Glycolysis module activated by hypoxia-inducible factor 1α is related to the aggressive phenotype of hepatocellular carcinoma

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Abstract. An increased level of glycolysis, an intracellular hallmark of neoplasms, enables cancer cells to survive under various conditions. To elucidate the role of increased glycolysis in the progression of hepatocellular carcinoma (HCC), we investigated the associations between the expression patterns of 14 glycolysis-related genes and clinicopathologic factors in 60 HCCs by using pooled transcriptome data. We then evaluated the therapeutic efficacy of the knockdown of *ENO1*, which is encoded by a glycolysis-related gene, in HCC cells. Among the 14 genes, levels of 8 genes (*GPI*, *ALDOA*, *TPI1*, *GAPD*, *PGK*, *PGAM*, *ENO1* and *PKM*), all of which can be transcriptionally activated by hypoxia-inducible factor 1α (HIF-1α), were significantly higher in HCC with venous invasion (VI) than in HCC without VI. Our cluster analysis showed that HCC patients with activation of the 8 HIF-1α-regulated genes had significantly shorter overall survival (P=0.023) than did HCC patients without increased expression levels of these genes. The association between the levels of *ENO1* and VI was confirmed in an independent sample set of 49 HCCs by real-time reverse-transcription PCR. The knockdown of *ENO1* by small-interfering RNA significantly inhibited the proliferation of an HCC cell line (HLE cells) in both the glucose-rich and glucose-free conditions, accompanied by a decreased S phase and increased G2/M phase of the cell cycle. Collectively, these data suggest that activation of an HIF-1α-regulated glycolysis module is closely related to the aggressive phenotype of HCC, and that *ENO1*, a glycolysis module gene, might serve as a new target to circumvent HCC metastasis.

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Introduction

An increased level of glycolysis in the energy production system of cancer cells was initially found by the renowned biochemist Otto Warburg (1). Since his discovery, many studies have revealed the molecular basis by which glycolysis is increased in cancer cells, particularly the regulation system of glycolysis-related enzymes, such as the hypoxic condition, oncogenic signals, and the Myc oncoprotein (2,3). As an example of the application of the Warburg effect to daily clinical practice, we can detect a cancerous nodule as an area where increased glucose uptake is visualized by positron emission tomography (PET) (4). However, applications of the Warburg effect to cancer therapy have not yet been found because of a lack of studies to comprehensively analyze glycolysis module genes or their encoded proteins in cancer progression.

Hepatocellular carcinoma (HCC) is the fourth most common malignancy in Japan (5) and the fifth most common malignancy worldwide (6). Despite advances in the conventional clinical treatment of HCC, the prognosis remains poor because of the high recurrence rate after resection (7). To clarify the molecular basis underlying the metastasis of HCC, we have performed genome-wide studies (8-10) in association with an aggressive phenotype of HCC. In the present study, we searched for glycolysis module genes among thousands of genes in the pooled data (8-10), and we investigated the relationship between the selected module genes and the clinical behavior of HCC. We discovered a new molecular subclass of HCC with poor prognosis; this subclass is characterized by the induction of several glycolysis module genes. Additionally, we show for the first time the therapeutic efficacy of α-enolase (ENO1), one of the glycolytic enzymes, on HCC cells by using small-interfering RNA.

Table I. Summary of glycolysis module genes and aggressive phenotype of HCC.

