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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Identification of the Consistently Altered Metabolic Targets in Human Hepatocellular Carcinoma

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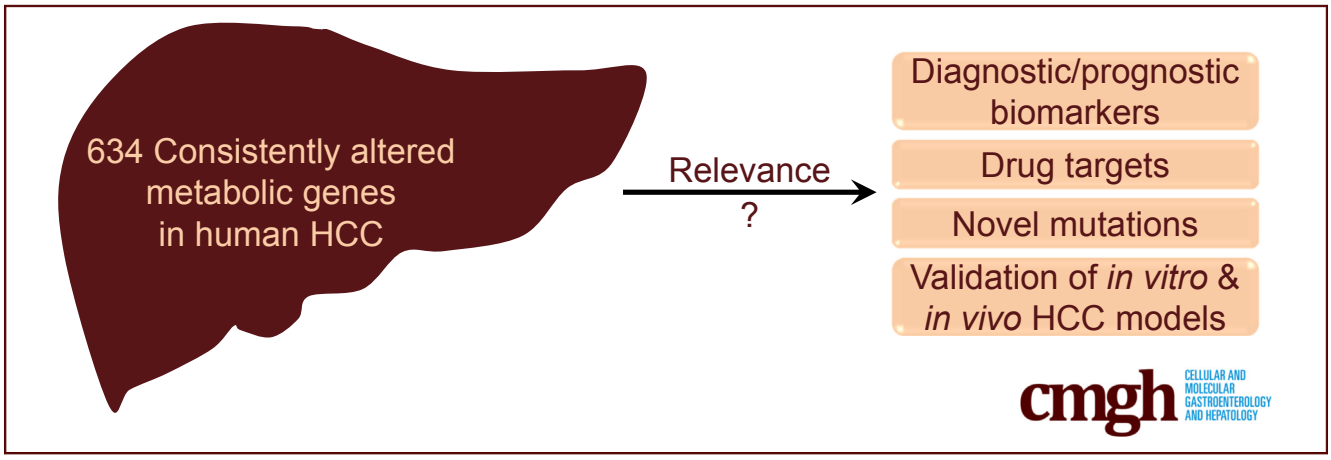

SUMMARY

We have identified metabolic targets that are consistently altered in human hepatocellular carcinoma, and are of potential clinical significance. This study exposes profound genomic dysregulation that could shed new light on how metabolism influences hepatocellular carcinogenesis.

BACKGROUND & AIMS: Cancer cells rely on metabolic alterations to enhance proliferation and survival. Metabolic gene alterations that repeatedly occur in liver cancer are largely unknown. We aimed to identify metabolic genes that are consistently deregulated, and are of potential clinical significance in human hepatocellular carcinoma (HCC).

METHODS: We studied the expression of 2,761 metabolic genes in 8 microarray datasets comprising 521 human HCC tissues. Genes exclusively up-regulated or down-regulated in 6 or more datasets were defined as consistently deregulated. The consistent genes that correlated with tumor progression markers (*ECM2* and *MMP9)* (Pearson correlation $P < .05$) were used for Kaplan-Meier overall survival analysis in a patient cohort. We further compared proteomic expression of metabolic genes in 19 tumors vs adjacent normal liver tissues.

RESULTS: We identified 634 consistent metabolic genes, ~60% of which are not yet described in HCC. The down-regulated genes (n = 350) are mostly involved in physiologic hepatocyte metabolic functions (eg, xenobiotic, fatty acid, and amino acid metabolism). In contrast, among consistently up-regulated metabolic genes (n = 284) are those involved in glycolysis, pentose phosphate pathway, nucleotide biosynthesis, tricarboxylic acid cycle, oxidative phosphorylation, proton transport, membrane lipid, and glycan metabolism. Several metabolic genes (n = 434) correlated with progression markers, and of these, 201 predicted overall survival outcome in the patient cohort analyzed. Over 90% of the metabolic targets significantly altered at the protein level were similarly up- or down-regulated as in genomic profile.

CONCLUSIONS: We provide the first exposition of the consistently altered metabolic genes in HCC and show that these genes are potentially relevant targets for onward studies in preclinical and clinical contexts. *(Cell Mol Gastroenterol Hepatol 2017;4:303–323; http://dx.doi.org/10.1016/j.jcmgh.2017.05.004)*

Keywords: Liver Cancer; HCC; Tumor Metabolism.

See editorial on page 283.

Metabolism is an indispensable process in normal and cancer cells. In the early 20th century, Otto Warburg discovered an alteration in tumor metabolic phenotype. He observed that cancer cells highly depend on aerobic glycolysis for energy production even when oxygen is abundantly available.[1–3] This discovery, complemented later by the dawn of the -omics era, has inspired several novel insights in cancer cell metabolism. Today, metabolic alteration is a recognized hallmark of cancer.[4] It is now known that in addition to relying on glucose (often called the Warburg effect), cancer cells also depend on other metabolites such as glutamine, serine, and fatty acids.[5–9] Furthermore, the accumulation of oncometabolites (eg, 2-hydroxyglutarate and fumarate), deregulated nutrient transporters (eg, glucose and monocarboxylate transporters), transcriptional regulators, epigenetic factors, and signaling molecules all prominently contribute to altered cancer metabolism.[2,10,11] In line with the rapidly evolving insights on tumor metabolism, a recent review has grouped the emerging alterations into 6 hallmarks, among which are deregulated uptake of glucose and amino acids, increased demand for nitrogen, and altered gene regulation caused by buildup of metabolites such as acetyl coenzyme A and 2-hydroxyglutarate.[12] Several molecular mediators of aberrant metabolism (eg, carnitine palmitoyltranferase 1, hexokinases, glucose transporter 1, glutaminase, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, and isocitrate dehydrogenases) have been studied in preclinical and clinical trials as potential cancer drug targets.[13,14] However, the extent and relevance of altered metabolism in cancer cells is still unclear.[12,14] This is partly due to the complex regulation of biochemical pathways as well as molecular heterogeneity within and across tumor entities. In addition, many studies have so far focused on the Warburg effect, thus narrowing the opportunities to identify novel and perhaps more relevant biochemical changes in cancer. Thus, as concluded by Pavlova and Thompson,[12] a detailed understanding of tumor metabolic features, especially for individual tumor types, will assist in better tumor classification and improve the prospects of exploiting metabolism in cancer therapy.

Liver cancer poses a global health challenge due to its rising incidence coupled with a low survival rate, especially in the developing world.[15,16] Hepatocellular carcinoma (HCC) accounts for over 80% of liver cancer cases, and is highly malignant, recurrent, drug resistant, and often diagnosed at the advanced stage.[17,18] For these reasons, the need to identify molecular features that uniquely define or contribute to HCC progression remains clinically urgent. To exploit metabolic alterations in HCC as diagnostic and prognostic indicators or as therapeutic targets, the alterations that distinguish cancerous liver cells from functionally normal hepatocytes must be known. Therapeutic interventions also need to consider that the liver is responsible for systemic metabolism and detoxification—functions that must not be compromised in an attempt to modulate pathways in adjoining cancerous liver. It is known that metabolic gene networks are heterogeneous in cancer (HCC inclusive).[19] Nevertheless, there are strong evidences that metabolic alterations have translational relevance in HCC. For instance, differences in acetate utilization have been reported as a possible phenotype for stratifying HCC patients.[20] Low betaine and propionylcarnitine have been proposed as combinatorial serum biomarkers in HCC.[21] Several metabolic targets are detectable by proteomic methods, and thus could serve as biomarkers in HCC.[22] Furthermore, all liver function parameters currently in clinical use reflect changes in either metabolic activities or enzymes. One notable liver function enzyme, aspartate transaminase, has also recently been shown to predict future risk of HCC development from primary biliary cirrhosis.[23] Therefore, identification of the consistently deregulated metabolic genes in HCC will accelerate future mechanistic studies aimed at exploiting specific candidates or pathways in diagnostic, prognostic or therapeutic contexts. In this study, we zoomed into the genomic landscape of human HCC with the aim of exposing consistently altered metabolic genes (hereafter also called targets) of potential clinical relevance. Across 8 datasets published in the last decade, we found that many metabolic genes are consistently deregulated regardless of the etiological background of the different patient cohorts. Many metabolic genes correlated with known markers of cancer progression, predicted survival outcome, and were similarly up- or down-regulated at the protein level in our analysis and other prior studies. We have revealed robust changes in metabolic gene expression in HCC to the extent that, to our knowledge, has not been previously acknowledged.

Methods

Collection of Liver Cancer Microarray Datasets, Processing and Identification of Consistently Altered Metabolic Genes

Eight liver cancer microarray datasets that have accompanying scientific publications (Table 1)[24–31] were assembled via online databases, namely ArrayExpress[32] and the Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information (NCBI).[33] To eliminate analytical bias that might arise from data reprocessing, the NCBI GEO2R tool was used to directly determine the differentially expressed genes between healthy or adjacent liver tissue control samples and HCC samples in each dataset. In total, 521 human HCC gene expression profiles

Abbreviations used in this paper: **EMT, epithelial to mesenchymal transition; FA, fatty acid; HCC, hepatocellular carcinoma; logFC, log of fold change; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NB, nucleotide biosynthesis; OXPHOS, oxidative phosphorylation; PPP, pentose phosphate pathway; TCA, tricarboxylic acid; TCGA, The Cancer Genome Atlas; XM, xenobiotics metabolism.**

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Table 1. Microarray Data Analyzed to Identify Altered Metabolic Targets in HCC Patients

Accession number	Data compared[a]	Number of samples		Main etiology reported	Reference
		Control	HCC		
GSE14520[b]	Paired NT vs HCC	220	225	HBV	[24]
GSE39791	Matched NT vs HCC	72	72	HBV	[25]
GSE57957	Adjacent NT vs HCC	39	39	HBV	[26]
GSE36376	AJCC Stage 3: Adjacent NT vs HCC	32	38	HBV	[27]
GSE60502	Adjacent NT vs HCC	18	18	NA	[28]
GSE14323[c]	Normal liver vs HCC	19	38	HCV	[29]
GSE6764	Normal liver vs very advanced HCC	10	10	HCV	[30]
GSE62232	Normal liver vs HCC	10	81	Mixed: alcohol, HBV, HCV, etc.[d]	[31]
Total arrays		420	521		

HBV/HCV, hepatitis B/C virus; HCC, hepatocellular carcinoma; NA, detail could not be accessed; NT, nontumor.
[a]Description of the data compared as documented in the National Center for Biotechnology Information Gene Expression Omnibus. Differential expression was analyzed with GEO2R tool. The overall design for each dataset can be found at https://www.ncbi.nlm.nih.gov/geo/.
[b]Data platform analyzed was GPL3921.
[c]Data platform analyzed was GPL571.
[d]Includes unknown etiology, hemochromatosis, metabolic syndrome and combinations of alcohol with the other etiologic factors.

