the endometrial tumor than in the non-squamous parts.* ***F****. pVHL staining which shows cytoplasmic staining in all the tumor glands, though there is an increase of membranous expression in the perinecrotic fields.* ***G****. pVHL staining of an endometrial cancer, which shows a remarkable mosaic-like expression pattern, possibly related to ciliated cell change.* ***H****. pVHL staining, with a cytoplasmic staining in the normal glands which is illustrative for the type of staining observed throughout the tumors, however in this cancer a diminished expression in the squamous parts (S) of the tumor is observed.*

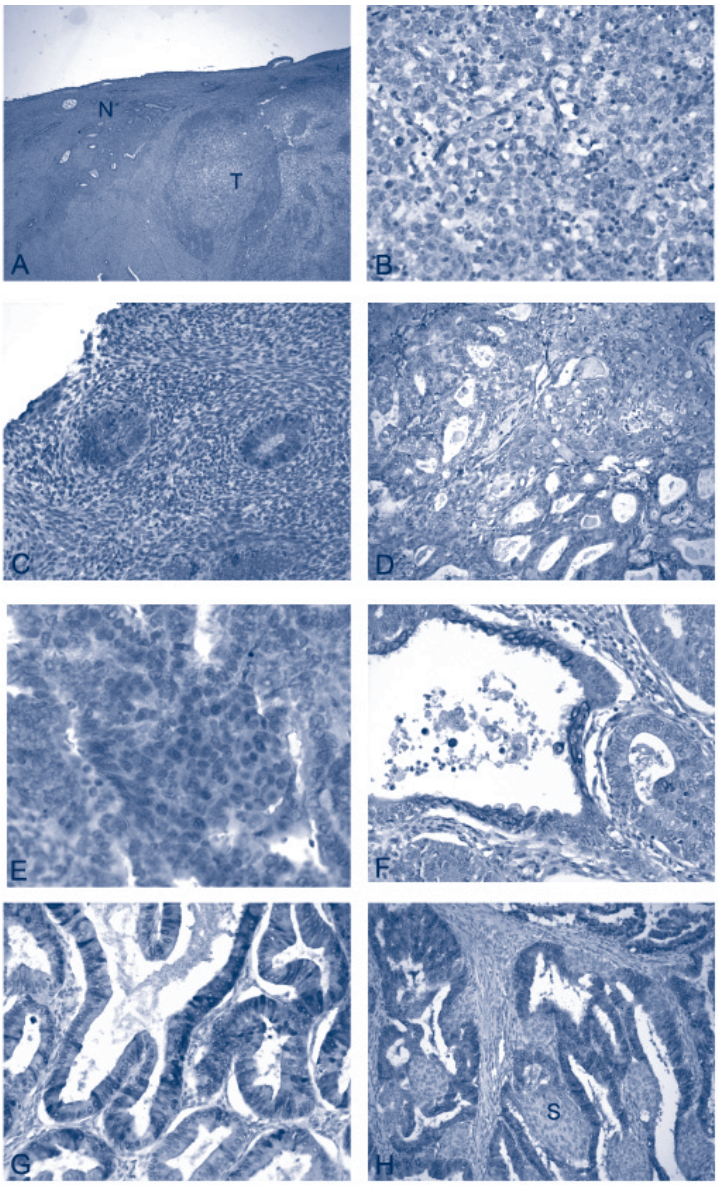
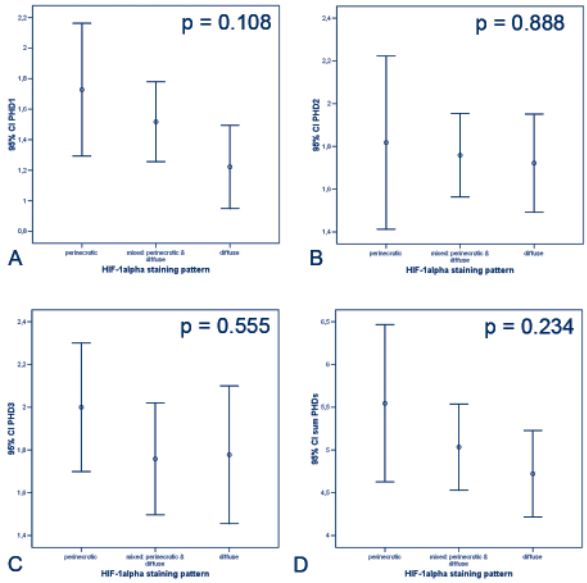


Figure 2. Intensity of cytoplasmic expression of PHD1, PHD2, PHD3 and total PHDs (with error bars) is shwon for the 3 different HIF-1α expression patterns: perinecrotic, mixed (perinecrotic and diffuse) and purely diffuse. p-values are shown for One-way anova tst. ***A.*** *PHD1.* ***B.*** *PHD2.* ***C.*** *PHD3.* ***D.*** *total PHD exprssion.*



Correlation of HIF-1α with PTEN and p-AKT

PTEN was not correlated to p-AKT expression in endometrial carcinomas (nuclear p-AKT cutoff 35, Fisher's exact test, p=0.512; intensity p-AKT, Fisher's exact test, p=0.255). Additionally, expression of PTEN (HIF-1α cutoff 5, Fisher's exact test, p=0.275) and p-AKT (Kendall'tau correlation coefficient -0.009, p=0.926) were not correlated to level of HIF-1α expression, or HIF-1α expression patterns (Fisher's exact, p>0.05).

Discussion

Recently, we described different HIF-1α staining patterns in endometrial carcinoma: expression directly around necrosis (perinecrotic staining) due to hypoxia, and a diffuse expression throughout the tumor thought to be related to aberrations in oncogenes and tumor suppressor genes rather (or a combination of these)[121]. In this study we evaluated different oncogenes and tumor suppressor genes that known to be involved in HIF-1α regulation for their role in diffuse HIF-1α expression in endometrial cancer. PTEN, p-AKT and pVHL were not associated with diffuse HIF-1α expression. However, there was a trend for less expression of PHD1 and total PHD expression in diffusely stained HIF-1α tumors. All three PHDs have the potential to hydroxylate HIF-1α *in vitro* so that it can be targeted for degradation by pVHL, although PHD2 is described to be most important[124,125]. It is shown *in vitro* that PHD2 and PHD3 (but not PHD1) are upregulated in response to hypoxia[124,126-132], probably HIF-1α-dependent[124,126,129,131]. *In vivo* experiments with rats have shown that systemic hypoxia elevates the

expression of PHD2 and HIF-1α in the brain[132], which makes sense as PHD2 contains a hypoxia-responsive element in the promoter[129]. Increased PHDs in hypoxic cells could be part of a negative feedback mechanism for the rapid hydroxylation and degradation of HIF-1α when oxygen supplies are restored[128]. Moreover, Siah1 and -2, the specific E3 ligases of PHD1 and 3, are transcriptionally up-regulated during hypoxia, thereby elevating the degradation of these PHDs by the proteasome[133]. It is therefore noteworthy that in endometrial carcinomas PHDs staining is not altered in necrotic regions (supposed to be hypoxic) compared to other parts of the tumor, although such a pattern is very clear for HIF-1α expression. However, we did observe increase of expression of PHD1 and the total expression of the PHDs in perinecrotic stained HIF-1α tumors. These tumors might be more hypoxic than the diffusely stained tumors and therefore may express overall more PHDs. However, only PHD2 and PHD3 seem to be directly upregulated by hypoxia, not PHD1. Possibly, hormone receptors play a role, as it is shown that PHD1 is estrogen inducible in breast cancer cell lines[134]. In addition, differences in PHD expression in response to hypoxia have been found between cell lines, tissues and organs, although endometrial cells have not been studied[114,135,136]. This could explain the increase of PHD1 we found, contrary to other results on hypoxia.

Of the prolyl hydroxylases, PHD1 was the one most expressed in the nucleus of endometrial cancers. This is in concordance with *in vitro* data, which showed that PHD1 was more expressed in the nucleus than PHD2 and PHD3[125,126]. Soilleux et al were the first to study PHD1 in several tissues, including endometrium, in which they observed some nuclear expression[137]. PHD2, thought to be the predominant isoform which regulates HIF-1α, was mainly expressed in the cytoplasm, which is in agreement with earlier *in vitro* results[125,126]. It might be that PHD2 is lost during carcinogenesis and malignant formation of the tumor, as we observed in one specimen less expression in the tumor than in the adjacent normal glands. This could be due to inactivating mutations or promoter methylation. Whether this could lead to higher HIF levels is not clear, as the PHDs show redundancy in function and the other PHDs might compensate for this loss. Furthermore, PHD2 was significantly less expressed in higher grades of endometrial cancer (data not shown), which is in contradiction to an earlier study in squamous cell carcinomes of the head&neck in which PHD2 was more expressed in higher grades[138]. In addition to possible loss of PHD2, mutations of these proteins may play a role as well. PHD2 was mutated in 60% of 20 endometrial cancers[139]. Introduction of wild-type PHD2 (EGLN1) into endometrial cancer cell lines that carry PHD2 gene mutations induced senescence. This was invoked through the negative regulation of HIF-1 expression[139]. For PHD3 we observed more cytoplasmic than nuclear staining, which has also been described for other tissues[126,137,140].

pVHL has not been studied in endometrial carcinomas before. In our samples pVHL staining did not correlate to HIF-1α staining as it did in colonic adenomas[141]. Although we observed differences in amount of pVHL staining, none of the tumors was pVHL-negative. Therefore we might conclude that pVHL loss does not seem to play a role in endometrial carcinogenesis. Cellular localisation of pVHL has been studied in cell culture: epitope-tagged pVHL has been observed in either the nucleus or the cytoplasm of cultured cells, depending on the density of the

cell culture and the study. Furthermore, Corless et al. reported mainly cytoplasmic subcellular localisation of pVHL *in vivo* in normal and neoplastic tissues[142], which is in contradiction to our observations: we also observed nuclear, perinuclear and membranous staining. Our observations might be more realistic as pVHL is considered to shuttle between the cytoplasm and nucleus, enabling HIF-1α degradation in both compartments[143]. The membranous staining observed by us, has also been shown in kidney tumors[144] and in adenocarcinomas of the gastroesophageal junction[145]. In the cytotrofoblast, subcellular localisation of pVHL (by electron microscopy) was observed in periplasma membrane vacuoles, considered to be the post-Golgi compartment[146]. In the kidney tumors, membranous staining was associated with missense mutations. We only observed clear membranous staining in 2 tumors, only around necrosis (thought to be hypoxic areas of tumors). In cytotrofoblasts, hypoxia upregulated pVHL expression *in vitro*, although *in vivo* opposite results were seen[146]. Furthermore, hypoxia upregulates VHL in renal cancer and pancreatic but not in glioblastoma cells[147]. We therefore propose that the perinecrotic membranous staining rarely seen in endometrial tumors, might be induced by hypoxia. Additionally, mosaic pVHL staining was observed in 2 tumors. As we observed such a pattern for p16 in atrophic endometrium with tubal metalpasia[148], and pVHL has been shown to be important for ciliogenesis[149], this pattern might be related to ciliated cell change.

PTEN is considered to be one of the most frequent early events in endometrioid endometrial carcinogenesis[117-120]. Although 60.7% of the carcinomas showed extensive loss of the tumor suppressor gene PTEN by immunohistochemistry, this was not correlated to increase of HIF-1α expression. PTEN's relation with cancer appears to be centered mainly around its ability to antagonize the phosphatidylinositol 3,4,5-trisphosphate (PIP_3) kinase activity, negatively modulating the PIP_3-AKT signaling pathway (AKT is dependent on PIP_3 for its activation). Normally, PTEN dephosphorylates PIP_3 at the 3 position of inositol, yielding PIP_2. Decreased activity of PTEN increases PIP_3 and AKT, leading to increased cell proliferation, cell survival and tissue growth[150]. Therefore, AKT has been implicated as a major factor in many types of cancer. In our samples PTEN did not correlate to p-AKT levels, for which we do not have a good explanation. We expected to find correlations between PTEN expression and HIF-1α, as loss of function of PTEN in a glioblastoma derived cell line increased HIF-1α levels and HIF-1α mediated gene expression[48]. Furthermore, in colorectal cancers, decreased levels of PTEN mRNA were significantly associated with increased expression of HIF-1α mRNA. Gort et al showed *in vitro* that dominant-active AKT restored HIF-1α expression, which was endorsed by results in human breast cancer tissue[49]. Therefore, it is notable that this correlation is not observed in our panel of endometrioid endometrial carcinomas. As there is a difference in degree of induction of HIF-1α between hypoxia and active AKT[50], this might be below the levels detectable by immunhistochemistry, especially, when hypoxia also plays a role. Secondly, there are studies indicating that stabilisation of HIF-1α by the PIP3-AKT pathway is cell type dependent[151,152], which could also help to explain our results. In summary, not PTEN, p-AKT, or VHL, but the PHDs might be related to the diffuse, non-hypoxia related expression of HIF-1α often seen in endometrial cancers.

Acknowledgements

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chapter 4

HIF-1alpha gene mutations are associated with higher microvessel density and AKT activation in endometrioid endometrial carcinomas

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Abstract

Purpose

Hypoxia-Inducible Factor 1 alpha (HIF-1α) is the key survival protein that is stabilised in response to hypoxia. In previous studies a C1772>T transition leading to Pro582>Ser substitution in the oxygen-dependent degradation domain (ODDD) of HIF-1α, has been associated with unfavourable clinicopathological features in different types of cancer. The aim of this study was to search for mutations in the ODDD of the HIF-1α gene in endometrioid endometrial cancer, and to associate these with clinicopathologic characteristics.

Methods

A series of 58 endometrioid endometrial cancers were examined for mutations within the ODDD of HIF-1α by PCR amplification and DNA sequencing. Non-wild type HIF-1α genotype in cancers was compared with normal tissue of the same patient. HIF-1α genotype in our patient groups was compared with literature controls. Furthermore, correlations between HIF-1α genotype variability and clinicopathologic features and downstream HIF-1α effects (microvessel density (MVD) and Vascular Endothelial Growth Factor (VEGF) expression) and p-AKT expression were studied.

Results

In 9/58 (15.3%) cases we identified a (known) polymorphism C1772>T present in tumor and normal tissue. In one patient we found the C1772>T transition as a de novo mutation which was not present in matched control DNA. When comparing the frequency of these HIF-1α mutations with those in healthy controls, P582S mutations were not associated with increased risk for endometrial cancer. Tumors with P582>S substitution did not show higher HIF-1α expression, but MVD and p-AKT activity was higher in these tumors.

Conclusion

In summary, the P582>S genotype variation in the ODDD of HIF-1α occurs at relatively normal frequency in our study and can occur as a *de novo* somatically acquired mutation in endometrial cancer. The P582>S genotype does not increase risk for endometrioid endometrial cancer, however, this genotype is significantly associated with a higher MVD and AKT activity.

Introduction

Hypoxia-Inducible Factor 1 (HIF-1) is a transcription factor complex which plays a key role in the hypoxic response and has been associated with tumor aggressiveness in many cancers, including endometrial cancer[35,36,101]. HIF-1α and HIF-1β together form the active HIF-1 complex which binds the hypoxia-response elements of various target genes[54-56] (e.g., glucose transporter, VEGF, growth factors, genes involved in apoptosis, gluconeogenesis and erythropoiesis) to induce their expression.

Under normoxic (4-21%O_2) conditions, the oxygen–dependent prolyl 4-hydroxylases (PHDs) hydroxylate HIF-1α protein on proline residues 402 and 564 within the oxygen dependent degradation (ODDD) domain. Proline hydroxylation of HIF-1α is required for binding, of the Von Hippel Lindau (VHL) E3 ligase complex, that polyubiquitinates HIF-1α marking it for proteosomal degradation[44,112,113]. The ODDD is encoded by aminoacids 401 – 603 of the HIF-1α protein. During hypoxia the activity of PHDs is attenuated leading to loss of VHL binding to HIF stabilization binding leading to stabilization of HIF-1 that can induce target gene expression[153].

In addition to hypoxia induced HIF-1α expression, a more diffuse, non-hypoxic HIF-1α staining has also been observed in tumors[101,121]. Diffuse HIF staining might be caused by oncogene activation or tumor suppressor gene loss of pathways regulating HIF stability such as seen in Von-Hippel Lindau disease[154,155]. For example, HIF-1α protein synthesis is enhanced upon activation of the phosphatidylinositol 3-kinase (PI3K)-AKT (protein kinase B)-pathway[48,51], however, this may be cell type restricted In contrast hypoxia has also been shown to induce AKT expression[151,156,157], and was detected after HIF-induction[151].

As has recently been shown that HIF-1α ODDD mutations increase risk for developing cancer[158-160] and are associated with unfavourable tumor features and a worse prognosis[161-163], we hypothesized that mutations in the ODDD of HIF-1α[47,164] affecting proline hydroxylation might also account for non-hypoxic HIF-1 activity in endometrial cancer. Therefore we screened endometrioid endometrial carcinomas for genetic alterations within the HIF-1α ODDD by PCR amplification and DNA sequencing. Correlations with tumor characteristics important for prognosis[6], p-AKT expression, microvessel density and expression of VEGF as major HIF-1α downstream gene were sought.

Materials and Methods

Patient and tissue selection

Paraffin-embedded clinical specimens from endometrioid endometrial carcinoma (n=61) were selected from the archives of the Department of Pathology of the University Medical Center, Utrecht, The Netherlands. These tissues were derived from patients operated between 1992 and 2005. None of the carcinoma patients received preoperative radio- or chemotherapy. Haematoxylin and eosin-stained sections were revised and histologically typed and graded (see table 1). The tumor stage and grade was defined by the International Federation of Gynecology and Obstetrics (FIGO) system[6]. Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our hospital[99].

Table 1. Main pathological features of 61 endometrioid endometrial cancers analyzed for HIF-1α ODDD mutations.

Pathological characteristics		N(%)	
Grade	Grade 1	15	(24.6%)
	Grade 2	30	(49.2%)
	Grade 3	16	(26.2%)
Myometrial invasion	<=50%	27	(44.3%)
	> 50%	34	(55.7%)
Stage	Stage I	28	(45.9%)
	Stage II	17	(27.8%)
	Stage III	12	(19.7%)
	Stage IV	4	(6.5%)

DNA isolation

For DNA isolation, an average of ten 10-µm sections were sectioned and placed on uncoated slides and dried overnight. After deparaffination, slides were washed twice in methanol, rehydrated by ethanol and hematoxylin stained. On the basis of a marked hematoxylin and eosin-stained serial section, regions containing carcinoma were scraped off (mesodissection) and collected in eppendorf tubes. Subsequently, DNA isolation was performed as described earlier[165] with the QIAamp DNA minikit (Qiagen, Valencia, CA, USA).

HIF-1α ODDD mutation analysis

Polymerase chain reaction (PCR) was performed in a 20 µL reaction containing 2.0 µL PCR buffer 10x, 1.2 µL 25 mmol/L $MgCl_2$, 0.2 µL 25 mmol/L dNTPs, 1.0 µL 10 µM each of forward and reverse primer, 0.15 µL 5 U/µL AmpliTaq DNA polymerase (Roche), 12.95 µL DEPC-treated water and 1.0 µL 20 ng/µL template genomic DNA. (Semi-) nested PCR (2.5 µL first product) was performed in 50 µL reactions containing the same concentrations of components.

For exon 9, a first PCR was performed with the forward primer 5'-gttgaaatggggcttgtagc-3' and reverse primer 5'-gcacgacttgattttctccc-3'. The nested PCR was performed with the forward primer 5'-caagtagcctctttgacaaac-3' and the reverse primer 5'-gcaacagacacaatttaggg-3'. Amplification was performed with an initial denaturation

step of 5 min at 95°C followed by 35 cycles of 92°C for 15 sec, 63°C for 1 min (first reaction) or 57°C (nested reaction), and 72°C for 1 min, followed by a final elongation step of 10 min at 72°C.
For exon 12 (codons 527–603), a first PCR was performed using forward primer 5'-acagatttagacttggagatg-3' and reverse primer 5'-cgttagggcttcttggatga-3'. The semi-nested PCR was performed with the same forward primer and reverse primer 5'-gtgatgatgtggcactagtag-3' .
Amplification was performed with an initial denaturation step of 5 min at 95°C followed by 35 cycles of 92°C for 15 sec, 60.5°C for 1 min and 72°C for 1 min, followed by a final elongation step of 10 min at 72°C.
PCR products were analyzed by 2% agarose gel electrophoresis. Negative controls (water) were run in each experiment. Before sequence analysis, the amplified fragments were purified using the Qiagen PCR purification kit. After a BigDye terminator reaction, sequencing was done on an ABI PRISM 3130xl Genetic Analyzer. Aberrations from wild type were always confirmed by a second experiment with DNA from a separate PCR as template. For cases with polymorphisms or mutations, normal tissue was isolated and also analyzed. The results were compared to the wild type HIF-1α DNA sequences using BLAST (available from URL http://www.ncbi.nlm.nih.gov/BLAST).

Immunohistochemistry

HIF-1α, VEGF-A, p-Akt and microvessels were immunohistochemically stained on serial 4 µm paraffin slides as described before[49,121]. Table 2 lists all antibodies, dilutions, incubation times and antigen-retrieval methods used. Immunohistochemical staining was carried out manually. Slides were deparaffinized with xylene and serial ethanol dilutions. Endogenous peroxidase activity was blocked for 15 minutes in a buffer solution pH 5.8 (containing 8.32 g citric acid, 21.52 g disodium hydrogen phosphate, 2 g sodium azide in 1 liter of water) with hydrogen peroxide (0.3%). This was followed by antigen retrieval. Next, slides were incubated with the primary antibody, followed by the secondary. Finally, peroxidase activity was developed with 3,3'-diaminobenzidine (DAB) for 10 minutes, slides were counterstained with hematoxylin and mounted. In between steps, slides were washed in PBS. Positive and negative controls were used throughout (see table 2).

Evaluation of Staining

Stainings were scored blinded to clinicopathologic data and results of other stainings. For HIF-1α, the percentage of dark, homogenously stained nuclei was estimated as before[121], ignoring cytoplasmic staining. Special attention was given to pattern of staining: diffuse (throughout the tumor), perinecrotic (only around a necrotic area, thought to be hypoxia induced) or a combination of both ("mixed").
MVD was scored as previously described[121]: the most hypervascular areas ("hot spots") were selected under low magnification in the CD31 stained slides. Herein, microvessels were counted in the 4 most hyper vascular adjacent fields at a magnification of 20x (the 'hot-spot'- method). The total area counted was 3.80 mm2, and MVD values were expressed per mm^2.

Table 2. Overview of the antibodies used and tissue processing details

Primary Antibody	Type	Source	Dilution	Antigen Retrieval	Second step	Positive control	Incubation time / temp (primary antibody)
HIF-1α	MoAb	BD Pharmingen	1:50	EDTA, pH 9.0, 20 min, 97°C	Powervision	Breast Cancer	Overnight / 4°C
VEGF	PoAb	R&D Systems	1:50	Citrate, pH 6.0, 20 minutes, 97°C	R-aG IgG + StrepAB	Endothelium (internal)	60 minutes / room temp
CD31	MoAb	Dako	1:40	Citrate, pH 6.0, 20 minutes, 97°C	Powervision	Endothelium (internal)	60 minutes / room temp
p-AKT	PoAb	Abcam	1:200	Citrate, pH 6.0, 20 minutes, 97°C	Powervision	Breast cancer	60 minutes / room temp

Abbreviations used: HIF-1α = hypoxia-inducible factor-1α, VEGF = vascular endothelial growth factor, MoAb = monoclonal antibody, PoAb = polyclonal antibody; Powervision = Powervision ready to use (Poly-HRP-anti Ms/Rb/RtIgG biotin free, ImmunoLogic, ImmunoVision Technologies, Brisbane, CA, USA); R-aG IgG = biotinylated Rabbit-anti Goat IgG (E0466, Dako) + StrepAB = Streptavidin peroxidase labeling (K0377, Dako). Sources: BD Pharmingen, BD Biosciences, San Diego, CA, USA; R&D Systems abingdon, Oxon, UK; Dako, Glostrup, Denmark; Abcam, Cambridge, UK.

Statistical Analysis

To compare genetic variations found, appropriate European controls (to diminish racial differences) were collected from the literature. To test for differences in genotype, the Fisher's exact test was used. χ2-Test was used to compare the alleles. To study differences between genotype and HIF-1α expression and downstream targets, the Kruskal-Wallis and Mann-Whitney U tests were used. Fisher's exact test was used to compare genotype with grade, stage, myometrial invasion and HIF-1α pattern. Two sided p-values <0.05 were considered significant. All statistical analyses were performed using SPSS for Windows version 12.0.1., 2003 (SPSS Inc., Chicago, IL, USA).

Results

HIF-1α ODDD mutations

Of 61 tumors with paraffin material available for DNA isolation, two and three tumors respectively did not yield sufficient high quality DNA for analysis of exon 9 and exon 12 leaving 59 and 58 tumors presented here.

In exon 9 (containing proline 402) no genetic variability (mutations or polymorphisms) was found in any of the 59 tumors. Table 3 and Figure 1 show the results of sequencing exon 12. No mutations were found at proline 564. Furthermore, the known polymorphism as result of nucleotide G>A substitution at position 1790,

was also not seen in this dataset. The A2024>G polymorphism not leading to an aminoacid change (L580L), is a known SNP (rs 34005929), and found in one case (see Figure 1).
6/58 cancers (10.3%) exhibited the heterozygous C/T 1772 polymorphism, resulting in an aminoacid change at position 582 changing a Proline to a Serine (i.e. P582>S), known as SNP rs11549465. 5 out of 6 cases matched control tissue (cervix or parametrium) carried the same c/t pattern. In one patient, however, the normal parametrial tissue was wild type, indicating that the tumor acquired the P582>S mutation somatically. In 3/58 (5.1%) of the cases the T/T 1772 allele was present homozygously in tumor and normal tissue. To determine whether the HIF P582>S SNP was associated with increased risk for endometrial cancer we compared the frequency of P582>S in our panel with its prevalence in the normal population by mining public SNP databases. In addition to the records from the NCBI (European reference) and Hapmap (Hapmap-CEU) (both available from URL: http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs =11549465) per May 2007, we included the controls of a British study on renal cell carcinoma with blood of renal patients as a control[166] and a Swedish study on colorectal cancer with blood from healthy controls[161].

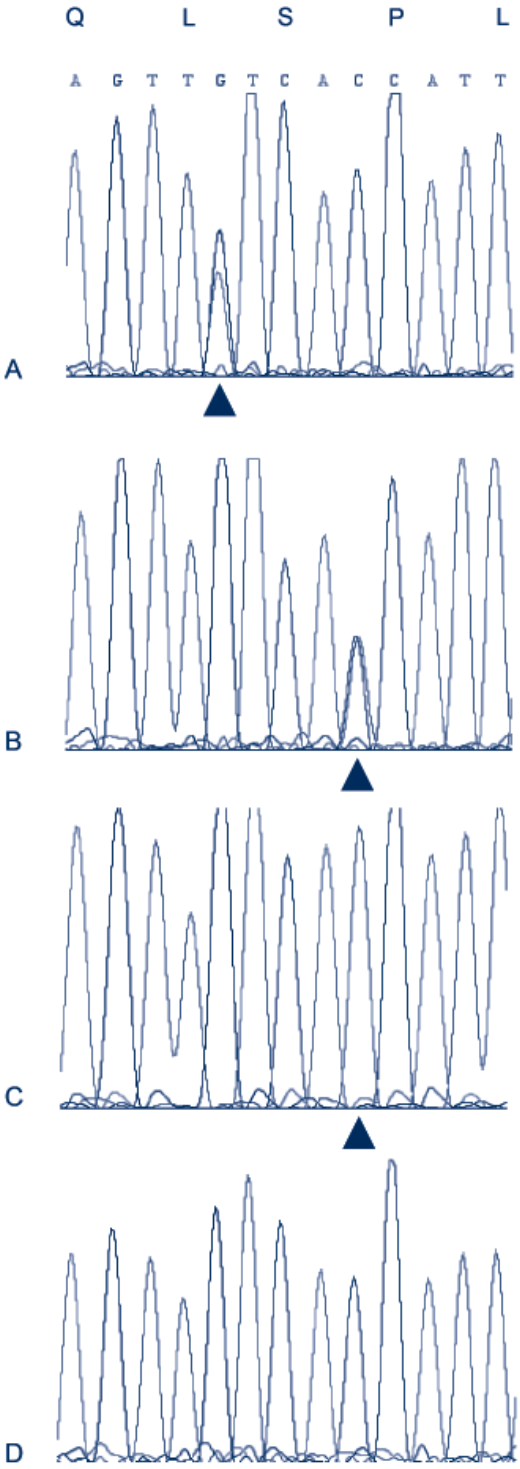
Table 3. *Frequency of heterozygous (C/T) and homozygous (T/T) substitutions on nucleotide 1772 of the HIF-1a gene to result in the P582>S mutation in endometrial carcinomas in comparison with controls from the literature.*

		Endometrial cancer (n=58)		Literature controls (n=559)		Test for differences
		N	(%)	N	(%)	
Genotype frequencies	C/C	50 *	(86.2 %)	463	(82.8 %)	Fisher's exact-test p=0.142
	C/T	5 **	(8.6 %)	84	(15 %)	
	T/T	3 **	(5.1 %)	12	(2.1 %)	
Allele frequencies	C	105	(90.5 %)	1010	(90.3 %)	χ2-test p= 1.0
	T	11	(9.5 %)	108	(9.7 %)	

** including 1 tumors with heterozygous c/t germline polymorphism, homozygous c/c1772 in the tumor due to a de novo somatic mutation. This patient was analyzed in the c/c group as the genetical change in the tumor was a mutation and not a SNP for the patient.*
*** genotype in tumor and non-cancerous tissue identical*

This resulted in a total of 559 controls. Table 3 shows the comparison. Because we were interested in the increased risk of P582>S mutations for endometrial cancer, the P582>S heterozygous case due to a *de novo* mutation in the tumor was analyzed as C/C (wild-type genotype), as we cannot exclude that this mutation has developed late in the carcinogenesis, and might therefore not be a first factor for increased 'risk'. Fisher's exact testing revealed no difference in genotype frequencies (p=0.142) and χ2-testing no difference in allele frequencies (p=1.0) between endometrial cancers and literature derived controls.

