Loss of the Mitochondrial Bioenergetic Capacity Underlies the Glucose Avidity of Carcinomas

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Running title: Aerobic glycolysis and the mitochondrial H+-ATP synthase

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Abstract

The down-regulation of the catalytic subunit of the mitochondrial H^+-ATP synthase (β-F1-ATPase) is a hallmark of most human carcinomas. This characteristic of the cancer cell provides a proteomic signature of cellular bioenergetics that can predict the prognosis of colon, lung and breast cancer patients. Here we show that the *in vivo* tumor glucose uptake of lung carcinomas, as assessed by positron emission tomography in hundred and ten patients using [^{18}F] 2-deoxy-2-fluoro-D-glucose as probe, inversely correlates with the bioenergetic signature determined by immunohistochemical analysis in tumor surgical specimens. Moreover, we demonstrate that the rate of aerobic glycolysis in cancer cells depends on the activity of oxidative phosphorylation and on the cellular expression level of the β-F1-ATPase protein of mitochondrial oxidative phosphorylation. The results highlight the relevance of the alteration of the bioenergetic function of mitochondria for glucose capture and consumption by aerobic glycolysis in carcinomas.

Introduction

The cellular uptake of the non-metabolizable [^{18}F] 2-deoxy-2-fluoro-D-glucose (FDG) used in positron emission tomography (PET) has emerged as a valuable tool for diagnosing and staging cancer patients (1). Tumor capture of FDG is expressed by the Standardized Uptake Value (SUV), a measure that provides an indication of the first committed step of glucose metabolism and thus, an estimation of glucose consumption by tumor cells. The increased glucose capture of tumor cells could result from an augmented demand of carbon skeletons to sustain uncontrolled proliferation and/or from a shift in the pathway used for energy provision during cellular proliferation. The latter was suggested by Otto Warburg many years ago (2), but remained largely unexplored until the recent renaissance of the so-called "Warburg effect" in cancer biology (3).

Over the past years, it has been shown that most types of cancers (4,5 and references therein) including lung carcinomas (6), fit the Warburg hypothesis since a decreased expression of β-F1-ATPase, which is the catalytic subunit of the mitochondrial H^+-ATP synthase and thus, a bottle-neck of mitochondrial oxidative phosphorylation, is linked to an increased expression of several markers of the glycolytic pathway in the cancer cell (4,5). This proteomic feature of cancer was defined as *"the bioenergetic signature"* of tumors and shown to provide a relevant marker of disease progression in colon, lung and breast cancer patients (4-6). Moreover, it has been predicted that the bioenergetic signature further provides a marker of the cellular response to chemotherapy (7).

On the basis of these findings, in this work we studied whether glucose uptake of lung carcinomas as assessed *in vivo* by FDG-PET imaging in the clinical setting correlates with proteomic markers of the bioenergetic signature determined in surgical specimens. Moreover, we demonstrate that the rate of aerobic glycolysis in cancer cells

depends on the activity of oxidative phosphorylation and on the expression level of markers of the bioenergetic signature. These results support in part that the increased glucose avidity of carcinomas results from a shift in the pathways of energy provision in the cancer cell and thus support Warburg's hypothesis.

Materials and Methods

Study Procedures and Clinical Information of Patients. Surgical specimens, study procedures and clinical information of patients with potentially respectable non-small cell lung cancer (NSCLC) included in this study were those described previously (8). The study was performed between February 1999 and May 2001 at the Hospital Universitario 12 de Octubre de Madrid, Spain. A summary of relevant clinico-pathological information of the cohort of patients analyzed is provided in Table 1.

Immunohistochemistry. Tissue microarrays containing triplicate 1 mm cores from selected tumor areas of formalin-fixed, paraffin-embedded tissue blocks of lung carcinomas were constructed using a tissue microarrayer (Beecher Instruments, Silver Spring, MD). For immunohistochemical analysis the following primary antibodies were used: anti-β-F1-ATPase (1:3,000) (4), anti-Hsp60 (SPA 807, Stressgene, Canada 1:400), anti-glyceraldehyde-3-phosphate dehydrogenase (GADPH 1:8,000) and anti-pyruvate kinase (PK 1:2,000) both from Abcam (Cambridge, UK). The expression level of the markers was scored as previously described (4) (see also Fig. 1).

