GLUT1 Expression Is Increased in Hepatocellular Carcinoma and Promotes Tumorigenesis

Accelerated glycolysis is one of the biochemical characteristics of cancer cells. The glucose transporter isoform 1 (GLUT1) gene encodes a key rate-limiting factor in glucose transport into cancer cells. However, its expression level and functional significance in hepatocellular cancer (HCC) are still disputed. Therefore, we aimed to analyze the expression and function of the GLUT1 gene in cases of HCC. We found significantly higher GLUT1 mRNA expression levels in HCC tissues and cell lines compared with primary human hepatocytes and matched nontumor tissue. Immunohistochemical analysis of a tissue microarray of 152 HCC cases revealed a significant correlation between Glut1 protein expression levels and a higher Ki-67 labeling index, advanced tumor stages, and poor differentiation. Accordingly, suppression of GLUT1 expression by siRNA significantly impaired both the growth and migratory potential of HCC cells. Furthermore, inhibition of GLUT1 expression reduced both glucose uptake and lactate secretion. Hypoxic conditions further increased GLUT1 expression levels in HCC cells, and this induction was dependent on the activation of the transcription factor hypoxia-inducible factor-1α. In summary, our findings suggest that increased GLUT1 expression levels in HCC cells functionally affect tumorigenicity, and thus, we propose GLUT1 as an innovative therapeutic target for this highly aggressive tumor. (Am J Pathol 2009, 174:1544–1552; DOI: 10.2353/ajpath.2009.080596)

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver and one of the most common tumors worldwide. Liver cirrhosis is the main predisposing condition, but the molecular pathogenesis of HCC is still not well understood. Morbidity and mortality correlate directly with surgical resectability of the primary tumor. However, outcome is mostly poor, because the majority of patients are diagnosed at an advanced stage, and only 10 to 20% of HCCs can be resected completely.

In the 1920s Otto Warburg made the observation that tumor cells use glycolysis instead of mitochondrial oxidative phosphorylation for energy production even under oxygen-rich conditions. Recently, the Warburg effect has experienced a revival because it has been shown that aerobic glycolysis governs tumor cell biology. Previous studies found differences in glycolytic capacity between HCC cells and hepatocytes, and positron emission tomography (PET) revealed the fluorine-18-fluorodeoxyglucose uptake value as an independent prognostic factor for HCC. Further, higher PET activity was shown to correlate with advanced tumor stages.

The glucose transporter isoform 1 (GLUT1, also known as SLC2A1; MIM no. 138140) is a key rate-limiting factor in the transport and metabolism of glucose in cancer cells. GLUT1 expression is primarily undetectable in normal epithelial tissues and benign epithelial tumors. However, GLUT1 is overexpressed in a significant proportion of human carcinomas. The apparent expression of a certain type of glucose transporter suggests an important role for this transporter in tumor biology. Therefore, it has been hypothesized that elevated GLUT1 expression by human carcinomas indicates an increased metabolic
state, enhanced utilization of energy, and an associated increase in aggressive, metastatic behavior. Actually, Glut1 protein expression confers poor prognosis in a wide range of solid tumors.\textsuperscript{11,12} Studies regarding GLUT1 expression in HCC have revealed inconclusive results, and the biological significance of GLUT1 expression in HCC remains unknown.\textsuperscript{13–19} Here, we show that GLUT1 expression is increased in a significant number of HCC cell lines and tissues, and high GLUT1 expression correlates with HCC proliferation and invasiveness. Furthermore, we found that siRNA-mediated abrogation of GLUT1 in HCC cell lines inhibits their proliferative and migratory potential. This suggests that increased GLUT1 expression in HCC does not only indicate an increased utilization of energy, which may correlate with an aggressive behavior, but directly causes tumorigenesis. Consequently, GLUT1 may serve as both a prognostic marker and a therapeutic target in HCC.

