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Hypoxia-regulated glucose transporter Glut-1 may influence chemosensitivity to some alkylating agents: Results of EORTC (First Translational Award) study of the relevance of tumour hypoxia to the outcome of chemotherapy in human tumour-derived xenografts

R.E. AIRLEY[1*], R.M. PHILLIPS[2*], A.E. EVANS[1], J. DOUBLE[2], A.M. BURGER[3], H.H. FEIBIG[3], C.M.L. WEST[4] and I.J. STRATFORD[5]

[1]Tumour Metabolism And Therapeutics Group, School of Pharmacy and Chemistry, John Moores University, Liverpool, L3 3AF; [2]Tom Connors Cancer Research Unit, University of Bradford, Bradford, UK; [3]Tumour Biology Centre, University of Freiburg, Freiburg, Germany; [4]CRUK Department of Radiation Oncology, Christie Hospital NHS Trust; [5]Experimental Oncology Group, School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester, M13 9PL, UK

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Abstract. Tumour hypoxia confers poor prognosis in a wide range of solid tumours, due to an increased malignancy, increased likelihood of metastasis and treatment resistance. Poorly oxygenated tumours are resistant to both radiation therapy and chemotherapy. However, although the link between radiation therapy and hypoxia is well established in a range of clinical studies, evidence of its influence on chemotherapy response is lacking. In this study, a panel of human tumour-derived xenografts that have been characterised previously for *in vivo* response to a large series of anti-cancer agents, and have been found to show chemosensitivities that correlate strongly with the parent tumour, were used to address this issue. Immunohistochemistry was carried out on formalin-fixed, paraffin-embedded sections of xenograft samples to detect expression of the intrinsic hypoxia marker Glut-1 and adducts of the bioreductive hypoxia marker pimonidazole. Glut-1 scores correlated significantly with T/C values for CCNU sensitivity (r=0.439, P=0.036, n=23) and showed a borderline significant correlation with dacarbazine T/C (r=0.405, P=0.076, n=20). However, there was no correlation between both Glut-1 and pimonidazole scores and T/C obtained for the bioreductive drug mitomycin C. The use of human tumour-derived xenografts offers a potentially useful way of using archival material to determine the influence of hypoxia and other tumour-microenvironmental factors on chemosensitivity without the direct use of human subjects.

Correspondence to: Dr Rachel Airley, School of Pharmacy and Chemistry, Liverpool John Moores University, Byrom Street, Liverpool, L3 3AF, UK
E-mail: r.airley@livjm.ac.uk

*Contributed equally

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Introduction

The tumour microenvironment is extremely hostile, being deprived of oxygen, glucose and more acidic than normal tissue. Many tumours contain regions of low oxygen tension generally thought to arise as a consequence of poor and disorganised blood supply, and rapid tumour cell proliferation (1). It is well established both experimentally and clinically that hypoxia leads to radiation resistance, and ultimately, poor local control and disease-specific survival in patients treated with radiation therapy. For example, measurements of O_2 tension in tumours prior to radiation therapy revealed hypoxia to be an independent prognostic indicator in head and neck cancer (2), soft tissue sarcomas (3) and advanced carcinoma of the cervix (4,5). Hypoxic tumours are also more malignant and more likely to metastasise, which may be due to the promotion of genomic instability associated with carcinogenesis and malignant progression (6,7).

The influence of tumour hypoxia on the sensitivity of tumours to many chemotherapeutic drugs may depend upon several factors. Firstly, hypoxia may increase the G_0 fraction, which will decrease sensitivity to phase-specific drugs. Secondly, whereas hypoxia-induced radiation resistance arises from the need for molecular oxygen to 'fix' radiation-induced DNA damage (8) certain conventional anti-cancer agents depend upon the presence of oxygen for activation. Finally, the lack of structurally and functionally normal tumour blood vessels may inhibit the delivery of chemotherapy (9). However, so far, although the connection between hypoxia and chemotherapy resistance is logical, there is a lack of experimental or clinical evidence supporting this hypothesis.