Figure 1. *Examples of sequencing of the oxygen dependent degradation domain in HIF-1a in endometrioid endometrial carcinomas, showing polymorphisms or mutations. At the top of the figure aminoacids and nucleotides are written of the part of the HIF-1a gene. Arrowheads indicate genetic variances.* ***A.*** *A2024>G which results in a silent SNP L580L*
B. *heterozygous C1772>T leading to P582>S* ***C.*** *homozygous T/T1772 leading to P582>S* ***D.*** *wildtype: homozygous C/C1772 P582 and A/A2024 L580.*

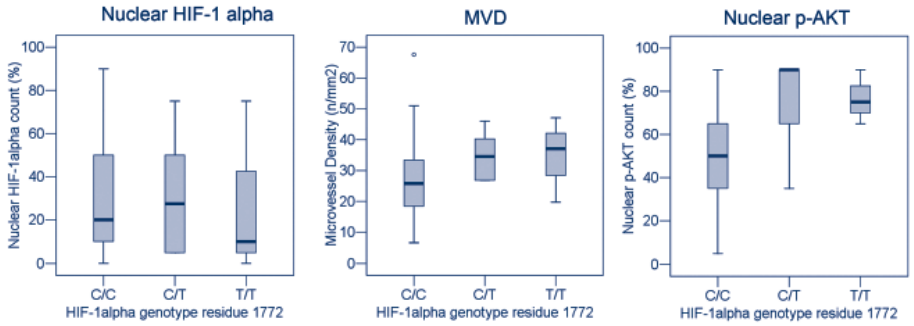

Q L S P L
A G T T G T C A C C A T T
A
B
C
D

MVD and expression of HIF-1α, VEGF and p-AKT in carriers of SNP P582>S and non-carriers (P582)

3/61 (4.9%) of cases were HIF-1α negative; 11/61 (18.0%) showed an exclusively perinecrotic staining pattern; 18/61 (29.5%) a diffuse and 29/61 (47.5%) showed mixed (diffuse and perinecrotic) patterns. Of 6 C/T tumors, 1 showed perinecrotic HIF-1α expression, 4 mixed and 1 diffuse. Of 3 T/T tumors, 1 tumor was negative for HIF-1α, 1 showed a mixed pattern and the other a diffuse pattern of HIF-1α expression. Although only 1 tumor with a non-wild type genotype showed perinecrotic staining, this was not statistically significant (p=0.951).

VEGF and MVD correlated significantly with one another (Kendall's tau-b 0.233, p = 0.023). Cancers with C/C1772, C/T1772 or T/T1772 showed no difference in amount of expression of HIF-1α (see Figure 2) nor in VEGF (p=0.144 for 3 groups and p=0.158 for 2 groups). However, we found that the P582>S allele (heterozygous and homozygous) conferred significantly higher microvessel densities than wild type (C/C) endometrial cancers (Mann-Whitney U-test, p=0.039), Figure 2) Furthermore, p-AKT expression was significantly higher in the groups with this SNP (see Figure 2). The clinicopathological characteristics grade, stage and myometrial invasion did not differ among genotypes.

Figure 2. Boxplots showing association of C to T substitution at position 1772 of exon 12 of HIF-1α (giving rise to a P582S) with nuclear HIF-1α and p-AKT expression and MVD in endometrioid endometrial carcinomas. p-values of tests for differences between the groups are shown. The Kruskall-Wallis test is used to analyze differences between the 3 groups, the Mann-Whitney U test is used to analyze for differences between C/C on one hand and C/T with T/T on the other hand.



C/C vs C/T vs T/T (Kruskall-Wallis test)	p = 0.825	p = 0.116	p = 0.024
C/C vs C/T and T/T (Mann-Whitney U test)	p = 0.948	p = 0.039	p = 0.006

Discussion

In patient studies, specific mutations and polymorphisms in the HIF-1α ODD domain have been investigated that may lead to HIF-1α expression under non-hypoxic conditions, especially at residue P582[164]. Therefore, we analysed whether polymorphisms or mutations in exons 9 and 12 of the HIF-1α gene were associated with the extent and pattern of HIF-1α expression in endometrial cancers, pathological

tumor characteristics, MVD, p-AKT and the HIF-1α downstream target VEGF, a potent angiogenic factor.
In none of the samples analyzed changes in exon 9 (including the regulatory Proline 402) were found. As far as we know, we are only the second group to search for mutations in exon 9 of HIF-1α. Percy et al could not find any mutations in exon 9 in 55 patients with idiopathic erythrocytosis[167]. The proline in this exon therefore does not seem to be a carcinogenetic target in these tumors.
In exon 12, all samples were wild type for P564, the regulatory proline for hydroxylation of HIF-1α. However, we found the silent exon 12 polymorphism A2024>G (L580L) in the tumor and noncancerous tissue of the patient, therefore indeed a SNP. Interestingly, heterozygous and homozygous C1772>T transitions were found in 6 cases in exon 12 of the HIF gene resulting in an amino acid change from Proline 582 to Serine. In 5 of these cases, the heterozygous change appeared to be germline inherited. Interestingly in one tumor the C1772>T transition was due to a somatic mutation. This has previously been found in 2 studies on prostate cancer[168,169], indicating that this mutation may be a tumorigenic event.
To assess whether P582>S carriers have an increased risk for endometrioid endometrial cancer, we compared genotype of our patients to control European data from the literature. Although we cannot exclude the possibility that some of these controls developed endometrial cancer, we found that the P582>S substitution was not more frequent in patients with endometrial cancer. Therefore we conclude that HIF P582>S carriers are not at increased risk for endometrial cancer. In 3 out of 7 previous studies it was demonstrated that P582>S genotype was associated with increased risk for prostate[158,159] and colorectal cancer[160], but in the 4 remaining studies, no increase in tumor incidence of P582>S genotype was shown in colorectal cancer cases[161], squamous cell cancers of the head and neck[162], esophagus[163], and renal cell carcinoma[166]. In one study on breast cancer, only homozygous wild type P582 cancers were found[165]. Furthermore, in idiopatic erytrocytosis, a proliferative disorder of red blood cells, the P582S substitution was not more frequent in patients compared to normal healthy controls[167].
Three of the four studies that did not show increased cancer risk of P582>S, did show correlations with adverse clinicopathological features such as larger tumors, higher rate of lymph node metastases, ulcerative growth and worse survival[161-163]. The 3 studies which found higher incidence of cancer in P582>S carriers, found no associations with pathological characteristics or survival[158-160].

In the present study, the P582>S genotype was not associated with an increase in expression of HIF-1α or its downstream target VEGF. However, we found a higher MVD and p-AKT expression in both heterozygous and homozygous C1772T carriers compared with wild type. The phosphatidylinositol 3-kinase (PI3K)-AKT (protein kinase B)-pathway is known to augment HIF-1α[48,51]. Conversely, hypoxia also leads to AKT activation[151,156,157]. It will be interesting to investigate whether the P582>S mutations have an impact on p-AKT activity in hypoxic endometrial cancers.
HIF proteins carrying the P582>S mutation have been shown to have increased transactivation capacity in different mammalian cell based systems[162,169,170]. Furthermore, Fu et al., showed that HIF-1 P582>S mutants are more stable under normoxic than wild type HIF[169]. Since P582 is not hydroxylated by PHD2 under the conditions where PHD2 hydroxylates P564 and not necessary for complex

formation with VHL[167] other mechanisms must account for the increased stability of HIF-1 P582>S. Also, it has been shown that P582 is not necessary for complex formation with VHL after hydroxylation of P564 by PHD2[167]. However, the other prolyl hydroxylases could play a role here.
Presently, we cannot explain the increased MVD, in the absence of VEGF induction. Possibly difficulties in scoring slides, or relative small sizes of groups play a role. However, increased MVD and activated AKT point to increased transactivation of HIF-1 in our study. The significance of P582>S substitution for tumorigenesis is highlighted by our finding of a de novo mutation only present in endometrial cancer suggesting selection for this variant during tumor progression .

In summary, the P582S genotype variation in the oxygen dependent degradation domain of the HIF-1α protein may occur as a *de novo* mutation in endometrial cancer, and is associated with higher microvessel density and AKT activation. This is possibly caused by a higher transactivation activity of HIF-1α. The P582>S substitution, however does not appear to increase risk for endometrioid endometrial cancer. It will be interesting to determine whether P582S carriers although not at higher risk for endometrial cancer develop more aggressive cancers with worse clinical outcome as wild type carriers.

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chapter 5

Progressive derailment of cell cycle regulators in endometrial carcinogenesis

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Abstract

Background

Derailments of the control mechanisms of the cell cycle can initiate carcinogenesis, and play a role in progression to cancer.

Aim

To explore the expression of cell cycle proteins in normal, premalignant and malignant endometrial lesions representing the morphologically well defined stepwise model of human endometrial carcinogenesis

Methods

Observational study. Paraffin-embedded specimens from inactive endometrium (n=16), endometrial hyperplasia (n=23) and endometrioid endometrial carcinoma (n=39) were stained immunohistochemically for cyclin A, cyclin B1, cyclin D1, cyclin E, cdk2, p16, p21, p27, p53 and Ki67(MIB-1)). Differences in expression between the tissues, and correlation with classical prognostic factors for the carcinomas were analysed.

Results

Expression of cyclin A and Ki67 gradually increased from normal through hyperplasia to carcinoma, indicating that proliferation increases over the carcinogenetic spectrum. cdk2, p16 and p21 gradually increased from normal through hyperplasia to carcinoma, indicating their potential importance in both early and late carcinogenesis. Cyclin D1, E and p53 especially increased and p27 decreased from hyperplasia to carcinoma, underlining their role in late carcinogenesis. In cancers, expression of cyclin A, p53 and Ki67 was positively correlated to grade, and cyclin A was positively correlated with cdk2, p21, Ki67, cyclin E and p53.

Conclusion

During (endometrioid) endometrial carcinogenesis, there is increasing proliferation paralleled by progressive derailment of cyclin B1, cyclin D1, cyclin E, p16, p21, p27, p53, and cdk2, indicating the importance of these cell cycle regulators in endometrial carcinogenesis.

Introduction

The normal premenopausal endometrium is characterized by a cyclic pattern of proliferative and secretory phases, controlled by ovarian steroid hormones. Cellular proliferation during these phases is a tightly controlled process[171,172]. After menopause, proliferation of the endometrium comes to a stop and turns to inactive endometrium, a resting state.

Aberrations in the normal cycling of cells lead to uncontrolled proliferation and hence, to hyperplasia and sometimes to cancer. The cell cycle consists of four phases, G1, S, G2, M, controlled by cyclins, cyclin-dependent kinases (CDKs), cyclin-dependent kinase inhibitors (CKIs) and several other tumor suppressor gene products. Cyclins form a complex with cdks enabling transition to the next phase of cell cycle. This will induce cell growth, unless inhibited by tumor suppressor gene products such as p53 or CDK-inhibitors (CKIs), such as $p16^{INK4A}$, $p21^{WAF-1/CIP-1}$ and $p27^{KIP-1}$. Cyclin D1 with its catalytic partner cdk4 functions as cells leave G0 and progress through G1. The transition of G1 to S is controlled by cyclin E/cdk2 and cyclin D1/cdk4 complexes, while p27 and p21 inhibit cyclin E, and p16 and p21 inhibit cyclin D1. In the transition of S to G2, cyclin A/cdk2 play a role, with the CKIs p27 and p21 exerting effects here as well. In G2/M cyclin B/cdk1 and cyclin A/cdk1 are functional.

Overexpression of cell cycle stimulating factors such as the CDKs and cyclins, and aberrant expression of inhibiting factors such as the cki's are frequently found in tumours. This is often associated with a more malignant phenotype, a higher proliferation rate, recurrence and a worse survival in different tumours, including endometrial carcinoma[59-67].

Endometrial cancer is the most common malignant tumor of the female genital tract. Estimated incidence of cancer in the uterine corpus in the US was 40,880 for 2005 (6% of all cancers), with an estimated probability of developing uterine cancer of 1 in 38[86]. Endometrial carcinoma is mainly a disease of postmenopausal women. Endometrioid adenocarcinoma is the most frequent type of endometrial cancer, thought to derive from the inactive endometrium through hyperplasia[13]. Hyperplasias comprise a heterogeneous group of lesions, some thought to be reversible and some to be truly neoplastic, so attempts have been made to subclassify these lesions in a biologically and clinically meaningful way. Studies have revealed a low reproducibility of classifying hyperplasias according to the World Health Organization (WHO) nomenclature[13,19,21]. An alternative classification method was therefore proposed making a distinction between endometrial hyperplasia caused by reversible estrogen effects and Endometrial Intraepithelial Neoplasia (EIN) considered to be a real precancerous lesion[21-23], with PTEN inactivation as an important event in progression[24].

Expression of cell cycle regulators has been studied before in endometrial carcinoma, but a comprehensive analysis of expression of cyclins, cdk's and cki's in a progression model of hyperplasia to endometrioid endometrial carcinoma has not been performed, especially not with regard to EIN. We therefore studied the expression of cyclin A, cyclin B1, cyclin D1, cdk2, p16, p21, p27, p53 and the proliferation marker Ki67(MIB-1) in normal endometrium, hyperplasia (subclassified as EIN/non-EIN) and endometrioid adenocarcinoma of the endometrium, and correlated expression with some relevant clinicopathologic features.

Materials and Methods

Patients and Tissues

Paraffin-embedded clinical specimens from inactive endometrium (IE, n=16, all postmenopausal), endometrial hyperplasia (EH, n=23, including 13 non-EIN and 10 EIN cases) and endometrioid adenocarcinoma (EC, n=39, one with a mucinous component and one with a serous component) were selected from the archives of the Department of Pathology of the University Medical Center, Utrecht, The Netherlands. All specimens from the years 1991-2004 with diagnosis inactive endometrium, hyperplasia or endometrioid endometrial cancer were selected and revised until enough consecutive samples for the group were retrieved.

Sample size was based on a power calculation (PS Power and Sample Size Calculation, version 2.1.30) that revealed that with an α of 0.010 (to correct for multiple comparisons), a power of 0.85, an expected difference in population means of 10, a within group standard deviation of 10 and an equal size over the groups, the estimated sample size was 28. As we expected the standard deviation in the IE group to be rather low, a sample size less than 28 was accepted for IE, and as standard deviation to be higher in EC, we enlarged the EC group size to 39.

None of the carcinoma patients received preoperative radio- or chemotherapy. Table 1 gives an overview of the patient demographics and main pathological features.

Haematoxylin and eosin-stained sections were revised and histologically typed by 2 experienced gynecopathologists (PvD, DSG). Endometrial hyperplasia was further subdivided according to the World Health Organization (WHO) nomenclature and recategorized as endometrial intraepithelial neoplasia (EIN) or non-EIN/hyperplasia[21,98]. For carcinomas, the tumor stage and grade were defined by the International Federation of Gynecologists and Obstetricians (FIGO) system, furthermore the presence of myometrial invasion was determined.

Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our hospital[99].

Immunohistochemistry

Cyclin A, Cyclin B1, Cyclin D1, cdk2, p16, p21, p27, p53 and Ki67(MIB-1) were immunohistochemically stained on 5 µm thick paraffin slides. Table 2 presents all antibodies, dilutions, incubation times and antigen-retrieval methods used. Slides were deparaffinized with xylene and rehydrated in serial ethanol dilutions, and endogenous peroxidase activity was blocked followed by antigen retrieval. After antigen retrieval, a cooling-off period of 20 minutes preceded the incubation of the primary antibodies. In between incubation steps, slides were washed in PBS. Finally, peroxidase activity was developed with DAB, and slides were counterstained with hematoxylin and dehydrated in alcohol and xylene. Positive controls were used throughout (see Table 2). Negative controls were obtained by omission of the primary antibodies from the staining procedures.

Table 1. Patient demographics and main pathological features of a group of lesions representing the endometrioid carcinogenetic spectrum (N=78)

Feature		N	(%)
Age	Mean	59.25	
	Median	58.57	
	Minimum	31	
	Maximum	85	
Histologic diagnosis	Inactive endometrium	16	(20.5%)
	Hyperplasia	23	(29.5%)
	Carcinoma	39	(50.0%)
Hyperplasia (WHO)	Simple without atypia	7	(30.4%)
	Simple with atypia	0	(0.0%)
	Complex without atypia	9	(39.1%)
	Complex with atypia	7	(30.4%)
EIN/hyperplasia	Non-EIN/hyperplasia	13	(56.5%)
	EIN	10	(43.5%)
Grade of carcinoma	Grade 1	6	(15.4%)
	Grade 2	21	(53.8%)
	Grade 3	12	(30.8%)
Stage of carcinoma	I	13	(33.3%)
	II	10	(25.6%)
	III	11	(28.2%)
	IV	5	(12.8%)
Myometrial invasion	< 50%	16	(41.0%)
	> 50%	22	(56.4%)
	Unknown	1	(2.6%)

Evaluation of Staining

Two authors (PvD, NH) scored all slides blinded to clinicopathologic data and results of other stainings. For cyclin B, cyclin D1, cyclin E, cdk2, p16, p21, p27, p53 and Ki67(MIB-1) the percentage of dark, homogenously stained nuclei was estimated as before, ignoring cytoplasmic staining[100]. In the inactive endometrium, areas of tubal metaplasia were skipped. In the two mixed carcinomas, only the endometrioid areas were considered.

Table 2. Overview of the antibodies used and tissue processing details

Primary Antibody*	Source**	Dilution	Antigen Retrieval	Detection†	Positive control	Incubation time / temp (primary antibody)‡	Procedure
cyclin A	Novocastra	1:100	Citrate pH 6.0	Strep AB(1)	Tonsil	o/n 4°C	Manual
cyclin B1	Abcam	1:400	Pepsin	Chemate Envision	Tonsil	o/n 4°C	Manual
cyclin D1	Novocastra	1:20	EDTA pH 9.0	Strep AB(2)	Mantle Cell Lymphoma	60 minutes / room temp	Autostainer
cyclin E	Novocastra	1:50	Citrate pH 6.0	Strep AB(1)	Placenta	o/n 4°C	Manual
Cdk2	Neomarkers	1:100	EDTA pH 9.0	PV	Tonsil	60 minutes / room temp	Manual
p16	Neomarkers	1:160	EDTA pH 9.0	PV	Cervix carcinoma	60 minutes / room temp	Autostainer
P21	Dako	1:25	EDTA pH 9.0	PV	Colon	60 minutes / room temp	Manual
P27	Transduction	1:500	Citrate pH 6.0	PV	Skin	o/n 4°C	Manual
P53	Dako	1:400	Citrate pH 6.0	Strep AB(1)	Serous endometrial carcinoma	o/n 4°C	Manual
MIB-1 (Ki67)	Immunotech	1:100	Citrate pH 6.0	Strep AB(2)	Tonsil	60 minutes / room temp	Autostainer

** All primary antibodies used are monoclonal antibodies.*
*** Novocastra, Newcastle upon Tyne, UK; Abcam, Cambridge UK; Neomarkers, Fremont USA; Dako, DakoCytomation, Glostrup, Denmark; Transduction, BD Transduction Laboratories, BD Biosciences, San Diego, CA, USA; Immunotech, Beckman Coulter, Fullerton, CA, USA.*
† Strep AB (1) = biotinylated rabbit-anti-mouse, diluted 1:500 in PBS, Dako, followed by streptavidin-biotin complex, diluted 1:200 in PBS, Dako; Strep AB(2) = biotinylated horse-anti-mouse, diluted 1:500 in PBS, Vector BA-2000, followed by streptavidin-biotin complex, diluted 1:1000 in PBS, Immunotech; PV = Powervision ready to use (Poly-HRP-anti Ms/Rb/RtIgG biotin free, ImmunoLogic, ImmunoVision Technologies, Brisbane, CA, USA); CheMate EnVision Detection kit, DakoCytomation, Glostrup, Denmark.
‡ o/n = overnight

Statistical Analysis

Cases were divided into 6 groups representing the progression spectrum of endometrial lesions: IE, non-EIN hyperplasia, EIN, and carcinoma grades 1 to 3. Level of expression of the cell cycle proteins throughout this progression spectrum was analysed with Spearman's rho correlation (SPSS for Windows version 12.0.1., 2003 (SPSS Inc., Chicago, IL)). To correct for multiple comparisons after Spearman's rank correlation we used the Bonferroni-Holm step down test with modification by Simes[173], with an α of 0.05.

Furthermore, to focus on differences between relevant adjacent groups, differences in medians and their 95% confidence intervals (and 99% CI if significant), based on the method proposed by Bonett and Price[174] were counted.

Spearman's rank correlation was used to correlate markers in endometrial carcinoma with each other, a p-value of ≤0.05 was considered significant.

Results

Expression of cell cycle proteins in inactive endometrium, hyperplasia and carcinoma

Figure 1 shows representative examples of the expression of all proteins stained in this study. For all stainings (table 3), differences between IE, non-EIN EH, EIN and EC were significant (Spearman's rho correlation, also after modified Bonferroni-Holm correction). For cyclin A, cyclin B1, cyclin D1, cyclin E, cdk2, p16, p21, p53 and Ki67 a stepwise increase in expression from IE via EH to EC was noticed. This is graphically shown in figure 2 by dotplots. Except for cyclin B1 and p16, differences in expression for all other stainings between EH and EC were significant, with a 95% confidence interval. p27 was significantly less expressed in EC than in EH, however, a trend towards higher expression in higher grades of cancer was noticed. Expression of p21 was higher in EC than in EH.

Table 3. *Expression of cell cycle-related proteins over the spectrum of lesions representing the endometrioid carcinogenetic route from inactive endometrium (IE), endometrial hyperplasia (EH, subdivided as non-Endometrial Intraepithelial Neoplasia (EIN) and EIN) and different grades of endometrioid endometrial carcinoma (EC). Medians (with interquartile range) are shown and Spearman rho correlation coefficient (R) and p-value are shown.*

	IE	EH		EC			correlation	
	(n=16)	*Non-EIN (n=13)*	*EIN (n=10)*	*grade 1 (n=11)*	*grade 2 (n=17)*	*grade 3 (n=11)*	*R*	*p-value***
cyclin A	1.5 (1-2)	5 (2-5)	2 (1-2)	5 (2–5)	20 (5-27.5)	20 (10-50)	0.785	<0.001
cyclin B1*	2 (0-5)	2 (0.5-10)	7.5 (1.5-31.25)	35 (10-50)	15 (2.75-35)	5 (5-20)	0.337	0.003
cyclin D1	0 (0-0)	0 (0-0.5)	0 (0-4)	2 (0-5)	2 (0.5-5)	1 (0-2)	0.494	<0.001
cyclin E	1 (0-5)	2 (0-2)	1 (0-5)	5 (0-10)	10 (5-20)	10 (5-20)	0.532	<0.001
cdk2	0 (0-2)	2 (1-5)	2 (0.75-8.75)	5 (2-10)	5 (5-20)	50 (2-65)	0.594	<0.001
p16	0 (0-0)	0 (0-3.5)	10 (5-20)	5 (2-35)	10 (5-10)	20 (1 – 35)	0.630	<0.001
p21	2 (1.25-5)	2 (2-2)	7.5 (2-10)	10 (5-20)	20 (5-35)	10 (2-35)	0.509	<0.001
p27	35 (6.25-50)	35 (10-57.5)	27.5 (8-67.5)	5 (2-20)	10 (2-20)	20 (5-50)	-0.259	0.022
p53	0 (0-0)	0 (0-2)	0 (0-2.75)	2 (0-2)	5 (0.5-5)	10 (5-75)	0.644	<0.001
Ki67 (MIB1)	2 (1-4.25)	5 (2-7.5)	2 (1-10)	5 (5-10)	20 (15-50)	20 (10-50)	0.632	<0.001

** 1 missing case for inactive and carcinoma*

*** All p-values significant after Bonferroni-Holm step down test with modification by Simes.*

Hyperplasia/EIN

Significant differences in expression between non-EIN/hyperplasia and EIN were seen for, cyclin A, p16 and p21 (Table 4). Cyclin A was higher expressed in non-EIN/hyperplasia than in EIN, and for p16 and p21 the reverse trend was seen. Cyclin A, cyclin E and Ki67 and p53 were significantly higher expressed in EC than in EIN.

Figure 1. *Representative examples of stained slides. Columns, from left to right: inactive endometrium, hyperplasia, endometrioid endometrial carcinoma. Rows: cyclin A (I), cyclin B1 (II), cyclin D1 (III), cyclin E (IV), cdk2 (V), p16 (VI), p21 (VII), p27 (VIII), p53 (IX), Ki67 (X).*

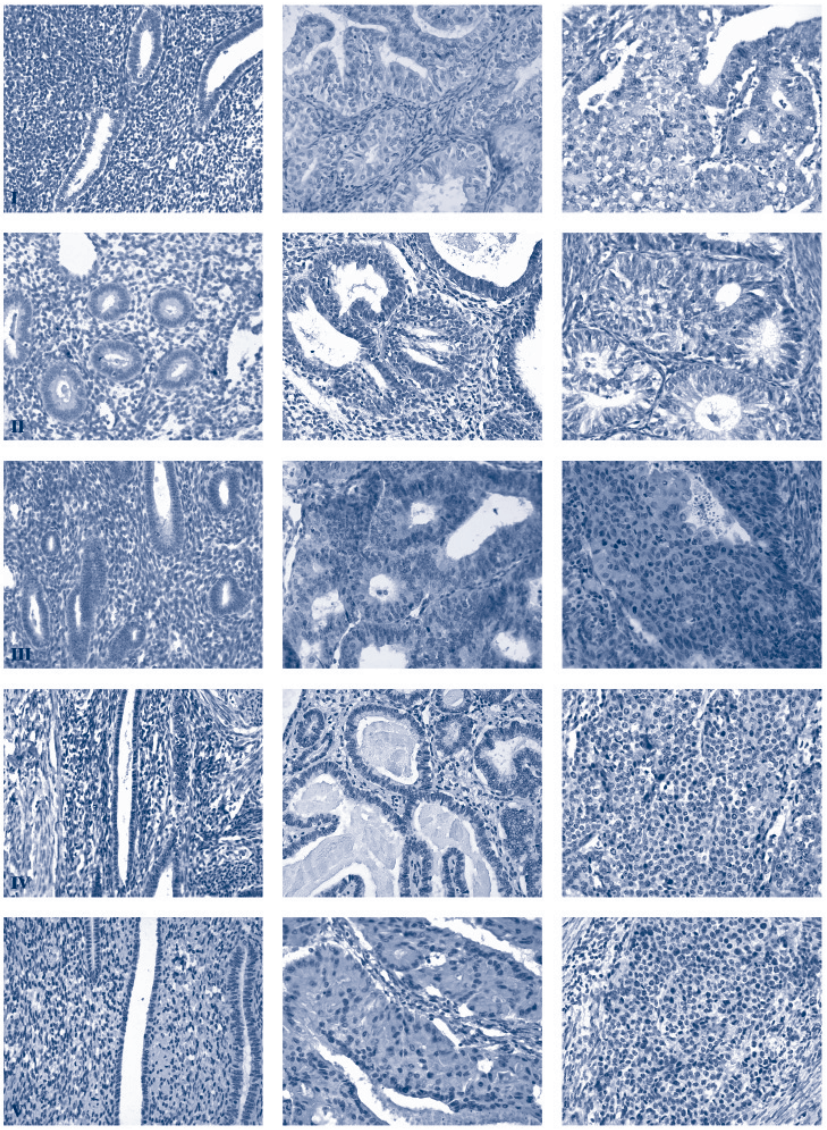

Fig. 1.1

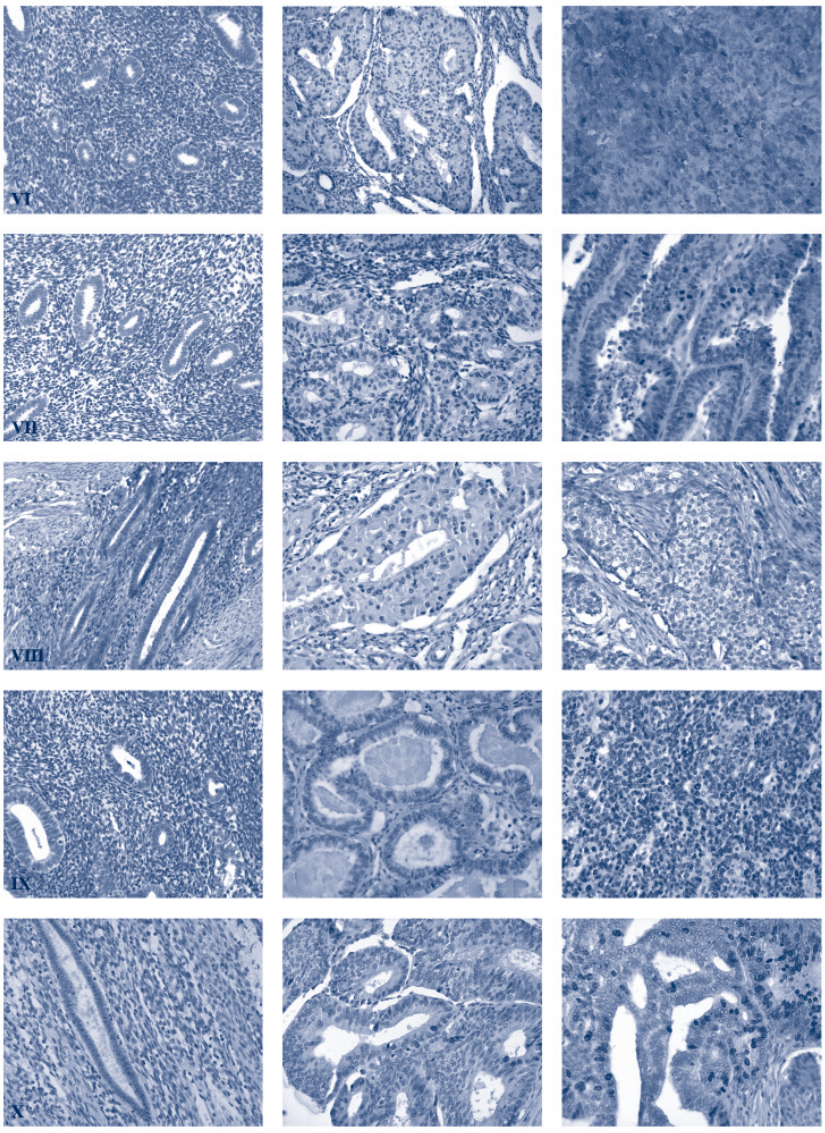

Fig. 1.2

Figure 2. Dotplots for expression of different cell cycle regulators over the spectrum of lesions representing the endometrioid endometrial carcinogenesis. Group 1 = inactive endometrium, 2 = non-EIN hyperplasia, 3 = EIN, 4 = carcinoma grade 1, 5 = carcinoma grade 2, 6 = carcinoma grade 3. Triangle: no myometrial invasion, Circle: < 50% myometrial invasion, Square: ≥ 50% myometrial invasion.

