P-glycoprotein expression affects ^{18}F-fluorodeoxyglucose accumulation in hepatocellular carcinoma *in vivo* and *in vitro*

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Abstract. ^{18}F-fluorodeoxyglucose (FDG) uptake in hepatocellular carcinoma (HCC) is associated with tumor differentiation and expression of P-glycoprotein (P-gp), a drug efflux pump that plays an important role in chemoresistance. The aim of the study was to clarify the factors that affects FDG uptake in HCC *in vivo* and *in vitro*. The standardized uptake value (SUV) and the tumor to non-tumor SUV ratio (TNR) for FDG uptake in HCC *in vivo* was determined by FDG-PET in 28 patients. Expression levels of glucose transporter-1 (GLUT-1), GLUT-2 and type II hexokinase (HK-II) were examined immunohistochemically in resected specimens. The glucose-6-phosphatase (G-6-Pase) activity was determined in tissue homogenates. *In vitro*, PLC/PRF/5 cells and doxorubicin-resistant PLC/DOR cells were used to examine the effect of P-gp on FDG uptake. The effects of two P-gp inhibitors, verapamil and cepharanthine, on accumulation of FDG were also examined. *In vivo*, GLUT-1 expression was low in HCCs, but was significantly higher in poorly differentiated HCCs than in moderately differentiated HCCs (P=0.043) and was positively correlated with SUV (r=0.75, P<0.0001) and TNR (r=0.7, P<0.0001). GLUT-2 and HK-II expression and G-6-Pase activity were not correlated with tumor differentiation, SUV or TNR. P-gp was over-expressed in PLC/DOR cells, and accumulation of FDG was significantly higher in PLC/PRF/5 cells than in PLC/DOR cells (P=0.04). Verapamil and cepharanthine restored FDG uptake in PLC/DOR cells, but not in PLC/PRF/5 cells. Collectively, our results show that FDG uptake in HCC is weakly correlated with GLUT-1 expression, and that FDG could be a substrate of P-gp, which may act as an efflux pump to reduce FDG accumulation.

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Introduction

Hepatocellular carcinoma (HCC) is a common tumor that ranks fifth in frequency of occurrence worldwide (1). Treatment of HCC using hepatic resection and liver transplantation give the best outcome in well-selected candidates, but the high rates of tumor recurrence and cancer-related mortality after potentially curative therapy for HCC have led to development of alternative therapies, including adjuvant chemotherapy. HCC is known for its poor chemosensitivity to anticancer agents, which is mainly due to multi-drug resistance (mdr). Mdr is often associated with the ATP binding cassette (ABC) transporter family, which mediates transport-based mdr mechanisms (2,3). The best understood member of this protein family is P-glycoprotein (P-gp), and a significant correlation between P-gp overexpression and chemoresistance has been reported in several tumor types (4-6).

Increased uptake of fluorine-18 fluorodeoxyglucose (^{18}F-FDG) based on enhanced glucose metabolism in cancer cells is a sensitive marker of tumor viability. ^{18}F-FDG is imported into tumor cells via the glucose transporter and converted to ^{18}F-FDG-6 phosphate, which is not a substrate for subsequent enzymatic reactions. Hot-spots reflect trapping of ^{18}F-FDG-6 phosphate, and detection of increased ^{18}F-FDG uptake by positron emission tomography (PET) has been used in diagnostic imaging of several tumor types, including HCC (7-9). Experimental studies show decreased glycogenesis and increased glycolysis during HCC carcinogenesis (10). Using dynamic PET, Torizuka *et al* found a constant increase in FDG uptake in poorly differentiated HCC with time, but a transient increase and then a gradual decrease in FDG uptake in well-differentiated HCC (11). This pattern of FDG uptake in well-differentiated HCC is similar to that in normal liver parenchyma, and is specific and different from those of other common cancers. We have previously shown that FDG uptake is associated with tumor differentiation and is inversely correlated with the level of P-gp in HCC (12). The reason for the inverse relationship between FDG uptake and

P-gp expression remains unclear, and therefore the goals of this study were to determine the relationships among FDG uptake, tumor differentiation and glucose metabolism, and to clarify the cellular mechanisms of FDG uptake and the role of P-gp in these mechanisms.

Patients and methods

Patients and tumor samples. Twenty-eight patients with HCC who underwent liver resection at the Department of Surgery, Kyoto University between September, 2003 and August, 2004 were enrolled in the study after giving written informed consent. None of the patients had hyperglycemia. All patients underwent liver resection within 2 weeks after the FDG-PET study and the diagnosis of HCC was confirmed histologically (well-differentiated in 8 patients, moderately differentiated in 14, and poorly differentiated in 6). Liver tissues from tumor and non-tumor regions were collected and partly fixed in 10% formalin. Tissues from 19 patients were also immediately frozen in liquid nitrogen for subsequent analysis.

PET study. ^{18}F was produced by a ^{20}Ne (d, alpha) ^{18}F nuclear reaction and ^{18}F-FDG was synthesized by nucleophilic substitution using an F-100 ^{18}F-FDG-synthesis instrument (Sumitomo Heavy Industries Co., Tokyo, Japan) and a CYPRIS-325R cyclotron (Sumitomo Heavy Industries). All patients were examined with a high-resolution, whole-body PET scanner with an 18-ring detector arrangement (Advance, General Electric Medical Systems, Milwaukee, WI). The patients fasted for >4 h before intravenous injection of ^{18}F-FDG (296±74 MBq) and acquisition of whole body PET images started 50 min after the injection. The patients lay supine on the PET table with their arms positioned beside their bodies, and were held in place by a holding belt fitted around the abdomen. Data acquisition (emission and transmission scans) was performed in two-dimensional imaging mode with septae in place. Emission images were acquired for 3 min per bed position and each post-emission transmission scan was obtained for 1 min per position. A whole body scan (from face to upper thigh) was performed in each patient using 5 or 6 bed positions according to the height of the patient. Data were reconstructed using the ordered subsets expectation maximization method (OSEM) using 16 subsets, 3 iterations, and a 128x128 array size.

