Lacking hypoxia-mediated downregulation of E-cadherin in cancers of the uterine cervix

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Background: Experimental studies have established a causal connection between tumour hypoxia, hypoxia-associated proteome changes and downregulation of E-cadherin, the final common pathway of epithelial-to-mesenchymal transition (EMT). Our study aimed at elucidating the interrelationship of these processes in cancers of the uterine cervix *in vivo*.

Methods: Tumour oxygenation was assessed in 48 squamous cell carcinomas (SCC) of the uterine cervix using polarographic needle electrodes. The expression pattern of E-cadherin was investigated by immunohistochemistry and western blotting, and was compared with that of the hypoxia-inducible proteins glucose transporter (GLUT)-1 and carbonic anhydrase (CA) IX in biopsy specimens of the oxygenation measurement tracks.

Results: The majority of cervical cancers (52%) were E-cadherin positive, with a complete absence of the antigen in only 10% of the tumours. No correlation was found between the level of E-cadherin expression and the oxygenation status (mean pO_2, median pO_2 and hypoxic fractions). In patients showing partial expression of E-cadherin (38%), staining was not preferentially diminished in GLUT-1- or CA IX-positive areas, and loss of E-cadherin occurred independently of tumour cell scattering.

Conclusion: Our data provide no evidence in favour of a hypoxia-induced EMT as a mechanistic basis of cervical cancer invasiveness.

Direct measurements of the oxygenation status using microelectrodes carried out in patients with cancers of the uterine cervix, the head and neck region and in soft tissue sarcomas have shown that critically lowered oxygen levels (hypoxia) within the tumour tissue are associated with a poor prognosis (Höckel *et al*, 1993, 1996, 1998; Brizel *et al*, 1996; Nordsmark *et al*, 2005). In one study on cervical cancer, this correlation with outcome was independent of the choice of treatment modality (Höckel *et al*, 1996), a finding that has indicated that tumour hypoxia may be a significant factor driving malignant progression (Höckel *et al*, 1996; Vaupel *et al*, 2004). With regard to the possible mechanisms underlying this phenomenon, hypoxia-induced proteome changes, which are coordinated primarily by the hypoxia-inducible factor (HIF)-1, may be of pivotal importance. This transcription factor is a heterodimeric protein whose α-subunit (HIF-1α) is directly regulated by oxygen availability (Wang *et al*, 1995). Under normoxic conditions, HIF-1α is modified by prolyl hydroxylase domain (PHD) enzymes and subsequently degraded via the proteasome, keeping its protein levels very low. Hypoxia interrupts this process and leads to its stabilisation, nuclear translocation and binding to its dimerisation partner HIF-1ß (Wenger, 2002). HIF-1 then activates the transcription of more than 800 genes (Semenza, 2012). Cellular processes driven by HIF-1 through this large-scale shift in gene expression are increasingly viewed as key mediators of hypoxia-associated malignant progression. This idea is supported by numerous studies that have shown clear correlations between elevated levels of hypoxia-inducible proteins and a poor patient prognosis (Vaupel and Mayer, 2007). Hypoxia-inducible factor -1-mediated mechanisms include the metabolic adaptation ('reprogramming') to hypoxic, energy-deprived conditions, enabling

cancer cells to survive in hypoxic niches, for example by the upregulation of glucose influx via glucose transporter (GLUT)-1 and proton efflux by a mechanism involving membranous carbonic anhydrase (CA) IX. Owing to their longer half-lives and greater protein abundance compared with HIF-1α, both proteins have been used as surrogate markers for the activation of the hypoxic response in numerous studies.