were compared with 420 control liver samples (Table 1). Thereafter, the GEO2R outputs were downloaded, and all genes differentially regulated at $P < .05$ were selected. Next, a previously published list of 2,752 metabolism-annotated genes[7] was updated with 9 additional genes (Supplementary Table 1), and used to extract only the deregulated metabolic genes in each of the 8 datasets (Table 2). For this, the COUNTIF function was applied in Microsoft Excel (Microsoft Corp, Redmond, WA), followed by the removal of duplicate probes (eg, whereby a gene has 4 up-regulated probes, the one with the highest expression value was retained). Furthermore, the average log of fold change (logFC) of all differentially expressed genes as determined by GEO2R was calculated, and used as reference to set cutoff threshold values for each dataset. This step ensured the exclusion of metabolic gene probes with very small expression changes—also including duplicate probes of genes that in the same dataset are already among the top differentially regulated. For onward analyses, metabolic genes with +logFC at or above the cutoff value in the respective datasets were selected as up-regulated, whereas those with –logFC at or below cutoff value were selected as down-regulated. Few genes that had 2 probes with strongly opposite expression patterns in the same dataset (ie, one probe is up-regulated and the other down-regulated) were left in the gene list and used to test for consistent alteration across datasets. Following these prior steps, a metabolic gene was identified as consistently altered if it has the same

Table 2. Selection of Metabolic Targets From the List of Deregulated Gene Probes in Each HCC Dataset Used in This Study

HCC microarrays	logFC generated via NCBI GEO2R ($P < .05$)			Number of metabolic genes selected	
	Mean	SD	T used for selecting metabolic genes	Up-regulated ($\geq$ +T)	Down-regulated ($\leq$ -T)
GSE14520	0.0295	0.598	0.2	542	654
GSE39791	0.0102	0.3772	0.15	551	653
GSE57957	0.0225	0.415	0.15	623	650
GSE36376	0.275	0.484	0.1	934	404
GSE60502	0.000321	0.976	0.4	340	597
GSE14323	–0.015	0.563	0.15	473	683
GSE6764	0.0196	1.00129	0.45	437	628
GSE62232	0.0104	0.6383	0.25	552	814

Mean and SD were calculated from all probe sets with logFC values at $P < .05$ (including metabolic and other genes). Metabolic genes with +logFC at and above threshold (T) were selected as up-regulated targets; those with –logFC at or below T selected as down-regulated targets.
HCC, hepatocellular carcinoma; logFC, log of fold change; NCBI, National Center for Biotechnology Information.

expression pattern (ie, exclusively in the up-regulated or down-regulated category) in at least 6 of the 8 HCC datasets.

Selection of Progression Markers

Known markers of tumor invasion or metastasis, specifically extracellular matrix proteins and matrix metalloproteinases as well as epithelial-to-mesenchymal (EMT) markers (eg, *SNAI*s, *TWIST*, *ZEB*s, cadherins, vimentin) were manually curated from literature.[34,35] The expression of these genes was compared across 8 liver cancer microarrays in Oncomine—an online repository of curated cancer transcriptomics data.[36] In the Oncomine platform, parameters were set as follows— Analysis type: Liver Cancer vs Normal Analysis, Threshold by: $P = .05$, Fold Change = All, and Gene Rank = All. Of the markers mentioned earlier, *ECM2*, *CDH1*, *VIM*, and *MMP9* were the most consistently deregulated. Differential regulation of *ECM2*, *MMP9*, *CDH1*, and *VIM* as observed in Oncomine was also confirmed in the GEO2R output from the HCC datasets used to identify the metabolic targets. Besides GSE6764 and GSE14323, the microarrays in Oncomine include The Cancer Genome Atlas (TCGA) and GSE14520 liver cancer data used in this study for correlation with progression markers and overall survival analyses, respectively. Based on their consistent expression, *ECM2*, *MMP9*, *CDH1*, and *VIM* were selected as progression markers for correlation analyses with the metabolic genes.

Correlation of Metabolic Genes With Progression Markers

Liver cancer gene expression data from TCGA was used as a reference for the correlation of metabolic genes with the selected progression markers (ie, *ECM2*, *MMP9*, *CDH1*, and *VIM*). The messenger RNA expression data for each metabolic gene and the progression markers were obtained for the completed tumor analysis (n = 190 patients) via the cBioPortal platform (http://www.cbioportal.org). The data were log transformed and each metabolic gene was correlated with each of the progression markers found to be down-regulated in HCC (ie, *ECM2* and *CDH1*) and those that are up-regulated (ie, *MMP9* and *VIM*). To be included for further analysis, up-regulated metabolic genes were expected to correlate inversely with down-regulated progression markers, and directly with those up-regulated—the reverse being the case for down-regulated metabolic genes. Subsequently, a metabolic gene was selected if its Pearson correlation with at least *ECM2* and *MMP9* was statistically significant ($P < .05$).

Kaplan-Meier Overall Survival Analyses

For each metabolic gene that correlated with the progression markers (n = 434), Kaplan-Meier overall survival analysis was performed with log-rank (Mantel-Cox) test in GraphPad Prism. The dataset GSE14520, which is the largest of the cohorts analyzed (Table 1), is available with published clinical data, and so was used for the survival analysis. Prior to the analysis, the expression pattern of a given gene was confirmed to be the same in GSE14520 as generally described (ie, whether also up- or down-regulated in GSE14520 as in the other datasets). Only 6 genes were excluded from the survival analysis due to 1) lack of expression data (*CAD* and *CES3*), 2) duplicate probes that were strongly regulated in opposite directions (*SLC16A3* and *SMOX*), 3) expression pattern that is not as generally described (*BCAT1*), or 4) probe identification issue (eg, *CYP4A22* was excluded because the probe, 217319_x_at, is identified as *LOC654164///CYP4A22///CYP4A11*). For all other genes, the range of their expression from patients with the lowest to those with highest values varied markedly, and was very narrow for some genes. Specifically, for some genes, several patients had expression values that were the same or different by a slight margin, especially in intermediate range, and yet had different survival outcomes. Therefore, to ensure that the analyzed overall survival can be attributed to a difference in the expression of a given gene, its expression values were used to rank the patients into lower, intermediate, and higher groups. Subsequently, patients with lower (n = 75) and higher (n = 75) expression values for a given gene were adopted as a uniform inclusion criterion for survival prediction. Based on this criterion, overall survival was assessed using a total of 150 patients for each gene separately analyzed, and a statistical significance was accepted at $P < .05$.

Proteomics Analysis

To assess protein level alterations, our recently published proteomics data were reanalyzed focusing on the candidates corresponding to the consistent metabolic genes. The data contained 2736 proteins derived from mass spectrometric analyses of 19 fresh-frozen HCC samples and adjacent liver tissue samples. For the current analyses, paired comparisons of tumor and liver tissue samples were conducted irrespective of tumor stage and grade (n = 19), or according to the tumor stages T1 (n = 11) and T2-3 (n = 8) as well as the histological gradings G1 (n = 5), G2 (n = 8), and G3 (n = 6). Statistical evaluation was performed as recently described using a 1-way analysis of variance. Further details regarding patient characteristics, sample preparation, mass spectrometry, and proteomic data analysis have been extensively described in our prior publications.[37,38]

Pathway Analysis

The Database for Annotation, Visualization and Integrated Discovery[39] was used to perform functional annotation analysis of the top differentially expressed genes (in metabolism and other processes) for each of the 8 datasets. For this analysis, the gene lists from each dataset were first ranked by logFC. Thereafter, the top 1,500 up-regulated and down-regulated genes per dataset were used for a functional annotation with reference to pathway database of the Kyoto Encyclopedia of Genes and Genome.

Other Analyses

GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA) was used for Pearson correlation, overall survival analysis, and analyzing the expression of the genes relative

to tumor size. For the latter, a multiple *t* test, 1 per row, was used. $P < .05$ was accepted as statistical significance throughout the study. Targets were highlighted as novel based on results from searching PubMed database for each of the consistent metabolic gene (total n = 634). The search terms used were *official gene symbol* plus *HCC* or *Liver cancer* or *Cancer*. All authors had access to the study data and had reviewed and approved the final manuscript.

Results

Metabolic Genes Are Consistently Altered in Human HCC

To gain a holistic insight on metabolic gene alterations in clinical HCC (Figure 1*A*, Tables 1 and 2), we assessed the expression pattern of almost all known human metabolic genes and transporters previously compiled by Possemato et al.[7] In the HCC patient cohorts, the main reported etiologies were hepatitis B and C, alcohol, metabolic syndrome, mixed etiologies, or unknown (Table 1). With the exception of GSE62232, none of the other cohorts included data on metabolic syndrome, which is associated with nonalcoholic fatty liver disease (NAFLD) that predisposes to HCC. We reasoned that regardless of etiology, the expression of certain metabolic genes could be a consistent feature of liver cancer. Accordingly, we identified 634 metabolic genes that were deregulated in 6 or more datasets investigated (Supplementary Table 1). A total of 350 of the genes were down-regulated, of which 107, 158, and 85 were present in 8, 7, and 6 datasets, respectively. Assortment of the genes by their associated biochemical pathways revealed a predominant suppression of candidates involved in gluconeogenesis, urea cycle, ketogenesis, and xenobiotic, glutathione, amino acid, and fatty acid (FA) metabolism (Figure 1*B*). Several of these pathways also emerged in functional annotation analyses of topmost down-regulated genes (involved in metabolism and other processes) in each of the 8 datasets (Figure 2). Similarly, we found 284 consistently up-regulated metabolic genes comprising of 53, 120, and 111 hits in 8, 7, and 6 datasets, respectively. The up-regulated metabolic genes in HCC notably belonged to processes such as glycolysis, pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle, glycan metabolism, nucleotide biosynthesis (NB), membrane lipid biochemistry, and several transporters (Figure 1*B*). NB and valine, leucine, and isoleucine biosynthesis were the metabolic processes that emerged in the functional annotation analysis of topmost up-regulated genes in each of the datasets.