Image analysis. PET images were interpreted by at least two experienced nuclear medicine physicians, using all available clinical information and correlative conventional imaging for anatomic guidance. For semi-quantitative analysis of ^{18}F-FDG uptake, regions of interest (ROIs) were manually defined on transaxial tomograms. In patients for whom no significant high uptake was detectable by PET, the ROI was drawn based on images from abdominal CT scans. The maximum standardized uptake value (SUV) was calculated for quantitative analysis of tumor ^{18}F-FDG uptake as follows:

$$SUV = C\ (kBq/ml)/ID\ [kBq/body\ weight\ (kg)]$$

where C is the tissue activity concentration measured by PET and ID is the injected dose.

The tumor to non-tumor ratio (TNR) was also calculated as follows:

$$TNR = Tumor\ SUV/non\text{-}tumor\ SUV$$

where the non-tumor SUV is the average of SUVs at 5 points in non-tumor liver tissues.

Histological examination and immunohistochemical staining. The specimens were fixed in 10% formalin and embedded in paraffin. Serial 5-μm sections were prepared for conventional light microscopy examination with hematoxylin-eosin staining and immunohistochemical analysis. Primary antibodies against glucose transporter (GLUT)-1 (A3536, Dako) and GLUT-2 (AB1342, Chemicon) were diluted 1:200 with 0.05 mol/l Tris-HCl buffer containing a carrier protein and 0.015 mol/l sodium azide (Dako). A primary antibody against hexokinase (HK)-II (AB1629, Chemicon) was diluted 1:500 with the same buffer. Paraffin was removed from sections of each tumor using xylene and ethanol, and unmasking treatments were performed on all sections before performance of immunohistochemistry. Sections for anti-GLUT-1, GLUT-2 and HK-II immunostaining were incubated with Target Retrieval Solution (Dako) using the water-bath method at 95-99˚C for 20 min. After 20 min for cooling, the sections were washed with phosphate-buffered saline (PBS) (Dako) containing 20 mM sodium phosphate and 150 mM NaCl (pH 7.0) for 15 min (5 min x 3 times). Endogenous peroxidase activities were blocked by incubation for 10 min at 25˚C with 0.3% hydrogen peroxide in distilled water and washing with PBS for 5 min. Non-specific binding was blocked by incubation for 30 min at 25˚C with blocking buffer (Dako) containing 10% normal bovine serum in PBS. In the next step, each section was incubated with anti-GLUT-1, anti-GLUT-2 or anti-HK-II antibody as primary antibody for 1 h at 25˚C. Parallel sections were incubated with rabbit IgG (20 μg/ml) as negative controls. All sections were washed with PBS containing 0.05% polyoxyethylene sorbitan monolaurate (Tween 20; Kanto Chemical Co., Tokyo, Japan) for 15 min (5 min x 3 times). In the following steps, each section was stained by the horseradish peroxidase (HRP)-labeled polymer method using an Envision Kit with HRP 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Dako). For linking, the sections were incubated with the labeled polymer for 60 min at 25˚C and washed with 0.05% Tween-20 for 15 min (5 min x 3 times). DAB diluted at 1 mg/ml with 0.05 M Tris-HCl buffer (pH 7.5) was used as a substrate-chromogen solution in an incubation performed at 25˚C for 10 min. All sections were then rinsed gently with distilled water and washed in flowing water for 5 min. In the final step, the sections were lightly counterstained with Gill's hematoxylin and then dehydrated, the alcohol was removed, and a coverslip was positioned with mounting medium. Slides were then examined by light microscopy. All chemicals used in the procedure were of the highest purity available.

Immunohistochemical analysis. Immunohistochemical analysis of anti-GLUT-1, anti-GLUT-2 and anti-HK-II antibodies was performed 3 times by an experienced researcher who was blinded to the SUV and TNR data. In each analysis, the percentages of strongly immunoreactive tumor cells

Table 1. Patient characteristics.

No.	Histological type	SUV	TNR	GLUT-1 index	GLUT-2 index	HK-II index	G-6-Pase activity (mU/mg)	G-6-Pase/HK-II HK-II ratio
1	Moderately	5.0	1.6	1.0	3.5	4.0	2.4	0.6
2	Moderately	10.3	2.8	1.5	4.0	5.0	7.8	1.56
3	Poorly	16.2	3.8	3.5	3.5	4.5	0.9	0.2
4	Moderately	5.9	2.0	3.0	4.5	4.5	1.2	0.27
5	Poorly	12.4	4.3	3.0	4.0	4.0		
6	Well	3.6	1.0	1.5	2.0	4.5	39.0	8.67
7	Well	3.7	1.0	1.0	3.5	4.5	6.0	1.33
8	Well	1.9	0.6	1.5	3.0	5.0		
9	Moderately	2.6	1.0	1.0	4.0	3.5	2.4	0.69
10	Well	5.6	1.7	2.5	2.5	4.0		
11	Poorly	16.3	5.0	3.0	2.0	5.0	2.4	0.48
12	Moderately	3.0	1.1	1.5	1.5	3.0		
13	Poorly	3.6	1.1	1.0	3.5	5.0		
14	Moderately	5.0	1.6	1.0	5.0	2.5	3.3	1.32
15	Moderately	4.0	1.0	1.0	5.0	4.5	9.0	2.0
16	Moderately	4.4	2.1	1.0	5.0	5.0		
17	Well	3.2	0.9	1.5	5.0	4.0	3.9	0.98
18	Well	3.6	1.1	1.0	4.0	5.0		
19	Moderately	3.0	1.8	1.0	3.5	5.0	9.6	1.92
20	Moderately	7.2	2.7	1.5	4.0	5.0	3.6	0.72
21	Moderately	5.7	2.0	1.0	2.5	5.0	16.2	3.24
22	Moderately	6.0	1.5	1.0	4.5	5.0	2.1	0.42
23	Moderately	5.4	2.2	1.0	4.0	4.0	2.4	0.6
24	Poorly	4.8	1.4	1.0	4.0	5.0		
25	Poorly	4.5	1.5	1.0	4.0	5.0	5.4	1.08
26	Well	6.1	2.0	2.5	3.0	5.0	3.0	0.6
27	Moderately	4.5	1.4	1.0	2.0	4.5		
28	Well	2.8	1.0	1.0	1.5	3.5	15.9	4.54

SUV, standardized uptake value; TNR, tumor to non-tumor ratio; GLUT, glucose transporter; HK, hexokinase; G-6-Pase, glucose-6-phosphatase.

among the total number of tumor cells were analyzed visually for each of 10 or more low-power fields (magnification 10x10; ≤30 fields) and the mean percentage was calculated and scored on a 5-point scale (1 = 0-20%, 2 = 20-40%, 3 = 40-60%, 4 = 60-80%, 5 = 80-100%) for each trial. The 3 mean scores from 3 independent trials were averaged again to give the GLUT-1, GLUT-2 and HK-II expression indices.