Hypoxia-associated proteome changes have also repeatedly been connected to increased cancer cell invasion and metastasis – core traits of malignancy – by means of the induction of epithelial-to-mesenchymal transitions (EMTs). As the term implies, this complex phenotypic transformation comprises the loss of epithelial and acquisition of mesenchymal characteristics, including an enhanced motility and local invasiveness associated with single-cell 'scattering'. Abolition of the tight cohesion found in normal epithelia absolutely requires the dissolution of mechanically stable physiological cell-to-cell contacts, in which the protein E-cadherin has a key role. Several *in vivo* and *in vitro* studies of tumours and tumour cells of different origins indicate that HIF-1α may be directly involved in the downregulation of E-cadherin. Mechanisms include upregulation of the EMT-inducing transcription factors SNAIL (Imai *et al*, 2003) and TWIST (Yang *et al*, 2008), as well as the induction of LOX (Erler *et al*, 2006). So far, numerous studies have already described a downregulation of E-cadherin in cervical cancer (Vessey *et al*, 1995; Jeffers *et al*, 1997; Narayan *et al*, 2003; Faleiro-Rodrigues and Lopes, 2004; van de Putte *et al*, 2004; Rodriguez-Sastre *et al*, 2005; Dursun *et al*, 2007; Caberg *et al*, 2008; Lee *et al*, 2008). In fact, a progressive downregulation of this protein has even been observed in cervical dysplasia (de Boer *et al*, 1999). As cancers of the uterine cervix are the tumour entity for which the best evidence for hypoxia-associated malignant progression exists, this study has examined the question of whether correlations between the directly measured oxygenation status (Eppendorf pO$_2$ histography), the expression of hypoxia-associated marker proteins (GLUT-1, CA IX) and the expression of E-cadherin can be identified.

MATERIALS AND METHODS

Patients. All of the patients in this study were part of a prospective clinical trial for the evaluation of the significance of tumour oxygenation in primary, locally advanced carcinomas of the uterine cervix at the Department of Obstetrics and Gynaecology, University Medical Centre, Leipzig, Germany. The study design was approved by the local Medical Ethics Committee, with patients giving informed written consent before being enrolled. All 48 patients with squamous cell carcinomas (SCCs) for whom biopsy specimens of the oxygen measurement tracks were available were included in this study (for patient and tumour characteristics see Table 1).

Tumour oxygen tension measurements. Tumour pO$_2$ was measured pretherapeutically with the computerised Eppendorf pO$_2$ histography system (Eppendorf, Hamburg, Germany), using a protocol that has been described in detail (Höckel *et al*, 1991). Briefly, pO$_2$ readings were performed in conscious patients along linear electrode tracks, first in the subcutaneous fat of the mons pubis followed by cervical measurements at the 12 and 6 o'clock sites in macroscopically vital tumour tissue. Within the tumour tissue, up to 35 pO$_2$ measurements were made along each electrode track (70 readings in total), starting at a tissue depth of approx. 5 mm. The individual pO$_2$ measurement points were situated 0.7 mm apart, resulting in an overall measurement track length of 25 mm. Immediately after pO$_2$ measurements, needle core biopsies (obtained using Biopty; Radioplast, Uppsala, Sweden) of 2 mm in diameter and 20 mm in length were taken

Table 1. Patient and tumour characteristics at the time of pretherapeutic pO$_2$ measurements

	No. of patients	%	Range
FIGO stage			
I B1	5	10.4	
I B2	4	8.3	
II A	2	4.2	
II B	8	16.7	
III B	19	39.6	
IV A	7	14.6	
IV B	3	6.3	
Grade			
1	4	8.3	
2	33	68.8	
3	11	22.9	
Largest tumour diameter (mm)[a]			20–120
<60	22	45.8	
⩾60	26	54.2	
Patient age (yr)[a]			29–73
< 50	21	43.8	
⩾50	27	56.3	
cHb (g/dl)[a]			8.2–16.1
<13.5	24	50	
⩾13.5	24	50	

[a]The median value was used as the cut-off point between the two groups. Sums of rounded percentages may not add to exactly 100 per cent.

from those tumour areas from which pO$_2$ readings had been obtained. Intravaginal temperature, arterial blood pressure, heart rate, haemoglobin concentration, haematocrit, and arterial oxyhaemoglobin saturation were monitored at the time when pO$_2$ readings were taken. The pretherapeutic pO$_2$ measurements were recorded 1–5 days before oncological treatment. The oxygenation status was described quantitatively by the parameters mean pO$_2$, median pO$_2$ and the percentage of pO$_2$ values below 2.5, 5 and 10 mmHg (i.e., 'hypoxic fractions', HF 2.5, HF 5 and HF 10, respectively).

