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Hypoxia in cancer: significance and impact on clinical outcome

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Abstract Hypoxia, a characteristic feature of locally advanced solid tumors, has emerged as a pivotal factor of the tumor (patho-)physiome since it can promote tumor progression and resistance to therapy. Hypoxia represents a "Janus face" in tumor biology because (a) it is associated with restrained proliferation, differentiation, necrosis or apoptosis, and (b) it can also lead to the development of an aggressive phenotype. Independent of standard prognostic factors, such as tumor stage and nodal status, hypoxia has been suggested as an adverse prognostic factor for patient outcome. Studies of tumor hypoxia involving the direct assessment of the oxygenation status have suggested worse disease-free survival for patients with hypoxic cervical cancers or soft tissue sarcomas. In head & neck cancers the studies suggest that hypoxia is prognostic for survival and local control. Technical limitations of the direct O_2 sensing technique have prompted the use of surrogate markers for tumor hypoxia, such as hypoxia-related endogenous proteins (e.g., HIF-1α, GLUT-1, CA IX) or exogenous bioreductive drugs. In many—albeit not in all—studies endogenous markers showed prognostic significance for patient outcome. The prognostic relevance of exogenous markers, however, appears to be limited. Noninvasive assessment of hypoxia using imaging techniques can be achieved with PET or SPECT detection of radiolabeled tracers or with MRI techniques (e.g., BOLD). Clinical experience with these methods regarding patient prognosis is so far only limited. In the clinical studies performed up until now, the lack of standardized treatment protocols, inconsistencies of the endpoints characterizing the oxygenation status and methodological differences (e.g., different immunohistochemical staining procedures) may compromise the power of the prognostic parameter used.

Keywords Tumor oxygenation · Hypoxia · Patient outcome · Oxygen needle electrode · Hypoxia marker · Hypoxia imaging

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1 Hypoxia in solid tumors

1.1 Evidence and characterization of tumor hypoxia

Clinical investigations carried out over the last two decades have clearly demonstrated that the prevalence of hypoxic tissue areas [i.e., areas with O_2 tensions (pO_2 values) ≤2.5 mmHg] is a characteristic pathophysiological property of locally advanced solid tumors and a relevant factor of the tumor (patho-)physiome. Such areas have been found in a wide range of malignancies: cancers of the breast, uterine cervix, vulva, head & neck, prostate, rectum, pancreas, lung, brain tumors, soft tissue sarcomas, non-Hodgkin's lymphomas, malignant melanomas, metastatic liver tumors and renal cell cancer [1–7].

Evidence has accumulated showing that up to 50–60% of locally advanced solid tumors may exhibit hypoxic and/or anoxic tissue areas that are heterogeneously distributed within the tumor mass. The pretherapeutic oxygenation status assessed in cancers of the breast, uterine cervix and head & neck is poorer than that in the respective normal tissues and is independent of clinical size, stage, histology, grade, nodal status and a series of other tumor characteristics

or patient demographics. The data do not suggest a topological distribution of pO_2 values within a tumor. Tumor-to-tumor variability in oxygenation is greater than intra-tumor variability. Local recurrences have a higher hypoxic fraction than the respective primary tumors. There is no clear-cut difference in the oxygenation status between primary and metastatic malignancies.

1.2 Pathogenesis of tumor hypoxia

Hypoxic (or anoxic) areas arise as a result of an imbalance between the supply and consumption of oxygen. Whereas in normal tissues or organs the O_2 supply matches the metabolic requirements, in locally advanced solid tumors, the O_2 consumption rate of neoplastic (as well as stromal) cells may outweigh a restricted oxygen supply and result in the development of tissue areas with very low O_2 levels.

Major pathogenetic mechanisms involved in the development of hypoxia in solid tumors are (a) severe structural and functional abnormalities of tumor microvessels (*perfusion-limited O_2 delivery*), (b) deterioration of diffusion geometry (*diffusion-limited O_2 delivery*), and (c) tumor-associated and/or therapy-induced anemia leading to a reduced O_2 transport capacity of the blood (*anemic hypoxia*). There is abundant evidence for the existence of substantial heterogeneity in the tissue oxygenation status, predominantly due to the first two mechanisms mentioned above.

Perfusion-limited O_2 delivery leads to *ischemic hypoxia* which is often transient. For this reason, this type of hypoxia is also called "acute hypoxia," a term that does not, however, take into account the mechanisms underlying this condition.

Hypoxia in tumors can also be caused by an increase in diffusion distances, so that cells far away (>70 μm) from a nutritive blood vessel receive less oxygen (and nutrients) than needed. This condition is termed *diffusion-limited hypoxia* and is also known as "chronic hypoxia." In addition to enlarged diffusion distances, an adverse diffusion geometry (e.g., concurrent vs. countercurrent tumor microvessels) can also cause hypoxia.

Tumor-associated or therapy-induced anemia can contribute to the development of hypoxia (*anemic hypoxia*). This type of hypoxia is particularly intensified in tumors or tumor areas exhibiting low perfusion rates. A similar condition can be caused by carboxyhemoglobin (HbCO) formation in heavy smokers which can lead to *toxic hypoxia*, since hemoglobin blocked by carbon monoxide (CO) can no longer transport oxygen.