Enzyme assay. Frozen samples from 19 patients were subjected to an enzyme assay for glucose-6-phosphatase (G-6-Pase) activity, which was determined under conditions of increasing concentration of G-6-Pase in liver tissue homogenates at -80˚C and pH 6.5, as previously described (13). Enzyme activities are expressed in mU per mg protein.

Cell lines and culture. The human HCC cell line, PLC/PRF/5, and a doxorubicin (DOX)-resistant HCC cell line, PLC/DOR, were donated by Dr K. Sakaguchi of the First Department of Internal Medicine, Okayama University, Japan. PLC/DOR cells were established by continuous exposure of gradually increasing concentrations of DOX (from 0.02 to 0.08 μg/ml) to PLC/PRF/5 cells (14). All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Gaithersburg, MD, USA) containing 10% heat-inactivated fetal calf serum (ICN Biochemicals, Aurora, OH, USA), 100 μg/ml of streptomycin, and 100 U/ml of penicillin-G. The human epidermoid carcinoma cell line, KB 3-1, and a vinblastine-resistant subline, KB V-1, isolated from KB 3-1 cells by stepwise selection with increasing vinblastine concentrations (15), were used as positive and negative controls, respectively, for P-gp expression. All cell lines were maintained in incubators at 37˚C with 5% CO_2.

Western blotting. Whole cell lysates were used in the analysis. In brief, cell suspensions in 1 ml of PBS were incubated on ice for 30 min after addition of 100 μl of 100% (w/v)

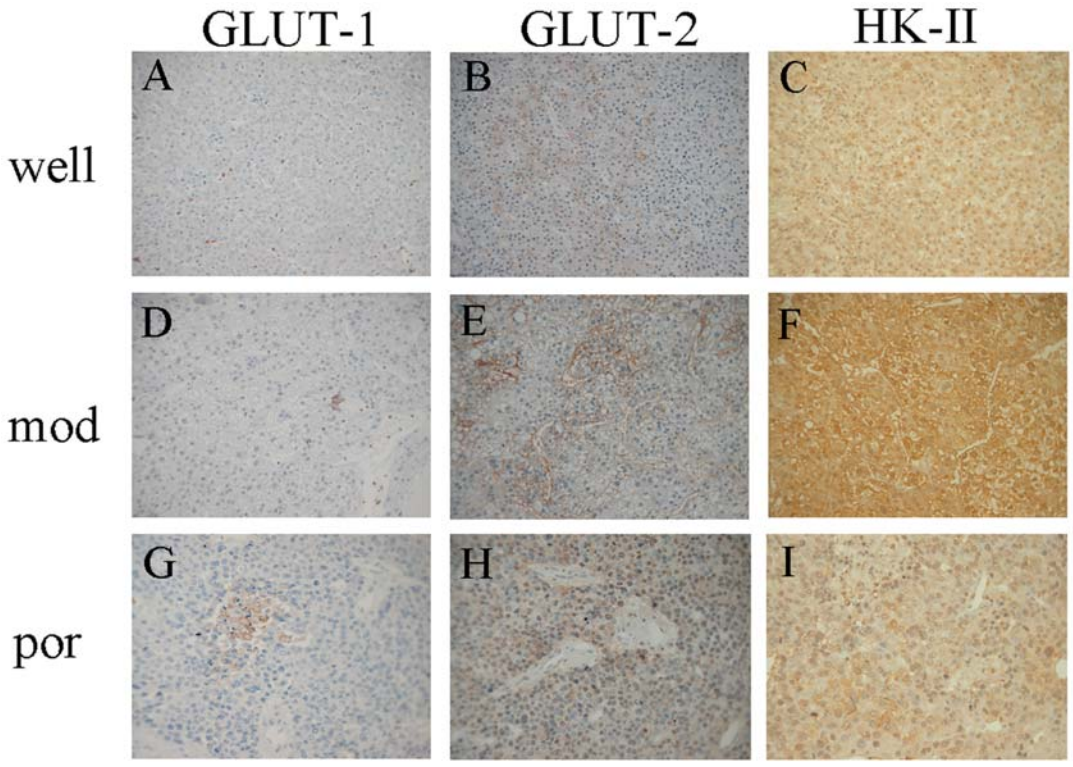

Figure 1. The expressions of GLUT-1 by immunohistchemical staining (x100) were low regardless of tumor differentiation. The expressions of GLUT-2 and HK-II by immunohistchemical staining (x100) were high regardless of tumor differentiation.

trichloroacetic acid. The pellet was collected after centrifugation at 15,000 rpm for 5 min, resuspended in 80 μl of solubilizing buffer and 20 μl of 10% sodium dodecylsulfate (SDS), and sonicated for 1 min using an ultrasonicator. Samples were transferred to a PDVF membrane (Millipore, Temecula, CA, USA), probed overnight with primary antibodies against P-gp (C219, Signet, Dedham, MA, USA, diluted 1:250), GLUT-1 (A3536, Dako, diluted 1:1,000), GLUT-2 (AB1342, Chemicon, diluted 1:1,000) or HK-II (AB1629, Chemicon, diluted to 1:1,000), and visualized with enhanced chemiluminescence detection (Amersham, Piscataway, NJ, USA) after incubation with anti-mouse IgG-HRP (Santa Cruz, diluted 1:2,000).