Glycolytic pathway	Enzymes	Symbol[a]	Probe number	Assessment	HIF-1α regulation	Tumor size	Tumor differentiation	Venous invasion	Stage[b]
Glucose									
↓	HK	*HK1*	M75126_at	Eligible	Yes	N.S.	N.S.	N.S.	N.S.
↓		*HK2*	Z46376_rna1_at	Ineligible	Yes				
↓		*HK3*	U51333_s_at	Eligible		N.S.	N.S.	N.S.	N.S.
↓		*HK4*	M90299_at	Eligible		N.S.	N.S.	N.S.	N.S.
Glucose-6-phosphate									
↓	GPI	*GPI1*	K03515_at	Eligible	Yes	N.S.	N.S	P=0.001	N.S.
Fructose-6-phosphate									
↓	PFK	*PFKL*	X15573_at	Ineligible					
↓		*PFKM*	U24183_s_at	Ineligible					
↓		*PFKP*	D25328_at	Ineligible					
Fructose-1,6-bisphosphate									
↓	ALDO	*ALDOA*	X12447_at	Eligible	Yes	N.S.	N.S.	P<0.001	P=0.022
↓		*ALDOB*	M15656_at	Eligible		N.S.	N.S.	N.S.	N.S.
↓		*ALDOC*	X05196_at	Eligible		N.S.	N.S.	N.S.	N.S.
Dihydroxyacetone phosphate									
↓	TPI	*TPI1*	HG2279	Eligible	Yes	N.S.	N.S.	P=0.014	P=0.033
Glyceraldehyde-3-phosphate									
↓	GAPD	*GAPD*	X01677_f_at	Eligible	Yes	N.S.	N.S.	P=0.014	N.S.
1,3-Bisphosphoglycerate									
↓	PGK	*PGK*	V00572_at	Eligible	Yes	N.S.	N.S.	P=0.007	N.S.
↓		*PGK2*	X05246_at	Ineligible					
3-Phosphoglycerate									
↓	PGAM	*PGAM-B*	J04173_at	Eligible	Yes	N.S.	N.S.	P=0.005	N.S
↓		*PGAM-M*	J05073_at	Ineligible					
2-Phosphoglycerate									
↓	ENO	*ENO1*	M14328_s_at	Eligible	Yes	N.S.	N.S.	P=0.002	P=0.01
↓		*ENO2*	X51956_rna1_at	Ineligible					
↓		*ENO3*	X16504_s_at	Ineligible					
Phosphoenolpyruvate									
↓	PK	*PKM*	X56494_at	Eligible	Yes	N.S.	P<0.001	P=0.02	N.S.
↓		*PKLR*	M15465_s_at	Eligible		N.S.	N.S.	N.S.	N.S.
Pyruvate									

N.S., not significant. HIF-1α hypoxia-inducible factor 1α. [a]Gene abbreviations are used based on Entrez Pubmed (http//www.ncbi.nlm.nih.gov/sites/entrez/). [b]Stage based on the UICC TNM classification.

Materials and methods

Patients and pooled transcriptome data. By using high-density oligonucleotide arrays (HuGeneFL Array, Affymetrix, Santa Clara, CA), we previously analyzed the expression levels of 7,070 genes in 60 HCC tissues from 60 HCC patients who underwent curative hepatectomy and then received follow-up care (8,9). The pooled data and the clinicopathologic features of the 60 HCCs are listed on our web site (http://surgery2.med.yamaguchi-u.ac.jp/research/DNAchip/hcc-recurrence/index.html) according to the Minimum Information About a Microarray Experiment (MIAME) (11) and TNM classification of the Union Internationale Contre le Cancer (UICC) (12). Written informed consent was obtained from all patients before surgery. The study protocol was approved by the Institutional Review Board for Human Use at the Yamaguchi University School of Medicine. All patients were followed up at least once every 3 months after surgery by ultrasonography (US), computed tomography (CT) scan, or magnetic resonance imaging (MRI), in addition to the measurement of

Table II. *ENO1* mRNA levels in newly enrolled 49 HCC samples.

		ENO1 mRNA levels (mean ± SD)	
Gender	No.		N.S. (ST)
Male	40	1.27±0.88	
Female	9	0.89±0.40	
Age			N.S. (ST)
<65 years	16	1.32±1.08	
≥65 years	33	1.14±0.68	
Tumor size			P=0.03 (ST)
<3 cm	15	0.82±0.49	
≥3 cm	34	1.37±0.89	
Tumor differentiation			N.S. (ANOVA)
Well	9	0.98±0.75	
Moderately	35	1.27±0.89	
Poorly	5	1.15±0.51	
Venous invasion			P=0.03 (MW)
Negative	22	0.88±0.35	
Positive	27	1.47±1.00	
Stage[a]			P=0.04 (MW)
I	17	0.86±0.36	
II and III	32	1.38±0.94	

N.S., not significant. ST, Student's t-test. Anova, analysis of variance. MW, Mann-Whitney's U test. [a]Stage based ov the UICC TNM classification.

serum α-fetoprotein and protein induced by vitamin K absence II (PIVKA-II), as described previously (13). When tumor recurrence was suspected, hepatic angiography was included in the follow-up examination.