To draw any conclusions relating to the level of tumour hypoxia, it is necessary to use a reliable marker of hypoxia. Although oxygen electrodes may be used to obtain direct oxygen measurements, this is expensive, invasive and some tumours are inaccessible. This problem may be partially overcome by the use of the 2-nitroimidazole bioreductive hypoxia marker pimonidazole, which, when administered approximately 16 h prior to biopsy, is activated in hypoxic areas of the tumour, forming intracellular adducts that may be detected immunohistochemically (10,11). Pimonidazole is used routinely in experimental approaches, and has been evaluated as a clinical marker of hypoxia in advanced carcinoma of the cervix and head and neck cancers (12,13). However, because the drug must be administered prospectively, if studies necessitate the use of archival material, the administration of pimonidazole is not possible.

To adapt and flourish in hypoxia, several classes of hypoxia-inducible genes may be upregulated. This gene expression is mediated via the transcription factor HIF-1 (hypoxia-inducible factor), although there may also be interaction with the recently discovered HIF-2α and HIF-3α (14,15). These genes include the hypoxia-regulated facilitative glucose transporter Glut-1, which mediates a switch to anaerobic glycolysis as the major energy source of the tumour cell, and which may be partially or wholly responsible for the increased glucose uptake observed in tumours. Glut-1 is overexpressed in a wide range of tumours (16), and predicts poor survival and poor metastasis-free survival after treatment with surgery or chemoradiotherapy (17-24). Glut-1 has been clinically validated as an intrinsic marker of hypoxia in advanced carcinoma of the cervix, and shows a significant statistical correlation with hypoxia as defined by oxygen electrodes (17) and pimonidazole binding (25). The major advantage of intrinsic markers of hypoxia is that whereas they are also detectable in biopsy material using immunohistochemical techniques; they require no invasive procedure other than routine diagnostic biopsy, which is highly desirable in a clinical setting. However, another advantage of intrinsic markers that does not apply to the use of oxygen electrodes or pimonidazole is that they may be used in archival material, which has applications in retrospective translational studies using clinical or xenograft material. A chemical marker such as pimonidazole may be used to reinforce data provided by intrinsic markers, but offers the advantage of being independent from the genetic changes associated with malignancy. These include the expression of the genes c-Myc or h-Ras, which mediate HIF-1-regulated genes in normoxic conditions, and may therefore confound any evaluation of tumour hypoxia (26,27). However, it must be considered that different hypoxia markers may reflect different populations of hypoxic cells, ranging from the acutely to chronically hypoxic. For example, whereas the extent of pimonidazole binding depends upon pharmacokinetic parameters such as the rate of accumulation in a tumour, and the enzymatic activation of bioreductive drugs by NADPH: cytochrome P_{450} reductase (P450R) (28), the hypoxia-induced upregulation of Glut-1 protein synthesis only occurs after activation and translocation of pre-existing glucose transporters (29). Therefore, in our experience, although pimonidazole and Glut-1 immunostaining may show similar spatial patterns, the area and intensity of staining may vary.

The Freiburg group have established a large panel of human tumour xenografts and around 250 of these human tumours at early passage have been well characterised for growth and shown typical features in terms of histology and differentiation consistent with the original tumour taken from patients. A subset of these tumours has also been characterised for their response to conventionally used cancer chemotherapeutic agents used either alone or in combination. When compared with patient response, a significant correlation was observed, with the xenografts giving a 96% prediction of resistance and a 90% prediction for tumour responsiveness. Drug sensitivity expressed as T/C (ratio of volumes of treated tumour to untreated tumour) for each drug have been characterised and described previously (30). Therefore, the examination of material from these xenografts allows important links to be drawn between the properties of this archival material and *in vivo* chemotherapy response, which can be in turn extrapolated back to the chemotherapy response of the respective human tumours. In this study, we have evaluated the extent of tumour hypoxia using pimonidazole and Glut-1 immunohistochemistry, in order to capture different hypoxic populations. In doing so, we aim to establish the effect of hypoxia on response to these commonly used anti-cancer agents. In lieu of a large-scale clinical study, which for the wide range of agents used in this study would present multiple problems; the use of this experimental model presents an attractive alternative.

