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Stromal expression of hypoxia regulated proteins is an adverse prognostic factor in colorectal carcinomas

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Abstract. *Background*: Hypoxia modifies the phenotype of tumors in a way that promotes tumor aggressiveness and resistance towards chemotherapy and radiotherapy. However, the expression and influence of hypoxia-regulated proteins on tumor biology are not well characterized in colorectal tumors. We studied the role of protein expression of hypoxia-inducible factor (HIF)-1α, HIF-2α, carbonic anhydrase 9 (CA9) and glucose transporter 1 (GLUT1) in patients with colorectal adenocarcinomas. *Methods*: Expression of HIF-1α, HIF-2α, CA9 and GLUT1 was quantified by immunohistochemistry in 133 colorectal adenocarcinomas. The expression of hypoxia markers was correlated with clinicopathological variables and overall patient survival. *Results:* Expression of these hypoxia markers was detected in the epithelial compartment of the tumor cells as well as in tumor-associated stromal cells. Although tumor cells frequently showed expression of one or more of the investigated hypoxia markers, no correlation among these markers or with clinical response was found. However, within the tumor stroma, positive correlations between the hypoxia markers HIF-2α, CA9 and GLUT1 were observed. Furthermore expression of HIF-2α and CA9 in tumor-associated stroma were both associated with a significantly reduced overall survival. In the Cox proportional hazard model, stromal HIF-2α expression was an independent prognostic factor for survival. *Conclusion*: These observations show, that expression of hypoxia regulated proteins in tumor-associated stromal cells, as opposed to their expression in epithelial tumor cells, is associated with poor outcome in colorectal cancer. This study suggests that tumor hypoxia may influence tumor-associated stromal cells in a way that ultimately contributes to patient prognosis.

Keywords: Hypoxia, stromal expression, epithelial expression, patient outcome

1. Introduction

Regulation of tissue oxygen homeostasis is critical for cell function, behavior and survival. Lack of oxygen (hypoxia), occurs early and remains a common feature of tumors throughout their development. Hypoxic areas in tumors contribute to a worse prognosis independent of treatment modality [8,23]. One of the underlying mechanisms proposed to account for this poor prognosis is a contribution of hypoxia to the malignant status of tumors through promotion of metastasis, angiogenesis, and selection of cells with defects in apoptosis [31,50]. In colorectal cancer, the importance of hypoxia has been demonstrated by clinical studies in which hypoxia predicts for worse outcome and resistance towards chemotherapy and radiotherapy [6,17, 18,38].

The majority of studies investigating hypoxia in human tumors has been carried out with oxygen sensing needle electrodes that are capable of direct oxygen measurements in tumors [51]. However, this procedure is invasive and restricted to accessible tumor sites [47]. This has led to the development and testing of alternative hypoxia markers including the 2-nitroimidazole compounds pimonidazole and EF-5 which undergo reduction upon binding within hypoxic cells [1]. Estimation of hypoxia by these so-called "exogenous" markers is made several hours following intravenous administration, by evaluating the degree of binding us-

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ing specific antibodies in biopsies [3]. In addition, several "endogenous" markers have also been proposed. These markers consist of hypoxia regulated gene products and include among others hypoxia inducible factor (HIF)-1α, HIF-2α, carbonic anhydrase 9 (CA9), glucose transporter 1 (GLUT1), glucose transporter 3 (GLUT3), and vascular endothelial growth factor (VEGF) [3,9,14,19,27,30,32,37,43].

HIF is a heterodimeric transcription factor composed of α and β subunits. HIF-1β is expressed constitutively and is not sensitive to the hypoxic status of the cells, whereas the alpha subunit accumulates rapidly inside the hypoxic cells primarily because of the prevention of ubiquitination and subsequent protein degradation by the proteasome complex, which usually takes place in normoxic cells. There are three homologues of the alpha subunit (HIF-1α, HIF-2α, HIF-3α) [39]. HIF-1α and HIF-2α are thought to play a significant role in tumor neovascularization, enhanced cellular proliferation, decreased apoptosis, and development of drug resistance to chemotherapeutic agents [38]. In colorectal cancer HIF-2α also seems to play an important role in angiogenesis, the combined expression of HIF-2α and HIF-1α may play a role in tumor progression and prognosis [19,53].

CA9 is a transmembrane glycoprotein and a member of the carbonic anhydrase family that is regulated by HIF [25]. Carbonic anhydrases are zinc metalloenzymes that catalyze the reversible conversion of carbon dioxide to carbonic acid and are involved in respiration, calcification, acid-base balance, the formation of cerebrospinal fluid, saliva, and gastric acid. Increased CA9 expression is induced by hypoxia, as was shown in a wide spectrum of tumor types including tumors of the uterine cervix, head and neck, lung, bladder, breast, esophagus, colon and rectum [2, 20,28,33,35,42,44,52]. CA9 expression has previously been demonstrated in colorectal tumors by Saarnio et al. (1998). CA9 staining intensity in colorectal tumors was higher in less advanced tumors (Dukes A + B) [24,35].

GLUT1 is a member of the glucose transporter family of proteins facilitating independent transport of glucose that is also regulated by HIF. Tumors have accelerated metabolism and increased requirements for ATP production, therefore cancer cells have high rates of glycolysis. Elevated GLUT1 expression under hypoxic conditions has been described for many cancers, including hepatic, pancreatic, breast, esophageal, brain, renal, lung, cutaneous, colorectal, endometrial, ovarian and cervical carcinoma [16,22]. In a study done by Haber et al in colorectal cancer, disease specific mortality was greater in patients with high expression of GLUT1 in tumors (>50% of cells were GLUT1 positive) [7]. Furthermore, GLUT1 expression in colorectal cancer was shown to be associated with a high incidence of lymph-node metastasis [36].