**Materials and Methods**

**Cells and Cell Culture**

The HCC cell lines HepG2 [American Type Culture Collection (Rockville, MD) HB-8065], PLC (American Type Culture Collection CRL-8024), and Hep3B (American Type Culture Collection HB-8064) were cultured as described.\textsuperscript{20} Primary human hepatocytes (PHHs) were isolated and cultured as previously described.\textsuperscript{21} Human liver tissue for cell isolation was obtained according to the guidelines of the charitable state-controlled foundation Human Tissue and Cell Research with the patient’s informed consent. Hypoxia was induced by incubation with 2,2'-dipyridyl (DP) (100 μM; Sigma Aldrich, Deisenofen, Germany) or exposure to 1% O\textsubscript{2} for the indicated periods of time. For pharmaceutical inhibition of hypoxia-inducible factor (HIF)-1 activity cells were incubated with 100 μM of 3-(5’-hydroxymethyl-2’-furyl)-1-benzylindazole (YC-1; Calbiochem, Darmstadt, Germany)\textsuperscript{22,23} or 10 nmol/L of echnomycin (Alexis Biochemicals, Lörrach, Germany).\textsuperscript{24}

**Human Tissues and HCC Tissue Microarray (TMA)**

HCC tissues and corresponding nonneoplastic liver tissues were obtained from HCC patients (child A/B cirrhosis) undergoing surgical resection at the university hospitals Regensburg (n = 85) and Erlangen (n = 67). TMAs were constructed as described.\textsuperscript{20} Further, tissue samples of 31 patients were immediately snap-frozen, stored at −80°C, and subsequently used for RNA isolation and analysis of mRNA expression. Clinicopathological patient characteristics are summarized in Table 1.

**Expression Analysis**

Isolation of total cellular RNA from cultured cells and tissues and reverse transcription were performed as de-

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**Table 1.** Glut1 Immunoreactivity (IR) in HCC Tissue of 152 Patients in Relation to Clinicopathological Characteristics and Proliferation Rate

<table>
<thead>
<tr>
<th>Variable</th>
<th>Categorization</th>
<th>n</th>
<th>Glut1 IR-negative</th>
<th>Glut1 IR-positive</th>
<th>(P^*)</th>
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<td>Clinicopathological characteristics</td>
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<td>4.6</td>
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</table>

*Fisher’s exact test (two-sided); bold face represents \(P\) values < 0.05.
nd, no data available; IR, immunoreactivity.
nally, the tissues were counterstained by hemalaun. Visualized using AEC-solution (LSAB2-Kit, DAKO). Further, cell number was determined by micro-

scopic counting after trypsinization of cells seeded in six-well plates (six per condition) at different time points. Migration assays were performed as previously described.20

PET

PET using 2-[18F]fluorodeoxyglucose (FDG-PET/CT) was performed by means of a Biograph16 PET/CT scanner (Siemens, Erlangen, Germany). After fasting for 4 to 6 hours to achieve blood glucose values <120 mg/dl, the patients received an intravenous dose of FDG (5 to 10 mCi, 185 to 379 MBq). Whole-body image acquisition from the skull base to the proximal thigh started ~60 minutes later (axial field 90 cm; seven bed positions for 3 minutes each, one head/neck position). The total time required was ~20 minutes (40 seconds CT scanning and subsequent CT attenuation-corrected PET scanning, low-dose CT).

Results

GLUT1 Expression in HCC

Initially, we analyzed GLUT1 mRNA expression in three different HCC cell lines (HepG2, PLC, and Hep3B) and PHHs by quantitative real-time PCR. In all three HCC cell lines a significantly increased GLUT1 mRNA expression was observed compared with PHHs (Figure 1A). This result was confirmed by Western blotting (Figure 1B).

Next, we analyzed a panel of 22 paired specimens obtained from patients with HCC. From each HCC patient, RNA was isolated from cancerous tissue and adjacent nontumorous liver tissue, and PHHs by quantitative real-time PCR. In all three HCC cell lines a significantly increased GLUT1 mRNA expression was observed compared with PHHs (Figure 1A). This result was confirmed by Western blotting (Figure 1B).