We observed that with the exception of few pathways (eg, proton transport, ketogenesis, gluconeogenesis, urea cycle), most others had a mixture of both consistently up-regulated as well as down-regulated genes (Figure 1*B*). To further highlight the consistency of metabolic gene alterations, we sought to identify the genes ranked in the top or bottom 25 in at least 6 datasets. Consequently, we found that the topmost down-regulated genes in HCC were *SLCO1B3*, an organic anion transporter of bilirubin; *CYPA12*, *CYP2A6*, *CYP2C8*, and *CYP3A4* all of which are involved in xenobiotics metabolism (XM); *FBP1* and *PCK1* involved in gluconeogenesis, *GLS2*, among others (Figure 1*C*). On the other hand, aldo-keto reductase family 1 member B10, *AKR1B10*, emerged as the topmost up-regulated metabolic gene, ranking first in all but 1 dataset (GSE60502). Other topmost up-regulated genes were *TKT*, *SQLE*, *ACLY*, *LYZ*, *TYMS*, *TXNRD*, *ACSL4*, *NQO1*, *FADS1*, *PLCB1*, and muscle isoform of *PKM* (Figure 1*C*). Of the 8 datasets analyzed, GSE14323 showed a slightly divergent gene expression pattern (Figure 1*C*). In this dataset, *SQLE* and *TKT* were not differentially expressed (ie, $P > .05$); *FADS1* was down-regulated, whereas *CYP3A4*, *CYP2A6*, and *GLS2* were all up-regulated instead of being suppressed as in other datasets (Figure 1*C*). Gene enrichment analysis also identified XM and drug metabolism in the up-regulated gene category only in the GSE14323 dataset (Figure 3). Nevertheless, topmost deregulated targets, including *AKR1B10*, *ACSL4*, *NNMT*, and *SLCO1B3* were also top hits in GSE14323 and were expressed in the same direction as in the other datasets (Figure 1*C*). Altogether, independent datasets reveal a strong deregulation of several metabolic genes in human HCC, and show that these alterations are broadly consistent across clinical cohorts.

Altered Metabolic Genes Show Similar Expression Patterns at Protein Level

We assessed the expression of metabolic genes at protein level in 19 human HCC tissue samples. A considerable number of the targets (n = 350), corresponding to 55% of the consistent metabolic genes, could be detected and quantified in the proteomics data (Figure 1*A*, Supplementary Table 2). Of those quantified, more than 90% (n = 252) were significantly expressed in the same direction as found at the gene level. For instance, of 207 down-regulated proteins, 167 were significantly decreased. Of these, 99% were also down-regulated at gene level with the exemption of BPGM and ATP5H, both of which are up-regulated in gene datasets. On the other hand, 143 targets were elevated at protein level—85 being significantly up. Of these, 79 (93%) were also consistently up-regulated at the gene level, with the exemption of PRG2, ST6GAL1, ACOX3, DHODH, TF, ABCD3 whose corresponding genes are consistently down-regulated in HCC. Next, based on common knowledge of biochemical pathways, we attempted to map the portrait of liver cancer metabolism using the consistently altered genes or their corresponding proteins where detected in our analysis. The snapshot clearly depicted the suppression of serine biosynthetic pathway, urea cycle, and transamination as striking features of HCC (Figure 4). Also represented were up-regulated targets in TCA cycle and mainly in NB, most of which were detected at protein level. In glycolysis, we found the novel hexokinase isoform, *HKDC1* to be up-regulated at gene and protein levels. Consistently, most other glycolytic targets were similarly expressed at gene and protein level, and have been identified and or mechanistically investigated in previous HCC studies (Supplementary Table 1). We show that notable genes that encode enzymes at the initial and terminal steps in commonly studied biochemical pathways are deregulated in HCC, and most reflected in our proteomics analysis. These include *HK2* and

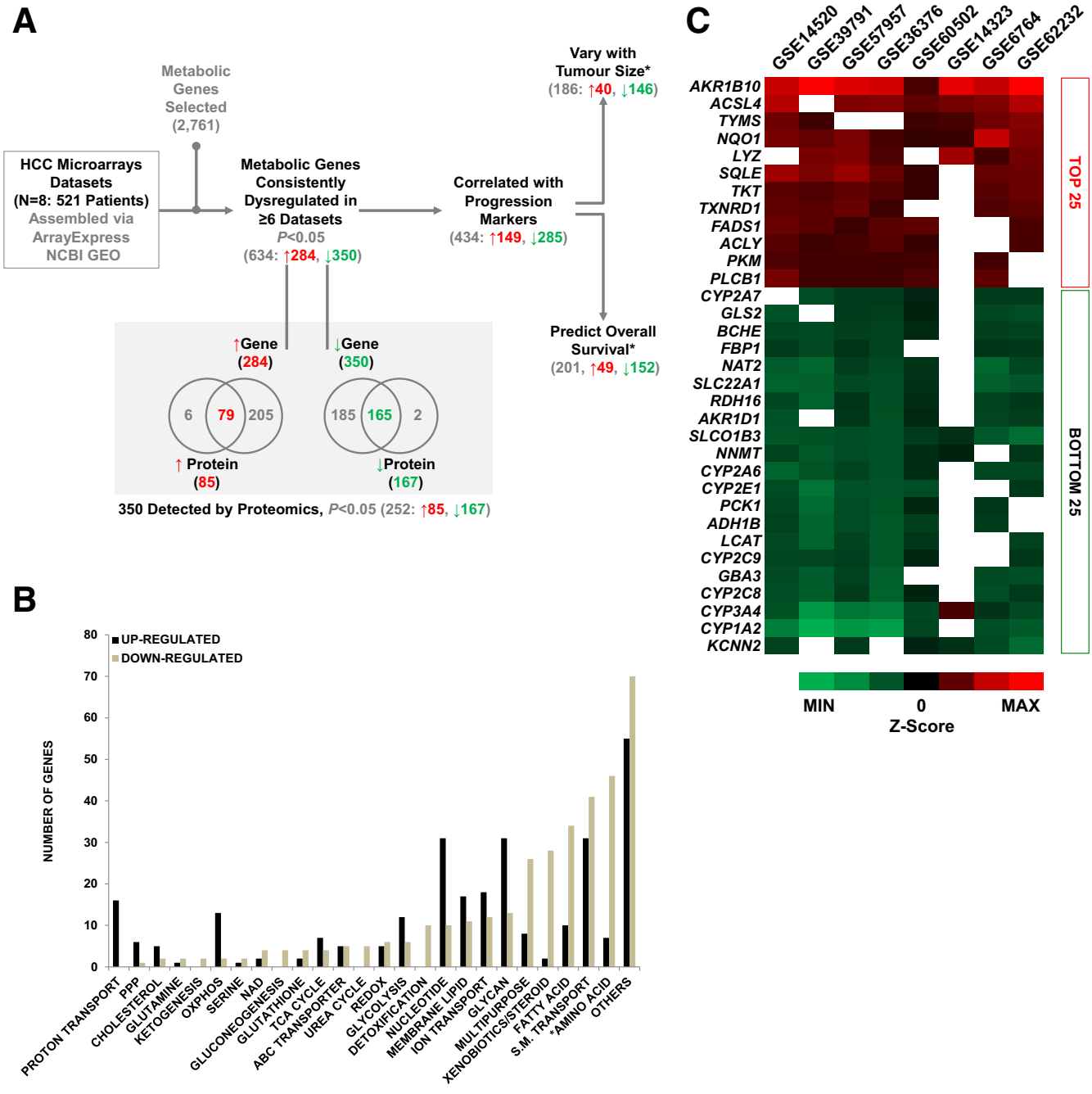


Figure 1. Summary of the study strategy used to identify the consistently altered metabolic targets in human HCC. (*A*) Flowchart of the steps adopted to identify metabolic targets of clinical relevance. Progression markers used are *ECM2*, *MMP9*, *CDH1*, and *VIM*. Genes considered to correlate with progression markers are only those that had significant Pearson correlation with at least *ECM2* and *MMP9* using The Cancer Genome Atlas liver cancer data as reference. *In the accompanying clinical data from GSE14520. Statistical significance were accepted at $P < .05$. See Supplementary Tables 1 and 2 for the metabolic genes and proteomics data, respectively. (*B*) Number of genes in each metabolic process that were consistently altered in 6 or more datasets. *Amino acid processes besides those already displayed in the graph (eg, glutamine). "Others" are mostly metabolic genes with other general functions beyond those displayed (see Supplementary Table 1). (*C*) Heatmap showing genes consistently among topmost 50 metabolic targets in 6 or more datasets. Blank means genes are not expressed at $P < .05$ or not expressed within the top 25 (up or down). ABC, adenosine triphosphate–binding cassette; HCC, hepatocellular carcinoma; NAD, nicotinamide adenine dinucleotide; NCBI GEO, National Center for Biotechnology Information Gene Expression Omnibus; OXPHOS, oxidative phosphorylation; PPP, pentose phosphate pathway; REDOX, reduction–oxidation reaction; S.M., small molecule; TCA, tricarboxylic acid.

