Low DMT1 expression associates with increased oxidative phosphorylation and early recurrence in HCC

Toshifumi Hoki, MD, PhD, Eriko Katsuta, MD, PhD, Li Yan, PhD, Kazuaki Takabe, MD, PhD, Fumito Ito, MD, PhD

Abstract

Background—Despite a high rate of recurrences, long-term survival can be achieved after the resection of hepatocellular carcinoma (HCC) with effective local treatment. Discovery of adverse prognostic variables to identify patients with high risk of recurrence could improve the management of HCC. Accumulating evidence showing a link between carcinogenesis and increased expression of iron import proteins and intracellular iron prompted us to investigate a role...
of divalent metal-ion transporter-1 (DMT1) that binds and regulates a variety of divalent metals in HCC.

**Materials and Methods**—Clinical and gene expression data from RNA-seq in 369 HCC patients were obtained from The Cancer Genome Atlas (TCGA). Disease-free survival (DFS) was compared between DMT1 high- and low-expressing tumors, and gene set enrichment analysis (GSEA) was conducted.

**Results**—Patients with lower expression of DMT1 exhibited significantly worse DFS compared with the DMT1 high group (P = 0.044), notably in advanced stage patients (P = 0.008). DMT1 expression did not differ in etiologies, stages, and differentiation status of HCC. Interestingly, DMT1 expression levels inversely associated with cellular respiratory function in HCC. Furthermore, GSEA revealed that metabolism-related gene sets such as glycolysis, oxidative phosphorylation, and reactive oxygen species pathway were significantly enriched in the DMT1 low-expressing HCC.

**Conclusions**—Low DMT1 expression associates with increased oxidative phosphorylation as well as glycolysis and identifies early recurrence in HCC patients after surgical treatment.

**Keywords**
Divalent metal-ion transporter-1; Hepatocellular carcinoma; Glycolysis; Oxidative phosphorylation; Mitochondria; The Cancer Genome Atlas

---

**1. Introduction**

Incidence of liver cancer has more than tripled since 1980. It has increased by about 3% per year in the U.S. from 2004 to 2013. An estimated 42,220 new cases of liver cancer will be diagnosed in the U.S. during 2018, and approximately three fourths of which will be hepatocellular carcinoma (HCC).\(^1\) HCC is the sixth most frequently diagnosed cancer and responsible for the second most common cause of cancer mortality worldwide with estimated 700,000 deaths per year.\(^2,3\)

HCC generally develops in patients with underlying chronic liver disease, which include cirrhosis from any cause such as chronic infection of hepatitis B virus (HBV) or hepatitis C virus (HCV), excessive alcohol intake, and nonalcoholic fatty liver disease (NAFLD). NAFLD and its advanced form, nonalcoholic steatohepatitis (NASH), which occurs with metabolic syndrome, obesity, and diabetes mellitus (DM), are now increasingly frequent underlying liver disease in patients with HCC.\(^4-8\) Furthermore, numbers of clinical observational studies have provided a link between obesity and risk of HCC.\(^9-13\) Several studies have indicated an association between the sequelae of NAFLD and the development of HCC even in the absence of cirrhosis;\(^8,14,15\) however, pathogenesis of HCC development in NAFLD/NASH remains to be elucidated.

For patients who are medically fit with healthy background liver function and have resectable disease, liver resection continues to be a mainstay treatment.\(^16,17\) However, even with complete surgical extirpation, recurrence rates within 5 years have been reported to exceed 70%.\(^18,19\) Since the majority of recurrences are intrahepatic due to local recurrence...
or a new second primary tumor, the goal of post-resectional surveillance is early detection of disease that might be amenable to subsequent local therapy such as repeat liver resection, liver transplantation, thermal ablation, and transarterial chemoembolization (TACE) or radioembolization.

Several tumor-related biologic as well as histologic factors such as high preoperative alpha fetoprotein (AFP) levels, tumor size, vascular invasion, resectional margin status, spontaneous tumor bleeding, and poor histologic grade of differentiation have been identified as potential predictors of recurrence, yet the management of surveillance remains challenging and additional indicators will improve the detection of recurrence in HCC. Recent advances in RNA-seq transcriptomics allow identification of novel transcripts and molecular mechanism governing carcinogenesis, progression, and prognosis in solid malignancies.

Divalent metal-ion transporter-1 (DMT1) is a transmembrane protein involved in transportation of divalent metals including cadmium, cobalt, copper, nickel, lead, manganese, zinc, and in particular iron. DMT1 is ubiquitously expressed, most notably in proximal duodenum, immature erythroid cells of the bone marrow, brain, and kidney. Dietary iron is imported into the enterocyte through DMT1 on its apical surface and enters into the blood stream through ferroportin 1 (Fpn1). Once absorbed, transferrin-bound iron is endocytosed and released into hepatocytes via DMT1. Non-transferrin–bound iron, in case of iron overload, is also taken up by hepatocytes through DMT1 directly. Some degrees of iron overload have been shown to be present in 10% to 30% of patients with chronic liver disease, and known as one of the causes of hepatocarcinogenesis through generating reactive oxygen species. We previously reported aberrant expression of DMT1 in the duodenum, where iron absorption takes place, led to liver iron accumulation in patients with NASH. Boult et al. investigated the expression of iron transport proteins in the premalignant lesions, Barrett’s metaplasia and esophageal adenocarcinoma, and found that progression to adenocarcinoma was associated with increased expression of iron transport proteins including DMT1. Moreover, Brookes et al. showed progression of normal colon and precancerous state such as low/high grade dysplastic adenomas to colorectal carcinoma was associated with increased expression of DMT1.

