Glucose transporter-1 (GLUT-1): a potential marker of prognosis in rectal carcinoma?

The aim of the study is to evaluate the pattern and level of expression of glucose transporter-1 (GLUT-1) in rectal carcinoma in relation to outcome as a potential surrogate marker of tumour hypoxia. Formalin-fixed tumour sections from 43 patients with rectal carcinoma, who had undergone radical resection with curative intent, were immunohistochemically stained for GLUT-1. A mean of three sections per tumour (range 1–12) were examined. Each section was semiquantitatively scored; 0, no staining; 1, <10%; 2, 10–50%; 3, >50% and a score given for the whole section, the superficial (luminal) and deep (mural) part of the tumour. Staining was seen in 70% of tumours. Increased staining was noted adjacent to necrosis and ulceration. A diffuse and patchy pattern of staining, with and without colocalisation to necrosis was seen. Patients with high GLUT-1-expressing tumours (score 3 vs 0–2) had a significantly poorer overall survival ($P = 0.041$), which was associated with poorer metastasis-free survival with no difference in local control. No significant correlation was seen with other prognostic factors. There was a strong correlation between the score for the superficial and deep parts of the tumour ($r = 0.81$), but a significant relationship with outcome was only found in the deep part ($P = 0.003$ vs $P = 0.46$). In conclusion, increased GLUT-1 expression in rectal tumours was an adverse prognostic factor and is worth further evaluation as a predictive marker of response to therapy.

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Keywords: GLUT-1; rectal cancer; hypoxia

The presence of hypoxia in tumours is known to lead to resistance to radiotherapy and chemotherapy and is associated with a more aggressive phenotype with an increased propensity for metastases (Hockel et al., 1996; Brown et al., 1998; Dachs et al., 1998). This latter finding is thought to be related to the increased expression of a number of proteins acting through the HIF-1 pathway, which allows tumour cells to survive the harsh tumour microenvironment (Maxwell et al., 1997; Semenza, 2000). The glucose transporter-1 (Glut-1) is one of the proteins upregulated in hypoxic conditions.

Tumours show increased uptake of glucose compared to normal tissue (Isslbacher, 1972), a response mediated by a number of facilitative glucose transporters located in the cell membrane (Zhang et al., 1999). Glut-1 is one of this family of facilitative glucose transporters. It is expressed variably in normal tissue (Mueckler et al., 1985) and is responsible for the passive transport of glucose across the cell membrane (Chen et al., 2001). The function and expression of Glut-1 is regulated by a number of physiological and pathophysiological conditions (Zhang et al., 1999). In the tumour microenvironment, hypoxia results in increased transcription of the Glut-1 gene, mediated through HIF-1, and reduction in oxidative phosphorylation, in the absence of hypoxia, leads to increased stabilisation of the Glut-1 mRNA (Behrroz et al., 1997). Glucose transporter-1 is overexpressed in several different tumour types (Younes et al., 1996). In addition, increased expression has been shown to correlate with a poor prognosis in a variety of tumours (Younes et al., 1997; Haber et al., 1998; Airley et al., 2001; Cantuaria et al., 2001). More recently, Glut-1 expression has been shown to correlate with the level of tumour hypoxia in carcinoma of the cervix measured using either Eppendorf needle electrodes (Airley et al., 2001) or pimonidazole staining (Airley et al., 2003). Therefore, the level of Glut-1 expression might be a suitable surrogate or intrinsic marker of hypoxia, which could be measured simply and inexpensively as part of the routine histological assessment of tumours. Little is known about the oxygenation of rectal tumours; however, it is likely that hypoxia plays a significant part in determining outcome (Haustermans et al., 2000; Wendling et al., 1994). Furthermore, determination of the level of hypoxia might be important for selecting appropriate preoperative radiotherapy or chemoradiotherapy regimes or determining which patients are more likely to develop distant metastases and therefore require systemic therapy. In this study, we report for the first time the level and pattern of expression of Glut-1, as a potential endogenous marker of hypoxia, in rectal carcinoma and relate the level of expression to outcome.