The present study was performed to evaluate the expression of hypoxia regulated proteins and their association with clinicopathological response in colorectal adenocarcinomas. We applied a panel of hypoxia-related endogenous immunohistochemical markers on a series of 133 colorectal cancer tissues, and correlated their expression with morphologic tumor parameters, clinicopathological features and overall patient survival.

2. Materials and methods

2.1. Patients

Patients were entered in two multi-center prospective clinical trials between 1979 and 1981 in the Netherlands. One trial was designed to compare patient survival after treatment of colonic cancer by conventional surgery or the no-touch isolation technique [49]. The other trial was conducted to compare survival in rectal cancer patients with or without preoperative radiotherapy. At the time the trial was conducted, only surgical removal of the tumors was performed, and adjuvant chemotherapy was not yet standard practice. Although the results from this study can therefore not be extrapolated to current practice, it does enable unbiased study of the influence of hypoxic conditions on tumor biology. In the current study, we included only the patients who did not undergo preoperative radiotherapy. For immunohistochemical analysis tumor tissues from 133 patients with primary colorectal cancer were available. The distribution of age, gender, tumor stage, location and type of tumor, frequency of events and mean follow-up time of the patients in this study are representative for the patients in the trial (see Table 1).

Follow up took place every 3 months during the first three years and every 6 months between three and five years after initial diagnosis and surgery. Standard protocols were followed, with routine blood counts and chemistry studies (including CEA levels) at each visit and liver ultrasound, chest x-ray and colonoscopy annually, to evaluate both recurrence of disease and disease-related death. After the initial five year fol-

Table 1
Clinicopathological variables of study population

Age	
<69 years	$n = 64$ (48%)
>69 years	$n = 69$ (52%)
Sex	
Male	$n = 55$ (41%)
Female	$n = 78$ (59%)
Tumor size	
<46 mm	$n = 57$ (43%)
>46 mm	$n = 76$ (57%)
Tumor location	
Proximal	$n = 52$ (39%)
Distal	$n = 81$ (61%)
Tumor	
Colon	$n = 91$ (68%)
Rectum	$n = 42$ (32%)
Differentiation	
Well	$n = 14$ (11%)
Moderate/poor	$n = 119$ (89%)
TNM	
1	$n = 0$ (0%)
2	$n = 83$ (63%)
3	$n = 35$ (26%)
4	$n = 15$ (11%)
Tumor type	
Exophytic	$n = 15$ (11%)
Sessile	$n = 19$ (14%)
Ulcerative	$n = 99$ (74%)

low up period, during the next years only the time and cause of death were registered. Follow-up was complete for all patients. In the present study, failure was defined as death due to recurrent disease, excluding postoperative mortality within 30 days and non-disease related death.

After surgery, tumor tissues and lymph nodes were fixed in buffered formalin, sectioned, and embedded in paraffin. Experienced pathologists documented the histopathological characteristics of the tumors, including tumor stage, differentiation grade, size, (lymph-) angioinvasion, perineural invasion and lymph node involvement. Tumor stage was defined according to the TNM staging system.

2.2. Immunohistochemistry

Formalin fixed, paraffin-embedded tissues consisting of both normal mucosa and tumor were used for immunohistochemical staining. Serial sections (4 μm) from each patient were stained for HIF-1α, HIF-2α, CA9 and GLUT1 after deparaffination. Endogenous peroxidase activity was blocked by pre-incubating in 0.6% hydrogen peroxide for 20 min.

The following staining protocols for the different antibodies were used:

HIF-1α staining: Antigen retrieval was performed by microwave 750 W for 2 min. followed by 20 min at 90 W in 1 mM TE buffer pH 9.0, followed by 30 min cooling in buffer. Slides were blocked in 25% normal serum for 10 min. Sections were incubated overnight (4°C) with primary antibody HIF-1α (1 : 120) (anti-HIF-1α monoclonal: 610958 BD, USA).

HIF-2α staining: Antigen retrieval was performed by microwave treatment (750 W for 20 min in 1 mM TE buffer pH 8.0), followed by 30 min cooling in buffer. Slides were blocked in 25% normal serum for 10 min. Sections were incubated with primary antibody HIF-2α (1 : 500) for 100 min (anti-HIF2 alpha monoclonal: ab8365 AbCam, UK).

CA9 staining: Slides were blocked in 25% normal serum for 10 min, then incubated for 45 min with primary CA9 antibody MoAb M75 (1 : 50) (anti-human CA9, kindly supplied by Dr. S. Pastorekova) at room temperature [27].

GLUT1 staining: Microwave treatment (750 W for 15 min in Citrate buffer pH 6.0), followed by 30 min cooling in buffer. Slides were blocked in 25% normal serum for 10 min. Sections were incubated (1 : 100) with primary antibody GLUT1 (rabbit polyclonal anti-human GLUT1, A3536, DakoCytomation, Denmark) for 2 hr followed by incubation with Swine Anti-Rabbit Immunoglobuline/Biotinylated (E0431, DakoCytomation, Denmark) (1 : 250) for 30 min at room temperature, and StreptABComplex/HRP (K0377, DakoCytomation, Denmark) (1 : 200) for 30 min at room temperature.

As a negative control for all antibodies, TBS buffer instead of primary antibody was used. Visualization was performed using Dako Envision, Peroxidase, mouse System (K4001, DAKO, Denmark). For GLUT1, Diaminobenzidine was applied for 10 min. The slides were counterstained with hematoxylin and mounted.