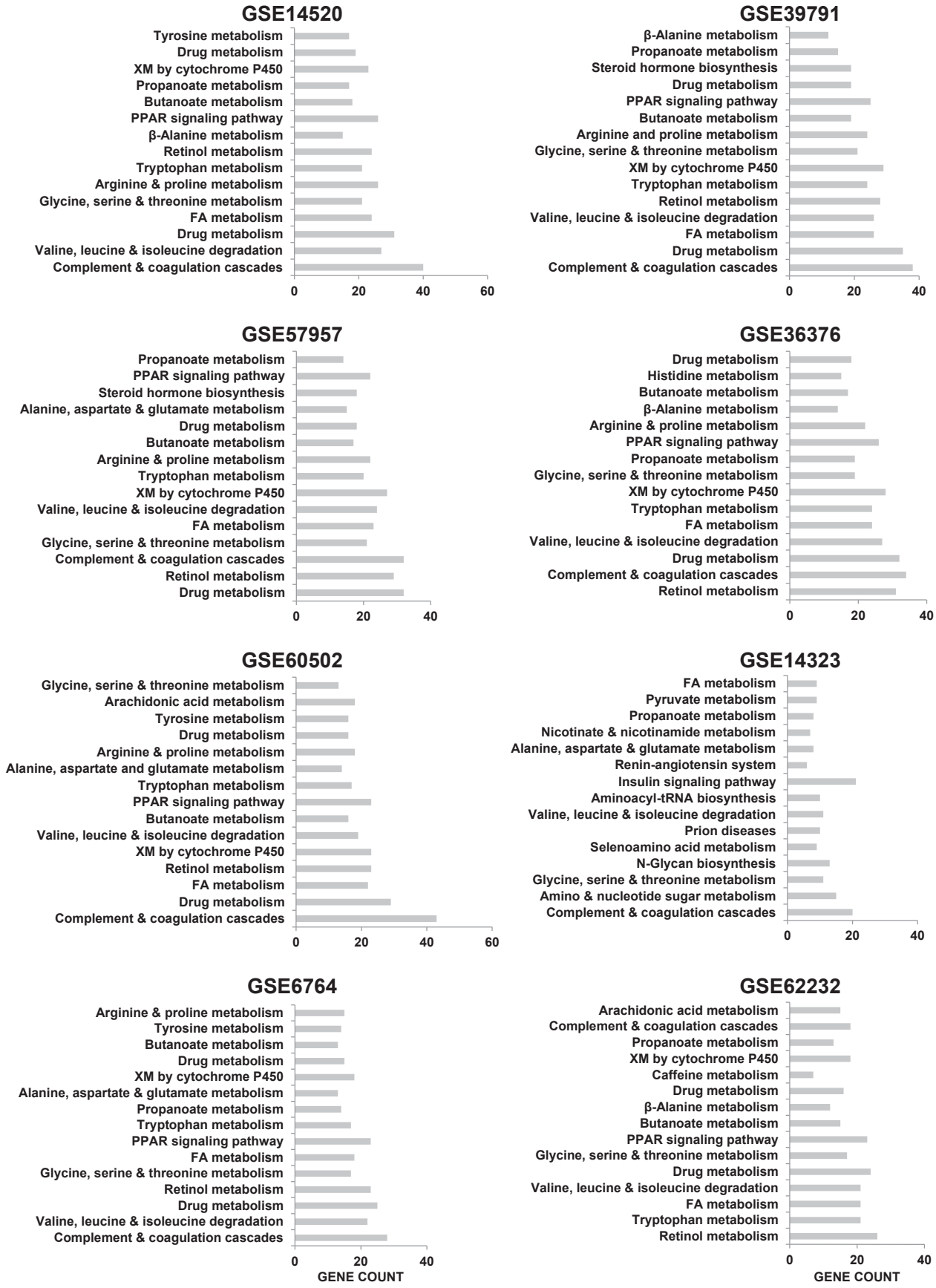


Figure 2. Functional annotation of the top 1500 down-regulated genes (whether in metabolic or other processes) in each hepatocellular carcinoma dataset. FA, fatty acid metabolism; PPAR, peroxisome proliferator-activated receptor; XM, xenobiotics metabolism.

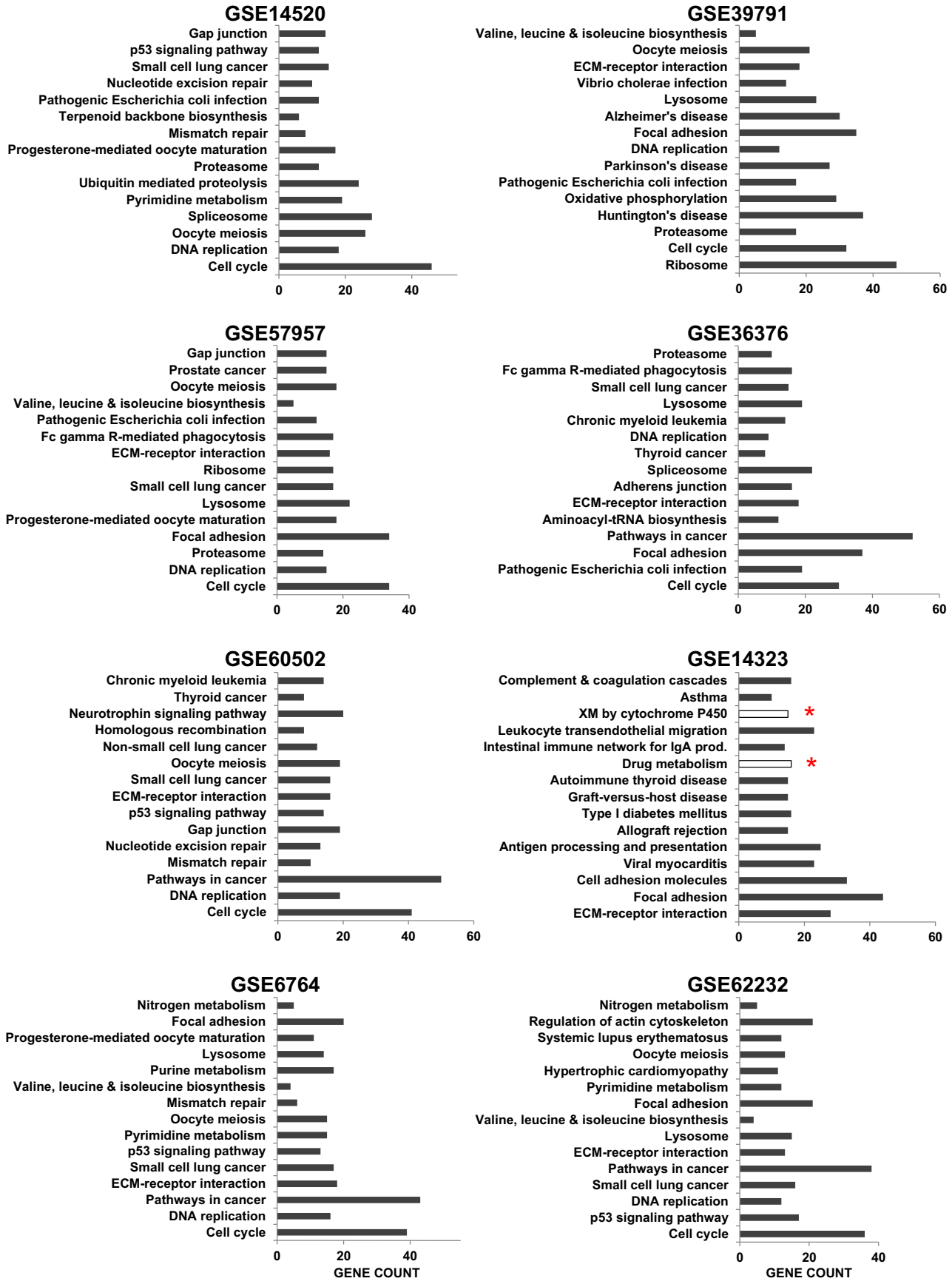


Figure 3. Functional annotation of the top 1500 up-regulated genes (whether in metabolic or other processes) in each hepatocellular carcinoma dataset. *Enriched among pathway annotations derived with down-regulated genes in the other datasets (see Figure 2). ECM, extracellular matrix; XM, xenobiotics metabolism.

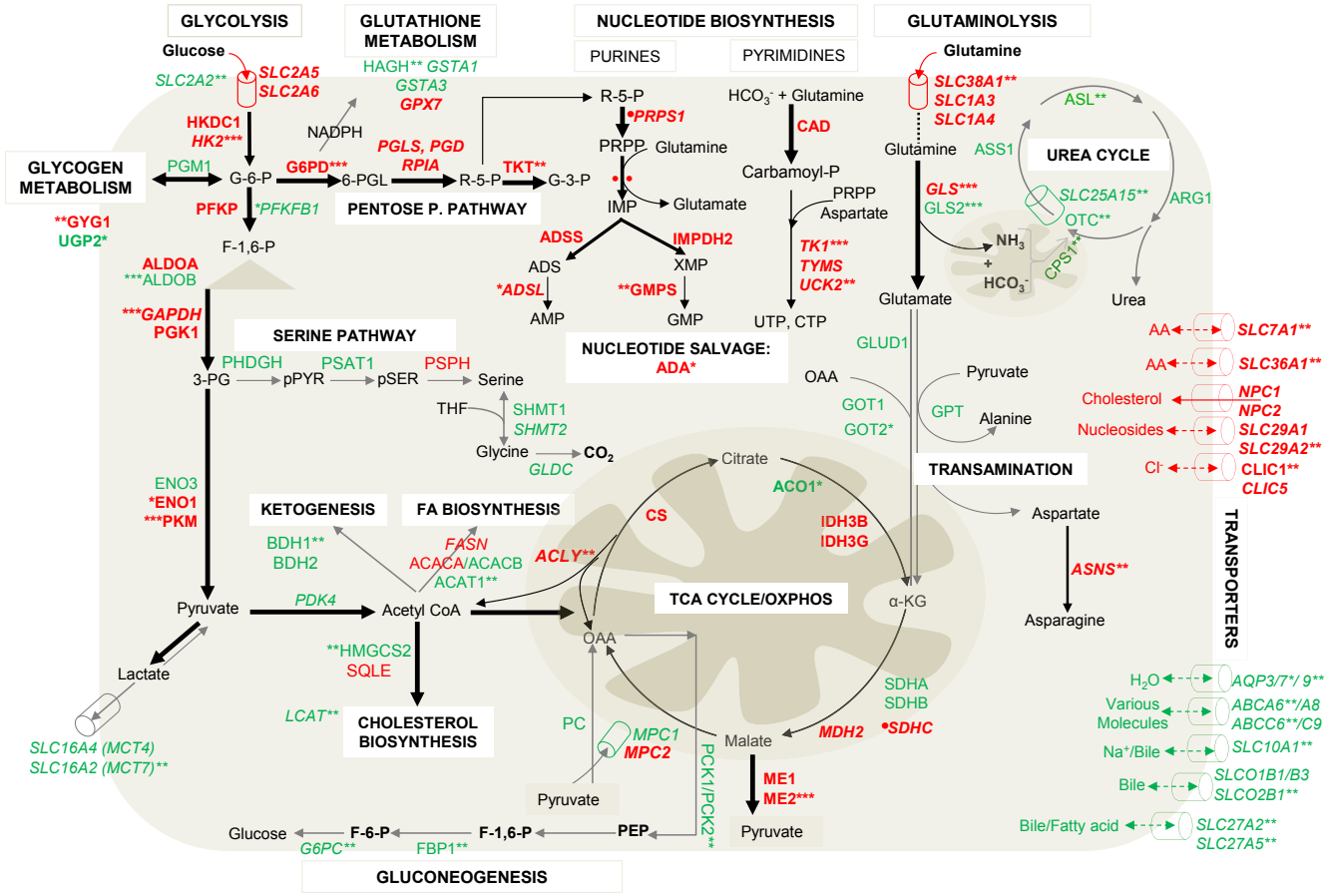


Figure 4. Schematic representation of biochemical pathways in HCC using consistently altered metabolic genes or their encoded proteins. Metabolic targets that are down-regulated are presented in green, those up-regulated are in red; metabolites are in black. Those we detected to be significantly altered at protein level are not in italics. *Predicted overall survival. **Predicted overall survival and varied with tumor size. ***Previously identified to be of clinical significance in HCC either as a drug target, biomarker, or prognostic indicator (see Supplementary Table 1). •Differentially expressed in 6 datasets, but did not reach selection threshold in 1 of the datasets. ••Within this axis are nucleotide biosynthesis targets PPAT, PAICS, ADSL, and GART, PFAS, and ATIC, all of which are up-regulated (the latter three were also detected to be up at protein level). 3-PG, 3-phoshoglycerate; 6-PGL, 6-phosphogluconolactone; α-KG, alpha ketoglutarate; AA, amino acids; ADS, adenylosuccinate; AMP, adenosine monophosphate; F-6-P, fructose-6-bisphosphate; G-3-P, glyceraldehyde-3-phosphate; G-6-P, glucose-6-phosphate; CTP, cytidine triphosphate; GMP, guanosine monophosphate; IMP, inosine monophosphate; NADPH, nicotinamide adenine dinucleotide phosphate; OAA, oxaloacetate; OXPHOS, oxidative phosphorylation; P, phosphate; PEP, phosphoenolpyruvate; pPYR, phosphohydroxypyruvate; PRPP, phosphoribosyl pyrophosphate; pSER, phosphoserine; R-5-P, ribose-5-phosphate; TCA, tricarboxylic acid cycle; THF, tetrahydrofolate; UTP, uridine triphosphate; XMP, xanthosine monophosphate.

