Abstract. The aim of the present study was to investigate hypoxia-inducible factor 1α (HIF-1α) and glucose transporter-1 (GLUT-1) expressions as predictors of response and survival after chemoradiotherapy in pretreatment biopsy specimens from patients with rectal cancer. Materials and Methods: The immunohistological expressions of HIF-1α and GLUT-1 were investigated in pretreatment biopsies from 86 patients with rectal cancer receiving long course preoperative chemoradiotherapy. The immunohistological stainings were scored semi-quantitatively (percentage of stained cells and staining intensity), and an immunoreactive score was calculated. The response to the chemoradiotherapy was assessed by the Mandard Tumour Regression Grade system (TRG). Results: No association was found between HIF-1α or GLUT-1 and clinicopathological variables. HIF-1α and GLUT-1 expression had no predictive impact regarding response to chemoradiotherapy measured by TRG and was not associated with overall survival. Conclusion: The present study did not suggest any predictive or prognostic value of pretreatment HIF-1α or GLUT-1 expression in patients with rectal cancer treated with preoperative chemoradiotherapy.

Tumour hypoxia is a well described phenomenon in solid tumours. Prolonged hypoxia leads to reduced proliferation, increased apoptosis and ultimately necrosis. Cancer cells, however, undergo adaptive changes, which make them able to survive and grow in a hypoxic environment. Hypoxia leads to genetic instability and impaired DNA repair, which leads to genomic changes and clonal selection towards a more malignant phenotype (1,2).

A central protein in cellular adaptation to hypoxia is hypoxia-inducible factor 1 (HIF-1), which activates the transcription of numerous genes associated with e.g. angiogenesis, metabolism, cell proliferation, and apoptosis (3). HIF-1 is a heterodimeric transcription factor consisting of an HIF-1α and HIF-1β subunit (4), where HIF-1α is regulated by the presence of oxygen. When oxygen is present, HIF-1α is degraded, whereas under hypoxia, HIF-1α is allowed to bind HIF-1β and become an active transcription inducer (5).

Under hypoxic conditions, tumour cells switch from oxygen-dependent glucose metabolism to oxygen-independent glycolysis. The transcription of several genes necessary for glycolysis is induced by HIF-1α, and one such target gene is glucose transporter-1 (GLUT-1) (6,7). GLUT-1 facilitates transport of glucose across the cellular membrane (8), providing the cell with substrate for glycolysis. GLUT-1 is present in few normal tissues, but like HIF-1α it is widely overexpressed in malignant tumours, including colorectal cancer (9-11). HIF-1α and GLUT-1 have been associated with aggressive tumour growth and poor prognosis in several types of malignant tumours including colorectal cancer (12-20). The presence of hypoxia in rectal tumours has been demonstrated (21,22), but the importance of hypoxia inducible factors in rectal cancer is rather poorly described.

Resistance to chemotherapy and radiotherapy is a well-known problem associated with hypoxia and studies of cancer cell lines link radiotherapy resistance to the HIF-pathway (23,24). Previous studies have shown a possible negative prognostic value of HIF-1α in rectal cancer with or without postoperative radiotherapy (15,16,20), but only one study has addressed the question of the role of HIF-1α as a predictive marker in preoperative radiotherapy with concomitant chemotherapy (25). The importance of GLUT-1 in relation to preoperative chemoradiotherapy of patients...
with rectal cancer has also been investigated (26). Taken together the two latter studies suggest an adverse association of HIF-1α and GLUT-1 with the outcome of chemoradiotherapy, as also indicated in studies of cervical and oropharyngeal cancer (27-30).

The aim of the present study was to investigate HIF-1α and GLUT-1 expression in pretreatment biopsies from rectal tumours as predictors of chemoradiotherapy response and their possible prognostic impact.

Materials and Methods

The study included 86 patients with locally advanced rectal cancer treated with long-course preoperative radiotherapy and concomitant chemotherapy at the Department of Oncology, Vejle Hospital, Denmark in the period 1998-2009, and from whom pretreatment biopsies were available.

