

Hypoxia and angiogenesis in endometrioid endometrial carcinogenesis

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Abstract. *Background:* Hypoxia-inducible factor 1 α (HIF-1 α) plays an essential role in the adaptive response of cells to hypoxia, triggering biologic events associated with aggressive tumor behavior. *Methods:* Expression of HIF-1 α and proteins in the HIF-1 α pathway (Glut-1, CAIX, VEGF) in paraffin-embedded specimens of normal ($n = 17$), premalignant ($n = 17$) and endometrioid endometrial carcinoma ($n = 39$) was explored by immunohistochemistry, in relation to microvessel density (MVD). *Results:* HIF-1 α 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-1 α 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-1 α 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-1 α in endometrial carcinogenesis.

Keywords: Endometrium, hypoxia, carcinogenesis, immunohistochemistry, HIF-1 α

1. Introduction

Solid tumors will outgrow their own vasculature beyond the size of several mm³, 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 [20,21]. Besides, hypoxic malignant cells are more resistant to radiotherapy and chemotherapy [11,32]. 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 [16]. HIF-1 is a transcription factor consisting of 2 subunits, HIF-1 α and HIF-1 β , which both contain basic helix-loop-helix and PAS (PER-aryl hydrocarbon nuclear translocator-SIM) domains to bind

to DNA [15,37]. HIF-1 α and HIF-1 β together form the active HIF-1 complex which binds the consensus sequence 5'-RCGTG-3' in the hypoxia-response elements of various target genes [29]. 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 [25,28,29]. Under normoxia, HIF-1 α protein has a very short half-life due to its continuous ubiquitination and proteasome mediated degradation, while in hypoxia this process is inhibited [13]. As HIF-1 β is constitutively expressed, HIF-1 α protein levels determine HIF-1 activity.

HIF-1 α is overexpressed in many cancers [40]. 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 [14]. HIF-1 α was associated with poor prognosis in stage 1 endometrial cancer in one study [30].

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Carbonic anhydrase IX (CAIX) is a membrane-associated carbonic anhydrase, that plays a role in pH regulation [33]. Its gene contains a hypoxia response element in the promoter region and is activated by HIFs [38]. 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 [22,38]. Elevated CAIX levels are predictive of hypoxia in various types of cancer and are related to poor prognosis [9,10] 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 up-regulated by HIF-1 α , appeared to be a useful indicator of high risk for development of endometrial carcinoma [36]. Increased angiogenesis, one of the effects of HIF-1 α through upregulation of vascular endothelial growth factor (VEGF), was a statistically significant predictor of decreased survival in endometrial cancer [17,19,24,26,35].

These results point to an important potential role of hypoxia and its key regulator HIF-1 α in endometrial carcinogenesis and progression. However, present data are fragmentary. The aim of this study was therefore to comprehensively explore the role of HIF-1 α 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 carcinogenic pathway in the endometrium.

2. Materials and methods

2.1. 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

	<i>N</i> = 79
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
Simple with atypia	0
Complex without atypia	9
Complex with atypia	7
Endometrial intraepithelial neoplasia	
Non-EIN/hyperplasia	13
EIN	10
Grade of carcinoma	
Grade 1	6
Grade 2	21
Grade 3	12
Stage of carcinoma	
Stage I	13
Stage II	10
Stage III	11
Stage IV	5

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 [12,23]. 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 [6].

2.2. Immunohistochemistry

HIF-1 α , Glut-1, CAIX, VEGF and CD31 were immunohistochemically stained on serial 5 μ m thick paraffin slides as extensively described before [4].

Table 2
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)	Manually / computer
HIF-1 α	MoAb, Mouse	BD Pharmingen	1:50	TRS, DAKO, 45 min, 97 $^{\circ}$ C	CSA	Mamma-carcinoma	60 minutes / room temp	By hand
Glut-1	PoAb, Rabbit	DAKO	1:200	Citrate, pH 6.0, 20 minutes, 93 $^{\circ}$ C	G-aR IgG+ Strep1	Red blood cells in slide	60 minutes / room temp	Autostaining
CAIX	PoAb, Rabbit	Novus-Biological	1:1000	Citrate, pH 6.0, 20 minutes, 93 $^{\circ}$ 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 $^{\circ}$ 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 $^{\circ}$ C	Powervision	Endothelium (internal)	60 minutes / room temp	By hand

HIF-1 α = hypoxia-inducible factor-1 α ; Glut-1 = glucose transporter-1; CAIX = carbonic anhydrase IX; VEGF = vascular endothelial growth factor, MoAb = monoclonal antibody; 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).

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.

For HIF-1 α , 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.