Selection of glycolysis-related genes. By using gene symbol (http://www.ncbi.nlm.nih.gov/sites/entrez/), we searched for genes whose products are involved in a series of reactions that metabolize glucose to pyruvate. As a result, we successfully extracted the raw data for 22 glycolysis-related genes from the data for 7,070 genes (Table I). Among the 22 genes, we omitted 8 genes whose levels were <20 in >15% of the HCC cases. The remaining 14 genes were statistically assessed for associations with an aggressive phenotype of HCC.

Quantitation of ENO1 mRNA by real-time reverse-transcription (RT)-PCR. RNA isolation from both tissues and cell lines was performed with TRIzol reagent according to the manufacturer's instructions (Invitrogen Corp., Tokyo, Japan). First-strand cDNA was synthesized from the total RNA with the ExScript RT Reagent kit (Takara, Otsu, Japan). cDNA template (10 ng) was used for real-time PCR. A Light Cycler-System and LightCycler FastStart DNA Master SYBR-Green I (Roche Diagnostics, Tokyo, Japan) were used for real-time PCR. Expression levels of *ENO1* mRNA were calculated as the ratio relative to ß-actin (internal control), as described previously (14). The primers were 5'-GAGCTCCGGGAC AATGATAA and 5'-CTGTTCCATCCATCTCGATC for *ENO1* and 5'-AACTCCATCATGAAGTGTGACG and 5-GATCCACATCTGCTGGAAGG for the ß-actin gene.

Validation group. To validate the performance of *ENO1*, we used real-time RT-PCR to analyze *ENO1* mRNA levels in 49 HCC tissues from 49 newly enrolled patients (Table II).

Cell culture. HLE cells, an HCC cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen) supplemented with penicillin (100 units/ml), streptomycin (100 Ag/ml), and sodium bicarbonate (1.5 g/l) at 37˚C in 5% CO_2 in air. To examine the effect of the inhibition of glucose metabolism on cell proliferation, we used glucose-free DMEM (Invitrogen) containing 10% dialyzed FBS (Invitrogen) instead of DMEM containing 10% FBS. The latter culture medium contains <5 mg/l of D-glucose.

Knockdown of ENO1 protein. Endogenous *ENO1* knockdown was performed by transfection of small-interfering RNA (siRNA) (sequence: 5'-AGCUGGUGCCGUUGAGAAG) specific for *ENO1* mRNA into HLE cells. Lipofectamine 2000 (Invitrogen Corp.) was used for siRNA transfection. Trypsinized HLE cells were resuspended in Opti-MEM I (Promega, Tokyo, Japan) containing 5% FBS. A complex of siRNA with Lipofectamine 2000 was administered to the suspended HLE cells. Twenty-four hours after transfection, the medium was replaced with glucose-containing or glucose-free DMEM, as mentioned above. A siRNA sequence (B-Bridge International Inc., Mountain View, CA) that does not match any human gene sequence was used as a negative control for *ENO1* siRNA.

Western blot analysis. Western blot analysis was performed as described previously (14,15). Anti-enolase polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-ß-actin polyclonal antibody (Sigma-Aldrich Co.) were used as the primary antibodies. Antigen detection was performed by using the ECL chemiluminescent assay (Amersham Biosciences, Tokyo, Japan).

Cell proliferation and cell cycle assays. Cell proliferation was determined by counting the number of viable cells. To determine the viability of cells, HLE cells were stained with Hoechst 33342 and propidium iodide (PI). Stained cells were counted under a fluorescence microscope (model IX71: Olympus Corp., Tokyo, Japan). HLE cells were harvested and fixed in cold 70% ethanol. Fixed cells were stained with PI. The cell-cycle stage was determined by measuring DNA content with flow cytometry (Epics XL, Beckman Coulter, Fullerton, CA).