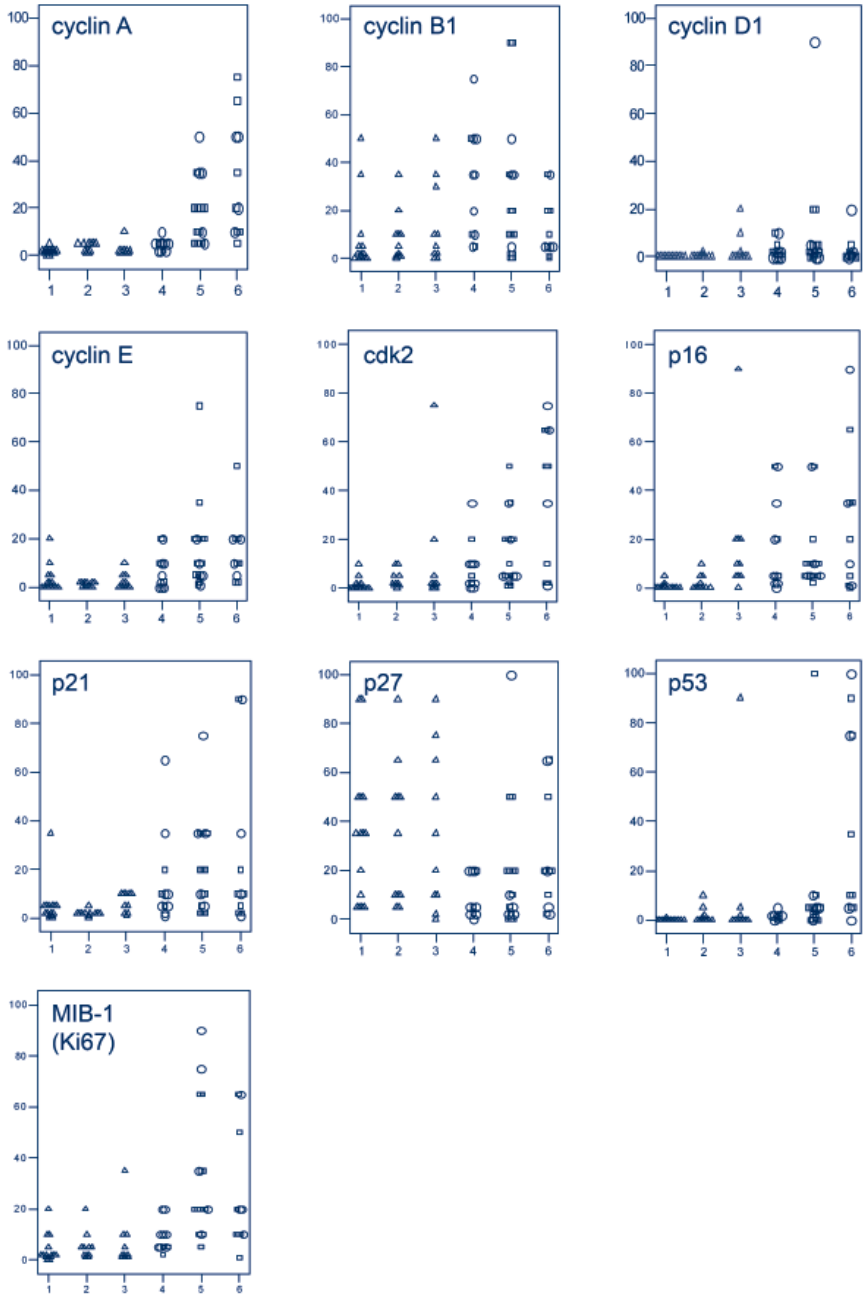

Table 4. Expression of cell cycle related proteins over the spectrum of lesions representing the endometrioid carcinogenetic route. Presented are the differences in medians (DM, with 95% confidence intervals) for the 3 most interesting transitions: between inactive endometrium (IE) and non-EIN (Endometrial Intraepithelial Neoplasia) hyperplasia, between non-EIN hyperplasia and EIN, and between EIN and endometrial carcinoma (EC) grade I. In addition, the differences between the 3 lumped groups (IE vs EH, EH vs EC) are given.

	IE and non-EIN EH	Non-EIN EH vs EIN	EIN and EC grade 1	IE vs EH	EH vs EC
cyclin A	3.5 (2.1 – 4.9)*	-3.0 (-4.4- -1.6)*	3.0 (1.35–4.65)*	0.5 (-0.96–1.96)	8 (0.27 – 15.73)
cyclin B1**	0 (-4.9 – 4.9)	5.5 (-10.0-21.0)	27.5 (1.5–53.5)	3.0 (-1.69– 7.69)	10 (-4.45 – 24.45)
cyclin D1	0 (-0.4 – 0.4)	0 (-4.3-4.3)	2.0 (-3.0–7.0)	0 (0-0)	2 (1.49 – 2.51)*
cyclin E	1 (-1.3 – 3.3)	-1.0 (-3.3-1.3)	4.0 (-1.7– 9.7)	1.0 (-1.32-3.32)	8 (0.34 – 15.66)
Cdk2	2 (0.08 – 3.9)	0 (-8.7-8.7)	3.0 (-6.5–12.5)	2.0 (-0.04-4.04)	8 (0.17 – 15.83)
p16	0 (-2.2 – 2.2)	10.0 (3.3 – 16.7)*	- 5.0 (-23.7–13.7)	5.0 (0.36-9.64)	5 (-3.91 – 13.91)
p21	0 (-1.3 – 1.3)	5.5 (2.1 – 8.9)*	2.5 (-6.2–11.2)	0 (-1.89-1.89)	8 (0.27 – 15.73)
p27	0 (-29.1-29.1)	-7.5 (-46.6 – 31.6)	- 22.5 (-55.1–10.1)	0 (-25.2-25.2)	-25 (-4.94 – -45.06)
p53	0 (-0.9 – 0.9)	0 (-2.3 – 2.3)	2.0 (-0.4 – 4.4)	0 (-0.93-0.93)	5 (3.22 – 6.78)*
Ki67 (MIB1)	3 (-0.5 – 6.5)	-3.0 (-8.2 – 2.2)	3.0 (-1.7 – 7.7)	3 (1.54-4.46)*	15 (9.74 – 20.26)*

** Show also significance at 99% confidence interval.*

*** 1 missing case for inactive and carcinoma*

Correlation of cell cycle proteins and clinicopathologic data in carcinomas

There were no significant relationships between the expression of cyclin B, cyclin D1, cyclin E, cdk2, p16, p21, and p27 on the one hand and tumor grade on the other. However, a significant positive relationship existed between grade on the one hand and p53, cyclin A and Ki67 on the other (Kruskal-Wallis test). For myometrial invasion, defined as less or more than 50% invasion of the myometrium, and for stage of cancer no associations were found with any of the cyclins, cdk's or cki's.

Correlation of the cell cycle regulatory proteins in endometrial carcinomas

In EC, significant positive correlations by Spearman's rank correlation were found for cyclin E with cdk2 (p=0.001), p21 (p=0.015) and with p16 (p=0.020). Additionally, borderline significance was noticed for the positive correlation between cyclin D1 and p21 (p=0.056). Cyclin A positively correlated with cdk2 (p<0.001), p21 (0.030), p53 (p=0.03), cyclin E (p=0.028) and Ki67 (<0.001). Furthermore, positive correlations were found for p21 with cdk2 (p<0.001) and Ki67 (p=0.042), and Ki67 with cdk2 (p=0.015, and p53 with p27 (p=0.050). One inverse association was found: between cyclin B1 and p27 (p=0.008).

Discussion

We investigated the expression of cell cycle proteins in the endometrioid carcinogenetic spectrum represented by IE, EH, and endometrioid EC. IE was used as the normal counterpart, as EC is a disease of postmenopausal women and cell cycle regulators will inherently be highly variable in proliferative and secretory

endometrium. Additionally, correlations with clinicopathological data were sought. We here also discuss changes found on the protein level with genomic changes in genes that were previously found to be involved in endometrioid endometrial carcinogenesis like PTEN, CTNNB1, ras and mismatch repair genes[28].

Expression of cyclin A and Ki67(MIB-1) gradually increased from normal through hyperplasia to carcinoma. As these proteins are expressed in proliferating cells and the underlying genes have not been proven to show alterations predisposing to cancer, these events should be regarded to reflect that proliferation increases over the carcinogenetic spectrum[175].

Cyclin B1, a regulator of G2/M transition, has previously been shown to be upregulated in EH[175] and EC[172,176-178], we also showed an increase, although not statistically significant. Cyclin D1 was absent in IE, in accordance with previous studies[177,179-181], and was as also found by others upregulated in EH[65,182] and EC[65,180-186]. Activated RAS, and β-catenin and PTEN mutations can cause upregulation of cyclin D1 and play a major role in endometrial cancer. In endometrial carcinomas, immunohistochemical nuclear expression of β-catenin indeed correlated positively with that of cyclin D1[187-189] This better explains cyclin D1 overexpression than amplification of CCND1, the cyclin D1 gene, as this occurs in only 2% of endometrioid endometrial carcinoma cases[182]. Moreno-Bueno et al.[189] did not find associations between β-catenin, K-RAS or PTEN mutations and cyclin D1 overexpression in endometrioid endometrial carcinomas, although they did observe that cyclin D1 tended to be expressed in areas where β-catenin nucleocytoplasmic expression also occurred. Microsatellite instability has also been associated with overexpression of cyclin D1 in endometrioid EC[189].

Cdk2, p16 and p53 gradually increased from normal through hyperplasia to carcinoma, indicating their potential importance in both early and late carcinogenesis.

Gradually increasing expression of p16 observed by us are in accordance with Tsuda et al.[68] Abnormally low but usually abnormally high expression of p16 mRNA and protein has been observed in carcinomas in other studies[68-71,177,190-193]. The p16 gene may be mutated or deleted or show promoter methylation in the endometrium[68-70,76,190,194] leading to loss or reduced immunohistochemical p16 expression[68,71,74]. Mechanisms leading to p16 overexpression are less clear, although Saegusa et al.[195] concluded, based on their *in vitro* and *in vivo* data, that p16 is under transcriptional control of nuclear β-catenin. Increased expression of β-catenin could therefore possibly explain the p16 overexpression.

Loss of wild-type p53 function predisposes to malignant transformation as damaged cells may enter the S-phase of the cell cycle without appropriate DNA repair. p53 mutations often result in conformationally altered and functionally defective protein with a longer half-life than the wild type counterpart, thus permitting immunohistochemical detection of the accumulated protein product[196]. However, the correlation between immunohistochemical nuclear p53 accumulation and gene mutations is not absolute, with a reported concordance of 76% for endometrial carcinomas[197]. Normal tissue is immunohistochemically (nearly) negative for p53 as we and others have shown[65,180,181,198]. No or few positive cells were found in EH by us and others[76,77,195-198], and our endometrioid endometrial carcinomas, like those in other studies[65,177,180,181,198,199] often showed p53 accumulation. Cdk2, less studied than the other cell cycle-related proteins, was found in the majority of endometrial tumors[65,177,184,186] We observed that cdk2 increased during carcinogenesis and was

associated with cyclin E, cyclin A, p21 and Ki67(MIB-1) in EC. Therefore, cdk2 might play an important role in inititation and progression of EC.

Cyclin E and p21 increased and p27 decreased from EH to EC, underlining their potential role in late endometrial carcinogenesis. Cyclin E complexes with cdk2 to move the cell cycle forward from late G1 to S phase. Earlier studies also found weak expression of cyclin E in normal endometrial glandular cells, but increased expression in some EH[59,77,186] and most EC[59,65,77,177,184,200,201].Increased cyclin E expression can result from amplification of the CCNE gene that has been found occasionally in endometrioid carcinomas[77] or mutations in the hCDC4 gene, coding for a protein which tags phosphorylated cyclin E for ubiquitination and proteosomal degradation; such mutations have been found in EC[77,202] p21 is a p53 and cyclin D1[203] regulated potent inhibitor of CDKs and can thereby inhibit the phosphorylation of Rb. Our finding of increased expression in EC is in accordance with the literature[65,177,179,181,184,204,205]. In view of its function, one would however expect loss of expression of p21 to be a carcinogenetic event. Likely, p21 overexpression in endometrial lesions is merely a reflection of a failing feedback attempt of a normal protein rather than the result of an alteration in the p21 gene. For the cki p27, loss of expression has been found in EH[206,207] but especially in EC[65,72,198,206-209]. Interestingly, we also noticed an increasing trend in expression of p27 in higher grades of carcinomas. p27 expression loss is rarely caused by structural alterations of its gene but rather posttranslationally regulated by ubiquitin-proteasome-dependent degradation mechanisms, e.g. caused by overexpression of skp2 that promotes degradation of p27 when it is not associated with cdk2, found in EH and EC[63,72]. Associations between p27 expression and clinicopathologic features and survival of EC have been sought by several studies, however, these have not been found in most studies[65,198,206-208,210], except for two[209,211]. Upregulation of p27 induced by PTEN has been reported in different cell lines, suggesting that p27 may be a target of the PTEN cell cycle arrest pathway in EC[208]. As mutations in the PTEN gene occur early in pathogenesis of endometrioid EC[212], this might be an explanation for decreasing p27.

We considered the EIN subclassification that resulted in two groups that were large enough for robust statistical analysis. p16 and p21 were significantly more expressed in EIN than in non-EIN/hyperplasia. This provides, in addition to PTEN, further biological evidence for the EIN classification that may help to discriminate between reversible and neoplastic EH.

In conclusion, during (endometrioid) endometrial carcinogenesis, there is increasing proliferation paralleled by progressive derailment of cyclin D1, cyclin E, p16, p53, and cdk2, indicating the importance of these cell cycle regulators in endometrial carcinogenesis. In practice, p16 and p21 may be useful to identify the dangerous EH lesions that require treatment.

Acknowledgements

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chapter 6

The invasive front in endometrial carinoma: higher proliferation and associated derailment of cell cycle regulators

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Abstract

Objective

To explore whether expression of proliferation and hypoxia-related proteins differs in the central parts and the invasive front in endometrial carcinomas.

Methods

Proliferation associated proteins Ki67 and cyclin A, cell cycle regulators p16, p21, p53, cyclin D1, cyclin E, and cdk2, and Hypoxia Inducible Factor 1α and its downstream factors Glucose transporter 1, Carboanhydrase IX, and Vascular Endothelial Growth factor, were immunohistochemically stained in paraffin-embedded specimens from endometrioid (n=33), mucinous (n=1) and serous (n=5) endometrial carcinomas. The percentages of positive cells at the invasive front and central tumor parts were scored and compared.

Results

Ki67 ($p<0.001$), cyclin E ($p=0.018$), p16 ($p=0.003$) and cdk2 (0.001) were higher expressed at the invasive front than centrally (Wilcoxon Signed Ranks test). Higher expression of these antigens at the invasive front was seen in 31/38 cases for Ki67, in 16/39 cases for cyclin E, in 15/39 cases for cdk2, and in 11/39 cases for p16. The other cell cycle proteins and the hypoxia related factors did not show significant differences in expression between the central parts and the invasive front.

Conclusion

Endometrial carcinomas clearly show an invasive front that is characterized by higher proliferation and progressive derailment of the cell cycle regulators cyclin E, p16 and cdk2, but not by an increased hypoxic response.

Introduction

For a tumor to become malignant, several phenomena are important of which the following are perhaps the most essential: proliferation outweighing cell death, cellular dedifferentiation, the ability to invade the environment and to metastasize. This ability of cancer cells to invade the surrounding stroma and vessels is linked to patient prognosis due to associations with advanced local invasive tumor growth and distant spread. Tumour invasion is a complex process that involves cell attachment, proteolysis of matrix components, migration of cells through the disrupted matrix, proliferation and angiogenesis[102].

As to the latter two phenomena, aberrations in the regulation of cell cycle control lead to uncontrolled proliferation, and angiogenesis is to a large extent regulated by the hypoxia response. The cell cycle, which is progressively deregulated during carcinogenesis, is controlled by cyclins like cyclins A, D1 and E, cyclin-dependent kinases (CDKs) like cdk2 and cdk4, cyclin-dependent kinase inhibitors (CKIs) like p16, p21 and p27, and tumor suppressor gene products like p53. Overexpression of cell cycle stimulating factors such as the CDKs and cyclins, and downregulation of inhibiting factors such as CKIs are frequently found in tumours, including endometrial carcinoma. It is described that they are correlated with a more malignant subtype, a higher proliferation rate, recurrence and a worse survival in different tumours[59-62,64-67].

HIF-1α is the key regulator of the hypoxia response[213], and has been implicated in carcinogenesis and cancer progression in many different epithelia including the female epithelia of the endometrium[35] and breast[100,214]. HIF-1α is a transcription factor upregulating many genes[215], that are involved in adaptation to low glucose levels like the glucose transporter Glut-1, carboanhydrase IX (CAIX) that regulates pH[87], and vascular endothelial growth factor (VEGF) that is one of the most potent inducers of angiogenesis[216].

Endometrial cancer is the most common malignant tumor of the female genital tract. Estimated incidence of cancer in the uterine corpus in the US was 40,880 for 2006 (6% of all cancers), with an estimated probability of developing uterine cancer of 1 in 38[86]. Endometrial cancer derives from the endometrial epithelial lining of the uterine corpus. During cancer progression, some endometrial cancers grow as polyps, but most invade downwards into the myometrium. Conceptually, the deepest parts of the tumors invading the myometrium must be the most active and aggressive ones showing highest proliferation, invasive behaviour and angiogenesis. Such an "invasive front" has been described in e.g. female breast cancer where highest proliferation is known to be in the periphery of the tumor[217], but has not been evaluated yet in endometrial cancer. While analysing expression of cell cycle related proteins during endometrial carcinogenesis in a previous study[218], we noticed a tendency for some proteins to be preferentially expressed at the invasive front of endometrial carcinomas. The aim of the present study was to perform a comprehensive analysis of the topographical expression of cell cycle regulators and proteins related to the hypoxia response, comparing central tumor parts with the invasive front.

Materials and Methods

Patients and Tissues

Paraffin-embedded specimens from endometrioid endometrial carcinoma (n=33), mucinous carcinoma (n=1) and serous endometrial carcinoma (n=5) with tumor and myometrium on one slide, were selected from the archives of the Department of Pathology of the University Medical Center, Utrecht, The Netherlands. These tissues were derived from patients operated between 1992 and 2004. None of the patients received preoperative radio- or chemotherapy.

Haematoxylin and eosin-stained sections were revised and histologically typed by 2 experienced gynecopathologists (PvD, DSG). Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our hospital[99].

Immunohistochemistry

MIB1 (against Ki67), p16, p21, p53, cyclin A, cyclin D1, cyclin E, cdk2, HIF-1α, Glut-1, CAIX and VEGF were immunohistochemically stained on 5 µm thick paraffin slides as extensively described before[100,214]. Table 1 presents all antibodies, dilutions, incubation times and antigen-retrieval methods used. For all stainings, slides were deparaffinized with xylene and rehydrated in serial ethanol dilutions, and endogenous peroxidase activity was blocked followed by antigen retrieval.

For HIF-1α, endogenous peroxidase was blocked by hydrogen peroxide (Dako CSA kit), after which antigen retrieval followed. A cooling off period of 30 minutes preceded blocking of the avidin by biotin block (Dako; 10 min) and protein block (Dako; 5 min). Then, the primary antibody was applied followed by the catalyzed signal amplification system (Dako CSA kit). Slides were washed in Tris buffer in between.

For all other stainings, endogenous peroxidase activity was blocked for 30 minutes, followed by antigen retrieval. Subsequently, slides were incubated with the primary antibody, followed by the secondary antibody. Finally, peroxidase activity was developed with DAB and counterstained with hematoxylin. In between steps, slides were washed in PBS.

Positive controls were used throughout, see table 1 for types of tissue. Negative controls were obtained by omission of the primary antibodies from the staining procedure.

Evaluation of Staining

The invasive front was defined as the deepest rim of cancerous tissue grown into the myometrium with a width of one full field of vision at a x20 magnification, corresponding with 1.22 mm. No difference regarding direction of growth, lateral or cranial, was made. Expression of MIB1, cyclin A, cyclin D1, cyclin E, p16, p21, p53, cdk2 and HIF-1α was assessed by a single observer (NH) as the mean percentage of estimated positive cells in 4 fields of vision in the center of the tumor and 4 fields at the invasive front, at a x20 magnification. In the same areas, membranous expression of CAIX and Glut-1 was scored as absent or present, and VEGF expression was semiquantitatively scored as -/+/++/+++.

Table 1. Overview of the antibodies used and tissue processing details

Primary Antibody*	Type Ab	Company**	Dilution	Antigen Retrieval†	Second step‡	Positive control	Incubation time / temp (primary antibody)	Procedure
MIB-1 (Ki67)	MoAb, Mouse	Immunotech	1:100	Citrate pH 6.0	Strep AB (1)	Tonsil	60 minutes / room temp	Autostainer
Cyclin A	MoAb, Mouse	Novocastra	1:100	Citrate pH 6.0	Strep AB (2)	Tonsil	o/n 4C	Manually
Cyclin D1	MoAb, Mouse	Novocastra	1:20	EDTA pH 9.0	Strep AB (1)	Mantle Cell Lymphoma	60 minutes / room temp	Autostainer
Cyclin E	MoAb, Mouse	Novocastra	1:50	Citrate pH 6.0	Strep AB (2)	Placenta	o/n 4C	Manually
p16	MoAb, Mouse	Neomarkers	1:160	EDTA pH 9.0	PV	Cervical Carcinoma	60 minutes / room temp	Autostainer
p21	MoAb, Mouse	Dako	1:25	EDTA pH 9.0	PV	Colon	60 minutes / room temp	Manually
p53	MoAb, Mouse	Dako	1:400	Citrate pH 6.0	Strep AB (2)	Serous endometrial carcinoma	o/n 4C	Manually
Cdk2	MoAb, Mouse	Neomarkers	1:100	EDTA pH 9.0	PV	Tonsil	60 minutes / room temp	Manually
HIF-1α	MoAb, Mouse	Pharmingen	1:50	TRS, Dako, 45 min, 97°C	CSA	Mamma-carcinoma	60 minutes / room temp	Manually
Glut-1	PoAb, Rabbit	Dako	1:200	Citrate, pH 6.0, 20 minutes, 93°C	G-aR IgG + Strep1	Red blood cells in slide	60 minutes / room temp	Autostainer
CAIX	PoAb, Rabbit	Novus Biologicals	1:1000	Citrate, pH 6.0, 20 minutes, 93°C	Powervision	Renal cell carcinoma	60 minutes / room temp	Manually
VEGF	PoAb, Goat	R&D Systems	1:50	Citrate, pH 6.0, 20 minutes, 93°C	R-aG IgG + Strep2	Endothelium (internal)	60 minutes / room temp	Manually

** HIF-1α = hypoxia-inducible factor -1α; Glut-1 = glucose transporter-1; CAIX = carbonic anhydrase IX; VEGF = vascular endothelial growth factor; MoAb = monoclonal anitbody; PoAb = polyclonal antibody*

*** Immunotech, Beckman Coulter Inc, Miami, USA; Novocastra, Newcastle upon Tyne, UK; Neomarkers, Fremont, USA; Dako, Glostrup, Denmark; Pharmingen, BD Biosciences, BD Pharmingen, San Diego, CA, USA; Novus Biologicals, Littleton, CO, USA; R&D Systems, Minneapolis, MN, USA.*

† TRS = Target Retrieval Solution, Dako S1700.

‡ PV = Powervision ready to use (Poly-HRP-anti Ms/Rb/RtIgG biotin free, ImmunoLogic, ImmunoVision Technologies, Brisbane, CA, USA); Strep AB(1) = biotinylated horse-anti-mouse, diluted 1:500 in PBS, Vector BA-2000, followed by streptavidin-biotin complex, diluted 1:1000 in PBS, Immunotech; Strep AB (2) = biotinylated rabbit-anti-mouse, diluted 1:500 in PBS, Dako, followed by streptavidin-biotin complex, diluted 1:200 in PBS, Dako; CSA = catalyzed amplification kit, Dako; G-aR IgG = biotinylated Goat-anti Rabbit IgG (BA-1000, Vector laboratories, CA, diluted 1:500) + Strep1 = Streptavidin peroxidase labeling (Streptavidin HRP, IM0309, Beckman Coulter, diluted 1:1000); R-aG IgG = biotinylated Rabbit-anti Goat IgG (E0466, Dako, Glostrup Denmark) + Strep2 = Streptavidin peroxidase labeling (K0377, Dako, Glostrup, Denmark)

Statistical Analysis

Mean percentages at the invasive front and center parts were compared with the Wilcoxon Signed Ranks test for related samples. Furthermore, means of cell cycle proteins and HIF-1α in the front and center of each sample were subtracted and the sample was classified as front>center, front=center or front<center. As CAIX, Glut-1 and VEGF were scored as ordinal variables, differences between front and center were compared with Fisher's exact testing.

Two sided p-values <0.05 were considered significant. All statistical analysis were performed with SPSS for Windows version 12.0.1., 2003 (SPSS Inc., Chicago, IL).

Table 2. *Expression of cell cycle related proteins and HIF-1α in the center parts and at the invasive front of endometrial carcinomas. Means of percentages of expression are shown with statistical testing. Minimum and maximum (range) of staining in the front and in the center are illustrated.*

	Invasive front		Center parts		p-value (Wilcoxon Signed Ranks test)
	mean	range	mean	range	
MIB-1/Ki-67*	25.62	1-91	15.74	0.5-68	<0.001
cyclin A	16.34	1-66	15.90	0-66	0.267
cyclin D1*	4.47	0-70	3.37	0-32	0.138
cyclin E*	12.04	0-70	9.92	0-43	0.018
p16	30.31	0-98	23.57	0-90	0.003
p21	14.58	0-61	13.03	0-50	0.085
p53	20.97	0-100	17.54	0-100	0.058
cdk2	17.72	0-64	14.71	0-61	0.001
HIF-1α	29.36	0-86	29.10	0-91	0.614

** 1 missing case for MIB-1, cyclin D1 and cyclin E staining.*

Results

Cell cycle proteins

Table 2 gives an overview of the expression of the cell cycle related proteins in the central parts of the tumor and at the invasive front. For MIB-1 (p<0.001), cyclin E (p=0.018), cdk2 (0.001) and p16 (p=0.003) differences between the percentages of positive cells in these areas of the tumor were statistically significantly different (Wilcoxon Signed Ranks test); further illustrated in Figure 1A-D. p53 showed a borderline significant difference between the invasive front and the center parts of the tumor (p=0.058). All these proteins were higher expressed at the invasive front than in the central parts of the tumor. Cyclin A (p=0.267), cyclin D1 (p=0.138), p21 (p=0.085) did not show topographical differences in expression.

This is further shown in table 3, wherein numbers of tumors are shown with a mean percentage of positive cells equal at the invasive front and in the center (front=center), higher in the center than at the invasive front (center>front) and

more at the invasive front than in the center (front>center). For MIB-1, cyclin E and cdk2, expression was frequently increased at the invasive front compared to the center, in respectively 31/38 (81.6%), 16/39 (41.0%) and 15/39 (38.5%) of cases. For the other markers this was less clear. However, for none of the markers the number of tumors expressing center>front was higher than front>center.