PKM (in glycolysis), *GLS* and *GLUD1* (in glutaminolysis), *CPS1* and *ASL* (in urea cycle), *ACACA* and *FASN* (in lipogenesis), *HMGCS2* and *SQLE* (in cholesterogenesis), and *PCK1* and *FBP1* in gluconeogenesis (Figure 4, Supplementary Tables 1 and 2). Furthermore, we uncovered about 40 family of metabolic targets (mostly paralogues), whose members are frequently expressed in the opposite direction in HCC (Table 3). Examples include *ALDO1* and *ALDO3*, *ENO1* and *ENO3*, and *ACACA* and *ACACB*, which were also detected at protein level. Besides strongly overlapping with genomic data, the protein level expression of several metabolic targets varied significantly with tumor stage and grade (Supplementary Table 2). Taken together, metabolic gene expression changes in HCC reflect at the protein level, and putting them in the contexts of biochemical pathways could enhance the understanding of their functional relevance.

Consistently Altered Metabolic Genes Correlate With Progression Markers and Predict Survival Outcome in HCC Patients

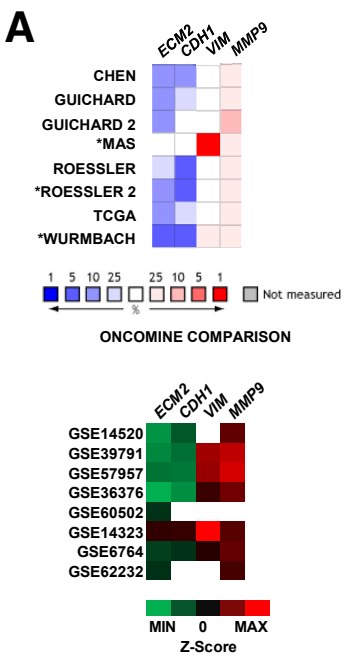
Correlation of metabolic targets with mediators of other cancer hallmarks can help uncover a mutual relationship. Through such analysis, Hu et al[19] observed a high mutual relationship between hypoxia inducible factor 1A and oxidative phosphorylation (OXPHOS) in cancer. Invasion or metastasis and EMT are crucial processes in tumor progression.[34,35,40] Whether metabolic alterations have

Table 3. Family of Metabolic Targets Consistently Expressed in the Opposite Direction in HCC

Metabolic processes	Up-regulated	Down-regulated
ABC transporters	*ABCC4*, *ABCC5*, *ABCC10*	*ABCC6*, *ABCC9*
Cholesterol trafficking	*NPC1*, *NPC2*	*NPC1L1*
Fatty acid biosynthesis	*ACACA*[a]	*ACACB*[a]
√	*ACSL3*[a], *ACSL4*[a]	*ACSL1*[a], *ACSL5*[a]
√	*ELOVL5*	*ELOVL6*
Fatty acid/phospholipids	*PLA2G4C*, *PLA2G7*	*PLA2G16*
Folate metabolism	*MTHFR*	*MTHFD1*[a], *MTHFS*[a]
Glutaminolysis	*GLS*	*GLS2*[a]
Glutathione metabolism	*GSTA4*	*GSTA1*, *GSTA3*, *GSTZ1*
Glycerophospholipid biosynthesis	*ABHD4*	*ABHD2*, *ABHD6*, *ABHD10*
Glycogenesis	*G6PC3*	*G6PC*
√	*GYG1*[a]	*GYG2*
Glycolysis	*ALDOA*[a]	*ALDOB*[a]
√	*ENO1*[a]	*ENO3*[a]
√	*PFKP*	*PFKFB1*
Glycolysis-TCA cycle junction	*MPC2*	*MPC1*
Glycoprotein/glycolipids Metabolism	*B4GALT3*, *B4GALT7*	*B4GALT1*
Glycosaminoglycan metabolism	*B3GAT3*	*B3GAT1*
√	*NDST1*	*NDST3*
√	*PAPSS1*	*PAPSS2*[a]
Lysophosphatidic acid synthesis	*ENPP2*, *ENPP4*	*ENPP1*
Oxidative stress	*PON2*	*PON1*[a], *PON3*[a]
Phospholipid	*AGPAT1*	*AGPAT2*
Purine biosynthesis	*NUDT1*, *NUDT2*	*NUDT7*
S-adenosylmethionine	*MAT1A*[a]	*MAT2A*
Sphingolipid metabolism	*PPAP2A*	*PPAP2B*
Steroid biosynthesis	*ACBD3*[a]	*ACBD4*
Second messenger molecule synthesis	*PDE6D*	*PDE2A*, *PDE7B*
√	*PLCG1*	*PLCG2*
Xenobiotics	*NAT9*, *NAT10*	*NAT2*
√	*SULT1C2*	*SULT1A1*[a], *SULT1A2*, *SULT2A1*[a]
Transporters	*SLC16A3*	*SLC16A2*, *SLC16A10*, *SLC16A4*
√	*SLC22A5*	*SLC22A1*
√	*SLC25A6*[a], *SLC25A3*[a]	*SLC25A15*, *SLC25A16* *SLC25A20*[a], *SLC25A37*
Glucose transporters	*SLC2A5*, *SLC2A6*	*SLC2A2*
Transporters	*SLC38A1*, *SLC38A6*	*SLC38A2*, *SLC38A4*
√	*SLC39A1*, *SLC39A6*	*SLC39A14*, *SLC39A8*
√	*SLC4A2*, *SLC4A7*	*SLC4A4*
√	*SLC6A8*	*SLC6A12*, *SLC6A13*, *SLC6A16*
√	*SLC7A1*, *SLC7A6*, *SLC7A11*	*SLC7A2*, *SLC7A8*
Organic anion transport	*SLCO2A1*	*SLCO2B1*
Lipid binding/unclear	*STARD7*	*STARD5*

ABC, adenosine triphosphate–binding cassette; HCC, hepatocellular carcinoma; TCA, tricarboxylic acid.
[a]Also detected to be significantly deregulated at protein level in our analysis.

association with tumor progression is largely unexplored in HCC. To reveal metabolic targets that may play a role in HCC progression, we correlated each of the 634 identified metabolic genes with 4 consistent progression markers, namely *ECM2* and *MMP9*, which are related to invasion or metastasis, and *CDH1* and *VIM*, which are related to EMT processes (Figure 5*A*). We selected metabolic targets that correlated at least with *ECM2* and *MMP9*, leading to the identification of 285 consistently down-regulated genes, and 149 hits in the up-regulated category (Pearson correlation



B

METABOLIC PROCESSES/PATHWAYS	CONSISTENTLY ALTERED GENES	CORRELATED WITH *ECM2* & *MMP9*	%
GLUCONEOGENESIS	4	4	100
GLUTAMINE	3	3	100
KETOGENESIS	2	2	100
UREA CYCLE	5	5	100
ABC TRANSPORTER	10	9	90
DETOXIFICATION	10	9	90
PPP	7	6	86
XENOBIOTICS/STEROID	30	25	83
*AMINO ACID	53	43	81
MULTIPURPOSE	34	27	79
FATTY ACID	44	34	77
TCA CYCLE	11	8	73
S.M. TRANSPORT	72	50	69
OTHERS	125	85	68
GLYCOLYSIS	18	12	67
GLUTATHIONE	6	4	67
REDOX	11	7	64
NUCLEOTIDE	41	26	63
MEMBRANE LIPID	28	16	57
ION TRANSPORT	30	16	53
NAD	6	3	50
GLYCAN	44	22	50
CHOLESTEROL	6	3	50
OXPHOS	15	7	47
PROTON TRANSPORT	16	7	44
SERINE	3	1	33

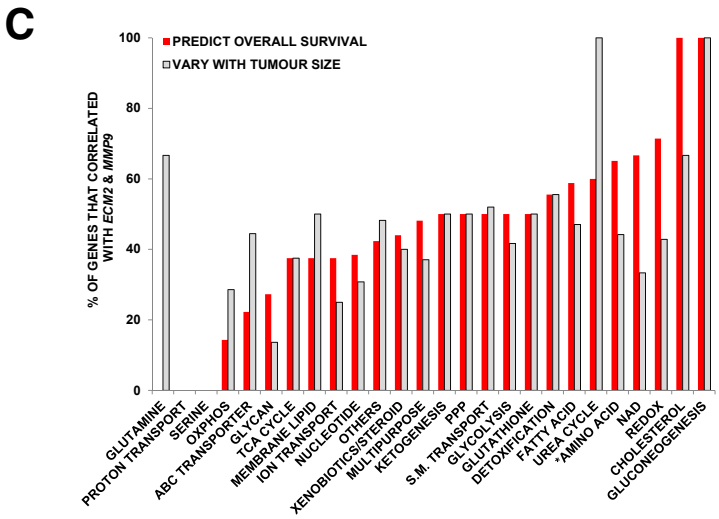


Figure 5. The proportion of genes that correlated with the progression markers, predicted overall survival, and varied with tumor size in the respective metabolic processes or pathways. (*A*, top) Expression of the progression markers in HCC datasets as observed in Oncomine database (https://www.oncomine.org/). "Hepatocellular carcinoma vs Normal" were compared in Oncomine. Mas, Roessler 2, and Wurmbach are represented in our analysis by GSE14323, GSE14520, and GSE6764, respectively. (*A*, bottom) Expression of the progression marker in the datasets used for identifying altered metabolic targets. (*B*) The proportion of consistent metabolic genes that correlated with *ECM2* and *MMP9* in The Cancer Genome Atlas liver cancer data, and were selected for subsequent analysis. (*C*) The proportion of selected targets that predicted survival outcome and varied with tumor size in the clinical data associated with GSE14520. *Amino acid processes besides those listed. ABC, adenosine triphosphate–binding cassette; NAD, nicotinamide adenine dinucleotide; PPP, pentose phosphate pathway; S.M., small molecule; TCA, tricarboxylic acid.