Very often, tumor microvessels are perfused (at least transiently) by plasma only [8]. Where this occurs, hypoxia develops very rapidly around these vessels since only a few tumor cells at the arterial end of the vessels can be adequately supplied under the given conditions. Metastatic liver lesions may be perfused (at least partially) by vessels arising from the portal vein system with a lower oxygen content of the blood. As a result, liver tumors may thus be confronted with a reduced O_2 supply (*hypoxemic hypoxia*).

2 Hypoxia as an adverse factor of the tumor (patho-) physiome

2.1 The Janus face of tumor hypoxia

Cells exposed to hypoxia respond by reducing their overall protein synthesis which in turn leads to *restrained proliferation* and subsequent *cell death*. Hypoxia can hinder or even completely inhibit tumor cell proliferation *in vitro*. Sustained hypoxia can also change the cell cycle distribution and the relative number of quiescent cells leading to alterations in the response to radiation and many drugs. The degree of inhibition depends on the severity and duration of hypoxia. Under anoxia, most cells undergo immediate arrest in whichever cell cycle phase they are presently in. Additionally, hypoxia can induce programmed cell death *(apoptosis)* both in normal and in neoplastic cells. p53 accumulates under hypoxic conditions through a HIF-1α-dependent mechanism and induces apoptosis. However, hypoxia also initiates p53-independent apoptosis pathways including those involving genes of the BCL-2 family. Below a critical energy state, hypoxia may result in *necrotic cell death*. Hypoxia-induced proteome changes leading to cell cycle arrest, differentiation, apoptosis, and necrosis, may explain delayed recurrences, dormant micrometastases, and growth retardation which can occur in large tumors.

In contrast, hypoxia-induced proteome and/or genome changes may *promote tumor progression* via mechanisms enabling cells to overcome nutritive deprivation, to escape from the hostile environment and to favor unrestricted growth. Sustained hypoxia may also lead to cellular changes resulting in a more clinically aggressive phenotype. During the process of hypoxia-driven malignant progression, tumors may develop an increased potential for local invasive growth, perifocal tumor cell spreading, and regional and distant tumor cell metastasis, resulting in a poor prognosis [3, 6].

2.2 Role of hypoxia in malignant progression

When tumors develop they often become more malignant with time, a process termed "tumor progression." Substantial data suggest that tumor hypoxia or anoxia (i.e., no measurable oxygen) and the HIF-system are intensly involved in processes conferring a growth advantage to tumor cells and the development of a more malignant phenotype [9–14]. Depending on the level and (possibly) the duration of hypoxia, three mechanisms may be involved in hypoxia-

induced tumor propagation: alterations in gene expression with subsequent changes of the proteome and/or changes in the genome [3, 15–17], and clonal selection [18–20].

2.3 Tumor hypoxia and acquired treatment resistance

Tumor hypoxia is classically associated with resistance to radiotherapy, but has also been shown to diminish the efficacy of certain forms of chemotherapy, of photodynamic therapy and immunotherapy (for reviews see [1, 3, 6, 21]).

3 Tumor hypoxia and clinical outcome

An adverse prognostic impact of tumor hypoxia in various tumor types—among them cancers of the uterine cervix, head and neck, and soft tissue sarcomas—has been repeatedly demonstrated. In the following sections this information is summarized for various tumor entities. In these studies, hypoxia has been assessed using different detection techniques.

3.1 Studies based on polarographic O_2 needle electrodes and prognostic value of hypoxia

The early study of Gatenby et al. [22] using O_2 needle electrodes in the clinical setting demonstrated hypoxia in head & neck tumors. These authors have convincingly shown that hypoxia in metastatic lesions was associated with a poor prognosis upon radiotherapy.

The first data suggesting that hypoxia could be a prognostic factor for patient outcome was published in 1993 by Höckel et al. [23]. In a first analysis of 31 *cervix cancer* patients, the authors could show that patients with hypoxic tumors (median pO_2<10 mmHg) had a significantly lower overall and recurrence-free survival. These observations were confirmed in a later study on 103 patients [24]. The survival differences were independent of stage, histology and grade. Differences in local control were not apparent on multivariate analysis (see Table 1).

Differences in survival were also observed in 106 patients at a cutoff of pO_2=5 mmHg by Fyles et al. [25]. The impact of hypoxia in this latter study, however, was observed only in node-negative patients. Again, hypoxia did not appear to be of prognostic value when local control was assessed. Two smaller studies by Knocke et al. [26] and Lyng et al. [27] also confirmed the prognostic impact of hypoxia on disease-free and overall survival in cervical cancers. Lyng et al. [27] demonstrated hypoxia to be a prognostic factor for local control also (for a review see [28]). In contrast, the prospective international multi-center study by Nordsmark et al. [29] involving 120 patients with cervical cancer yielded conflicting data with no impact of hypoxia on the outcome. The reason for these conflicting results is not clear.

Hypoxia also appears to be prognostic for outcome in *head & neck cancers*, with data suggesting that hypoxia is prognostic for survival and local control (see Table 2). The international multi-center study by Nordsmark et al. [30] involving 397 patients with head & neck tumors provided further evidence that tumor hypoxia is associated with a poor prognosis in patients with advanced head & neck cancer following primary radiotherapy. In head & neck cancers, hypoxia not only predicts for disease-free survival and overall survival (as is also the case in cervical cancers) but also for local control, suggesting hypoxia-induced radiation resistance as a major factor for local failure. In the study of Terris [31] only a small number of patients was assessed and hypoxia did not appear to be a prognostic factor in disease-free survival and local control.