2.3. Evaluation of staining

Two authors (PvD, NH) scored all slides blinded to clinicopathologic data and results of other stainings. For HIF-1 α , the percentage of dark, homoge-

neously stained nuclei was estimated as before [34], 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-1 α , 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 [34], 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 20 \times , the 'hot-spot'-method. The total area counted was 3.80 mm 2 , 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.

2.4. 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-1 α 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-1 α 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).

3. Results

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

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%)	$p < 0.001^*$	$p < 0.001^*$
Membrane positive	0	(0%)	0	(0%)	37	(94.9%)		
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		$p < 0.001^\dagger$	$p = 0.009^\ddagger$
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%)	$p < 0.001^*$	$P = 0.027^*$
\geq 5% positive	0	(0%)	14	(60.9%)	34	(87.2%)		
Median	0		5		20		$p < 0.001^\dagger$	$p = 0.025^\ddagger$
Range	0–0		0–75		0–90			

* Fisher's exact test. † Kruskal–Wallis test. ‡ Mann–Whitney test.

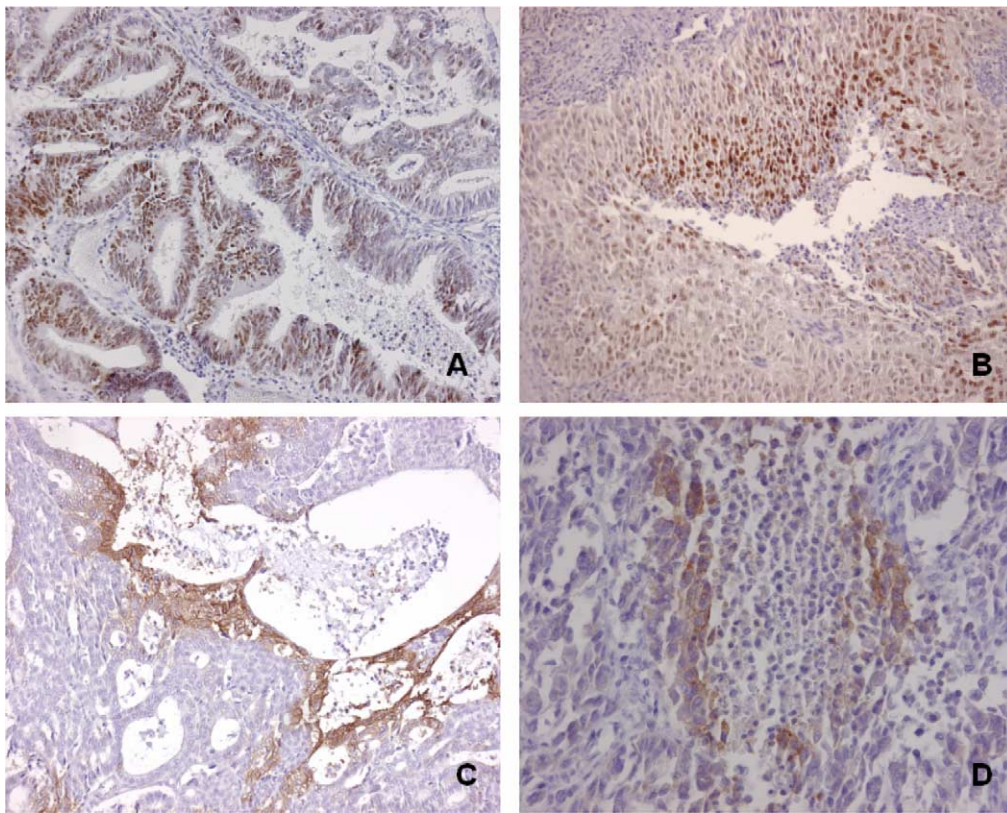


Fig. 1. Expression of HIF-1 α , Glut-1 and CAIX in endometrial carcinomas. (A) Diffuse nuclear expression of HIF-1 α (10 \times magnification). (B) perinecrotic nuclear expression of HIF-1 α (20 \times magnification). (C) Perinecrotic membrane CAIX expression (10 \times magnification). (D) Perinecrotic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

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

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 (Fig. 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 (Fig. 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 pattern 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.