To assess GLUT1 expression in HCC in situ, we performed immunohistochemical staining for Glut1 protein. A representative immunohistochemical staining of a Glut1-positive tumor is presented in Figure 1D. Immunohistochemistry revealed a strong membranous signal in HCC.
GLUT1 was detectable \((n = 24; 3.4 \pm 1.1\text{-fold}; P = 0.0015)\). This finding indicates that highly increased Glut1 expression is accurately detected by immunohistochemistry (IHC). However, lower expression may be below the detection limit of IHC. Herewith, differences between HCC and nontumorous liver may be missed, and probably, Glut1 protein is increased in even more cases than now shown by IHC.

For descriptive data analysis, clinicopathological characteristics were compared with GLUT1 mRNA and protein expression. GLUT1 mRNA expression correlated significantly with tumor stage \((r = 0.37, P = 0.039)\), grading \((r = 0.48, P = 0.007)\), and proliferation rate (MiB-1 index; \(r = 0.62, P = 0.002\)). Immunohistochemistry confirmed these data on the protein level (Table 1). Glut1 expression was significantly associated with higher tumor stage \((P = 0.023)\) and tumor grading \((P < 0.0001)\). Furthermore, Glut1-positive HCCs had a significantly higher proliferation rate (MiB-1 index) compared with Glut1-negative HCCs \((P = 0.006, \text{Figure } 1E)\). No correlation was found between Glut1 expression and age, gender, tumor size, and the existence of liver cirrhosis. The etiology of the underlying liver disease was known in only approximately half of the patients \((73 \text{ of } 152)\). In most cases, HCC had developed in alcohol-related cirrhosis \((52 \text{ of } 73, 71\%)\), and this percentage was similar in the groups of Glut1-negative \((45 \text{ of } 64, 70\%)\) and -positive \((7 \text{ of } 9, 78\%)\) HCCs.

**Molecular Mechanisms of GLUT1 Expression in HCC**

The transcription factor HIF-1\(\alpha\) is an important mediator of hypoxic adaptation of tumor cells and controls several genes that have been implicated in tumor growth including GLUT1. Therefore, we analyzed HIF-1\(\alpha\) protein expression in three different HCC cell lines \((\text{HepG2, PLC, and Hep3B})\) grown under aerobic conditions in vitro. Interestingly, no HIF-1\(\alpha\) expression could be detected in HCC cells by Western blotting (Figure 2A). In contrast, after pharmacological HIF induction by DP, strong HIF-1\(\alpha\) protein expression was detected in all three HCC cell lines by Western blotting (Figure 2A).

In line with this finding, transfection of Hep3B cells with a luciferase reporter plasmid containing six copies of a functional hypoxia-responsive element (HRE) revealed only baseline activity under aerobic conditions (Figure 2B). In contrast, strong HRE reporter gene activity was observed in Hep3B cells under DP-induced hypoxia, and this activity was completely abrogated by pharmacological inhibition of HIF-1\(\alpha\) activity with YC-1,22,23 or echinomycin.24 Similar results were obtained with HepG2 and PLC cells (data not shown). Interestingly, GLUT1 expression was further increased in HCC cells under DP-induced hypoxia, and this induction was strongly repressed by inhibition of HIF-1\(\alpha\) activity with YC-1 or echinomycin (Figure 2C). Similarly as DP-induced hypoxia, also culture of HCC cells under hypoxic conditions led to a significant increase of GLUT1 mRNA expression in all three HCC cell lines (Figure 2D).

Previous studies in glia cells have shown that hypoxia alters GLUT1 expression post-transcriptionally by en-
exposed to normoxic or hypoxic conditions, actinomycin D (Act.D, 7.5 μM) in 1% oxygen for 16 hours. * 
the transcriptional level by HIF-1 under hypoxic conditions, and this induction is regulated on

A:

hypoxic conditions, indicating that hypoxia does not affect mRNA expression in HCC cells compared with PHHs (data not shown). In summary, these data indicate that constitutively high GLUT1 mRNA expression in HCC cells under normoxic conditions is at least in part dependent on the transcription factor MAZ, and that GLUT1 mRNA expression is further increased under hypoxic conditions by HIF-1α activation.