PATIENTS AND METHODS

Patients

The study was retrospective. Formalin-fixed tumour sections from 43 consecutive patients (29 male, 14 female) with rectal carcinoma who had undergone curative intent treatment at a single institution
A rabbit IgG fraction (DAKO) at an identical protein concentration was applied and the sections were incubated for 1 h at 37°C. Rabbit anti-human Glut-1 (Alpha Diagnostic International, TX, USA) was applied again. A 1 : 100 (10 protein) concentration of affinity-pure goat anti-rabbit/HRP was applied to the sections for 30 min at room temperature. A substrate–chromogen solution containing 3,3’-diaminobenzidine in a buffered substrate solution, supplied in the kit, was applied at room temperature for 7 min. After rinsing in water, the slides were counterstained with Gill’s haematoxylin, dehydrated and mounted. The batch to batch variation was excluded by running sections from the same biopsy through more than one batch, and running one biopsy section through all the batches.

**Scoring method**

Glucose transporter-1 expression in tumour cells was evaluated using a semiquantitative scoring method: score 0 = absence of immunostaining; score 1 = 1–10% of cells stained; score 2 = 10–50% of cells stained; and score 3 = >50% of cells stained. No account was taken of the intensity of staining, and ulcerated or necrotic areas were excluded from the evaluation. For each tumour section, three areas were scored: a general score for the whole tumour section, a score for the superficial part of the tumour (approximately nearest one-third from the luminal surface) and a score for the deep part (approximately deepest one-third from the luminal surface). Intraobserver reliability was tested by the same pathologist re-evaluating 20 slides chosen at random after a 4-week gap.

**Statistical analysis**

Previous studies have analysed Glut-1 expression as either positive (score 1–3) vs negative (score 0) or strongly positive (score 3) vs the rest (score 0–2) (Younes et al, 1997; Haber et al, 1998; Airley et al, 2001). It was therefore decided to analyse our patients using these two categories. Correlations between tumour characteristics and the expression of Glut-1 were obtained using a two-tailed Spearman’s rank correlation and Fisher’s exact test. Survival analysis was by the Kaplan–Meier method, and prognostic factors were assessed by log-rank statistics. Analyses were carried out for overall, metastasis-free and local recurrence-free survival. Bivariate analyses were used to test for the independence from other potential prognostic factors including T stage, nodal stage and tumour size.

**RESULTS**

**Expression of GLUT-1**

Glucose transporter-1 expression was only observed as membranous staining in tumour cells. Erythrocytes within blood vessels were also seen to stain strongly for Glut-1, which served as an internal control. Perinecrotic and periulcerative areas stained strongly. Two patterns of staining were observed, a focal pattern and a diffuse pattern. Although staining was increased around necrotic areas, the patchy staining pattern was not consistently localised to necrosis. In addition, more cells were positive in the deeper portion of the tumour compared to the superficial luminal part. Normal cells and tumour stroma did not stain for Glut-1. There was a significant correlation between the first and the second score for the general (r = 0.87, P < 0.0001), superficial (r = 0.59 P = 0.03) and deep (r = 0.82, P < 0.0001) parts of the tumour. Figure 1 illustrates sections that were scored 1,2 and 3.

**Distribution of patients according to GLUT-1 expression**

Of the 43 cases examined, 70% expressed Glut-1. The distribution of scores is shown in Table 2. There was a strong correlation between the overall tumour score and the scores for the deep

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**Table 1** Summary of patient and tumour characteristics

| Characteristic | Value |
|---------------|-------|
| **Age (years)** | Median 57, Range 21–80 |
| **T stage** | T2 9, T3 29, T4 5 |
| **Nodal status** | Negative 27, Positive 16 |
| **Tumour size (pathological)** | Median (cm) 5, Range (cm) 2–11, <5 cm 19, ≥5 cm 24 |
| **Grade** | 1 10, 2 24, 3 3, Not classified 6 |

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**Immunohistochemistry**

Formalin-fixed, paraffin-embedded tumour sections (5 μm thick) were placed on poly-L-lysine-coated slides. Sections were dewaxed in xylene and rehydrated using a series of ethanol solutions of decreasing concentration. After rinsing in water, the slides were counterstained with Gill’s haematoxylin, dehydrated and mounted. The field to field variation was excluded by running sections from the same biopsy through more than one batch, and running one biopsy section through all the batches.

