

Metabolic Signature Genes Associated with Susceptibility to Pyruvate Kinase, Muscle Type 2 Gene Ablation in Cancer Cells

Yuri Jung^{1,2}, Ye Jin Jang¹, Min Ho Kang¹, Young Soo Park^{1,2}, Su Jin Oh^{1,2}, Dong Chul Lee¹, Zhi Xie³, Hyang-Sook Yoo¹, Kyung Chan Park^{1,*}, and Young Il Yeom^{1,2,*}

Pyruvate kinase, muscle type 2 (PKM2), is a key factor in the aerobic glycolysis of cancer cells. In our experiments, liver cancer cell lines exhibited a range of sensitivity to PKM2 knockdown-mediated growth inhibition. We speculated that this differential sensitivity is attributable to the variable dependency on glycolysis for the growth of different cell lines. Transcriptome data revealed overexpression of a glucose transporter (GLUT3) and a lactate transporter (MCT4) genes in PKM2 knockdown-sensitive cells. PKM2 knockdown-resistant cells expressed high levels of the lactate dehydrogenase B (LDHB) and glycine decarboxylase (GLDC) genes. Concordant with the gene expression results, PKM2 knockdown-sensitive cells generated high levels of lactate. In addition, ATP production was significantly reduced in the PKM2 knockdown-sensitive cells treated with a glucose analog, indicative of dependency of their cellular energetics on lactate-producing glycolysis. The PKM2 knockdown-resistant cells were further subdivided into less glycolytic and more (glycolysis branch pathway-dependent) glycolytic groups. Our findings collectively support the utility of PKM2 as a therapeutic target for high lactate-producing glycolytic hepatocellular carcinoma (HCC).

INTRODUCTION

During tumorigenesis, alterations in cellular metabolism occur to prime cells for transformation in a manner that facilitates their survival and anabolic growth (Daye and Weller, 2012; Ward and Thompson, 2012). Whereas the ATP-producing process of normal cells usually depends on oxidative phosphorylation in mitochondria, cancer cells depend mainly on glycolysis, even under aerobic conditions (the “Warburg effect”). Pyruvate kinase, muscle type 2 (PKM2), is a key metabolic enzyme in aerobic glycolysis, producing energy and pyruvate (eventually lactate) from phosphoenolpyruvate (PEP). PKM2, differing from PKM1

only by the differential splicing of one exon, is the predominant form of pyruvate kinase in cancer cells. The increased expression of PKM2 confers substantial benefits on the rapidly growing tumorigenic cells. In contrast with the constitutively active PKM1, PKM2 is allosterically regulated either as a dimer with low affinity for PEP or as a tetramer with high affinity for PEP (Mazurek et al., 2005). The oncogenic signal-mediated allosteric change of PKM2 leads to accumulation of various glycolytic metabolites for macromolecular biosynthesis to support cell growth, providing a selective growth advantage for tumor cells (Christofk et al., 2008a; 2008b; Hitosugi et al., 2009; Yang et al., 2011).

The mechanisms by which PKM2 facilitates lactate production and blocks mitochondrial oxidative phosphorylation in cancer cells have been investigated in a recent report focusing on a non-metabolic function of PKM2. The prolyl hydroxylase 3 (PHD3)-mediated prolyl hydroxylation of PKM2 stimulates the function of PKM2 as a coactivator of HIF-1 transactivation, leading to an HIF-dependent increase of metabolic gene expression, such as lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase 1 (PDK1), resulting in increased lactate production (Kim et al., 2006; Luo et al., 2011; Semenza, 2011).

In addition to producing lactate by glycolysis via overexpression of PKM2, cancer cells develop other glucose metabolic processes by promoting the expression of genes involved in associated pathways. Serine/glycine metabolism using 3-phosphoglyceride, a branch of glucose metabolism that generates pyrimidine, has been implicated in the development of prostate and non-small cell lung cancers (Sreekumar et al., 2009; Zhang et al., 2012). Overexpression of either glycine dehydrogenase (GLDC) or any of the other serine/glycine metabolic enzymes induces glycolysis, leading to increased pyrimidine metabolism for cancer cell proliferation and cellular transformation in a metabolic activity-dependent manner (Zhang et al., 2012). Hexosamine biosynthesis pathway is another arm of glucose metabolism, which is the process for the O-linked

¹Medical Genomics Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Korea, ²Department of Functional Genomics, University of Science and Technology, Daejeon 305-350, Korea, ³Pfizer Global Research and Development, 10770 Science Center Drive, San Diego, California 92121, USA

*Correspondence: kpark@kribb.re.kr (KCP); yeomyi@kribb.re.kr (YIY)

Received December 6, 2012; revised February 28, 2013; accepted March 4, 2013; published online March 18, 2013

Keywords: glycolysis, glycolysis-dependent, lactate, PKM2, SLC16A3

glycosylation of the serine or threonine residues in proteins that play a role in controlling fuel metabolism and cell growth (Butkinaree et al., 2010). Glutamine-fructose-6-phosphate transaminases (GFPT1 and GFPT2) are the first and rate-limiting enzymes of the hexosamine biosynthesis pathway.