Cell cultures. Human liver (HepG2), lung (HOP62) and colon (KM12 and HCT116) cancer cells were grown at 37ºC in culture medium supplemented with 10% FCS. To change the bioenergetic phenotype of HCT116 cells (9), cells were incubated with 6 μM oligomycin or 10mM 2-desoxiglucose during 2 days. Cells were recovered from the plates by trypsin treatment and cellular lysates prepared. For Western blotting, cellular

proteins (10 or 15 μg of protein) were fractionated on 10% SDS-PAGE and membranes blotted with anti-β-F1-ATPase (1:20,000), anti-hsp60 (1:2,000), anti-GAPDH (1:20,000) and anti-PK (1:1,000). Secondary horseradish peroxidase conjugated goat anti-rabbit or anti-mouse or anti-goat antibody (1:3,000 dilution) were used for detection, which was accomplished using an enhanced chemiluminescence detection method (Amersham Pharmacia Biotech, U.K.). For the determination of aerobic glycolysis, cells were seeded and allowed to grow until reaching 60 % confluence. Cells were incubated with or without 6 μM oligomycin to assess the relevance of oxidative phosphorylation in the rates of aerobic glycolysis. At various times, 0.1 ml aliquots of the culture media were collected and precipitated in 6% perchloric acid, neutralized and used for the enzymatic determination of lactate as described previously (10).

Statistical analysis. Pearson's correlation coefficient, Spearman's Rho and Kendall's Tau were used as measures of linear correlation among the variables. Simple and multiple linear regression models were fitted to the data. Response variables were SUV and TSN-SUV. Explanatory variables were the expression level of mitochondrial and glycolytic markers. In all the cases the global significance of the models and the individual significance of each explanatory variable were assessed using standard F and Student's t tests. One-way analysis of variance was used to detect differences in SUV for different levels of β-F1-ATPase/GAPDH ratio in the tumors. Standard F tests were used to assess significance. To determine the degree of association of SUV and β-F1-ATPase expression with survival, cutoff points of both variables were used to define potentially "high" and "low" risk groups. For each situation, survival curves were computed using Kaplan-Meier estimates and compared using the log-rank test. All the computations were carried out using the statistical software package SPSS 13.0.

Results

A set of 110 lung carcinomas with full clinical annotation including PET data were studied (Table 1) (8). Tissue sections were reviewed by two pathologists to reclassify lung tumors according to the 2004 WHO Classification. Sections were processed for immunohistochemical analysis of mitochondrial (β-F1-ATPase and Hsp60) and glycolytic (GAPDH and PK) markers of the bioenergetic signature of the cell (Fig. 1) (4,5). The maximum FDG uptake of the tumor assessed by SUV was also normalized for the tumor size (TSN-SUV), using for this purpose the largest diameter (cm) of the surgical specimen.

We observed that in our cohort of patients a lower SUV of the tumor was a significant prognostic factor of survival (Fig. 2a), consistent with recent findings (11). A direct linear correlation was observed between tumor SUV data and the expression level of GAPDH ($R= 0.196$, $P= 0.044$) suggesting that glucose capture by the tumor is linked to its rate of utilization by glycolysis. Although no correlation was observed between tumor SUV data and PK expression, both glycolytic markers (PK and GAPDH) significantly correlated in the surgical specimens ($R= 0.368$, $P<0.001$), supporting a concerted adaptation of lung tumors to a glycolytic phenotype (4,5). A significant inverse correlation was found between TSN-SUV values and the expression level of β-F1-ATPase in the tumors ($R= - 0.308$, $P= 0.004$). In contrast with this finding, no correlation was observed between the expression of Hsp60, a structural marker of mitochondria, and SUV or TSN-SUV data, suggesting that the loss of the bioenergetic marker of mitochondria underlies the increased glucose uptake of the cancer cell. Multiple linear regression models for the response variables SUV and TSN-SUV as a function of the markers of the bioenergetic signature confirmed the significance of GAPDH ($P=0.019$) and β-F1-ATPase ($P=0.005$) expression, respectively. In agreement with previous findings (6), it was found that patients with a higher tumor expression