Inhibition of GLUT1 Expression in HCC Cells

To gain insight into the functional role of increased GLUT1 in HCC, we inhibited GLUT1 expression in HCC cells by transient transfection with two different GLUT1 siRNAs. Quantitative real-time PCR analysis revealed a strong down-regulation of GLUT1 mRNA in Hep3B cells transfected with GLUT1 siRNA (siRNA1 and siRNA2) as compared with Hep3B cells transfected with control siRNA and nontransfected cells, respectively (Figure 3A). Down-regulation of GLUT1 expression in Hep3B cells transfected with GLUT1 siRNA was also confirmed at the protein level (Figure 3B).

FACS analysis of Alexa Fluor 488-labeled control siRNA revealed a transfection efficiency of ~90.6 ± 0.8% (data not shown). Furthermore, we studied the duration of the inhibitory effect of GLUT1 siRNA on GLUT1 mRNA expression in HCC cells and found that GLUT1 mRNA remained reduced for at least 4 days after transfection (data not shown). Comparable results regarding efficiency of transfection and GLUT1 expression with GLUT1 siRNA were obtained in PLC and HepG2 cells (data not shown).

Effect of GLUT1 Inhibition on Migration and Proliferation of HCC Cells

To further characterize the role of GLUT1 in HCC cells, we performed functional in vitro assays with HCC cells by suppressing GLUT1 expression (GLUT1 siRNA1 and GLUT1 siRNA2) in comparison with HCC cells transfected with control siRNA and nontransfected HCC cells. Inhibition of GLUT1 expression caused significantly impaired migration as analyzed in Boyden chamber assays (Figure 3C). Next, we analyzed whether GLUT1 expression affected the proliferation of HCC cells in vitro. HCC cells with suppressed GLUT1 expression grew significantly slower compared with controls cultured in monolayers (Figure 3D). In addition, we compared the growth of HCC cells with suppressed GLUT1 expression and control cells in a three-dimensional cell culture model. Transfection with GLUT1 siRNA resulted in the formation

hancing GLUT1 mRNA stability. Therefore, we analyzed GLUT1 mRNA expression in HCC cells at different time point after exposure to DP-induced hypoxia with or without pretreatment with actinomycin D (ActD), an inhibitor of transcription. ActD treatment resulted in a decline of GLUT1 mRNA levels with time indicating a GLUT1 mRNA half-life larger than 12 hours, similarly as previously reported in neurons (Figure 2E). No significant difference was found between HCC cells grown under normoxic and hypoxic conditions, indicating that hypoxia does not affect mRNA stability in HCC cells. Together, these data indicate that basal GLUT1 expression in HCC is further increased under hypoxic conditions, and this induction is regulated on the transcriptional level by HIF-1α activation.

In search for the molecular mechanisms that cause the increased expression of GLUT1 in HCC cells under normoxic conditions we performed in silico promotor studies using the Genomatix software. Alignment of the promotor sequence of the murine, rat, and human GLUT1 gene revealed that a binding site for the transcription factor MAZ (Myc-associated zinc finger protein, located 310 bp upstream of the transcriptional start site of the human GLUT1 gene) was highly conserved in all three species. Similarly as previously described we found increased MAZ expression in HCC cells compared with PHHs (data not shown), and noteworthy, transient transfection with two different MAZ siRNAs significantly inhibited GLUT1 mRNA expression in Hep3B cells (Figure 2F) as well as in HepG2 and PLC cells (data not shown). In summary, these data indicate that constitutively high GLUT1 mRNA expression in HCC cells under normoxic conditions is at least in part dependent on the transcription factor MAZ, and that GLUT1 mRNA expression is further increased under hypoxic conditions by HIF-1α activation.

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of significantly smaller spheroids compared with spheroids of control cells (Figure 3E).