Studies of *soft tissue sarcomas* also suggest worse disease-free survival for patients with hypoxic tumors (see Table 3). In these studies, however, the small number of patients did not allow multivariate analysis. For this reason,

Table 1 Pretherapeutic oxygenation status of locally advanced cancers of the uterine cervix and prognostic significance of tumor hypoxia (*n*=number of patients)

Authors	*n*	Median pO_2 (mmHg)	HF 2.5 (%)	HF 5 (%)	HF 10 (%)	Prognostic significance of tumor hypoxia (multivariate analysis)	
						Endpoint	Oxygenation parameter
Höckel et al. [23]	31	10	11	26	50	OS, DFS	pO_2<10 mmHg
Höckel et al. [24]	103						
Fyles et al. [25]	106	5		47		PFS, DS, DFS	pO_2<5 mmHg
Knocke et al. [26]	51	10	22	28	50	DFS	pO_2<10 mmHg
Lyng et al. [27]	40	4	47	64	76	DFS, OS, LC	pO_2<5 mmHg
Nordsmark et al. [29]	120	4	38	59	72	no evidence	

Empty spaces indicate a lack of suitable information.
HF 2.5 hypoxic fraction (pO_2<2.5 mmHg), *HF 5* hypoxic fraction (pO_2<5 mmHg), *HF 10* hypoxic fraction (pO_2<10 mmHg), *PFS* progression-free survival, *DFS* disease-free survival, *OS* overall survival, *DS* distant spread, *LC* local control

Table 2 Pretherapeutic oxygenation status of locally advanced head & neck tumors (measurements in neck nodes and/or primary tumors) and prognostic significance of tumor hypoxia (*n*=number of patients)

Authors	*n*	Median pO_2 (mmHg)	HF 2.5 (%)	HF 5 (%)	Prognostic significance of tumor hypoxia (multivariate analysis)	
					Endpoint	Oxygenation parameter
Dunst et al. [122]	125	9		33	OS	HSV
Brizel et al. [123, 124]	86	5		51	DFS, OS, LC	pO_2<10 mmHg
Nordsmark et al. [125, 126]	67	13	22	32	LC	pO_2<2.5 mmHg
Rudat et al. [127, 128]	44	7	25	44	OS	pO_2<2.5 mmHg
Nordsmark et al. [30]	397	9	19	38	OS	pO_2<2.5 mmHg
Terris et al. [31]	25	18	0	2	no evidence	

HF 2.5 hypoxic fraction (pO_2<2.5 mmHg), *HF 5* hypoxic fraction (pO_2<5 mmHg), *DFS* disease-free survival, *OS* overall survival, *LC* local control, *HSV* hypoxic subvolume

the impact of hypoxia on local control and distant spread is not clear.

The interpretation of the data presented on these three tumor types is complicated by a series of unresolved issues: (a) the selection of the optimal endpoints characterizing the oxygenation status of tumors (e.g., median pO_2, HF 2.5, HF 5, HF 10, hypoxic subvolume [28]), (b) the role of heterogeneity in tumor oxygenation [32], (c) the impact of heterogeneous treatment protocols [29], (d) insufficient sample sizes [29], (e) pronounced inter-institutional (inter-observer) differences for the same tumor type (7), and (f) pO_2 readings in necrotic regions (7). In this context, it has to be mentioned that a combination of the O_2 microsensor technique with other existing techniques (see below) has also not yet been proven to be helpful.

3.2 Hypoxia detection with endogenous markers and patient outcome

Difficulties with the polarographic O_2 needle electrode as mentioned above have prompted a search for other markers of tumor hypoxia, particularly endogenous (intrinsic) ones, which can define patient outcome using archived tissue specimens. The use of the term "endogenous hypoxia markers" should however, in the strict sense, be avoided, because it is now evident that none of the markers discussed below are regulated exclusively by oxygen availability *in vivo* (discussed in detail in [33, 34]). Most, if not all endogenous proteins show considerable variability of basal and inducible expression levels between different cell types, which is a reflection of cellular differentiation. In the context of malignant disease, this kind of variability is known to be greatly enhanced, owing to high genetic instability ("mutator phenotype" [35]) and strong selection pressures. Both processes are known to yield clonal heterogeneity. Additionally, multiple factors pertaining to the microenvironment, e.g., local pH and metabolite concentrations have been shown to modify hypoxia-responsive protein expression [36, 37].

3.2.1 The hypoxia-inducible factor (HIF) system and patient outcome

Of all proteins induced by hypoxic conditions, hypoxia-inducible factors (HIF) and their downstream target genes have been studied most intensively. The prognostic impact of HIF-1α expression has been the subject of numerous studies (see Table 4). *Higher expression of HIF-1α* has been shown to be almost unequivocally correlated with a poorer survival in breast [38–41], head & neck [42], esophagus [43], stomach [44], lung cancers (NSCLC, [45]) and other tumor types. In cervical cancer, however, the prognostic impact of HIF-1α expression is less clear. While Burri et al. [46] found an independent influence of HIF-1α on overall survival, other studies [47–49] could not confirm this result. The only available study in endometrial carcinomas [50] also did not find a prognostic impact of HIF-1α expression.