Together with increased aerobic glycolysis, cancer cells develop specific changes in mitochondrial function to supply the intermediates required for rapid cell growth. Citrate, a tricarboxylic acid (TCA) cycle metabolite, is released into the cytosol and used as a precursor for lipid biosynthesis, together with acetyl-CoA (Metallo et al., 2012). In some cancer cells, glutamine is a major source for replenishing the intermediates of the TCA cycle required by the continual loss of citrate and malate (another source of acetyl-CoA) from the cycle. Therefore, glutamine uptake and oxidation are important factors for the bioenergetics and biomaterial supplies in cancer cells (Le et al., 2012; Wise and Thompson, 2010). Myc stimulates glutamine metabolism by inducing the expression of glutamine metabolism genes, such as the glutamine transporter SLC1A5 and carbamoyl phosphate synthetase II (CAD) genes (Bush et al., 1998; Wise et al., 2008), or by suppressing the expression of the glutaminase (GLS)-targeting miR-23a/b gene (Gao et al., 2009). Up-regulation of Myc in cancer cells triggers glutamine addiction, resulting in cellular apoptosis upon glutamine deprivation (Gao et al., 2009; Wise et al., 2008).

In the current study, we evaluated the molecular features of hepatocellular carcinoma (HCC) cell lines sensitive or resistant to PKM2 knockdown-mediated growth inhibition. Lactate-producing glycolysis-addicted cells, which exhibit a greater efflux of lactate, were extremely sensitive to PKM2 knockdown-mediated growth inhibition. The PKM2^{KD}-resistant cells were further subdivided into two groups: less glycolytic and more glycolytic (glycolysis arm pathway-dependent). Based on the collective results, we propose the utility of PKM2 as a therapeutic target for high lactate-producing glycolytic HCC.

MATERIALS AND METHODS

HCC cell lines, cultures and materials

The SNU cell lines were purchased from Korean Cell Line Bank (KCLB), the JHH series were purchased from JHSF (Japan Health Sciences Foundation, www.jhsf.or.jp), and the other cells were purchased from American Type Culture Collection (ATCC). The SNU cells were cultured in Roswell Park Memorial Institute (RPMI) medium, the JHH cells in MEM, and the remaining cell types in Dulbecco's Modified Eagle Medium (DMEM) at 37°C in 5% CO₂. All of the media contained 10% heat-inactivated FBS. The glucose analog, 2-deoxy-D-glucose (2-DG, Cat. No. D6134), and the glutamine analog, 6-diazo-5-oxo-L-norleucine (DON, Cat. No. D2141), were obtained from Sigma, USA. PKM2 siRNA (sense 5'-AACATCAAGATTATC AGCAA-3', anti-sense 5'-TTTGCTGATAATCTTGATGTT-3') was acquired from Genolution, Korea (www.genolution1.com). All of the primers used in this study are listed in the Supplementary Table (sTable 1).

Illumina BeadChip array assay

The total RNAs of the six HCC cell lines (Huh-1, JHH-4, SNU-449, SNU-886, SNU-739 and SNU-423) were isolated using the Qiagen RNA preparation kit, and their transcriptomes were analyzed using Illumina's Human HT-12 v3 Expression BeadChips. The RNA quality was verified using an Agilent Bioanalyzer (Agilent Technologies, USA), and microarray analyses were performed as previously described (Bany et al., 2012).

The results were normalized using publicly available transcriptome data for non-tumor liver tissues (n = 193) of HCC patients (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36376>).

Reverse transcriptase (RT)-PCR assay

Total RNAs were extracted using TRIzol reagent (Cat. No. TR 118, Molecular Research Center, Inc.) according to the manufacturer's instructions. The RT-PCR assays were performed according to standard protocols. Aliquots (3 µg) of total RNA were employed for cDNA synthesis using reverse transcriptase (MBI Fermentas Inc.). PCRs were performed under the following optimized PCR conditions: 1 cycle at 95°C for 30 s; 18 to 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; with a final extension cycle at 72°C for 10 min. The gene-specific primer sequences are listed in the Supplementary Table 1.