Immunohistochemistry and morphometry. Histologic slides were prepared from the paraffin blocks and dried overnight at 37 °C. On the next day, specimens were dewaxed in xylene and then rehydrated in a descending alcohol series. Retrieval of antigenic binding sites was performed by heating specimens in 10/1 mM Tris/EDTA buffer (pH 9.0) in a steamer (Braun FS 10, Braun, Kronberg, Germany) for 40 min. Primary antibodies against E-cadherin, GLUT-1 and CD34 were obtained from DAKO (DAKO, Hamburg, Germany, cat.-no. M3612, A3536 and M7165, respectively), and antibodies against CA IX and Ki67 were obtained from Abcam (Abcam, Cambridge, UK, cat.-no. ab15086 and ab16667). Detection of the first antigen, CD34, was carried out using overnight incubation with the primary antibody at 4 °C, appropriate biotinylated secondary antibodies followed by a standard streptavidin/biotin/horseradish peroxidase reagent and colour development using DAB (brown reaction product; DAKO Duet kit). Detection of the second target protein (E-cadherin, GLUT-1 or CA IX) was preceded by a second round of antigen retrieval to prevent cross-reactions with the first antigen and to enhance unmasking efficiency. After primary antibody incubation for 1 h at 37°, the micropolymer-based, biotin-free Vector Impress detection system was applied and Vector VIP (purple reaction

product) was used as the peroxidase substrate (Vector Laboratories, Burlingame, CA, USA). Negative control specimens were incubated in PBS without the primary antibody under the same conditions. A tumour specimen with a known strong expression of each antigen was run as a positive control with every staining batch. Slides were dehydrated in an ascending alcohol series, and covered with a coverslip using Eukitt mounting medium (Riedel-de Haen, Seelze, Germany). Digital images of the specimens were acquired with a Zeiss AxioImager microscope and AxioCam HRc using the software AxioVision (Zeiss, Oberkochen, Germany). Diffusion distances were measured in 2 to 15 areas in each biopsy using measure tools in AxioVision.

Quantitative assessment of antigen expression. Only immunostaining compatible with the known biological function and corresponding subcellular localisation of each antigen was considered as being evaluable as marker expression: membranous staining in the case of E-cadherin, GLUT-1 and CAIX, and nuclear staining for Ki67. A semiquantitative scoring system was used. For E-cadherin, score 0, negative; score 1, partially negative; score 2, all cells positive. For GLUT-1, CA IX and Ki67, score 0, no staining ('absent'); score 1, $<10\%$ positive ('weak'); score 2, 11% to 50% positive ('moderate'); and score 3, $>50\%$ positive ('strong'). Because of limited amounts of tissue, only 45 (of 48) biopsies stained for CA IX and 47 (of 48) stained for Ki67 could be analysed.