Figure 1. *Immunohistochemical expression of cell cycle regulators in endometrial carcinoma. In all figures an increase in expression at the invasive front of the tumor compared to the tumor center is noticed.* ***A.*** *MIB-1 expression in endometrioid endometrial carcinoma.* ***B.*** *Cyclin E expression in endometrioid endometrial carcinoma. Inset corresponds to area left of asterisk (*).* ***C.*** *Cdk2 expression in serous carcinoma of the endometrium.* ***D.*** *p16 expression in endometrioid endometrial carcinoma. TC = tumor center; IF = invasive front; M = myometrium. Brown staining depicts positive expression of the proteins.*

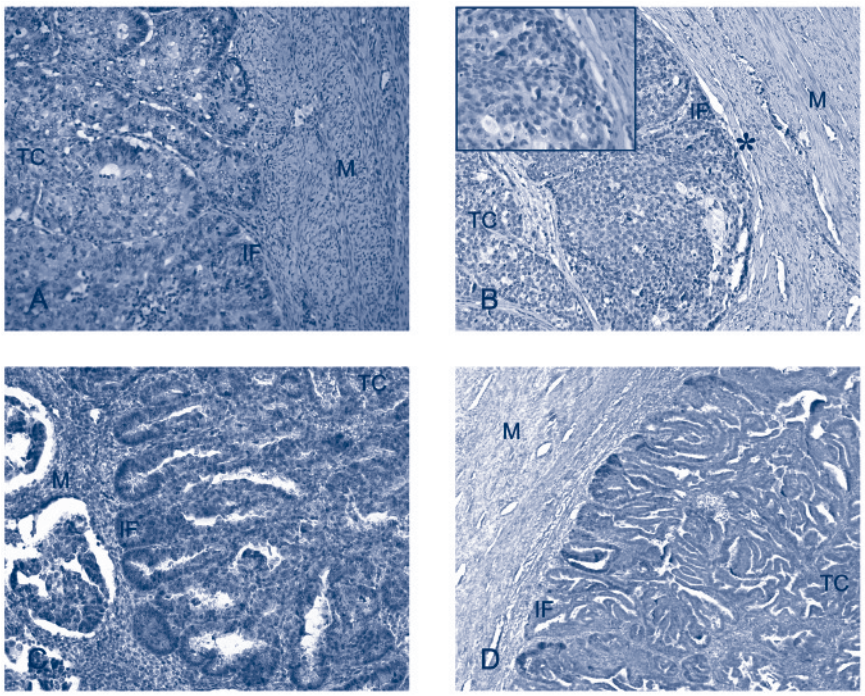

Table 3. *Expression of cell cycle proteins and HIF-1α at the invasive front and the center parts of endometrial carcinomas. Numbers of tumors are shown with a mean percentage of positive cells equal in the front and the center (front=center), higher in the center than in the front (center>front), higher in the front than in the center (front>center).*

	MIB1	cyclin A	cyclin	cyclin E	p16	p21	p53	cdk2	HIF-1α
front = center	4	21	33	20	28	20	30	24	19
center > front	3	5	1	3	0	6	2	0	10
front > center	31	13	4	16	11	12	7	15	9
total	38	39	38	39	39	38	39	39	38

HIF-1α and its downstream factors CAIX, Glut-1, and VEGF

HIF-1α did not show an increased percentage of positive cells at the invasive front (table 2) and 29/38 cases lacked increased expression at the invasive front compared to the center parts (table 3). Table 4 gives an overview of the expression of the HIF-1α downstream factors in the center parts and at the invasive front. For CAIX 30/39 cases lacked increased expression at the invasive front, comparable to Glut-1 for which 28/39 showed no difference between front and center. Glut-1 and CAIX were only expressed in a true perinecrotic pattern, a more diffusely staining as we noticed for HIF-1α in some tumor borders was not seen for these 2 HIF-1 downstream factors. For VEGF, 32/39 cases lacked increased expression at the front.

Table 4. *Expression of HIF-1α downstream factors at the invasive front and the center parts of endometrial carcinomas.*

		Invasive front	Center parts	p-value (Fisher's exact test)
CAIX	Negative	13	6	0.112
	Positive	26	33	
Glut-1	Negative	11	5	0.098
	Positive	27	34	
VEGF	Negative	3	1	0.714
	+	18	16	
	++	14	16	
	+++	4	6	

Discussion

The purpose of this study was to objectively investigate differences in topography of proliferation (MIB-1), aberrant expression of cell cycle regulators and HIF-1α with three of its downstream factors, Glut-1, CAIX and VEGF. This is the first study considering this subject in endometrial carcinoma. The concept of an "invasive front" has been proposed in other cancers, but differences in definitions and methods in these studies make comparisons difficult[219-227]. We used as a definition of the invasive front the deepest rim of cancerous tissue into the myometrium with a width of 1.22 mm, which was one field of vision with the microscope that we used for quantitation.

Differences in protein expression at the invasive front versus the center parts of the tumor might be caused by clonal selection or by interactions with the defending "normal" tissue. Since the edge becomes the inner tumour area when the tumour continues its growth and a new invading tumour front is formed by multiplying malignant cells which outflank the initial edge[228], clonal selection is less likely and interactions with the surrounding extracellular matrix components that regulate growth and differentiation of epithelial cells[229] may be more important.

The main focus regarding the invasive front in cancers has been on matrix metalloproteinases, 'stromatogenesis' and angiogenesis[220,228,230-232], which is understandable as the first provides the prerequisite for cancer cells to invade by breaking down tissue barriers and the last one allows survival of cells by the increased supply of oxygen and nutrients, and generates a pathway to form metastases. Sivridis et al.[231,332] proposed the term 'stromatogenesis' to indicate the existence of a specific stroma in endometrial cancer, after the proliferaton of peritumorous fibroblasts and the disruption of normal tissue continuity facilitating tumour cell invasion. They stated that it is probable that at the edge of the tumor important interactions occur between cancer cells, endothelial cells and the supporting stroma. The same group proposed, after their study on differences in vascularization in the front and the inner area of the non-small cell lung tumor, the immunostained vasculature to be considered not as a static image but as the result of a dynamic process occurring during tumor growth[233].

Little further attention has been paid to the concept of an invasive front in endometrial carcinoma. In the present study we focused for the first time on the topographical expression of cell cycle regulators and proteins related to the hypoxia response, as aberrant expression of these proteins is frequently found in tumours and is associated with a more malignant phenotype[59-62,64-67,101].
We noticed especially MIB-1, cyclin E, p16 and cdk2 expression to be increased at the invasive front compared to the center. For cyclin A, cyclin D1, p21 and p53 this was less pronounced, although a higher expression in the front compared to the center was more often seen than more expression in the center than in the front of the tumor. As MIB-1 and cyclin A are proteins expressed in proliferating cells without the genes being a target of carcinogenetic changes, this indicates that in most endometrial cancers the invasive front is more rapidly proliferating than the central tumour parts.
Difference in expression between infiltrating part and the remainder of the tumor has been described in vulva carcinomas, with a basal, diffuse and infiltrating type[221]. Higher proliferation in the front has been found in oral squamous cell carcinomas[234]. However, these results are in conflict with results in colorectal and gastric cancers[222,223,225,235] where proliferative activity was significantly higher at the luminal border compared with the invasive margin[223]. Furthermore, tumors with low proliferation at the invasive margin had worse prognosis[223]. They propose that the influence of for example bile acids and bacteria might play a role in increased proliferation at the luminal border in colorectal cancers[223]. This might explain differences between endometrial and colorectal cancer, although both tumors are polarised with a superficial (inner) localization facing a cavity and deeper structures facing a muscular wall.
The cell cycle regulators cyclin E, p16 and cdk2 seem to be most involved in the increased proliferation at the invasive front. Cyclin E contributes to proliferation in normal cells, by accelerating G1-S phase turnover of the cell cycle. However, in cancer cells the tight control of cyclin E is often disrupted, leading to aberrant expression of cyclin E. In endometrial carcinoma, for example, expression of cyclin E is correlated with higher grade[59]. Cdk2, a CDK which is activated by cyclin A and cyclin E, controls the G1-S phase of the cell cycle by phosphorylation of pRB. The increased expression of cyclin E and cdk2 in the invasive front might explain the increased expression of

MIB1, and reflects the malignant behavior of the border of the tumor.
p16, a cyclin dependent kinase inhibitor (CKI) which inhibits cyclin in complex with its kinase, should therefore be a brake for the cell cycle. However, we observed higher
p16 expression at the front in many cases, in line with observations in colorectal cancer where 40% do not express p16 in central parts of the tumor but re-express it at the invasive front[222]. They propose that this may be due to promoter methylation in the central parts. Svensson et al[236] showed by immunohistochemistry and Western blots of microdissected tumors that p16 was upregulated at the invasive front of the majority of basal cell carcinomas with infiltrative growth patterns, but with ceased proliferaton (low MIB-1 in invasive margins). Previously we showed that upregulation of p16 is a frequent event in endometrial cancer[218] as in cervical[237] and ovarian cancer[238], indicating that p16 is not functional as a cell cycle inhibitor but rather is a reflection of a bystander effect of the dysregulated cell cycle. p16 is obviously in the invasive front in endometrial carcinoma unsuccessful in ceasing cell cycle progression, as MIB-1, a reliable marker of proliferation, is highly expressed here.

Since angiogenesis was previously shown to be increased at the invasive front in non-small cell lung cancer[233], and RNA levels of HIF-1 were shown to be higher in the invading front compared to the centre in thyroid papillary carcinoma[226], we expected HIF-1α, the key regulator of the hypoxic response and a potent inducer of VEGF as a major angiogenetic growth factor, to be increased at the invasive front. However, we failed to demonstrate this except in a few cases. In line with this, its downstream factors CAIX, Glut-1 and VEGF were also not higher expressed at the invasive front. Possibly, hypoxia in the center of the often fairly solid growing endometrial cancers may induce hypoxia related proteins which may balance the expression at the invasive front.

Apart from being interesting from a biological point of view, the observed differences in expression of proteins between the invasive front and central tumor parts in endometrial cancer may be clinically quite relevant. First, when creating tissue arrays from endometrial cancer sampling both the central and deepest invading parts needs to be considered. Second, even when whole tumor sections are being analyzed by the pathologist for proliferation related phenomena, heterogeneity between central parts and the invasive front needs to be taken into account. Third, thymidine phosphorylase expression in fibroblasts and myometrial cells confronting the tumor edge of endometrial carcinoma appeared to be correlated with adverse prognostic factors in endometrial cancer such as high tumour grade, deep myometrial invasion, and advanced stage of disease[227], so the invasive front concept may even have prognostic implications.
In summary, endometrial carcinomas clearly show an invasive front that is characterized by higher proliferation and progressive derailment of the cell cycle regulators cyclin E, p16 and cdk2, but not by an increased hypoxic response. This seems to have implications in daily clinical and research settings.

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chapter 7

P16 is consistently expressed in endometrial tubal metaplasia

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Abstract

Background

Cell cycle proteins and HIF-1α with downstream factors are often aberrantly expressed in (pre)neoplastic tissue.

Methods

Paraffin-embedded specimens of inactive endometrium with TM (n=15), ovarian inclusion cysts (n=6), cervix with TM (tubal metaplasia) (n=3), Fallopian tubes (n=7), cycling endometrium (n=9) and a ciliated cell tumor of the ovary were stained for p16 and LhS28. 39 Endometrioid endometrial carcinomas and 5 serous endometrial carcinomas were stained for p16. Additionaly, inactive endometrium (n=15) was immunohistochemically stained for p21, p27, p53, cyclin A, cyclin D1, cyclin E, HIF-1α, CAIX, Glut-1 and MIB-1.

Results

A mosaic pattern of expression of p16 was seen throughout in all cases of endometrial TM (15/15), in 2/6 of the ovarian inclusion cysts with TM, in all (3/3) cervical TM and focal in 5/7 of Fallopian tube cases. Mosaic expression was also seen in a ciliated cell tumor of the ovary and in 18/39 of endometrioid endometrial carcinomas, and diffuse p16 expression was seen in 5/5 serous carcinomas. In comparison with normal endometrium, TM areas in the endometrium showed significantly increased expression of HIF-1α, cyclin E, p21 and cyclin A, and decreased expression of p27. Membranous expression of CAIX and Glut-1 was only seen in TM areas, pointing to functional HIF-1α.

Conclusion

As p16 is consistently expressed in TM, less and only patchy expressed in the nomal Fallopian tube, is paralleled by aberrant expression of cell cycle proteins, HIF-1α, CAIX and Glut-1 and resembles the pattern of p16 expression frequently seen in endometrial carcinomas, we propose endometrial TM to be a potential premalignant endometrial lesion.

Introduction

The tumor suppressor gene p16, also known as INK4A, or MTS1, is located on chromosome 9p21. It encodes the cell cycle regulatory protein p16, a cyclin-dependent kinase inhibitor, which binds to cyclin D1-cyclin-dependent kinase (CDK) 4 and 6 complexes to control the cell cycle at the G1-S transition. $p16^{INK4A}$ is considered to be a tumor suppressor gene as it inhibits cell proliferation by regulating cdk4 activity to prevent pRb phosphorylation, leading to G1 arrest. Therefore, inactivation of the $p16^{INK4A}$ gene could lead to uncontrolled cell growth.
Alterations of $p16^{INK4A}$ gene have been detected in various human tumors, but the role of this gene as a tumor suppressor *in vivo* appears to be dependent on tumor type. Loss of p16 function by point mutation, homozygous deletion or hypermethylation of the promoter region of the gene has been implicated in carcinogenesis of several human malignancies. p16 loss per se is not sufficient to induce tumorigenesis, but rather cooperates with exogenous stimuli (e.g. carcinogens) or other spontaneous mutations to facilitate malignant transformation[239]. On the other hand, overexpression of p16 has been described as well, for example in cervical intraepithelial neoplasias associated with oncogenic human papilloma virus (HPV) infections. In clinical practice, p16 is therefore used as a marker of dysplastic cervical cells [237,240-242].
The ciliated cell is a normal constituent of the human endometrium, increasing in number during age[14]. However, since the function of these cells within the endometrium is not clear, one could argue that these cells have actually undergone metaplastic differentiation to resemble those of the Fallopian tube showing a mosaic pattern of secretory and ciliated type cells, usually referred to as tubal metaplasia (TM) when it does not concern just scattered individual cells but glands or a stretch of surface epithelium completely composed of ciliated cells. There are more ciliated cells, tubal type secretory cells and reserve or intercalary cells than are normally present[13]. Endometrial TM may be extensive, may almost line the surface of the endometrium, especially in peri- and postmenopausal women. Ciliated (tubal) change is seen in normal endometrium, hyperplasia and carcinomas of the endometrium[15,16]. All these changes are thought to reflect a mild degree of estrogenic stimulation[13,14].
This type of differentiation is frequently found in the gynaecological tract. In the cervix, TM refers to endocervical glands that are lined by a Müllerian-type epithelium that closely resembles that of the Fallopian tube. In the ovary, there are often epithelial inclusion cysts, that are traditionally thought to arise from cortical invaginations of the ovarian surface epithelium that have lost their connection with the surface. Recently, it has however been proposed that tubal cells may seed to the ovarian surface, are uptaken and form cysts. These cysts are typically lined by a single layer of columnar cells that are often ciliated, mimicking tubal epithelium[243-245]. Ovarian inclusion cysts are likely the site of origin for most common epithelial tumors of the ovary. Several studies have found that patients with ovarian carcinoma have an increased number of inclusion cysts in the contralateral ovary compared to controls[246,247]. Others could not confirm that, but noticed as well more cortical invaginations in ovaries from women with contralateral ovarian cancer compared to controls[248,249]. In patients at high hereditary risk of ovarian cancer undergoing prophylactic adnectomy, a high frequency of TM inclusion cysts showing aberrant expression of bcl-2, the progesterone receptor, Ki67 and p53 was found[245,248].

Interestingly, several authors have written about 'scattered patterns' or 'focal expression' of p16 positive cells in tubal or tubo-endometrial tissue of the cervix[250-254,237]. They describe a pattern which is not seen in normal tissue, and is different from p16 immunohistochemically negative tissue and diffuse positivity, as is seen in high-risk HPV positive cervical intraepithelial lesions. Inoue considered in a review metaplasia in general to be a precursor of the variant types of endometrial carcinomas, based on the p53 accumulation and PCNA staining, and the fact that endometrial carcinomas are often accompanied by adjacent metaplastic epithelium[255]. However it is, for now, not a generally accepted view that endometrial metaplasias are a precursor of endometrial cancer.

To our knowledge, this observation has never been compared to staining for LhS28, a ciliated cell marker[256]. Furthermore, p16 expression in TM has never been placed in a broader context and the expression of p16 has never been studied extensively in TM in the endometrium or in inclusion cysts of the ovary. Interestingly, TM is not merely a process of terminal differentiation that should conceptually be absent in malignancy, as the uncommon endometrial ciliated cell tumour even shows exceptionally high tubal differentiation and may be malignant[257,258]. Ciliated cell tumors of the ovary have also been described[259]. HIF1-α, the key regulator of the hypoxia response, has been shown to be expressed in endometrial carcinomas[35], was seen to be expressed in TM areas in our study on the hypoxia response during endometrial carcinogenesis[121].

This study was therefore undertaken to investigate p16 expression in TM in the endometrium, and to compare it to TM in the cervix and ovary, and to the normal Fallopian tube. It is generally thought that endometrial TM is a benign disease, although molecular studies are scarce. Aberrant expression of the proliferation marker MIB-1 (Ki67) and proteins that are often involved in carcinogenesis such as cell cycle proteins and increased expression of hypoxia inducible factor (HIF)-1 alpha (the key regulator of the carcinogenetic hypoxic response) and its target genes Glut-1 and CAIX in endometrial TM, might necessitate to change the common view that endometrial TM is a purely benign lesion.

Materials and Methods

Patients and Tissues

Paraffin-embedded clinical specimens from inactive endometrium (n=15), all showing areas of tubal metaplasia, normal Fallopian tubes (n=7), ovaries with TM in inclusion cysts (n=6), cervix (n=3) with TM, secretory phase endometrium (n=3), interval phase (n=1), proliferative phase endometrium (n=5), and serous (n=5) and endometrioid carcinoma of the endometrium (n=39), and one borderline ciliated cell tumor of the ovary were collected from the archives of the Department of Pathology of the University Medical Center, Utrecht, The Netherlands.

These tissues were derived from patients operated between 1992 and 2005. Haematoxylin and eosin-stained sections were revised by 2 experienced gynecopathologists (PvD, DSG). Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our hospital[99].

Immunohistochemistry

All slides were immunohistochemically stained for p16 and LhS28. Additionally, slides with inactive endometrium were further analysed for p21, p27, p53, cyclin A, cyclin D1, cyclin E, HIF-1α, Glut-1, CAIX, and MIB-1 (Ki67) expression.

Immunohistochemistry was performed on 5 µm thick paraffin slides. Table 1 presents all antibodies, dilutions, incubation times and antigen-retrieval methods used. Slides were deparaffinized with xylene and rehydrated by serial ethanol dilutions.

For HIF-1α, endogenous peroxidase was blocked by hydrogen peroxide (Dako CSA kit), after which antigen retrieval followed. A cooling off period of 30 minutes preceded blocking of the avidin by biotin block (Dako; 10 min) and protein block (Dako; 5 min). Then, the primary antibody was applied followed by the catalyzed signal amplification system (Dako CSA kit). Slides were washed in Tris buffer in between.

For all other stainings, endogenous peroxidase activity was blocked for 30 minutes, followed by antigen retrieval. Additionally, slides were incubated with the primary antibody, followed by the secondary antibody after washing slides in between in PBS. Finally, peroxidase activity was developed with DAB and counterstained with hematoxylin. In between steps, slides were washed in PBS.

Positive controls were used throughout, see table for types of tissue. Negative controls were obtained by omission of the primary antibodies from the staining procedure.

Scoring of staining

Two authors (PvD, NH) scored all slides blinded to clinicopathologic data and results of other stainings. For p16, cytoplasmic and nuclear staining were both considered positive. Each slide was examined for staining pattern, which was classified as negative (no positive cells), focal (some positive cells, though much less than "mosaic staining"), a "mosaic staining" pattern of secretory and ciliated cells, or diffuse staining. A mosaic staining was defined as a typical pattern of positive staining of 50% - 80% of cells, in a checkerboard pattern of positive and negative cells.

LhS28, a ciliated cell marker, only stained the apical side of the cell, where the basal bodies and cilia are situated; this type of staining was considered positive[256]. LhS28 staining was compared to p16 for colocalization in all slides.

For Glut-1 and CAIX membranous staining was considered positive staining. For all other proteins, the percentage of dark, homogenously stained nuclei was estimated as before[101], ignoring cytoplasmic staining.

Statistics

Slides with parts of normal inactive endometrium and parts with TM were scored (% of positive nuclei) separately, and differences in expression were analyzed with non-parametric paired testing (Wilcoxon Signed Ranks Test). Two sided p-values <0.05 were considered significant. All statistical analysis were performed with SPSS for Windows version 12.0.1., 2003 (SPSS Inc., Chicago, IL).

Table 1. Overview of the antibodies used and tissue processing details

Primary	Source**	Dilution	Antigen Retrieval†	Second step‡	Positive control	Incubation time / temp	Procedure
LhS28	Abcam	1:500	Citrate pH 6.0	Strep AB(2)	Cilliated cell Fallopian tube	60 minutes / room temp	Manual
p16	Neomarkers	1:160	EDTA pH 9.0	PV	Cervical Carcinoma	60 minutes / room temp	Automatic staining device
p21	Dako	1:25	EDTA pH 9.0	PV	Colon	60 minutes / room temp	Manual
p27	Transduction	1:500	Citrate pH 6.0	PV	Skin	o/n 4C	Manual
p53	Dako	1:400	Citrate pH 6.0	Strep AB(2)	Serous endometrial carcinoma	o/n 4C	Manual
Cyclin A	Novocastra	1:100	Citrate pH 6.0	Strep AB(2)	Tonsil	o/n 4C	Manual
Cyclin D1	Novocastra	1:20	EDTA pH 9.0	Strep AB(3)	Mantle Cell Lymphoma	60 minutes / room temp	Automatic staining device
Cyclin E	Novocastra	1:50	Citrate pH 6.0	Strep AB(2)	Placenta	o/n 4C	Manual
HIF-1α	Pharmingen	1:50	TRS, DAKO, 45 min, 97°C	CSA	Breast Carcinoma	60 minutes / room temp	Manual
Glut-1	Dako	1:200	Citrate pH 6.0	G-aR IgG + Strep AB(1)	Red blood cells in slide	60 minutes / room temp	Automatic staining device
CAIX	Novus Biologicals	1:1000	Citrate pH 6.0	PV	Grawitz tumor	60 minutes / room temp	Manual
MIB-1 (Ki-67)	Immunotech	1:100	Citrate pH 6.0	Strep AB (3)	Tonsil	60 minutes / room temp	Automatic staining device

** All primary antibodies used are monoclonal antibodies, except for Glut-1 and CAIX. HIF-1α = hypoxia-inducible factor -1α.*

*** Neomarkers, Fremont, USA; Abcam, Cambridge, UK; Pharmingen, BD Biosciences, BD Pharmingen, San Diego, CA, USA; Dako, DakoCytomation, Glostrup, Denmark; Novus Biologicals, Littleton, CO, USA; Transduction, BD Biosciences, BD Transduction Laboratories, San Diego, CA, USA; Novocastra, Newcastle upon Tyne, UK.*

† TRS = Target Retrieval Solution, DAKO S1700.

‡ CSA = catalyzed amplification kit, Dako; G-aR IgG = biotinylated Goat-anti Rabbit IgG (BA-1000, Vector laboratories, CA, diluted 1:500) + Strep AB (1) = Streptavidin peroxidase labeling (Streptavidin HRP, IM0309, Beckman Coulter, diluted 1:1000); Strep AB (2) = biotinylated rabbit-anti-mouse, diluted 1:500 in PBS, Dako, followed by streptavidin-biotin complex, diluted 1:200 in PBS, Dako; Strep AB(3) = biotinylated horse-anti-mouse, diluted 1:500 in PBS, Vector BA-2000, followed by streptavidin-biotin complex, diluted 1:1000 in PBS, Immunotech; PV = Powervision ready to use (Poly-HRP-anti Ms/Rb/RtIgG biotin free, ImmunoLogic, ImmunoVision Technologies, Brisbane, CA, USA).

Results

p16

All (15/15) cases of endometrial TM showed a mosaic p16 expression pattern in the TM areas (Fig. 1A). In the normal Fallopian tube, some focal p16 expression was seen in 5/7 cases (Fig. 1D), 2 cases were negative. 3/6 cases of TM in ovarian inclusion cysts were p16 negative, while 1 case showed some focal positivity and 2 showed a mosaic pattern of p16 expression. One of the positive ovaries harboured 4 inclusion cysts (Fig. 1E,F), one being p16 negative not showing much TM, but the other 3 smaller cysts were strongly p16 positive in a mosaic pattern and showed extensive TM. In the cervix all (3/3) cases with TM showed a mosaic expression of p16 expression (Fig. 1H).

All normal parts of the proliferative endometrium (n=5) stained negative for p16, the parts with ciliated cell change (n=5) showed p16 positivity in a mosaic pattern. Interval phase endometrium (n=1) was p16 negative. In the 3 slides with secretory endometrium 2 showed parts with TM, both of these showed p16 in a mosaic pattern in the TM, the normal parts were p16 negative (n=1) or focally p16 positive (n=1); one slide showed only normal secretory endometrium, which was p16 negative.

Serous carcinoma of the endometrium showed a diffuse pattern of p16 expression in 5/5 cases. Of 39 endometrioid endometrial carcinomas 1 was p16 negative, 7 were focally positive, 18 showed a mosaic pattern of expression, and 13 were diffusely positive.

In 2 endometrioid carcinomas, expression was particularly pronounced in the squamous parts. One cilliated cell tumor of the ovary showed the typical mosaic p16 expression pattern (Fig. 1J).

LhS28 expression colocalized with p16 mosaic pattern in TM in endometrium (Fig. 1A, B), cervix (Fig 1G, H), ovary and Fallopian tubes (Fig 1C, D) and in ciliated cell tumor of the ovary (Fig 1I, J).

HIF-1α,Glut-1, CAIX, p21, p27, cyclin A, cyclin D1, cyclin E

The TM areas in the endometrial cases were further analyzed for aberrant expression of cell cycle proteins (other than p16) and HIF-1α (table 2). In comparison with normal parts of the endometrium, TM areas in the endometrium showed increased expression of HIF-1α (p=0.005). The HIF-1α downstream genes Glut-1 and CAIX showed no expression in inactive endometrium, whereas TM areas showed positive cell membranes for Glut-1 in 4/15 cases and for CAIX in 2/15 cases, which colocalized with the HIF-1 α positive areas. TM areas also showed increased expression of cyclin E (p=0.039), cyclin A (p=0.001) and p21 (p=0.005), and decreased expression of p27 (p=0.007) compared to normal parts of the endometrium. For MIB-1 (Ki67) no difference in staining between inactive endometrium and tubal metaplasia (p=0.344) was noticed.

Figure 1. *Ciliated cell change in various parts of the gynecological tract. A) Endometrium with tubal metaplasia stained by LhS28, a ciliated cell marker, and in B) for p16. C) Normal Fallopian tube showing LhS28 staining, and D) focal p16 expression. E) Subcortical ovarian inclusion cysts showing varying degrees of tubal metaplasia, with a mosaic pattern of p16 expression in the cysts with the most pronounced ciliated cell change (F). G) Cervix with tubal metaplasia showing positivity for LhS28 and H) p16 in a mosaic pattern. I) Endometrial ciliated cell tumor of the ovary showing positivity for LhS28 and J) p16 in a mosaic pattern.*

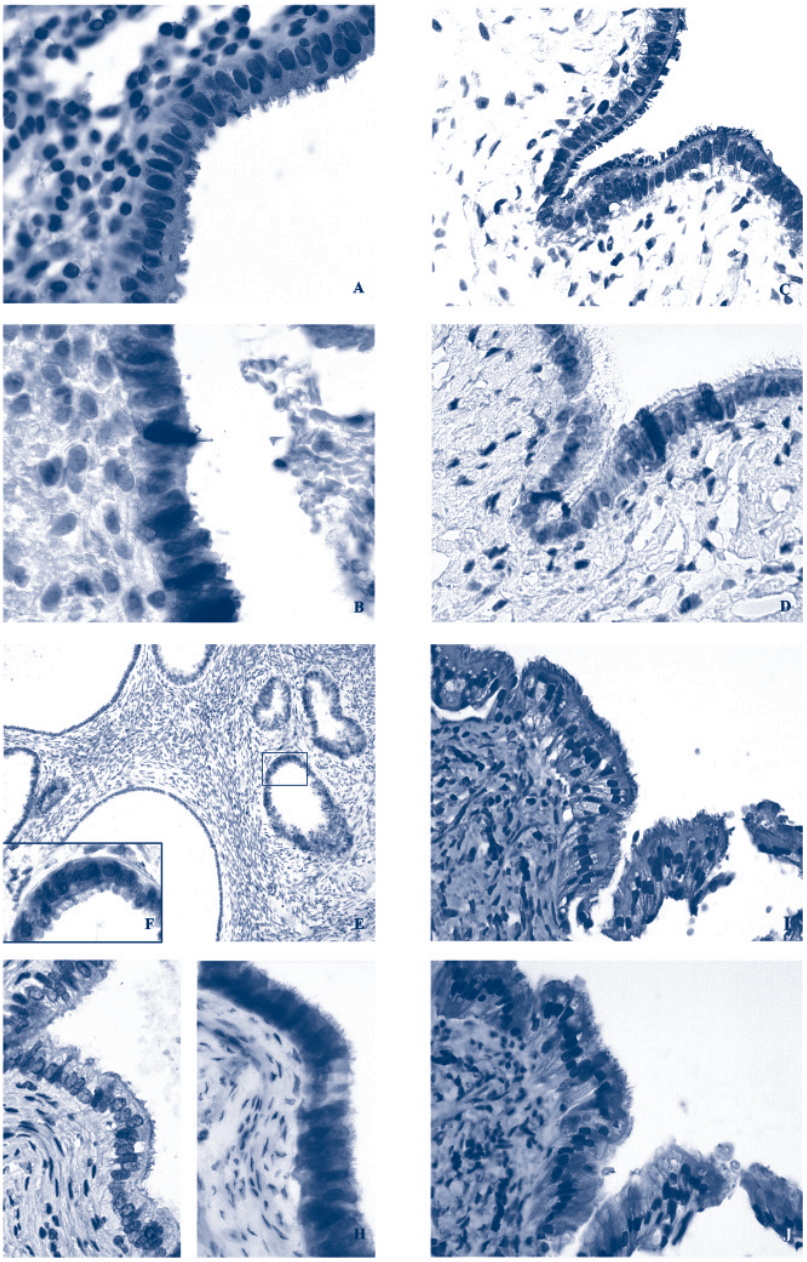

Table 2. Expression of cell cycle proteins (% of positive cells) in normal inactive endometrium and tubal metaplasia (TM) of the endometrium.