In vitro ^{18}F-FDG accumulation. To determine FDG uptake *in vitro*, cells were plated at a density of 1x10^6 cells/well in 6-well plates 3 days prior to the experiments. These conditions allowed for the cells to be close to confluence at the time of the assay. On the day of the experiment, 1 ml of medium containing 300 μCi of ^{18}F-FDG was added to each well. ^{18}F-FDG was allowed to accumulate in cells in the incubator over times ranging from 30 to 120 min. The medium was removed and immediately washed four times with 3 ml of ice cold PBS, and the cells were collected from the wells by trypsin-EDTA treatment. The ^{18}F-FDG radioactivity was immediately determined using a Packard 5600 gamma counter (Packard, Meriden, CT), with non-specific background assessed in identically treated cells that were not incubated with ^{18}F-FDG.

The P-gp inhibitors verapamil and cepharanthin were used to evaluate the effect of P-gp on influx and efflux of FDG (16). Cells were plated at a density of 1x10^6 cells/well in 6-well plates 3 days prior to the uptake experiments. On the day of the experiment, the cells were preloaded for 60 min with 1 ml of uptake medium containing 10 μm verapamil or 3 μM cepharanthin added to each well. ^{18}F-FDG was allowed to accumulate in cells in the incubator for 30-120 min. The subsequent washing and measurement procedures were the same as those described above.

Statistical analysis. All values are expressed as means ± SD. Differences between two groups were analyzed using Student's t-test for unpaired data. Analyses of correlations were carried out using a Spearman's rank test. The kinetics of FDG uptake was analyzed by analysis of variance (ANOVA). All statistical analyses were performed using StatView (PowerPC version). A $P<0.05$ was considered statistically significant.

Results

Relationship of tumor differentiation with GLUT, HK-II, G-6-Pase and G-6-Pase/HK-II ratio. Tumor differentiation, FDG-PET findings, immunohistochemical expression indices and enzymatic activity are summarized in Table I. The average GLUT-1 expression index in all HCCs was very low (1.6±0.8, Fig. 1A, D and G), but was significantly higher in poorly differentiated HCCs than in moderately differentiated HCCs (2.1±1.2 vs. 1.3±0.6, P=0.043) (Fig. 2A). GLUT-1 expression was positively correlated with SUV (r=0.75, P<0.0001) (Fig. 3A) and TNR (r=0.7, P<0.0001) (Fig. 4A). The average GLUT-2 expression index in all HCCs was high (3.5±0.8, Fig. 1B, E and H), but was not correlated with tumor

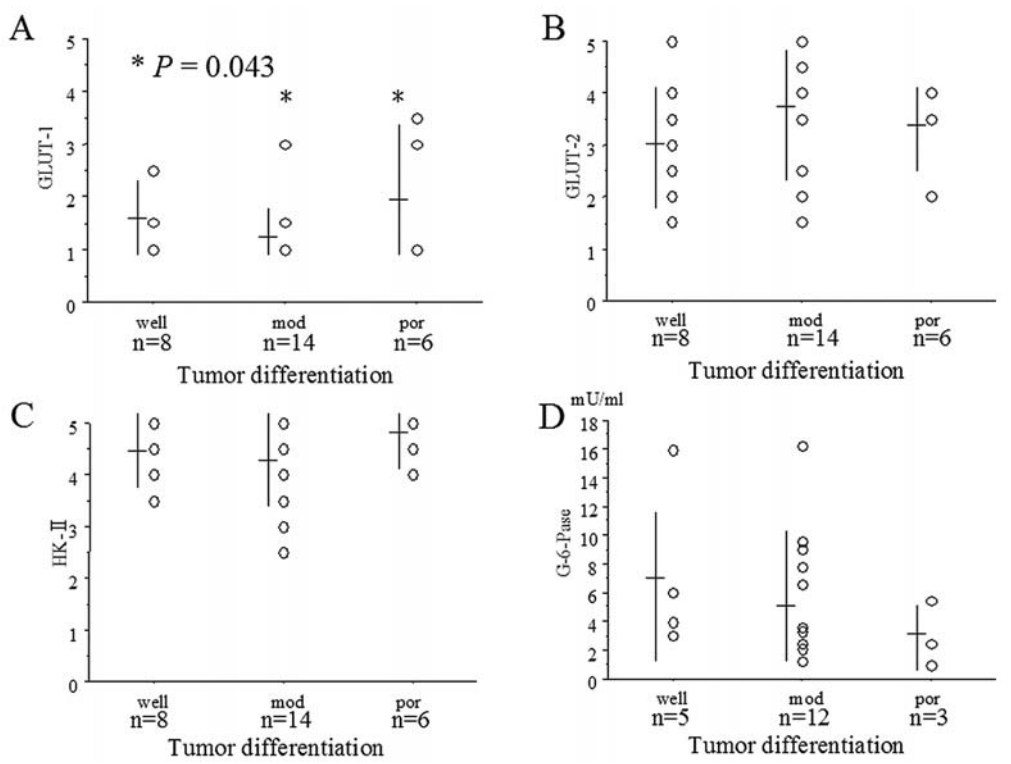

Figure 2. (A) The average GLUT-1 expression was very low (1.6±0.8), but was significantly higher in poorly differentiated HCCs (2.1±1.2) than in moderately differentiated HCCs (1.3±0.6, P=0.043). (B) The average GLUT-2 expression was high (3.5±0.8), but was not correlated with tumor differentiation. (C) The average HK-II expression was very high (4.4±0.8), but was not correlated with tumor differentiation. (D) The G-6-Pase activities were not correlated with tumor differentiation.