Statistical analysis. Data for clinicopathologic characteristics were analyzed by the Student's t-test, Mann-Whitney U test,

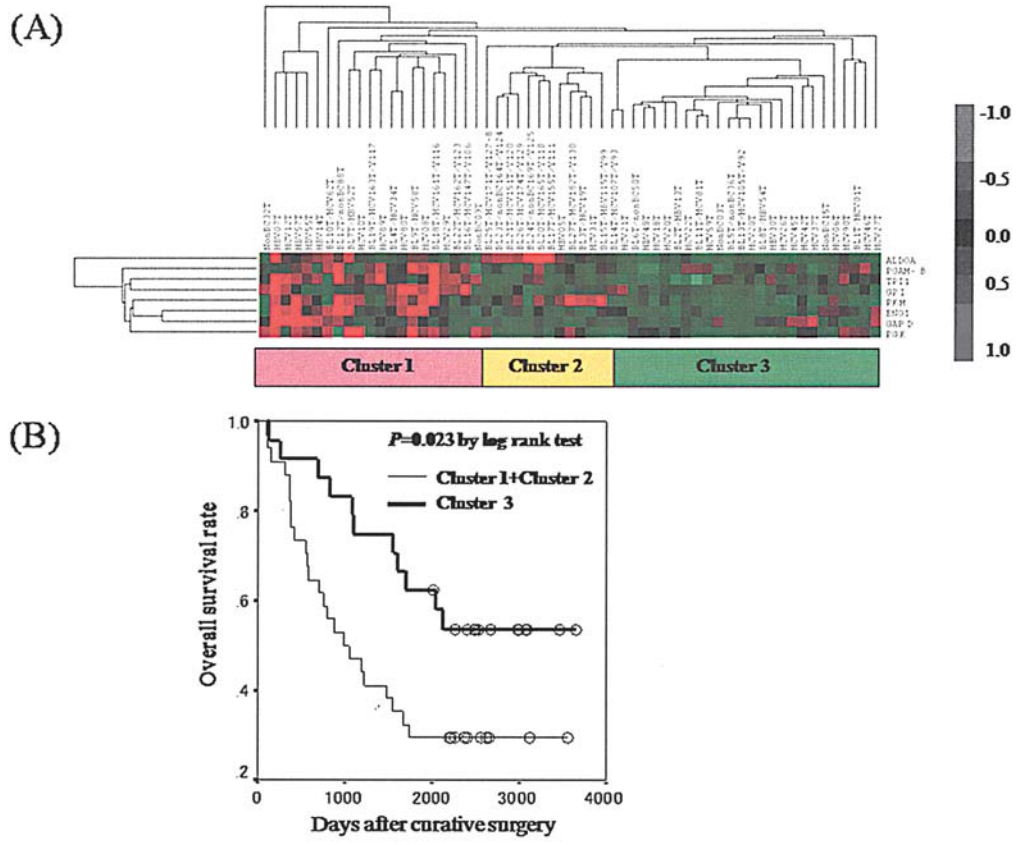


Figure 1. New molecular subclasses of HCC based on the expression patterns of 8 glycolysis module genes activated by HIF-α. (A) Color displays of expression of 8 glycolysis module genes in 60 HCC samples (http://surgery2.med.yamaguchi-u.ac.jp/research/DNAchip/) created with Cluster software and Tree View software (16). There are 3 major clusters according to the 8 gene expression levels. HCC in cluster 1 shows activation patterns of the 8 HIF-α-regulated genes, HCC in cluster 3 shows their inactivation patterns, and HCC in cluster 2 shows intermediate patterns of their expression levels. (B) The relationship between the new molecular subclasses and patient outcome. HCC patients in clusters 1 and 2 had significantly shorter overall survival than did HCC patients in cluster 3.

analysis of variance (ANOVA), and the log-lank test by using SPSS 11.0J software (SPSS, Inc., Chicago, IL). P<0.05 was accepted as statistically significant. A cluster analysis with 8 genes in 60 patients was performed according to the method of Eisen *et al* (16).