	Normal inactive endometrium (n=15)			TM endometrium (n=15)			Test for difference between groups: Wilcoxon Signed Ranks test
	Mean	Median	Range	Mean	Median	Range	p-value
cyclin A	0.6	0	0-2	2.73	2.0	0-5	0.001
cyclin D1	0	0	0	0.47	0	0-2	0.083
cyclin E	2.67	0	0-35	7.07	0	0-35	0.039
P21	1.07	1.0	0-5	6.87	5.0	0-35	0.005
P27	59.67	65.0	5-100	42.00	35.0	5-100	0.007
P53	0.07	0	0-1	0.07	0	0-1	1.0
HIF-1α	0	0	0	18.33	5.0	0-90	0.005
MIB1	2.80	2.0	0-20	3.53	2.0	0-10	0.344

Discussion

The objective of the study was to assess p16 expression in TM in the endometrium in comparison with normal endometrium, normal Fallopian tube and TM in ovarian inclusion cysts and cervix; and to verify whether cell cycle proteins, HIF-1α, Glut-1 and CAIX were expressed equally in TM and normal parts of the endometrium.

On the basis of the data presented in this study, the comparison with the ciliated cell marker LhS28 and the published literature, we conclude that mosaic expression of p16 is a consistent phenomenon of TM in the female genital tract[237,250-254], especially in the endometrium. We also noticed, as others did before[14,121], that ciliated cells are more present during the proliferative phase compared to secretory endometrium. p16 expression has been observed before in cyclic endometrium, more in the proliferative than the secretory phase, but has not been studied and linked incessantly to TM as we did[68,191,252-254,261]. The typical expression of p16 which we observed in TM was not seen that extensively in the normal Fallopian tube. In the ovary, p16 expression paralleled the degree of tubal metaplasia. A ciliated cell tumor of the ovary showed the typical mosaic pattern of p16 expression we observed in TM. We propose that TM of the endometrium could be a potentially premalignant lesion, based on the following arguments.

Firstly, aberrant expression of p16 is regarded as a carcinogenetic event in many tumors, including those in the gynecological tract. p16 is very frequently aberrantly expressed in cervical dysplasia and carcinoma[192,193,250,251] where it may however be a bystander effect of HPV E6/E7 cell cycle activation without an inherent contribution to carcinogenesis, but in other malignancies the carcinogenetic role of p16 alterations are much better defined[260,261]. Further, Umezaki[262] suggested that tubal metaplasia should be considered a neoplastic entity of uterine cervical glandular lesions that may have the potential to undergo malignant transformation, although this is up

to now not a widespread view. Secondly, in the present study, p16 is also aberrantly expressed in TM in some ovarian inclusion cysts. These cysts have been proposed to be precursors of ovarian cancer[248]. In ovarian cancer, especially the serous type, p16 is often aberrantly expressed[238]. Furthermore, in endometrial carcinoma of the endometrium, p16 positivity rates vary from 30-94%[68-71,191,192,239,263], in endometrioid carcinoma especially in the squamous areas[251], and aberrant p16 expression has also been found in endometrial hyperplasia[68,263]. In serous and clear cell endometrial cancers, p16 positivity is also frequent[70,71,192,238], although the number of cases studied so far is low.

Additionally, we found that the TM areas in the endometrium showed aberrant expression of many cell cycle proteins, HIF-1α, Glut-1 and CAIX compared to normal tissue. The cell cycle consists of four phases: G1, S, G2, M. Cyclins form a complex with cyclin-dependent kinases and by doing this they make transition to the next phase of cell cycle possible. This will induce cell growth, unless inhibited by tumor suppressor gene products such as p53 or cdk-inhibitors (cdki's), such as p16, p21 and p27. The transition of G1/S is partly controlled by cyclin E and cyclin D1; p27 and p21 inhibit cyclin E; p16 and p21 inhibit cyclin D1. In the transition of S/G2 cyclin A plays a role, with the cdki's p27 and p21. Overexpression of cell cycle stimulating factors such as the cdk's and cyclins, and underexpression of inhibiting factors such as cdki's are frequently found in tumors. In general, aberrant expression of cell cycle regulators correlated with a more malignant subtype, a higher proliferation rate, recurrence and a worse survival in different tumors. We found a significantly higher expression of cyclin A, cyclin E and p21, and a lower expression of p27 in TM parts with TM compared to the normal endometrium, which points to disturbance of the G1/S, S/G2 and G2/M transitions in endometrial TM, consistent with potential premalignant change. Cyclin A and MIB-1 are markers for proliferation, the first being more expressed in TM, but this was not apparent for MIB-1. The changes in cell cycle regulators are however not accompanied with obvious morphological changes like in other dysplastic lesions. It is therefore not in this stage possible to morphologically discriminate potentially premalignant from harmless TM. As obvious dysplastic changes are lacking, it is also difficult to indicate to what kind of cancer TM could progress. In view of the p16 expression patterns seen in endometrioid and serous carcinomas, we suggest that both these cancers but especially serous cancer could be at the far end of the progression spectrum of TM.

It is unclear why HIF-1α is overexpressed in TM. HIF-1α is the key regulator of the hypoxia response, and has been implicated in carcinogenesis in many different epithelia including the female epithelia of the endometrium[35,121] and breast[100]. In the absence of necrosis, it is unlikely that hypoxia causes HIF-1α overexpression. Therefore, the observed HIF-1α overexpression is possibly caused by aberrant expression of oncogenes and tumor suppressor genes that are known to be able to upregulate HIF-1α[213]. This deserves to be further studied.

Although HIF-1α is well-known as the key regulator for survival of hypoxic tumor cells, another direct effect of HIF-1α in hypoxia is proposed to be promotion of cell cycle arrest, for example by influencing p21 or p27[264,265], however, this has not been studied in endometrial cells. Therefore, whether the increase in expression of p21 in TM in this study is HIF-1α-driven remains to be proven. The decrease of p27 in TM is inexplicable in relation to HIF-1α.

Glut-1 and CAIX are well characterized downstream targets of HIF-1α, which facilitate

survival of cells in acid and low glucose circumstances. Carbonic anhydrase IX (CAIX) is a membrane-associated carbonic anhydrase, that plays a role in pH regulation[87]. A role for this enzyme in the adaptation of tumor cells to hypoxic conditions and in tumor cell progression is suggested by a significant overlap between CAIX expression and regions of hypoxia in solid tumors[88,89]. In endometrial hyperplasia, expression of Glut-1, a glucose transporter upregulated by HIF-1α, appeared to be associated with (pre)neoplastic stages of the endometrium and is therefore proposed to be a useful indicator of high risk for development of endometrial carcinoma[92,266,267]. On the whole, these studies reveal an indication to preneoplastic progress in tissue expressing CAIX and Glut-1 in the membranes. This is remarkable as we noticed this type of expression in parts of TM.

In conclusion, ciliated cells are regarded by some to be normal constituents of the endometrium[89]. We show here that endometrial TM, the far morphological end of tubal differentiation, shows aberrant expression of several cell cycle regulators HIF-1α, the key regulator of the hypoxia response, Glut-1 and CAIX. This implies that TM might not be as benign as generally accepted, and may in fact be a potential premalignant lesion. This warrants further molecular studies on genetic aberrations in TM to better found these preliminary results. In view of the consistent expression of p16 in a characteristic mosaic pattern in TM, p16 immunohistochemistry may help to identify TM areas in the gynecological tract as an alternative for the ciliated cell marker LhS28.

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chapter 8

Hypoxia-Inducible Factor 1alpha is essential for hypoxic p27 induction in endometrioid endometrial carcinoma

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Abstract

Background

Hypoxia-inducible factor 1α (HIF-1α) plays an essential role in the adaptive response of cells to hypoxia. The cyclin-dependent kinase inhibitor $p27^{kip1}$ is highly expressed in the normal endometrium but is lost during endometrial carcinogenesis. However, in high grade cancers p27 re-expression is observed.

Aim

As there were contradictory results concerning the role of HIF-1α in hypoxia-induced expression of $p27^{kip1}$, we analysed their relationship *in vitro* and *in vivo* in endometrial cancer.

Methods Paraffin-embedded specimens from endometrioid endometrial carcinoma (n=39) were stained immunohistochemically for HIF-1α, $p27^{kip1}$ and Ki67. HEC-1B, an endometrial carcinoma cell line, was cultured under normoxic or hypoxic conditions in the presence or absence of transiently expressed short hairpin RNAs targeting HIF-1α. Protein expression of $p27^{kip1}$ and HIF-1α was assessed by Western blotting.

Results

Immunohistochemical staining revealed in 26 out of 39 (67%) perinecrotic HIF-1α expression and in 18 out of 39 (46%) p27 emphasized staining central in the tumor islands, mostly around necrosis. In 13/26 tumors both p27 and HIF-1α perinecrotic/central staining was observed. In these tumor sections hypoxia-associated p27 expression showed less proliferation around necrosis. Analysis of cultured endometrial carcinoma cells demonstrated that p27 protein expression is induced by hypoxia. This induction was abrogated by transient knockdown of HIF-1α using RNAi. Further, hypoxia induced cell cycle arrest in HEC-1B cells.

Conclusions

We conclude that in endometrioid endometrial carcinoma, p27 re-expression by hypoxia is HIF-1α dependent and leads to cell cycle arrest. This may contribute to survival of cancer cells in hypoxic parts of the tumor.

Introduction

Lack of oxygen supply to tumor tissue results in hypoxic areas. This is important in carcinogenesis because this results in a more aggressive phenotype with increased invasiveness and proliferation, formation of metastases and poorer survival[268,269]. Moreover, hypoxic tumors are more resistant to radiotherapy and chemotherapy[268]. In reaction to hypoxia, cells will alter their metabolism and activate survival genes. Hypoxia-inducible factor 1 (HIF-1) plays an essential role in the adaptive cellular response to hypoxia. HIF-1 is a transcription factor and consists of an alpha and a beta subunit. Both subunits are basic helix loop helix proteins that contain a PAS (PER-Aryl hydrocarbon nuclear translocator-SIM) domain[38]. Under normoxia, the HIF-1α protein has a very short half-life due to oxygen-dependent polyubiquination by the Von Hippel-Lindau (VHL) tumor suppressor protein, which targets HIF-1α for proteasomal degradation[270]. Lack of oxygen abrogates this process, which results in stable HIF-1α protein. HIF-1β is constitutively expressed, and therefore HIF-1α protein levels determine the amount of HIF-1 formed. Target genes of HIF-1 control glucose transporters, glycolytic enzymes, gluconeogenesis, high-energy phosphate metabolism, growth factors, apoptosis, erythropoiesis, haem metabolism, iron transport, vasomotor regulation and nitric oxide synthesis[111].

During carcinogenesis, the cell cycle is progressively deregulated. Overexpression of cell cycle stimulating factors such as the cyclins and cyclin dependent kinases (CDKs), and reduced activity of cyclin dependent kinase inhibitors (CDKIs) are frequently found in tumours. Cell cycle deregulation is correlated with a more malignant subtype, a higher proliferation rate, recurrence and a worse survival in different tumors.

Hypoxia leads to cell cycle arrest, but the role of HIF-1 is not fully understood. Hypoxia reduces proliferation and increases apoptosis in wild-type embryonic stem (ES) cells, but not in HIF-1α knock-out ES cells. Furthermore, some genes involved in cell cycle control are regulated by hypoxia either in a HIF-1α-dependent (p53, $p21^{waf, cip1}$, Bcl-2) or HIF-1α-independent (GADD153) manner, suggesting that there are at least two different adaptive responses to oxygen deprivation[264]. The cdki $p27^{kip1}$ inhibits the kinase activity of cyclin-cdk complexes, particularly cyclinE-cdk2 activity, resulting in cell cycle arrest[271]. It is a matter of debate whether the induction of p27 by hypoxia is HIF-1α dependent[264,265,272,273].

Endometrial cancer is the most common malignant tumor of the female genital tract. Estimated incidence of cancer in the uterine corpus in the US is 39,080 for 2007 (6% of all cancers)[1]. HIF-1α is overexpressed in many cancers[85], including endometrial cancer[121] and is associated with poor prognosis in stage 1 endometrial cancer[35].

In a recent study on cell cycle regulation during endometrial carcinogenesis[218], we observed a decrease in p27 expression in endometrial cancer compared to normal tissue and precursor lesions. However, p27 protein was highly re-expressed in perinecrotic (i.e hypoxic) areas of endometrial carcinomas. Here we analyzed the involvement of p27 in the hypoxic response *in vitro* and *in vivo* in endometrial cancer.

Our results demonstrate a critical role for $p27^{kip1}$ in the HIF-1 mediated hypoxic response of endometrial cancer cells *in vitro* and suggest a similar mechanism for cell survival in perinecrotic endometrial cancers.

Materials and Methods

Patients and Tissues

Paraffin-embedded clinical specimens from endometrioid adenocarcinoma (EC, n=39) were selected from the archives of the Department of Pathology of the University Medical Center, Utrecht, The Netherlands. These tissues were derived from patients operated between 1991 and 2004. None of the carcinoma patients received preoperative radio- or chemotherapy. Table 1 gives an overview of the patient demographics and main pathological features. Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our hospital[99].

Table 1. Patient characteristics and demographics

Clinicopathologic features of patients with endometrioid endometrial carcinoma		N=39	
		n	(%)
Age (yrs)	Mean	62.5	
	Minimum	40	
	Maximum	85	
Grade	1	11	(28.2%)
	2	17	(43.6%)
	3	11	(28.2%)
Myometrial Invasion	< 50%	17	(43.6%)
	≥ 50%	22	(56.4%)
Stage	1	13	(33.3%)
	2	10	(25.6%)
	3	11	(28.2%)
	4	5	(12.8%)

Immunohistochemistry

HIF-1α[121], p27 and Ki67[218] were immunohistochemically stained on 5 µm thick paraffin slides. Slides were deparaffinized with xylene and serial ethanol dilutions. For HIF-1α, target retrieval solution (Dako, Glostrup, Denmark) was used for antigen retrieval with all slides placed in a water bath for 45 minutes at 97°C. A cooling off period of 20 minutes preceded the incubation of the HIF-1α mouse monoclonal (BD Biosciences, Pharmingen, Lexington, USA), at a dilution of 1:500. The catalysed signal amplification system (Dako) was used according to the manufacturer's instructions to detect HIF-1α.

For p27 and Ki67 staining, endogenous peroxidase activity was blocked for 30 minutes with a buffer solution containing peroxide, followed by antigen retrieval (citrate buffer, pH 6.0, 20 minutes). Next, for p27, slides were incubated with the primary antibody (anti-kip1/p27, BD Transduction Laboratories, USA; dilution 1:500, overnight 4°C), followed by the secondary antibody (Powervision (Poly-HRP-anti Ms/Rb/RtIgG biotin free), ImmunoVision Technologies, Brisbane, CA, USA; ready to use). For Ki67, slides were further processed in an automatic staining device. Incubation

with the primary antibody (anti-Ki67, Immunotech, Beckman Coulter, Fullerton, CA, USA; dilution 1:100, 60 minutes, room temperature) was followed by the secondary antibody (biotinylated horse-anti-mouse, diluted 1:500, Vector BA-2000, Vector Laboratories, Burlingame, CA, USA), followed by streptavidin-biotin complex, diluted 1:1000, Immunotech).

All slides were developed with diaminobenzidine followed by haematoxylin counterstaining. Before the slides were mounted all sections were dehydrated in alcohol and xylene Appropriate positive and negative controls were used throughout.

Evaluation of Staining

Two authors (PvD, NH) scored all slides blinded to clinicopathologic data and results of the other stainings. The percentage of dark, homogenously stained nuclei was estimated as before[100,101,121], ignoring cytoplasmic staining. The pattern of HIF-1α staining was described as perinecrotic, diffuse, or mixed. For p27, the pattern of staining was noted as perinecrotic, centrally in tumor islands, diffuse, or mixed. A perinecrotic staining is a type of staining in which cells only stain in the close vicinity of a necrotic field. As central and perinecrotic patterns were seen in combination and both likely represent staining in hypoxic areas, these were further grouped. The pattern of Ki67 staining was classified as diffuse (no association with necrosis) or anti-perinecrotic (less staining around necrosis).

HIF-1α/p27 double staining

To show colocalization of HIF-1α and p27, immunohistochemical double staining was performed. Slides were deparaffinized and dehydrated with xylene and serial ethanol dilutions. Endogenous peroxidase was blocked with a buffer containg peroxide for 15 minutes. Then, antigen retrieval was performed with EDTA buffer pH 9.0 for 20 minutes. After cooling down, slides were incubated with anti-HIF-1α antibody (mouse monoclonal, BD Transduction, dilution 1:50, overnight 4°C), followed by incubation with the secondary antibody Powervision (Poly-HRP-anti Ms/Rb/RtIgG biotin free, ImmunoLogic, ImmunoVision Technologies, Brisbane, CA, USA, ready to use) for 30 minutes. All slides were developed with diaminobenzidine followed by incubation with anti-p27 monoclonal antibody (Kip1, Transduction, dilution 1:500, overnight 4°C). Afterwards, slides were incubated with Fast Red substrate (Dako). Before the slides were mounted all sections were air-dried and put through xylene.

Cell culturing

Endometrial carcinoma cells, HEC-1B, obtained from American Type Culture Collection (LGC Promochem, Middlesex, UK) were cultured in minimal eagles medium (ATCC, LGC Promochem, Middlesex, UK) containing penicillin-streptomycin (Gibco BRL, UK) and 10% fetal calf serum (Gibco BRL, UK). For hypoxic treatment cells were incubated at 1% 0_2 in a hypoxia working station (Ruskin Technology, Leeds, United Kingdom).

Plasmids and transfection

Cells were transfected using Lipofectamin 2000© (Invitrogen, CA, USA) according to manufacturer's protocol. The HIF-1α knockdown plasmid was generated by ligation

of annealed oligo sequences 5'-GATCCCCGGACAAGTCACCACAGGACTTCAAGAGA GTCCTGTGGTGACTTGTCCTTTTTGGAAA-3' and 5'-AGCTTTTCCAAAAAGGACA AGTCACCACAGGACTCTCTTGAAGTCCTGTGGTGACTTGTCCGGG-3' into Bgl II and Hind III digested pSuper-puro (kindly provided by Dr. Reuven Agami, Dutch Cancer Institute, Amsterdam).

Western blotting

20 µg protein/lane was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) followed by Western blot analysis. The following antibodies were used; anti-HIF-1α (1:250) and $p27^{kip1}$ (1:1.000) (BD Transduction Laboratories, CA, USA), anti-β-actin (1:5.000) (US Biological, MA, USA), and goat anti-mouse IgG + IgM HRP conjungate (1:10.000) (Biosource, CA, USA). Enhanced Chemo Luminescence (ECL, Amersham Biosciences, UK) was used for signal detection according to the manufacturer's protocol.

FACS analysis

Cells were labelled with BrdU (Sigma-Aldrich Co., MO, USA) for two hours prior to trypsinisation and collection in 1ml of medium. Subsequently, cells were fixated by gently adding 4.5ml of 100% ethanol and left overnight. Cells were permeabilized and DNA was denatured by adding 500µl of a solution containing 5M HCl and 0.5% TritonX-100 for 20 minutes and were stained with anti-BrdU-FITC (Roche, IN, USA) (1:100) in PBS containing 1% BSA and 0.1% Tween-20 for 30 minutes. After washing the cells in PBS, they were stained with Propidium Iodide (Sigma-Aldrich Co., MO, USA) for 20 minutes and analyzed using a FACS Calibur system (BD Transduction Laboratories, CA, USA).

Statistical Analysis

To assess correlations between p27 expression and Ki67 patterns and clinicopathological data the Fisher's exact test was used. Two sided p-values <0.05 were considered significant. All statistical analyses were performed by using SPSS for Windows version 12.0.1., 2003 (SPSS Inc., Chicago, IL).

Results

p27 en HIF-1α are perinecrotically co-expressed in endometrial cancers

To study the relationship between HIF-1α and p27 in endometrial carcinoma, we analyzed 39 tumor tissue sections for HIF-1α and p27 protein expression. We observed three patterns of HIF-1 immunostaining: exclusively perinecrotic (representing areas with low oxygen levels) (6/39, 15.4%), diffuse (11/39, 28.2%) or mixed (20/39, 51.3%), the remaining 2 (5.1%) were HIF-1α negative[121]. Expression of p27 showed differential types of staining patterns: a central/ perinecrotic pattern (14/39, 35.9%), a diffuse pattern (17/39, 43.6%), or a mixed pattern (4/39, 10.3%), the remaining 4 (10.3%) were p27 negative. In 50% of the tumors (13/26) with perinecrotic HIF-1α expression, we observed central/perinecrotic p27 staining. In 9 of these, p27 expression was confined to these perinecrotic areas, suggesting hypoxia-induced p27 re-expression. Co-expression of p27 and HIF-1α in perinecrotic areas was confirmed by HIF-1α/p27 double immunohistochemistry as shown in figure 1F.

Clinicopathological correlations

p27 staining pattern did not correlate with the clinicopathological survival parameters, age, stage or myometrial invasion. However, p27 in a central/perinecrotic pattern was more often seen in higher grade endometrial cancers ($p = 0.039$).

p27 expression under hypoxia is HIF-1α dependent

Since we observed a co-expression of p27 and HIF-1α in a significant fraction of endometrial cancer, we addressed whether p27 is induced by hypoxia. Therefore, we analyzed p27 expression in HEC1B human endometrial carcinoma cells cultured for different time periods under hypoxic conditions. Normoxic HEC1B cells did not express HIF-1α and had modest p27 levels. Induction of HIF-1α levels was seen after 1 hour of hypoxia, which peaked at 6 hours and persisted up to 48 hours of hypoxia (Figure 2A). The expression of p27 was increased after 24 hours of hypoxia and after 48 hours of hypoxia. To study whether this induction is dependent on HIF-1α, we used RNA interference (RNAi) to knock down HIF-1α in HEC1B cells. RNAi against HIF1a markedly reduced hypoxia-induced HIF-1α levels (Figure 2B). This reduced HIF-1α completely blocked the induction of p27 by hypoxia, indicating that hypoxia-induced p27 expression is HIF-1α dependent.

Hypoxia induces a G1 and G2/M arrest

Expression of p27 induces cell cycle arrest by blocking the G1 to S-phase transition [19]. As hypoxia induces p27, we wondered whether hypoxia can induce G1 arrest in endometrial carcinoma cells. Therefore we compared the cell cycle profile of HEC1B cells cultured for 24 hours under hypoxic or normoxic conditions by FACS analysis. Hypoxia resulted in a decrease in amount of cells in the S-phase from 41,52% to 13,77%, whereas both G1 and G2/M phase were enriched from 41,89% to 55,80 and from 16,59 to 30,43% respectively (Figures 3A and B). This demonstrates that hypoxic endometrial carcinoma cells undergo a block in G1 and G2/M in response to hypoxia.

p27 re-expression leads to diminished proliferation around necrosis

Previous results suggest that one of the roles of p27 re-expression after oxygen deprivation in endometrial carcinomas is the induction of cell cycle arrest. To support this hypothesis we investigated the expression of cell division marker Ki67 in the perinecrotic tumor areas. 39 tumor sections were immunohistochemically stained for Ki67 and scored for patterns of staining. In 5 tumors Ki67 count was too low to be assessed for staining pattern and therefore not considered in this analysis. Table 2 shows staining results. An anti-perinecrotic staining was observed in 12 tumors, a diffuse staining in 22 cases. Perinecrotic p27 expression colocalized significantly with anti-perinecrotic staining of Ki67 in 9/14 cases (64.3%, $p=0.012$) (Figures 3C, D and E). These data indicate that hypoxic p27 re-expression functions to cease cell growth in perinecrotic tumor fields.

Table 2. Cross table of patterns of immunohistochemical p27 and Ki67 staining of endometrial cancer.

		Ki67		Total
		Diffuse	Anti-perinecrotic	
p27	Negative	3	0	3
	Diffuse and mixed	14	3	17
	Central and perinecrotic	5	9	14
Total		22	12	34

Fisher's exact test: p=0.012

Figure 1. *Immunohistochemical staining of p27 and HIF-1α in endometrial carcinoma. Typical patterns are shown: A and B same tumor and consecutive slide with perinecrotic staining of p27 (A), perinecrotic staining of HIF-1α (B); C and D same tumor and consecutive slide with perinecrotic staining of p27 (C), perinecrotic staining of HIF-1α (D); tumor slide with 3 normal glands (E) the normal glands and the stroma around the glands express p27 and the tumor glands show loss of p27 staining; double staining p27 and HIF-1α (F). Asterisk implies necrosis in the tumor.*

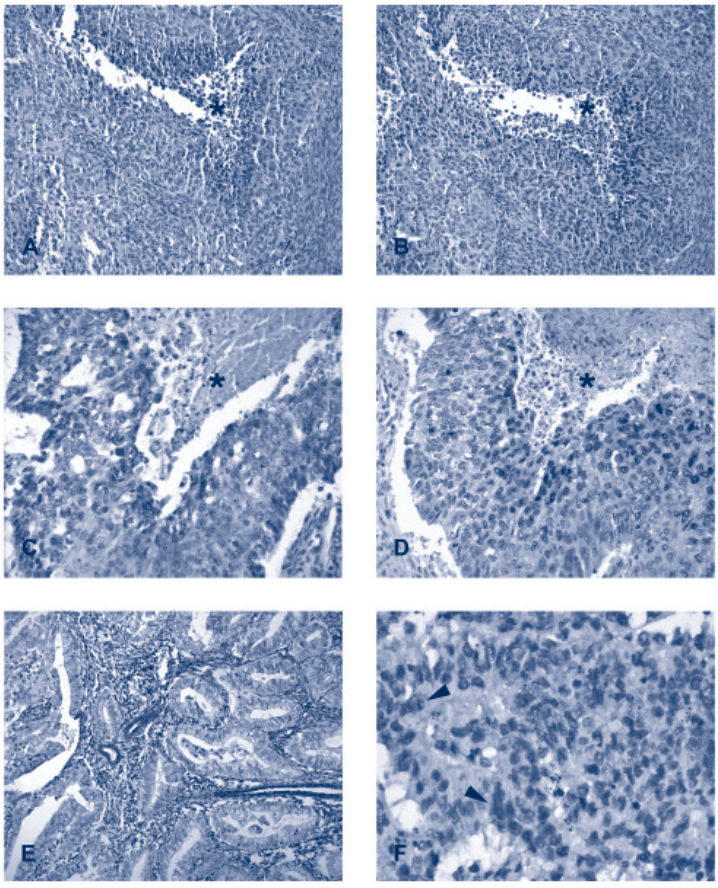


Figure 2. *Hypoxia induces p27 expression in a HIF-1 dependent manner.*
A. *Western blot analysis of HIF-1a, p27 and β-actin in HEC-1B cells cultured under hypoxic conditions for indicated periods of time.*
B. *Western blot analysis of HIF-1a, p27 and β-actin in HEC-1B cells transiently transfected with (lanes 2 and 4) and without (lanes 1 and 3) a knockdown vector targeting HIF-1a, under normoxic (lanes 1 and 2) and hypoxic conditions (lanes 3 and 4). Control cells were transfected with an empty vector. When indicated cells were treated for 24 hours under hypoxic conditions. These blots are representative of at least two independent experiments.*

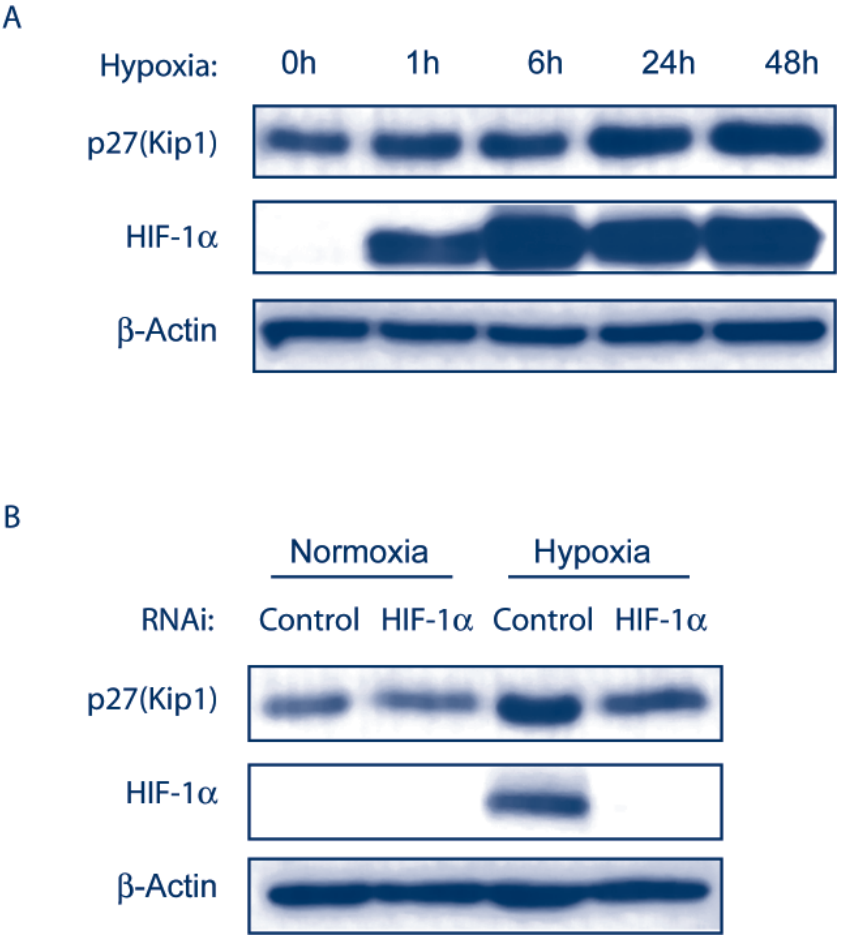
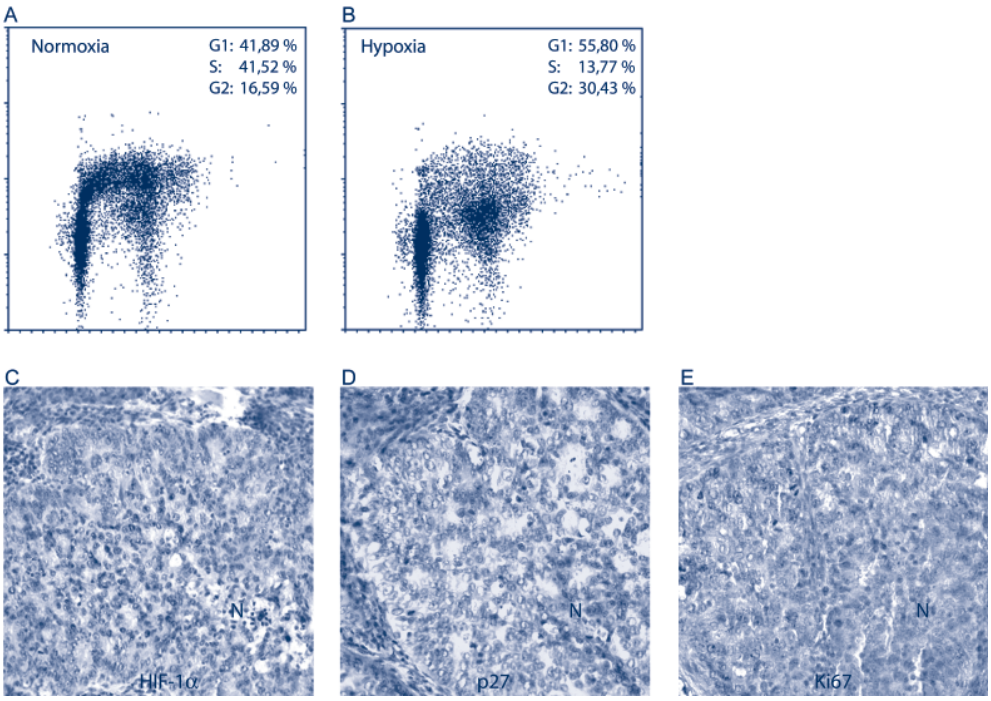


Figure 3. *FACS analysis and Ki67 staining.*
(A-B) HEC1B cells were cultured for 24 hours under normoxic or hypoxic conditions, labeled with BrdU (y-axis) and Propidium Iodide (x-axis) and subjected to FACS. Per experiment 10.000 cells were counted. G1, G2 and S-phase content were calculated by mannual gating. The same gates were used for both the normoxia (A) and hypoxia (B) experiments. Results were independently reproduced three times and a representative experiment is shown.
(C-E) Consecutive slides of the same tumor showing perinecrotic HIF-1α staining (C), central/perinecrotic p27 staining (D) and anti-perinecrotic Ki67 staining (E). N = Necrosis.