2.3. Evaluation of staining

Immunohistochemical staining was evaluated on the basis of (1) localisation in tumor epithelial or stromal cells and (2) subcellular localisation. For the tumor stroma, only the tumor-associated stromal cells were

taken into account, not the tumor infiltrating inflammatory cells or the lamina propia of the normal mucosa.

If nuclear staining was present in >5% of the tumor epithelial cells or tumor-associated stromal cells, the sample was considered positive for HIF-1α and HIF-2α. If membranous staining occurred in >5% of the tumor epithelial cells or stromal cells, samples were considered positive for CA9 and GLUT1. Staining results were also checked by determining the extent and intensity of staining. Because this did not change the categorization, we used the 5% cut-off value for further analysis [52,53].

2.4. Data analysis

For the data analyses we used three groups of expression patterns for HIF-1α, HIF-2α, CA9 and GLUT1 expression, namely: 1 Stromal expression (=defined as purely stromal or a combination of stromal and epithelial expression), 2 Epithelial expression (=defined as epithelial only or a combination of stromal and epithelial expression), and 3 Negative.

The correlations between HIF-1α, HIF-2α, CA9, GLUT1 and various clinicopathological parameters were determined by the Pearson Chi-Square and Fisher's exact test as appropriate. To evaluate the relationship between HIF-1α, HIF-2α, CA9, GLUT1 and patient survival, Kaplan–Meier survival curves were calculated. Statistical differences between groups were determined by using the Log-rank test. The endpoint for analyses was overall survival starting from the day of surgery. Independent variables predicting survival were evaluated by the multiple stepwise regression analyses using Cox Regression. The Cox-regression model included the variables: sex, age, tumor size, tumor location, TNM stage, differentiation grade, HIF-1α, HIF-2α, CA9 and GLUT1 (epithelial and stromal expression separately). All p-values are two sided and p-values <0.05 were considered statistically significant. SPSS 10.0 software was used for data analyses.

To check for differences between colon and rectum tumors, all survival analyses were performed separately for these two categories.

3. Results

We evaluated the staining characteristics of the potential hypoxia markers HIF-1α, HIF-2α, CA9 and GLUT1 in 133 human colorectal adenocarcinomas. On analyzing these four hypoxia associated proteins, it became clear that many tumors displayed positive staining in the tumor epithelial cells as well as positive staining in tumor- associated stromal cells. We therefore separately evaluated the staining patterns of HIF-1α, HIF-2α, CA9 and GLUT1 in tumor epithelial cells and tumor-associated stromal cells. All tumors showed immunohistochemical expression of at least one of the analysed hypoxia markers. Overall HIF-1α was positive in 43% of cases, whereas 83% of the tumors were positive for HIF-2α, 89% of the tumors showed CA9 protein expression and GLUT1 expression was positive in 85% of the cases.

3.1. Expression of hypoxia proteins in tumor epithelial cells

In order to evaluate the association of hypoxia and the clinical outcome of colorectal cancer, quantification of the expression of hypoxia markers within the tumor epithelial cell compartment was performed. Overall, 29% of the patients showed HIF-1α protein expression within the tumor epithelial cells (Fig. 2A). In these cells, HIF-1α expression was observed exclusively in the nuclei, often in areas surrounding tumor necrosis (Fig. 1A), a pattern that is typical of hypoxia induced expression. Surprisingly, none of the 133 tumors showed any staining for HIF-2α in tumor epithelial cells. This lack of expression may be associated with the intestinal cell lineage as expression was also not detected in any adjacent normal intestinal epithelial cells. Epithelial CA9 expression was observed in 78% of all tumors (Fig. 2C). Expression in the tumor cells was restricted to a membranous type of expression (Fig. 1E) but was typically not perinecrotic. GLUT1 was also present in tumor epithelial cells in 83% of the cases (Fig. 2D), where it showed a clear and strong membranous staining, typically surrounding areas of tumor necrosis (Fig. 1G).

3.2. Expression of hypoxia proteins in the tumor-associated stromal cells

Stromal HIF-1α expression was observed in 32% of tumors, of which 18% showed combined staining of tumor epithelium and stroma and 14% showed exclusive staining in the stromal cells (Fig. 2A). Staining was observed surrounding areas of tumor necrosis (Fig. 1B) and was confined to the nuclei.

In contrast with the lack of HIF-2α expression in tumor epithelial cells, HIF-2α staining was frequently seen within the tumor stroma, both in tumor-associated