The patients included had histologically verified adenocarcinoma of the rectum located less than 10 cm from the anal verge. They all had T3 tumours with a distance less than 5 mm to the mesorectal fascia or T4 tumours. Staging was supplemented by magnetic resonance imaging (MRI) and transrectal ultrasound, where lymph nodes >5 mm were categorized as lymph node metastasis. Computerized tomography of the chest and abdomen or chest X-ray and ultrasound of abdomen were performed to exclude distant metastasis.

Different treatment schemes were used: external radiotherapy 60 Gy/30 fractions or 50.4 Gy/28 fractions, combined with or without endorectal brachytherapy, and concomitant chemotherapy with UFT (Uracil/Tegafur, molar ratio 4:1), 300 mg/m² and isovorin 22.5 mg on treatment days.

The patients were scheduled for operation 8 weeks after finishing radiotherapy, and response to the treatment was evaluated according to the Mandard Tumour Regression Grade (TRG) (31). The tumours were evaluated by multiple step sections of the residual lesion and classified TRG 1 if no residual carcinoma cells were present, TRG 2 when rare residual carcinoma cells were seen, TRG 3 when fibrosis was outgrowing the residual carcinoma cells, TRG 4 when the residual carcinoma cells outgrew fibrosis, and TRG 5 if no regressive changes were seen.

Immunohistochemistry. The immunohistological analyses were performed using 4-μm-thick tissue sections from formalin-fixed paraffin-embedded endoscopic biopsies from the rectal tumours at

Table I. HIF-1α and GLUT-1 expression as related to patient and tumour characteristics.

<table>
<thead>
<tr>
<th></th>
<th>HIF-1α</th>
<th></th>
<th>GLUT-1</th>
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<tbody>
<tr>
<td></td>
<td>Total, n (%)</td>
<td>Low, n (%)</td>
<td>High, n (%)</td>
<td>p-value</td>
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<tr>
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<td>49 (57)</td>
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<td>37 (43)</td>
<td>19 (41)</td>
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<tr>
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<td>13 (15)</td>
<td>9 (20)</td>
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<td>10 (22)</td>
<td>7 (18)</td>
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<td>69 (80)</td>
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<tr>
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<td>Total</td>
<td>86</td>
<td>46</td>
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Sum of the percentages does not always equal 100% due to rounding of data. The total number of patients with sections stained for HIF-1α is 85 and for GLUT-1 it is 83 due to exclusion of sections not evaluable.
the time of diagnosis. Heat-induced epitope retrieval was carried out in 10 mM Tris/EGTA (ethylene glycol tetraacetic acid) buffer using a microwave oven. HIF-1α staining was carried out using an EnVision (Dako; Glostrup, Denmark) protocol with incubation overnight with primary monoclonal antibody (1:75, 610958 Clone 54 BD Transduction Laboratories; Franklin Lakes, NJ, USA) and GLUT-1 staining using Super Sensitive (BioGenex Laboratories Inc; Fremont, CA, USA.) protocol with incubation for 30 minutes with polyclonal anti-GLUT-1 (1:250, DP128 Acris; Herford, Germany) according to the manufacturer’s recommendations. The sections were counterstained with Mayer’s haematoxylin.

HIF-1α was primarily visualized as nuclear staining in tumour and stromal cells, whereas GLUT-1 was seen only in the tumour cells as cytoplasmic and membranous staining. HIF-1α expression was scored by semi-quantitatively assessing the percentage of cells (P) stained and by evaluating the staining intensity (I). Intensity was scored as: 1: weak or no staining and 2: strong. The percentage of cells was rated as follows: 0: no cells staining, 1: <10%, 2: 10-50% and 3: >50 % of cells having nuclear staining at ×100 magnification and higher when intensity was low. An immunoreactive score (IRS) was calculated by adding I to P, resulting in values of 1-5.

GLUT-1 expression was determined by scoring the overall intensity (I) (1: weak, 2: strong) and semi-quantitatively assessing the percentage of cells with membranous staining (P) (1: <10%, 2: 10-50%, 3: >50 %) at ×100 magnification. As for HIF-1α, an IRS was calculated for GLUT-1 by adding I to P, resulting in values of 2-5.

The staining was scored by one observer, and intraobserver reproducibility was evaluated by scoring 50 sections twice. The same 50 sections were reviewed by two independent observers to evaluate the interobserver reproducibility. Consensus was obtained, when observers disagreed, for use in further statistical analysis.