Table 3 Pretherapeutic oxygenation status of soft tissue sarcomas and prognostic significance of tumor hypoxia (*n*=number of patients)

Authors	*n*	Median pO_2 (mmHg)	Prognostic significance of tumor hypoxia (univariate analysis)	
			Endpoint	Oxygenation parameter
Brizel et al. [129]	45	10	DFS, OS, DS	pO_2<10 mmHg
Nordsmark et al. [130]	31	19	DFS, OS, DS	pO_2<19 mmHg

DFS disease-free survival, *OS* overall survival, *DS* distant spread

Both the biological complexity of the HIF system and methodological difficulties in its experimental assessment are likely to account for conflicting data. All of the studies mentioned in the previous paragraph depicted the typical HIF-1α expression pattern of increasing staining intensity with enlarging distance from microvessels and in the viable cell layers surrounding necroses. Other studies, however, did not show this pattern and this dissimilarity may be associated with the application of different antibody clones and immunohistochemical staining procedures. Importantly, these differences cannot be entirely ascribed to the well-known existence of a "hypoxia-independent" or "diffuse" staining pattern for HIF-1α [40, 42] which is commonly attributed to oncogene- or growth factor-mediated HIF-1α expression, being independent of local oxygen concentrations. Comparability of results is further hampered by the fact that neither criteria for marker positivity nor methods for marker quantification are standardized: Since it is a transcription factor, most authors agreed that only nuclear expression of HIF-1α should be assessed (e.g., [39, 40, 42, 49]). Nevertheless, other investigators chose to also or even predominantly assess cytoplasmic expression [51, 52]. Some studies used the visual estimation of cell numbers [46, 48] while others performed an image analysis based counting of positive cells. Comparability of image analysis results is also limited by the fact that either fractions of positive nuclei [49] or tumor tissue [47] were scored. Since the immunohistochemical protocols were very similar in both of the latter studies, the large differences in "marker-positive" values (0.2–98.6% vs. 0–10.7%) illustrate the high relevance of these methodological considerations. Swinson et al. [45] pointed out that different cut-off levels of HIF-1α expression are widely used. This also applies to the previously cited studies. Burri et al. [46], in cancers of the uterine cervix, found a poorer survival for strong HIF-1α staining in >50% of all tumor cells, while Bos et al. [38] reported poorer survival in lymph node-negative breast cancers using a cut-off of 5%. Our study of HIF-1α expression in cervix cancer [49] used the median (24%) as the cut-off and found no association with patient outcome. All cervical cancer studies cited here analyzed HIF-1α expression in biopsy specimens. When comparing results obtained from biopsies with expression in surgical specimens, one has to be aware of the possible influence of surgery-induced ischemia, which has been shown to lead to significant HIF-1α induction in rectal cancer [53]. Furthermore, Haugland et al. mentioned that extended fixation times can markedly reduce HIF-1α expression [47].

Despite such methodological problems, the majority of these studies agree that HIF-1α signalling is a positive factor which has an impact on tumor growth, a finding which was to have been expected considering results previously obtained from *in vitro* experiments [54, 55]. Some conflicting results have however been reported. For example, HIF-1α$^{-/-}$ ES cells have been shown to have both higher apoptosis and proliferation rates [56] whereas higher proliferation was found to be combined with lower apoptosis in HIF-1β-deficient (a functional HIF-1 knockout) Hepa-1/c4 cells [57]. Even so, the proposed mechanisms by which HIF-1α target genes are thought to lead to poorer patient survival are enhanced cancer cell survival, decreased apoptosis and induction of angiogenesis. A simultaneous assessment of surrogate markers of cell proliferation, apoptosis and microvessel density in prognostic studies could therefore have the potential to add significantly to (a) an estimation of the overall plausibility of the results, and (b) a verification of the pathophysiological models. Unfortunately, studies of this kind (e.g., [58]) are very rare. One such example is the study of Bos et al. who found higher proliferation in HIF-1α-positive tumors [38].

Less data are available regarding the prognostic significance of *HIF-2α expression* in tumor cells. In head & neck cancer, the earlier study of Beasley et al. [59] could not identify a correlation of tumor or tumor-associated macrophage expression of HIF-2α with a poorer outcome. A more recent study by Koukourakis et al. [60] did however find a shorter overall survival and worsened locoregional control in cases with higher HIF-2α expression in tumor cells. None of these studies identified a predominantly perinecrotic pattern of HIF-2α expression, a finding which is in contrast to that of an extensive methodological study of the HIF-2α expression pattern across a large panel of tumors [61]. Additionally, Onita et al. [62] not only described a predominantly perinecrotic expression pattern, but also found HIF-2α expression to be located exclusively in stromal cells and to be entirely absent in tumor cells in a series of 67 cases of bladder cancer. Again, as was the case for HIF-1α, these differences may be, at least partially, the consequence of different immunohistochemical protocols (e.g., type of heat pre-treatment in the immunohistochemical staining procedure). Unfortunately, methodological details (e.g., antibody concentrations, pre-treatment buffers) have not always been clearly described.