Protein extraction, western blotting and band densitometry. Whole-cell protein extracts (including membrane proteins) were generated using a FastPrep-24 tissue homogeniser (MP Biomedicals, Solon, OH, USA). Cryospecimens were placed in 2.0 ml of impact-resistant tubes containing 250 µl of RIPA buffer (C-C-pro, Oberdorla, Germany) supplemented with 1 mM fresh DTT and Lysing Matrix D (i.e., 1.4 mm ceramic spheres, MP Biomedicals), homogenised for 10–20 sec, incubated on ice for 30 min and centrifuged at 15 000 U/min for 30 min at 4 °C. An aliquot of the supernatant was used for the determination of the protein concentration using Bradford's reagent (Roti-Quant, Roth, Karlsruhe, Germany), BSA as a protein standard (Sigma-Aldrich, St Louis, MO, USA) and a Tecan F200 microplate reader equipped with a 595-nm filter (Tecan, Crailsheim, Germany). A measure of 50 µg of protein was separated by SDS-PAGE using a Bio-Rad Mini-Protean 3 electrophoresis chamber and transferred to PVDF-membranes using a Bio-Rad transblot SD transfer cell (Bio-Rad, Hercules, CA, USA). The primary antibody against human E-cadherin (DAKO M3612), identical to the one used for IHC, was incubated overnight at 4 °C and detected using an HRP-conjugated secondary antibody (NXA931, GE Healthcare, Waukesha, WI, USA). To control for equal protein loading, membranes were stripped and reprobed with antibodies against ß-actin (sc-1616r, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and appropriate anti-rabbit secondary antibodies (sc-2054, Santa Cruz). Bands were developed using ECL reagent (GE Healthcare) and images were captured with a CCD camera (Fujifilm LAS-3000, Fujifilm, Tokyo, Japan). Molecular-weight determination was carried out using the Fujifilm Multigauge software and the Bio-Rad Precision Plus Dual Colour Standards, which were run on each gel.

Statistical analysis. All of the statistical tests were performed using SPSS (Version 20, IBM, Armonk, NY, USA). The significance level was set at $\alpha = 5\%$ for all comparisons. Linear correlations between two parameters were described by Spearman's rank correlation coefficient (ρ). Two-sided Mann–Whitney U tests and Kruskal–Wallis tests were used for comparison of categorised variables.

Oxygenation status was correlated with FIGO stages and with tumour size, which was dichotomized using the median tumour diameter (60 mm) as the cut-off value. Diagrams were generated using SigmaPlot 11.

RESULTS

Prevalence of E-cadherin in SCC of the uterine cervix. On performing immunohistochemistry (IHC), the majority (52%) of 48 squamous cell carcinomas of the uterine cervix showed strong membranous E-cadherin expression in all tumour cells. In all, 38% of SCCs showed a partial loss (ranging from no more than focal areas of absence of the antigen to its expression in only selected groups of cells). In the remaining 10%, E-cadherin was not detectable at all. Conversely, 9 of 37 (24%) specimens available for western blot (WB) analyses of E-cadherin expression were negative, whereas the remaining showed 120-kDa bands of different intensities (Supplementary Figure S1). A significant positive correlation between both assays was found ($P = 0.01$), but individual cases showed significant disagreement; for example, one tumour showed E-cadherin expression in all cells in IHC but was tested negative using WB. Owing to the lower sensitivity of the WB assay, all further results refer to the IHC data.

E-cadherin and pO₂-histography data. The Kruskal–Wallis Test yielded no significant differences in the values of any of the oxygenation status parameters (mean pO₂, median pO₂, HF 2.5, HF 5, and HF 10) between the three categories of E-Cadherin expression in the IHC analysis (i.e., the presence of the antigen in no, some or all cancer cells within the tumour biopsy). Boxplots depicting these data are presented in Figure 1.

E-cadherin and levels of GLUT-1, CA IX and Ki67. All tumours showed a varying degree of GLUT-1 expression, whereas one-third were negative for CA IX (see Table 2). Higher levels of E-cadherin were correlated with higher expression scores of the endogenous hypoxia-related markers GLUT-1 ($\rho = 0.51$, $P < 0.001$) and CA IX ($\rho = 0.33$, $P = 0.03$). Expression levels of GLUT-1 and CA IX were also correlated with each other ($\rho = 0.48$, $P = 0.001$). Correlations with proliferation (Ki67) were not found.

