Glucose Transporter Glut-1 Expression Correlates with Tumor Hypoxia and Predicts Metastasis-free Survival in Advanced Carcinoma of the Cervix[1]

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ABSTRACT

Hypoxic tumors are known to be more malignant, to be more likely to metastasize, and to have a poor prognosis. They are also radio- and chemoresistant. For this reason, it is desirable that a clinically useful marker of hypoxia is found, so that treatment with radiotherapy and bioreductive chemotherapy can be rationally applied to individual patients. Glut-1 is a facilitative glucose transporter that is ubiquitously expressed in normal tissue and expressed at higher levels in a number of tumors. Its potential as an intrinsic hypoxia marker arises from its dual control in hypoxic conditions by reduced oxidative phosphorylation and the hypoxia-inducible factor (HIF-1) oxygen-sensing pathway. Eppendorf histography, by virtue of its proven predictive qualities, is a suitable gold standard used in our laboratory to validate new hypoxia markers. Using this technique, pretreatment pO_2 measurements were performed on 54 patients with locally advanced cervical carcinoma. Then, immunohistochemical staining was used to detect Glut-1 protein in individual tumor biopsy sections. Both measurements were made before initiation of treatment. By using a low-tech scoring system, pO_2 was found to correlate weakly with Glut-1 score ($r = 0.28$; $P = 0.04$). To extrapolate this correlation to the known adverse effects of tumor hypoxia on outcome, we examined the prognostic significance of Glut-1 staining in a retrospective series of 121 patients. An absence of Glut-1 significantly increased the likelihood of metastasis-free survival ($P = 0.022$) but did not significantly effect disease-free or recurrence-free survival. These findings suggest that Glut-1 be an intrinsic marker of hypoxia that can easily be applied in a clinical setting.

INTRODUCTION

Tumor hypoxia gives rise to a poor prognosis, a more malignant phenotype, and an increased likelihood of metastasis (1–3). Poor availability of molecular oxygen and the metabolic changes occurring in these conditions also confer resistance to both chemo- and radiotherapy, leading to treatment failure (4, 5). Bioreductive drugs are differentially toxic to hypoxic cells within a tumor and include tirapazamine (6) and AQ4N (7), which are presently undergoing clinical trials. They have shown promise when used in combination with other cytotoxic drugs, such as cisplatin (8, and with radiation (9). Bioreductive activation has a strong dependence upon cellular oxygen tension, and pro-drugs vary in the extent and duration of hypoxia necessary for bioreduction (10). The level of oxygenation varies inter- and intratumorally, as well as between tumor types (11). An accurate, inexpensive, and minimally invasive means of measuring tumor hypoxia is therefore highly desirable to enable the selection of patients into the appropriate treatment arms of clinical trials and to facilitate the future rational application of bioreductive drugs to individual patients.

At present, the "gold standard" for the assessment of tumor hypoxia consists of direct pO_2 measurements using Eppendorf polarographic needle electrodes as described by Vaupel *et al.* (12). The method has been validated as a predictor of radiation response in murine tumor models (13) and successfully used to evaluate hypoxia in human cancers (4, 14, 15). The advantages of using oxygen electrodes are the immediate availability of data and the ability to measure the effects of acute, perfusion-related changes in oxygenation (16) as well as different hypoxic subpopulations (2, 17). Disadvantages that hinder the use of the oxygen electrode in routine practice are the invasive nature of the procedure and that use is restricted to accessible tumors. There is, therefore, a recognized need for a method of measuring tumor hypoxia that is suitable for more widespread clinical use. An intrinsic marker of hypoxia, which would necessitate no additional intervention beyond an initial pretreatment biopsy, is very attractive.

Potential markers may be those that are produced with changes in oxidative status. These include ATP, certain oxygen-regulated stress or heat shock proteins (18), and proteins up-regulated via an oxygen-sensing pathway involving the HIF-1[3] transcription factor. Examples of the latter are vascular endo-

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[3] The abbreviation used is: HIF-1, hypoxia-inducible factor-1.

Table 1 Distribution of putative prognostic factors of age, stage, and grade in prospective (oxygen measurements obtained) and retrospective series

	O_2 measurements available	Retrospective series (prognosis)
Stage		
I	2	35
II	20	38
III	26	41
IV	6	7
Age	Median = 58.5	Median = 50
<Median	27	62
>Median	27	59
Grade (differentiated)		
Well	3	18
Moderate	32	68
Poor	10	17
Unknown	9	18

thelial growth factor, erythropoietin, various glycolytic enzymes, and the facilitative glucose transporter, Glut-1 (19, 20).