3.2.2 Immunohistochemical detection of GLUT-1 and patient outcome

The immunohistochemical detection of the HIF-1α target gene *glucose transporter-1 (GLUT-1)* is much more straightforward than the detection of HIF-1α itself. Since erythrocytes and perineural tissue express the GLUT-1 antigen in large quantities, these structures are widely used as internal positive controls for staining consistency. Accordingly, studies agree—almost without exception—on a predominantly membranous expression pattern. In univariate analyses, higher GLUT-1 expression has been

Table 4 Immunohistochemical detection of HIF-1α in selected tumor types and patient outcome

Authors	No. of patients	Antibody clone/ pretreatment/ detection system	HIF-1α positive (%)	Tumor Stage/LN status	Therapy	Prognostic impact of hypoxia marker expression[a]	
						Univariate	Multivariate
Cancer of the uterine cervix							
Bachtiary et al. [131]	67	BD H72320/Citrate pH 6/NS	72	IB: 9%, II: 51%, III: 42% LN−: 69%, LN+: 31%	RT	PFS, CCSS	PFS, CCSS
Birner et al. [132]	91	H1α67/Citrate pH 6/labelled SA	81	pT1b	Surg. (+RT if LN+)	OS, DFS	OS, DFS
Burri et al. [46]	78	H1α67/DAKO TRS pH 6.1/CSA (mod.)	94	IB/IIA: 12%, IIB/IIIA: 55%, IIIB/IVA: 33%, LN−: 62%, LN+: 38%	RT (+CT in 23%)	LPFS	OS
Haugland et al. [47]	41	H1α67/Citrate pH 6/CSA	100	FIGO IB/IIA: 40%, IIB: 27%, III: 33%, LN−: 76%, LN+: 24%	RT	No evidence	
Hutchison et al. [48]	99	H1α67/EDTA/TSA	96	IB: 27%, II: 30%, III: 36%, IVA: 6%	RT	No evidence	
Ishikawa et al. [133]	38	OZ12/Zinc citrate/ABC	NS	All patients: IIIB, LN−: 37%, LN+: 63%	RT	MFS, RFS	ND
Mayer et al. [49]	38	H1α67/Citrate pH 6/CSA	100	IB/IIA: 15%, IIB/IIIA: 62%, IIIB–IVB: 24%, LN−: 26%, LN+: 43%, LN NK: 29%	Heterogeneous	No evidence	
Breast cancer							
Bos et al. [38]	81 (150)	H1α67/DAKO TRS pH 6.1/CSA	75	AJCC I–II	Surg.	OS, DFS, LN− only	OS and DFS, LN− only
Dales et al. [134]	745	H-206/NS/Ventana	100	NS, LN−: 50%, LN+: 50%	Surg.	OS, MFS	OS, MFS (only in LN negative tumors)
Gruber et al. [39]	77	H1α67/DAKO TRS pH 6.1/CSA	56	pT1/pT2: 71, pT3/pT4: 29 all LN+	Surg. + RT + CT	DFS, DMFS	DFS, DMFS (only in T1/T2 tumors)
Schindl et al. [135]	206	H1α67/Citrate pH 6/ABC	76	pT1: 52%, pT2: 41%, NK: 7%	Surg. + aCT in ~83%	OS, DFS	OS, DFS
Schoppmann et al. [136]	119	H1α67/Citrate pH 6/ABC	76	pT1: 59%, pT2: 41%, LN+: all patients	Surg. + RT + CT (50%), Tamoxifen (43%)	OS, DFS	OS, DFS
Trastour et al. [41]	132	rabbit polyclonal (antiserum 2087)/Citrate pH 7.3/ Polymer-HRP	45	LN−: 64%, LN+: 36%	Surg. + heterogenous combinations of RT, CT and Tamoxifen in 91%	OS, DFS, DMFS	DFS, DMFS
Vleugel et al. [40]	166	BD H72320/DAKO TRS pH 6.1/CSA	40	NS, LN−: 57.5%, LN+: 36%, LN NK: 6.5%	Surg. + aCT	DFS (worse for perinecrotic expression pattern)	ND
Head & neck cancer							
Aebersold et al. [42]	98	H1α67/DAKO TRS pH 6.1/CSA (mod.)	94	T1/2: 12%, T3/4: 88%, 66% LN+	RT + CT (26%)	OS, DFS, LFFS	OS, DFS, LFFS

Beasley et al. [59]	69	ESEE122/Tris/EDTA pH 9/ABC	64	T1/2: 37%, T3/4: 73% LN−: 48%, LN+: 52%	Surg. (+RT in 35%)	improved OS, DFS	improved OS, DFS
Koukourakis et al. [51]	75	ESEE122/NS/ APAAP or ABC	NS	T2: 13%, T3: 44%, T4: 43%, N0: 32%, N1/2a: 32%, N2b/3: 36%	RT + CT	OS, LRFS	No evidence
Kyzas et al. [137]	81	Sc-13515/Citrate pH 6/Polymer-HRP	NS	I/II: 69%, III/IV: 31%, LN−: 74%, LN+: 26%	Surg.	No evidence	
Winter et al. [138]	140	ESEE122/Tris/ EDTA pH 9/Polymer-HRP	NS	T1:17%, T2: 24%, T3: 19%, T4a,T4b: 40%, N0: 36%, N1: 21%, N2: 38%, N3: 5%	Surg. + RT in 85%	DFS, DSS	DFS, DSS