While DMT1 is involved in the carcinogenesis, its contribution to tumor progression and relapse of HCC remains unclear. Interestingly, DMT1 expression was found to be up-regulated in iron-loaded, non-cirrhotic, and non-tumorous liver tissues compared with normal liver controls. However, Deugnier et al. described lack of iron accumulation within HCC in hereditary hemochromatosis patients, indicating the role of DMT1 differs between hepatocarcinogenesis and tumor progression. To date, no study has been reported on the significance of DMT1 gene expression in the context of recurrence following liver resection. In the present study, we employed publicly available database, The Cancer Genome Atlas (TCGA) for genomic analysis to elucidate a role of DMT1 in recurrence after liver resection for HCC and performed gene set enrichment analysis (GSEA) to interpret genome-side expression profile.
2. Materials and Methods

Data acquisition and pre-processing

The National Cancer Institute (NCI) and National Human Genome Research Institute (NHGRI) obtained human data from doctors who collected tissue for TCGA after gaining approval with informed consent documents and local Institutional Review Boards (IRBs). The TCGA Biospecimen Core Resource laboratory extracted RNA from all samples and distributed the RNA to their Genome Sequencing Centers, where the uniform sequencing technique was used by TCGA researchers. There were 440 HCC cases of liver resection in TCGA cohort (http://cancergenome.nih.gov). RNA seq v2 z-scores and clinical data were obtained through the cBioportal for Cancer Genomics (http://cbioportal.org) on January 15th, 2018, and processed as previously described. Of 440 cases, there were 369 primary tumors available for gene expression data from RNA-sequence. Two recurrent tumor samples from two patients whose primary tumors were also registered were excluded from our study to avoid duplication of patients. We also excluded two patients with neoadjuvant therapy to eliminate the gene expression affected by therapy prior to surgery. Patients were divided into DMT1 high and DMT1 low groups according to their gene expression levels with a cutoff being determined as the lower quartile value.

GSEA

GSEA was performed comparing DMT1 high and low patients utilizing the Java GSEA implementation version 3.0 provided by the Broad Institute (http://software.broadinstitute.org/gsea/index.jsp) as described previously. Statistical analyses were performed using unpaired two-sided Student’s t test or one-way ANOVA followed by Tukey’s test for continuous variables when appropriate and Fisher’s exact test for categorical variables. Disease-free survival (DFS) was estimated by the Kaplan–Meier (KM) method and the log-rank test. Censoring times were designated with vertical tick-marks on the KM curves. Correlations between DMT1 and the other gene mRNA expression levels were assessed with Pearson’s correlation coefficients. P values of less than 0.05 were considered statistically significant. Data analyses and creation of graphs were carried out using R version 3.4.3 (http://www.r-project.org) and Bioconductor packages (http://www.bioconductor.org).

3. Results

TCGA liver hepatocellular carcinoma patient cohort

Of 440 HCC cases in the whole TCGA hepatocellular carcinoma cohort, gene expression data from RNA-seq of primary tumor without neoadjuvant therapy were available for 369 patients. The mean age of the cohort was 59.4 ± 27.0 years-old. The prevalence of HBV, HCV, dual HBV-HCV, and non-B non-C were 96 (26.0%), 48 (13.0%), 7 (1.9%), and 198 (53.7%), respectively (Fig. 1A). There were also 7 (1.9%) hemochromatosis patients. Of the 369 patients who underwent liver resection, the numbers of single segmentectomy, multiple segmentectomy, lobectomy, extended lobectomy, and liver transplantation were 88 (23.8%),
86 (23.3%), 140 (37.9%), 25 (6.8%), and 1 (0.3%), respectively (Fig. 1B). The proportions of patients with AJCC stage I, II, III, and IV were 169 (45.8%), 85 (23.0%), 85 (23.0%), and 5 (1.4%), respectively (Fig. 1C). The median observation period was 19.3 months (range, 0–120.7m). The estimated 3- and 5-year DFS rates were 38.1% and 28.7%, and the 3- and 5-year OS rates were 62.5% and 47.8%.

**Decreased DMT1 expression is associated with worse DFS in HCC patients after liver resection**

Next, 369 patients were divided into DMT1 high and DMT1 low groups. DFS data were available in 317 HCC patients who underwent surgical resection, and there were 242 DMT1 high and 75 DMT1 low patients. We found the DMT1 low group showed significantly worse DFS compared with the DMT1 high group (P = 0.044) (Fig. 2A). To explore potential correlation between DFS and DMT1 expression in different stages, subgroup analyses were conducted in early-stage (AJCC stage I) patients and advanced-stage (AJCC stage II, III, and IV) patients. The DFS of DMT1 high and low groups were similar in 148 early-stage patients (P = 0.815), whereas the DMT1 low group showed significant worse DFS compared with the DMT1 high group in 148 advanced-stage patients (P = 0.008) (Fig. 2B and C). High expression of DMT1 has been reported to contribute to carcinogenesis, while our data illustrated low expression of DMT1 caused worse prognosis, suggesting the role of DMT1 might be different between carcinogenesis and tumor progression.

**DMT1 expression does not differ in etiologies, stages, and differentiation status of HCC**

To identify the features of the low DMT1-expressing HCC associated with worse DFS, we evaluated the expression of DMT1 in etiologies, stages, and differentiation status of HCC. We found that DMT1 expression levels were independent of virus infectious status (Fig. 3A), and comparable in different stages (Fig. 3B). The comparison of clinicopathological characteristics between DMT1 high (n = 277) and low (n = 92) groups were shown in Table 1. There was no difference in the any features listed between DMT1 high and low groups. There was also no difference in DMT1 expression levels among grade 1 (well differentiated), grade 2 (moderately differentiated), and grade 3/4 (poorly differentiated/ undifferentiated) (Fig. 3C). As NAFLD is a growing cause of HCC and a manifestation of metabolic syndrome, we sought to investigate the relation between DMT1 expression and metabolic status. There was little information available on metabolic syndrome including diabetes in this cohort. We compared 66 patients with BMI > or = 30, which indicates high risk for diabetes, with 265 patients with BMI < 30, and there was no difference of DMT1 expression (Fig. 3D). Taken together, we found DMT1 expression levels were not associated with underlying disease, or any known factors related to prognosis or recurrence of HCC. Of note, K-ras and H-ras are known as oncogenes found in less than 7% of human liver cancers, and the activation of Ras pathway has a role in HCC initiation and progression. Loss of TP53 function, which is a frequently mutated tumor suppressor gene in HCC, has been shown to be associated with hepatocellular carcinogenesis and poor prognosis. Therefore, we looked at P53 pathway, K-ras, and H-ras signaling in the GSEA; however, we did not find any significant enrichments of those gene sets (Fig. 3E).
**DMT1 expression is not associated with iron regulatory or heme transport genes in HCC**