Materials and methods

Tissue collection. Sixty tumours were selected from the Freiburg panel of human tumour-derived xenografts that were representative of the common human malignancies, and included lung, breast and colon tumours. For this study, tumours were previously characterised for chemosensitivity/resistance to a range of anti-cancer agents, where T/C values (ratio of treated tumour volume to untreated tumour volume), where determined, and are shown in Table I. Xenografts were generated from tumour pieces obtained from early passage stock implanted subcutaneously into the flanks of female nu/nu mice and allowed to grow to an approximate volume of 200 mm^3. The mice could then treated with the chosen agents, and at an appropriate time after treatment, the mice sacrificed, tumours excised and sliced into two pieces. Of these, one piece was immediately frozen in liquid N2 to enable analysis of bioreductive enzymes, and the other was formalin-fixed for subsequent immunohistochemical analysis of markers of hypoxia.

Measurement of tumour hypoxia. Tumour hypoxia was assessed by immunohistochemical methods, which were used to detect hypoxia markers in formalin-fixed, paraffin-embedded xenograft material. These included the 2-nitroimadazole bioreductive hypoxia marker, pimonidazole (31) and the HIF-1-regulated 'intrinsic' marker of hypoxia Glut-1 (17,25).

Pimonidazole binding. Pimonidazole HCl (Hypoxyprobe-1, Natural Pharmacia International Inc.) was administered to mice at a single dose of 100 mg/kg followed by sacrifice and excision of tumours 6 h later. Immunostaining for pimonidazole adducts was carried out as described by Raleigh

Table I. Spearmans rank correlations between T/C (ratio of volumes of treated to untreated tumour) and hypoxia marker scores; correlations derived from mean and separate scores are shown.

Drug	Pimonidazole (mean score of duplicates)	Pimonidazole (duplicates as separate values)	Glut-1 (mean score of duplicates)	Glut-1 (duplicates as separate values)
Adriamycin	r=0.166 P=0.371 n=31	r=-0.65 P=0.587 n=72	r=0.100 P=0.585 n=32	r=-0.020 P=0.862 n=75
Bleomycin	r=0.000 P=1.000 n=10	r=-0.328 P=0.199 n=17	r=0.025 P=0.945 n=10	r=-0.048 P=0.849 n=18
Mitomycin C	r=-0.141 P=0.493 n=26	r=-0.080 P=0.531 n=64	r=0.315 P=0.116 n=26	r=0.183 P=0.144 n=65
Mitoxantrone	r=0.021 P=0.965 n=7	r=-0.265 P=0.210 n=24	r=-0.039 P=0.934 n=7	r=-0.220 P=0.292 n=25
Vincrististine	r=0.018 P=0.944 n=18	r=0.056 P=0.730 n=40	r=0.436 P=0.071 n=18	r=0.218 P=0.165 n=42
Vinblastine	r=0.087 P=0.778 n=13	r=-0.290 P=0.159 n=25	r=0.228 P=0.454 n=13	r=-0.101 P=0.623 n=26
Vindesine	r=0.072 P=0.691 n=33	r=0.065 P=0.577 n=77	r=0.157 P=0.384 n=33	r=0.018 P=0.872 n=79
Etoposide	r=0.094 P=0.749 n=14	r=-0.262 P=0.128 n=35	r=0.314 P=0.275 n=14	r=-0.158 P=0.351 n=37
CCNU	r=0.069 P=0.761 n=22	r=0.347 P=0.014 n=50	r=0.439[a] P=0.036 n=23	r=0.414[b] P=0.002 n=52
Cyclophosphamide	r=-0.188 P=0.338 n=28	r=-0.125 P=0.304 n=70	r=0.213 P=0.267 n=29	r=0.127 P=0.288 n=72
Dacarbazine	r=0.285 P=0.237 n=19	r=0.277 P=0.079 n=41	r=0.405 P=0.076 n=20	r=0.615[b] P≤0.001 n=44
HECNU	r=0.519 P=0.153 n=9	r=0.448[a] P=0.048 n=20	r=0.604 P=0.085 n=9	r=0.687[b] P≤0.001 n=22
Ifosfamide	r=0.104 P=0.724 n=14	r=0.215 P=0.273 n=28	r=0.418 P=0.137 n=14	r=0.436[a] P=0.018 n=29
Cisplatin	r=0.036 P=0.865 n=25	r=-0.014 P=0.915 n=63	r=0.208 P=0.319 n=25	r=0.160 P=0.199 n=66
Fluorouracil	r=0.121 P=0.633 n=18	r=0.002 P=0.992 n=38	r=-0.121 P=0.634 n=18	r=-0.148 P=0.368 n=39
Methotrexate	r=0.816 P=0.184 n=4	r=0.200 P=0.493 n=14	r=-0.816 P=0.184 n=4	r=-0.156 P=0.595 n=14

[a]Correlation is significant at the 0.05 level (two-tailed). [b]Correlation is significant at the 0.01 level (two-tailed).