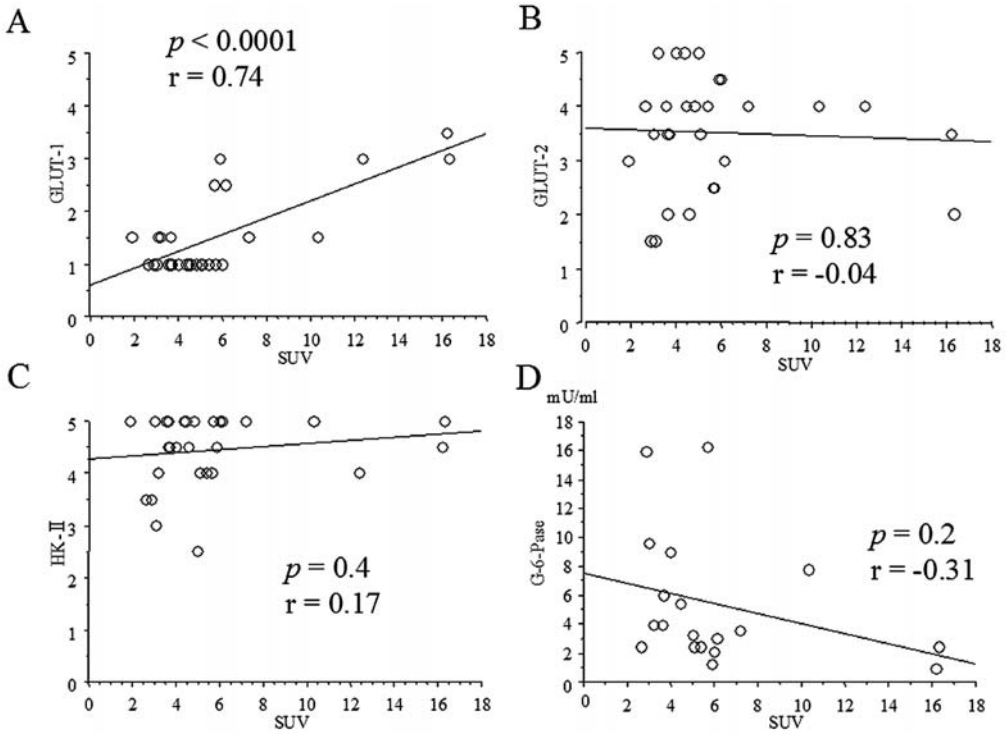

Figure 3. (A) The average GLUT-1 expression was positively correlated with SUV (r=0.75, P<0.0001). (B) The average GLUT-2 expression was not correlated with SUV. (C) The average HK-II expression was not correlated with SUV. (D) The G-6-Pase activities were not correlated with SUV.

differentiation (Fig. 2B), SUV (Fig. 3B) or TNR (Fig. 4B). The average HK-II expression index in all HCCs was very high (4.4±0.8, Fig. 1C, F and I) and also showed no correlation with tumor differentiation (Fig. 2C), SUV (Fig. 3C)

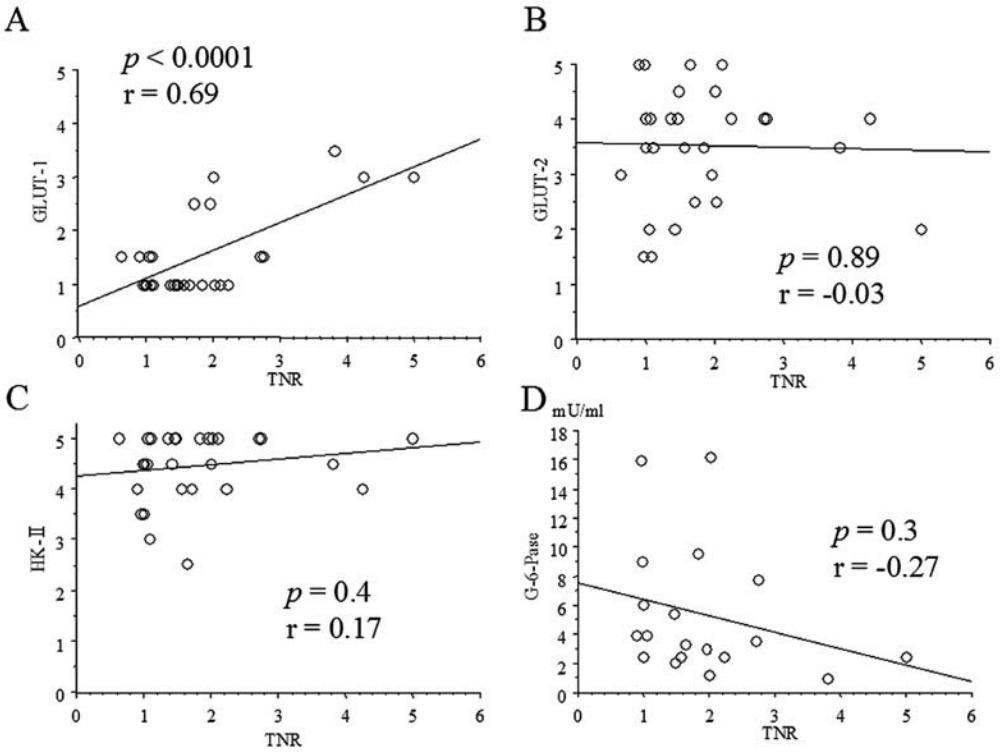

Figure 4. (A) The average GLUT-1 expression was positively correlated with TNR (r=0.7, P<0.0001). (B) The average GLUT-2 expression was not correlated with TNR. (C) The average HK-II expression was not correlated with TNR. (D) The G-6-Pase activities were not correlated with TNR.