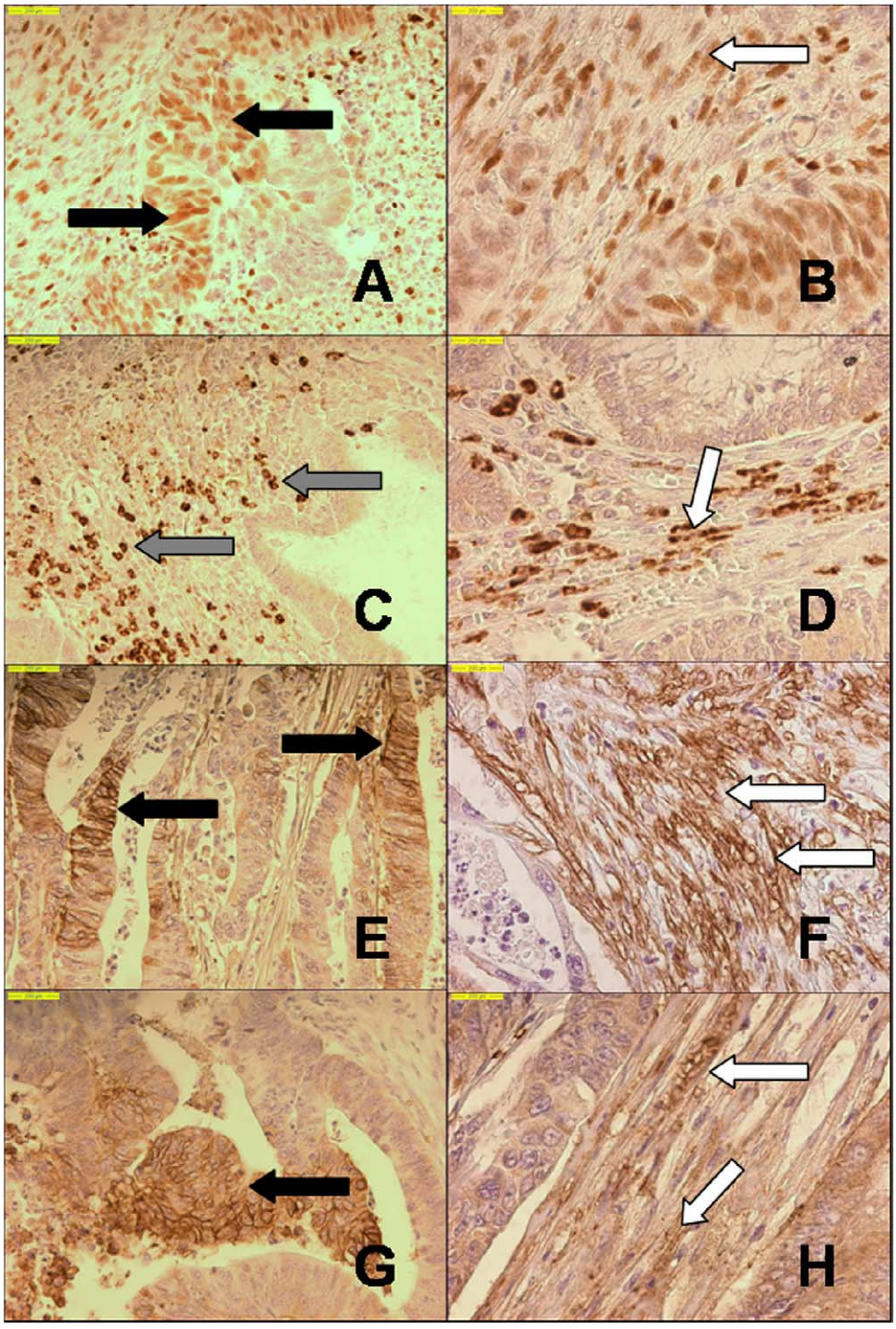

Fig. 1. Expression patterns of hypoxia regulated proteins in colorectal tumors. (A–H) black arrow: epithelial tumor cell expression, white arrow: tumor-associated stromal cell expression, gray arrow: inflammatory cell expression. (A) HIF-1α expression in the nuclei of epithelial tumor cells. (B) HIF-1α expression in the nuclei of tumor-associated stromal cells. (C) HIF-2α expression in inflammatory cells located within the tumor-associated stroma. (D) HIF-2α expression in tumor-associated stromal cells. (E) CA9 expression in epithelial tumor cells. (F) CA9 expression in tumor-associated stromal cells. (G) Expression of GLUT1 in tumor epithelial cells. (H) GLUT1 expression in tumor-associated stromal cells. (A, B, D, F) and (H) original magnifications $\times$ 40; (C, E) and (G) original magnifications $\times$ 20.