Cell viability assay

Cells were seeded (1 × 10³ cells per well) in 96-well plates, and siPKM2 was transiently transfected using Lipofectamine® RNAiMAX (Cat. No. 13778-150, Invitrogen). After incubation for 0, 1, 3, or 5 days, the cells were incubated with 20 µl of Cell Titer-Blue® (CTB) reagent from Promega (Cat. No. G8081) for 2 h at 37°C in 5% CO₂. Fluorescence was measured at 530 nm excitation and 590 nm emission.

Assays for determining lactate and ATP concentrations

To determine the amount of lactate in the culture media, we seeded the cells (3 × 10⁵ cells per well) in 6-well plates. After 24 h of growth at 37°C in a 5% CO₂ incubator, the cell culture media were collected and centrifuged at 10,000 × g for 10 min. The supernatants of the samples were processed for lactate determination using the EnzyChrom™ L-lactate assay kit (Cat No ECLC-100, BioAssay Systems) according to the manufacturer's protocol, and the absorbance at 565 nm was measured using a microplate reader.

The total ATP content was determined using the ATP fluorometric assay kit (Cat. No. K354-100, BioVision). Briefly, cells were seeded (3 × 10⁵ cells per well) in 6-well plates and incubated for 24 h at 37°C in 5% CO₂. The cells were pelleted and lysed in 100 µl of the ATP assay buffer supplied in the kit, followed by centrifugation at 10,000 × g for 10 min. An aliquot of the supernatant (5 µl) was mixed with the ATP Probe, ATP Converter and Developer Mix, and incubated for 30 min at room temperature in the dark. Fluorescence intensity at 530 nm excitation and at 590 nm emission was recorded.

MYC reporter assay

Transient transfection was conducted using Lipofectamine® Transfection Reagent (Cat. No. 18324-012, Invitrogen), and the Firefly and Renilla luciferase activity assays were performed according to the manufacturer's instructions (Promega, Madison, WI). The day before transfection, the cells (5 × 10⁴ per well) were seeded into 12-well culture plates. The cells were co-transfected with a reporter plasmid (200 ng) and the internal control (CMV-Renilla, 50 ng) in 12-well plates and incubated for 24 h. Subsequently, the cells were harvested in Promega lysis buffer, and the luciferase and Renilla activities were measured using the appropriate substrates in the reporter assay systems (Promega). The luciferase activity was normalized to that of Renilla.

Table 1. DEGs associated with glycolysis-related processes

Grp	#	Gene (Alias)	$\Delta(z\text{-score})_{\text{Average}}$		$\Delta(z\text{-score}) (z_{\text{sens}} - z_{\text{resis}})$	<i>t</i> -Test	Ref. Seq.
			Sensitive	Resistant			
Sensitive	1	SLC16A3 (MCT4)	2.32	-0.40	2.72	0.0097	NM_001042422.2
	2	SLC2A3 (GLUT3)	1.34	-0.20	1.54	0.0264	NM_006931.2
	3	GFPT2	0.65	-0.74	1.39	0.0239	NM_005110.2
	4	HK2	0.48	-0.49	0.97	0.0092	NM_000189.4
Resistant	1	LDHB	-0.67	2.49	-3.16	0.0001	NM_001174097.1
	2	CPS1	-0.47	1.73	-2.21	0.0336	NM_001122633.2
	3	GLDC	-0.20	1.84	-2.04	0.0038	NM_000170.2
	4	FOXA1 (HNF3A)	-0.07	1.78	-1.85	0.0311	NM_004496.3
	5	SLC27A5 (FATP5)	-0.60	0.73	-1.34	0.0011	NM_004496.4
	6	RBKS	0.03	0.68	-0.65	0.0363	NM_022128.1
	7	SLC25A10 (DIC)	0.56	1.19	-0.63	0.0406	NM_001270888.1
	8	DCXR	1.03	1.64	-0.61	0.0086	NM_001195218.1