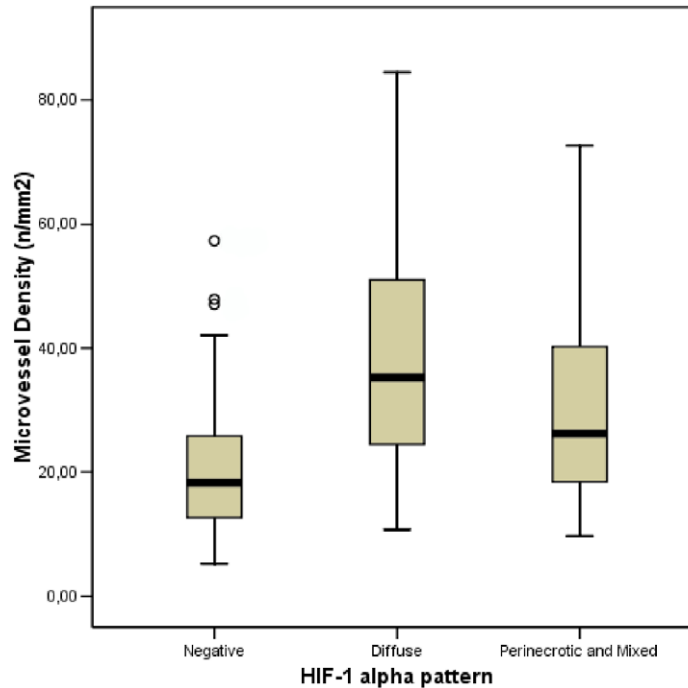


Fig. 2. Microvessel Density (MVD) for different expression patterns of HIF-1 α . Diffuse expression of HIF-1 α was associated with highest MVD; perinecrotic and mixed patterns were associated with an intermediate MVD (Kruskal–Wallis test, $p < 0.05$).

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

In the hyperplasias (non-EIN and EIN), diffuse HIF-1 α 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-1 α 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-1 α all showed perinecrotic CAIX expression and perinecrotic Glut-1, without diffuse staining. In these perinecrotic HIF-1 α stained carcinoma cases, VEGF was positive in all (2 perinecrotic, 2 diffuse and 2 mixed). The 20 tumors with a mixed expression of HIF-1 α 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-1 α in carcinoma ($n = 9$) was accompanied by CAIX expression (1 diffuse, 5 perinecrotic,

3 mixed), Glut expression (2 diffuse, 4 perinecrotic, 1 mixed) and VEGF expression (6 diffuse, 3 mixed).

Low HIF-1 α 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-1 α was associated with highest MVD; perinecrotic and mixed patterns were associated with an intermediate MVD ($p < 0.05$).

4. Discussion

The purpose of this study was to investigate the expression of HIF-1 α , its downstream genes Glut-1, VEGF and CAIX, and angiogenesis in the endometrioid carcinogenic 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-1 α in endometrial hyperplasia.

HIF-1 α showed increasing overexpression from inactive endometrium through hyperplasia to endometrioid carcinoma. Perinecrotic, hypoxia associated HIF-1 α overexpression was absent in inactive endometrium, rare in endometrial hyperplasia and frequent in en-

ometrioid carcinoma. This largely confirms previous studies on HIF-1 α in endometrial carcinogenesis. Acs et al. [2] found 74% of carcinoma cases to be HIF-1 α positive with significantly more expression in tumor samples containing areas of necrosis, and only negative benign endometrium cases. Sivridis et al. [30] found 49% of carcinoma cases to be HIF-1 α 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 [27,36].

Interestingly, diffuse HIF-1 α expression (thought to be especially due to non-hypoxic stimuli such as HIF-1 α 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-1 α (that is thought to be hypoxia driven) was in general associated with Glut-1 and CAIX expression [34]. The activation of CAIX and Glut-1 in diffuse and perinecrotic HIF-1 α expressing endometrial carcinomas may point to diffuse HIF-1 α expression being functional too. This is further evidenced by the fact that highest MVD was seen in cases with diffuse HIF-1 α expression compared to cases with perinecrotic/mixed expression. Although mild hypoxia cannot be excluded as reason for diffuse HIF-1 α expression, especially non-hypoxic stimuli are thought to be involved. These include HIF-1 α mutations and amplifications, and mutations in p53, PTEN, and VHL, Her/2*neu* 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 [1,8]. 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. [7] found no correlation either, although others [5,8,26] 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 per-

inecrotic areas where also HIF-1 α 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-1 α and VEGF expression. In our study, HIF-1 α 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 [18,39]. The EIN classification was introduced as a potentially better reproducible alternative system and is also used in diagnosis [12,23]. 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 [31]. 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 [3]. Functional inactivation of the PTEN gene is associated with stabilization of HIF-1 α [41]. 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-1 α upregulation in endometrial tissues.

In conclusion, HIF-1 α 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-1 α in endometrial carcinogenesis. Besides, detecting HIF-1 α may identify subgroups of patients that could benefit from hypoxia targeting therapeutic strategies and may be resistant to radiotherapy.

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