Expression patterns and interrelationships of E-cadherin, GLUT-1, CA IX and Ki67. Focal downregulation of E-cadherin in GLUT-1- or CA IX-positive areas, as expected from the concept of hypoxia-induced EMT, was not observed. In subregions of most biopsies (41 of 48), expression of GLUT-1 was found to increase beyond an average distance of 76 µm from the nearest microvessel
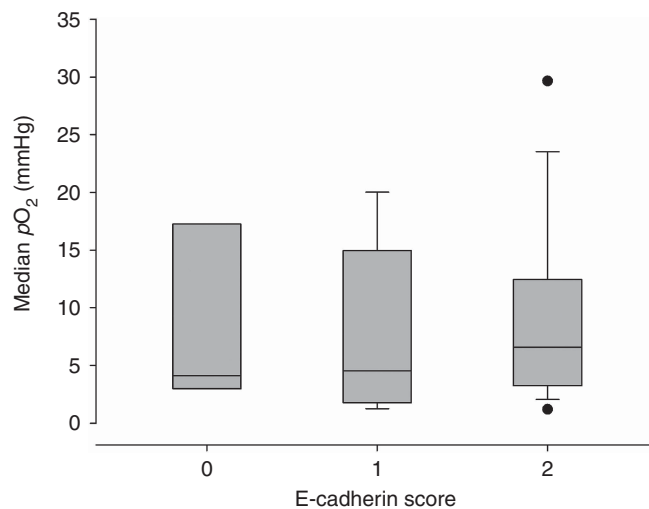

Figure 1. No correlation between E-cadherin expression and the oxygenation status of cervix cancers. Boxplots show equal distributions of median pO₂ values in the three categories of E-cadherin expression. Similar distributions were found for mean pO₂ values and hypoxic fractions (see text for details).

(CD34 expression), as evaluated by multiple manual measurements in each double-stained section (see Figure 2A for an example). Of 30 CA IX-positive biopsies, 23 showed increased antigen expression at a mean distance of 83 µm from the nearest microvessel. In the remaining seven positive tumours, a relationship between CA IX expression and diffusion distances could not be identified. Conversely, no spatial relationship between focal areas of E-cadherin downregulation and microvessels was observed in any of the positive biopsies (Figure 2B). E-cadherin expression was also independent of the local growth pattern. To illustrate this observation, Figure 3A shows an example of strongly E-cadherin-positive cells that exhibit a reticular pattern of infiltration. Conversely, Figure 3B shows compact and coherent tumour cell aggregates that completely lack the expression of E-cadherin.

Other findings. No correlation was found between clinical tumour size and the oxygenation status when both parameters were tested as continuous variables. When tumour size was dichotomized using the median value as the cut-off, larger tumours showed a trend towards a slightly poorer oxygenation status, but only regarding the mean pO_2 ($P = 0.054$). Higher FIGO stages, however, did not show a poorer oxygenation status.

DISCUSSION

Beginning with the experiments of Coman (1944), who was able to show almost 70 years ago that scrapings obtained from squamous cell carcinomas of the uterine cervix exhibit a reduced intercellular cohesion, numerous studies have investigated the importance of loosened cell–cell contacts for the invasiveness of cancers of this and other tumour types. It had been recognised even earlier that extracellular Ca^{2+} levels are important for cell-to-cell coherence, and it is now established that the (calcium-dependent) homotypic interaction of the extracellular domains of E-cadherin molecules, located in adherens junctions, is crucial for the mechanical stability of intercellular contacts (Perez-Moreno et al, 2003). Possible mechanisms mediating a specific downregulation of these structures in hypoxic tumour areas are of great interest, as numerous studies have demonstrated a significant prognostic importance of hypoxia and hypoxia-associated proteome changes. Our study is the first to investigate this question in SCCs of the uterine cervix. Our results clearly demonstrate that hypoxic tumours or tumours with a high expression of hypoxia-associated proteins do not show downregulation of E-cadherin or other characteristic signs of EMT. On the contrary, we have even

Table 2. Expression scores of GLUT-1 and CA IX in squamous cell carcinomas of the uterine cervix