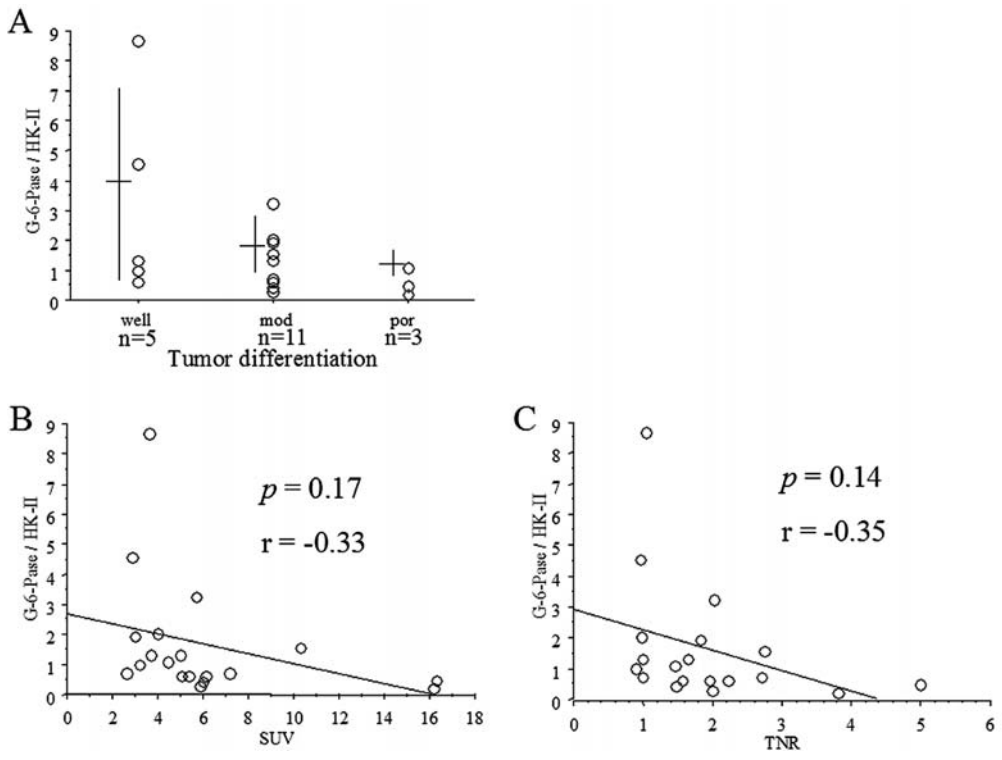

Figure 5. (A) G-6-Pase/HK-II ratio was not correlated with tumor differentiation. (B) G-6-Pase/HK-II ratio was not correlated with SUV. (C) G-6-Pase/HK-II ratio was not correlated with TNR.

or TNR (Fig. 4C). Similarly, the G-6-Pase activity (Figs. 2D, 3D and 4D) and the G-6-Pase/HK-II ratio (Fig. 5) were not correlated with tumor differentiation, SUV or TNR.

Western blot analysis of P-gp, GLUT-1, GLUT-2 and HK-II. Expression of P-gp, GLUT-1, GLUT-2 and HK-II was assessed by Western blotting (Fig. 6). P-gp was expressed very weakly

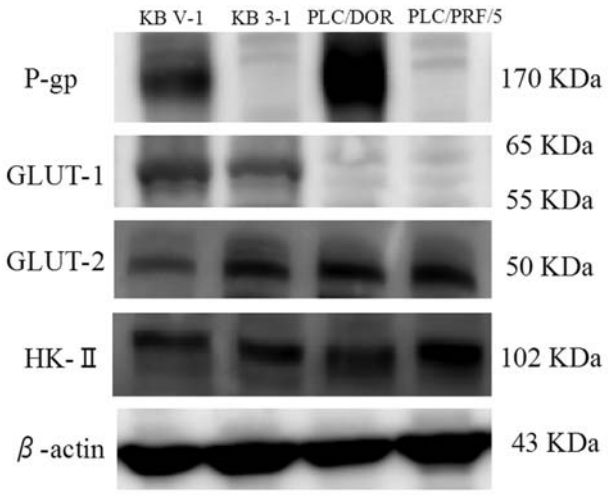

Figure 6. The expression of P-gp, GLUT-1, GLUT-2 and HK-II was assessed by Western blot analysis. The parental cell lines, PLC/PRF/5, expressed P-gp only slightly while their DOX-resistant cell lines, PLC/DOR, expressed at much higher levels. KB V-1 and KB 3-1 were used for positive and negative controls, respectively. No expression of GLUT-1 was recognized in either PLC/DOR or PLC/PRF/5. The expressions of GLUT-2 and HK-II were strong in both PLC/DOR and PLC/PRF/5.

in the parental cell line PLC/RPF/5, but very strongly in the DOX-resistant cell line PLC/DOR. KB V-1 and KB 3-1 were used as positive and negative controls for P-gp expression, respectively. No expression of GLUT-1 was detected in PLC/DOR or PLC/PRF/5 cells, whereas expression of GLUT-2 and HK-II was high in both cell lines. The expression levels of GLUT-1, GLUT-2 and HK-II did not differ significantly between PLC/DOR and PLC/PRF/5 cells.

In vitro ^{18}F-FDG accumulation. The kinetics of ^{18}F-FDG uptake in KB V-1 [P-gp (+)] and KB 3-1 [P-gp (-)] cells (Fig. 7A) indicated that ^{18}F-FDG uptake was significantly higher in KB 3-1 cells than in KB V-1 cells (P=0.0005). Similarly, ^{18}F-FDG uptake in PLC/PRF/5 [P-gp (-)] cells was significantly higher than that in PLC/DOR cells [P-gp (+)] (P=0.0003) (Fig. 7B).

Effect of P-gp inhibitors on ^{18}F-FDG accumulation. ^{18}F-FDG uptake in KB V-1 cells [P-gp (+)] in the presence of 10 μM verapamil was significantly higher than in untreated cells (P<0.0001), whereas verapamil did not influence ^{18}F-FDG uptake in KB 3-1 cells [P-gp (-)] (Fig. 8A). Similarly, ^{18}F-FDG uptake in PLC/DOR [[P-gp (+)] cells in the presence of 10 μM verapamil was significantly higher than in untreated cells (P=0.02), whereas verapamil did not influence ^{18}F-FDG uptake in PLC/PRF/5 cells [P-gp (-)] (Fig. 8B). Similar results were obtained with 3 μM cepharanthine: ^{18}F-FDG uptake was significantly higher in treated KB V-1 cells than in untreated cells (P=0.002), but did not differ in KB 3-1 cells (Fig. 8C); and significantly higher in treated PLC/DOR cells than in untreated cells (P=0.0001), but did not differ in PLC/PRF/5 cells (Fig. 8D).