Effect of GLUT1 on Glucose Uptake and Glycolytic Rate in HCC

HCC cells secreted significantly more lactate into the supernatant than PHHs (Figure 4A). In Hep3B cells transiently transfected with two different GLUT1 siRNAs (siRNA1 and siRNA2), and cells transfected with control siRNA and nontransfected HCC cells (ctrl.), glucose uptake is reported as glucose utilization per cell within 24 hours. To that end, glucose concentration was measured in the supernatant and cells were counted 24 hours after seedings. All experiments have been performed at least three times. Data are given as mean ± SEM (*P < 0.05 compared with controls).

Discussion

The aim of this study was to analyze the expression and function of GLUT1 in HCC. Previous studies on the expression of GLUT1 in human and murine HCC tissue and cells had revealed discrepant results. In some studies a strong and increased expression of GLUT1 mRNA had been found in HCC as compared with adjacent nonneoplastic tissue in >80% of cases. Other studies, in contrast, failed to detect Glut1 protein expression by immunohistochemical staining in HCC tissue in most, if not all cases. However, these previous studies had been performed in only a small number of cases and...
GLUT1 expression had been measured only at the mRNA or the protein level.

In the present study, we investigated GLUT1 mRNA and protein expression in 152 HCC cases by quantitative real-time PCR and immunohistochemical analysis, respectively. We found an increased expression of GLUT1 mRNA and protein in all HCC cell lines compared with PHHs. Furthermore, 68.2% of human HCC tissues revealed higher GLUT1 mRNA levels than adjacent noncancerous liver tissue. By immunohistochemical analysis Glut1 was detectable in only 13.2% of the HCC tissues, but in none of the noncancerous liver tissues analyzed. An explanation for the discrepant GLUT1 mRNA and protein expression data may be, that the antibodies and/or the immunodetection methods used here and in the previous studies lacked sufficient sensitivity to detect Glut1 in HCC, in which it was expressed at low level. Supportive for this hypothesis, we found a close correlation between GLUT1 mRNA and protein expression, and only HCCs with the highest GLUT1 mRNA levels revealed a visible Glut1 immunosignal, suggesting that there is a threshold that allows the detection of Glut1 protein by immunohistochemistry. In addition, in all three HCC cell lines analyzed treatment with GLUT1 siRNA significantly suppressed both GLUT1 mRNA and protein expression, further suggesting that GLUT1 expression in HCC is at least in part regulated at the transcriptional level.

Biological Significance of GLUT1 Expression in HCC

The expression of GLUT1 in a significant number of HCCs and its lower or undetectable expression in corresponding normal and benign hepatic tissue indicate that this transporter probably plays an important role in the uptake of glucose by HCC cells. Glucose is a major source of energy, and increased GLUT1 expression may indicate an increased utilization of energy, which in turn may correlate with aggressive behavior of cancer cells. Indeed, GLUT1 overexpression was associated with parameters conferring more aggressive behavior in several solid tumor types.11,12 Actually, we found that GLUT1 expression was a rate-limiting factor for the uptake of glucose and glycolysis in HCC cells. We also showed for the first time, that GLUT1 expression was significantly associated with tumor growth and invasiveness in HCC.

It is well known that proliferation of transformed cells is accompanied by an accelerated uptake and metabolism of glucose.4,5 GLUT1 was shown to be up-regulated during development/embryogenesis, and is more abundant in fetal hepatocytes than in adult hepatocytes.30,31 Hence, one may speculate that the GLUT1 overexpression observed in cancerous tissue fosters rapid tumor growth. Indeed, we have found that inhibition of GLUT1 expression in HCC cells reduced proliferation rate. In addition, it also reduced migratory potential, thus suggesting a direct role of GLUT1 in the tumorigenicity of HCC.

Interestingly, two previous studies have found higher PET activity in advanced HCC stages,7,8 and in a preliminary study we found a positive FDG-PET signal only in two HCC patients with positive Glut1 immunohistochemical staining of the primary tumor but none of the four patients with undetectable Glut1 immunosignal (data not shown). Certainly, this finding has to be confirmed in a larger cohort of patients but puts forward the hypothesis that high GLUT1 expression leads to increased glucose metabolism (as indicated by a positive FDG-PET signal), and herewith, promotes tumorigenicity of HCC also in vivo.