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was used as a negative control. Following washing, the secondary antibody (goat anti-rabbit/HRP) was applied to the sections for 30 min at room temperature. A substrate–chromogen solution containing 3,3’-diaminobenzidine in a buffered substrate solution, supplied in the kit, was applied at room temperature for 7 min. After rinsing in water, the slides were counterstained with Gill’s haematoxylin, dehydrated and mounted. The batch to batch variation was excluded by running sections from the same biopsy through more than one batch, and running one biopsy section through all the batches.

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Correlation of GLUT-1 expression in relation to clinical factors
The distribution of Glut-1 expression according to Duke’s stage and nodal status is shown in Table 3. There was no correlation between the degree of Glut-1 immunostaining and tumour stage \((r = -0.047, P = 0.77)\). The patients were also grouped according to the presence or absence of nodal metastasis; however, there was no significant difference in the metastasis status of patients whose tumours showed negative (score 0) or positive (scores 1–3) Glut-1 staining \((P = 0.52, \text{Fisher’s exact test})\). Likewise, there was no significant difference in the metastasis status of patients whose tumours had weak (score 0–2) or heavy (score 3) Glut-1 staining \((P = 0.30, \text{Fisher’s exact test})\). Furthermore, there was no significant association between Glut-1 expression and either tumour grade \((r = 0.10, P = 0.58)\) or pathological size \((r = -0.02, P = 0.89)\).

Relationship between Glut-1 expression and outcome
To examine whether the group of patients studied had similar characteristics to larger groups of patients with rectal cancer, we analysed the relationship between treatment outcome and established clinical prognostic factors (tumour stage, nodal status and tumour size). As expected, advanced stage was a significant adverse prognostic factor \((P = 0.023)\). In addition, the 5-year actuarial survival was higher for patients with node negative (89%) vs positive (56%) tumours \((P = 0.009)\), and tumours that were pathologically <5 cm (89%) rather than ≥5 cm (67%) in diameter \((P = 0.06)\). Grade was a borderline significant prognostic factor for survival \((P = 0.06)\).

There was no significant difference in the overall survival of patients with Glut-1 positive (score 1–3) vs negative (score 0) tumours \((71\% \text{ vs } 92\% \text{ at } 5 \text{ years}; P = 0.17)\). There was, however, a significant difference in overall survival for patients with strongly positive tumours (score 3) compared to those with a score of 0–2 \((33\% \text{ vs } 80\%, \text{respectively}; P = 0.041)\). Survival curves are shown in Figure 2. Strong Glut-1 expression in tumours appeared to be associated with a poor metastasis-free survival (Figure 3) rather than local control (Figure 4).

Bivariate analyses were performed to test for the independence from tumour stage, nodal status and tumour size. Glucose transporter-1 expression remained a significant prognostic factor for overall survival after allowing for tumour stage \((P = 0.0052)\) and tumour size \((P = 0.05)\), but not nodal status \((P = 0.16)\).
Glut-1 expression in the superficial vs deep part of the tumour

Scores from the deep part of the tumour were also prognostic for overall survival (82% vs 25% actuarial survival at 5 years for score 0–2 vs score 3, respectively; \( P = 0.003 \)). However, the Glut-1 expression of the superficial part of the tumour was not prognostic for survival (actuarial survival at 5 years 78% vs 50%, \( P = 0.46 \) for score 0–2 vs score 3, respectively).

Independence of Glut-1 staining to known prognostic factors

In order to assess the degree of Glut-1 staining as an independent prognostic marker, all factors that had been significant for overall survival on log-rank univariate analysis (tumour stage, nodal status, strongly positive vs the rest for the whole tumour section and the deep part of the tumour) were included in a multivariate Cox regression analysis. The only factor that was prognostic for survival was the presence of Glut-1 staining in the deep portion of the tumour (\( P = 0.013 \)).

