Microregional Expression of Glucose Transporter-1 and Oxygenation Status: Lack of Correlation in Locally Advanced Cervical Cancers

Arnulf Mayer,1 Michael Höckel,2 Alexander Wree,1 and Peter Vaupel1

Abstract Purpose: Glucose transporter-1 (GLUT-1), a target gene of hypoxia-inducible factor-1, has been considered a candidate endogenous marker of tumor hypoxia. Expression of GLUT-1 may also serve as an indicator for the induction of the transcriptional response to hypoxia, which has been linked to enhanced proliferation, resistance to therapy, and metastatic propagation of cancer cells. Overexpression of GLUT-1 has been shown to correlate with poor prognosis in several tumor entities, among them cancers of the uterine cervix. The validity of these hypotheses is investigated.

Experimental Design: The expression of GLUT-1 was assessed in 80 biopsies of Eppendorf oxygenation measurement tracks from locally advanced cervical cancers in 47 patients using immunohistochemistry.

Results: No correlation was found between the expression of GLUT-1 and oxygenation variables (median pO2, HF 2.5 and HF 5). Expression of GLUT-1 was found greater in larger tumors (P = 0.0001) and to exhibit a linear increase with Fédération Internationale de Gynécologie et d’Obstétrique stage (P = 0.002). Overall survival (P = 0.004) and recurrence-free survival (P = 0.007) were significantly shorter for patients with expression of GLUT-1. In the subgroup of patients treated with surgery, this effect on prognosis was not independent when pT stage or pN stage were included in a multivariate Cox proportional hazards model.

Conclusions: The suitability of GLUT-1 as an endogenous marker of tumor hypoxia seems questionable. The association with prognosis may partially depend on confounding factors.

Materials and Methods

Patients. All patients in this study were enrolled in a prospective clinical trial for the evaluation of the significance of tumor oxygenation in primary, locally advanced carcinomas of the uterine cervix that commenced at the Department of Obstetrics and Gynecology, University of Mainz Medical School, in June 1989. The study design was approved by the local Medical Ethics Committee, with patients...
giving informed written consent before being enrolled. All 47 patients from the former study, for whom one or two tumor biopsy specimens of the oxygen measurement tracks were available, were included in the present study. Patients of this subgroup had been recruited between August 1991 and April 1997. Table 1 shows relevant patient and tumor characteristics at the time of pretreatment oxygen tension (oxygen partial pressure, $pO_2$) measurements.

For correlations involving survival, only patients treated with curative intent were included ($n = 42$). In 31 of these, the primary therapy was surgical. Abdominal radical hysterectomy and pelvic/periaortic lymph node dissection was the standard surgical procedure. In 5 of 31 patients, suprapelvic exenteration had to be done instead of radical hysterectomy. The remaining 11 cases were treated by radiation instead of surgery because their tumors were fixed to the pelvic wall or their comorbidity excluded extensive operations. Primary radiotherapy was given as combined teletherapy and brachytherapy. External beam irradiation was applied with 10 MV photons produced by a linear accelerator at the Division of Radiotherapy. For brachytherapy, a high–dose rate 192Ir afterloading machine at the Department of Obstetrics and Gynecology was used (for details, see ref. 24). Adjuvant chemotherapy regimens are described in detail in Ref. 31. Median follow-up time was 28 months (SD 28), ranging from 4 to 95 months.

**Tumor oxygen tension measurements.** Tumor $pO_2$ was measured pretherapeutically with the computerized Eppendorf histography system (Eppendorf, Hamburg, Germany), using a protocol that has been described in detail previously (25). Briefly, $pO_2$ readings were done in the conscious patient along linear tracks, first in the s.c. fat of the mons pubis followed by cervical measurements at the 12 and 6 o’clock sites in macroscopically vital tumor tissue. Within the tumor tissue, up to 35 $pO_2$ measurements were made along each electrode track (70 readings in total) starting at a tissue depth of 5 mm. The individual $pO_2$ measurement points were situated 0.7 mm apart, resulting in an overall measurement track length of ~25 mm. Immediately following $pO_2$ measurements, needle core biopsies (obtained using Biopsy, Radioplast, Uppsala, Sweden) of ~2 mm in diameter and 20 mm in length were taken from those tumor areas where $pO_2$ readings had been obtained. Both the $pO_2$ readings and the needle core biopsies were done without general anesthesia in all patients. Intravaginal temperature, arterial blood pressure, heart rate, hemoglobin concentration, hematocrit, and arterial oxyhemoglobin saturation were monitored at the time when $pO_2$ readings were taken. The pretherapeutic $pO_2$ measurements were usually done 1 to 5 days before oncological treatment. After histologic examination of the biopsy specimens, $pO_2$ measurements in necrotic tissue areas were excluded from analysis.