Glut-1 expression is dually controlled via HIF-1 and in response to reduced oxidative phosphorylation (21). It is one of eight structurally related membrane-bound facilitative glucose transporter proteins whose structural and ultrastructural location have been extensively studied and well-characterized (22, 23). Glut-1 has been detected immunohistochemically in a variety of malignant and normal tissues, including tumors of the breast (24), thyroid (25), head and neck (26), bladder (27), lung (28) and in juvenile hemangiomas (29). In all cases, expression is increased relative to corresponding normal tissue, and overexpression of Glut-1 is a marker for poor prognosis in colorectal (30) and non-small cell lung (31) carcinomas. The increase in glucose transport seen in malignancies has also been detected using 18-fluorodeoxyglucose positron emission tomography (32, 33), and raised glucose uptake has shown potential value as a prognostic indicator, high 18-fluorodeoxyglucose uptake predicting poorer survival in head and neck tumors treated with radiation therapy (34). A hypoxia-related increase in glucose transport has been demonstrated *in vitro* using positron emission tomography (35), and additional experiments have shown that an oxygen concentration of $\leq$1.5% up-regulates cellular glucose uptake independent of glucose deprivation (36).

The aim of our study was to examine the relationship between tumor hypoxia and Glut-1 expression in human cervical carcinoma and to relate this to prognosis after treatment of these tumors with radiation therapy. To our knowledge, this is the first example of the use of Glut-1 as a marker of tumor hypoxia in human subjects.

PATIENTS AND METHODS

Patients. Ethical approval was granted before work involving the use of the Eppendorf oxygen electrode. All patients included in this study gave prior consent to allow tumor biopsies to be taken for research purposes at the time of their staging examination under anesthesia.

Measurement of pO_2 in Tumors Using Eppendorf Oxygen Electrode. Fifty-four patients with advanced squamous carcinoma of the cervix were included in this study, and clinical details on age, stage, and grade are listed in Table 1. Patients underwent pretreatment tumor oxygenation measurements using the Eppendorf pO_2 histograph system, as described by Cooper *et al.* (37). All measurements were performed under general anesthesia, which consisted of propofol infusion and nitrous oxide. To measure tumor oxygenation, measurements were taken at the 12- and 6-o'clock positions, starting at a depth of ~4 mm, with at least 5 mm between measurement tracks. A median of 4 oxygen electrode tracks (range, 1–7) was made per tumor, resulting in a median of 128 oxygen measurements (range, 32–300) per tumor. Data were expressed as the hypoxic fraction below 2.5, 5, and 10 mm Hg ($HP_{2.5}$, HP_5, and HP_{10} respectively).

Optimization of Biopsy Number. For a number of patients in the above series, multiple biopsies were taken, including 13 tumors with four biopsies, 4 tumors with three biopsies, 4 tumors with two biopsies, and 33 tumors with one biopsy. To test the hypothesis that obtaining an overall score using multiple biopsies from individual tumors might be a better representation of tumor hypoxia than one biopsy alone, sections from all available biopsies were stained and scored. Multiple biopsies from the same tumor were either given a single score representative of all of the sections or, for the purpose of assessing tumor heterogeneity, assigned individual scores.

Retrospective Analysis of Glut-1 as a Prognostic Indicator. Biopsy material from 121 patients treated at the Christie Hospital between April 1987 and June 1993 was analyzed for Glut-1 protein expression. Clinical information on tumor characteristics is given in Table 1. All patients received radiation therapy with curative intent according to the Manchester school (38). Data on patient outcome were obtained from specialist oncology clinics and complemented by additional follow-up information received via questionnaires sent to general practitioners. Any incidence of disease relapse was identified clinically and radiologically and, where appropriate, confirmed by biopsy. Recurrences were defined as either local or metastatic, occurring within or outside the radiation therapy field, respectively.