Since DMT1 is known as an iron importer, we evaluated correlation between DMT1 expression and iron regulatory genes or heme transport gene expressions in HCC. These include ceruloplasmin, cytochrome b reductase (Dcytb), Fpn1, hepcidin, hephaestin, iron-responsive element-binding protein 1 (IRP1), iron-responsive element-binding protein 1 (IRP2), transferrin receptor 1 (Tfr1), transferrin receptor 2 (Tfr2), transferrin (Tfr), and solute carrier family 39 member 14 (Zip14) as iron regulatory genes, and hemoglobin scavenger receptor (CD163) and low density lipoprotein receptor-related protein 1 (LRP1/CD91) as heme transport genes. Despite its function known to be as an iron importer, DMT1 expression correlated with neither of the iron regulatory genes nor heme transport genes (Fig. 4A and B). Furthermore, GSEA revealed that none of iron regulatory or heme transport-related gene sets were enriched in DMT1 high group (Fig. 4C).

**DMT1 expression level inversely associates with oxidative phosphorylation and glycolysis in HCC**

In order to identify the features of the low DMT1-expressing HCC, which have worse DFS after liver resection, GSEA was conducted based on the 50 hallmark gene sets.44 Intriguingly, oxidative phosphorylation and glycolysis gene sets were found to be enriched in DMT1 low tumors in addition to DNA repair and reactive oxygen species gene sets (Table 2). Respiratory metabolism-related gene sets, such as glycolysis (NES = -1.547, P = 0.022), oxidative phosphorylation (NES = -2.166, P < 0.001), which generates adenosine triphosphate (ATP) using pyruvate, a product of glycolysis, and reactive oxygen species pathway (NES = -1.766, P = 0.011), were enriched in the low DMT1-expressing HCC among 50 hallmark gene sets (Fig. 5A and B). Furthermore, respiratory chain known to be a major source of reactive oxygen species and the site of oxidative phosphorylation was also enriched in DMT1 low group (NES = -2.148, P < 0.001) (Fig. 5B). Our unexpected findings of inverse correlation between DMT1 and oxidative phosphorylation and glycolysis in HCC patients prompted us to further examine mitochondrial metabolism-related gene sets by GSEA. Since oxidative phosphorylation is a process that takes place in mitochondria, as we expected, “Mitochondrion pathway”, a broad function of mitochondria, was found to be significantly enriched in DMT1 low tumor (NES = -2.099, P < 0.001) (Fig. 5C). Next, we further evaluated roles of DMT1 in regulation of other mitochondrial functions. Despite increased oxidative phosphorylation and mitochondrial pathway in DMT1 low group, other known mitochondrial functions including citric acid cycle (Kreb pathway; NES = -1.302, P = 0.193), fatty acid beta-oxidation (mitochondrial fatty acid beta oxidation; NES = -1.199, P = 0.332), storage of calcium ions (calcium ion homeostasis; NES = -0.990, P = 0.428), or apoptosis (apoptotic mitochondrial changes; NES = -1.381, P = 0.053) were not enriched (Fig. 5D). These findings suggest that DMT1 low tumors with higher risk of recurrence have increased glycolysis and oxidative phosphorylation facilitating higher ATP production.

Although glycolysis is known to be induced by HIF-1α in hypoxia,50 hypoxia was not enriched in GSEA (NES = 0.688, P = 0.856) (Fig. 5E). HIF-1α and its upstream regulator, prolyl hydroxylase domain-containing protein 1 (PHD1) did not show strong correlations with DMT1 expression (Fig. 5F). While protein secretion gene set was significantly enriched in the DMT1 high group (Normalized enrichment score (NES) = 1.782, P = 0.008) (Table 2),
expression of ALB, which encodes the major protein exclusively synthesized in the liver, was not associated with DMT1 expressions ($r = -0.125$, $P = 0.016$) (Fig. 5G).

4. Discussion

Despite a higher recurrence rate, long-term survival can be achieved after resection of HCC because in most cases recurrences are confined to the liver and may be amenable to local therapies.\textsuperscript{17,20,21} Thus identification of risk factors, close follow-up evaluation, and early detection are of great importance.

DMT1 was first identified as a transmembrane iron-transport protein, and found to play an important role in intestinal iron absorption.\textsuperscript{51} DMT1 is also expressed in the liver; however, a role of DMT1 in hepatocytes remains unclear. Although transferrin-bound iron as well as free iron is uptaken by hepatocyte via DMT1, Wang \textit{et al.} showed that DMT1 in hepatocytes is dispensable for hepatic iron accumulation and non-transferrin-bound iron uptake using mice with the Dmt1 gene selectively inactivated in hepatocytes (Dmt1\textsuperscript{liv/liv}).\textsuperscript{52} In line with this, expression of DMT1 did not correlate with iron regulatory genes or heme transport genes.

While iron deposition in hepatocyte has been shown to induce hepatocarcinogenesis, how iron and its importer, DMT1, are associated with cancer recurrence remains to be elucidated. In this study, we employed gene expression data from TCGA HCC cohort, and found that higher expression of DMT1 in HCC was associated with significantly longer DFS after resection (Fig. 2A). This was somewhat an unexpected finding given the previous studies suggesting potential role of DMT1 in iron overload frequently seen in chronic liver disease and one of causes of hepatocarcinogenesis,\textsuperscript{33,35} but might suggest potentially unrecognized roles of DMT1 in HCC.