DISCUSSION

The presence of hypoxia in tumours has been shown to correlate with resistance to therapy and reduced survival. Therefore, pretreatment assessment of tumour hypoxia might enable prediction of patients likely to have a poor outcome, and who would be suitable for hypoxia-modifying treatment. Polarographic needle electrodes have been used to give a quantitative, pretreatment measurement of tumour hypoxia that correlates with outcome for patients with carcinoma of the cervix (Hockel et al, 1996; Fyles et al, 1998), head and neck (Nordsmark et al, 1996; Brizel et al, 1997) and soft tissue sarcoma (Brizel et al, 1996). However, the method is invasive and limited to accessible tumours greater than 2 cm in diameter. Furthermore, any measurement will also include areas of necrosis that can bias the results to falsely low values. An alternative method is the immunohistochemical assessment of bound nitroimidazoles, such as pimonidazole, injected prior to biopsy (Kennedy et al, 1997; Nordsmark et al, 2001). This requires an added intervention and, as yet, there are only limited data on their relationship with treatment outcome. This has prompted a number of groups to examine potential endogenous markers of hypoxia. The transcription factor HIF-1 \( \alpha \) has been the most widely studied potential endogenous marker of hypoxia (Aebersold et al, 2001; Haugland et al, 2002; Janssen et al, 2002; Koukourakis et al, 2002), along with its downstream proteins such as carbonic anhydrase IX (CAIX) (Giatromanolaki et al, 2001; Koukourakis et al, 2001; Loncaster et al, 2001), vascular endothelial growth factor (Loncaster et al, 2000; Koukourakis et al, 2002) and Glut-1 (Airey et al, 2001, 2003). The advantage of using intrinsic markers...
of hypoxia is that the approach is simple and quick, and could be potentially applied to a wide variety of solid tumour types.

In the present study, we examined the pattern and extent of Glut-1 expression in adenocarcinoma of the rectum as a potential endogenous marker of hypoxia. A total of 70% of tumours expressed Glut-1 to a varying degree. Glucose transporter-1 expression was seen to localise around areas of necrosis and ulceration, while little staining was seen in stromal or normal tissue. In general, two patterns of staining were observed, diffuse and patchy, the latter showing both colocalisation with necrosis and non-necrotic areas. Other studies have reported similar patterns of staining (Haber et al., 1998; Airley et al., 2001).

In addition, two recent studies have shown that the levels of Glut-1 expression in carcinoma of the cervix correlates with the level of hypoxia measured using either polarographic needle electrodes (Airley et al., 2001) or pimonidazole staining (Airley et al., 2002). In the former study, the strongest correlation was seen with the most hypoxic fraction of polarographic needle electrode measurements, leading to the suggestion that Glut-1 scores might represent the level of chronic or diffusion-limited hypoxia. While in the second study, there was a good, but not exact, colocalisation of pimonidazole staining and Glut-1 expression. Taken together, these findings suggest a link between hypoxia and Glut-1 expression in human carcinoma of the cervix. However, several factors could reduce the usefulness of Glut-1 as a hypoxic marker and explain the diffuse pattern of staining observed in some of the tumours. Glucose transporter-1 expression is known to be stimulated by a number of other stimuli including growth factors, thyroid hormone, alkaline pH and oncogenic transformation (Flier et al., 1987; Hakimian et al., 1991; Kuruvilla et al., 1991). Furthermore, it is dually controlled by both hypoxia and reduced oxidative phosphorylation in the absence of hypoxia (Behrroz et al., 1997). Chronic hypoxia leads to increased production of Glut-1 that is mediated via the transcription factor HIF-1. Although it was previously thought that HIF-1 was only stabilised under hypoxia, it is now known to be constitutively expressed in some tumours due to genetic alterations occurring during malignant transformation (Jiang et al., 1997; Feldser et al., 1999; Zhong et al., 2000). This might therefore explain the diffuse pattern of staining and the patchy noncolocalisation with necrosis observed in some of the tumours in the present study.