[a] higher marker expression is correlated with poorer survival, except where stated otherwise
ABC avidin-biotin complex® (DAKO), *aCT* adjuvant chemotherapy, *AJCC* American Joint Committee on Cancer, *CCSS* cervical cancer-specific survival, *CSA* Catalyzed signal amplification system® (DAKO), *CT* chemotherapy, *DAKO TRS* DAKO Target retrieval solution®, *DFS* disease-free survival, *DMFS* distant metastasis-free survival, *DSS* disease-specific survival, *LFFS* local failure-free survival, *LN−* lymph node negative, *LN+* lymph node positive, *LPFS* local progression-free survival, *LRFS* Local relapse-free survival *MFS* metastasis-free survival, *mod.* modified procedure, *ND* not determined, *NK* not known, *NS* not stated, *OS* overall survival, *RFS* recurrence-free survival, *RT* radiotherapy, *SA* streptavidin, *TSA* Tyramide signal amplification

shown to correlate with a poorer survival in many tumor entities, among them breast [63], head & neck [64], esophagus [65], bladder [66], stomach [67], colorectal [68], ovarian [69] and lung cancer (NSCLC) [70] (see Table 5). Two studies on cervical cancer have assessed the prognostic impact of GLUT-1 expression. Airley et al. [71] found a correlation of high GLUT-1 expression with metastasis-free survival in both uni- and multivariate analyses. Our own results [72] indicated a strong influence on prognosis only in univariate analysis, but inclusion of either pT- or pN-stage in a multivariate analysis resulted in no prognostic information being obtained from GLUT-1 expression. GLUT-1 expression was correlated with FIGO and pT stage in our study and similar associations have been described for other tumor types [64, 65, 67].

3.2.3 Immunohistochemical detection of CA IX and patient outcome

The second target gene of HIF-1α that has been extensively studied with regard to its prognostic significance is *carbonic anhydrase IX (CA IX)*. As with HIF-1α and GLUT-1, most studies agree on a negative impact of high CA IX expression on patient survival for various tumor entities (e.g., [60, 66, 73–76], see Table 6). Surprisingly, a study of 321 patients with renal cell cancer showed exactly the opposite, with poorer survival being found in patients with lower CA IX expression [77]. The reason for this data conflict is not clear.

CA IX expression has been implicated as playing a role in tumor cell survival [78] and invasiveness [79]. Harris and Potter were able to show that overexpression of CA IX leads to an up to six-fold increase in proton-extrusion capacity [80]. Intracellular acidification is known to be linked to apoptosis induction [81], suggesting an anti-apoptotic role for CA IX activity. Parkkila et al. [79] have shown that invasiveness of renal cell cancer cell lines can be inhibited by up to 74% with acetazolamide (an inhibitor of carbonic anhydrases) as assessed by the Matrigel invasion assay.

3.2.4 Detection of other HIF-1α target genes and patient outcome

Vascular endothelial growth factor (VEGF) plays a key role in tumor angiogenesis. The independent prognostic significance of increased VEGF expression has been proven for most types of solid tumors and also for some hematological malignancies [82]. However, in addition to its inducibility by hypoxia, other microenvironmental factors have been shown to influence VEGF expression, among them glucose depletion [83], glutamine deprivation (leading to endoplasmic reticulum stress [84]) and an acidic extracellular pH [85]. The relevance of these hypoxia-independent induction mechanisms is illustrated

Table 5 Immunohistochemical detection of GLUT-1 in selected tumor types and patient outcome

Authors	No. of patients	Antibody clone/ pretreatment/ detection system	GLUT-1 positive (%)	Tumor Stage/LN status	Therapy	Prognostic impact of hypoxia marker expression	
						Univariate	Multivariate
Cancer of the uterine cervix							
Airley et al.[71]	121	Rabbit polyclonal/ none/Polymer-HRP	77	I: 29%, II: 31%, III: 34%, IV: 6%	RT	MFS	MFS
Mayer et al. [72]	42	Rabbit polyclonal/ Citrate pH 6/ABC	74	I: 14%, II: 64%, III: 16%, IV: 5%	Surg.: 74%, RT: 26% aCT: heterogenous	OS, RFS	No evidence
Breast cancer							
Kang et al. [63]	100	Rabbit polyclonal/ none/ABC	47	LN-: 53%, LN+: 47%	Surg. + CT/Tamoxifen "according to risk factors"	OS, DFS	DFS
Head & Neck cancer							
De Schutter et al. [139]	67	Rabbit polyclonal (DAKO)/Citrate pH 6/Polymer-HRP	NS	T1: 3%, T2: 25%, T3: 40%, T4: 31%, N0: 22%, N1: 18%, N2: 46%, N3: 13%	RT + CT in 5%	No evidence	
Jonathan et al. [140]	58	Rabbit polyclonal/ none/ABC	NS	T1: 7%, T2: 28%, T3: 47%, T4: 19%, N0: 42%, N1: 28%, N2: 29%	ARCON	OS	NS
Kunkel et al. [64]	118	Chemicon AB 1351/ EDTA pH 8/ABC	100	I: 24%, II: 22%, III: 5%, IV: 49%, N0: 60%, N1: 8%, N2: 31%	Surg. Post-OP RT, when resection margins histopathologically +	OS	OS
Oliver et al. [141]	54	Rabbit polyclonal/ none/ABC	91	NS	Surg., no additional details given	RFS	ND

AB antibody, *ARCON* accelerated radiotherapy with carbogen and nicotinamide (see Table 4 for further abbreviations)

by the fact that no correlations have been found for either VEGF and oxygen electrode measurements [86] or for pimonidazole [87].