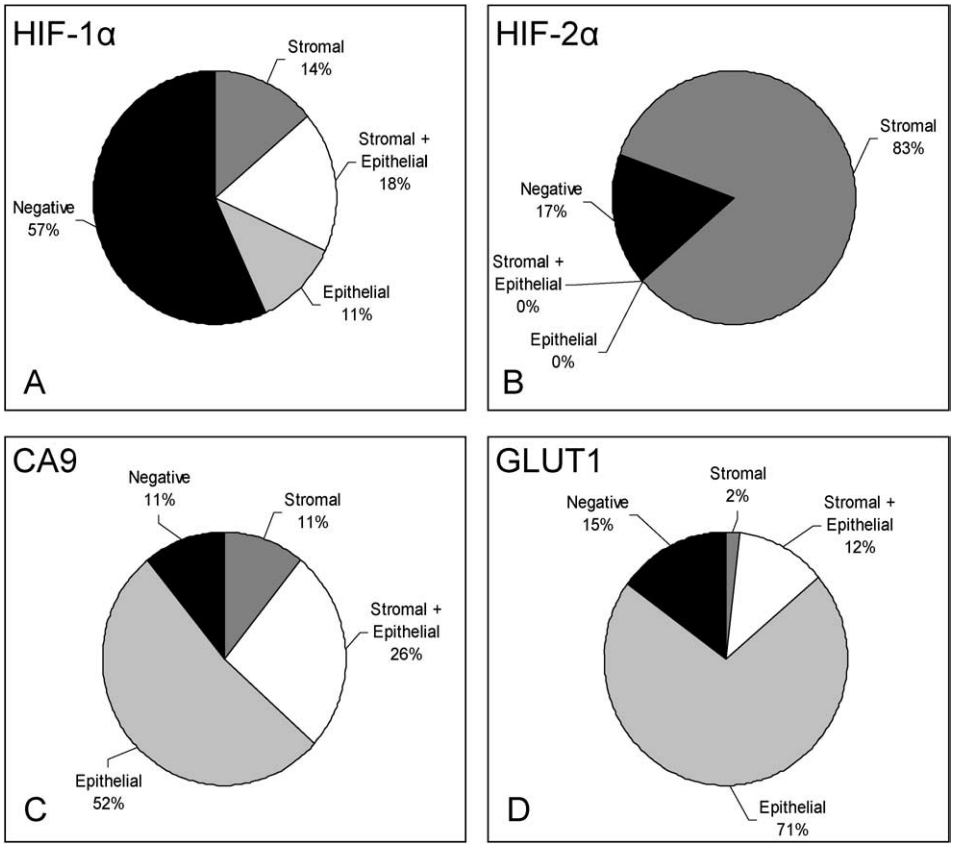

Fig. 2. Quantification of expression patterns of hypoxia associated proteins in 133 colorectal cancers. (A) Quantification of HIF-1α expression patterns. (B) Quantification of HIF-2α expression patterns. (C) Quantification of CA9 expression patterns. (D) Quantification of GLUT1 expression patterns.

stromal cells and inflammatory cells (Fig. 1C and 1D). Expression was predominantly nuclear, only occasionally accompanied by additional cytoplasmic staining. In further analysis, HIF-2α staining of inflammatory cells in the tumor stroma was disregarded, because HIF-2α expression in inflammatory cells did not influence the effect of HIF-2α expression in tumor-associated stromal cells on patient outcome (Fig. 3A). Overall, 83% of the tumors showed positive staining for HIF-2α in stromal cells, typically surrounding areas of necrosis (Fig. 2B). In the remaining 17% of the tumors where no expression of HIF-2α was observed, some staining was found in inflammatory cells in the lamina propria of the normal mucosa. Stromal membranous CA9 expression (Fig. 1F) was observed in 37% of all tumors. Interestingly, of the tumors that lacked CA9 expression in tumor epithelial cells, half were positive for CA9 in the stroma (Fig. 2C). GLUT1 expression was only occasionally detected in stromal cells and restricted to a membranous type of expression (Fig. 1H). Stromal cell expression of GLUT1 was present in 14% of the cases (Fig. 2D).

No staining of stromal cells within the lamina propria of surrounding normal mucosa was found for any of these hypoxia inducible proteins.

3.3. Correlations between HIF-1α, HIF-2α, CA9, GLUT1 and clinicopathological variables

To investigate the functional relevance of HIF-1α and HIF-2α expression, we tested the correlations of the expression of these transcription factors with the expression of their downstream gene products CA9 and GLUT-1. HIF-1α expression showed no correlation with HIF-2α, GLUT1 or CA9 when analyzed by either epithelial or stromal expression patterns. This suggests that HIF-1α expression is not primarily responsible for induction of CA9 and GLUT1 expression and that these genes are regulated by factors other than HIF-1α. In contrast, a significant correlation was found