In 1950s, Otto Warburg observed that cancer cells preferentially relied on aerobic glycolysis, which is less efficient than mitochondrial oxidative phosphorylation in terms of ATP generation, even in the presence of abundant oxygen.\textsuperscript{53,54} For decades, the respiratory alteration had been regarded as a result of compensation for mitochondrial dysfunction, while recent studies have revealed oncogenes, such as $K$-ras and $c$-Myc, and hypoxia environment induce glycolysis.\textsuperscript{50,55} Of note, iron is required as a cofactor for PHD1 to hydroxylate proline residues on HIF-α in regulation of hypoxia;\textsuperscript{56} however, hypoxia pathway was not associated with DMT1 expression levels in HCC in hallmark gene set (Fig. 5E), and we did not find significant correlation between DMT1 expression and PHD1 (Fig. 5F) in the present study. Low DMT1 group with worse DFS showed enrichments of both glycolysis and oxidative phosphorylation, suggesting ATP production depends on both glycolysis and oxidative phosphorylation. Although it is well known that glycolysis is enhanced in most malignant tumors, contribution of oxidative phosphorylation for ATP production to HCC growth remains elusive.\textsuperscript{57} In this study, we found that HCC with low DMT1 correlates not only with increased glycolysis but also with enhanced oxidative phosphorylation. While it is unclear whether oxidative phosphorylation is augmented in the process of an adaptation of hepatocellular carcinoma cells to meet energy demands in the tumor microenvironment, HCC usually develops blood supply predominantly from the
arterial system during progression, and can provide more oxygen for oxidative phosphorylation than portal vein.

Some tumors are relatively glycolytic, the others have high rates of mitochondrial respiration. Inactivation of the mitochondrial transcription factor has been shown to impair lung tumor cell proliferation, suggesting mitochondrial function is inevitable for cancer survival. Recently, Tan et al. have shown that tumor microenvironment instructs cancer cells to restore respiratory function, recover mitochondrial function, and reestablish tumor-initiating efficacy. ATP is known as the main energy fuel and also reported to promote cell proliferation and drug resistance in cancer cells. Since we have observed inverse association between DMT1 and oxidative phosphorylation, our results might indicate that DMT1 plays a role in directly or indirectly inhibiting oxidative phosphorylation, or vice versa. Indeed, our findings of strong correlation between increased mitochondrial metabolism and worse prognosis are in line with the profound influence of mitochondrial metabolism on all steps of oncogenesis such as malignant transformation, tumor progression and response to treatment.

Given cancer cells require higher levels of cytosolic ATP than normal tissue to sustain elevated rates of growth and division, we assume elevated ATP is associated with worse prognosis in HCC. ATP is used for acquiring drug resistance in cancer cells through ATP-dependent efflux pump. ATP also increases metastatic efficiency by improving ability of cancer cells to withstand traumatic deformation in the microvasculature of target organs. Cancer cells secrete ATP into their microenvironment at high concentrations than healthy tissues. The extracellular ATP is known to be unstable and can be hydrolyzed to ADP, AMP, and eventually adenosine, and the final degradation product suppresses cellular immunity in the tumor environment and stroma.

DMT1 has thus far not been reported to be associated with activation of mitochondrial metabolism. Recently, DMT1 was found to be located in outer mitochondrial membrane and play roles in mitochondrial iron import and other metals. The fact that iron is of vital importance for mitochondrial energy metabolism in oxidative phosphorylation as electron carriers contradicts our data where low DMT1 expression associates gene enrichment of mitochondrial function. Since our study investigated oxidative phosphorylation between high and low DMT1 expression and did not compared with DMT1 expression in normal liver, the relatively low DMT expression does not necessarily reflect a shortage of DMT1 and iron. The involvement of other metals may underlie the association between DMT1 low and up-regulated mitochondrial respiration.

The present study provides novel links between low DMT1 and increased mitochondrial and respiratory function in HCC with worse DFS, yet there are some limitations. First, this study was conducted using only one publicly available data without being validated using other cohorts. Second, this study is based on the gene expression of the primary tumor in TCGA cohort, that is, we have not verified through any in vitro or in vivo studies. To determine the role of DMT1 gene in association with oxidative phosphorylation and glycolysis, further studies would be warranted.
5. Conclusions

Lower DMT1 mRNA expression associates with poor DFS in patients with HCC regardless of underlying disease, stages, and differentiation status, and is associated with increased mitochondrial oxidative phosphorylation and glycolysis. Further studies are warranted to uncover how DMT1 affects the respiratory chain in mitochondria.

Acknowledgments

This work was supported by institutional funds from Roswell Park Comprehensive Cancer Center and National Cancer Institute (NCI) grant, P30CA016056. F.I is a Young Investigator supported by the Melanoma Research Alliance, and the NIH/NCI K08CA197966.