level of β-F1-ATPase had a higher life expectancy (Fig. 2b), whereas other markers provided no meaningful correlations with survival.

The β-F1-ATPase/GAPDH ratio is a proteomic index of the overall mitochondrial capacity of the cell (4-6). Consistent with the above observations it was found that squamous cell carcinomas displayed a lower β-F1-ATPase/GAPDH ratio than lung adenocarcinomas (Fig. 2c) and had significantly higher SUV values (Fig. 2c). Moreover, the stratification of lung carcinomas based on the overall mitochondrial capacity of the cell also significantly related ($P= 0.048$) with the rates of glucose consumption as assessed by FDG capture (Fig. 2d).

To illustrate the relevance of oxidative phosphorylation in the rate of glucose consumption by aerobic glycolysis various human cancer cell lines were treated with oligomycin, an inhibitor of the mitochondrial H^+-ATP synthase. Oligomycin treatment promoted a rapid burst in glucose consumption in the cells as assessed by the rapid increase in their rates of aerobic glycolysis (Fig. 3a). Likewise, the regulation of the overall mitochondrial activity of HCT116 cells (9), that results in cells expressing different levels of β-F1-ATPase (Fig. 3b), also showed a significant inverse relationship ($P< 0.001$) between the rates of glucose consumption by aerobic glycolysis and the mitochondrial capacity of the cell (Fig. 3b). Taken together, these results highlight both *in vivo* and *in vitro* the relevance of the bioenergetic function of mitochondria for cellular glucose uptake and consumption and support that an altered oxidative phosphorylation, by affecting the expression level of β-F1-ATPase, is one of the determinants that underlies the abnormal aerobic glycolysis of the cancer cell.

Discussion

The onset of the Warburg effect in the cancer cell, i.e., the shift to a glycolytic phenotype, has been explained on the grounds of (i) metabolic adaptation to the hypoxic

environment where the tumor develops (12) and/or by a direct effect of HIF1α on mitochondrial bioenergetics (13), (ii) mutations in oncogenes and proteins related to signal transduction pathways (myc, Akt, mTOR) that in turn promote changes in the expression of genes involved in cellular energetic metabolism (10,14) or directly interfere with mitochondrial bioenergetics by affecting the biogenesis of specific respiratory complexes (15) and finally, (iii) by mutations on mtDNA (16) or in nuclear genes involved in the metabolic and bioenergetic function of the organelle (17). However, superimposed to any of these genetic and/or epigenetic alterations, it is possible that the shift to a glycolytic phenotype is hard-wired in cancer cells because glycolysis is the metabolic pathway required for cellular proliferation. In fact, it has been described that lymphocytes shift to glycolysis during cellular proliferation (18). Moreover, progression through the cell cycle is supported by non-respiratory modes of energy generation as a result of the inhibition of mitochondrial function by cyclin D1 (19). In this regard, the late cell-cycle biosynthesis of β-F1-ATPase, that mostly occurs at G2/M by a mechanism that requires the internal ribosome entry site translational activity of the 3'UTR of the transcript (20), could suggest that differences in the bioenergetic signature of tumor specimens might also arise as a result of diverse rates of cellular proliferation in the tumor.

Overall, we provide for the first time a link between the expression of a mitochondrial protein involved in energy transduction (β-F1-ATPase) and the functional estimation of the rate of glucose capture and consumption in cancer cells. These results support Warburg's hypothesis and afford a mechanistic explanation for FDG-PET imaging in oncology.