**Immunohistochemistry.** Expression of GLUT-1 was assessed in 80 biopsy specimens taken from the tumor $pO_2$ measurement tracks obtained directly after $pO_2$ measurement in 47 patients. Two biopsies, corresponding to the 6 and 12 o’clock positions of the tumor center were available for each of 33 patients and one biopsy for each of the remaining 14 cases. All material was fixed in formalin before being embedded in paraffin. Histologic slides were prepared from the paraffin blocks and dried overnight at 37°C. Subsequently, specimens were dewaxed in two changes of fresh xylene and rehydrated in a descending alcohol series. Retrieval of antigenic binding sites was done by heating specimens in 10 mmol/L citrate buffer (pH 6.0) in a microwave oven for 17 minutes. GLUT-1 polyclonal rabbit anti-human GLUT-1 clone MYM (DakoCytomation, Hamburg, Germany) was used as the primary antibody at a concentration of ~1 ng/mL in antibody diluent (DakoCytomation, 1:200 dilution). Incubation took place overnight at 4°C. A biotinylated goat anti-mouse/rabbit secondary antibody was applied for 30 minutes at room temperature and further detection was carried out using a streptavidin-biotinhorseradish peroxidase system (Duet-Kit, DakoCytomation). In accordance with the manufacturer’s instructions. Negative control specimens were treated with antibody diluent without the primary antibody under the same conditions. Slides were counterstained with Mayer’s hematoxylin, dehydrated in an ascending alcohol series and covered with a coverslip using Eukitt mounting medium (Riedel-de Haen, Seelze, Germany).

**Assessment of GLUT-1 expression.** A semiquantitative scoring system was used to assess the degree of GLUT-1 expression in entire biopsy sections: score 0, no staining or only very few positive cells (“absent”); score 1, <10% positive (“weak”); score 2, 11% to 50% positive (“moderate”); score 3, >50% positive (“strong”). Each specimen was scored by two independent observers (A.M. and A.W.). Discordant cases were reevaluated and discussed using a conference microscope.

**Statistical analysis.** All statistical tests were done using the SPSS software package (version 11.5; SPSS, Inc., Chicago, IL). The

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Table 1. Patient and tumor characteristics at the time of pretherapeutic oxygen tension measurements

<table>
<thead>
<tr>
<th>FIGO stages</th>
<th>No. patients</th>
<th>Median</th>
<th>Range</th>
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<tr>
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<td></td>
<td></td>
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<tr>
<td>IVB</td>
<td>2</td>
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<tr>
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<td>3</td>
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NOTE: Numbers in brackets indicate deviations for the subgroup of patients treated with curative intent (survival correlations).

Abbreviations: ND, not documented; NA, not applicable, no surgical treatment (radiation only).
significance level was set at \( \alpha = 5\% \) for all comparisons. Linear correlations between two variables were described by Spearman’s rank correlation coefficient \((\rho)\). Two-sided Mann Whitney \(U\) tests and Kruskal Wallis tests were used for comparison of categorized variables. Survival estimates were calculated using the Kaplan-Meier method and differences between groups were assessed with log-rank statistics. The Cox proportional hazards model was used for the multivariate analysis of the effect of individual factors on survival.