References

1. American Cancer Society. Cancer Facts & Figures 2018. Atlanta: American Cancer Society; 2018.
2. Ferenci P, Fried M, Labrecque D, et al. Hepatocellular carcinoma (HCC): a global perspective. J Clin Gastroenterol. 2010; 44(4):239–245. DOI: 10.1097/MCG.0b013e3181d46ef2 [PubMed: 20216082]
3. Tang A, Hallouc O, Chernyak V, Kamaya A, Sirlin CB. Epidemiology of hepatocellular carcinoma: target population for surveillance and diagnosis. Abdom Radiol (NY). 2018; 43(1):13–25. DOI: 10.1007/s00261-017-1209-1 [PubMed: 28647765]
4. Bugianesi E, Leone N, Vanni E, et al. Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. Gastroenterology. 2002; 123(1):134–140. DOI: 10.1053/gast.2002.34168 [PubMed: 12105842]
5. Hashimoto E, Yatsuji S, Tobari M, et al. Hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. J Gastroenterol. 2009; 44(Suppl 19):89–95. DOI: 10.1007/s00535-008-2262-x [PubMed: 19148800]
6. Ascha MS, Hanouneh IA, Lopez R, Tamimi TA-R, Feldstein AF, Zein NN. The incidence and risk factors of hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. H epatol ogy. 2010; 51(6):1972–1978. DOI: 10.1002/hep.23527 [PubMed: 2029604]
7. Yasui K, Hashimoto E, Komorizono Y, et al. Characteristics of patients with nonalcoholic steatohepatitis who develop hepatocellular carcinoma. Clin Gastroenterol Hepatol. 2011; 9(5):428–33. quize50. DOI: 10.1016/j.cgh.2011.01.023 [PubMed: 21320639]
8. Mittal S, EL-Serag HB, Sada YH, et al. Hepatocellular Carcinoma in the Absence of Cirrhosis in United States Veterans is Associated With Nonalcoholic Fatty Liver Disease. Clin Gastroenterol Hepatol. 2016; 14(1):124–31 e1. DOI: 10.1016/j.cgh.2015.07.019 [PubMed: 26196445]
9. W ezel TM, Graubard BI, Zeuzem S, El-Serag HB, Davila JA, McGlynn KA. Metabolic syndrome increases the risk of primary liver cancer in the United States: a study in the SEER-Medicare database. Hepatology. 2011; 54(2):463–471. DOI: 10.1002/hep.24397 [PubMed: 21538440]
10. Loomba R, Yang H-I, Su J, et al. Synergism between obesity and alcohol in increasing the risk of hepatocellular carcinoma: a prospective cohort study. Am J Epidemiol. 2013; 177(4):333–342. DOI: 10.1093/aje/kws252 [PubMed: 23355498]
11. Campbell PT, Newton CC, Freedman ND, et al. Body Mass Index, Waist Circumference, Diabetes, and Risk of Liver Cancer for U.S. Adults. Cancer Res. 2016; 76(20):6076–6083. DOI: 10.1158/0008-5472.CAN-16-0787 [PubMed: 27742674]
12. Lauby-Secretan B, Scoccianti C, Loomis D, et al. Body Fatness and Cancer--Viewpoint of the IARC Working Group. N Engl J Med. 2016; 375(8):794–798. DOI: 10.1056/NEJMsr1606602 [PubMed: 27557308]
13. Seyda Seydel G, Kucukoglu O, Altinbasv A, et al. Economic growth leads to increase of obesity and associated hepatocellular carcinoma in developing countries. Ann Hepatol. 2016; 15(5):662–672. DOI: 10.5604/16652681.1212316 [PubMed: 27493104]
14. Marchesini G, Bugianesi E, Forlani G, et al. Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. Hepatology. 2003; 37(4):917–923. DOI: 10.1053/jhep.2003.50161 [PubMed: 12668987]

15. Takamatsu S, Noguchi N, Kudoh A, et al. Influence of risk factors for metabolic syndrome and non-alcoholic fatty liver disease on the progression and prognosis of hepatocellular carcinoma. Hepatogastroenterology. 2008; 55(82–83):609–614. [PubMed: 18613418]

16. Llovet JM, Fuster J, Bruix J. Intention-to-treat analysis of surgical treatment for early hepatocellular carcinoma: resection versus transplantation. Hepatology. 1999; 30(6):1434–1440. DOI: 10.1002/hep.510300629 [PubMed: 10573522]

17. Poon RT-P, Fan ST, Lo CM, Liu CL, Wong J. Long-term survival and pattern of recurrence after resection of small hepatocellular carcinoma in patients with preserved liver function: implications for a strategy of salvage transplantation. Ann Surg. 2002; 235(3):373–382. DOI: 10.1097/00000658-200203000-00009 [PubMed: 11882759]

18. Kianmanesh R, Regimbeau JM, Belghiti J. Selective approach to major hepatic resection for hepatocellular carcinoma in chronic liver disease. Surg Oncol Clin N Am. 2003; 12(1):51–63. DOI: 10.1016/S1055-3207(02)00090-X [PubMed: 12735129]

19. Bruix J, Sherman M. Management of hepatocellular carcinoma. Hepatology. 2005; 42(5):1208–1236. DOI: 10.1002/hep.20933 [PubMed: 16250051]

20. Portolani N, Coniglio A, Ghidoni S, et al. Early and late recurrence after liver resection for hepatocellular carcinoma: prognostic and therapeutic implications. Ann Surg. 2006; 243(2):229–235. DOI: 10.1097/01.sla.0000197706.21803.a1 [PubMed: 16432356]

21. Shah SA, Cleary SP, Wei AC, et al. Recurrence after liver resection for hepatocellular carcinoma: risk factors, treatment, and outcomes. Surgery. 2007; 141(3):330–339. DOI: 10.1016/j.surg.2006.06.028 [PubMed: 17349844]

22. Bruix J, Llovet JM. Prognostic prediction and treatment strategy in hepatocellular carcinoma. Hepatology. 2002; 35(3):519–524. DOI: 10.1053/jhep.2002.32089 [PubMed: 11870363]

23. Torzilli G, Belghiti J, Kokudo N, et al. A snapshot of the effective indications and results of surgery for hepatocellular carcinoma in tertiary referral centers: is it adherent to the EASL/AASLD recommendations?: an observational study of the HCC East-West study group. Ann Surg. 2013; 257(5):929–937. DOI: 10.1097/SLA.0b013e31828329b8 [PubMed: 23426336]

24. Tabrizian P, Jibara G, Shrager B, Schwartz M, Roayaie S. Recurrence of hepatocellular cancer after resection: patterns, treatments, and prognosis. Ann Surg. 2015; 261(5):947–955. DOI: 10.1097/SLA.0000000000000710 [PubMed: 25010665]

25. Yu SJ. A concise review of updated guidelines regarding the management of hepatocellular carcinoma around the world: 2010–2016. Clin Mol Hepatol. 2016; 22(1):7–17. DOI: 10.3350/cmh.2016.22.1.7 [PubMed: 27044761]

26. Hubert N, Hentze MW. Previously uncharacterized isoforms of divalent metal transporter (DMT)-1: implications for regulation and cellular function. Proc Natl Acad Sci USA. 2002; 99(19):12345–12350. DOI: 10.1073/pnas.192423399 [PubMed: 12209011]

27. Ludwiczek S, Theurl I, Muckenthaler MU, et al. Ca2+ channel blockers reverse iron overload by a new mechanism via divalent metal transporter-1. Nat Med. 2007; 13(4):448–454. DOI: 10.1038/nm1542 [PubMed: 17293870]