Immunohistochemical Staining for Glut-1/Endothelial Cell Markers CD31 and CD34. Formalin-fixed, paraffin-embedded tumor sections were dewaxed in xylene and rehydrated using a series of ethanol solutions of increasing dilution. Staining for Glut-1 and CD31/CD34 was then carried out using the Envision Doublestain kit according to protocol. This kit consists of two staining systems. The first uses a horseradish peroxidase conjugate and 3,3′-diaminobenzidine substrate system that enabled visualization of Glut-1 protein as a brown stain. The second uses an alkaline-phosphatase conjugate and Fast Red substrate that stained the CD31 and CD34 endothelial proteins an intense red, facilitating visualization of the tumor vasculature. This procedure included the use of three primary antibodies. For Glut-1 staining, a 1/100 (10 μg/ml protein) concentration of affinity-pure rabbit antihuman Glut-1 (Alpha Diagnostic International, Texas) was used. For CD31 and CD34, a combination of two monoclonal antibodies, mouse antihuman CD31 (1/70) and mouse antihuman CD 34 (1/70; DAKO, United Kingdom) were used. An incubation time of 1 h at 37°C was used for both primary antibody steps, whereas an incubation time of 30 min at room temperature was chosen for each secondary antibody. Negative controls included the use of a rabbit IgG fraction (DAKO) used at an identical protein concentration to the rabbit Glut-1 antibody, and Tris-buffered saline was used

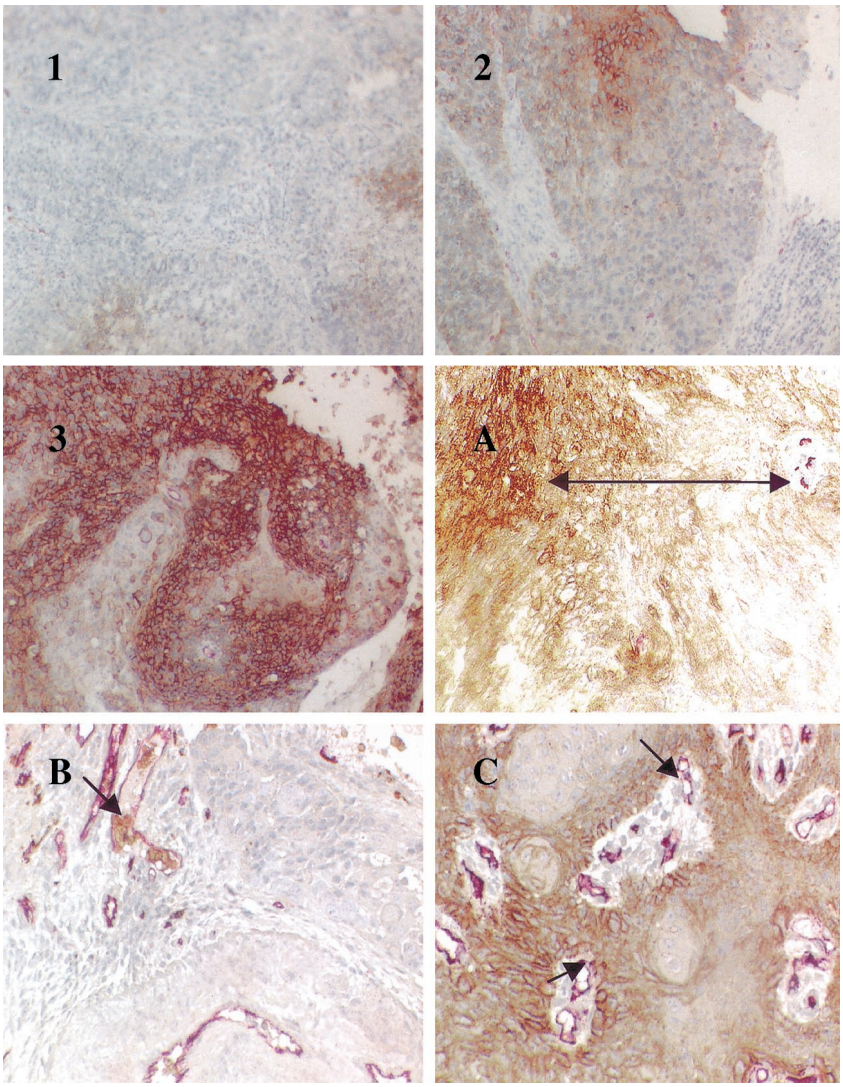

Fig. 1 Glut-1 staining intensities (*1*, light staining; *2*, moderate staining; *3*, heavy staining). Glut-1 staining occurs in two forms, either cytoplasmic or membranous, the latter occurring at a greater distance from perfused blood vessels, shown by *arrow* (*A*). Positive Glut-1 staining is seen in erythrocytes, which may be used as evidence of perfused blood vessels, particularly as surrounding tissue shows no Glut-1 staining (*B*). Where blood vessels appear to be free of erythrocytes, the intense Glut-1 staining in the surrounding tissue is consistent with resulting ischemia (*C*).

alongside the mouse anti-CD31/CD34 antibodies. Two batch controls were included in each subsequent run. After staining, sections were rinsed with water, counter-stained with 1× Gill's hemotoxylin, and coverslipped using an aqueous mountant.