Statistics. Kappa statistics were used to test inter- and intraobserver reproducibility, and a kappa value >0.6 was considered acceptable (32). Fisher’s exact test and Mann-Whitney U-test were used for analysis of associations between the immunohistological stains and clinicopathological variables, while the correlation between immunohistological staining and response to radiotherapy was analyzed by Fisher’s exact test and Kendall’s Tau-B.

Univariate survival analyses were performed by log rank tests, illustrated by Kaplan Meier curves, and overall survival (OS) was defined as the time from operation until death from any cause. All tests were two-sided and the result was considered significant if p<0.05. NCSS 2007 (NCSS, Kaysville, UT, USA), and STATA 10 (StataCorp Lp, College Station, TX, USA) statistical software were used for all analyses.

Results

Patient characteristics. Patient and treatment characteristics are shown in Table I. It appears that 73 (85%) of the 86 patients had T3 and 13 (15%) had T4 tumours, while 69 (80%) had regional lymph node metastases by imaging before the start of treatment. Fifty-seven percent were males while 43% were females and the median age was 66.7 years. The rate of patients achieving a good pathological response (TRG1 and 2) was 31% (27/86).

Immunohistochemistry. In one of the histological sections stained for HIF-1α and three of the sections stained for GLUT-1, no invasive tumour was present, and these were accordingly withdrawn from the study.
The immunohistological staining of HIF-1α was seen primarily in the nucleus of malignant tumour cells, but also in those of adenomas and normal epithelial cells as well as in stromal fibroblasts and inflammatory cells (Figure 1A). Often a heterogeneous staining pattern was visualized and no general pattern in extent of tumour and stromal staining was documented. In most cases, an increasing staining gradient towards necrotic areas was observed.

GLUT-1 staining was seen in malignant epithelial tumour cells as a diffuse or granular cytoplasmic and membranous staining (Figure 1B). In many cases, we found an increasing gradient of the staining towards the lumen of the tubular glandular structure. Erythrocytes stained strongly and served as an internal control.

The inter- and intraobserver reproducibility of both GLUT-1 and HIF-1α was acceptable, with kappa values >0.6 as shown in Table II.

For statistical analysis of the immunohistological staining of HIF-1α and GLUT-1, the IRS was used. The median of HIF-1α IRS (=3) was used as cut-off point, creating two equally sized groups: HIF-1α-high and HIF-1α-low. The median of GLUT-1 IRS (=4) divided the cohort unequally (73.5%/26.5%), while IRS=3 gave a more equal division and was thus used as the cut-off point between GLUT-1-high (47%) and GLUT-1-low (53%). We found no association between HIF-1α or GLUT-1 and the clinicopathological variables (Table I), and no correlation between the IRS of HIF-1α and GLUT-1.

**Response and survival.** A good response (TRG1 and TRG2) was achieved in 30% of the patients with low HIF-1α expression and 33% of patients with high HIF-1α expression, while 36% of the GLUT-1-low and 25% of the GLUT-1-high group obtained good response. No significant association was found between good response and high versus low expression of HIF-1α (p=0.8) or GLUT-1 (p=0.3) (Figure 2).

The dichotomization of IRS and TRG simplifies the grading, and information may be missed. No correlation, however, was found between the original values of TRG and IRS of HIF-1α (p=1.0, Kendall Tau-B=-0.03) or GLUT-1 (p=0.4, Kendall Tau-B=0.09).

For the survival analysis, 4 patients were excluded because of previous malignancies. The median follow-up of patients who were still alive at the time of analysis was 49 months (9.9-105 months). Univariate OS analyses showed no prognostic impact of HIF-1α (p=0.9) or GLUT-1 (p=0.8) (Figure 3).

The patients in the study had different treatment schemes and were therefore divided into two therapy groups according to the total radiation dose given to the tumour: <60 Gy and >60Gy. The lack of association of HIF-1α or GLUT-1 with TRG and survival applied to both therapy groups (p>0.1).

In 82 patients both HIF-1α and GLUT-1 expression was evaluable, and 19 of these patients had high expression of both GLUT-1 and HIF-1α. However, there was no association between the dual expression and TRG or OS.

**Discussion**

Tumour hypoxia remains a major problem in the treatment of solid tumours, and reliable hypoxia-related markers are essential to address the problem in a rational way. There are, however, only few studies dealing with the clinical importance of HIF-1α and GLUT-1 in rectal cancer.