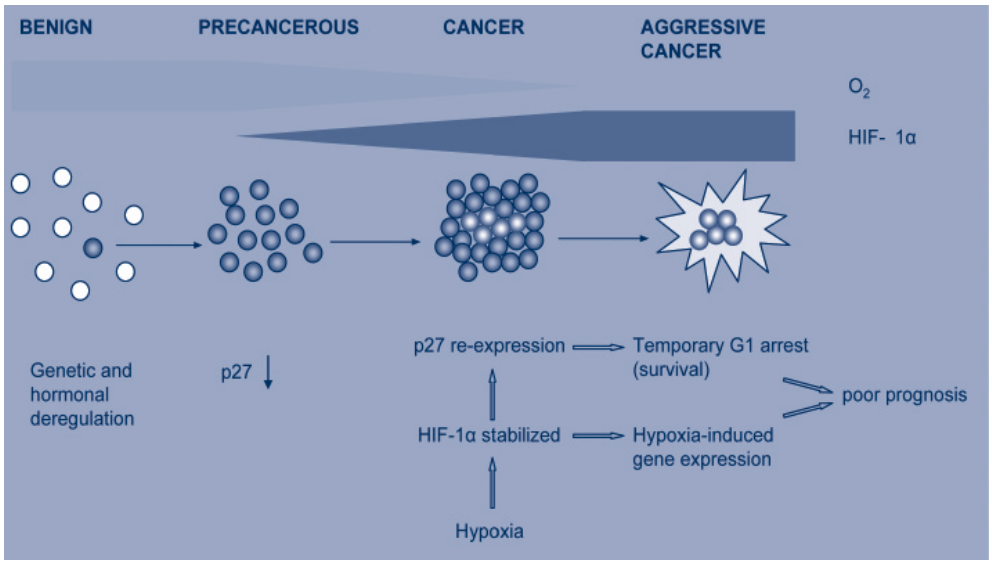
Discussion

HIF-1α is a key regulator for survival of hypoxic tumor cells. However, the function of HIF-1α in hypoxia-induced cell cycle arrest is less well understood. We investigated associations between HIF-1α and p27 in endometrioid endometrial carcinoma, the most frequent histological subtype of endometrial cancer. p27 expression was often seen around necrotic, hypoxic areas in otherwise p27 negative cancers. As p27 is highly expressed in the normal endometrium but gets down regulated during carcinogenesis[218], this points to hypoxia induced perinecrotic re-expression of p27. Perinecrotic re-expression of p27 was more often seen in higher grade endometrial cancers. We propose that the central expression of p27 that was sometimes observed in solid tumor groups in tumors with perinecrotic p27 expression may be an early indicator of hypoxia. This perinecrotic expression of p27 has not been described before in endometrial carcinomas but has been noted in melanomas[275] and in gliomas[276], although these studies did not compare p27 to HIF-1α expression.

Hypoxia is known to upregulate p27 but there are some conflicting data on the role of HIF-1 in this process and implications for G1/S cell cycle arrest[264,265,272,277,278]. Hypoxic regulation of p27 has been shown to be regulated via a HIF-1 independent region of the proximal p27 promoter in mouse embryonic fibroblasts (MEFs)[272], although it has also been shown that gene silencing of HIF-1α by small interfering RNA reduces p27 protein and mRNA levels[273]. Furthermore, in HIF-1α wild-type splenic B lymphocytes induction of p27 was seen after hypoxia, which was absent in HIF-1α double knock out cells. Thus, these studies are contradicting. Green et al showed that p27 double knock-out mice are still able to initiate a G1/S arrest[278], however Mack et al showed that in VHL double knock out MEFs increase of p27 was associated with diminished proliferation[273].
Differences between other studies and our results might be explained by different experimental cell systems used, which is endorsed by data from Box et al who showed that different cell lines differed in their ability to upregulate p27 and to undergo G1/S cell cycle arrest in response to hypoxia within one study with equal experimental environmental conditions[277].
However, our results provide evidence for *in vivo* dynamics apart from *in vitro* results. We show that the induction of p27 during hypoxia is HIF-1α-dependent in the endometrial carcinoma cell line HEC1B. Our study provides further insight into the mechanism governing the re-expression of p27 in perinecrotic areas of endometrial cancer. It remains to be investigated whether HIF-1α dependent re-expression of p27 is a direct effect. Since we show p27 induction after 24 hours of hypoxia, an indirect effect is not excluded.
Recently, p27 was shown to be regulated by the energy sensing LKB1-AMPK pathway, via AMPK-dependent phosphorylation, which increased stability of p27[279]. The stable form of p27 was sufficient to induce autophagy, an evolutionarily conserved process wherein catabolism of intracellular organelles generates energy. Hypoxia can also induce LKB1-AMPK activity because of failure to generate sufficient ATP required for cellular functions[280-281]. This process turned out to be independent of HIF-1α in MEFs[281]. In DU145 cells (prostate cancer) however, it has been shown that AMPK activity itself is critical for the HIF-1 transcriptional activity and its target expression[282], which implicates that HIF-1 is situated downstream in this pathway. It might be of significance to search for the role of AMPK in hypoxic, HIF-1α dependent induction of p27 in (endometrial) cancer development.

We conclude that in endometrioid endometrial carcinoma, p27 up regulation by hypoxia is HIF-1α dependent. Furthermore, hypoxic HEC1B cells undergo a partial G1 arrest, corroborated by lack of staining for the proliferation marker Ki67 in perinecrotic p27/HIF-1α expressing cells in tumor sections. This cell cycle arrest may contribute to survival of cancer cells in hypoxic tumor areas. We propose a model for endometrioid carcinogenesis, which involves early loss of p27, which facilitates proliferation, and late HIF-1-mediated re-expression of p27, that results in dormancy of these hypoxic cells (Figure 4). Combined with the hypoxia induced expression of genes regulating supply of energy, growth factors and other survival factors, this may promote survival and adaptation of sub clones within the tumor that may contribute to metastatic disease and poor clinical outcome.

Figure 4. Schematic illustration of the HIF-1α – p27 pathway in endometrioid endometrial carcinogenesis.



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chapter 9

General discussion and Summary

In this thesis the HIF-1α pathway and cell-cycle deregulation were studied to determine their potential involvement in endometrial carcinogenesis. The relevance and clinical implications of the findings and the future perspectives are discussed in this chapter.

Although the role of HIF-1α has been studied extensively *in vitro* and tissue studies on different types of cancer, research on endometrial cancer is underrepresented. HIF-1 is a transcription factor induced by hypoxia, but also by some oncogenes, tumor suppressor genes, and growth factors.[47-53] Regulation of HIF-1α is a delicate equilibrium in which prolyl hydroxylases, Von Hippel Lindau protein, and changes in the HIF-1α gene itself are essential.[44,45,116,154,155] HIF-1, a dimer consisting of HIF-1α and HIF-1β, can induce angiogenesis, glycolysis, erythropoiesis, and other processes that allow cells to survive during hypoxia and hypoglycemia.[54-57] Because these processes are also involved in endometrial carcinogenesis and are likely to influence malignant potential of endometrial cancer, we first tried to unravel the role of HIF-1 in endometrial carcinogenesis, and then analyzed potential causes of an increased expression in endometrial cancer.

Furthermore, we studied aberrations in cell-cycle regulation in preneoplastic and neoplastic endometrial tissue compared with normal endometrium. In this analysis, our attention was attracted to an increased expression of p27 around necrotic parts of the tumor, which prompted us to study p27 regulation by hypoxia and its influence on cell-cycle arrest in endometrial cancer.

As has been described in other cancer types,[217] the deepest endometrial cancer parts invading the myometrium must be the most active and aggressive ones showing highest proliferation, invasive behavior, and angiogenesis. Because HIF-1α expression has been associated with proliferation and angiogenesis, we studied the topographic expression of cell-cycle regulators and proteins related to the hypoxia response.

It is generally thought that endometrial tubal metaplasia is a benign disease,[14] although molecular studies are scarce. Therefore, we determined expression patterns of cell-cycle proteins and HIF-1α in tubal metaplasia of the endometrium in comparison with tubal metaplasia in other organs.

HIF-1α in Endometrial Cancer

In **Chapter 2** expression patterns of **HIF-1α and its downstream genes** Glut-1, VEGF, and CAIX, as well as angiogenesis, was assessed in the **endometrioid carcinogenetic spectrum** represented by inactive endometrium, endometrial hyperplasia, and endometrioid endometrial carcinoma. HIF-1α showed increasing overexpression from inactive endometrium through hyperplasia to endometrioid carcinoma. Perinecrotic, hypoxia-associated HIF-1α overexpression was absent in inactive endometrium, rare in endometrial hyperplasia, and frequent in endometrioid carcinoma. This largely confirms previous studies on HIF-1α in endometrial carcinogenesis. Indeed, one earlier study found only negative benign endometrium[102]: 49% to 74% of endometrial cancers are considered to overexpress HIF-1α.[35,102] For CAIX and Glut-1 we noticed an increasing overexpression from

normal to malignant endometrium too. Glut-1 was often (94.9%) and exclusively expressed in carcinomas, in line with previous studies.[92,103]

In contrast with previous findings in breast cancer,[101] in which only perinecrotic HIF-1α (that is thought to be hypoxia driven) was, in general, associated with Glut-1 and CAIX expression. In endometrial carcinoma diffuse HIF-1α expression (thought especially to be due to nonhypoxic stimuli) was often accompanied by activation of the HIF-1α downstream genes CAIX, Glut-1, and VEGF. This may point to functional diffuse HIF-1α expression, which is further evidenced by the fact that highest mean vascular density (MVD) was seen in cases with diffuse HIF-1α expression compared with cases with perinecrotic/mixed expression. MVD differed significantly between inactive (lowest), hyperplasia, and carcinoma, in line with previous studies. The angiogenic switch during endometrioid endometrial carcinogenesis therefore seems to lie between inactive and hyperplastic endometrium.

Because we found no correlation between VEGF and MVD, VEGF might not be the only angiogenic factor in endometrial carcinoma. On the other hand, we observed VEGF in perinecrotic areas where HIF-1α is also preferentially expressed. This observation points to a biological relation between hypoxia, HIF-1α, and VEGF expression. In our study, HIF-1α was associated with VEGF expression, which underlines this idea.

The findings of a diffuse (attributed to nonhypoxic causes) HIF-1α staining pattern in a substantial amount of endometrial cancer guided us to study HIF-1α gene alterations in the oxygen-dependent domain and the coregulatory molecules: VHL, prolyl hydroxylase (PHD)1, PHD2, and PHD3. In addition, correlations with PTEN and p-AKT expression were analyzed as **possible explanations for diffuse HIF-1α expression**. This study is described in **Chapter 3**. In short, type of HIF-1α expression did not correlate with PTEN, p-AKT, and VHL expression. However, total expression of the PHDs (assuming redundancy in function[114,123]) was less in diffusely HIF-1α-expressing tumors than in tumors with pronounced perinecrotic HIF-1α expression, largely caused by reduced expression in PHD1. This indicates that PHD expression may be involved in diffuse HIF-1α expression. However, because correlations were weak, the search for other factors responsible for diffuse HIF-1α expression in endometrial cancer must continue.

In **Chapter 4** we described our sequence analysis for possible **mutations in the ODDD of HIF-1α**. All tumors showed wild-type sequence in exon 9 (containing proline 402). One patient showed the known silent a2024g polymorphism on exon 12 in tumor and normal tissue, which was therefore thought to be a SNP. The polymorphism P582>S (c1772>t) was heterozygously found in 6 of 58 (10.3%) tumors and homozygously in 3 of 58 (5.1%). One of the patients with the heterozygous polymorphism in the tumor showed wild-type normal tissue, pointing to a new somatic mutation. When we compared these results with European controls from the literature, we concluded that P582>S variations do not increase risk for endometrial cancer. Tumors with P582>S variation did not show higher HIF-1α expression (as is shown for lung cancers[164]); however, MVD and p-AKT expression were higher in these tumors. These findings are possibly induced by a higher transactivation of HIF-1α.[162,169,170] Therefore, sequencing of HIF-1α is not necessary as a screening method

for increased risk for endometrial cancer but might be correlated to survival. It is, therefore, in that specific view interesting for further research.

Cell-cycle Proteins and Proliferation in (Pre)neoplastic Tissue of the Endometrium

Since derailments of the control mechanisms of the cell cycle are associated with malignant tissues, they may play a role in carcinogenesis. **Chapter 5** describes our study regarding the expression of **cell-cycle proteins in the endometrioid carcinogenetic spectrum**. Indeed, differences in expression between normal, preneoplastic and neoplastic tissue were found. Markers of proliferation, cyclin A and Ki67(MIB-1), gradually increased from normal through hyperplasia to carcinoma.

Activated RAS, microsatellite instability, and β-catenin and PTEN mutations can cause upregulation of cyclin D1 and play a major role in endometrial cancer. Immunohistochemical associations have been found for β-catenin and cyclin D1 in endometrial carcinoma.[187-189] That cyclin D1 plays a role not only in cancers, but also in the progression of precursor lesions towards cancer is shown by the fact that cyclin D1 was absent in inactive endometrium and was upregulated in endometrial hyperplasia and carcinoma .

Increased expression of β-catenin could also possibly explain the p16 overexpression we found in the spectrum from normal through endometrial hyperplasia to endometrial carcinoma.[195] Increased expression of p16 is a remarkable observation because low p16 has also been observed in tumors, as a result of mutations, deletions, or promoter methylation of the p16 gene. Cdk2 and p53 also gradually increased from normal through endometrial hyperplasia to carcinoma, indicating their potential importance in both early and late carcinogenesis.

Loss of wild-type p53 function predisposes to malignant transformation since damaged cells may enter the S-phase of the cell cycle without appropriate DNA repair. Mutations of p53 often result in conformationally altered and functionally defective protein with a longer half-life than the wild-type counterpart, thus permitting immunohistochemical detection of the accumulated protein product. However, the correlation between immunohistochemical nuclear p53 accumulation and gene mutations is not absolute, with a reported concordance of 76% for endometrial carcinomas.[197] Normal tissue is immunohistochemically (nearly) negative for p53 as we and others have shown.[65,180,181,198] No or few positive cells were found in endometrial hyperplasia by us and others, and our endometrioid endometrial carcinoma, like those in other studies often showed p53 accumulation.[65,177,180,181,198,199]. Cdk2, less studied than the other cell-cycle–related proteins, was found in the majority of endometrial tumors, increased over the spectrum of (pre)neoplasias, and was associated with cyclin E, cyclin A, p21, and Ki67(MIB-1) in endometrial carcinoma. Therefore, cdk2 may play an important role in initiation and progression of endometrial carcinoma.

Cyclin E and p21 increased and p27 decreased from hyperplasia to carcinoma, underlining their potential role in late endometrial carcinogenesis. Cyclin E complexes with cdk2 to move the cell cycle forward from late G1 to S phase. Earlier studies also found weak expression of cyclin E in normal endometrial glandular cells, but increased expression in some hyperplasia[59,77,186] and most endometrial carcinoma.[59,65,77,177,184,200,201] Increased cyclin E expression can result from amplification of the CCNE gene that has been found occasionally in endometrioid endometrial carcinoma or mutations

in the hCDC4 gene, coding for a protein, which tags phosphorylated cyclin E for ubiquitination and proteosomal degradation; such mutations have been found in endometrial carcinoma.[77,202] The CKI p21 is a p53- and cyclin D1-regulated potent inhibitor of CDKs and can thereby inhibit the phosphorylation of Rb. Our finding of increased expression in endometrial carcinoma is in accordance with the literature.[65,177,179,181,184,204,205] In view of its function one would, however, expect loss of expression of p21 to be a carcinogenetic event. Likely, p21 overexpression in endometrial lesions is merely a reflection of a failing feedback attempt of a normal protein rather than the result of an alteration in the p21 gene. For the CKI p27, loss of expression has been found in endometrial hyperplasia and especially in endometrial carcinoma. Interestingly, we also noticed an increasing trend in expression of p27 in higher grades of carcinomas. A possible explanation for this is elaborated in Chapter 8 in which we focus on HIF-1α and p27 interactions. Upregulation of p27 induced by PTEN has been reported in different cell lines, suggesting that p27 may be a target of the PTEN cell-cycle arrest pathway in endometrial carcinoma. As mutations in the PTEN gene occur early in pathogenesis of endometrioid endometrial carcinoma, this might be an explanation for decreasing p27 in early stages of preneoplasias.

From this study, important challenges arise for the future. The EIN classification has been shown to be more objective than the WHO classification for preneoplastic endometrial tissues. CKIs p16 and p21 were significantly more expressed in EIN than in non–EIN/hyperplasia. This study provides, in addition to PTEN, further biological evidence for the EIN classification that may help to discriminate between reversible and neoplastic hyperplasia. More research should focus on more precisely identifying the real preneoplastic lesion.

Differences in expression are seen not only between normal and (pre)neoplastic tissues, but also in the cancer itself **(Chapter 6)**. A heterogeneous expression of cell-cycle proteins was observed in endometrial carcinomas. These cancers clearly show an **invasive front** that is characterized by higher proliferation (more Ki67) and progressive derailment of the cell-cycle regulators cyclin E, p16, and cdk2, but not by an increased hypoxic response. This was the first study considering this subject in endometrial carcinoma. The concept of an "invasive front" has been proposed in other cancers, but differences in definitions and methods in these studies make comparisons difficult. We used as a definition of the invasive front the deepest rim of cancerous tissue in the myometrium with a width of 1.22 mm, which was one field of vision with the microscope that we used for quantitation.

Differences in protein expression at the invasive front versus the center parts of the tumor might be caused by clonal selection or by interactions with the defending normal tissue. Since the edge becomes the inner tumor area when the tumor continues its growth and a new invading tumor front is formed by multiplying malignant cells that outflank the initial edge, clonal selection is less likely and interactions with the surrounding extracellular matrix components that regulate growth and differentiation of epithelial cells may be more important.

The main focus regarding the invasive front in cancers has been on matrix metalloproteinases, stromatogenesis, and angiogenesis,[220,228,230-232] which is

understandable since the first provides the prerequisite for cancer cells to invade by breaking down tissue barriers, and the last allows survival of cells by the increased supply of oxygen and nutrients and generates a pathway to form metastases. The term stromatogenesis indicates the existence of a specific stroma in endometrial cancer, after the proliferation of peritumorous fibroblasts and the disruption of normal tissue continuity facilitates tumor cell invasion. It has been stated that it is probable that at the edge of the tumor, important interactions occur between cancer cells, endothelial cells, and the supporting stroma.[233] This same group of investigators proposed after their study on differences in vascularization in the front and the inner area of the non–small-cell lung tumor, that the immunostained vasculature be considered not as a static image but as the result of a dynamic process occurring during tumor growth.[233]

We observed higher p16 expression at the front in many cases, in line with observations in colorectal cancer in which 40% of the tumors do not express p16 in central parts of the tumor in contrast with the invasive front.[222] Jung et al proposed that this may be due to promoter methylation in the central parts.[222] Svensson et al showed by immunohistochemistry and Western blots of microdissected tumors that p16 was upregulated at the invasive front of the majority of basal cell carcinomas with infiltrative growth patterns, but with ceased proliferation (low MIB-1 in invasive margins).[236] The CKI p16 is obviously—in the invasive front in endometrial carcinoma—unsuccessful in ceasing cell-cycle progression, since MIB-1, a reliable marker of proliferation, is highly expressed here.

We failed to demonstrate elevated HIF-1α levels in the invasive front. Possibly, hypoxia in the center of the often fairly solid growing endometrial cancers may induce hypoxia-related proteins, which may balance the expression at the invasive front.

Apart from being interesting from a biological point of view, the observed differences in expression of proteins between the invasive front and central tumor parts in endometrial cancer may be clinically quite relevant. First, when creating tissue arrays from endometrial cancer, sampling from both the central and deepest invading parts needs to be considered. Second, even when whole tumor sections are being analyzed by the pathologist for proliferation-related phenomena, heterogeneity between central parts and the invasive front needs to be taken into account. Third, thymidine phosphorylase expression in fibroblasts and myometrial cells confronting the tumor edge of endometrial carcinoma appeared to be correlated with adverse prognostic factors in endometrial cancer such as high tumor grade, deep myometrial invasion, and advanced stage of disease, so the invasive-front concept may even have prognostic implications.

In a paper written by Sivridis and Giatromanolaki,[283] the postmenopausal endometrium was studied to search for the potential histologic state that forms the (as they called it) "lurking potential for giving rise to an endometrial adenocarcinoma." These researchers concluded that the "lurking potential" is the proliferative state, still present in postmenopausal tissue, probably triggered by continuous low levels of estrogens. They did not mention tubal metaplasia, although this must have been

present because tubal differentiation is also caused by estrogens. Tubal metaplasia is generally considered to be a harmless entity. However, a thorough search of the literature shows that this conclusion has been doubted by some authors. Umezaki suggested that tubal metaplasia should be considered a neoplastic entity of uterine cervical glandular lesions that may have the potential to undergo malignant transformation.[262] Up to now, however, this is not a widespread view.

In **Chapter 7** p16 expression in **tubal metaplasia** (TM) in the endometrium was assessed in comparison with normal endometrium, normal Fallopian tube, and TM in ovarian inclusion cysts and the cervix. Additionally, cell-cycle proteins, HIF-1α, Glut-1, and CAIX were verified. These proteins were aberrantly expressed in TM compared with normal parts of the endometrium. LhS28, a ciliated cell marker, was used to improve visualization of TM. Our observations showed that mosaic expression of p16 is a consistent phenomenon of TM in the female genital tract, especially in the endometrium.

We also noticed, as others did before,[14,121] that ciliated cells are present more during the proliferative phase compared with secretory endometrium. Expression of p16 has been observed before in cyclic endometrium, more in the proliferative than the secretory phase, but has not been studied and clearly linked to TM as we did. We propose that TM of the endometrium could be a potentially premalignant lesion, based on the following arguments.

First, aberrant expression of p16 is regarded as a carcinogenetic event in many tumors, including those in the gynecological tract. p16 is very frequently aberrantly expressed in cervical dysplasia and carcinoma where it may, however, be a bystander effect of human papillomavirus (HPV) E6/E7 cell-cycle activation without an inherent contribution to carcinogenesis. In other malignancies the carcinogenetic role of p16 alterations are much more clearly defined.[260,261] As we showed in our study on cell-cycle expression described in Chapter 5, it was also our observation that p16 expression is increased during carcinogenesis. Other studies revealed that in endometrial carcinoma of the endometrium, p16 positivity rates vary from 30% to 94%, especially in the squamous areas.[251] Aberrant p16 expression has also been found in endometrial hyperplasia.[68,263] In serous and clear cell endometrial cancers, p16 positivity is also frequent, although the number of cases studied so far is low.[70,71,192,238]

Second, in the present study, p16 is also aberrantly expressed in TM in some ovarian inclusion cysts. These cysts have been proposed to be precursors of ovarian cancer.[248] In ovarian cancer, especially the serous type, p16 is often aberrantly expressed.

Additionally, we found that the TM areas in the endometrium showed aberrant expression of many cell-cycle proteins, HIF-1α, Glut-1, and CAIX compared with normal tissue. The observed HIF-1α overexpression is possibly caused by aberrant expression of oncogenes and tumor suppressor genes that are known to upregulate HIF-1α. Glut-1 and CAIX expression in membranes is often associated with preneoplastic progress. This is remarkable in that we noticed this type of expression in parts of TM. Overexpression of cell-cycle–stimulating factors such as the CDKs and cyclins, and underexpression of inhibiting factors such as CKIs are frequently found in tumors,[59-67] and during endometrial carcinogenesis (see Chapter 5). In general, aberrant expression of cell-cycle regulators correlated with a more malignant subtype, a higher proliferation rate, recurrence, and worse survival in

different tumors.
The observed changes in cell-cycle regulators in TM are, however, not accompanied with obvious morphologic changes like in other dysplastic lesions. At this stage, it is not possible to morphologically discriminate potentially premalignant from harmless TM. Since obvious dysplastic changes are lacking, it is also difficult to indicate to which kind of cancer TM could progress. In view of the p16 expression patterns seen in endometrioid and serous carcinomas, we suggest that both these cancers could be at the far end of the progression spectrum of TM.

Linking HIF-1α to the Cell Cycle in Endometrioid Endometrial Cancer

Activity of HIF-1 provokes not only prosurvival (CAIX, VEGF, Glut-1) but also antiproliferative (proapoptotic) decisions. As a result a "stop-and-go" strategy as a dynamic balance to maintain overall cell growth and survival has been proposed.[284] However, the function of HIF-1α in hypoxia-induced cell-cycle arrest is not well understood and has never been studied in endometrial cancer.

Therefore, associations between **HIF-1α and p27** in endometrioid endometrial carcinoma are studied and described in **Chapter 8**. This study in human endometrioid endometrial carcinoma revealed that p27 expression is often induced around necrotic areas of the tumor. Because p27 is highly expressed in the normal endometrium but often gets downregulated during carcinogenesis and was often expressed perinecrotically in cancers that were otherwise p27 negative, this indicates that p27 gets re-expressed in hypoxic tumor regions. This perinecrotic re-expression of p27 was more often seen in higher grade endometrial cancers, which are more likely necrotic. It might be that in a hypoxic zone, upregulation of p27, which inhibits proliferation, is part of the cellular survival program.
Hypoxia is known to upregulate p27, but there is some discrepancy concerning the function of HIF-1 in this process.[264,265,272,273] We have shown *in vitro* using the human endometrium carcinoma cell line HEC1B with PTEN loss, analogous to most endometrioid endometrial carcinomas, that the induction of p27 in hypoxia is HIF-1α dependent. It remains to be investigated whether this HIF-1α–dependent re-expression of p27 is a direct effect. Since we show p27 induction after 24 hours of hypoxia, an indirect effect is possible. Hypoxic regulation of p27 has been shown before to be regulated via a HIF-1–independent region of the proximal p27 promoter[272]. Other HIF-1–controlled processes might therefore govern p27 regulation. In addition, p27 was recently shown to be regulated by the energy-sensing LKB1-AMPK pathway, via AMPK-dependent phosphorylation, which increased stability of p27.[279] The stable form of p27 was sufficient to induce autophagy (an evolutionarily conserved process wherein catabolism of intracellular organelles generates energy). Because hypoxia can activate the LKB1-AMPK pathway by deregulation of the AMP/ATP balance, it might be of significance for hypoxic regulation of p27.

In addition, the PTEN status might influence p27 expression. PTEN mutations are frequent in human endometrioid endometrial carcinomas.[117-120] We therefore chose to use a PTEN-mutated cell line for our *in vitro* experiments to stay as close as possible to the ***in vivo*** situation. Further study will be necessary to uncover the involvement of both the AMPK and PTEN pathways in hypoxic regulation of p27.

In short, we found that in endometrial carcinogenesis, the hypoxic response is turned on relatively late and not early in hyperplasias, which makes HIF-1α–targeted therapies unattractive for hyperplasias but feasible for cancers. Although one study described HIF-1α expression in the proliferative phase, the researchers used other staining methods than we did.[35] Furthermore, because CAIX, Glut-1, and HIF-1α are very much associated with cancer, these immunohistochemical stainings might be used in diagnostics in cases of doubt for preneoplastic or neoplasias.

Thus far, studies in which HIF-1α is used as a biomarker in endometrial cancer have revealed that HIF-1α was significantly higher in recurrent tumors when compared with their primary tumors, although it was not an independent marker for recurrent endometrial carcinoma.[285] In only one study of stage 1 endometrial cancers, HIF-1α was associated with a worse prognosis in multivariate analysis in combination with easy available clinicopathologic characteristics.[35] In two other studies a role was shown for HIF-1α in progression of cancer. However, HIF-1α was not an independent predictor.[286,287] These studies were limited by relatively small numbers of patients.