Scoring System. Sections were viewed at a magnification of ×10 and given a score according to the intensity of Glut-1 staining (0, no staining; 1, light staining; 2, medium staining; and 3, heavy staining). Edge effects and necrotic and stromal areas were ignored.

Statistical Analysis. Correlations between variables such as Glut-1 and pO_2 measurements were obtained using a two-tailed Spearman's rank correlation. Survival analysis was by the Kaplan-Meier method, and prognostic factors were assessed by log-rank statistics. Analyses were made of disease-free, metastasis-free, and local recurrence-free survival. Bivariate analyses were used to test independence from age, stage, and grade.

RESULTS

Immunohistochemical Expression of Glut-1. Glut-1 expression in tumors was easily apparent as brown staining and occurred in both the cytoplasm and the plasma membrane of the tumor cells (Fig. 1). Typical examples of staining intensities, denoting scores of 0 (no staining), 1 (light staining), 2 (moderate staining), or 3 (heavy staining) are shown. The presence of Glut-1 in RBCs provided an internal control as well as a simple means of distinguishing perfused blood vessels (Fig. 1, *B* and *C*). Glut-1 was consistently seen at a distance from perfused blood vessels, and where both the cytoplasmic and the membranous form of the protein were visible, the membranous form occurred distally (Fig. 1*A*). Heavy Glut-1 staining was seen both in and around necrotic foci, but no Glut-1 staining was seen in stromal areas.

Inter- and Intra-observer Variability of Scoring System. Archival material consisting of the 121 stained tumor sections used in retrospective analyses was scored by both R. E. A and C. M. L. W., and a highly significant correlation was found ($r = 0.89$; $P = < 0.001$). A series of 30 tumor sections was rescored by R. E. A approximately 3 weeks after first scoring, and the scores correlated significantly ($r = 0.85$; $P = < 0.001$).

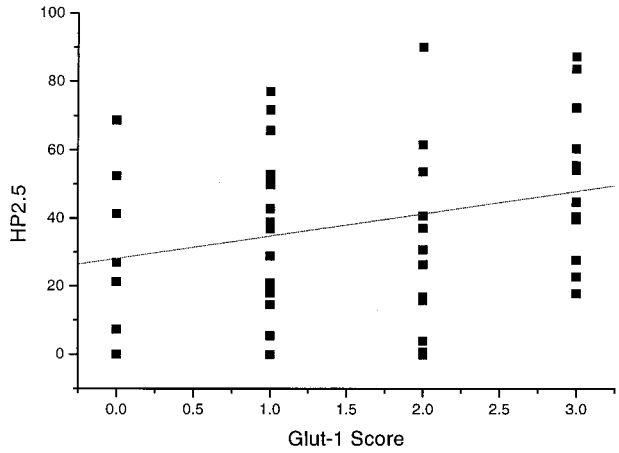

Fig. 2 Correlation between Glut-1 score and $HP_{2.5}$. The Eppendorf pO2 histograph system was used to measure $HP_{2.5}$ in 54 patients with advanced squamous carcinoma of the cervix. ($r = 0.277$; $P = 0.04$).

Correlation between Glut-1 Expression and pO_2 Measurements. In 54 tumors, the median $HP_{2.5}$ was 40% (range, 0–91), the median HP_5 was 52% (range, 0–93), and the median HP_{10} was 63% (range, 16–98). Fig. 2 shows a weak but significant correlation between Glut-1 score and the $HP_{2.5}$ ($r = 0.28$; $P = 0.04$) in 54 tumors. However, there was no significant correlation between Glut-1 score and HP_5 ($r = 0.25$; $P = 0.066$) or HP_{10} ($r = 0.23$; $P = 0.089$). For 21 of the tumors, multiple biopsies were available. For these 21 tumors, scoring multiple biopsies strengthened the correlation (Table 2). There was also a significant correlation between the scores obtained from one and multiple biopsies ($r = 0.45$, $P = 0.043$; $n = 21$). For the subset of 21 tumors, a one-way ANOVA test was performed on individual scores obtained from multiple biopsies within the same tumor, yielding a coefficient of variation of 42%. This compared with a coefficient of variation between tumors of 85% when scoring one section from each of the 21 tumors. These analyses showed that there is more variability between than within tumors.