28. Montalbetti N, Simonin A, Kovacs G, Hediger MA. Mammalian iron transporters: families SLC11 and SLC40. Mol Aspects Med. 2013; 34(2–3):270–287. DOI: 10.1016/j.mam.2013.01.002 [PubMed: 23506870]

29. Ba Q, Hao M, Huang H, et al. Iron deprivation suppresses hepatocellular carcinoma growth in experimental studies. Clin Cancer Res. 2011; 17(24):7625–7633. DOI: 10.1158/1078-0432.CCR-10-3099 [PubMed: 22052937]

30. Ciechanover A, Schwartz AL, Dautry-Varsat A, Lodish HF. Kinetics of internalization and recycling of transferrin and the transferrin receptor in a human hepatoma cell line. Effect of lysosomotropic agents. J Biol Chem. 1983; 258(16):9681–9689. [PubMed: 6309781]

31. Dautry-Varsat A, Ciechanover A, Lodish HF, pH and the recycling of transferrin during receptor-mediated endocytosis. Proc Natl Acad Sci USA. 1983; 80(8):2258–2262. DOI: 10.1073/pnas.80.8.2258 [PubMed: 6309093]
32. Canonne-Hergaux F, Gruenheid S, Ponka P, Gros P. Cellular and subcellular localization of the Nramp2 iron transporter in the intestinal brush border and regulation by dietary iron. Blood. 1999; 93(12):4406–4417. [PubMed: 10361139]

33. Shindo M, Torimoto Y, Saito H, et al. Functional role of DMT1 in transferrin-independent iron uptake by human hepatocyte and hepatocellular carcinoma cell, HLF. Hepatol Res. 2006; 35(3):152–162. DOI: 10.1007/s00535-012-0739-0 [PubMed: 23329365]

34. Tanaka S, Miyanishi K, Kobune M, et al. Increased hepatic oxidative DNA damage in patients with nonalcoholic steatohepatitis who develop hepatocellular carcinoma. J Gastroenterol. 2013; 48(11):1249–1258. DOI: 10.1007/s00535-012-0739-0 [PubMed: 23329365]

35. Hoki T, Miyanishi K, Tanaka S, et al. Increased duodenal iron absorption through up-regulation of divalent metal transporter 1 from enhancement of iron regulatory protein 1 activity in patients with nonalcoholic steatohepatitis. Hepatology. 2015; 62(3):751–761. DOI: 10.1002/hep.27774 [PubMed: 25753988]

36. Boult J, Roberts K, Brookes MJ, et al. Overexpression of cellular iron import proteins is associated with malignant progression of esophageal adenocarcinoma. Clin Cancer Res. 2008; 14(2):379–387. DOI: 10.1158/1078-0432.CCR-07-1054 [PubMed: 18223212]

37. Brookes MJ, Hughes S, Turner FE, et al. Modulation of iron transport proteins in human colorectal carcinogenesis. Gut. 2006; 55(10):1449–1460. DOI: 10.1136/gut.2006.094060 [PubMed: 16641131]

38. Tan MGK, Kumarasinghe MP, Wang SM, Ooi LLPJ, Aw SE, Hui KM. Modulation of iron-regulatory genes in human hepatocellular carcinoma and its physiological consequences. Exp Biol Med (Maywood). 2009; 234(6):693–702. DOI: 10.3181/0807-RRM-227 [PubMed: 19307463]

39. Deugnier YM, Charalambous P, Le Quilleuc D, et al. Preneoplastic significance of hepatic iron-free foci in genetic hemochromatosis: a study of 185 patients. Hepatology. 1993; 18(6):1363–1369. DOI: 10.1002/hep.1840180613 [PubMed: 7902316]

40. Kim SY, Kawaguchi T, Yan L, Young J, Qi Q, Takabe K. Clinical Relevance of microRNA Expressions in Breast Cancer Validated Using the Cancer Genome Atlas (TCGA). Ann Surg. 2017; 24(10):2943–2949. DOI: 10.1245/s10434-017-5984-2

41. Ramanathan R, Olex AL, Dozmorov M, Bear HD, Fernandez LJ, Takabe K. Angiopoietin pathway gene expression associated with poor breast cancer survival. Breast Cancer Res Treat. 2017; 162(1):191–198. DOI: 10.1007/s10549-017-4102-2 [PubMed: 28062977]

42. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci USA. 2005; 102(43):15545–15550. DOI: 10.1073/pnas.0506580102 [PubMed: 16199517]

43. Liberzon A, Birger C, Thorvaldsdóttir H, Ghandi M, Mesirov JP, Tamayo P. The Molecular Signatures Database (MSigDB) hallmark gene set collection. Cell Syst. 2015; 1(6):417–425. DOI: 10.1016/j.cels.2015.12.004 [PubMed: 26771021]

44. Katsuta E, Yan L, Nagahashi M, et al. Doxorubicin effect is enhanced by sphingosine-1-phosphate signaling antagonist in breast cancer. J Surg Res. 2017; 219:202–213. DOI: 10.1016/j.jss.2017.05.101 [PubMed: 29078833]

45. Terakawa T, Katsuta E, Yan L, et al. High expression of SLCO2B1 is associated with prostate cancer recurrence after radical prostatectomy. Oncotarget. 2018; 9(18):14207–14218. DOI: 10.18632/oncotarget.24453 [PubMed: 29581838]

46. Karnoub AE, Weinberg RA. Ras oncogenes: split personalities. Nat Rev Mol Cell Biol. 2008; 9(7):517–531. DOI: 10.1038/nrm2438 [PubMed: 18568040]

47. Ye H, Zhang C, Wang B-J, et al. Synergistic function of Kras mutation and HBx in initiation and progression of hepatocellular carcinoma in mice. Oncogene. 2014; 33(43):5133–5138. DOI: 10.1038/onc.2013.468 [PubMed: 24213574]