Regulation of GLUT1 in HCC

It has been described that GLUT1 expression is regulated by the transcription factor HIF-1α. HIF-1α is induced in response to stress and hypoxia. In cancerous tissue, it may also be up-regulated under aerobic conditions. Of note, HIF-1α was not detectable in HCC cells in vitro and GLUT1 expression in these cells was not dependent on HIF-1α under aerobic conditions. Interestingly, we newly identified MAZ as regulator of GLUT1 expression. MAZ is a six-Cys2-His2 zinc finger transcription factor, and by performing in silico analysis we found a potential MAZ binding site conserved in the GLUT1 promoter of three different species. In accordance with a previous study we found increased expression of MAZ in HCC,29 and suppression of MAZ in HCC cells inhibited GLUT1 expression under normoxic conditions. Further studies have to be performed to demonstrate whether MAZ regulates GLUT1 directly by promoter binding, however, our data indicate that increased MAZ expression accounts at least in part for the up-regulation of GLUT1 in HCC under normoxic conditions. Induction of hypoxia further induced GLUT1 expression in HCC cells in vitro, and this induction was dependent on HIF-1α. Interestingly, hypoxia-independent overexpression of HIF1α through enhanced PI3 kinase/Akt signaling has been reported as an early event during hepatocarcinogenesis.32 Further, HIF-1α expression has been shown to correlate with HCC survival, and to play a role in tumor progression after induction of hypoxia in HCC.33,34 Altogether, these findings suggest that in vivo in addition to GLUT1 overexpression secondary to the tumorigenic transformation of the tumor cells, chronically hypoxic tumor cells will further enhance glucose transport via a HIF-dependent increase of GLUT1 synthesis. It has been shown that hypoxia promotes HCC cell growth and resistance to therapy,34,35 and it is sometimes observed that surviving cells in HCC nodules pretreated with transarterial chemoembolization (TACE) grow faster than those in neighboring nodules, and become resistant to subsequent TACE. The molecular basis of this phenomenon was in part explained by hypoxia-induced hexokinase II (HK II) expression in human HCC cells in a HIF-1α-dependent manner, and this enhanced HK II expression accelerates HCC cell proliferation.35 Based on the results of the present study it can be speculated that hypoxia-induced Glut1 expression increases HCC cell growth and motility and accelerates the progression of HCC.
GLUT1 as a Therapeutic Target

Pharmacological inhibition of glucose metabolism has been shown to exhibit promising anticancer activity in vitro and in vivo, alone or in combination with other therapeutic modalities.\(^{36,38}\) Inhibition of expression or functionality of GLUT1, rather than inhibiting glucose metabolism in its entirety may more specifically target those cells within the tumor that depend on a high rate of glucose uptake and glycolysis. There has been significant progress in the theoretical and experimental characterization of the crystal structure of Glut1, which may prove useful for the rational design of Glut1-inhibiting agents.\(^{37,38}\) Furthermore, lessons learned from the treatment of patients with Glut1 deficiency may open a way for overcoming potential adverse effects of such agents.\(^{38}\) Studies investigating the use of glucose analogues or glucose conjugates that are likely to be taken up into target cells through Glut1 offer compelling evidence that the difference of Glut1 expression between the brain and tumors is large enough to allow targeting of Glut1. For instance, the PET tracer FDG has recently been investigated in mouse models of breast cancer as a radiomolecular therapy, and doses up to 5 mCi proved to be nonradiotoxic to normal organs.\(^{38}\)

In summary, this study revealed increased GLUT1 expression in a subset of HCC and suggests that this increased GLUT1 expression functionally affected proliferation and invasiveness of HCC cells. Herewith, GLUT1 expression in HCC appears as a potential innovative therapeutic target for this highly malignant tumor.

Acknowledgments

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