Discussion

FDG-PET is a less invasive diagnostic tool for detection of malignant tumors (17) and for staging and monitoring of chemotherapeutic response (18,19) in several cancer types. This technique can be used to evaluate glucose metabolism *in vivo* by measuring uptake of the glucose analog, FDG. Two steps are required for accumulation of FDG in cancer cells: facilitated diffusion through glucose transporter proteins and subsequent phosphorylation by a hexokinase. FDG-6-P, a phosphorylated form of FDG, is not transported out of cells and does not undergo glycolytic breakdown; that is, it is trapped metabolically inside cells (Fig. 9). Proliferation rate (20,21), cell viability (22), hexokinase activity (23-25) and GLUT expression level (23,26-28) are thought to influence uptake of FDG.

Several studies have focused on expression of glucose transporters and hexokinases to define the role of these two classes of genes in regulation of FDG uptake, and it has been concluded that FDG uptake in malignant tumors depends largely on the presence of the facilitated glucose transporter, GLUT-1, and the rate-limiting glycolytic enzyme, HK-II (29). More than 11 subtypes of human facilitative glucose transporters have been described (30). Many of these subtypes have been detected in human cancers and cancer cell

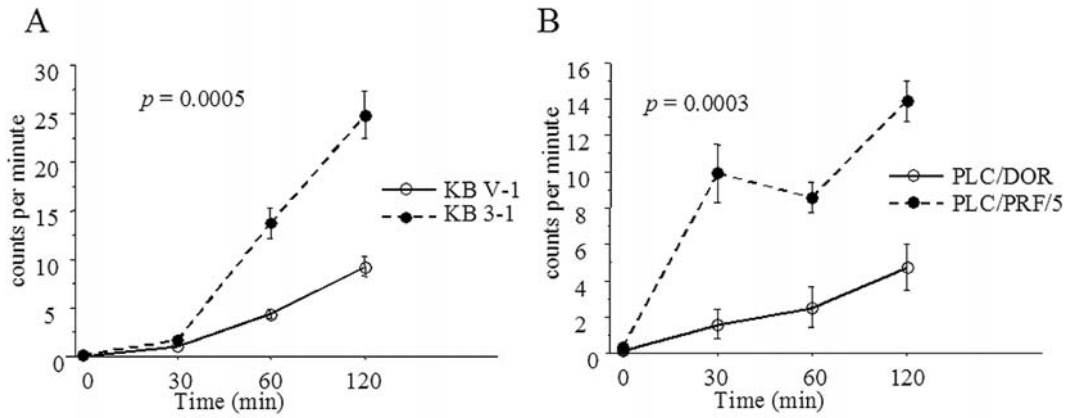

Figure 7. The kinetics of ^{18}F-FDG uptake in KB V-1, KB 3-1, PLC/DOR and PLC/PRF/5 cell lines. ^{18}F-FDG uptake in PLC/PRF/5 cells was significantly higher than that in PLC/DOR cells (P=0.0003).

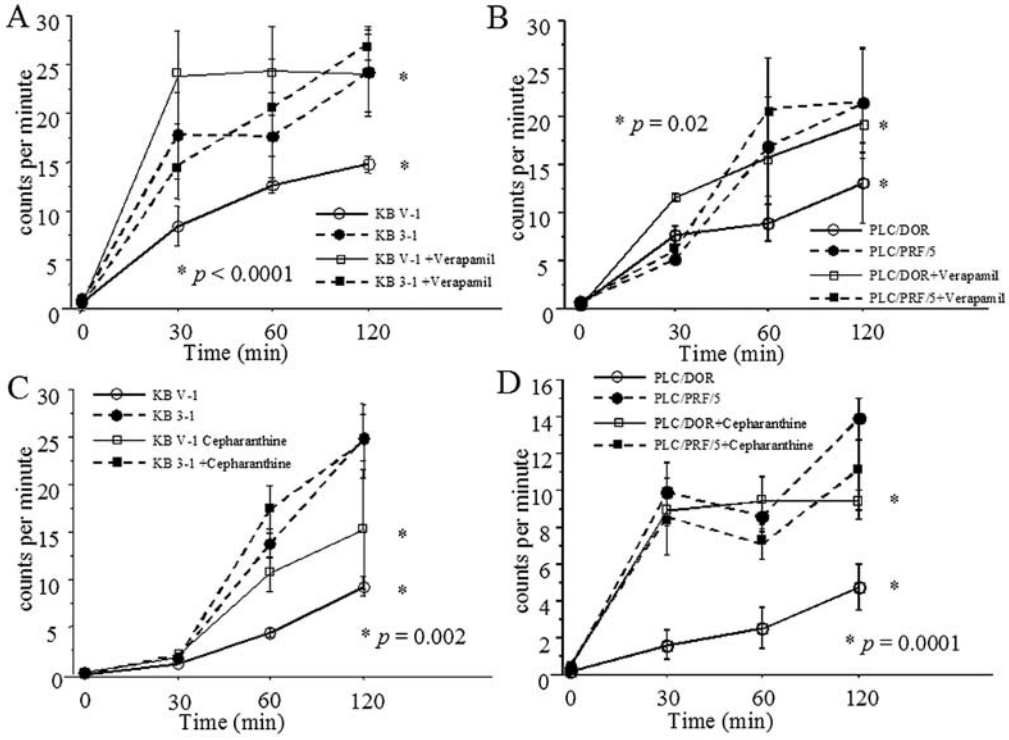

Figure 8. The kinetics of ^{18}F-FDG uptake in KB V-1, KB 3-1, PLC/DOR and PLC/PRF/5 cell lines in the presence or absence of verapamil or cepharanthin. In the presence of 10 μM verapamil, ^{18}F-FDG uptake in PLC/DOR cells was significantly higher than that in verapamil-untreated cells (P=0.02), while verapamil did not influence ^{18}F-FDG uptake in PLC/PRF/5 cells. In the presence of 3 μM cepharanthine, ^{18}F-FDG uptake in PLC/DOR cells was significantly higher than that in cepharanthine-untreated cells (P=0.0001), while cepharanthine did not influence ^{18}F-FDG uptake in PLC/PRF/5 cells.