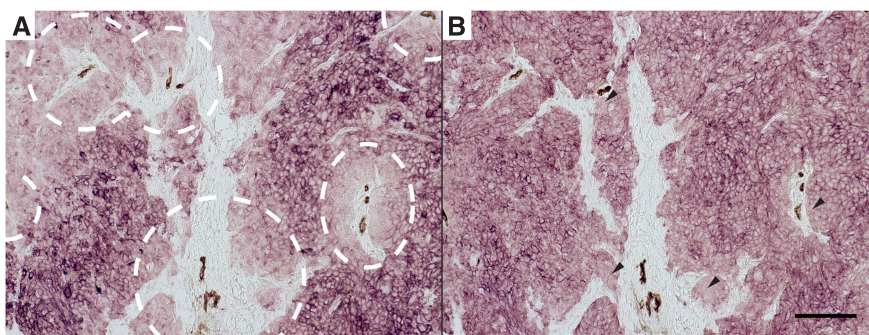| Score	GLUT-1 number of patients	%	CA IX number of patients	%
0	0	0	15	33.3
1	11	22.9	10	22.2
2	13	27.1	16	35.6
3	24	50.0	4	8.9
Total	48	100.0	45	100.0

Figure 2. **Expression pattern of GLUT-1, but not E-cadherin, is related to diffusion gradients in cancers of the uterine cervix.** GLUT-1 expression (purple) shows a gradual increase with increasing distance from the microvessels (brown) in panel (**A**). Circular areas of low GLUT-1 expression (marked by white dashed lines) can be identified around selected microvessels. No such pattern is evident for E-cadherin (purple) in panel (**B**). 200 x magnification, scale bar in **B** = 100 µm.
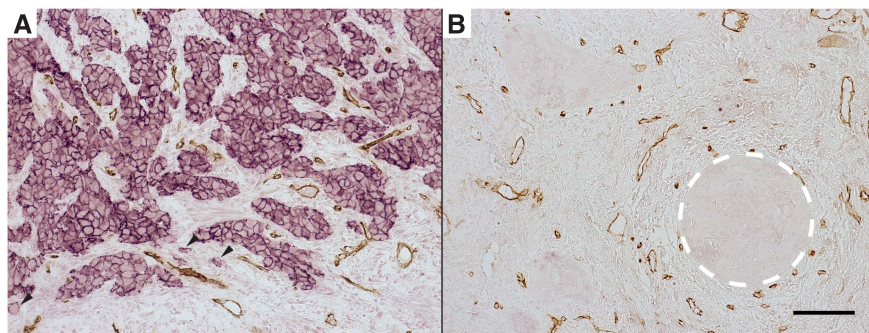

Figure 3. **Expression of E-cadherin is not related to the microregional growth pattern of cancers of the uterine cervix.** A reticular, highly branched pattern of invasion, including apparently scattered single cells (arrowheads), can be observed in tumours with strong membranous E-cadherin expression (**A**). Conversely, compact, spheroid-like configurations of tumour cell clusters (demarcated by a white dashed line) can be observed in tumours completely negative for E-cadherin (**B**). 200 x magnification, scale bar in **B** = 100 µm.

observed significant positive correlations between the expression of the hypoxia-associated proteins (GLUT-1 and CA IX) and E-cadherin. Such a correlation would seem plausible if a reduced E-cadherin expression were to be accompanied by increased cell scattering, which, in turn, would lead to the 'reoxygenation' of tumour cells upon their infiltration into the microvessel-carrying and thus 'oxygen-providing' tumour stroma. This possibility was, however, excluded in this study, as in terms of E-cadherin expression no systematic differences between highly fragmented tumour cell clusters and isolated tumour cells on the one hand and coherent tumour cell aggregates on the other could be identified. As shown in Figure 3, examples both of tumour areas in the first category (scattered phenotype), which consistently showed strong expression of E-cadherin, and areas in the second category (cohesive phenotype), which completely lacked the expression of this antigen, have been found. These observations confirm earlier findings in cancers of the uterine cervix (Jeffers *et al*, 1997) and breast cancer (Colpaert *et al*, 2003), which also did not show an impact of E-cadherin expression on the invasion pattern. With regard to the positive correlations between E-cadherin and hypoxia-related markers, we currently have no biologically plausible explanation for this finding, but would assume that it is unlikely that these findings are the result of technical shortcomings of our immunohistochemical protocols, as these were already validated extensively in prior studies (e.g., Mayer *et al* (2008)).