Results

We identified 22 glycolysis-related genes among the 7,070 genes on the DNA array platform (8). Fourteen of the 22 genes were judged to be eligible because of their abundant expression level (Table I). *PKM* gene expression was significantly higher in less-differentiated tumors than in well-differentiated tumors (P<0.0001 by ANOVA). The expression levels of 3 genes (*ALDOA*, *TPI1*, and *ENO1*) were significantly higher in advanced HCC (stages II and III) than in stage I HCC (Table I). We found that 10 of the 14 eligible glycolysis-related genes were transcriptional targets of hypoxia-inducible factor 1α (HIF-1α) (3). Except for HK1 and HK2, levels of the remaining 8 HIF-1α-regulated genes (*GPI*, *ALDOA*, *TPI1*, *GAPD*, *PGK*, *PGAM*, *ENO1* and *PKM*) were significantly higher in HCC with venous invasion (VI) than in HCC without VI (Table I).

Because all of the 8 downstream genes of HIF-1α were related to VI of HCC, we used a cluster analysis (16) to define a new subclass of HCC with induction of HIF-1α-regulated glycolysis module genes. Our cluster analysis classified the 60 HCC patients into 3 clusters (Fig. 1A). HCC in cluster 1 showed activation of the 8 HIF-1α-regulated genes, HCC in cluster 3 showed their inactivation, and HCC in cluster 2 showed an intermediate pattern of expressions. HCC patients in clusters 1 and 2 had a significantly shorter overall survival than did HCC patients in cluster 3 (P=0.023 by log-rank test, Fig. 1B). The median disease-free survival periods of HCC patients in clusters 1 and 2 (combined) and cluster 3 were 419 days and 924 days, respectively; however, there was no statistically significant difference between the disease-free survival periods of the 2 groups (data not shown).

To confirm the reproducibility of our microarray data, we used real-time RT-PCR to investigate the *ENO1* mRNA levels in an independent group of 49 HCC samples. We found that *ENO1* mRNA levels were significantly higher in HCCs with a larger tumor size (>3 cm), HCCs with VI, and HCCs of stages II and III than in those with smaller tumor size (<3 cm), those without VI, and those of stage I (P=0.03, P=0.03 and P=0.04, respectively, Table II).

Transfection of a small-interfering RNA (siRNA), which was designed to inhibit the transcription of *ENO1*, into HLE cells markedly decreased both the mRNA and protein levels of *ENO1* (Fig. 2A and B). The knockdown of *ENO1* significantly inhibited the proliferation of HLE cells in both the

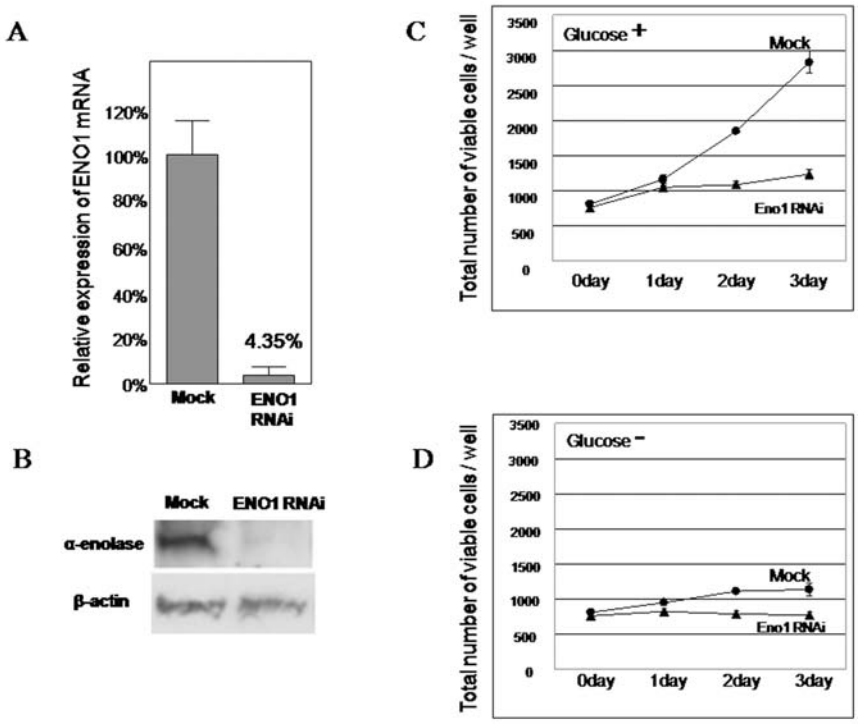
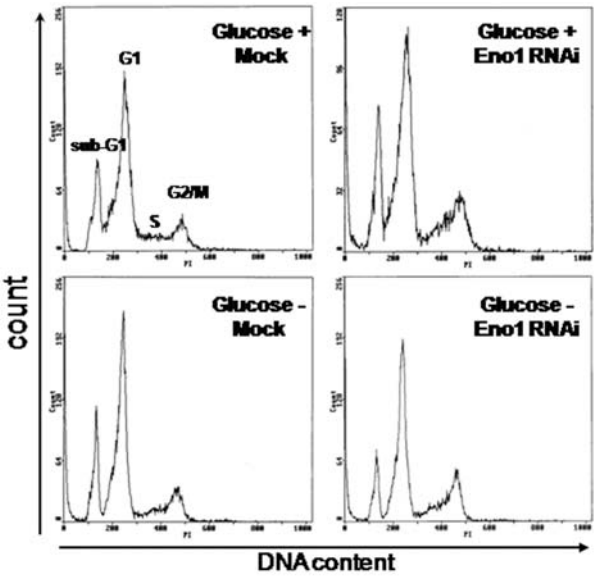