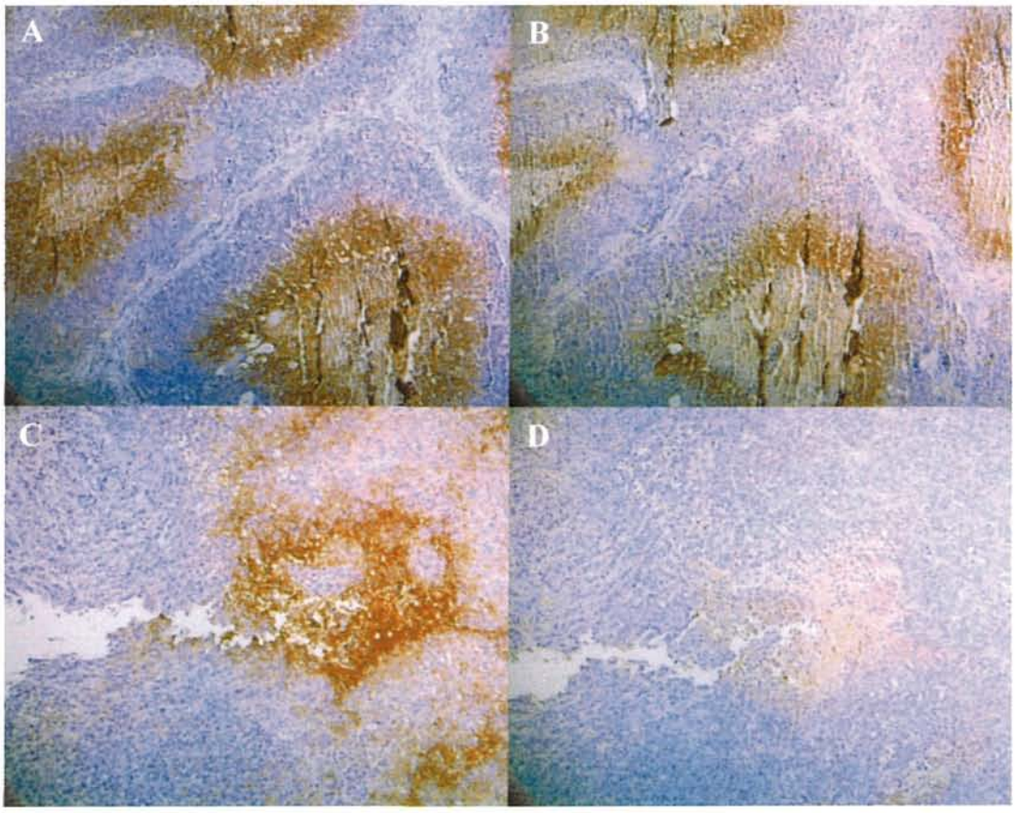


Figure 1. Hypoxia marker staining in formalin-fixed, paraffin-embedded sections from xenograft material. (A) and (C) show Glut-1 expression and (B) and (D), pimonidazole adduct binding is peri-necrotic, i.e. in areas likely to be hypoxic. Although there was considerable spatial co-localisation, the intensity of staining often varied between the two markers. Magnification, x100.

et al (11). Briefly, immunohistochemical staining was performed, according to protocol, using an Envision kit (Dako) containing an anti-mouse secondary antibody labelled polymer conjugate. An additional antigen retrieval step was carried out after blocking of endogenous peroxidases, where sections were incubated in 0.05% pronase (Dako) in TBS (pH 7.2) for 5 min at room temperature. The primary antibody step consisted of incubation for 30 min, at room temperature, in the presence of a 1/100 dilution of mouse monoclonal antibody raised against intracellular pimonidazole protein adducts (Natural Pharmacia International Inc.).

Glut-1 protein expression. Immunostaining for Glut-1 expression was carried out as described previously (25), according to protocol using Envision kits containing rabbit secondary antibodies (Dako). Primary antibody step was carried out for 1 h at 37°C with a 1/100 dilution of affinity purified anti-rabbit Glut-1 (Alpha Diagnostic International, TX, USA). For each subsequent run, two batch controls were used to rule out any inter-batch variation.