**Results**

**GLUT-1 expression.** GLUT-1 expression exhibited a characteristic pattern, with staining intensity increasing as a function of distance from the vascularized tumor stroma (Fig. 1), being particularly strong in the viable cell layers immediately adjacent to necrotic areas. Erythrocytes and perineural tissue invariably stained positive. Variation of erythrocyte staining intensity was

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Fig. 1. Expression patterns and score of GLUT-1 expression (histologic photographs, 20× magnification) with respective oxygen tension \((pO_2)\) histograms for hypoxic (top) and normoxic tumors (bottom). Examples of high and low expression of GLUT-1 are depicted; \(n\) = number of \(pO_2\) readings in the respective measurement track. Note that positive staining (bottom right) is found almost entirely in erythrocytes.
very low, indicating a neglectable batch to batch variation in overall GLUT-1 immunoreactivity. No positive staining was seen in the vascular tumor stroma. GLUT-1 expression was present in 59 of 80 biopsies (~74%). Of these, GLUT-1 expression was weak in 37 cases (~63%), moderate in 18 cases (~30%), and strong in four cases (~7%).

GLUT-1 expression, clinical, and pathohistologic data. GLUT-1 expression increased linearly with Fédération Internationale de Gynécologie et d’Obstétrique (FIGO) stage ($r = 0.35, P = 0.002$; see Fig. 2). Significantly higher expression of GLUT-1 was also found in larger tumors ($r = 0.42, P = 0.0001$) and in tumors with a higher pT stage ($r = 0.34, P = 0.015$). GLUT-1 expression showed no correlation with histologic grading, pN stage, patient age, parity, or pretherapeutic hemoglobin level.

GLUT-1 expression and oxygenation data. The Kruskal-Wallis test showed no differences in median $pO_2$, hypoxic fraction $\leq$2.5 mm Hg (HF 2.5) and hypoxic fraction $\leq$5 mm Hg (HF 5) between the four GLUT-1 expression scores. There were also no statistically significant differences in GLUT-1 expression between individual categories (e.g., absent expression versus strong expression; Fig. 3). A subgroup analysis of squamous cell carcinomas only (n = 60) also showed no differences in any of the oxygenation variables between the four classes of intensity of GLUT-1 expression. A weak trend ($r = 0.34, P = 0.14, n = 20$) was seen for higher values of HF 5 in non–squamous cell histology cases with higher GLUT-1 expression. As Fig. 3 shows, some severely hypoxic tumors had weak to absent expression of GLUT-1 and normoxic tumors repeatedly showed moderate to strong GLUT-1 expression.

GLUT-1 expression and survival. Univariate Kaplan-Meier survival analysis showed significantly improved overall ($P = 0.004$) and recurrence-free ($P = 0.007$) survival for patients whose tumors entirely lacked (both biopsies negative in cases where two biopsies were available) expression of GLUT-1 (see Fig. 4). In addition, correlations with poorer overall and recurrence-free survival, respectively, were found for the presence of lymph node metastasis ($P = 0.0002$ and $P = 0.0001$), higher pT stage ($P = 0.02$ and $P = 0.0367$) and higher FIGO stage ($P = 0.0067$ and $P = 0.0248$). When either pT stage or pN stage were included in a multivariate Cox proportional hazards analysis (only applicable in cases treated with surgery), there was no significant independent influence of GLUT-1 expression on prognosis. Only pN stage remained a significant prognostic factor for overall ($P = 0.015$) and recurrence-free ($P = 0.007$) survival.