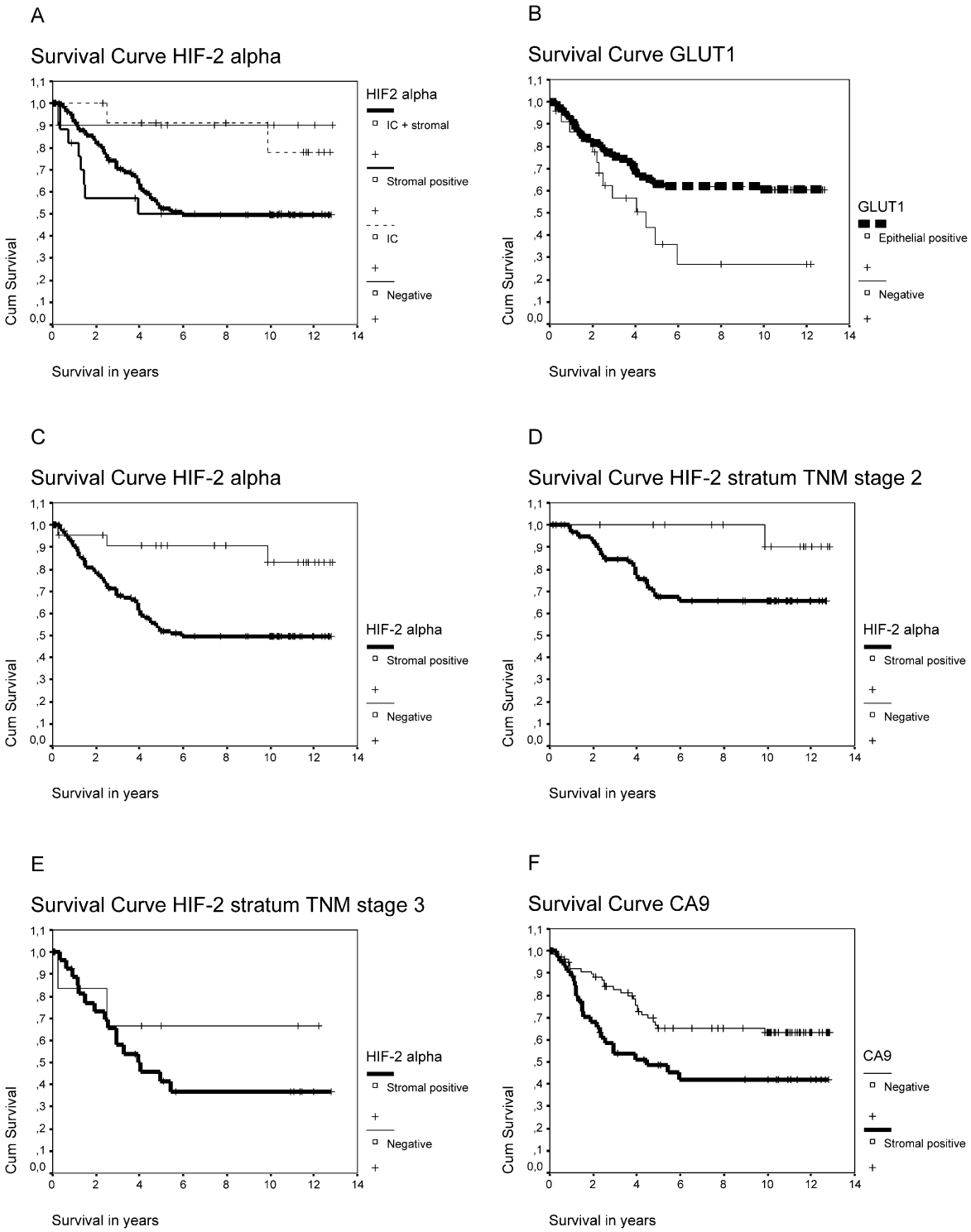

Fig. 3. (A) Survival curve HIF-2α expression including inflammatory cells (IC) $p = 0.06$ ($n = 133$). (B) Survival curve GLUT1 epithelial expression $p = 0.02$ ($n = 133$). (C) Survival curve HIF-2α (without inflammatory cells) $p = 0.008$ ($n = 133$). (D) Survival curve HIF-2α stratified for TNM stage 2 $p < 0.05$ ($n = 83$). (E) Survival curve HIF-2α stratified for TNM stage 3 $p = 0.4$ ($n = 35$). (F) Survival curve CA9 $p = 0.01$ ($n = 133$).

Table 2
Correlations between stromal expression of HIF-1α, HIF-2α, CA9, GLUT1

	HIF-2α		P-value		HIF-1α		P-value
	Stroma	Negative			Stroma	Negative	
HIF-1α				GLUT1			
Stroma	37/43 (86%)	6/43 (14%)		Stroma	5/19 (26%)	14/19 (74%)	
Negative	74/90 (82%)	16/90 (18%)	0.6	Negative	38/114 (33%)	76/114 (67%)	0.5
	HIF-2α				HIF-1α		
	Stroma	Negative			Stroma	Negative	
GLUT1				CA9			
Stroma	17/19 (89%)	2/19 (11%)		Stroma	18/48 (38%)	30/48 (62%)	
Negative	94/114 (82%)	20/114 (18%)	0.7	Negative	25/85 (29%)	60/85 (71%)	0.3
	HIF-2α				GLUT1		
	Stroma	Negative			Stroma	Negative	
CA9				CA9			
Stroma	45/48 (94%)	3/48 (6%)		Stroma	13/48 (27%)	35/48 (73%)	
Negative	66/85 (78%)	19/85 (22%)	0.02	Negative	6/85 (7%)	79/85 (93%)	0.002

Table 3
Cox proportional hazard model

	Event: CRC mortality		P-value
	HR	(95%CI)	
HIF-2α Positive*	4.61	(1.40–15.50)	0.01
GLUT1 Epithelial†	0.44	(0.22–0.86)	0.02
TNM 3‡	2.74	(1.42–5.29)	0.003
4‡	16.09	(7.00–39.95)	0.000
Differentiation moderately/poorly**	3.12	(0.93–10.48)	0.07
Age >69 years††	2.09	(1.16–3.74)	0.01

Abbreviations: CRC, colorectal cancer; HR, hazard ratio = relative risk.
* Reference group = negative HIF-2α cases;
† Reference group = negative GLUT1 cases;
‡ Reference group = TNM stage 2;
** Reference group = well differentiated;
†† Reference group = Age <69 years.

between HIF-2α and CA9 ($p = 0.02$, Table 2) within the tumor stroma. Furthermore, GLUT1 and CA9 expression within the tumor stroma were significantly correlated ($p = 0.002$, Table 2). These data suggest that HIF-2α upregulation in tumor stroma may be responsible for upregulation of CA9.

No significant correlation was found between stromal or epithelial expression of HIF-1α, HIF-2α or GLUT1 and any of the following clinicopathological characteristics: age, sex, tumor size, location in the colon (proximal, distal), differentiation (well, moderately and poorly), TNM stage (2, 3 and 4). Only CA9 epithelial expression showed a significant correlation with TNM stage, with reduced CA9 expression in the more advanced tumors. These results suggest that the biological responses to hypoxia in colorectal cancer are largely independent of these clinicopathological variables.

3.4. Relationship between hypoxia markers and patient survival

An important objective of our study was to analyze the relationship between hypoxia regulated proteins and patient survival. Previous studies in other tumor types have indicated that expression of the hypoxia induced proteins investigated here correlate with poor overall survival. This is presumed to be due to an adverse effect of hypoxia on the tumor cells themselves. Since we also observed frequent staining within the tumor stroma, we further investigated the potential relationship between stromal hypoxia and overall survival.

HIF1-α expression in tumor-associated stromal cells and epithelial tumor cells showed no significant correlation with patient survival. Similarly, CA9 epithelial expression had no impact on patient survival. GLUT1 epithelial negative cases showed a significantly poorer survival compared to GLUT1 positive epithelial cases ($p = 0.02$, Fig. 3B).

In contrast to the lack of association between markers of hypoxia in the tumor cells and clinical response, we found strong evidence that stromal hypoxia may be associated with poor outcome. Patients with stromal HIF-2α expression showed a statistically significant poorer survival compared to HIF-2α negative pa-

tients ($p = 0.008$, Fig. 3C). This trend was maintained in TNM stage 2 and stage 3 (Fig. 3D and 3E). The same trend was observed for stromal CA9 expression ($p = 0.01$, Fig. 3F). Patients with stromal GLUT1 expression also showed a tendency for poorer overall survival compared to patients negative for stromal GLUT1 expression, although this did not reach statistical significance. There was no difference in survival between cases with exclusive stromal expression and cases with combined stromal and epithelial expression for any of the four applied markers (data not shown).