Jeffers *et al* (1997) also reported E-cadherin expression in all 20 tumours investigated in their study, i.e., even more than the 90% positive tumours identified in our work (in the IHC assay). Nevertheless, both studies stand out among the literature, which commonly reported much lower E-cadherin levels. For example, the largest study to date, the work of van de Putte *et al* (2004), which comprised 219 patients, reported a high expression of E-Cadherin (in more than 50% of the tumour cells) being restricted to only 10% of the patients analysed, whereas our own results show 100% positive tumour cells in more than half of our patient cohort. Furthermore, as opposed to our study, which found only 10% of patients to have completely negative tumours, relatively high rates of negative tumours (34 to 100%) have been reported in the majority of prior studies (e.g., Ancuta *et al* (2009); Faleiro-Rodrigues and Lopes (2004); Lee *et al* (2008); Rodriguez-Sastre *et al* (2005)). These differences may partly be explained by the use of different tissue fixation protocols, primary antibody clones and detection systems or a combination of these factors. Indeed, the use of the purple peroxidase substrate VIP for the detection of E-cadherin - which in our study was performed to enable double staining with CD34 - resulted in much stronger (yet decisively specific) staining results than DAB, as tested during the pilot phase of our study (data not shown). Particular incongruities exist between our study and the report from Lee *et al* (2008), who studied SCC of the uterine cervix using immunofluorescence detection of E-cadherin and vimentin in specimens, which, according to the interpretation of these authors, represented an ascending order of local spread (i.e., normal squamous epithelium, superficial tumour tissue, tumour cell nests in the parametrium and tumour cells in pelvic lymph nodes). They found a progressive decrease of E-cadherin and an opposed induction of vimentin, suggesting *bona fide* EMT in 10 out of 10 patients analysed. Interestingly, their results agree with our data in one important aspect: E-cadherin downregulation obviously did not necessarily lead to single-cell scattering, as is evident from the E-cadherin-negative, but still compact, tumour cell nodule found in the parametrium in the study of Lee *et al* (see Figure 1A in Lee *et al* (2008) compared to Figure 3B of this study). Nevertheless, we are currently unable to explain the substantial differences between these two studies.

Detection of E-Cadherin using western blotting of tissue extracts confirmed the finding of maintained E-cadherin expression in the vast majority of tumours. However, in our hands, western blots turned out to be significantly less sensitive than IHC, which is why we relied on data from the latter assay for all further analyses. The inability to check for the amount of stromal tissue present in the portion of the biopsy lysed for protein extraction is a significant drawback of this method and may well be decisive for the quantitative differences between the results of these two methods. We are aware of the fact that the finding of preserved E-cadherin expression in the cell membrane of the majority of cancer cells investigated in our study does not necessarily imply that these molecules are fully functional. However, this aspect was not investigated in our work, as the hypothesis explicitly implied a downregulation of protein abundance. This topic may, however, be an interesting subject for further studies. E-Cadherin expression levels were also not correlated with the proliferation rate, but knowledge of the expression pattern of this antigen in consecutive sections enabled us to ascertain that our analyses were carried out in proliferating, and hence vital, areas of the tumours.