Figure 2. Inhibitory effect of *ENO1* knockdown on the proliferation of HLE cells. (A) Assessment of the knockdown of the *ENO1* gene by real-time RT PCR. Forty-eight hours after transfection of a small-interfering RNA (siRNA) targeting *ENO1* or a mock siRNA into HLE cells, total RNA was isolated from those cells. Levels of *ENO1* mRNA were measured by real-time RT-PCR. Results are shown as the mean ± SE of triplicate cultures. (B) Assessment of the knockdown of the *ENO1* protein by Western blot. Cell lysates of *ENO1* siRNA-transfected or control cells were prepared at 72 h after transfection. (C) Growth curves of *ENO1* siRNAi-transfected cells and mock cells. Twenty-four hours after siRNA transfection, *ENO1* siRNA-transfected and mock cells were cultured in glucose-containing medium (glucose$^+$). The total number of viable cells was counted with the use of propidium iodide and Hoechst 33342 at each time indicated. Results are shown as the mean ± SE of 5 cultures. (D) The same procedure was performed in glucose-free medium (glucose$^-$). Note that the knockdown of *ENO1* significantly inhibited the proliferation of HLE cells in both the glucose-rich and glucose-free conditions (C and D).



Glucose	RNAi	sub G1	G1	S	G2/M % (mean ± S.E.)
+	Mock	20.4 (± 0.69)	47.37 (± 0.41)	9.89 (± 0.16)	9.33 (± 0.46)
	ENO1	17.97 (± 0.44)	42.43 (± 0.79)	**5.93 (± 0.16)***	**15.7 (± 0.46)***
−	Mock	16.33 (± 0.09)	51.13 (± 0.79)	5.97 (± 0.13)	13.73 (± 0.30)
	ENO1	12.17 (± 0.20)	47.57 (± 0.61)	5.92 (± 0.06)	**22.33 (± 0.44)***

Figure 3. Effect of *ENO1* knockdown on the cell cycle of HLE cells. Cells were cultured in glucose-containing or glucose-free medium (glucose$^+$ or $^-$) 24-72 h after *ENO1* or mock transfection. Subsequently, cells were harvested, fixed in cold 70% ethanol, and stained with propidium iodide. The cell cycle stage was determined by measuring DNA content with flow cytometry. *ENO1* knockdown in HLE cells significantly decreased the S phase and increased the G2/M phase of the cell cycle in the glucose-rich condition, and it significantly increased the G2/M phase of the cell cycle in the glucose-free condition.

glucose-rich and glucose-free conditions (Fig. 2C and D). The knockdown of *ENO1* in HLE cells significantly decreased the S phase and increased the G2/M phase of the cell cycle in the glucose-rich condition, and the knockdown significantly increased the G2/M phase of the cell cycle in the glucose-free condition (Fig. 3).