Lysyl oxidase (LOX) has been shown to be associated with poorer survival in head & neck and breast cancer [88]. LOX co-localizes with pimonidazole and has been implicated in metastasis formation [89], although the exact mechanism is unclear.

Overexpression of the HIF-1α target gene *lactate dehydrogenase isoenzyme-5 (LDH-5)* has been linked to a poor prognosis in non small-cell lung cancer [90] and colorectal cancer [91]. However, these correlations rely largely on nuclear LDH-5 expression. Since localisation of this glycolytic enzyme in the nucleus is atypical, the functional relevance of this expression pattern remains questionable.

Several other HIF-1α target genes have an indisputed role in cancer pathophysiology and have been demonstrated to be associated with patient survival. An in depth discussion of urokinase plasminogen activator receptor (UPA-R), plasminogen activator inhibitor-1 (PAI-1), Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3), erythropoietin (EPO) and others, however, is beyond the scope of this article.

In conclusion, members of the HIF-cascade may not be seen as ideal direct surrogate markers for the assessment of tumor hypoxia, but are nevertheless likely to represent the molecular substrates of a large part of the pathophysiological impact of oxygen deficiency. The verification of their supposed actions (e.g., anti-apoptotic) in samples obtained from patients may present an interesting subject for further investigations.

3.2.5 Hypoxia-induced proteins independent of HIF and patient outcome

Many other proteins have been implicated in the genetic response to hypoxia, among them *nuclear factor κB (NF-κB)*, *activator protein-1 (AP-1)* and *members of the unfolded protein response (e.g., GRP78)*. Their exact role in hypoxia-induced tumor progression is less clear. For example, "hypoxic" induction of NF-κB is likely to be the consequence of reoxygenation-induced reactive O_2 species [92].

Overexpression of *osteopontin (OPN)* has been demonstrated to be involved in metastasis formation [93] by virtue of its integrin and CD44 binding sites. OPN is overexpressed during hypoxia and the transcriptional control

seems to be independent of HIF-1α [94]. High OPN plasma levels have been shown to be associated with a poorer prognosis in head & neck [95] and non-small cell lung cancers [96]. The immunohistochemical expression of OPN in tumor cells was however not found to be correlated with prognosis [97].

3.3 Hypoxia detection using exogenous bioreductive compounds and patient outcome

Certain 2-nitroimidazoles (e.g., pimonidazole, EF5) are referred to as "exogenous hypoxia markers" because these substances are chemically modified in hypoxic cells to yield hydroxylamine derivatives which covalently bind to sulfhydryl residues of proteins. These adducts may then be detected using immunohistochemical methods. The resulting staining pattern typically shows increasing signal intensity as a function of increasing distance from the microvessel-carrying stroma together with expression in the viable cell layers surrounding necrosis. Areas of necrosis do not become labeled since the substance can only be metabolized within viable cells. This point may be decisive regarding the lack of correlation between the staining of 2-nitroimidazoles and O_2 microelectrode measurements found in most studies [98–101]. Using the Eppendorf O_2 needle electrode technique, exclusion of necrosis can only be carried out *post hoc* by examination of biopsies containing the electrode measurement track [23, 49]. The measurement may then be discarded as a whole, if the proportion of necrosis is regarded as being too high. Exclusion of the influence of multiple micronecroses—a common trait of many cancer growth patterns—is not feasible.

Table 6 Immunohistochemical detection of CA IX in selected tumor types and patient outcome

Authors	No. of patients	Antibody clone/ pretreatment/ detection system	CA IX positive (%)	Tumor Stage/ LN status	Therapy	Prognostic impact of hypoxia marker expression	
						Univariate	Multivariate
Cancer of the uterine cervix							
Hedley et al. [142]	102	M75/none/ Fluorescence	70	IB/IIA: 27%, IIB: 40%, III/IV: 32%, LN−: 48%, LN+: 28%, LN NK: 24	RT alone: 56% RT + CT : 44%	No evidence	
Loncaster et al. [73]	130	M75/none/ Polymer-HRP	71	I: 28%, II: 29%, III: 36%, IV: 7%	RT	DSS, MFS	OS, MFS
Breast cancer							
Brennan et al. [143]	400	M 75/none/ NS	11	All patients: II	Surg. + RT, CT: <2% Tamoxifen: 50%	OS, RFS, BCSS	BCSS
Chia et al. [74]	103	M75/none/ Polymer-HRP	48	NS, LN−: 44%, LN+: 56%	Surg. + RT, CT: 26%, Tamoxifen: 78%	OS, RFS	OS
Hussain et al. [144]	144	M75/EDTA/ Polymer-HRP	26	NS, LN−: 56%, LN+: 35%, LN NK: 9	Surg., details NS	OS	OS
Trastour et al. [41]	132	M75/Citrate pH 7.3/ Polymer-HRP	29	LN−: 64%, LN+: 36%	Surg. + heterogenous combinations of RT, CT and Tamoxifen in 91%	DFS	DFS, DMFS
Head & neck cancer							
De Schutter et al. [139]	67	M75/none/ Polymer-HRP	NS	T1: 3%, T2: 25%, T3: 40%, T4: 31%, N0: 22%, N1: 18%, N2: 46%, N3: 13%	RT + CT in 5%	No evidence	
Jonathan et al. [140]	58	Mouse anti-CA IX/ Citrate pH 6/ ABC	NS	T1: 7%, T2: 28%, T3: 47%, T4: 19%, NO: 43%, N1: 28%, N2:29%	ARCON	better LC and FDM for patients with high CA IX expression	NS
Koukourakis et al. [60]	198	M75/NS/ ABC	NS	T1: 5%, T2: 46%, T3: 31%, T4: 19%, N0: 64%, N1: 19%, N2: 13%, N3: 5%	RT (CHART: 59%, conventional: 41%)	OS, LC	OS, LC
Winter et al. [138]	149	M75/none/ Polymer-HRP	62	T1:17%, T2: 24%, T3: 19%, T4a,T4b: 40%, N0: 36%, N1: 21%, N2: 38%, N3: 5%	Surg. + RT in 85%	No evidence	