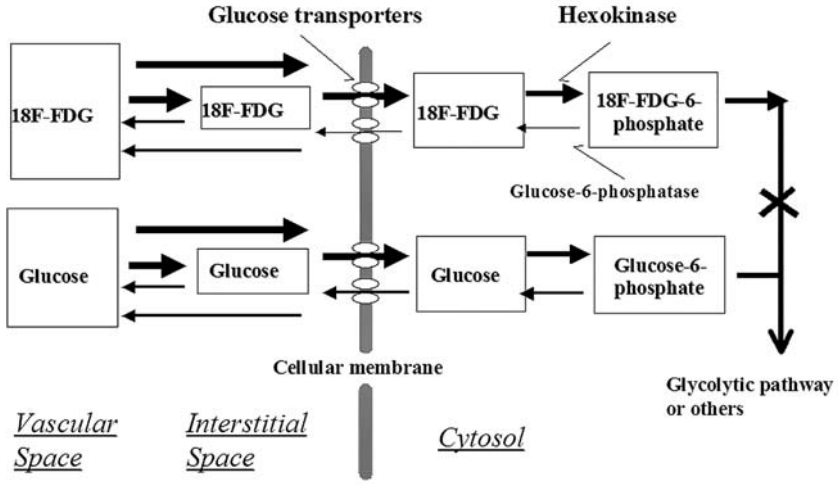

Figure 9. Metabolic trapping of FDG in tumor cells.

lines, but GLUT-1 is the only subtype found in almost all cell lines (31) and is overexpressed in many human cancers (32). However, Roh *et al* found that GLUT-1 expression is not increased in HCC (33). The low expression of GLUT-1 in HCC might be explained by the apparent glutamine-dependence of growth of HCCs (34,35) and the role of fatty acids as the major energy source in HCC cells (36). Our results indicated a positive correlation of the GLUT-1 expression index with SUV and TNR, but it is unlikely that GLUT-1 affects FDG uptake in HCC because the average GLUT-1 expression index was very low.

GLUT-2 is selectively expressed in liver, pancreatic ß cells, and absorptive renal and intestinal epithelial cells (37). During rat liver development a shift in the expression of glucose transporters from GLUT-1, which is present in high amounts in embryonic liver, to GLUT-2 occurs 2 days after birth (38). Expression of GLUT-2 is reduced markedly in preneoplastic hepatic foci and hepatocellular neoplasm,

whereas GLUT-1 is often expressed in hepatocellular adenomas and carcinomas (39). In this study, the GLUT-2 expression index was high, but was not correlated with tumor differentiation, suggesting that HCC cells can transport glucose or FDG regardless of the extent of differentiation. These data are in contrast to those for the GLUT-1 expression index, which was significantly higher in poorly differentiated HCCs than in moderately differentiated HCCs, and the role of GLUT-1 and GLUT-2 in FDG uptake in HCCs remains unclear.

Hexokinases also play an important role in FDG uptake and the glycolytic pathway in malignant cells. Among the 4-hexokinase isozymes in mammalian tissues, HK-II is increased markedly in rapidly growing, highly malignant tumors, including HCCs (40,41), and hexokinases (and HK-II in particular) are thought to regulate glucose metabolism in cancer cells (42). Consistent with these results, we found high expression of HK-II in HCCs. However, our data also showed that HK-II expression did not correlate with FDG uptake. This indicates that FDG uptake may not always occur in parallel with HK expression, and it is of note that hexokinase is inhibited by an increased concentration of intracellular G-6-P in hepatoma cells (43).

The liver is known to have increased G-6-Pase activity (44), but few reports have examined this activity in HCC. Nelson *et al* showed that the lack of G-6-Pase activity in tumors plays a role in FDG retention by preventing dephosphorylation of FDG-6-P to FDG (45), but Torizuka *et al* found that G-6-P activities did not differ significantly between high grade and low grade HCCs (11). In the current study, G-6-Pase activities did not correlate with tumor differentiation, SUV or TNR, which is consistent with previous reports and suggests that G-6-Pase activity may not have a strong influence on FDG retention in HCC. Our data for GLUT-1, GLUT-2, HK-II and G-6-Pase suggest that these proteins do not affect FDG uptake in HCC and that the specific pattern of FDG uptake is dependent on other factors.

P-gp is an ATP-dependent plasma membrane transporter that is responsible for innate and/or acquired drug resistance in tumor cells due to its drug efflux function (46). Immunohistochemical studies indicate that 40-80% of HCC tumors are P-gp-positive and that the response of HCC to chemotherapy is inversely related to P-gp expression (47-50). We have shown that FDG uptake in HCC is inversely correlated with the level of P-gp (12). Lorke *et al* found that FDG uptake of a P-gp-positive tumor was reduced compared to that of a P-gp-negative tumor in an animal study (51), and Bentley *et al* showed that 2-deoxy-D-glucose accumulation *in vitro* is reduced in MDR cell lines with strong P-gp expression and reduced GLUT-1 expression (52). A recent study also showed that FDG may be a substrate of MDR proteins in some melanoma cells and that MDR may influence melanoma imaging by FDG-PET (53), and the present study is the first to suggest that P-gp may act as an efflux pump to reduce FDG accumulation in HCC cells.

In conclusion, our results show that GLUT-1 expression in HCC is low, but weakly correlated with FDG uptake, whereas GLUT-2 expression is high, but not correlated with FDG uptake or tumor differentiation. Therefore, the role of GLUT-1 and GLUT-2 expression in FDG uptake by hepatocellular carcinoma cells remains unclear. However, FDG may be a substrate of P-gp, which may act as an efflux pump to reduce FDG accumulation. Clinically, FDG-PET is a promising diagnostic approach for rapid characterization of P-gp expression in human tumors *in vivo* and ultimately may facilitate optimization of individualized cancer therapy.

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