48. Teramoto T, Satonaka K, Kitazawa S, Fujimori T, Hayashi K, Maeda S. p53 gene abnormalities are closely related to hepatoviral infections and occur at a late stage of hepatocarcinogenesis. Cancer Res. 1994; 54(1):231–235. [PubMed: 8261444]

49. Villanueva A, Hoshida Y. Depicting the role of TP53 in hepatocellular carcinoma progression. J Hepatol. 2011; 55(3):724–725. DOI: 10.1016/j.jhep.2011.03.018 [PubMed: 21616106]
50. Semenza GL. Oxygen-dependent regulation of mitochondrial respiration by hypoxia-inducible factor 1. Biochem J. 2007; 405(1):1–9. DOI: 10.1042/BJ20070389 [PubMed: 17555402]
51. Gunshin H, Mackenzie B, Berger UV, et al. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature. 1997; 388(6641):482–488. DOI: 10.1038/41343 [PubMed: 9242408]
52. Wang C-Y, Knutson MD. Hepatocyte divalent metal-ion transporter-1 is dispensable for hepatic iron accumulation and non-transferrin-bound iron uptake in mice. Hepatology. 2013; 58(2):788–798. DOI: 10.1002/hep.26401 [PubMed: 23508576]
53. Warburg O. On respiratory impairment in cancer cells. Science. 1956; 124(3215):269–270. DOI: 10.1126/science.124.3215.267 [PubMed: 13351639]
54. Warburg O. On the origin of cancer cells. Science. 1956; 123(3191):309–314. DOI: 10.1126/science.123.3191.309 [PubMed: 13298683]
55. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science. 2009; 324(5930):1029–1033. DOI: 10.1126/science.1160809 [PubMed: 1160809]
56. Schofield CJ, Zhang Z. Structural and mechanistic studies on 2-oxoglutarate-dependent oxygenases and related enzymes. Curr Opin Struct Biol. 1999; 9(6):722–731. DOI: 10.1016/S0959-440X(99)00036-6 [PubMed: 10607676]
57. Zu XL, Guppy M. Cancer metabolism: facts, fantasy, and fiction. Biochem Biophys Res Commun. 2004; 313(3):459–465. DOI: 10.1016/j.bbrc.2003.11.136 [PubMed: 14697210]
58. Sezai S, Sakurabayashi S, Yamamoto Y, Morita T, Hirano M, Oka H. Hepatic arterial and portal venous oxygen content and extraction in liver cirrhosis. Liver. 1993; 13(1):31–35. DOI: 10.1111/j.1600-0676.1993.tb00602.x [PubMed: 8454523]
59. Weinberg F, Hamanaka R, Wheaton WW, et al. Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. Proc Natl Acad Sci USA. 2010; 107(19):8788–8793. DOI: 10.1073/pnas.1003428107 [PubMed: 20421486]
60. Tan AS, Baty JW, Dong L-F, et al. Mitochondrial genome acquisition restores respiratory function and tumorigenic potential of cancer cells without mitochondrial DNA. Cell Metab. 2015; 21(1):81–94. DOI: 10.1016/j.cmet.2014.12.003 [PubMed: 2556207]
61. Qian Y, Wang X, Li Y, Cao Y, Chen X. Extracellular ATP a New Player in Cancer Metabolism: NSCLC Cells Internalize ATP In Vitro and In Vivo Using Multiple Endocytic Mechanisms. Mol Cancer Res. 2016; 14(11):1087–1096. DOI: 10.1158/1541-7786.MCR-16-0118 [PubMed: 27578770]
62. Wallace DC. Mitochondria and cancer. Nat Rev Cancer. 2012; 12(10):685–698. DOI: 10.1038/nrc3365 [PubMed: 23001348]
63. Vyas S, Zaganjor E, Haigis MC. Mitochondria and Cancer. Cell. 2016; 166(3):555–566. DOI: 10.1016/j.cell.2016.07.002 [PubMed: 27471965]
64. Keibler MA, Wasylenko TM, Kelleher JK, Iliopoulos O, Vander Heiden MG, Stephanopoulos G. Metabolic requirements for cancer cell proliferation. Cancer Metab. 2016; 4(1):16.doi: 10.1186/s40170-016-0156-6 [PubMed: 27540483]
65. Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer. 2002; 2(1):48–58. DOI: 10.1038/nrc63194 [PubMed: 11902585]
66. Furlow PW, Zhang S, Soong TD, et al. Mechanosensitive pannexin-1 channels mediate microvascular metastatic cell survival. Nat Cell Biol. 2015; 17(7):943–952. DOI: 10.1038/ncb3194 [PubMed: 26098574]
67. Raffaghello L, Chiozzi P, Falzoni S, Di Virgilio F, Pistoia V. The P2X7 receptor sustains the growth of human neuroblastoma cells through a substance P–dependent mechanism. Cancer Res. 2006; 66(2):907–914. DOI: 10.1158/0008-5472.CAN-05-3185 [PubMed: 16424024]
68. Pellegratti P, Raffagello L, Bianchi G, Piccardi F, Pistoia V, Di Virgilio F. Increased level of extracellular ATP at tumor sites: in vivo imaging with plasma membrane luciferase. PLoS ONE. 2008; 3(7):e2599.doi: 10.1371/journal.pone.0002599 [PubMed: 18612415]
69. Ohta A. A Metabolic Immune Checkpoint: Adenosine in Tumor Microenvironment. Front Immunol. 2016; 7:109.doi: 10.3389/fimmu.2016.00109 [PubMed: 27066002]
70. Wolff NA, Garrick LM, Zhao L, Garrick MD, Thévenod F. Mitochondria represent another locale for the divalent metal transporter 1 (DMT1). Channels (Austin). 2014; 8(5):458–466. DOI: 10.4161/19336950.2014.956564 [PubMed: 25483589]
Fig. 1. The distribution of etiologies, surgical procedures, and stages
(A) Pie chart showing the distribution of etiologies in 369 patients with HCC from The Cancer Genome Atlas (TCGA) Liver Hepatocellular Carcinoma (LIHC) dataset. (B) Pie chart showing the types of surgical procedures performed. (C) Pie chart showing AJCC stages.
Fig. 2. Decreased DMT1 mRNA expression indicates poor disease-free survival (DFS) in patients with HCC