Acknowledgements

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Table 1. Summary of clinicopathological characteristics of the cohort of patients studied.

Characteristics	No.	Percentage
Age, years		
Range	43-82	--
Mean	65	--
Gender		
Male	104	96
Female	6	4
Histology		
SCC	63	57
AC	26	24
Others	21	19
T stage		
T1	25	23
T2	65	59
T3	12	11
T4	8	7
N stage		
N0	65	59
N1	17	15
N2	23	21
N3	5	5
M stage		
M0	93	85
M1	17	15
SUV		
$\leq$ 5.0	40	36
> 5.0	68	62
Unknow	2	2

Figure 1. Expression of markers of the bioenergetic signature in lung carcinomas. Tissue microarray sections were cut and processed for immunohistochemical analysis of the markers of the bioenergetic signature. Representative examples of low (panels to the left) and high (panels to the right) expression levels of the mitochondrial β-F1-ATPase and Hsp60 as well as of the glycolytic GAPDH and PK markers are shown. Note the preferential granular perinuclear staining of the cytoplasm revealing mitochondria in β-F1-ATPase and Hsp60 and the diffuse staining of the cytoplasm in GAPDH and PK.

Figure 2. Tumor glucose uptake and the bioenergetic signature of NSCLC. **a** and **b,** Kaplan-Meier survival analysis shows the association of FDG uptake (SUV) and β-F1-ATPase expression in lung cancer with overall patients' survival (OS), respectively. **c**, The bioenergetic signature (β-F1-ATPase/GAPDH ratio) and FDG uptake (SUV) of adenocarcinomas (AC) and squamous carcinomas (SCC) of the lung. The results shown are the mean ± SEM of 26 and 63 tumors for AC and SCC, respectively. *, $P<0.05$ and **, $P<0.01$ when compared with AC. **d**, The bioenergetic signature of lung carcinomas defines the rates of glucose uptake (SUV) as assessed by FDG-PET. The results shown are the mean ± SEM of 33/32, 38/37 and 37/37 tumors for β-F1-ATPase/GAPDH ratio and SUV data, respectively. The relationship between both parameters is significant ($P=0.048$) as assessed by standard F test in a one-way Anova model.

Figure 3. The activity of oxidative phosphorylation and the bioenergetic signature of the cell define the rate of glucose consumption by aerobic glycolysis. **a**, The flux of aerobic glycolysis of cancer cells (HepG2, HOP62 and KM12) increases when the activity of the mitochondrial H^+-ATPase is inhibited by incubation of the cells with

oligomycin (+). The results shown are the means ± SEM of 3-6 determinations. *, $P<0.05$ and **, $P<0.005$ when compared with non-treated (-) cells. **b**, The bioenergetic signature (β-F1-ATPase/GAPDH ratio) of HCT116 cells defines the rates of glucose utilization by aerobic glycolysis. HCT116 cells were incubated with 10mM 2-desoxiglucose (hatched symbol), 6 μM oligomycin (open symbol) or left untreated (closed symbol) during 48h to promote changes in the cellular bioenergetic phenotype. After removal of the drugs, the cells were maintained in culture for the determination of the bioenergetic signature and rates of glycolysis. The results shown are the mean ± SEM of 4 (hatched), 9 (closed) and 5 (open) independent determinations of the β-F1-ATPase/GAPDH ratio and 6 (hatched), 15 (closed) and 8 (open) determinations of the glycolytic flux. The relationship between both parameters is highly significant ($P<0.001$) as assessed by standard F test in a one-way Anova model.