3.5. Multivariate analysis

An overall model to test the most relevant prognostic factors for patient outcome was performed using a multistep Cox-regression model. After stepwise multivariate analysis, stromal HIF-2α positivity (Hazard Ratio (HR) 4.61), tumor epithelial GLUT1 positivity (HR 0.44), TNM stage 3 (HR 2.74) and stage 4 (HR 16.09) and age >69 years (HR 2.09) were statistically significant independent prognostic markers. Poorly and moderately differentiated tumors had a HR of 3.12 compared to well differentiated tumors, although this did not reach statistical significance ($p = 0.07$, Table 3). Multivariate analysis restricted to stage 2 and 3 cases, revealed stromal HIF-2α positivity as the strongest independent prognostic marker, with a HR 4.87 ($p = 0.01$), followed by TNM stage 3 (HR 2.29, $p = 0.01$), stromal CA9 expression (HR 1.95, $p < 0.05$), age >69 years (HR 1.94, $p < 0.05$) and epithelial GLUT1 expression (HR 0.26, $p = 0.001$).

There were no differences in univariate or multivariate survival analysis between colon and rectum tumors.

4. Discussion

This is the first comprehensive study analyzing the expression of endogenous hypoxia markers HIF-1α, HIF-2α, CA9 and GLUT1 in colorectal cancer. All 133 tumors in this study showed expression of at least one of the hypoxia-regulated proteins, suggesting that all tumors may exhibit at least some degree of hypoxia. In contrast to what was initially expected, our data suggest that HIF-1α immunostaining does not appear to be a reliable indicator of hypoxia in colorectal cancer and furthermore does not correlate with patient outcome. Although HIF-1α did display staining characteristics consistent with hypoxia (peri-necrotic staining of cell nuclei), its expression in either stromal or tumor cells did not correlate with the expression of its downstream genes CA9 and GLUT1. Consistent with our results, HIF-1α staining was previously reported to be positive in 44.8% of patients with colorectal cancer, but had no correlation with HIF-2α expression and no prognostic value [18,53]. Several explanations for the lack of correlation between HIF-1α and CA9 or GLUT1 in the tumor cells are possible. HIF-1α is susceptible to degradation after prolonged hypoxia due to upregulation of the Prolyl Hydroxylase Domain proteins [45]. *In vitro* studies suggest that nutrient deprivation may also decrease HIF-1α expression. Furthermore, the half-life of CA9 is sufficiently long ($\gg$24 hrs) that once formed it remains present for many days in the absence of continued HIF-1α expression [41].

As for HIF-1α, our data indicate that tumor epithelial cell expression of CA9, although frequently occurring, is not related to poorer survival either. In fact, we found that epithelial CA9 expression decreased with advancing TNM stage, with 78% of CA9 epithelial positive cases in TNM stage 2 compared to 53% of CA9 epithelial positive cases in TNM stage 4 ($p = 0.02$). It is well known that in colorectal cancer TNM Stage 2 tumors have a better overall survival than Stage 4 tumors [34]. The lack of correlation between HIF-1α and CA9 expression in tumor epithelial cells suggests that the CA9 positivity may also be unrelated to hypoxia. This is supported by the finding that in cases with only tumor epithelial CA9 staining (52%), the expression was usually not perinecrotic. On the other hand, tumor epithelial cell expression of GLUT1 in our study was typically found in perinecrotic zones, and therefore may indeed be indicative of tumor hypoxia. However, GLUT1 epithelial positivity turned out to be a marker for good prognosis compared to epithelial negative GLUT1 cases. This is somewhat surprising, since GLUT1 expression has been previously reported to be associated with a high incidence of lymph-node metastases [54]. Although further analyses is clearly necessary to determine the role for epithelial GLUT1 expression within different TNM stages of colorectal cancer, our data do not support the premise that epithelial GLUT1 expression is a poor prognostic factor. Taken together, the immunostaining patterns of these four markers within the tumor epithelial cell compartment do not consistently reflect tumor hypoxia, and tumor epithelial cell hypoxia does not seem to contribute to a poorer patient prognosis in colorectal cancer.

In direct contrast to these results, our study did indicate that the tumor stroma may be frequently hypoxic. We found that 83% of all tumors demonstrated

staining of HIF-2α within the tumor-associated stromal cells. HIF-2α appears to be biologically active in stromal cells because HIF-2α correlated significantly with stromal CA9 expression ($p = 0.02$) in these tumors. Most importantly, tumors showing stromal HIF-2α expression also had a worse overall survival when compared with HIF-2α negative cases. After stratification for other factors in a multivariate analysis (sex, age, TNM stage, differentiation grade, HIF-1α, GLUT1 and CA9), stromal HIF-2α expression was a strong (HR 4.61, $p = 0.01$) independent unfavorable prognostic factor. The negative impact of HIF-2α expression on patient survival in colorectal cancer was recently also described by others. However, in this study of curatively resected colorectal carcinoma patients, the authors did not study the contribution of the epithelial and stromal tumor compartments separately [53].

As might be expected given the correlation between HIF-2α and CA9 in tumor-associated stromal cells, our data also indicated that stromal CA9 expression was associated with a worse overall survival. Furthermore, stromal CA9 expression proved to be an independent unfavorable prognostic factor in cases having stage 2 or 3 colorectal cancer. This finding may not be unique to colorectal cancer. In invasive breast cancer, stromal CA9 expression levels have been associated with a higher relapse rate and a worse overall survival [5].