Glut-1 as an Indicator of Disease-free Survival. Fig. 3 shows survival as a function of Glut-1 staining intensity. There is evidence of a nonsignificant trend for increased survival when tumor Glut-1 expression was absent ($P = 0.31$). When patients were stratified according to the presence or absence of Glut-1 staining, statistical significance was slightly increased ($P = 0.29$). Bivariate analysis was undertaken to exclude tumor stage, grade, and patient age-dependent effects. Allowing for these factors did not notably improve statistical significance. However, when stratification by stage took place and significance values were considered separately, it was noticed that Glut-1 expression, as an indicator of poor survival, became more relevant when considering stage one and two tumors in isolation ($P = 0.063$).

Glut-1 as an Indicator of Metastasis-free Survival. The influence of Glut-1 staining intensity on metastasis-free survival is shown in Fig. 4. Again, there was a trend for absence of Glut-1 staining favoring metastasis-free survival ($P = 0.081$). This became significant when the absence or presence of Glut-1 staining was considered ($P = 0.022$). Bivariate analysis was again undertaken to exclude the effect of tumor stage and grade and patient age. Glut-1 expression remained a significant prognostic factor for metastasis-free survival after allowing for patient age ($P = 0.018$), tumor stage ($P = 0.019$), and disease grade ($P = 0.049$).

Table 2 Using multiple biopsies from the same tumor enhanced statistical significance of correlations[a]

Glut-1 score	$HP_{2.5}$	HP_5	HP_{10}
One biopsy	$r = 0.077$	$r = 0.106$	$r = 0.235$
	$P = 0.74$	$P = 0.65$	$P = 0.30$
	$n = 21$	$n = 21$	$n = 21$
Multiple biopsies	$r = 0.286$	$r = 0.391$	$r = 0.439$
	$P = 0.21$	$P = 0.08$	$P = 0.05$
	$n = 21$	$n = 21$	$n = 21$

[a] Data from 21 patients with two to four biopsies.

Glut-1 as an Indicator of Recurrence-free Survival (Local Control). Fig. 5 shows local control as a function of Glut-1 staining intensity. Although the trend is not statistically significant in the number of patients studied ($P = 0.24$), tumors exhibiting heavy Glut-1 staining were most likely to recur. Furthermore, bivariate analysis undertaken to exclude stage effects improved statistical significance ($P = 0.13$).

DISCUSSION

Validation of any novel means of detecting tissue hypoxia logically necessitates comparison with other methods. This provided the rationale behind the statistical correlations described in this paper and with other putative hypoxia markers currently being investigated in our laboratory. Another approach has been to compare various indicators of hypoxia with bioreductive markers (39, 40). These include the 2-nitroimidazoles, which at nontoxic doses form intracellular adducts in hypoxic cells (41–44). However, the action of these agents may not be entirely dependent upon tumor hypoxia, inasmuch as the extent of bioreduction, and hence binding, is influenced by the level of reducing enzymes expressed in individual tumors (45). Eppendorf histography is a proven means of measuring human tumor pO_2 and its influence on prognosis (14). The view in our laboratory is that a comparison with a direct means of measuring oxygen tension is more informative in the research environment.

We have shown a correlation between Glut-1 and tumor hypoxic fraction, which lends validity to its suitability as a surrogate marker of hypoxia. We have also demonstrated that the scoring system may be more representative of tumor pO_2 if multiple biopsies are used because of the existence of tumor heterogeneity. Although the pattern of Glut-1 expression matches that of the expression observed previously in hypoperfused regions of lung carcinomas (46), the correlation with pO_2 measurements is weak. This may be attributable to differing sensitivities to acute and chronic hypoxia. Although Eppendorf histography may be more sensitive to acute changes in oxygenation, Glut-1 expression is more likely to indicate the presence of chronic hypoxia or an overall lower pO_2. Stimulation of Glut-1 in hypoxic conditions consists of a series of changes relating to intrinsic activity, kinetics and expression, and has