Scoring system. Semi-quantitative scoring was carried out as previously, where the extent of staining was designated as 0, no staining; 1, light staining; 2, moderate staining; and 3, heavy staining. Areas of stroma, necrosis and normal tissue were excluded. To exclude the effects of intra- and inter-observer variation, a series of 30 sections stained with Glut-1 was scored on two occasions by the same observer, a minimum of 6 weeks apart, and by two different observers.

Results

Xenografts derived from 38 human tumours were generated, consisting of 5 melanomas, 1 hepatoma, which was of mixed histology, 2 lung adenocarcinomas, 3 non-small cell lung, 3 epidermoid lung, 2 pancreatic, 5 renal, 3 colon, 7 breast, 1 CNS, 1 prostate, 1 bladder and 2 gastric tumours. For each tumour designation, between 1 and 9 xenografts were generated (mean 2.59, median 1) so that analysis of hypoxia marker staining could be carried out in duplicate to give either a mean score or several scores derived from the same tumour designation.

Hypoxia markers. Examples of typical patterns of Glut-1 and pimonidazole staining are shown in Fig. 1. Glut-1 staining was membranous and typically expressed peri-necrotically, whereas pimonidazole, although showing substantial co-localisation with Glut-1, was cytoplasmic. The pattern of staining was spatially similar, although the total area of staining varied between the two markers. However, there were significant correlations between Glut-1 and pimonidazole scores, where either mean scores ($r=0.416$, $P=0.012$, $n=36$) or separate scores from duplicate tumour designations ($r=0.336$, $P=0.002$, $n=79$) were used (Fig. 2). To rule out inter-observer

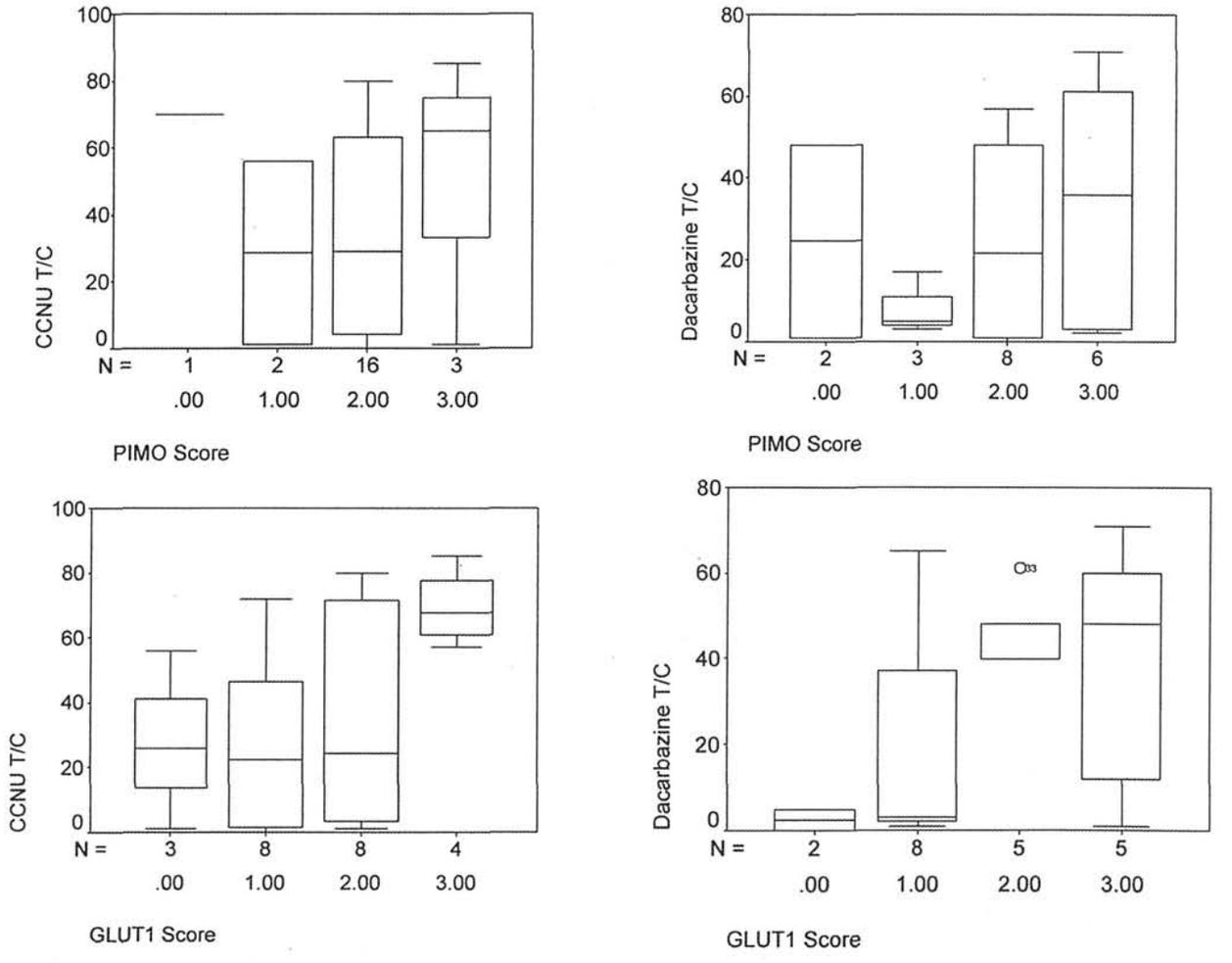
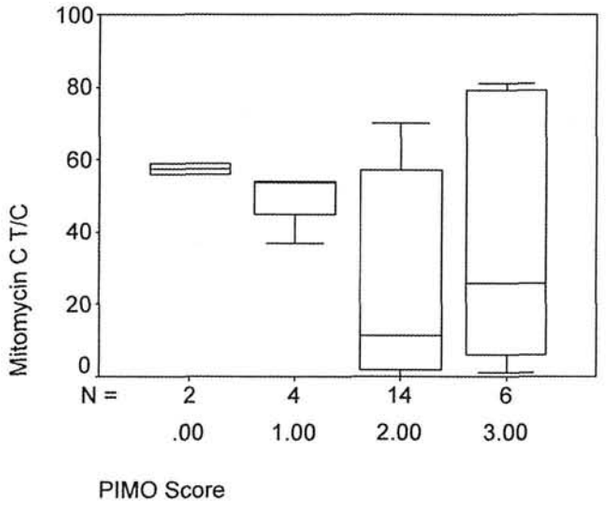