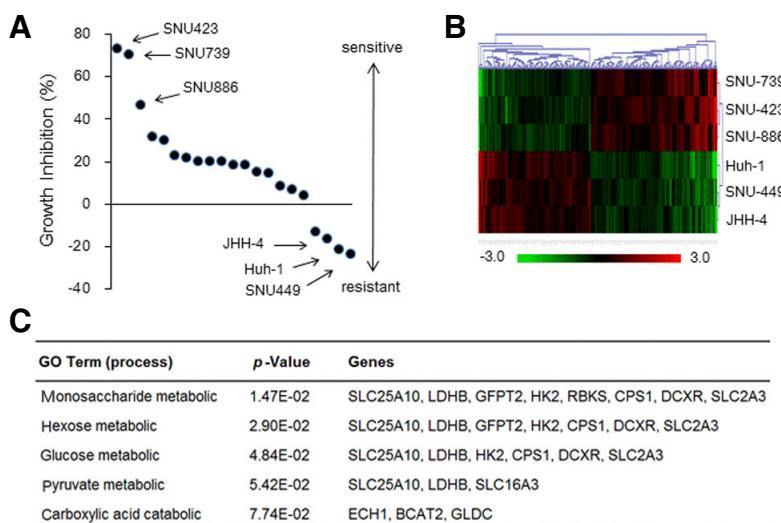


Fig. 1. Glycolysis-related processes are involved in the different sensitivities of HCC cell lines to PKM2 knockdown. (A) PKM2 knockdown differentially affects the viability of 21 HCC cell lines, which range from very sensitive to resistant (not affected). Cell growth was determined on day 5 using the CTB assay kit. (B) Heatmap of DEGs. The total RNAs of six cell lines from the extremely sensitive or resistant groups (three cell lines per end) were subjected to an Illumina BeadChip array. The DEGs ($n = 231$) displaying different average expression levels with criteria $|\Delta z\text{-score}| > 0.5$ were selected among the genes satisfying the *t*-test criterion ($p < 0.05$). (C) Glycolysis-related pathways and genes selected from the ontology analysis of the DEGs that distinguish the differentially sensitive cell lines.

RESULTS AND DISCUSSION

HCC cell lines exhibit a range of sensitivity to PKM2 knockdown

The cell viability assays were performed using PKM2 siRNA to estimate the value of PKM2 as a therapeutic target. We observed inhibition of cancer cell growth upon PKM2 knockdown in some, but not all, of the HCC cell lines tested. To further validate this finding, the viability assay was performed with more of the HCC cell lines. During the 5-day assay, the growth of 21 HCC cell lines was examined at days 0, 1, 3 and 5 using the CTB reagent (Supplementary Fig. 1A). Our experiments revealed a range of growth inhibitory effects in the HCC cell lines examined, from very sensitive to not responsive to PKM2 knockdown. In contrast to the SNU-449, Huh-1 and JHH-4 cells that showed no growth inhibition in response to the PKM2 knockdown, the SNU-886, SNU-739 and SNU-423 cell lines displayed very restricted cell growth by day 5 (Fig. 1A). For those six cell lines, the efficiency of PKM2 knockdown was

confirmed by RT-PCR analyses (Supplementary Fig. 1B). Therefore, we investigated the effect of PKM2-overexpression on cell growth using PKM2 knockdown-sensitive or -resistant cells that were stably transduced with shRNA PKM2. The results demonstrated that PKM2-overexpression significantly rescued the retarded growth phenotype of the SNU739/shPKM2 cells but did not change the growth of SNU449/shPKM2 cells, indicating the physiological relevance of PKM2 expression for cell viability (Supplementary Fig. 1C). We hypothesized that the differential sensitivities may be attributable to the different cellular contexts of the cell line-specific expression of specific genes on which the cells depend for continued growth or survival.

To ascertain the underlying causes of the different sensitivities to PKM2 knockdown, we further examined the differentially expressed genes (DEGs) based on the transcriptome data for the cell lines in the different sensitivity groups. Accordingly, we performed Illumina DNA chip assays of six of the cell lines (three from each extreme end of sensitivity) grown under normal culture conditions. The Illumina chip data for the cell lines