In order to exploit the measurement of Glut-1 expression as a marker of hypoxia in different types of tumours, there is a need to demonstrate that the marker can provide prognostic information in each tumour type of interest. Previous studies have shown a relationship between Glut-1 expression and overall survival in colon cancer (Haber et al., 1998) and lung cancer (Younes et al., 1997), and disease-free survival in ovarian cancer (Cantuaria et al., 2001). In addition, a significant association between Glut-1 expression and metastasis-free survival, but not disease-free survival or local recurrence, has been reported in patients with cervical carcinoma (Airley et al., 2001). In the present study, we were able to show in rectal cancers that strong expression of Glut-1 (> 50% of tumour cells) was a significant prognostic factor for overall survival independent of tumour stage and tumour size, and on multivariate analysis the presence of strong staining in the deep part of the tumour was the only significant factor for overall survival. We also observed a nonsignificant relationship with metastases-free survival, but not local recurrence-free survival, for the presence of staining (1 – 3) vs non (0). However, larger numbers of patients would be required to confirm this.

It is of interest that in the study of Airley et al. (2001), where Glut-1 expression was prognostic for metastasis-free survival, but not overall or local recurrence-free survival, patients with cervical carcinoma were treated with radiotherapy. In our study, patients were predominantly treated with surgery, and Glut-1 was predictive for overall and metastasis-free survival but again not local recurrence-free survival. It might be that Glut-1 expression reflects more severe and longer duration hypoxia, especially as in our study intense staining (score 3) showed the best correlation with outcome and heavy staining is more indicative of de novo Glut-1 synthesis which only occurs after prolonged hypoxia (Shetty et al., 1992; Behrroz et al., 1997). This in turn might be more reflective of the tumours propensity to form distant metastases, associated with hypoxia rather than resistance to radiotherapy and is consistent with previous experimental and clinical data (Fyles et al., 2002).

We found no significant relationship between the expression of Glut-1 in tumours and the accepted clinical prognostic factors of tumour stage, depth of invasion (T stage), nodal status, tumour size and grade of differentiation. In contrast, a previous study in patients with mainly colon cancer showed that extensive staining (>50% of cells) for Glut-1 correlated significantly with the presence of nodal metastases (Haber et al., 1998). Similarly, no consistent picture has emerged for any relationships between clinical prognostic factors and the level of hypoxia measured using polarographic needle electrodes (Hockel et al., 1996; Nordsmark et al., 1996; Pitson et al., 2001). The fact that no correlation was found in our study might reflect the low number of patients included and/or the inhomogeneous treatment given. However, the lack of association and the fact that only strong staining in the deep part of the tumour was a significant factor for overall survival on Cox regression analysis suggests that Glut-1 expression in rectal tumours might give additional information over and above that provided by the established clinical prognostic factors. Studies on a larger group of patients are required to clarify this issue.
Of interest in our study was the finding that more cells were seen to express Glut-1 in the deeper compared to the superficial (corresponding with the luminal surface) parts of tumours. In addition, no relationship was found between patient outcome and Glut-1 expression in the superficial part of the tumours. The latter observation is difficult to interpret because of the lack of published data on the oxygenation status of rectal tumours. However, the data do highlight the potential problem of intratumour heterogeneity and the need to take multiple biopsies from different parts of rectal tumours. Taking a mean of three biopsies per patient, as used in our study, is recommended for other studies in this area.

In the results presented here, Glut-1 was expressed in two-thirds of the rectal tumours examined, and high expression was associated with a poor treatment outcome. In addition to Glut-1, several potential endogenous markers of hypoxia are currently under investigation including HIF-1α, HIF-2α, CAIX and VEGF. Studies suggest that different levels and/or durations of tumour hypoxia are required to upregulate each protein (Giartromanolaki et al., 2001; Olive et al., 2001). It is possible, therefore, that a combination of markers might be required to fully evaluate hypoxia within a given tumour type. Furthermore, several studies have shown that combining protein expression with other factors, such as the level of vascularity, improves the definition of patient groups with differing prognoses (Koukourakis et al., 2001, 2002).

This is the first study of Glut-1 expression in purely rectal carcinoma. Rectal cancer is an interesting area as preoperative radiotherapy or chemoradiotherapy is being increasingly used as part of the treatment. Furthermore, there is still debate as to which node-negative patients should receive adjuvant chemotherapy. As several different regimens are being used, a better understanding of the individual tumour microenvironment might enable a more rational application of these therapies along with the incorporation of hypoxia-modifying treatments for those patients with hypoxic tumours.

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