Figure 2. Correlation between pimonidazole/Glut-1 and the alkylating agents CCNU and dacarbazine (compiled using mean scores from several xenografts of the same tumour designation).

error, series of samples were scored for Glut-1 by two independent observers (RA and AE) (r=0.882, P≤0.001, n=28). To rule out intra-observer error, RA scored a series of samples stained for pimonidazole on two occasions approximately 6 weeks apart (r=0.841, P≤0.001, n=20).

Expression of hypoxia markers according to tumour type. Pimonidazole staining was determined in 36/38 xenografts. Of these, using mean scores, there was an absence of pimonidazole binding (0) in 2 cases, whereas 4 (11.1%) showed light staining (1); 21 (58.3%) were moderately stained (2) and 9 (25%) were heavily stained (3) for pimonidazole adducts. Glut-1 expression was determined in 37/38 xenografts. Of these, 4 (10.8%) showed no Glut-1 staining, 12 (32.4%) light staining, a further 12 (32.4%) moderate levels of staining and 9 (24.3%) cases stained heavily for Glut-1.

Correlations between hypoxia markers and T/C. Spearman's rank correlations of Glut-1 and pimonidazole with drug sensitivity using scores obtained from all samples and mean scores derived from duplicate xenografts were, in most cases, insignificant at the P=0.05 level (Table I). The use of scores from all available samples increased the statistical significance of the resulting correlations, although this was likely to be a consequence of this method of analysis. Using mean scores, there were no statistically significant correlations between hypoxia markers and sensitivity to either adriamycin or mitomycin C, which are known to be bioreductively activated. Interestingly, though, whereas negative correlations existed between T/C values obtained for mitomycin C and pimonidazole (r=-0.141, P=0.493, n=26), the correlation with Glut-1 was positive (r=0.315, P=0.116, n=26) (Fig. 3). Additionally, Glut-1 score correlated significantly with T/C obtained for CCNU (lomustine) (r=0.439, P=0.036, n=23), and showed borderline statistical significance with T/C values obtained for dacarbazine (r=0.405, P=0.076, n=20) and HECNU (r=0.604, P=0.085, n=9). However, there were no significant correlations between response to these drugs and pimonidazole score, unless scores from all available xenograft samples were used (Fig. 2). Thus, the data suggested a putative link between Glut-1 and resistance to nitrosourea alkylating agents, which may or may not be hypoxia-regulated.