Together our data suggest the intriguing possibility that hypoxia within the tumor associated stroma may contribute to a poor outcome in patients with colorectal cancer who are treated by surgery alone. Although it is commonly accepted that the genetic and epigenetic changes in tumor cells are responsible for the differences in patient response, there are also data to suggest that non-tumor cells may play an important role as well. The induction of CA9 by hypoxia could help in maintaining normal intracellular pH, preventing apoptosis, and thus provide a survival advantage to the stromal cells, and indirectly be of benefit to the adjacent tumor cells. A reduction in extracellular pH might also provide an advantage to tumor cells as it helps in breakdown of extracellular matrix, migration and invasion of tumor cells, as well as induction and expression of growth factors. This explanation is consistent with a report from Sivridis et al., who showed that cancer-stromal cell interactions may be favoured by the altered microenvironmental conditions of hypoxia and acidity [40]. The strong association of markers of intratumoral hypoxia and pH within the tumor stroma indicates an interesting link between cancer cell metabolism and the induction of a supportive stroma that favors cancer cell invasion and migration [11,15,16,21,24,46,48].

Recent data have provided further compelling evidence that tumor-associated stromal cells are important in cancer growth and metastasis [29]. In colorectal tumors not only epithelial but also stromal elements demonstrate genetic instability and that such stromal alterations might influence the genesis of sporadic colorectal carcinomas [12]. Weinberg et al. showed that co-injection of carcinoma associated stromal cells (myofibroblasts) promoted the growth of invasive breast carcinomas. This effect was mediated in part through increased secretion of stromal cell-derived factor-1 (SDF-1). This is particularly interesting since SDF-1 and its receptor have been shown to be upregulated by hypoxia [4]. The Weinberg study also indicated that the tumor associated stromal cells could promote angiogenesis by recruiting endothelial progenitor cells (EPC's) into carcinomas [26]. Stromal cells within the tumor microenvironment have been shown to acquire new properties, including the capacity to promote phenotypic and genetic progression in adjacent epithelial cells [13,24]. For example, epigenetic changes have been shown to occur in stromal cells of breast cancer during tumorigenesis, in a tumor stage- and cell type-specific manner [10]. Alterations of stromal cells may therefore be either a consequence or contributor to an abnormal tumor micro-environment. Our study indicates that hypoxia may be a potential mechanism influencing the phenotype of tumor stroma, ultimately leading to a worse patient prognosis.

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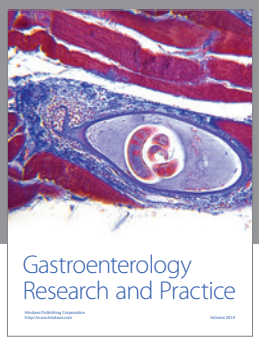

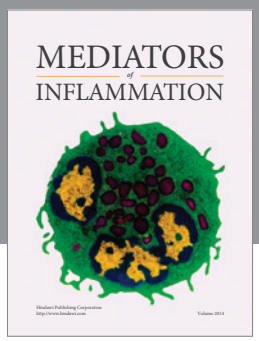

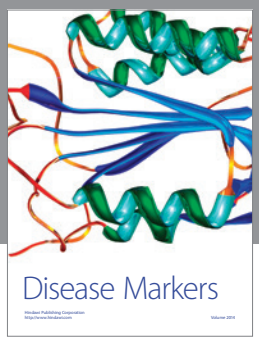

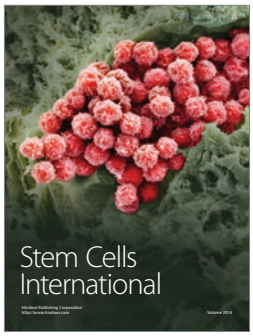

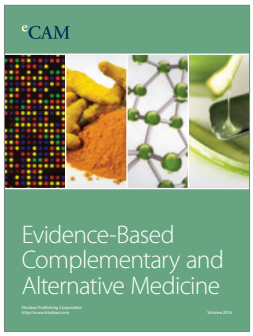

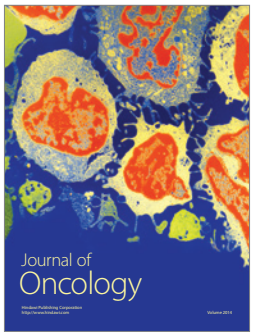

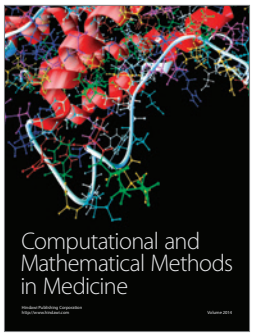

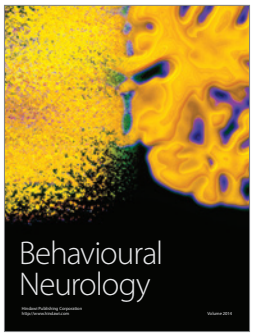

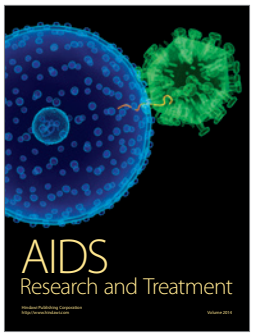

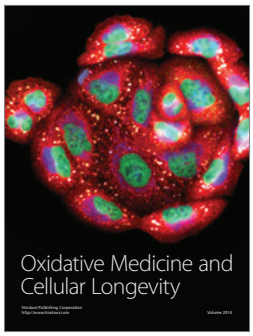

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