Brophy et al. investigated membranous GLUT-1 expression in pretreatment biopsies from T3-T4 rectal...
tumours confirmed by MRI or transrectal ultrasound. The study included 69 patients who all received long-course preoperative chemoradiotherapy. The results suggested a relationship between GLUT-1 immunohistological staining and response rate (RR), as a good response (TRG1+2) was found in 70% of patients with GLUT-1-negative tumours versus 32% of patients with GLUT-1-positive tumours (p=0.004). However, no association was found between GLUT-1 and local recurrence or disease-free survival (DFS) (26). The patient population, the treatment given and the tissue used in this study were comparable to the present study. We were unable to reproduce an association between GLUT-1 and treatment response using the grouping into GLUT-1-high (RR 25%) and -low (RR 36%), nor when using the division used by Brophy et al. into GLUT-1-negative (RR 30%) and GLUT-1-positive (RR 30%). The lack of prognostic impact of GLUT-1 was, however, consistent with the results of the present study.

Korkeila et al. investigated 75 patients with rectal cancer (T1-T4 tumours classified by MRI) receiving preoperative radiotherapy, where 39 patients had long-course radiotherapy comparable to the treatment given in the present study. The results of this study, dealing with these 39 patients, indicate an association between HIF-1α expression and poorer response to chemoradiotherapy, while 33% of the patients with poor response had strong intensity of HIF-1α staining as opposed to only 11% of the patients with moderate/excellent response (p=0.04). This difference was not significant when considering HIF-1α-positive tumours versus HIF-1α-negative tumours. Furthermore, they reported a significantly more favourable disease-specific survival (DSS) for patients with HIF-1α-negative tumours (3-year DSS rate 75%) compared to those with HIF-1α-positive tumours (3-year DSS rate 20%) (p=0.001). This difference did not hold true in a multivariate analysis and was not reproduced regarding DFS analysis (25). It should be noted that the Korkeila et al. had a small number of patients. Furthermore, the study population is not comparable with that in the present study, which included only T3 and T4 tumours, and the tissue used for the analysis was from operative specimens after radiotherapy.

The importance of HIF-1α and GLUT-1 has also been investigated in operative specimens from patients with rectal cancer treated without preoperative chemoradiotherapy. Theodoropoulos et al., Rasheed et al., and Lu et al. have suggested a prognostic role of HIF-1α in patients radically operated for rectal cancer treated with or without postoperative radiotherapy (15, 16, 20), while Cooper et al. suggested a prognostic importance of GLUT-1 in 43 patients with rectal cancer treated with or without radiotherapy (33). Comparison with these studies is however problematic because the patients received a treatment different from that in the present study, and the tissue used for immunohistological staining was collected from operative specimens in contrast to pretreatment biopsies. Atkin et al. have shown that immunohistological stains of several proteins, including HIF-1α and GLUT-1, differ in biopsies obtained before and after surgery (34). Consequently, comparison of protein expression in small preoperative biopsies from the tumour in vivo and that in operative specimens cannot be accepted without reservation. It should also be noticed that radiotherapy influences the protein.

Figure 3. A: Overall survival curves for patients with HIF-1α-high versus HIF-1α-low tumours. B: Overall survival curves for patients with GLUT-1-high versus GLUT-1-low tumours.
 expression of HIF-1α in tumours (24, 25), and therefore the role of HIF-1α is not necessarily the same in tumours treated with or without radiotherapy.

The available studies addressing the predictive and prognostic role of HIF-1α and GLUT-1 in patients with rectal cancer treated with preoperative long-course chemoradiotherapy are few and no definitive conclusion can be drawn in light of the existing studies.

In conclusion, the present study does not suggest any predictive or prognostic impact of HIF-1α or GLUT-1 expression in pretreatment biopsies from patients with locally advanced rectal cancer treated with preoperative chemoradiotherapy.

Conflict of Interest Statement

The authors declare no conflict of interest.

Acknowledgements

Supported by CIRRO – The Lundbeck Foundation Center for Interventional Research in Radiation Oncology, and Desiree and Niels Ydes foundation.

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Received January 25, 2011
Revised March 29, 2011
Accepted April 1, 2011