In keeping with the results of our prior studies in the same tumour entity (Mayer *et al*, 2005a, b), we again did not observe correlations between endogenous hypoxia-related markers GLUT-1 and CA IX and any parameter of the oxygenation status in this completely different and more recent cohort of patients. As our protocol specified biopsy acquisition from tumour microregions identical to the areas in which oxygenation measurements had been made, the important influence of tumour heterogeneity on the expression of hypoxia-related markers has already been minimised by our approach. However, tumour heterogeneity alone is probably not sufficient to explain these discrepancies, as even the elaborate IHC analysis protocol of systematically investigating 3 levels of two different biopsies used in the study of Iakovlev *et al* (2007) did not result in the identification of correlations between the oxygenation status and the expression of CA IX. Correlations between HIF-dependent markers and oxygen microsensor measurements may also be distorted by the occurrence of tumour necroses, which are severely hypoxic using needle electrode measurements (Lyng *et al*, 1997; Jenkins *et al*, 2000) but do not necessarily go hand-in-hand with extensive expression of HIF-related markers in the same specimens. The influence of this factor is also expected to be rather modest in our study, as pO_2 measurements and corresponding biopsies were excluded from analyses in cases in which histopathological examination showed widespread tumour necrosis. Conversely, it has long been known that the expression levels of both HIF-1α and its target genes GLUT-1 and CA IX, as investigated in this study, are influenced by a variety of factors other than hypoxia, such as glucose deprivation (Boado and Pardridge, 2002; Kwon and Lee, 2005), acidosis (Mekhail *et al*, 2004), oncogenes (e.g., c-MYC (Osthus *et al*, 2000)) and oncogene-associated signalling pathways (e.g., PI3K/AKT (Kaluz *et al*, 2002)). These significant hypoxia-independent influences have been summarised in a review (Mayer *et al*, 2006). In addition, recent research has demonstrated a role for reactive oxygen species and NO in stabilizing HIF-1α (Fong and Takeda, 2008; Dewhirst, 2009).

Beyond mere quantitative correlations between E-cadherin, GLUT-1 and CA IX expression *levels*, we were able to carry out systematic computer-aided classifications of antigen expression *patterns* with regard to the local diffusion geometry (by measuring distances from marker-positive cells to the nearest microvessel), as sections of all three antigens had each been double-stained for the marker CD34 (microvascular endothelium). Results of this analysis showed that most of the (antigen-positive) tumours exhibited the expected pattern of expression of GLUT and CA IX beyond a mean distance of approx. 80 μm (76 μm for GLUT-1 and 83 μm for CA IX) from the nearest microvessels in subregions of the biopsies. In contrast, such a constellation could not be identified for E-cadherin, which was typically expressed both in the immediate

vicinity of blood vessels and areas further down the oxygen diffusion gradients. The absence of any systematic spatial relationship between E-cadherin and the hypoxia-associated proteins clearly contradicts the model of a hypoxia-mediated downregulation of E-cadherin in cancers of the uterine cervix.

The results obtained in this study indicate that downregulation of E-cadherin in cancers of the uterine cervix does not show associations with the 'physical' oxygenation status of the tissue or with the locoregional expression levels of hypoxia-related markers. Hence, other mechanisms leading to E-cadherin downregulation may predominate in this tumour entity. However, the possibility that the general importance of E-cadherin downregulation (and of the associated EMT) may be smaller in SCCs of the uterine cervix than in other tumour entities should also be considered. It is well known that alternative patterns of tumour invasion exist (Friedl and Wolf, 2003) and, according to one study (Wicki et al, 2006), a collective migration pattern may in fact predominate in SCCs of the uterine cervix, consistent with our finding of widespread E-cadherin expression in this entity. In addition, in a recent study, we have identified recurrent histological signs indicating an active role of the tumour stroma in mediating invasiveness in the form of cell collectives in this tumour entity (Mayer et al, 2011). Indeed, prior experimental findings have directly visualised how fibroblasts may enable the invasion of cancer cells derived from SCCs (e.g., Gaggioli et al, (2007)). Future work is needed to clarify to what extent hypoxia-mediated mechanisms may have a role in the context of these alternative types of cancer invasion.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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