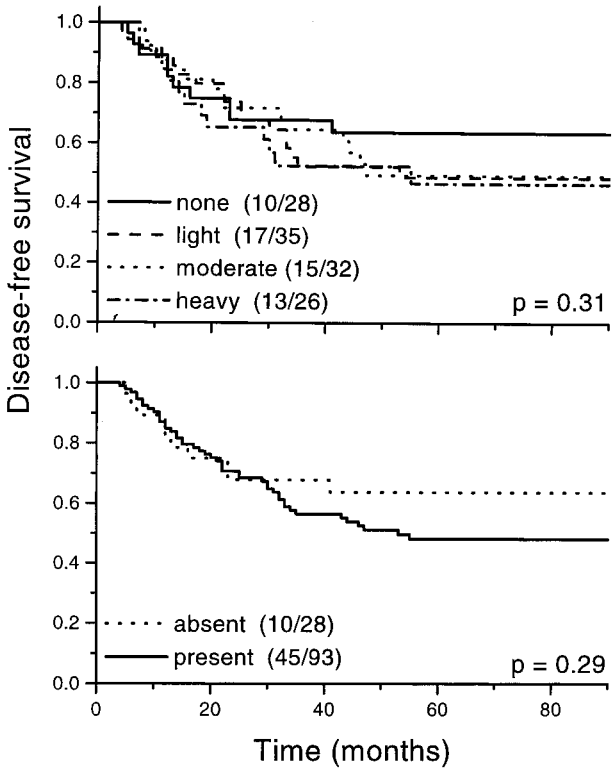

Fig. 3 Disease-free survival as a function of Glut-1 staining intensity ($P = 0.31$) and absence or presence of Glut-1 ($P = 0.29$). Although the number of patients is too small to show a significant difference, a trend is apparent whereby those patients staining negatively for Glut-1 are more likely to survive.

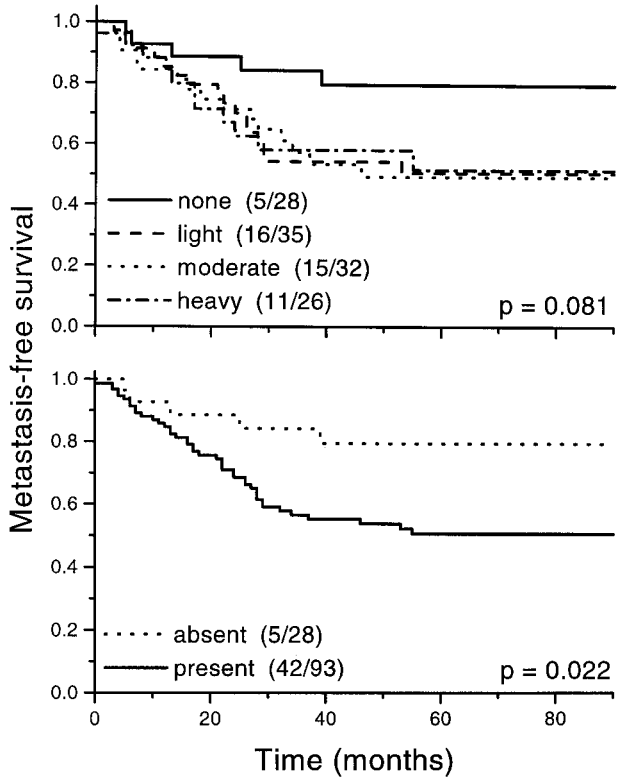

Fig. 4 Metastasis-free survival as a function of Glut-1 staining intensity ($P = 0.081$) and presence or absence of Glut-1 ($P = 0.022$), the latter showing that absence of Glut-1 staining is significantly prognostic for metastasis-free survival.