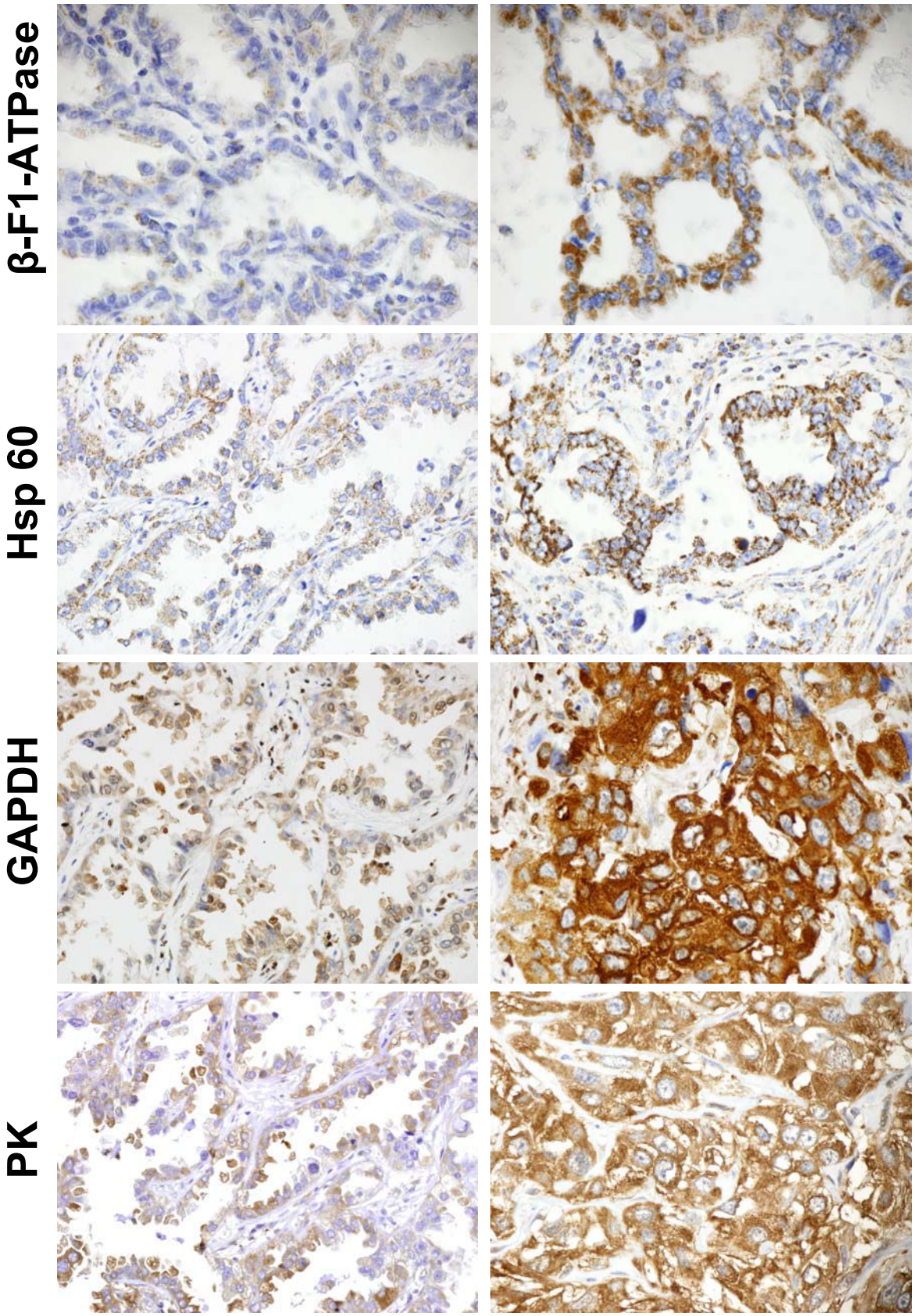

Figure 1

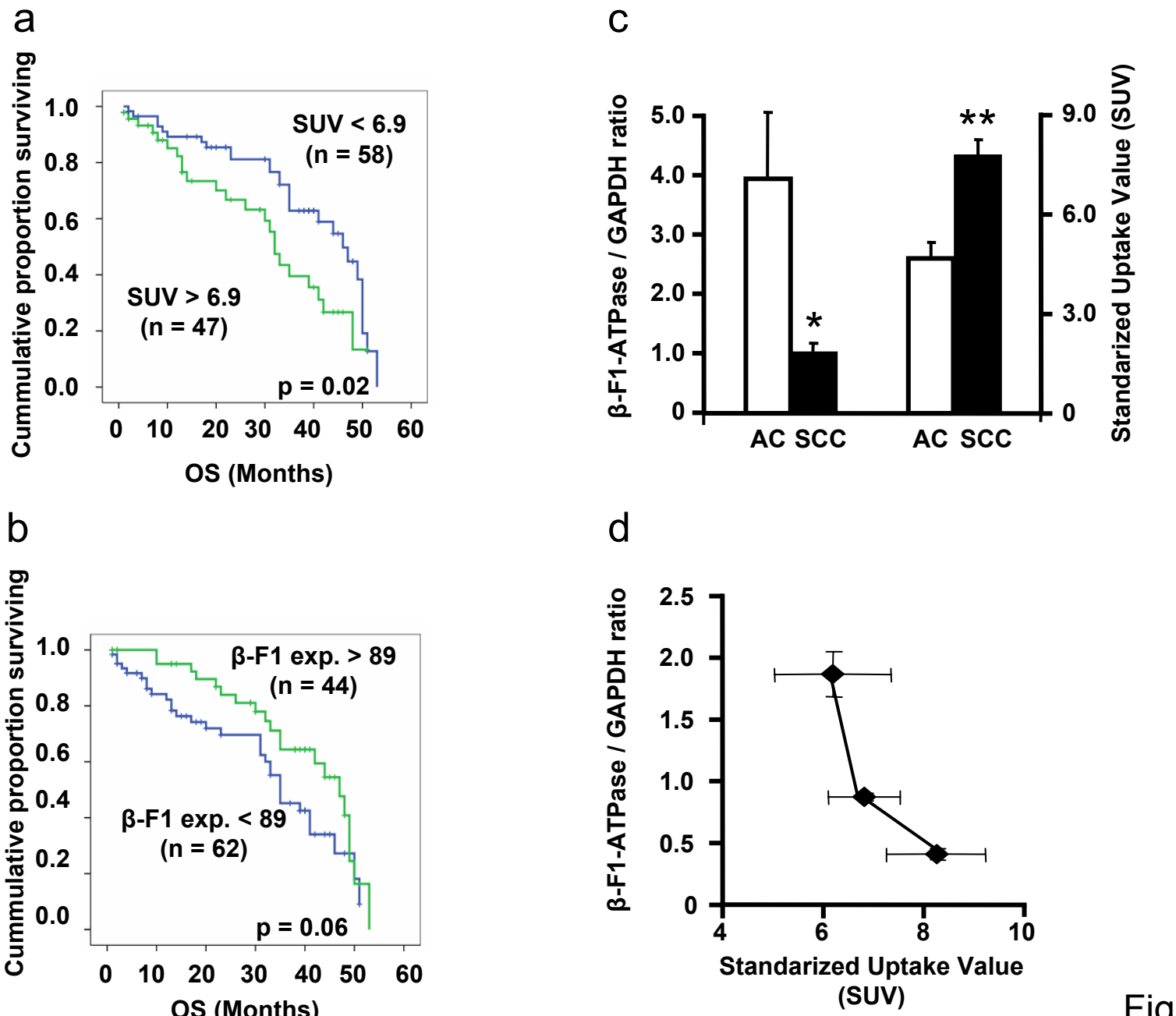
a
Cummulative proportion surviving
1.0
0.8
0.6
0.4
0.2
0.0
SUV < 6.9
(n = 58)
SUV > 6.9
(n = 47)
p = 0.02
0 10 20 30 40 50 60
OS (Months)
b
Cummulative proportion surviving
1.0
0.8
0.6
0.4
0.2
0.0
β-F1 exp. > 89
(n = 44)
β-F1 exp. < 89
(n = 62)
p = 0.06
0 10 20 30 40 50 60
OS (Months)
c
β-F1-ATPase / GAPDH ratio
5.0
4.0
3.0
2.0
1.0
0
*
**
9.0
6.0
3.0
0
Standarized Uptake Value (SUV)
AC SCC
AC SCC
d
β-F1-ATPase / GAPDH ratio
2.5
2.0
1.5
1.0
0.5
0
4 6 8 10
Standarized Uptake Value
(SUV)