BCSS breast cancer specific survival, *CHART* Continuous Hyperfractionated Accelerated Radiotherapy, *LC* locoregional control (see Table 4 for further abbreviations)

Studies reporting a prognostic significance of 2-nitroimidazole markers have been published for soft tissue sarcoma [102], brain tumors [103] and head & neck cancer [104]. In contrast, a correlation with prognosis has never been demonstrated for cervical cancer [29]. It is not clear why polarographic O_2 electrode measurements seem to yield more relevant data regarding the biology of malignant disease in patients than 2-nitroimidazole markers. The systematic exclusion of necrosis, as is the case with 2-nitroimidazoles, cannot however be considered to be a methodological advantage *a priori*. Exogenous markers may therefore even systematically miss an important part of the tumor, since necrotic areas are often infiltrated by tumor-associated macrophages (TAM) and macrophage-derived (e.g., angiogenic) cytokines may actually be of high pathophysiological relevance [105, 106]. In breast cancer, a significant positive correlation between high vascular density, high numbers of TAMs and reduced survival has been described [107]. Additionally, unspecific or hypoxia-independent marker binding may mitigate prognostic associations of 2-nitroimidazole staining. Pimonidazole binding to keratinizing tissue areas has been shown to be at least partially responsible for unspecific staining and a role for varying intertumor levels of endogenous nitroreductases cannot be ruled out [108]. In conclusion, exogenous hypoxia markers are a valuable tool, but—on the basis of currently available data—their prognostic relevance appears to be limited.

3.4 Non-invasive hypoxia imaging and patient outcome

Nitroimidazole derivatives (see Section 3.3) can also be used as tracers for positron emission computed tomography (PET) imaging. *[^{18}F]Fluoromisonidazole* (^{18}F-MISO) is the most widely used agent of this group. Maximum tumor/blood ratios for ^{18}F-MISO standard uptake values lying above the median were associated with poorer overall survival in a study of 73 head & neck cancer patients treated with different treatment protocols [109]. Rischin et al. [110] demonstrated higher locoregional failure rates in head & neck cancer patients who were treated with conventional chemotherapy, whose tumors were classified as being hypoxic using ^{18}F-MISO scanning. Additionally, these authors found a significantly lower failure rate in patients treated with a chemotherapeutic regime including tirapazamine. Similar results were obtained with a further nitroimidazole compound, *[^{18}F]Fluoroerythronitroimidazole* ([^{18}F]FETNIM) by Lehtio et al. [111] in 21 head & neck cancer patients. Fractional hypoxic volumes lying above the median were correlated with impaired local control and shorter overall survival. *^{18}F-labelled EF5* [112] has not been used for prognostic studies in humans, but may be of interest since it can be used for both invasive imaging and immunohistochemical detection in the same subject. As was the case for the nitroimidazoles, *^{60}Cu- or ^{64}Cu-labeled diacetyl-bis (N^4-methylthiosemicarbazone)* (Cu-ATSM) is retained in hypoxic tissue following its reduction. Cu-ATSM may yield a higher signal-to-noise ratio, because of a quicker washout in normal, non-hypoxic tissue [113]. However, a hypoxia-independent retention of Cu-ATSM has also been reported [114]. Nevertheless, a prognostic significance of ^{60}Cu-ATSM PET has been reported, e.g., in cancers of the uterine cervix [115]. Li et al. showed poorer overall survival for 32 patients with non-small cell lung cancer using *^{99}mTc-labeled 4,9-diaza-3,3,10,10-tetramethyldodecan-2,11-dione dioxime* (^{99}mTc-HL91), which can be detected with single photon emission computed tomography (SPECT) [116]. *Dynamic contrast-enhanced MRI* using gadolinium has been shown to correlate with Eppendorf O_2 electrode measurements in cancers of the uterine cervix [117] and a subsequent study of 50 patients with cervix cancers treated with radiotherapy indicated that low Gadolinium enhancement is correlated with poorer disease-specific survival [118]. *Blood oxygenation level-dependent (BOLD) MRI* uses paramagnetic deoxyhemoglobin as an endogenous hypoxia tracer. This approach is, however, only applicable in tissue areas perfused with red blood cells, a prerequisite which is not always fulfilled in tumor tissue, owing to the pathological vessel architecture and function [119, 120]. A prognostic impact of this technique has only been demonstrated in rodent tumors [121].

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