### Discussion

The primary aim of this study was to evaluate the suitability of GLUT-1 as an endogenous marker of tumor hypoxia. The expression of GLUT-1 was analyzed using immunohistochemistry in biopsy specimens taken from oxygenation measurement tracks done with the Eppendorf microsensor system. The fact that both measurements originate from identical tissue micro-areas is a novel feature of this study. Using this methodology, no correlation of GLUT-1 expression and oxygenation variables (median $pO_2$, HF 2.5 and HF 5) were found. Several severely hypoxic tissue biopsies showed weak or no expression of GLUT-1, whereas moderate to strong expression was repeatedly found in normoxic specimens. In a recent study, Airley et al. (4) described a weak, albeit statistically significant, correlation of higher GLUT-1 expression in cases with higher values of HF 2.5. This finding may in the first instance be interpreted as being contradictory to our results. On closer examination however, the suitability of GLUT-1 as an endogenous hypoxia marker seems highly questionable from the data of both studies. In agreement with our own results, Airley et al. (4) found no correlation of GLUT-1 expression with HF 5 and the study also does not mention a correlation with the median $pO_2$. Both studies show that GLUT-1 expression may be absent in a significant amount of severely hypoxic tumors and that well-oxygenated tumors may exhibit very strong expression of GLUT-1. Two recent studies compared GLUT-1 (and carbonic anhydrase IX) expression with the accumulation of the “hypoxia-marker” pimonidazole and found a strong spatial colocalization and correlation between the two variables, concluding that both proteins may be regarded as endogenous hypoxia markers (17, 26). This interpretation is problematic.
found no correlation between a further Similar to our findings, Hedley et al. (31) in a recent study necrotic tissue areas can be excluded as done in our study. hypoxia (acute and chronic), provided measurements in technique, assessing all pathophysiologically relevant types of picture of hypoxia acquired with the Eppendorf microsensor correlations have thus far only been shown for the "snapshot" (27–30). It also has to be kept in mind that prognostic role of GLUT-1 as an another "extrinsic" hypoxia marker, EF5, have been shown not to correlate with the oxygenation status, as directly measured with microelectrodes (27–30). It also has to be kept in mind that prognostic relevance of GLUT-1 expression on T stage and N stage has been described for other tumor entities (e.g., breast carcinoma; ref. 23) and colorectal cancer (16, 22). The only study that evaluated the prognostic effect of GLUT-1 expression on cancers of the uterine cervix found a significant correlation with prognosis only for metastasis-free survival. A possible dependency of this correlation on nodal status could not be analyzed, as only patients treated with radiotherapy were examined (4).

A remarkable finding in the present study is the correlation of GLUT-1 expression with FIGO stage, T stage, and maximum clinical tumor size. Correlations between GLUT-1 expression and tumor size have also been described by others (14, 39–41) and may have important pathophysiologic implications. Because it is known (24) that tumor oxygenation is independent of tumor size, the finding is consistent with a partial GLUT-1 activation by factors other than hypoxia. Because the expression pattern of GLUT-1 shows increasing intensity towards areas of necrosis and with increasing distance from the stroma (containing the microvessels), it is probable that environmental factors (e.g., glucose deprivation) are important for the expression of the protein. Activated oncogenes may have an effect on the degree of activation by these factors. This influence is likely to become more relevant in higher stages and larger tumors, because oncogenic mutations, accumulated during malignant progression, may become more prevalent.

In conclusion, from the data of the present study as well as from recent findings by other groups, the role of GLUT-1 as an endogenous marker of tumor hypoxia is questionable, at least for cancers of the uterine cervix. There is an association of GLUT-1 expression with prognosis, although correlations were not independent, but instead were due to the association of GLUT-1 expression with established factors of dominant prognostic relevance.

Expression of GLUT-1, much like HIF-1α, is induced by a variety of stimuli besides hypoxia. For GLUT-1, established inducing factors are glucose deprivation (33), oncogenic transformation (e.g., overexpression of c-MYC; ref. 34), inhibition of oxidative phosphorylation (35), angiotensin II (in mesangial cells; ref. 36), and osmotic stress (37, 38). According to our interpretation of the data, induction of HIF-1α and subsequent transactivation of GLUT-1 by hypoxia, although undoubtedly present, cannot be selectively identified due to the heterogeneous occurrence of the other above-mentioned factors in human cancer specimens.

Another important issue is the prognostic effect of GLUT-1 as a marker of the hypoxic response. In univariate analysis, an improved overall and recurrence-free survival in patients with completely absent GLUT-1 expression (i.e., two negative biopsies) was found. Multivariate Cox regression analysis revealed that both correlations were independent of FIGO stage, clinical tumor size, histologic grading, patient age, and peratherapeutic hemoglobin concentration. Inclusion of pT stage or pN stage into the model, however, abrogated the independent prognostic effect of GLUT-1 expression. The dependency of the prognostic relevance of GLUT-1 expression on T stage and N stage has been described for other tumor entities (e.g., breast carcinoma; ref. 23) and colorectal cancer (16, 22). The only study that evaluated the prognostic effect of GLUT-1 expression in cancers of the uterine cervix found a significant correlation with prognosis only for metastasis-free survival. A possible dependency of this correlation on nodal status could not be analyzed, as only patients treated with radiotherapy were examined (4).

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Fig. 4. Kaplan-Meier plots for overall (top) and recurrence-free (bottom) survival of GLUT-1-negative and GLUT-1-positive cases.
References


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