Figure 2

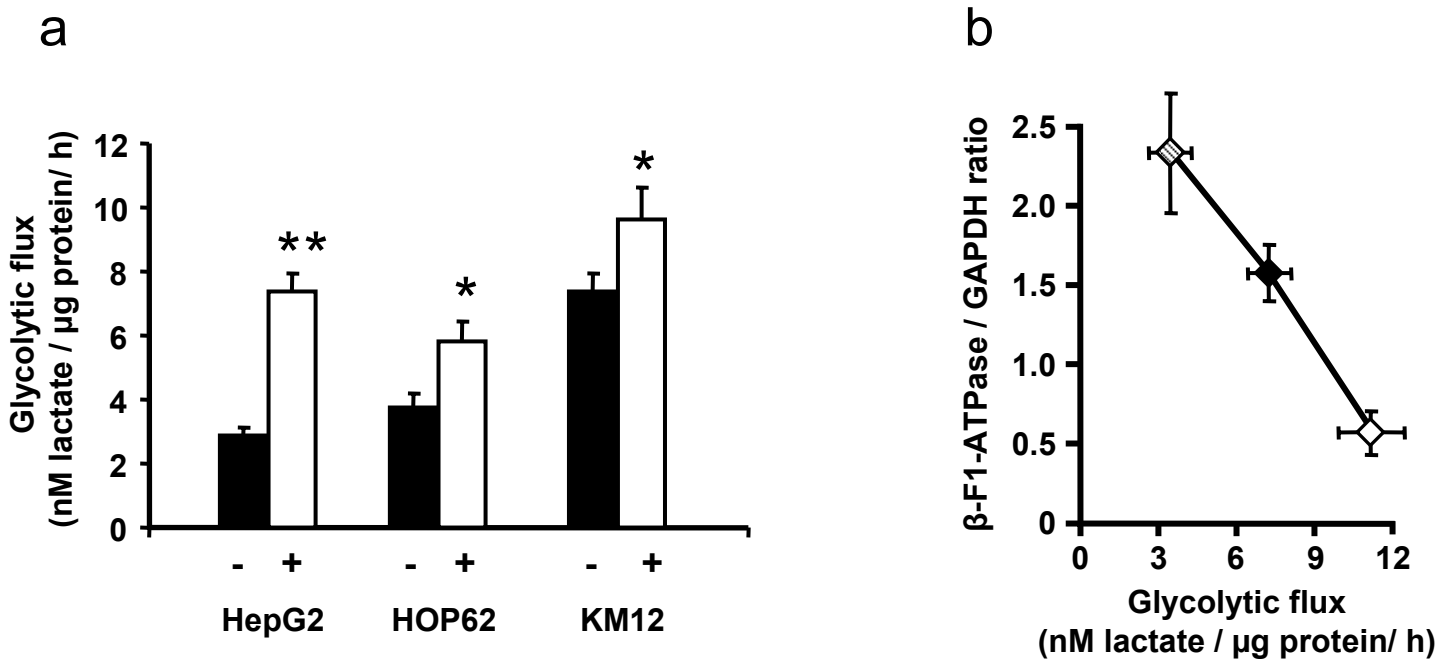
a
Glycolytic flux
(nM lactate / µg protein/ h)
12
10
8
6
4
2
0
**
*
*
- + - + - +
HepG2
HOP62
KM12
b
β-F1-ATPase / GAPDH ratio
2.5
2.0
1.5
1.0
0.5
0
0 3 6 9 12
Glycolytic flux
(nM lactate / µg protein/ h)

Figure 3