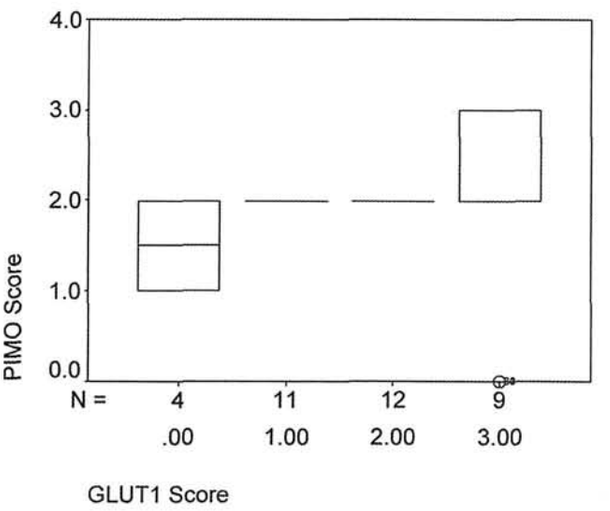


Figure 4. Correlation between Glut-1 and pimonidazole scores is significant (r=0.416, P=0.012, n=36 obtained from mean score values is shown here).

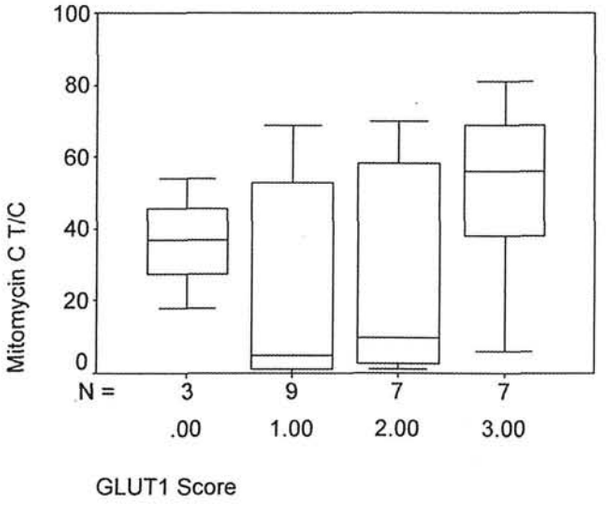


Figure 3. There was no significant correlation between T/C obtained for mitomycin C and hypoxia markers, although there were non-significant trends where pimonidazole score corresponded to chemosensitivity, whereas Glut-1 score corresponded to chemotherapy resistance, reflecting the possible hypoxia-independent effects associated with Glut-1 (correlations obtained from mean scores).

Discussion

The individualization of chemotherapy depends upon a reliable and clinically feasible means of detecting the heterogeneity that exists between tumours. In this study, we add to the understanding of the extent to which chemotherapy response in individual patients may be dependent upon inter-tumour variation of oxygenation. This study had a number of potential outcomes. Firstly, hypoxia or hypoxia-regulated genes would confer resistance to certain drugs. Oxygen is needed for the activation of bleomycin, and phase-specific drugs such as methotrexate depend upon the presence of cycling cells, therefore tumour hypoxia, indicated by high pimonidazole or Glut-1 scores would correlate with T/C values for these drugs (9). Secondly, hypoxia, along with expression of reducing enzymes such as DT-Diaphorase or P450R (32) might cause activation of bioreductive drugs, in this case mitomycin C. Finally, Glut-1 may confer resistance or sensitivity in a mechanism that is independent of hypoxia. In this study, the key findings are that Glut-1 may correlate with resistance to alkylating agents, particularly CCNU and dacarbazine. Glut-1 expression may mediate chemotherapy resistance via a range of mechanisms dependent on an increased rate of glucose uptake or the modified glucose metabolism taking place in tumours. One such change involves alterations in the relative rate of glycolysis and pentose phosphate pathway activation. It has been shown recently that a higher flux of glucose-derived carbon through the pentose phosphate pathway is prevalent in tumour cells, and that this is necessary to achieve the increased rate of DNA synthesis and therefore an increased proliferation rate (33).