$P < .05$) (Figure 1*A*, Supplementary Table 1). Genes that performed best in the correlation analysis were those in gluconeogenesis, metabolism of glutamine and other amino acids, ketogenesis, urea cycle, adenosine triphosphate–binding cassette transporters, PPP and XM (Figure 5*B*), suggesting metabolic processes most likely associated with cancer progression. On the other hand, fewer genes in proton transport and OXPHOS correlated with the progression markers. Next, we wondered whether genes that correlated with the progression markers could also predict overall survival of liver cancer patients. We searched for currently available literature on each correlated gene, and found that although many of the genes are not yet described in HCC, about 20 candidates were previously reported to

predict prognostic outcome (Supplementary Table 1). We analyzed patient overall survival data from 1 of the HCC cohorts, and identified 201 genes whose expression predicted survival outcome (Tables 4 and 5). Of these genes, 61% were also among those whose expression significantly varied with tumor size (n = 186), implying that consistently altered metabolic genes are strongly associated with clinicopathological variables. In terms of the genes associated with overall survival and tumor size, fewer targets in OXPHOS, glycan metabolism as well as adenosine triphosphate–binding cassette transporters were statistically significant— none were significant among proton transport genes (*ATP5SL*, *ATP6V1E1*, *ATP5E*, *ATP5G2*, *ATP6AP1*, *ATP6V0B*, and *ATP6V1F*) (Figure 5*C*, Supplementary Table 1). In contrast, the metabolic processes with the best-performing candidates were notably gluconeogenesis, urea cycle, detoxification, amino acid metabolism, FA metabolism, PPP, and small molecule transport (Figure 5*C*, Supplementary Table 1). Taken together, our study reveals that altered expression of metabolic genes are broadly consistent in HCC, correlate with clinical parameters, and hold yet untapped prospects in liver cancer research.

Discussion

Identification of the consistently altered metabolic targets is an indispensable step toward exploiting metabolism in basic, translational, and clinical cancer studies. We have exposed for the first time, metabolic genes that are consistently deregulated in human HCC. The metabolic genes, when put in the context of their associated biochemical pathways, reveal the suppression of well-known hepatocyte metabolic functions (eg, XM), and the up-regulation of energy-yielding processes (eg, glycolysis), as consistent features of HCC. XM genes are among the topmost down-regulated candidates in HCC and prominently emerged in pathway annotation analysis that took into account non-metabolic genes in each dataset. Previous genomic study reported down-regulation of XM genes across 22 cancers, HCC inclusive, and suggested it may be associated with sensitivity to chemotherapies.[19] Therapeutic resistance is currently an intractable problem in cancer, and has contributed to the failure of several drug trials in HCC.[18,41,42] Although it is still unclear how down-regulated XM genes may influence drug sensitivity, studies suggest they are induced by therapy and cause a depletion of the systemic drug level. One example is cytochrome P450 3A4 (CYP3A4), which is down-regulated in HCC. In non–small cell lung cancer, it has been suggested that to ensure bioavailability, Erlotinib should not be used in combination with inducers of CYP3A4.[43] In a xenograft model of HCC, treatment with Sorafenib caused the induction of CYP3A4, which coincided with reduced systemic level of the drug and the onset of resistance.[44] Thus, our study could help in further identification of targets in XM or other metabolic processes that are down-regulated, but are prone to be re-expressed to mediate resistance.

Besides XM, the predominant down-regulation of genes in urea cycle, glutathione, FA, amino acid, gluconeogenesis, ketogenesis, and transamination are also consistent features of HCC (Figure 2). Notably, genes in urea cycle and gluconeogenesis scored very high in their correlation with progression markers, variation with tumor size, and prediction of overall survival. Reasons for the profound down-regulation of critical biochemical pathway targets could be multifactorial, including, among others, 1) lack of key pathway substrates; 2) products that are detrimental to HCC cell proliferation or survival, hence warranting pathway inhibition; 3) diversion of substrates into other pathways of higher priority for the tumor; or 4) transcriptional and epigenetic controls, or mutations that repress gene expression. Using urea cycle as an example, the supplementation of HCC cells with recombinant arginase, which hydrolyzes arginine to produce ornithine and urea, inhibited proliferation and induced cell cycle arrest.[45] This implies a possible availability of substrate (in this case, arginine), but a lack of the enzymatic machinery for urea production. It also offers hint that intracellular urea as a product is detrimental to HCC cells. However, detrimental products may not explain why gluconeogenesis genes are suppressed given that cancer cells rely on its end product (glucose). As such, there is currently no molecular information to sufficiently explain why the down-regulation of these genes is crucial for orchestrating the global metabolic activities of HCC. Regarding FA biosynthesis, it is known that conditions such as nonalcoholic steatohepatitis (NASH) arise from FA accumulation and can lead to HCC. In line with our finding in human HCC, the down-regulation of FA genes has been reported in mice exposed to chronic choline-deficient high-fat diet in which NASH transited to HCC.[46] Thus, impaired FA metabolism may represent an early event in HCC development that is consistent even across species, but yet unappreciated. In amino acid metabolism, we uncovered a striking suppression of serine pathway genes (*PHGDH*, *PSAT1*, *SHMT1*, *SHMT2*, *GLDC*) in HCC —with the exception of *PSPH*. The serine pathway branches from glycolysis at the level of 3-phosphoglycerate, with PHGDH catalyzing the first step.[6,7] Serine deficiency, and the down-regulation of the serine pathway genes, has been reported in patients with NAFLD.[47] Thus, similar to FA metabolism discussed above, the down-regulation of serine pathway may also represent an early event in HCC development or progression. Suppressed serine pathway in HCC could also expose interesting contrasts when compared with breast cancer, where up-regulated serine pathway via PHGDH is essential in tumorigenesis.[6,7] *PSPH*, the only member of serine pathway we found to be up-regulated in HCC, has been reported to be critical for cMyc-driven cancer progression upon nutrient deprivation.[48] Interestingly, the serine pathway genes mentioned earlier—except *GLDC* that was not shown—were all induced in HCC cell lines upon glucose or glutamine deprivation.[48] Downstream of glycolysis, the down-regulation of the mitochondrial pyruvate carrier *MPC1* is also notable in HCC. *MPC1* has been reported to be down-regulated in NASH patients,[47] and has been identified as a repressor of Warburg effect in cancer.[49] *MPC1* was also previously shown to be consistently down-regulated in HCC and several other cancers, and is induced upon glutamine

Table 4. Consistently Down-Regulated Genes Associated With Hepatocyte Metabolic Functions Correlate With Expression of Progression Markers and Predict Survival Outcome in HCC Patients

				Correlation[a]		
Metabolic process	Gene symbol	# of datasets	Expression	*CDH1*	*VIM*	Predicts overall survival[b]
Xenobiotics	*CYP4A11*	7	Down		[c]	.0001
	CYP4F3	8	Down	[c]	[c]	.0007
	CYP3A4	7	Down		[c]	.001
	CYP2J2	7	Down		[c]	.0012
	■*HSD17B6*	8	Down		[c]	.0018
	CYP2C8	8	Down		[c]	.0022
	■*SRD5A1*	7	Down		[c]	.0038
	CYP2A6	7	Down		[c]	.0051
	HSD11B1	7	Down		[c]	.0307
	CYP2A7	6	Down			.0346
	CYP3A43	7	Down		[c]	<.0001
Detoxification	■*FMO4*	6	Down		[c]	.0011
	■*EPHX2*	7	Down	[c]	[c]	.0032
	■*TPMT*	8	Down		[c]	.0032
	CAT	7	Down		[c]	.0053
	FMO3	6	Down		[c]	.0065
Urea cycle	*OTC*	7	Down		[c]	.0055
	ASL	6	Down	[c]	[c]	.0074
	CPS1	7	Down		[c]	.011
Redox	■*FDX1*	7	Down		[c]	.0081
	■*DHRS12*	6	Down		[c]	.0141
	■*DHTKD1*	6	Down		[c]	.0167
	■*DHRS1*	7	Down		[c]	<.0001
	CYB5A	8	Down		[c]	.0026
Glutathione	■*HAGH*	7	Down		[c]	.0015
	■*MGST2*	6	Down		[c]	.0493
Fatty acid	*ACOX2*	7	Down		[c]	.0003
	■*ECHDC2*	8	Down		[c]	.0004
	■*ACADM*	7	Down		[c]	.0007
	■*ACBD4*	8	Down		[c]	.0012
	■*FAAH*	6	Down		[c]	.0015
	■*ACAA2*	7	Down		[c]	.0018
	■*ACSM3*	7	Down		[c]	.002
	■*PECR*	6	Down		[c]	.0022
	■*ETFDH*	8	Down		[c]	.0028
	■*ACSM5*	7	Down		[c]	.0104
	EHHADH	7	Down		[c]	.0115
	ACADVL	6	Down			.0127
	MTTP	6	Down		[c]	.0134
	■*ACADSB*	8	Down		[c]	.0212
	■*SCP2*	8	Down		[c]	.0227
	■*HADH*	7	Down		[c]	.0228
	■*ACAT1*	8	Down			.0264
	■*PHYH*	7	Down	[c]	[c]	.0302
	■*ACSL3*	6	Up			.0426
	■*MLYCD*	8	Down		[c]	<.0001
Gluconeogenesis	*G6PC*	6	Down	[c]	[c]	.0015
	PCK1	7	Down		[c]	.0245

Table 4. Continued

Metabolic process	Gene symbol	# of datasets	Expression	Correlation[a]		Predicts overall survival[b]
				CDH1	*VIM*	
	PCK2	7	Down		[c]	<.0001
	FBP1	7	Down	[c]		.0013
Ketogenesis	■*BDH1*	7	Down		[c]	.0082
Amino acid	■*GCDH*	8	Down		[c]	.0005
	■*PIPOX*	7	Down		[c]	.0007
	■*AGXT*	8	Down		[c]	.0011
	CDO1	7	Down		[c]	.0014
	■*FAHD2A*	8	Down			.0015
	■*AASS*	7	Down		[c]	.0018
	FTCD	7	Down		[c]	.002
	ADI1	6	Down		[c]	.0029
	DAO	6	Down		[c]	.0038
	CTH	7	Down		[c]	.0039
	■*HAAO*	7	Down		[c]	.0039
	HPD	7	Down		[c]	.0052
	HGD	7	Down		[c]	.0052
	BHMT	7	Down		[c]	.0057[d]
	SARDH	8	Down		[c]	.0079
	■*BCKDHA*	7	Down			.0081
	MSRA	8	Down			.0086
	■*HIBCH*	7	Down		[c]	.009
	■*BHMT2*	6	Down		[c]	.0128
	■*BCKDHB*	8	Down		[c]	.0135
	CBS	8	Down		[c]	.0141[d]
	MAT1A	8	Down		[c]	.0167[d]
	PAH	7	Down		[c]	.0205
	■*THNSL1*	6	Down		[c]	.0256
	FAH	7	Down		[c]	.0444
	SUOX	7	Down		[c]	<.0001
	■*ALDH18A1*	7	Up		[c]	.02
	■*ASNS*	7	Up		[c]	.0103[d]
ABC transporter	■*ABCA6*	7	Down		[c]	.0015
	■*ABCC6*	8	Down	[c]	[c]	.0106
Ion transport	■*KCNJ8*	7	Down		[c]	.0109
	■*CNGA1*	7	Down	[c]	[c]	.0002
	■*ATP1B3*	7	Up		[c]	.0008
	■*P2RX4*	7	Up		[c]	.0466
	■*SLC39A1*	7	Up			.0235
	CLIC1	8	Up		[c]	<.0001
S.M. transport	■*SLC25A15*	7	Down	[c]	[c]	.0003
	■*SLC27A5*	7	Down		[c]	.0003
	■*SLC16A2*	8	Down		[c]	.0007
	■*SLC25A20*	8	Down		[c]	.001
	■*AQP7*	7	Down			.0013
	■*SLC6A12*	7	Down		[c]	.0015
	■*STARD5*	8	Down			.0023
	■*SLCO2B1*	7	Down		[c]	.0042
	■*SLC46A3*	8	Down			.0057
	■*SLC1A1*	6	Down	[c]		.0266