Discussion

DNA microarray technology started a revolution in medical science upon its introduction in 1995 (17). This sophisticated technique has enabled a comprehensive genomic search to discover modules responsible for the metastatic potential of HCC (7). These modules include the immune response module (8,9,18), detoxification module including *CYP* family genes (19), the cell adhesion module such as *CD44*, *ITGA6*, *SPP1* (10) and *Rho* family genes (20,21), the cell growth module such as *TAF4B*, *SLC4A7*, *RAB38* and *RYR1* genes (22), the oncogenic module such as *MET* (23), and the *ID2*-related module (12,13). Additionally, our current study shows that a glycolysis module has a significant role in HCC metastasis.

Notably, levels of the 8 glycolysis module genes, all of which were transcriptionally activated by HIF-1α, were significantly higher in HCC with VI than in HCC without VI. Since VI is a hallmark of the intrahepatic spread of HCC cells and of poor outcome (24), we hypothesized that a molecular subclass of HCC determined by the 8-gene signature might be related to the outcome. Indeed, our cluster analysis showed that HCC patients with upregulation of the 8 glycolysis module genes had significantly shorter overall survival than did HCC patients without upregulation. It is known that HIF-1α activation plays a key role in the maintenance of the glycolytic phenotype (25,26), favors cancer invasion and metastasis via increased angiogenesis (27), and promotes cell proliferation (28). Thus, in conjunction with these reports and our present findings, it is reasonable to assume that HIF-1α can be involved in the metastatic potentials of HCC, and the downstream glycolysis module is a potent therapeutic target for preventing HCC metastasis.

The comprehensive analysis of cellular proteins is a central area of oncology research that is developing in the post-genome era (29,30). Our previous proteomics study found that ENO1, one of the HIF-1α-regulated glycolytic enzymes, was overexpressed in HCC and that its level was associated positively with tumor progression (31). Many proteomics studies have reported increased levels of various glycolysis-related enzymes in malignant tumors such as lung squamous cell carcinoma (32), pancreatic carcinoma (33), esophageal squamous cell carcinoma (34), HER-2/neu-positive breast carcinoma (35), and prostate carcinoma (36). More importantly, *ENO1* is the only upregulated protein common to all of the cancer tissues in these studies (31-36). In the present study, we used an independent cohort of HCC patients to confirm that *ENO1* mRNA levels were positively associated with VI of HCC. These findings prompted us to highlight the inhibitory effect of *ENO1* knockdown on the proliferation of HCC cells. Our *ENO1* knockdown strategy may have the advantage of fewer adverse effects, because we previously confirmed that the *ENO1* protein is overexpressed in HCC tissues compared with non-cancerous liver tissues (31). By contrast, the targeting of HIF-1α would cause severe effects because of its broad range of biological function and its wide distribution in various organs (27,28).

We also found that *ENO1* knockdown significantly inhibited the proliferation of HLE cells even in glucose-free conditions. This finding suggests that *ENO1* has other non-glycolytic functions, which may yield another tactic against HCC cells in addition to the metabolic advantage of the increased glycolysis in cancer cells. The *ENO1* gene intrinsically encodes both α-enolase and a shorter monomeric structural lens protein, tau-crystallin. α-enolase is a full-length form that is found in the cytoplasm. The shorter tau-crystallin protein is produced from an alternative translation start of *ENO1*, and it is localized to the nucleus. This protein has been found to bind to an element in the c-myc promoter (37), and has been designated as the c-myc promoter-binding protein (MBP-1). Ghosh *et al* reported that knockdown of MBP-1 in prostate cancer cells perturbs cell proliferation by inhibiting cyclin A and cyclin B1 expression (37). This report supports our present finding of the non-glycolytic action of *ENO1* in cell proliferation. Thus, we must investigate whether the non-glycolytic action of *ENO1* is specific for HCC cells or not. It also remains unclear why *ENO1* knockdown allowed HLE cells to arrest in the G2/M phase of the cell cycle without an increase in apoptosis.

Our present study shows the significance of the HIF-1α-regulated glycolysis module in HCC progression by showing its predictive power for patient outcome and the therapeutic efficacy of ENO1 from among the HIF-1α-regulated glycolysis module genes. This HIF-1α-regulated glycolysis module could be important for the development of first-line treatments during the multiple metastatic cascades of HCC. Further studies are needed for deeper insights into our present findings and to develop a new therapy against HCC.

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