Glut-1 is known to be a glucose-regulated protein as well as a hypoxia-regulated glucose transporter (34,35), and its characterization has been paralleled to a large extent by studies relating to glucose regulated stress proteins GRP78 and GRP94. The regulation of GRP78 and Glut-1 in particular have shown similarities in response to glucose deprivation and exposure to calcium ionophores (36). GRP78 and GRP94 are expressed in response to hypoxia and glucose deprivation (37) and their induction leads to resistance to doxorubicin (38). GRP-inducing conditions also lead to resistance to the topoisomerase II inhibitor etoposide, most notably via decreased expression of target enzyme (39), and partial resistance to vincristine, actinomycin D and mitomycin C (40,41). GRP78 expression correlates with multi-drug resistance proteins such as lung resistance protein LRP56 in lung tumours and is overexpressed in resistant lung tumour cell lines (42). In this present study, there was no significant correlation between Glut-1 expression and resistance to etoposide or vincristine, although Glut-1, in contrast to pimonidazole, showed a non-significant positive trend with T/C. Therefore, Glut-1 may reflect chemotherapy resistance independently of hypoxia via mechanisms common to both Glut-1 and GRP's.

While the adverse effect of Glut-1 on prognosis is widely known, little work has been carried out to examine the link between glucose transporter expression and chemoresistance. One study carried out by Cantuaria *et al* (43) in a series of patients with ovarian carcinoma, whilst showing Glut-1 to be a predictor of poor survival, also revealed counter intuitively that high Glut-1 expression predicts complete clinical response. However, any resistance mechanisms conferred by deregulated glucose transport and metabolism may be heavily dependent upon the type of agent used. In this present study, Glut-1 expression correlated with resistance to the nitrosourea alkylating agents dacarbazine and, to a certain extent, CCNU. However, expression of GRP78 is associated with hypersensitivity to these alkylating agents and also cisplatin, an effect which is believed to be due to impaired DNA cross-link repair (44,45), and is exacerbated by deficient poly (ADP-ribose) [p(ADPR)] metabolism (46). Recent work has also shown a link between the expression of GRP58 and mitomycin C-induced cross linking (47). Important to consider, though, is that the functions of Glut-1 and GRP78 are different: whereas Glut-1 leads directly to cellular glucose uptake and therefore a local increase in glucose metabolism, GRP78 functions as a molecular chaperone that is inactivated once the inducing conditions are removed (37). Also, the extent and duration of oxygen and glucose deprivation to induce GRP's and glucose transporters may differ, and in turn, the response to GRP-inducing stress by a tumour may be different to that observed *in vitro*.

The preferential activation of anti-cancer agents by hypoxia was most likely to occur with mitomycin C. To overcome the problems presented by the hypoxia-independent regulation of an intrinsic hypoxia marker such as Glut-1, it was essential to include a chemical marker of hypoxia in any assessment of the influence of hypoxia on the activation of this drug. In this study, there was no significant negative correlation between hypoxia marker staining and mitomycin C T/C i.e. hypoxia was not likely to reflect chemosensitivity. However, any activation by hypoxia would be dependent upon the level of bioreductive enzymes, therefore low pimonidazole binding or low expression of intrinsic markers of hypoxia may not have been sufficient to indicate chemosensitivity unless combined with considerable levels of bioreductive enzymes. Current investigations within our laboratories aim to uncover the combined effects of bioreductive enzymes and hypoxia on mitomycin C sensitivity using these models.

We have shown previously that each hypoxia marker may detect populations of cells within a tumour that have a varying depth and duration of hypoxia. This is reflected by the significant, but not strong, correlations between pimonidazole, Glut-1, and CAIX scores in a series of patients with advanced carcinoma of the cervix (25). However, this study was carried out using xenografts derived from a wide variety of tumour types, and was therefore subject to the statistical limitations of using a small and heterogeneous sample size. Despite these limitations, the correlations between the hypoxia markers used in this study persisted, strengthening the assertion that intrinsic markers of hypoxia are a reliable means of evaluating tumour hypoxia that will continue to be useful in future investigations involving archival material from a range of sources.

Acknowledgements

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