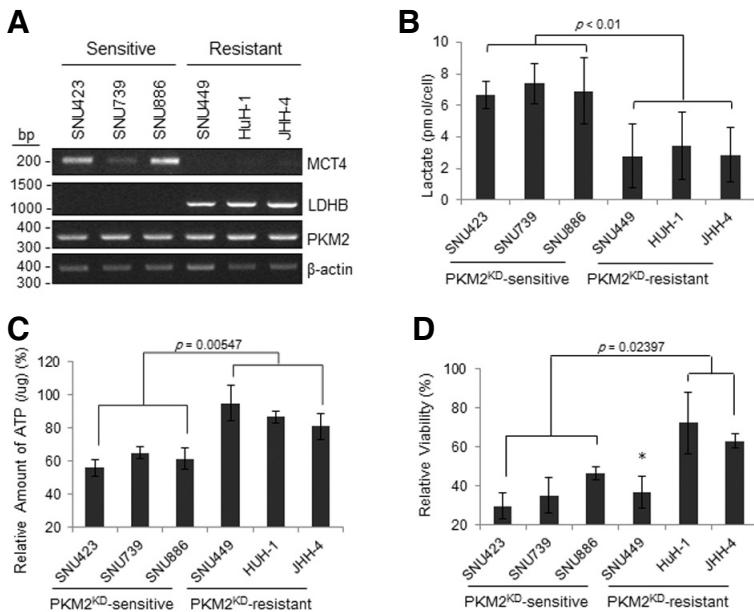


Fig. 2. PKM2 knockdown-sensitive cells display glucose-dependent growth. (A) The expression of the MCT4 and LDHB genes, which were among glycolysis-related pathway DEGs, was determined via RT-PCR analysis of the total RNA from the six cell lines. The whole-gel images are shown in the supplementary data (Supplementary Fig. 3a). (B) The amount of lactate in the culture medium was determined using the EnzyChrom™ L-lactate assay kit. Differences in the lactate levels of the two groups were analyzed using the *t*-test ($p < 0.01$). (C) Cells were treated with a glucose analog, 2-DG (10 mM), for 18 h, and the amount of ATP in the cell lysates was determined using the ATP fluorometric assay kit. Differences in the ATP amounts of the two groups were analyzed using the *t*-test ($p = 0.00547$). (D) Cells were treated with 2-DG (10 mM), and their growth rates were determined using the CTB assay kit on day 4. Differences in the viability of the two groups, excluding the SNU449 cell line, were analyzed using the *t*-test ($p = 0.02397$).

were normalized with publicly available transcriptome data for non-tumor tissues ($n = 193$) of HCC patients (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36376>), and the z-scores were plotted (Supplementary Fig. 2A). Compared with the transcriptome data from the Samsung Medical Center, the PKM2 level in all six cell lines was consistently high relative to that of non-tumor liver tissues, implying that PKM2 expression was significantly more upregulated in HCC than in non-tumor liver tissues (Supplementary Fig. 2B). To identify the DEGs of the PKM2 knockdown-sensitive and -resistant cell lines, we calculated the average difference and *t*-test value of the z-scores of the two sensitivity groups. Among the genes satisfying the *t*-test criteria ($p < 0.05$), 234 DEGs displaying different average expression levels with criteria $|\Delta z\text{-score}| > 0.5$ were selected (Fig. 1B and Supplementary Table 2).

The two sensitivity groups show significant differences in the expression patterns of genes involved in glycolysis-related processes

To elucidate the specific mechanisms underlying the differential sensitivities of the cell lines, we initially analyzed the ontology of DEGs using the DAVID bioinformatics resource (<http://david.abcc.ncifcrf.gov/>). The results included metabolic ontology terms, such as the monosaccharide, hexose, glucose and pyruvate metabolic processes, that are directly related to glycolysis (Fig. 1C and Supplementary Table 3). Expression levels of specific genes, such as SLC16A3 (MCT4) and SLC2A3 (GLUT3), were high in the PKM2 knockdown-sensitive cell lines, whereas high expression of the LDHB and GLDC genes was observed in the PKM2 knockdown-resistant cell lines (Table 1). In view of the function of PKM2 in glycolysis, we were interested in determining whether differentially expressed glycolysis-related genes contribute to the variable dependencies of HCC cell lines on PKM2 expression for growth.

To investigate this effect, we clarified the known biological functions of the above genes using PubMed searches. Initially, we focused on the MCT4 and LDHB genes, which ranked high among the DEGs between the sensitive and resistant groups.

The DNA chip data were confirmed using RT-PCR with the total RNAs isolated from the six cell lines (Fig. 2A). The MCT4 gene product exports lactate across the plasma membrane to the outside of the cell and may thus accelerate the metabolic conversion of glucose to lactate (Supplementary Fig. 2C). This process may be facilitated by low levels of LDHB, which catalyzes the transition of lactate to pyruvate. Unlike PKM2 knockdown-sensitive cells, the high level of expression of LDHB and the low level of expression of MCT4 in the PKM2 knockdown-resistant cells may downregulate the transition of pyruvate to lactate (Supplementary Fig. 2C), possibly resulting in high levels of cellular pyruvate, inhibition of glucose consumption, and, consequently, cells that are less glycolytic. LDHA, which catalyzes the transition of pyruvate to lactate, was upregulated in all six of the HCC cell lines as well as in tumor tissues, as compared with non-tumor tissues, but was not differentially expressed between the PMK2 knockdown-sensitive and -resistant cell lines (Supplementary Table 4). This finding suggests that PKM2 knockdown-resistant cells also depend on glycolysis for their survival, but not to the same extent as highly glycolytic cells, resulting in minor effects, if any, on cell growth upon PKM2 knockdown-mediated blocking of glycolysis. We propose that the growth of PKM2 knockdown-sensitive cells is inhibited as a result of their addiction to PKM2-dependent glycolysis for the production of energy and cellular building blocks for survival or growth, in contrast to the PKM2 knockdown-resistant cells that are less glycolytic.