Immunohistochemical studies are difficult to compare because of a variation in definition of HIF-1α positivity. Possibly prognostic relevance of HIF-1α must be seen more in the background of its biological behavior. For example, these studies did not focus on differences of expression patterns throughout the tumor and correlations with prognosis as Vleugel et al did in breast cancer.[101] Also, according to Zhou et al,[288] HIF-1 shows a "Janus face." In one condition, HIF-1 endeavours survival; on the other, not. This is underlined by activation of downstream targets like CAIX and Glut-1 (cell survivors) on one hand and p27 on the other hand in this cancer. We should take this knowledge into account in prognostic studies, which may increase diagnosis and treatment for the individual patient.

Major Conclusions of This Thesis

1. HIF-1α and its downstream genes are increasingly expressed from normal through premalignant to endometrioid adenocarcinoma of the endometrium, paralleled by activation of downstream genes and increased angiogenesis.
2. The angiogenic switch during endometrioid endometrial carcinogenesis seems to lie between inactive and hyperplastic endometrium.
3. Tubal metaplasia might be a potential premalignant lesion in the endometrium.
4. Immunohistochemistry of p16 may help to identify TM areas in the gynecologic tract as an alternative for the ciliated cell marker LhS28.
5. During endometrioid endometrial carcinogenesis, there is increasing proliferation paralleled by progressive derailment of cyclin B1, cyclin D1, cyclin E, p16, p21, p27, p53, and cdk2, indicating the importance of these cell-cycle regulators in endometrial carcinogenesis.
6. In endometrioid endometrial carcinoma, p27 re-expression in hypoxic tumor areas is HIF-1α dependent.
7. Endometrial carcinomas clearly show an invasive front that is characterized by higher proliferation and progressive derailment of the cell-cycle regulators cyclin E, p16, and cdk2, but not by an increased hypoxic response.

8. Genotype variations of HIF-1α, and staining of PTEN, p-AKT, and VHL do not explain the non–hypoxia-related expression of HIF-1α that is seen in endometrioid endometrial carcinomas.
9. The decrease in expression of the prolyl hydroxylases, especially PHD1, may account for a diffuse HIF-1α expression in endometrioid endometrial cancers.
10. Genotype variation of amino acid P582 does not increase risk for endometrioid endometrial carcinoma.
11. An amino acid change of Proline 582 to Serine 582 of the HIF-1α protein is associated with increased MVD and p-AKT expression.

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Addendum

Nederlandse samenvatting voor niet-ingewijden

Baarmoederslijmvlieskanker is de meest voorkomende gynaecologische kanker, met 1400 nieuw gediagnosticeerde patiëntes per jaar. Het baarmoederslijmvlies is de binnenste bekleding van de baarmoeder, waarvan een deel maandelijks wordt afgestoten: de menstruatie. De functie van het baarmoederslijmvlies is het mogelijk maken van de innesteling van een embryo voor de zwangerschap. Echter, in dit weefsel kan een deel van de cellen ongeremd gaan groeien, zodat er een voorstadium van kanker ontstaat, hyperplasie genoemd. Hieruit kan kanker ontstaan.
In dit proefschrift worden studies beschreven die de bijdrage bestuderen van de 'HIF-1α pathway' en de ontsporing van regulatoren van de celdeling aan het ontstaan en verergeren van baarmoederslijmvlieskanker. Wat de term HIF-1α pathway is en wat regulatoren van de celdeling zijn wordt verderop in dit hoofdstuk uitgelegd.

Zuurstoftekort en Hypoxia-Inducible Factor-1α in baarmoederslijmvlieskanker

Kanker van de weefsels wordt gekenmerkt door een te snelle deling van cellen, ingroei van deze kankercellen in het omliggende weefsel en uitzaaiingen op afstand via de lymfebanen of de bloedvaten. In veel soorten kanker is de zuurstofvoorziening onvoldoende omdat er te weinig of kwalitatief te slechte bloedvaten aanwezig zijn. Dit zorgt voor een zuurstoftekort, waarvoor de medische term 'hypoxie' wordt gebruikt.
Normaliter worden er in cellen voortdurend eiwitten geproduceerd, waaronder Hypoxia-Inducible Factor-1α (HIF-1α). Dit eiwit zal in een cel met voldoende zuurstof snel worden afgebroken. Is er echter sprake van zuurstoftekort, dan wordt het eiwit niet afgebroken, maar verplaatst het zich naar de celkern, waar het samenvoegt met HIF-1β, en zo HIF-1 vormt. Dit HIF-1 stuurt via het DNA in de celkern processen aan die de zuurgraad in de cel verhogen, de cel meer energie geven en de energie op een andere manier gebruiken. Ook worden processen gestimuleerd die zorgen voor bloedaanmaak (het welbekende EPO) en de aanmaak van bloedvaten zodat er meer energie en zuurstof naar de cel kan komen.
Een manier om de hoeveelheid HIF-1α in een tumor te onderzoeken is door dit eiwit op hele dunne plakjes weefsel (coupe) te 'kleuren'. Deze veel gebruikte techniek heet immunohistochemie.
Er is veel onderzoek verricht naar HIF-1α, waardoor duidelijk is geworden dat de aanwezigheid van dit eiwit (bepaald met behulp van immunohistochemie) correleert met een slechtere levensverwachting voor de patiënt in diverse soorten kanker, waaronder baarmoederslijmvlieskanker.

Voorheen was het nog onbekend hoeveel HIF-1α er aanwezig is in hyperplasie in het baarmoederslijmvlies. In dit onderzoek is daarom begonnen met het ontrafelen van de rol van HIF-1α in het ontstaan van baarmoederslijmvlieskanker, met behulp van opgeslagen weefsel verzameld tijdens operaties. Normaal weefsel, hyperplasie en kankerweefsel werd immunohistochemisch gekleurd voor HIF-1α. Om te onderzoeken of dit HIF-1α ook functioneel was (dus de eerder besproken effecten veroorzaakt na samenvoegen met HIF-1β), werden kleuringen gedaan voor eiwitten die door HIF-1 worden aangestuurd en die de zuurgraad van de cel reguleren (CAIX), die de hoeveelheid suiker die de cel ingaat reguleren (Glut-1) en die de bloedvat aanmaak bevorderen (VEGF). Ook werden de bloedvaten in de tumor aangekleurd

en geteld. Dit onderzoek staat beschreven in **hoofdstuk 2**. Er was meer HIF-1α in hyperplasie dan in normaal weefsel, maar HIF-1α was het hoogst in kankerweefsel. Wanneer weefsel te weinig zuurstof en voeding krijgt, zal het doodgaan. Wanneer dit ongereguleerd (in tegenstelling tot geregeluleerd) gebeurt, wordt dit dode weefsel necrose genoemd. Aankleuring rondom necrose (perinecrotisch) van HIF-1α werd niet gezien in normaal weefsel, was zeldzaam in hyperplasie, maar kwam veelvuldig voor in baarmoederslijmvlieskanker. Dit komt overeen met een eerdere studie, waarin men echter geen hyperplasie heeft onderzocht. In twee studies zag men HIF-1α positiviteit in 49 – 74% van de baarmoederslijmvlieskankers. De suiker en zuurgraad regulatoren, Glut-1 en CAIX namen ook toe van normaal tot kanker. Glut-1 werd vaak (94,9%) en alleen in kankers gezien.
Naast de aankleuring van HIF-1α rondom necrose (perinecrotisch) is in dit onderzoek ook een meer diffuus (door de tumor heen) patroon, of een mix van beiden gevonden. Zowel in de perinecrotische HIF-1α positieve tumoren als in de tumoren met diffuse aankleuring zagen we positiviteit voor de eiwitten die aangestuurd worden door HIF-1α, namelijk CAIX, Glut-1 en VEGF. Dit duidt erop dat HIF-1α hier functioneel is. Dit wordt ook onderbouwd door het gemiddeld hogere aantal vaatjes in de diffuse HIF-1α positieve tumoren hoger dan bij de perinecrotische HIF-1α positieve tumoren. Dit duidt namelijk op geactiveerde bloedvat aanmaak in tumoren met HIF-1α eiwit door de hele tumor (diffuus) heen. Overigens, het moment van geactiveerde bloedvataanmaak bij het ontstaan van baarmoederslijmvlieskanker lijkt tussen normaal weefsel en hyperplasie in te liggen.
Omdat geen correlatie is gevonden tussen VEGF (belangrijk voor aanmaak bloedvaten) en het gemiddeld aantal bloedvaatjes, kan geconcludeerd worden dat VEGF in baarmoederslijmvlieskanker niet de enige faktor is die tot aanmaak van vaten leidt. Wel was VEGF verhoogd aanwezig was in gebieden rondom necrose. Dit duidt inderdaad op een relatie tussen hypoxie, HIF-1α en VEGF in baarmoederslijmvlieskanker.

De diffuse aankleuring van HIF-1α, die mogelijk niet veroorzaakt wordt door een zuurstofgebrek, gaf aanleiding voor een studie naar verklarende faktoren voor dit diffuse patroon.
Bij de afbraak van HIF-1α wanneer er voldoende zuurstof aanwezig is, spelen vele moleculen een rol, waaronder VHL en de prolyl hydroxylasen (PHD): PHD1, PHD2 en PHD3. Daarnaast is het beschreven dat in cel-modellen in het laboratorium verlies van PTEN zorgt voor activatie van een 'pathway' met uiteindelijk activatie van AKT (p-AKT), en daardoor toename van HIF-1α. Zodoende onderzochten we in een groep van 61 baarmoederslijmvlieskankers de correlatie tussen aanwezigheid van VHL, PHD1, PHD2, PHD3, PTEN en p-AKT met de aanwezigheid van HIF-1α, door middel van immunohistochemie, beschreven in **hoofdstuk 3**. Het patroon van HIF-1α expressie correleerde niet (positief dan wel negatief) met PTEN, p-AKT, VHL, PHD2 en PHD3 expressie. Er werd echter wel verminderde hoeveelheid van PHD1 en van de PHDs samen gevonden in tumoren met diffuus HIF-1α. Echter, deze correlatie was zwak, dus het is goed mogelijk dat er andere factoren zijn die belangrijker zijn om dit diffuse HIF-1α patroon te verklaren.
Zodoende zijn genetische afwijkingen in het HIF-1α gen bestudeerd. Afwijkingen in het gen zorgen vervolgens voor afwijkingen in het eiwit. Wanneer er slechts één kleine bouwsteen (nucleotide) in een gen veranderd is (dit heet een "single nucleotide

polymorphism" of SNP), kan daardoor een ander aminozuur gevormd worden dan eigenlijk bedoeld is. In het geval van HIF-1α betekent dit dat, als het HIF-1α gen een beetje veranderd is, het HIF-1α eiwit ook veranderd is. Dit kan implicaties hebben voor de samenwerking van HIF-1α met andere moleculen. Uit diverse studies is gebleken dat wanneer er veranderingen voorkomen in het zuurstofgevoelige deel van het HIF-1α gen (het ODD domein), deze gepaard kunnen gaan met langdurige stabilisatie van HIF-1α. En dan is er dus meer HIF-1α in de cel aanwezig. Een andere studie laat zien dat de genen die door HIF-1 geactiveerd worden, vervolgens ook meer in de cel aanwezig zijn. **Hoofdstuk 4** beschrijft het onderzoek naar afwijkingen in het DNA van 58 baarmoederslijmvlieskankers. Dit onderzoek was gericht op twee stukken (exonen) van het ODD domein van het HIF-1α gen: exon 9 en exon 12. In deze exonen zitten belangrijke aminozuren (Prolines). Deze aminozuren zorgen ervoor dat HIF-1α afgebroken kan worden. In exon 9 vonden wij geen afwijkingen, dit heet 'wild type'. In exon 12 werd één reeds bekende SNP gevonden, maar deze nucleotide verandering leidt per definitie niet tot een aminozuurverandering. Proline 564, een belangrijk proline in exon 12, vertoonde bij geen enkele patiënt afwijkingen. Echter, Proline 582 was in 9 (15,4%) van de 58 tumoren veranderd in Serine (P582>S) door de SNP C1772>T. In 6 (10,3%) gevallen was dit in één van beide genen (van elk gen heeft een mens er twee), dit heet heterozygoot. In de overige drie (5.1%) gevallen lieten beide genen deze verandering zien (homozygoot). Van één van de patiënten met een heterozygote HIF-1α P582>S-kanker, liet het normale weefsel (zonder kanker) wild type HIF-1α zien. Deze afwijking is dus ontstaan tijdens het veranderen van normaal weefsel in kanker weefsel.
De nucleotide verandering (SNP) die leidt tot P582>S is reeds bekend en komt bij een deel van de bevolking in alle (dus ook normale) cellen voor en blijkt geassocieerd met verhoogde kans op prostaat en darmkanker. Om te onderzoeken of dit ook geldt voor baarmoederslijmvlieskanker werd het vóórkomen van deze genetische variatie in de kankers vergeleken met het vóórkomen in normale Europese controles. Dit werd gedaan met een controle groep samengesteld uit personen in bekende databases en de internationale literatuur. Hieruit hebben wij kunnen concluderen dat HIF P582>S genotype geen duidelijk verhoogde kans op baarmoederslijmvlieskanker oplevert.
Kankers met deze afwijking bleken meer bloedvaten te hebben en verhoogd p-Akt te vertonen, mogelijk doordat HIF-1α in deze vorm actiever is. Het duidt er aldus op dat deze tumoren biologisch anders zijn dan tumoren zonder dit genotype. Er zou meer onderzoek gedaan moeten worden om uit te zoeken of dit ook van invloed is op de overleving van patiëntes met baarmoederslijmvlieskanker.

De activiteit van HIF-1 zorgt niet alleen voor een betere overleving van de cel, maar kan er ook juist voor zorgen dat de cel 'geprogrammeerd' (i.t.t necrose) dood gaat. Een mogelijk gevolg hiervan is een 'stop-and-go' strategie die ervoor zorgt dat er een dynamische balans is waardoor de tumor kan groeien en kan overleven op de lange duur. De rol van HIF-1α in de celdeling tijdens hypoxie is nog niet geheel duidelijk en is bij baarmoederslijmvlieskanker nog niet eerder onderzocht.

In *hoofdstuk 5* wordt beschreven dat p27 (een eiwit dat zorgt voor remming van de celdeling) veel aanwezig is in normaal baarmoederslijmvlies, verminderd aanwezig is in hyperplasie en nog minder in baarmoederslijmvlieskanker. Echter, rondom de

necrose in de kankers is het p27 weer verhoogd aanwezig. Dit zijn de gebieden met zuurstofgebrek. Het is reeds bekend dat zuurstofgebrek leidt tot verhoging van p27, maar of dit proces door HIF-1$^{\alpha}$ gereguleerd wordt was vooralsnog discutabel. **Hoofdstuk 8** beschrijft de studie waarin dit wordt onderzocht. Met de baarmoederslijmvlieskanker-cellijn (in leven blijvende cellen buiten het lichaam) HEC1B werd in het laboratorium aangetoond dat de verhoging van p27 in een ruimte met een laag zuurstofgehalte afhankelijk is van HIF-1α. Uit eerder laboratorium onderzoek is gebleken dat ook een andere activatie door hypoxie mogelijk is, namelijk via de LKB1-AMPK pathway. Of deze manier ook belangrijk is in het kanker weefsel (i.t.t. losse cellen van cellijnen), is niet bekend. Het bleek echter wel dat op de plekken in baarmoederslijmvlieskanker waar zuurstofgebrek is, meer HIF-1α is en ook meer p27, én dat op die plekken minder celdeling aanwezig is.

Samengevat, tijdens de vorming van baarmoederslijmvlieskanker wordt de respons op zuurstofgebrek relatief laat geactiveerd, en niet al in hyperplasie. Daarom is therapie gericht op HIF-1α niet mogelijk in hyperplasie, maar wel in baarmoederslijmvlieskanker. Omdat CAIX, Glut-1 en HIF-1α aankleuring op coupes met name plaatsvindt in kanker, zou dit mogelijk gebruikt kunnen worden om onderscheid te kunnen maken tussen hyperplasie en kanker.
Uit eerdere studies waarbij onderzocht is of de aanwezigheid van verhoogd HIF-1α eiwit iets zegt over de kans op overleving voor de patiënt, heeft men kunnen vaststellen dat dit inderdaad het geval was. Dit had echter geen toegevoegde waarde boven op andere, makkelijker te verkrijgen gegevens van de tumor. Helaas is het verband tussen HIF-1α en levensverwachting bij baarmoederhalskanker maar één keer onderzocht. Dit was alleen bij de niet-uitgezaaide vorm en helaas was de groep relatief klein. Het is interessant om te onderzoeken of dit ook in andere patiëntengroepen (in andere ziekenhuizen en ook vrouwen met uitzaaiingen) het geval is. Daarnaast zou het interessant zijn om te kijken naar de voorspellende waarde van HIF-1α patronen, omdat die mogelijk meer zeggen over het biologisch gedrag van de tumor. Daarbij is het ook interessant om te kijken of de re-activatie van p27 rondom de necrose in tumoren, die afhankelijk is van HIF-1α, voorspellend is. Door dit soort onderzoek is het mogelijk de patiënt beter in te lichten, een beter begrip van de tumor te krijgen en op die manier de behandeling voor de individuele patiënte, met de individuele kanker te verbeteren.

Celdeling en ongebreidelde groei van cellen in (voorstadia) van baarmoederslijmvlieskanker

Aangezien afwijkingen in de regulerende mechanismen van de celdeling geassocieerd zijn met kwaadaardig weefsel, kan dit ook mogelijk een rol spelen in het ontstaan hiervan. **Hoofdstuk 5** beschrijft de ontsporing van regulatoren van de celdeling in normaal baarmoederslijmvlies, in hyperplasie en in baarmoederslijmvlieskanker. In de Engelstalige discussie wordt uitgebreid ingegaan op de diverse biologische routes die de eiwitten die de celdeling reguleren beïnvloeden. Gezien de complexiteit zal hier alleen ingaan worden op een korte beschrijving van de resultaten. In de celdeling spelen vele eiwitten een rol, waaronder cyclines die een complex vormen met hun 'kinase' (oftewel cyclin-dependent–kinase, CDK) en zo zorgen voor progressie van de celdeling. Dit complex kan weer geremd worden door cyclin-dependent-kinase-inhibitors (CKI's). p16, p21 en p27 behoren tot de CKI's en ook

p53 kan de celdeling remmen. Uit eerder onderzoek is gebleken dat een toename van p53 (gemeten met immunohistochemische kleuring) in kankerweefsel meestal duidt op disfunctionerend of 'fout' p53.
Tijdens het ontstaan van baarmoederslijmvlieskanker nemen cycline A en Ki67 (indicatoren voor de mate van celdeling) toe. Er was namelijk meer van deze eiwitten aanwezig in hyperplasie in vergelijking met normaal weefsel en nog meer in kanker. De hoeveelheid cycline D1, cdk2 en p53 nam toe in het spectrum van normaal naar hyperplasie naar kanker.
Deze eiwitten zijn dus al in een vroeg stadium betrokken bij het veranderen van normaal weefsel in kanker en blijven ook betrokken. Cycline E en p21 namen toe vanaf hyperplasie tot kanker en p27 verminderde over dit traject. Met andere woorden, deze drie regulatoren spelen later dan de andere eiwitten een rol in het ontstaan van baarmoederslijmvlieskanker. Gezien de functie van p21, zou je eerder een afname dan een toename verwachten, net als bij p27. Mogelijkerwijs is de toename van p21 naast een toename van de celdeling-markers Ki67 en cyclin A meer een reflectie van het mislukken om de celdeling te remmen, dan dat er een verandering van het p21 gen is. Ditzelfde fenomeen was ook zichtbaar bij p16. Deze opmerkelijke observatie is voor p16 in meer tumoren gezien.

Uit deze studie naar celdeling-regulatoren komt een belangrijke vraag voort: wat is het échte voorstadium van baarmoederslijmvlieskanker. Uit eerder onderzoek bleek dat de Wereld Gezondheidszorg Organisatie (WHO) Classificatie (waarbij gebruik wordt gemaakt van de genoemde term hyperplasie) minder objectief was dan de EIN classificatie die gebaseerd is op meer objectieve criteria en de echte kanker voorstadia probeert te onderscheiden van de 'reactieve' afwijkingen. Deze laatste classificatie is relatief nieuw en nog niet algemeen geaccepteerd, ondanks dat het een betere voorspeller is voor associaties met en progressie naar kanker. Uit het onderzoek beschreven in dit proefschrift blijkt ook dat p16 en p21 significant hoger zijn in EIN afwijkingen dan in niet-EIN afwijkingen binnen de groep die de WHO hyperplasie noemt. Dit geeft naast het bekende doorgroei naar kanker en een verlies van het PTEN eiwit nog meer redenen om te kiezen voor het gebruik van de EIN classificatie.
Deze classificatie zou dan ook meer in de dagelijkse praktijk toegepast moeten worden. En er zou ook meer onderzoek moeten komen om te kunnen differentiëren tussen de diverse weefsels die tot nu toe ervan verdacht worden een voorstadium van kanker te zijn.

Tijdens het beschreven onderzoek in *hoofdstuk 5* komt naar voren dat tussen verschillende plekken in één en dezelfde tumor verschillen bestaan in de aanwezigheid van bepaalde eiwitten. **Hoofdstuk 6** beschrijft de studie waarin we deze subjectieve observatie objectiveren. Baarmoederslijmvlieskankers hebben een buitenste rand die het omliggende normale weefsel (de spierlaag van de baarmoeder) ingroeit. Deze buitenste, ingroeiende rand (een soort front-linie) wordt gekarakteriseerd door meer celdeling en een verandering van de regulatoren van de celdeling ten opzichte van het binnenste gedeelte van de tumor. De regulatoren die verhoogd aanwezig waren, waren: cycline E en cdk2 (zorgen voor voortgang van de deling) en p16 (zorgt voor remming van de deling). Dit is de eerste studie waarin dit onderwerp binnen baarmoederslijmvlieskanker wordt beschreven.

De toename van p16 in de rand van de tumor vergeleken met het centrum komt overeen met observaties gedaan in dikke darm kanker. Deze onderzoekers geven aan dat de toename in rand (of de afname in het centrum) mogelijk veroorzaakt wordt door methylering van het gen in het centrum van de tumor. Door methylering wordt een gen als het ware 'uit gezet', en daardoor wordt er minder eiwit geproduceerd. Wij zagen een toename van de celdeling in de rand ten opzichte van het centrum, ondanks de hoeveelheid p16, wat eigenlijk zou moeten leiden tot een celdeling stop. Dit is in tegenstelling met waarnemingen van anderen in huidkanker, waarin ook een toename van p16 in de rand werd gezien, maar dit ging juist gepaard met mínder celdeling. p16 lijkt dus in baarmoederslijmvlieskanker niet succesvol in het stoppen van de celdeling in de rand van de tumor, ondanks dat het verhoogd aanwezig is.
Een verhoging van het HIF-1α niveau in de rand van de tumor kon niet worden aangetoond. Mogelijk komt dit doordat zuurstofgebrek in het centrum van de tumor HIF-1α verhoogt en dit zodoende niet te onderscheiden is van een toename van HIF-1α aan de rand.
Verschillen in eiwit expressie tussen de buitenste rand en het binnenste gedeelte van de tumor kan veroorzaakt worden door 'clonale' selectie van de meest kwaadaardige cellen, remming van groei in het midden van de tumor of door interacties met het 'normale' zich 'verdedigende' weefsel. Omdat de rand tijdens het groeien van de tumor vanzelf meer een binnenste gedeelte van de tumor wordt, en er een nieuwe buitenste rand wordt gevormd, is het idee van clonale selectie minder aannemelijk, in tegenstelling tot de invloed van het omliggende weefsel.
Behalve dat kennis over verschillende delen van een tumor interessant is vanuit biologisch oogpunt, kan het ook klinisch relevant zijn. Ten eerste omdat er bij weefselonderzoek meer en meer gebruik gemaakt van 'tissue arrays'. Hierbij worden zeer kleine stukjes weefsel (en dus ook kanker) verzameld van een operatief verwijderd stuk weefsel. Vaak wordt hiervoor het middelste stuk gebruikt, om zeker te weten dat er niet per ongeluk niet-representatief weefsel wordt meegenomen. Uit dit onderzoek blijkt echter dat zowel van het binnenste gedeelte als van de buitenste rand weefsel meegenomen zou moeten worden om een representatief beeld te krijgen van de kanker. Ten tweede ook al neemt de patholoog een groter gedeelte, dan nog zouden bij het beoordelen van de hoeveelheid eiwit (dit gebeurt in de dagelijkse praktijk bij diagnostiek) de verschillen binnen tumoren beoordeeld en vastgelegd moeten worden. Ten derde, prognostische studies zijn op dit gebeid niet verricht, maar zouden wel zeer interessant kunnen zijn. Mogelijk hebben veranderingen in de rand van de tumor betere voorspellende waarde dan factoren die nu gebruikt worden.

In eerder onderzoek door anderen werd het baarmoederslijmvlies van vrouwen na de overgang bestudeerd. Men concludeerde dat de staat van het weefsel waarin vóór de overgang het baarmoederslijmvlies is opgebouwd, van invloed is, waarschijnlijk aangezet door oestrogenen (de vrouwelijke hormonen). Deze oestrogenen kunnen er ook voor zorgen dat de baarmoederslijmvliescellen een beetje veranderen en eruit gaan zien als cellen in de eileiders: namelijk met kleine trilhaartjes. Deze verandering wordt met een medische term 'tubaire metaplasie' genoemd, en wordt behalve in het baarmoederslijmvlies ook in de baarmoederhals en in de eierstokken gezien. Vooralsnog werd aangenomen dat tubaire metaplasie een ongevaarlijke afwijking

van de norm is. Dit is echter nooit goed onderzocht, maar wordt in enkele artikelen in de internationale literatuur voorzichtig in twijfel getrokken.
Zodoende werden eiwitten onderzocht die betrokken zijn bij de HIF-1α pathway (HIF-1α, Glut-1 en CAIX) en eiwitten die een rol spelen bij de celdeling in normaal baarmoederslijmvlies met en zonder tubaire metaplasie. Dit staat beschreven in **hoofdstuk 7**. Zowel eiwitten betrokken bij de celdeling als HIF-1α, Glut-1 en CAIX waren verstoord in de tubaire metaplasie. De verhoogde aanwezigheid van HIF-1α wordt waarschijnlijk niet veroorzaakt door zuurstofgebrek, maar door andere genen (oncogenen en tumor suppressor genen) die HIF-1α ook kunnen verhogen. De aanwezigheid van Glut-1 en CAIX, en de afwijkende hoeveelheden eiwitten betrokken bij de celdeling zijn vaak geassocieerd met (voorstadia van) kanker (zie *hoofdstuk 2* en *5*).
Met name één van de eiwitten betrokken bij de celdeling: p16, een remmer, vertoonde onder de microscoop een opvallend mozaïek patroon in weefsel met tubaire metaplasie. Vervolgens onderzochten we of dit ook gezien werd in de eileiders (met veel trilhaar-dragende cellen), in de baarmoederhals met tubaire metaplasie en in eierstokken met cysten (kleine holtes) met tubaire metaplasie. Ook in deze andere weefsels werd dit opvallende patroon waargenomen, doch net iets minder uitgesproken dan in het baarmoederslijmvlies. Door dit opvallende patroon kan de p16 immunohistochemische kleuring gebruikt worden in de dagelijkse praktijk van de patholoog om tubaire metaplasie duidelijker te maken, in plaats van of naast de LhS28 kleuring.
Het is al eerder beschreven dat aanwezigheid van trilhaar dragende cellen in holtes in de eierstokken geassocieerd is met eierstokkanker. In het baarmoederslijmvlies van vrouwen vóór de overgang (en dus met een menstruele cyclus) waren trilhaar-dragende cellen meer aanwezig in de opbouwende (proliferatieve) dan in de afbrekende (secretoire) fase van de cyclus. Ook p16 expressie was hoger. Ondanks dat het onduidelijk is waarom p16 expressie verhoogd is en zo'n mozaïek patroon vertoont, zou dit mogelijk kunnen bijdragen, of is een weerspiegeling van de afwijkende natuur van tubaire metaplasie, mede gezien de associatie van verhoogd p16 met (voorstadia van) baarmoederhalskanker. Daarnaast laten wij zelf in **hoofdstuk 5** zien dat de hoeveelheid p16 toeneemt van normaal weefsel, via hyperplasie, naar baarmoederslijmvlieskanker. Ook in zeldzame subtypes van baarmoederslijmvlieskanker (clear cell en sereus) is beschreven dat p16 positiviteit verhoogd is.
Kortom, tubaire metaplasie vormt mogelijk een verhoogd risico op dan wel is een voorstadium van baarmoederslijmvlieskanker. Ten eerste omdat er afwijkingen werden gezien in de expressie van HIF-1α pathway eiwitten en celdelings-eiwitten; ten tweede door het patroon van p16 positiviteit dat gezien werd bij tubaire metaplasie op meerdere plaatsen in het vrouwelijk genitaalstelsel. Ten derde omdat tubaire metaplasie in holtes in de eierstokken ook geassocieerd is met eierstokkanker en dit hetzelfde p16 patroon vertoont.
Deze moleculaire veranderingen worden echter niet vergezeld van morfologische ("op het oog") veranderingen zoals in andere voorstadia. Het is daarom op dit moment niet mogelijk om potentieel schadelijke van onschadelijke tubaire metaplasie te onderscheiden. Ook is nog onduidelijk tot welk type kanker tubaire metaplasie in het baarmoederslijmvlies kan doorgroeien.