(A) Kaplan-Meier (KM) estimates of DFS in whole patients (n=317) from TCGA. (B) KM estimates of DFS in patients with AJCC stage I (n=148). (C) KM estimates of DFS in patients with stage II/III/IV (n=148).
Fig. 3. *DMT1* expression does not differ in etiologies, stages, and differentiation status of HCC

(A) Box-plot diagram showing the *DMT1* mRNA expression levels in 96 patients with HBV, 48 patients with HCV, and 198 patients without HBV or HCV (NBNC) from TCGA. Seven patients with dual HBV and HCV were excluded from this analysis. (B) Box-plot diagram showing the *DMT1* mRNA expression levels of 169 patients with stage I, 85 patients with stage II, 85 patients with stage III, and 5 patients with stage IV. (C) Box-plot diagram showing the *DMT1* mRNA expression levels in 55 patients with Edmondson-Steiner grade 1, 175 patients with grade 2, and 133 patients with grade 3/4. (D) Box-plot diagram showing the *DMT1* mRNA expression levels of 66 patients with BMI $\geq 30$ and 265 patients with BMI $< 30$. (E) Enrichment plots of HCC-related gene sets comparing *DMT1* high and low.
Fig. 4. DMT1 mRNA expression correlates with no other iron regulatory genes or heme transport genes

(A) Scatter plots between DMT1 and iron regulatory gene mRNA expressions in HCC patients from TCGA. (B) Scatter plots between DMT1 and heme transport gene mRNA expressions in HCC patients. (C) Enrichment plots of iron metabolism/heme metabolism-related gene sets comparing DMT1 high and low.
Fig. 5. Low DMT1-expressing HCC is associated with oxidative phosphorylation as well as glycolysis

(A) Enrichment plot of glycolysis gene set, (B) oxidative phosphorylation-related and reactive oxygen species gene sets, (C) mitochondrion gene set, (D) other mitochondrial function-related gene sets, (E) and hypoxia-related gene set comparing DMT1 high and low HCC tumors from TCGA. (F) Scatter plots between DMT1 and hypoxia-related gene mRNA expressions. (G) Scatter plots between DMT1 and ALB gene mRNA expressions in HCC patients.
### Table 1

Clinicopathological characteristics of patients from The Cancer Genome Atlas Liver Hepatocellular Carcinoma

|                          | DMT1 High (n = 277) | DMT1 Low (n = 92) | P value |
|--------------------------|---------------------|-------------------|---------|
| Age, years, mean (range) | 60(16–90)           | 59(17–81)         | 0.565   |
| Gender (%)               |                     |                   |         |
| Male                     | 189(68)             | 59(64)            | 0.444   |
| Female                   | 87(31)              | 33(36)            |         |
| Race (%)                 |                     |                   |         |
| White                    | 143(52)             | 39(42)            | 0.278   |
| Black                    | 14(5)               | 3(3)              |         |
| Asian                    | 112(40)             | 45(49)            |         |
| BMI, kg/m², mean (range) | 26(15–62)           | 25(17–38)         | 0.103   |
| Child-Pugh (%)           |                     |                   |         |
| A                        | 156(56)             | 58(63)            | 1.000   |
| B/C                      | 16(6)               | 5(5)              |         |
| Etiology (%)             |                     |                   |         |
| HBV                      | 70(25)              | 26(28)            | 0.515   |
| HCV                      | 39(14)              | 9(10)             |         |
| NBNC                     | 160(58)             | 57(62)            |         |
| Serum albumin, g/dL, mean (range) | 3.9(1.2–8.0) | 3.9(2.0–5.3) | 0.946   |
| AFP, ng/mL, median       | 15                  | 11                | 0.066   |
| Pathology stage (AJCC 6th) (%) |             |                   |         |
| Stage I                  | 128(46)             | 41(45)            | 0.711   |
| Stage II/III/IV          | 129(47)             | 46(50)            |         |
| Pathology T stage (%)    |                     |                   |         |
| T1/2                     | 203(73)             | 69(75)            | 1.000   |
| T3/4                     | 70(25)              | 23(25)            |         |
| Vascular invasion (%)    |                     |                   |         |
| No                       | 158(57)             | 47(51)            | 0.335   |
| Yes                      | 77(28)              | 30(33)            |         |
| Residual tumor (%)       |                     |                   |         |
| R0                       | 240(87)             | 82(89)            | 0.397   |
| R1/2                     | 11(4)               | 6(7)              |         |
| Ishak fibrosis score (%) |                     |                   |         |
| 0                        | 47(17)              | 27(29)            | 0.061   |
| 1/2                      | 24(9)               | 7(8)              |         |
| 3/4                      | 23(8)               | 5(5)              |         |
| 5/6                      | 62(22)              | 14(15)            |         |
| Edmondson-Steiner grade (%) |                 |                   |         |
| G1/2                     | 177(64)             | 54(58)            | 0.259   |
| G3/4                     | 95(34)              | 38(41)            |         |
# Table 2

Summary of gene set enrichment analysis (GSEA) in hallmark gene sets

| Gene sets enriched in $DMT1$ high | Size | ES    | NES     | p-value |
|-----------------------------------|------|-------|---------|---------|
| Protein secretion                 | 95   | 0.474 | 1.782   | 0.008   |
| Androgen response                 | 96   | 0.391 | 1.520   | 0.039   |
| Oxidative phosphorylation         | 195  | −0.709| −2.166  | < 0.001 |
| DNA repair                        | 142  | −0.472| −1.828  | 0.006   |
| Reactive oxygen species pathway   | 46   | −0.525| −1.766  | 0.011   |
| Glycolysis                        | 195  | −0.324| −1.547  | 0.022   |
| UV response up                    | 153  | −0.282| −1.376  | 0.030   |

ES = enrichment score; NES = normalized ES