PKM2 knockdown-sensitive cells display glucose-dependent growth

To confirm our hypothesis, we initially assessed the amount of lactate released by the cells into the medium. As expected, all three PKM2 knockdown-sensitive cell lines released significantly more lactate into the culture medium than did the resistant cell lines (Fig. 2B). Because higher lactate release is indicative of greater glucose use, we investigated the effect of the glucose analog, 2-deoxy-D-glucose (2-DG), which inhibits the activity of hexokinases and blocks cellular glycolysis, on ATP

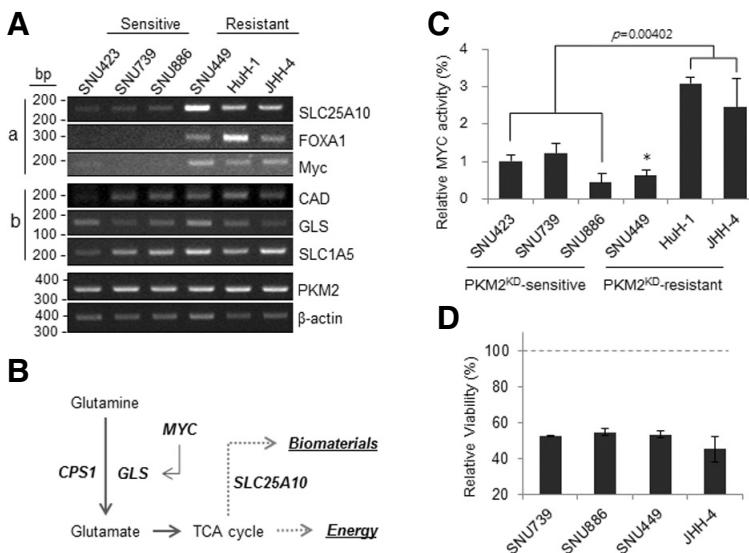


Fig. 3. Glutaminolysis is not directly involved in the sensitivity of cancer cells to PKM2 knockdown. (A) Expression of DEGs in the glutaminolysis pathway was confirmed via RT-PCR using total RNA samples from the six cell lines. The upper section (a) depicts glutaminolysis-related genes that were highly expressed only in the resistant cells. The lower section (b) shows the glutaminolysis-related genes that did not show differential expression between the sensitive and resistant groups. The whole-gel images are shown in the supplementary data (Supplementary Figs. 3a-3c). (B) Schematic diagram of the glutaminolysis pathway and related genes. (C) Cellular MYC activities of the six cell lines were determined by following the protocol described in “Materials and Methods.” Differences in the viability of the two groups, excluding the SNU449 cell line, were analyzed using the *t*-test ($p = 0.00402$). (D) Cells were treated with a glutamine analog, DON (10 μ M), and cell growth was determined on day 4 using the CTB assay kit.

production. As we expected, 2-DG significantly affected ATP production in the PKM2 knockdown-sensitive cell lines (Fig. 2C). In accordance with the effect of 2-DG on ATP production, the PKM2 knockdown-sensitive cells were very susceptible to 2-DG-induced growth inhibition, clearly indicative of their glucose addiction for survival or growth (Fig. 2D). However, different results were obtained with the cell lines of the PKM2 knockdown-resistant group, which were classified as sensitive, moderately sensitive or resistant to 2-DG-mediated growth inhibition (Fig. 2D). Several cancer cell types use cellular metabolic processes other than lactate-producing glycolysis, including glutaminolysis, to supply the biomaterials required for continued cell growth and survival (Le et al., 2012; Wise and Thompson, 2010). These findings are consistent with our hypothesis that PKM2 knockdown-resistant cells are less dependent on glucose than are the PKM2 knockdown-sensitive cells that demonstrate glucose addiction.