been extensively reviewed by Zhang *et al.* (47). It has been shown that early changes consist of the "unmasking" of the protein, which accompanies an increase in the affinity for glucose. Additional stimulation by hypoxia or ischemia induces translocation of existing glucose transporters from cytoplasmic vesicles to the plasma membrane, and eventually an increase in the synthesis of Glut-1 mRNA. We are currently investigating the hypothesis that the cytoplasmic and membranous forms of Glut-1 seen in these tumor sections correspond to the duration and extent of hypoxia existing in different areas of the tumor. The weakness of the correlation is also likely to be attributable to the alternative function of Glut-1 as a glucose-regulated protein (48); hence, protein expression is a consequence of both oxygen and glucose starvation. Thus, it is logical that these areas of Glut-1 staining, particularly the areas of intense staining perceived round necrotic foci, are both oxygen- and nutrient-deprived. It is well established that a reduction in oxidative phosphorylation, which might be a consequence of the increased proliferation seen in tumors, enhances Glut-1 expression (21, 49). Sensitivities to changes in glucose availability, utilization, metabolism, and HIF-1 expression will all determine the extent of Glut-1 expression in these tumors. Insulin, thyroid-stimulating hormone and thyroid hormones also control the expression and functionality of Glut-1 (50–52). Therefore, although Glut-1 expression is expected to be a surrogate marker for hypoxia, this may be confounded in patients with diabetes mellitus and hypo- or hyperthyroidism.

A prospective surrogate hypoxia marker, like Glut-1, is validated further if the prognostic significance of tumor hypoxia can be extrapolated to its expression. The absence of Glut-1 is significantly prognostic for metastasis-free survival. This is consistent with observations that hypoxia is associated with the formation of metastases in human (53) and experimental tumors (54). Previous work has demonstrated that Glut-1 expression is an indicator of poor prognosis in colorectal (30) and non-small cell lung (31) carcinomas. Because death in these cases can be attributed to metastatic spread, our findings reinforce these observations and are attributable to hypoxia-induced malignant changes as well as the possible effects of glucose starvation on metastatic potential and invasive capacity (55, 56).

There is increasing evidence that the more transiently hypoxic cells and/or those cells at more intermediate O_2 tensions determine treatment response (57), a finding that might partially explain the lack of prognostic significance shown between recurrence-free survival and Glut-1 expression. The correlation of Glut-1 positivity with pO_2 measurements is most significant at $HP_{2.5,}$ additional evidence that Glut-1 expression less clearly represents acute hypoxia and, therefore, radiotherapy outcome. Thus, this lack of correlation with local control will impact on any relationship with disease-free survival. However, this will not necessarily affect metastasis-free survival, because the ra-

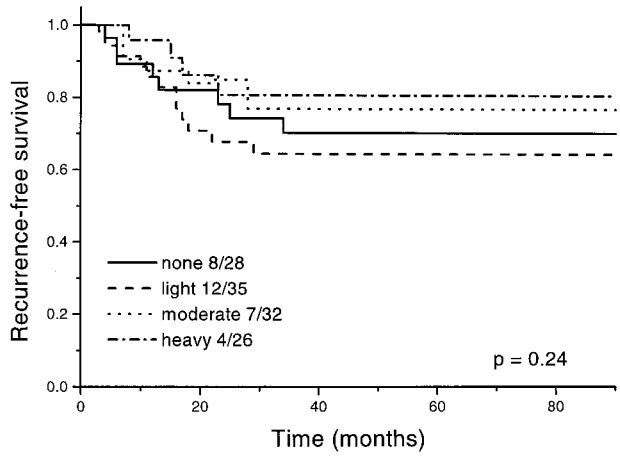

Fig. 5 Recurrence-free survival (local control) as a function of Glut-1 staining intensity ($P = 0.24$).

diation chemical events determining O_2-dependent radiosensitivity are quite different from those O_2-dependent molecular processes leading to metastatic spread.

Bivariate analysis has shown Glut-1 expression to be independent from other prognostic factors, and it is interesting to note that exclusion of stage 3 and 4 tumors increases the prognostic significance of Glut-1 to overall survival rate. It has to be considered, therefore, that tumors that have progressed beyond stage 2 respond differently to treatment, and that other factors such as radiosensitivity are now affecting survival.

In conclusion, this paper demonstrates the use of a simple, low-tech scoring system to assess Glut-1 expression in individual tumors that can be easily applied in a clinical setting. Although the correlation between Glut-1 and tumor pO2 measurements is weak, the assessment of tumor Glut-1 expression may be justified in the identification of changes in gene expression associated with chronic tumor hypoxia and metastatic spread. This might have an experimental or clinical application in selecting patients suitable for gene therapy approaches involving hypoxia responsive elements in gene therapy (58). The use of Glut-1 in the clinic will be validated further by the use of multiple biopsies and in combination with other hypoxia markers.

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