Table 4. Continued

Metabolic process	Gene symbol	# of datasets	Expression	Correlation[a]		Predicts overall survival[b]
				CDH1	*VIM*	
	■*SLC27A2*	8	Down		[c]	.0421
	■*SLC47A1*	7	Down		[c]	.0467
	SLC38A4	8	Down		[c]	.0066
	SLC28A1	6	Down		[c]	.0467
	AQP9	6	Down		[c]	.0001
	SLC2A2	7	Down		[c]	.0002
	SLC10A1	7	Down		[c]	.0005
	SLC22A7	7	Down	[c]	[c]	.0008
	SLC23A2	7	Down		[c]	.0096
	SLC22A1	8	Down			.0129[d]
	■*SLC2A6*	6	Up		[c]	.0094
	■*SLC36A1*	7	Up		[c]	.0338
	SLC38A1	6	Up		[c]	.0047
	SLC29A2	6	Up			.0142
	SLC7A1	8	Up		[c]	.0163
Multipurpose	■*ADH6*	7	Down		[c]	.0001
	■*GOT2*	7	Down	[c]		.0001
	■*MAOB*	6	Down	[c]	[c]	.001
	■*ALDH7A1*	6	Down		[c]	.0011
	■*CBR4*	8	Down			.004
	ALDH2	8	Down		[c]	.0062
	■*ALDH9A1*	7	Down		[c]	.0477
	ADH1B	7	Down		[c]	.0008
	ALDH6A1	8	Down		[c]	.0062
	ADH1C	6	Down		[c]	.0071
	AKR7A3	7	Down		[c]	.0169
	■*CA12*	8	Up		[c]	.0027
Others	■*SORD*	7	Down		[c]	.0003
	■*MMACHC*	6	Down		[c]	.0006
	■*HAO1*	7	Down		[c]	.0009
	■*DCXR*	7	Down		[c]	.002
	■*QDPR*	7	Down		[c]	.0035
	■*PCCB*	6	Down		[c]	.0058
	■*GFOD2*	7	Down			.0059
	■*AGL*	8	Down		[c]	.009
	■*GNE*	8	Down		[c]	.0238
	■*MTHFD1*	7	Down		[c]	.0254
	■*RBKS*	7	Down		[c]	.0351
	■*ADCY1*	8	Down	[c]		.0375
	■*GAMT*	8	Down		[c]	.0386
	UGP2	7	Down		[c]	.0014
	ALAS1	6	Down		[c]	.0027
	PON3	7	Down		[c]	.0047
	RDH16	7	Down		[c]	.0089
	ABAT	8	Down		[c]	.0093
	HAO2	8	Down			.0152[d]
	GRHPR	7	Down		[c]	<.0001
	MTHFS	8	Down	[c]	[c]	.0028
	■*NNT*	6	Down	[c]		.0046

Table 4. Continued

Metabolic process	Gene symbol	# of datasets	Expression	Correlation[a]		Predicts overall survival[b]
				CDH1	*VIM*	
	■*MUT*	7	Down	[c]	[c]	.0067
	GLYAT	7	Down		[c]	.0002
	SULT2A1	6	Down		[c]	.0002
	CRYL1	7	Down		[c]	.0045
	GNMT	8	Down		[c]	.0009
	PON1	7	Down		[c]	.0072
	■*NANS*	6	Up	[c]		.0009
	■*DDAH2*	8	Up	[c]		.001
	■*MFSD10*	7	Up		[c]	.0057
	■*LTA4H*	8	Up			.0087
	■*GYG1*	7	Up		[c]	.0267
	SULT1C2	8	Up			.0064
	AACS	7	Up		[c]	.0069
	NOX4	7	Up		[c]	.0422
	SMS	6	Up	[c]	[c]	.0328
	SRM	7	Up	[c]		.0006

Genes categorized as other or multipurpose are those with functions either not yet well defined or not directly connected to a particular metabolic process. Kaplan-Meier overall survival was calculated for each gene and statistical significance analyzed by log-rank (Mantel-Cox) test. Square (■) indicates novel targets in liver cancer as determined by searching PubMed.
#, number of datasets in which each gene is expressed at $P < .05$.
[a]All listed metabolic genes correlated with *ECM2* and *MMP9*.
[b]Denotes *P* value.
[c]Indicates those that also correlated with *CDH1* and or *VIM* (Pearson correlation was considered significant at $P < .05$).
[d]Previously identified as a survival predictor in hepatocellular carcinoma (HCC) (see the referenced study and other similarly described targets in Supplementary Table 1).

deprivation as observed in colon cancer cells.[49] These evidences strongly support that HCC have a predominant down-regulation of metabolic genes involved in physiologically relevant biochemical pathways. It further offers hint that several of the down-regulated targets could be re-induced to mediate resistance or stress response when cellular homeostasis is challenged, thus highlighting novel aspects of liver cancer metabolism for further investigation.

The consistently up-regulated metabolic genes in HCC mostly belong to processes such as glycolysis, PPP, NB, TCA cycle, OXPHOS, proton transport, membrane lipid, glycan metabolism, and small molecule transport. Whereas the prospects of novel insights still abound in known alterations (eg, glycolysis, TCA, OXPHOS, NB),[12] similar opportunities also exist in processes least studied in HCC (eg, NB, proton transport, membrane lipid, glycan metabolism). For instance, nucleoside transporters and metabolizing enzymes (eg, uridine-cytidine kinases) modulate sensitivity to nucleoside analogues in leukemia.[50–53] *UCK2* is up-regulated in HCC, but there are currently no data on its function. In addition, we identified other NB genes with yet unclear role in HCC (eg, *TYMS* [up-regulated], *CDA* and *DPYD* [both down-regulated]). In colorectal cancer, sequence variants of *TYMS*, *CDA*, and *DPYD* were found to be clinically relevant predictors of toxicity to fluoropyrimidine drugs (eg, 5-fluorouracil) and the prodrug capecitabine.[53] Therefore, it might be interesting to investigate the role of NB targets in drug sensitivity in HCC. Similarly, the up-regulation of genes in glycan metabolism could broaden the chances of finding new drug targets, given that glycans have been considered prospective agents in cancer therapy.[54] In addition, given their strategic expression on cell membranes, the proteins encoded by genes in glycan metabolism may represent important biomarkers in HCC. Altogether, the consistently up-regulated targets and their associated pathways could shed light on drug resistance mechanisms as well as the molecular mechanisms of metabolic reprogramming. Indeed, as was previously noticed in cancer,[19] we observed a mixture of both up-regulated and down-regulated genes in most of the metabolic pathways. For example, although glycolytic targets are predominantly up-regulated, *ALDOB*, *ENO3*, and *PFKFB1* are down-regulated, as are some glucose transporters (Figure 4). Although it is unclear if HCC actually require the down-regulation of these genes for optimal glycolysis, their suppressed expression seem to be beneficial for cancer cells. For instance, previous study show that low *ALDOB* expression influence aggressiveness and predict patients survival outcome in HCC.[55] In our analysis, low expression of *ALDOB* and also *PFKFB1* predicted overall survival. We detected ALDOB at protein level and its expression varied with tumor size. Therefore, the relevance of the up-regulated genes in metabolic pathways with

Table 5. Genes in Mainly Up-Regulated Pathways That Correlated With the Expression of Progression Markers and Predicted Survival Outcome in HCC Patients

				Correlation[a]		
Metabolic process	Gene symbol	# of datasets	Expression	*CDH1*	*VIM*	Predicts overall survival[b]
Glycolysis	■*PFKFB1*	6	Down		[c]	.0202
	ALDOB	8	Down	[c]	[c]	.0022[d]
	ALDOA	8	Up		[c]	.0001[d]
	HK2	6	Up		[c]	.0001[d]
	PKM	8	Up		[c]	.0003[d]
	PDK4	7	Down			.0177
PPP	■*DERA*	7	Down		[c]	.0075
	TKT	7	Up	[c]		.0132
	G6PD	7	Up			.0021[d]
TCA cycle	■*ACO1*	7	Down	[c]	[c]	.0211
	ACLY	7	Up			.0054
	ME2	7	Up		[c]	.0257
OXPHOS	*NDUFA4L2*	7	Up			.0003
Nucleotide	■*DPYS*	7	Down		[c]	.0455
	XDH	7	Down	[c]	[c]	.0047
	UPB1	7	Down		[c]	.0053
	■*GMPS*	7	Up			.0056
	■*ADSL*	7	Up	[c]		.0183
	■*IMPDH2*	8	Up	[c]		.0085
	■*NT5DC2*	8	Up		[c]	.0206
	RRM2	8	Up			.0422
	UCK2	7	Up	[c]		.0005
	ADA	6	Up		[c]	.0328
Membrane lipid	■*PLCB1*	8	Up			.0006
	■*GPD1L*	8	Up		[c]	.0029
	■*LPIN2*	7	Down	[c]		.0154
	■*PLCE1*	8	Up		[c]	.0459
	LPCAT1	7	Up		[c]	.0001
	PTDSS2	6	Up			.0005
Glycan	■*CTBS*	8	Down			.0203
	■*GAL3ST1*	6	Up		[c]	.0026
	■*B3GALNT1*	8	Up		[c]	.013
	■*NAGPA*	7	Up	[c]		.0325
	■*DDOST*	7	Up			.0401
	SULF1	7	Up		[c]	.0291
Cholesterol	■*HMGCS2*	7	Down		[c]	.0002
	LCAT	8	Down			.0165
	■*LBR*	7	Up			.0269

Kaplan-Meier overall survival was calculated for each gene and statistical significance analyzed by log-rank (Mantel-Cox) test.
Square (■) indicates novel targets in liver cancer as determined by PubMed search.
#, number of datasets in which each gene is expressed at $P < .05$.
[a]All listed metabolic genes correlated with *ECM2* and *MMP9*.
[b]Denotes *P* value.
[c]Indicates those that also correlated with *CDH1* and or *VIM* (Pearson correlation was considered significant at $P < .05$).
[d]Previously identified as a survival predictor in hepatocellular carcinoma (HCC) (see the referenced study and other similarly described targets in Supplementary Table 1).

predominantly down-regulated targets and vice versa cannot be overlooked. Similarly, isoforms of metabolic genes that are consistently expressed in the opposite direction (eg, *GLS* and *GLS2*, *MPC1* and *MPC2*, *MAT1A* and *MAT2A*) (see Table 3, Supplementary Table 1) represent alterations that could be of clinical importance in liver cancer.