Glutaminolysis is not directly involved in the PKM2-knockdown sensitivity of cancer cells

Previous reports have suggested that cancer cells are addicted to glutamine for energy, redox homeostasis and biomaterials for survival and growth (Le et al., 2012; Wise and Thompson, 2010). Cells take up glutamine through SLC1A5 (also called ASCT2) and supply a component of the TCA cycle, α -ketoglutarate, through glutaminolysis (Nicklin et al., 2009). SLC25A10 (also known as dicarboxylate ion carrier, or DIC) carries the malate or citrate required for the synthesis of amino acids and lipids via the mitochondrial TCA cycle (Mizuarai et al., 2005). FOXA1 is a transcription factor for the citrate carrier gene in mitochondria (Iacobazzi et al., 2009). MYC enhances glutamine metabolism as a transcription factor for certain glutaminolysis-related genes that supply the TCA cycle compound, α -ketoglutarate (Gao et al., 2009; Wise et al., 2008). Here, we showed markedly higher expression of MYC and other glutaminolysis-related target genes, including carbamoyl phosphate synthetase I (CPS1), SLC25A10 and FOXA1, in the resistant cells than in the sensitive cells (Table 1, Figs. 3Aa and 3B), suggesting greater glutamine dependency in the resistant group. We confirmed this using the MYC-luciferase reporter assays, which demonstrated greater cellular MYC activities in

the resistant cell lines ($p = 0.00402$), except for the SNU449 line (Fig. 3C). Considering this result and the different effect of 2-DG on SNU449 cells (see Fig. 2D), this cell line may have a gene signature distinct from that of the other resistant cell lines (this is mentioned below, in the result of Fig. 4).

However, in the experiment performed to establish the extent of dependency of cancer cells on glutamine, the growth of all the cell lines tested was susceptible to the glutamine analog, 6-diazo-5-oxo-L-norleucine (DON), with no discernible differences between the cell lines of the two groups (Fig. 3D). This finding may be attributed to the fact that the majority of cancer cells are addicted to glutaminolysis, even those sensitive to glycolysis. To validate this assumption, we investigated the expression patterns of other glutaminolysis-related genes, including GLS, SLC1A5 and CAD, using an Illumina chip assay and RT-PCR analysis. These genes were upregulated in all the cancer cell lines, as compared with non-tumor tissues, with no significant difference among the cell lines (Supplementary Table 4, Figs. 3Ab and 3B). The results indicated that glutamine addiction does not directly contribute to the differential sensitivity of the cancer cells to PKM2 knockdown.

Metabolic arms of glycolysis may be important for resistance of cancer cells to PKM2 knockdown effects

We further investigated the expression patterns of other DEGs associated with glycolysis-related processes to establish the mechanisms underlying the resistance to the effects of PKM2 knockdown (see Table 1). Interestingly, unlike the SLC2A3 (GLUT3) and HK2 genes, the glycolysis-related gene GLDC was highly expressed specifically in the resistant cells (Fig. 4A). A recent report showed that overexpression of GLDC or genes in the glycine synthesis pathway induces glycolysis in a metabolic activity-dependent manner (Zhang et al., 2012). It is possible that GLDC-induced glycolysis provides a consistent supply of biomaterials for cell growth, leading to the relative independence from PKM2 observed in the resistant cells.

The SNU-449 cells were resistant to the effects of PKM2 knockdown, but were sensitive to 2-DG treatment (see Fig. 2D), signifying their requirement for glucose, but not PKM2, for growth and survival. The RT-PCR experiments provided robust evidence for this inference, showing high expression of SLC2A3