De belangrijkste conclusies van dit proefschrift

1. HIF-1α en de processen die aangestuurd worden door dit eiwit zijn meer aanwezig in hyperplasie (het kanker-voorstadium) en nog meer in baarmoederslijmvlieskanker vergeleken met normaal weefsel.
2. Het moment van vernieuwde vaataanmaak tijdens het ontstaan van baarmoederslijmvlieskanker ligt tussen het stadium van normaal weefsel van vrouwen na de overgang en hyperplasie in.
3. Genetische afwijkingen van HIF-1α, alsmede aankleuring van PTEN, p-Akt en VHL geven geen verklaring voor de verhoogde aanwezigheid van HIF-1α die wordt gezien in baarmoederslijmvlieskanker onafhankelijk van zuurstofgebrek. Een verminderde aanwezigheid van PHD1 (een eiwit dat ervoor zorgt dat HIF-1α afgebroken kan worden) zou wel een van de factoren kunnen zijn die deze diffuse HIF-1α positiviteit kan verklaren.
4. Drager zijn van de genetische variatie 'P582>S', een verandering van het HIF-1α gen, lijkt de kans op baarmoederslijmvlieskanker niet te verhogen. Deze genverandering is echter wel geassocieerd met meer bloedvaten en meer actief Akt in de tumor.
5. Tubaire metaplasie (verkrijgen van 'trilhaartjes' op de cellen) is mogelijk geassocieerd met het krijgen van kanker.
6. De immunohistochemische p16 kleuring kan gebruikt worden om tubaire metaplasie zichtbaarder te maken in de vrouwelijke geslachtsorganen als alternatief voor de specifieke marker LhS28.
7. Tijdens de vorming van baarmoederslijmvlieskanker is er een toename van de celdeling, en parallel daaraan werden ontsporingen gezien van cycline D1, cycline E, p16, p21, p27, p53 and cdk2, duidend op het belang van celcyclus regulatoren in de genese van baarmoederslijmvlieskanker.
8. In baarmoederslijmvlieskanker wordt rondom necrose, in zuurstofarme gebieden, p27 opnieuw opgereguleerd, via HIF-1α. Dit leidt vervolgens tot vermindering van de celdeling.
9. Baarmoederslijmvlieskanker laat duidelijk een buitenste, infiltrerende rand zien, dat gekarakteriseerd wordt door een verhoogde celdeling en ontsporingen van regulatoren van de celcyclus (cycline E, p16 en cdk2). De HIF-1α pathway is hier niet verhoogd geactiveerd in vergelijking met de rest van de tumor.

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Petra, Arjan, Eelke, Marc, Karijn, Theo, Geert en Ingrid, de echte hard-core laboratorium onderzoekers van het MRL. Nadat ik ruim een jaar bezig was met mijn onderzoek

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Ik kom nog graag een keertje vrijdagmiddagborrelen en schieten op het lab!

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Marian, wat gezellig dat we precies tegelijk aan de opleiding beginnen! De Vogelenbuurt is leuk met jou en Erik!
(S)OHHH, het weekend na het inleveren van het manuscript was goed gepland ;-) Snel weer naar Lamelemele.
D11, de woensdag en zondag waren heerlijk! Thanks!
Karin en Remco: dankjulliewel voor het onderdak in de eerste maanden van het onderzoek!
Familie Wolffenbuttel, voor jullie was het afgelopen jaar ook niet fijn, hopelijk vanaf nu alleen verbetering! Dankjewel voor de interesse in het boekje en persoonlijke dingen.

Lieve pap, mam en Rick. Wat heerlijk om zo'n fijn 'thuis' te hebben. Het is zo fijn om altijd de mogelijkheden te hebben gekregen om mijn eigen weg te bepalen. Zonder jullie zorg en liefde was ik nu nog niet zo ver gekomen. Pap, dankjewel voor hulp bij het boekje. Mam, het waren gezellige middagjes zo afentoe in Utrecht. Hopelijk vanaf nu wat minder telefoontjes vanaf de fiets, maar ik ben bang…
Lisa, je bent een aanwinst!

Wouter, lieve lieve Wouter, het is moeilijk op te schrijven hoe ontzettend dankbaar ik ben voor al je steun in mijn onderzoekstijd. We werken in twee heel verschillende werelden, maar zijn allebei zo gedreven dat zelfs 'de verplichte dinsdagavond vanaf 8 uur' ingevoerd moest worden. Maar dat was slechts van korte duur, totdat je het ziekenhuis en de oncologie wel op een zéér vervelende manier leerde kennen. Het werd wel des te duidelijker wat ik al wel wist: je bent ontzettend sterk (nee, niet in de trant van Clark) en dapper. Jouw optimisme en humor heeft ons door deze moeilijke tijd heen gekregen. Er waren meer tranen van de grappen dan van het verdriet. (al mag dat laatste ook hoor!) Heerlijk om te zien hoe snel je weer beter aan het worden bent. Ik hoop dat je over een jaar weer net zo gezond bent als je een jaar geleden was!
Je bent echt de aller-allerliefste! Ik ben zo trots op jou.
(en dankjewel voor je hulp met de referentie-lijst en Nederlandse samenvatting)

Nicole

Juli 2007

Curriculum Vitae

Nicole Horrée was born on April 10, 1980 in Arnhem, the Netherlands. Following her graduation from the Marnix College in Ede in 1997, she started her medical study at the University of Groningen.
During her medical study she followed internships at the Tribhuvan University Teaching Hospital in Kathmandu, Nepal (student-internship; Emergency Medicine and General Practice), Sint Elisabeth Hospital, Curaçao (first half year of internships), the University Medical Center Groningen and allied peripheral hospitals (second part of internships) and the Leyenburg Hospital, The Hague (extra internship in Gynaecology and Obstetrics). In 2003 she resided in Surinam for four months to conduct research in screening methods for cervical cancer in developing countries in cooperation with the Female Cancer Program of the Leiden University Medical Center (Prof. dr. A.A.W. Peters) and the Lobi Foundation.
Following her graduation she worked for four months as an AGNIO at the Department of Obstetrics at the Wilhelmina Children's Hospital (University Medical Center Utrecht) before she started her PhD program under supervision of Prof. dr. A.P.M. Heintz and Prof. dr. P.J. van Diest in October 2004. From September 2005 she combined her PhD with a Master of Science in Clinical Epidemiology, which she hopes to finish at the end of 2007. As of October 2007 Nicole will start her specialization in Gynaecology and Obstetrics. During her PhD program Nicole fell in love with Wouter, they now live together in Utrecht.

Marieke Verberg, paranimf

Color Figures

Figure 1, Chapter 2. *Expression of HIF-1a, Glut-1 and CAIX in endometrial carcinomas. A) Diffuse nuclear expression of HIF-1a (10x magnification). B) perinecrotic nuclear expression of HIF-1a (20x magnification). C) Perinecrotic membrane CAIX expression (10x magnification). D) Perinecrotic membrane Glut-1 expression (20x magnification).*

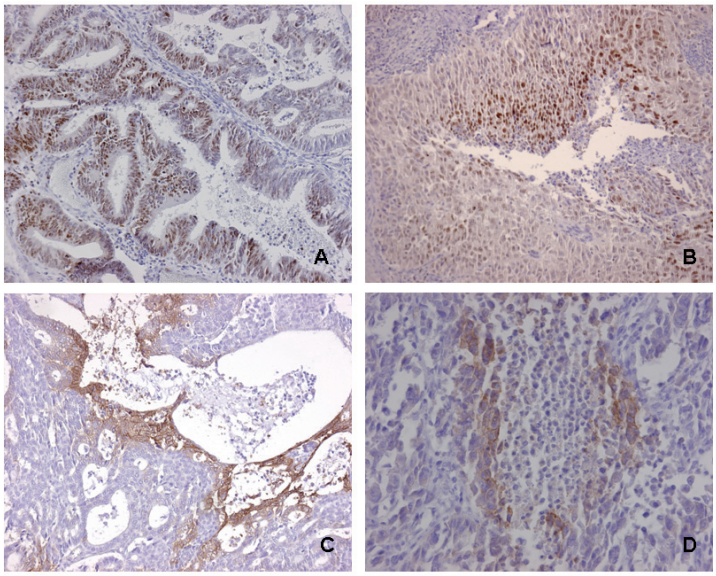


Figure 1, Chapter 3. *PHD en VHL immunohistochemical staining on endometrioid endometrial cancers.* ***A.*** *PHD2 staining. Overview of uterus with tumor (T) and normal (N) tissue, showing less staining of PHD2 in the tumor compared to the normal tissue. This is further illustrated in picture* ***B****, showing higher magnification of the tumor and in picture* ***C****, showing higher magnification of the normal endometrial glands.* ***D.*** *PHD2 staining showing more expression in the squamous part of the endometrial tumor than in the non-squamous parts.* ***E.*** *PHD1 staining showing more expression in the squamous part of the endometrial tumor than in the non-squamous parts.* ***F.*** *pVHL staining which shows cytoplasmic staining in all the tumor glands, though there is an increase of membranous expression in the perinecrotic fields.* ***G.*** *pVHL staining of an endometrial cancer, which shows a remarkable mosaic-like expression pattern, possibly related to ciliated cell change.* ***H.*** *pVHL staining, with a cytoplasmic staining in the normal glands which is illustrative for the type of staining observed throughout the tumors, however in this cancer a diminished expression in the squamous parts (S) of the tumor is observed.*

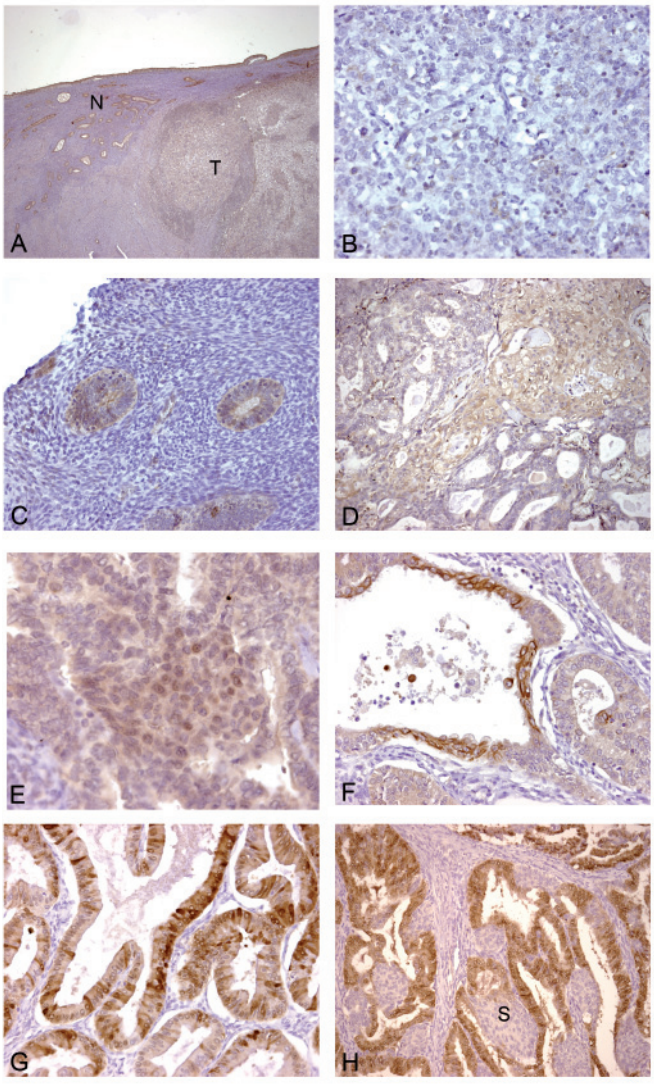
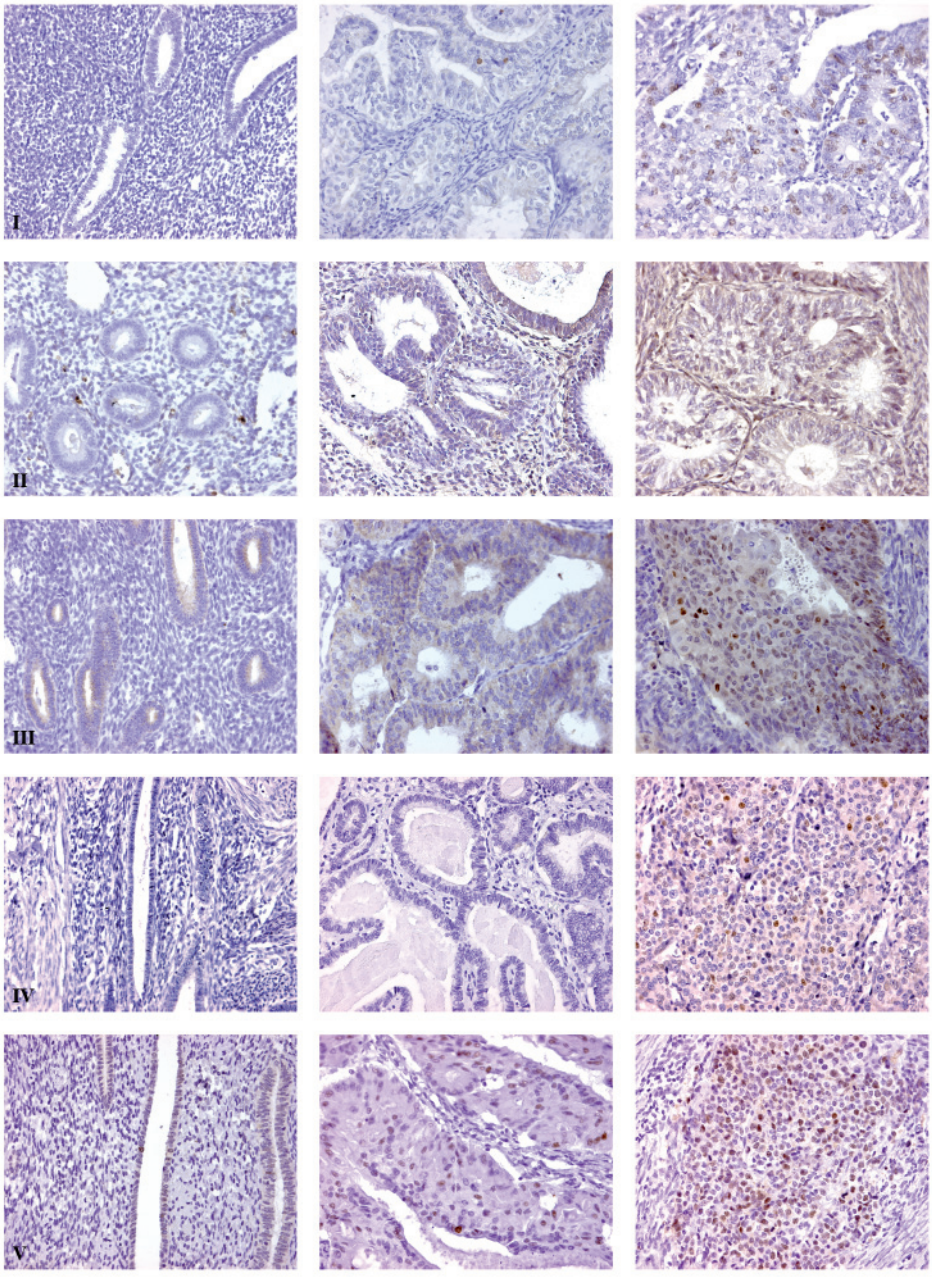
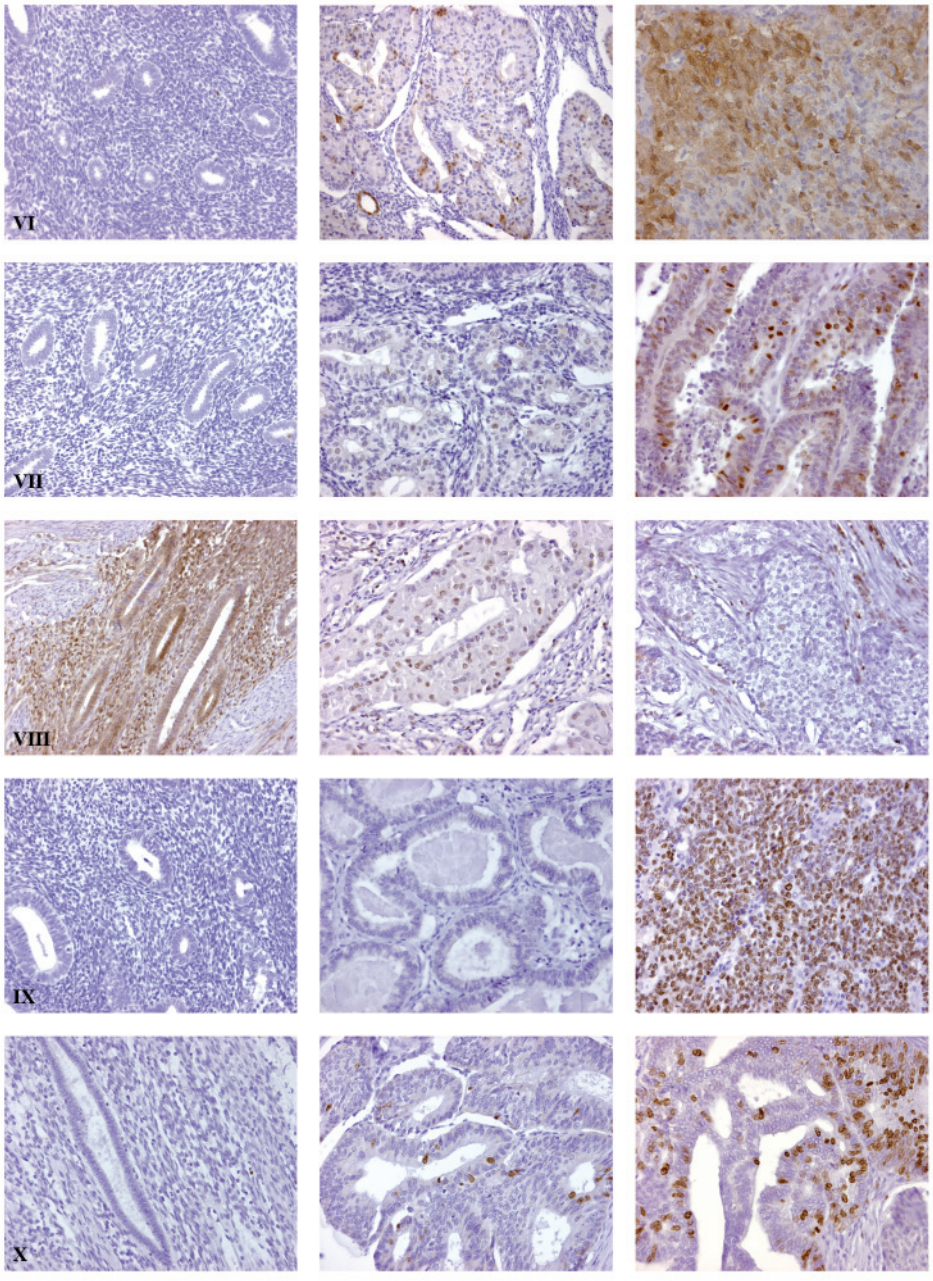


Figure 1, Chapter 5. *Representative examples of stained slides. Columns, from left to right: inactive endometrium, hyperplasia, endometrioid endometrial carcinoma. Rows: cyclin A (I), cyclin B1 (II), cyclin D1 (III), cyclin E (IV), cdk2 (V), p16 (VI), p21 (VII), p27 (VIII), p53 (IX), Ki67 (X).*




VI
VII
VIII
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X

Figure 1, Chapter 6 *Immunohistochemical expression of cell cycle regulators in endometrial carcinoma. In all figures an increase in expression at the invasive front of the tumor compared to the tumor center is noticed.* ***A.*** *MIB-1 expression in endometrioid endometrial carcinoma.* ***B.*** *Cyclin E expression in endometrioid endometrial carcinoma. Inset corresponds to area left of asterisk (*).* ***C.*** *Cdk2 expression in serous carcinoma of the endometrium.* ***D.*** *p16 expression in endometrioid endometrial carcinoma. TC = tumor center; IF = invasive front; M = myometrium. Brown staining depicts positive expression of the proteins.*

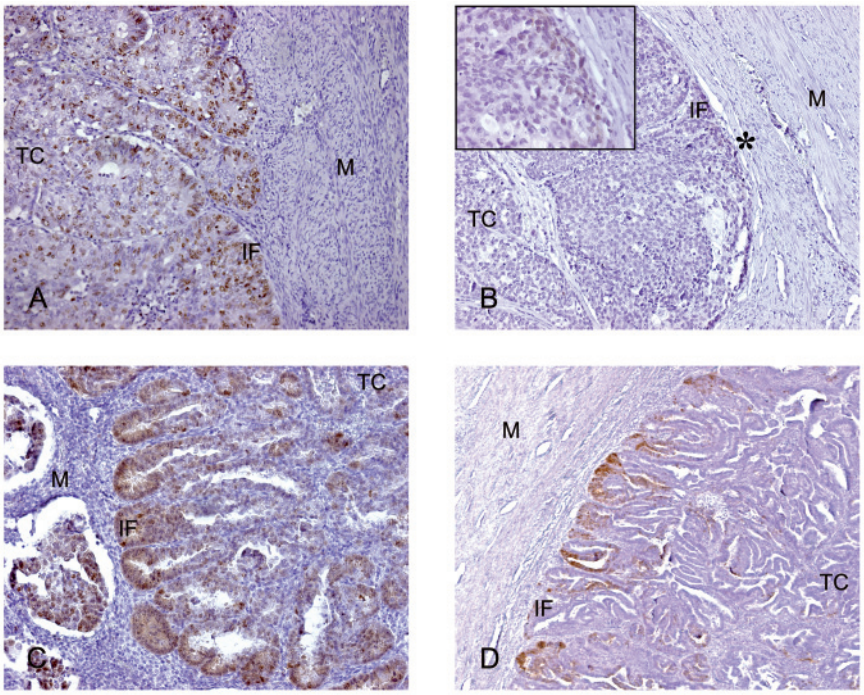

Figure 1, Chapter 7. *Ciliated cell change in various parts of the gynecological tract. A) Endometrium with tubal metaplasia stained by LhS28, a ciliated cell marker, and in B) for p16. C) Normal Fallopian tube showing LhS28 staining, and D) focal p16 expression. E) Subcortical ovarian inclusion cysts showing varying degrees of tubal metaplasia, with a mosaic pattern of p16 expression in the cysts with the most pronounced ciliated cell change (F). G) Cervix with tubal metaplasia showing positivity for LhS28 and H) p16 in a mosaic pattern. I) Endometrial ciliated cell tumor of the ovary showing positivity for LhS28 and J) p16 in a mosaic pattern.*

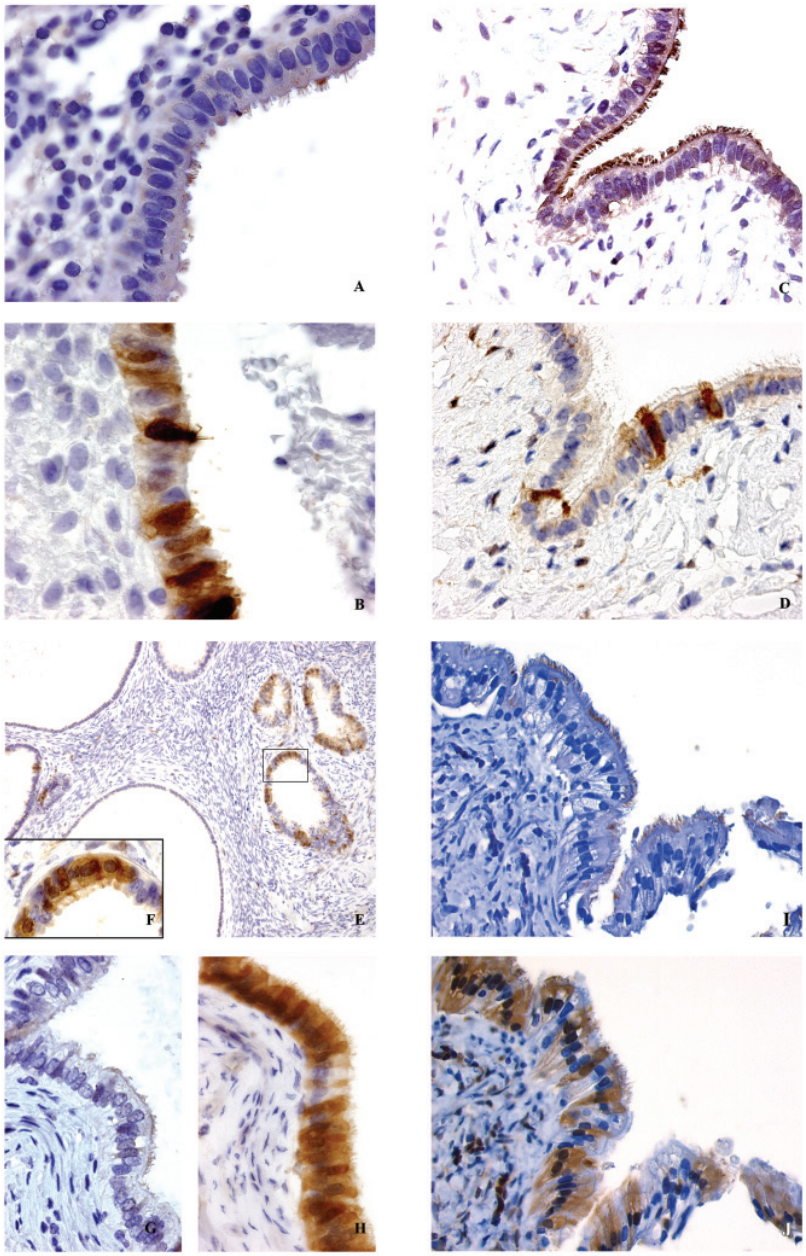

Figure 1, Chapter 8. Immunohistochemical staining of p27 and HIF-1α in endometrial carcinoma. *Typical patterns are shown: A and B same tumor and consecutive slide with perinecrotic staining of p27 (A), perinecrotic staining of HIF-1α (B); C and D same tumor and consecutive slide with perinecrotic staining of p27 (C), perinecrotic staining of HIF-1α (D); tumor slide with 3 normal glands (E) the normal glands and the stroma around the glands express p27 and the tumor glands show loss of p27 staining; double staining p27 and HIF-1α (F). Asterisk implies necrosis in the tumor.*

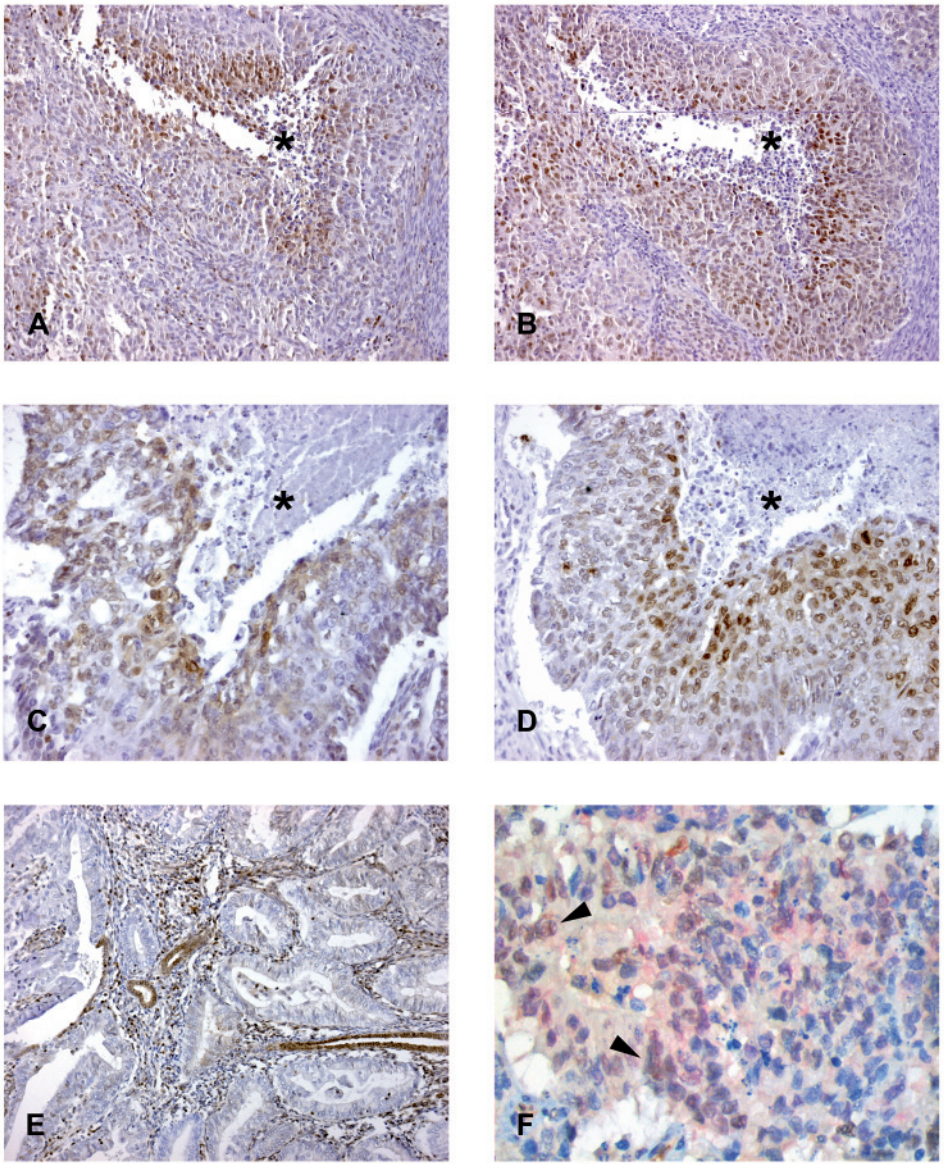

Figure 3, Chapter 8. FACS analysis and Ki67 staining.

(A-B) HEC1B cells were cultured for 24 hours under normoxic or hypoxic conditions, labeled with BrdU (y-axis) and Propidium Iodide (x-axis) and subjected to FACS. Per experiment 10.000 cells were counted. G1, G2 and S-phase content were calculated by mannual gating. The same gates were used for both the normoxia (A) and hypoxia (B) experiments. Results were independently reproduced three times and a representative experiment is shown.

(C-E) Consecutive slides of the same tumor showing perinecrotic HIF-1α staining (C), central/perinecrotic p27 staining (D) and anti-perinecrotic Ki67 staining (E). N = Necrosis.