Conclusions

We have revealed metabolic targets that are consistently deregulated and so can be further studied as potential clinical biomarkers, therapeutic targets, or prognostic indicators in liver cancer. Several of the targets reflect at protein level, correlate with the progression markers, vary with tumor size, and predicted patient overall survival. Moreover, 54% (n = 343) (Supplementary Figure 1) were represented in the recent list of gene mutations identified in HCC by exome sequencing analysis.[31] We believe these metabolic targets are broadly of promising clinical relevance in HCC. Consistent with this notion, most of the identified metabolic targets already described in the literature were proposed as biomarkers, therapeutic targets, or prognostic indicators in HCC. Examples include *AKR1B10*, *CLIC1*, *PKM*, *ASNS*, *GLS*, *LPCAT1*, *NDUFA4L2*, *SLC39A6*, and *VDAC1*, which are all up-regulated, and *CYP1A2*, *ASS1*, *MAT1A*, *GLS2*, and *ALDH1L1*, which are all down-regulated in HCC (see Supplementary Table 1). Hence, our findings are in agreement with several independent studies that have relied on various HCC patient cohorts. It gives strong impetus for detailed mechanistic studies on metabolic targets and their associated pathways in HCC. It is worthy to note that, a potential limitation of our work is the probability that some metabolic genes were not captured, for instance, due to gene probes not currently annotated. Also, although several targets are well known for their involvement in specific biochemical pathways, our assortment of some others to pathways should be used as a guide especially for those with yet unknown biological roles. Furthermore, due to technical limitations, we did not detect all proteins corresponding to the metabolic genes. Nevertheless, given the consistency of the genes we identified from several independent HCC datasets, and their correspondingly similar expression pattern where detected at proteomic level, this study is to date the most extensive exposition of the metabolic genes often deregulated in human HCC. Whether these alterations are specific to liver cancer or also present in other liver diseases, especially those related to metabolism such as NASH, should be an interesting subject of future investigation contingent on the accumulation of a comparable amount of genomic data for the clinical disease in question. For such study, the targets herein reported will serve as useful reference.

Our findings are also important in other aspects of liver cancer metabolism. For example, it will assist future studies in deciding on specific metabolic pathways to modulate therapeutically, and could increase the chances of identifying alternative metabolic pathways or targets that are used by HCC to evade therapy. It will also assist in identifying unique metabolic gene pattern in liver cancer compared with other cancers. In addition, the consistently altered targets represent a powerful tool for determining the in vitro or in vivo experimental HCC models that best depict the human HCC situation, especially from metabolism perspective—this knowledge is currently lacking and if obtained can help fine-tune future prospects of understanding liver cancer metabolism. In the context of personalized medicine, we hope that the consistently altered targets, including those most deregulated, as shown in Figure 1*C*, will be relevant for the identification of patients whose liver tumors have divergent expression patterns that might warrant individualized interventions.

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The published datasets that were analyzed in this study are freely accessible from the National Center for Biotechnology Information Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/) under the Accession numbers GSE6764, GSE14323, GSE14520, GSE36376, GSE39791, GSE57957, GSE60502, and GSE62232. In addition, The Cancer Genome Atlas liver cancer data were accessed via cBioPortal database (http://www.cbioportal.org/).

Author contributions
ZCN conceived the study, performed data analyses and wrote manuscript. SH and CM performed PubMed Search for the identification of novel metabolic targets and also discussed manuscript outline. SR discussed method and presentation of data. DAM and BS performed proteomics analysis. MPE performed critical revision of the manuscript. SD provided supervisory support and corrected manuscript. All authors read the final version of the paper.

Conflicts of interest
The authors declare no conflicts.

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Supplementary Material

FASN	*CAD*	*SLC33A1*	*MFSD1*
ABCC4	*CHKA*	*SLC38A6*	*NAGPA*
NPC1	*CHPF2*	*SLC4A7*	*NDUFA4L2*
ACACA	*DOLK*	*SPTLC1*	*NEU1*
OAS3	*GNPTAB*	*AACS*	*NQO1*
B4GALT7	*HK2*	*ABCC5*	*NUDT1*
CDS1	*IMPAD1*	*ABCF2*	*NUDT2*
LBR	*LPCAT1*	*ACBD3*	*P2RX4*
SLC4A1AP	*SLC2A6*	*ACLY*	*PIGF*
ABCC10	*SLC4A2*	*ACOT9*	*PIGO*
ACSL3	*SLCO2A1*	*ACSL4*	*PIGT*
ATP2C1	*SULF1*	*ADA*	*PIK3CB*
CTPS2	*ABCF1*	*ALG3*	*PLA2G7*
FLAD1	*ACOT7*	*ASNS*	*PPAT*
GART	*ALDH3A1*	*ATIC*	*PPOX*
GLS	*ALG8*	*ATP1B3*	*PTDSS2*
GNS	*ATP2A2*	*ATP5H*	*PYCR1*
PI4KB	*ATP6V1H*	*ATP6AP2*	*PYGB*
SLC2A5	*ATP8B2*	*ATP6V1C1*	*RRM1*
SLC36A1	*BCAT1*	*ATP6V1D*	*RRM2*
SLC7A11	*CA12*	*B3GAT3*	*SCD*
STARD7	*CLIC5*	*BCAT2*	*SEPHS1*
ALDH18A1	*ELOVL5*	*BPGM*	*SLC10A3*
ALG6	*ENOX2*	*CLCC1*	*SLC1A4*
COX11	*ENTPD1*	*COX6C*	*SLC22A5*
ENPP2	*FADS1*	*CYB561*	*SLC29A2*
PGK1	*GFPT1*	*DCK*	*SLC38A1*
SLC26A6	*GMDS*	*DDOST*	*SLC39A6*
UGDH	*IDH3B*	*ENOPH1*	*SLC7A6*
CS	*IDI1*	*ENTPD6*	*SMOX*
PLCE1	*IP6K1*	*FDPS*	*SMS*
EXT2	*LPGAT1*	*GALNT11*	*SQLE*
HMBS	*PAPSS1*	*GAPDH*	*SRM*
IDUA	*PFKP*	*GLTP*	*SULT1C2*
NDST1	*PIGC*	*GSTA4*	*SYNJ2*
ADSL	*PIP4K2C*	*GUK1*	*TALDO1*
ADSS	*PLA2G4C*	*GYG1*	*TAP1*
ALG13	*PLCB1*	*HEXB*	*TXNRD1*
ATP1A1	*PLCG1*	*HKDC1*	*TYMS*
ATP5G2	*PYCRL*	*MDH2*	*VDAC3*

HMGCS2	*HPD*	*B3GAT1*	*LIPG*
FTCD	*MTHFS*	*BAAT*	*MAN1C1*
GPHN	*NNT*	*BCKDHA*	*MMACHC*
MAOB	*SLC16A4*	*CDA*	*MME*
RDH16	*A1CF*	*CES3*	*MUT*
TF	*ACAA2*	*CHST7*	*NADK*
ACOX3	*ADCY1*	*COMT*	*NAT2*
ENPP1	*ADK*	*CYP1A2*	*NIT2*
PCK1	*BCHE*	*CYP26A1*	*PAH*
SLC47A1	*BHMT2*	*CYP2A7*	*PAOX*
ACSL1	*CTH*	*CYP2C18*	*PAPSS2*
AKR7A3	*CYP2C8*	*CYP2C19*	*PC*
ALAS1	*DAK*	*CYP4F3*	*PCBD1*
ALDH9A1	*DPYD*	*DDAH1*	*PCK2*
AOX1	*DPYS*	*DHRS1*	*PDE2A*
CNGA1	*EDEM1*	*DSE*	*PDK4*
CYP2E1	*GNE*	*FAAH*	*PGM1*
CYP4A11	*GRHPR*	*FAH*	*PIK3C2G*
EHHADH	*HSD17B2*	*FDX1*	*PIK3R1*
KCNMA1	*KDSR*	*FMO4*	*PON1*
ALDOB	*NDST3*	*FOLH1*	*PON3*
CP	*NPC1L1*	*FUT6*	*PPAP2B*
CYP2C9	*PCCA*	*GATM*	*PRG2*
GYS2	*PECR*	*GGT5*	*PTS*
HIBCH	*PTGS2*	*GK*	*SCP2*
SARDH	*RBKS*	*GLDC*	*SDS*
SDHA	*SLC25A16*	*GLUD1*	*SHMT1*
SLC22A1	*SLC37A4*	*GNMT*	*SLC16A10*
SLC6A16	*SLC39A14*	*HAAO*	*SLC17A1*
ADH1A	*SLCO2B1*	*HAL*	*SLC17A2*
CPT2	*ABCA8*	*HAO1*	*SLC22A7*
CYP2A6	*ABCC6*	*HAO2*	*SLC31A1*
CYP4F2	*ABCC9*	*HGD*	*SLC38A2*
ECHS1	*ABCD3*	*HMGCL*	*SLC38A4*
HSD11B1	*ABHD6*	*HPGD*	*SLC6A12*
NNMT	*ACADM*	*IVD*	*SLC6A13*
PCCB	*ACO1*	*KCNAB1*	*SLC7A8*
SLC27A5	*ACOX1*	*KCNJ16*	*SOD1*
UPB1	*ACSM5*	*KCNN2*	*ST6GAL1*
PLCG2	*ADH1B*	*LGSN*	*SULT1A2*
SLC2A2	*ADH6*		*SULT2A1*
SRD5A1	*AKR1D1*		*SUOX*
ABCA6	*ALDH2*		*UGP2*
ACACB	*ALDH6A1*		
ACADVL	*ALDH7A1*		
AGL	*ALDH8A1*		
ALDH1L1	*AQP7*		
CFTR	*ASL*		
CPS1	*ASPA*		
DHTKD1	*ASS1*		

Supplementary Figure 1. Consistently Altered Metabolic Targets Among List of Mutations in Published Exome Sequencing Analysis of Hepatocellular Carcinoma.