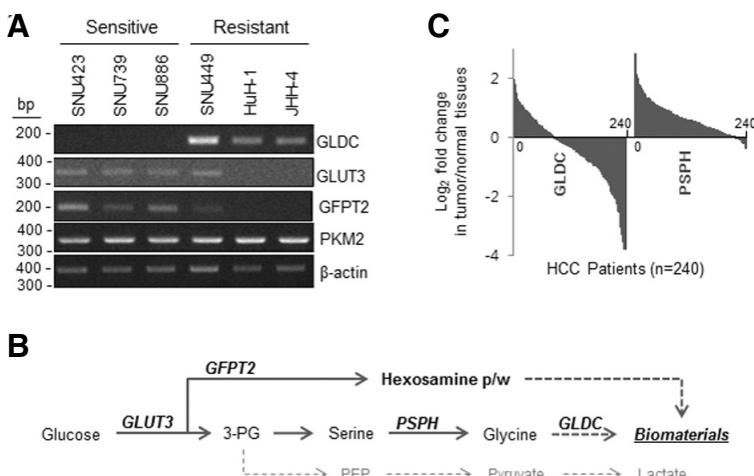


Fig. 4. Metabolic arms of glycolysis may be important for the resistance of cancer cells to PKM2 knockdown effects. (A) Expression patterns of glycolysis genes or those involved in alternative arms of the glycolysis pathway were confirmed via RT-PCR analysis using total RNA samples from the six cell lines. All whole-gel images are shown in the supplementary data (Supplementary Figs. 3a-3c). (B) Schematic diagram of the glycolysis side-arm pathways and related genes. (C) Expression of the PSPH gene, instead of the GLDC gene, was upregulated in most of the HCC patient samples from the Samsung Medical Center.

(GLUT3) in SNU-449 cells, comparable to that in sensitive cells, in contrast to the extremely low expression in the other resistant cell lines, Huh-1 and JHH-4 (Fig. 4A). In addition, all the sensitive cells and the SNU-449 cells commonly expressed a high level of GFPT2, a catalytic enzyme in the hexosamine biosynthetic pathway, an arm of glucose metabolism that generates UDP-N-acetyl-d-glucosamine (UDP-GlcNAc), a donor substrate for glycosylation reactions (Figs. 4A and 4B) (Hanover et al., 2010). A recent study showed that glucose availability modulated cellular uptake of glutamine through the hexosamine biosynthetic pathway (Wellen et al., 2010). This may explain why both the sensitive cells and the SNU-449 cells are susceptible to blockage of glucose use as well as of glutamine use by 2-DG and DON, respectively.

Identification of the PKM2-sensitive gene signature from transcriptome data for clinical HCC tissues

We investigated whether clinical samples had gene signatures similar to those of sensitive or resistant cell lines. To identify the samples displaying gene signatures similar to those of the sensitive cells, we used the publicly available DNA chip data for HCC tumors (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36376>). Initially, we analyzed the expression patterns of the genes highlighted in this study. Owing to the complex cellular context, we did not expect the signatures of a limited number of genes to explain all the different sensitivity levels. To simplify the analysis, we employed three genes whose expression was clearly distinguishable between the two sensitive and resistant cell lines. High levels of MCT4 and GLUT3 expression and a low net level of LDHB ([LDHB]-[LDHA]) expression were considered the gene signature of PKM2 knockdown-sensitive cells, whereas low expression of the MCT4 gene and high expression of the phosphoserine phosphatase (PSPH) gene (instead of the GLDC gene; see below) and a high net LDHB expression level were the resistant cell gene signature. Based on these analyses, we identified 26 (10.8%) and 27 (11.2%) HCC tissues with sensitive or resistant gene signatures, respectively (Supplementary Table 5). Upregulation of the GLDC gene was not common in HCC patient tissues (n = 240), whereas PSPH, another gene for serine/glycine synthesis, was overexpressed (Fig. 4C). A previous report also revealed that the GLDC gene is aberrantly upregulated in only a small proportion of the tissues of colon cancer patients and that overex-

pression of any of other serine/glycine metabolic enzymes induced glycolysis and cellular transformation in a metabolic activity-dependent manner (Zhang et al., 2012).

In conclusion, we categorized 21 HCC cell lines as sensitive or resistant to PKM2 knockdown-mediated growth inhibition and identified potential metabolic gene signatures on the basis of transcriptome data for both groups. Although the majority of cancer cells are dependent on both glutamine and glucose, they exhibit different dependencies on the processes of glucose usage. The sensitive cells were significantly dependent on lactate-producing glycolysis for energy and the building blocks for cell growth, and overexpression of MCT4 was identified as their representative gene signature. The resistant cells were further subdivided into those that were more dependent on glucose (SNU-449) and those that were less dependent (Huh-1, JHH-4). Because these gene signatures were also identified in the clinical samples, we propose that our findings are critical for selecting which patients would benefit from PKM2-targeting cancer treatment, although the detailed mechanisms remain to be established.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

This work was supported by National Research Foundation (NRF) grants funded by the Ministry of Education and Science Technology of Korea (MEST) (nos. 2010-0010465 and 2011-0028171), the National R&D Program (no. 0720050) for Cancer Control from the Ministry of Health and Welfare of Korea (MHW), and the Korea Research Institute of Bioscience and Biotechnology (KRIIBB) Research Initiative Program. We thank Dr. Cheol-Keun Park, Samsung Medical Center, for the clinical transcriptome data on the public website.

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