Published in final edited form as:

Gastroenterology. 2013 May ; 144(5): 1066–1075.e1. doi:10.1053/j.gastro.2013.01.054.

Integrated Metabolite and Gene Expression Profiles Identify Lipid Biomarkers Associated with Progression of Hepatocellular Carcinoma and Patient Outcomes

Anuradha Budhu¹, Stephanie Roessler¹, Xuelian Zhao¹, Zhipeng Yu¹, Marshonna Forgues¹, Junfang Ji¹, Edward Karoly², Lun-Xiu Qin³, Qing-Hai Ye³, Hu-Liang Jia³, Jia Fan³, Hui-Chuan Sun³, Zhao-You Tang³, and Xin Wei Wang^{1,*}

¹Liver Carcinogenesis Section, Laboratory of Human Carcinogenesis, Center for Cancer Research, National Cancer Institute, Bethesda, MD, USA

²Metabolon Inc., Durham, NC, USA

³Liver Cancer Institute and Zhongshan Hospital, Fudan University, Shanghai, China

Abstract

BACKGROUND & AIMS—We combined gene expression and metabolic profiling analyses to identify factors associated with outcomes of patients with hepatocellular carcinoma (HCC).

METHODS—We compared metabolic and gene expression patterns between paired tumor and non-tumor tissues from 30 patients with HCC, and validated the results using samples from 356 patients with HCC. A total of 469 metabolites were measured using liquid chromatography/mass spectrometry and gas chromatography/mass spectrometry. Metabolic and genomic data were integrated, and Kaplan-Meier and Cox proportional hazards analyses were used to associate specific patterns with patient outcomes. Associated factors were evaluated for their effects on cancer cells in vitro and tumor formation in nude mice.

RESULTS—We identified 28 metabolites and 169 genes associated with aggressive HCC. Lipid metabolites of stearoyl-CoA-desaturase (SCD) activity were associated with aberrant palmitate signaling in aggressive HCC samples. Expression of gene products associated with these metabolites, including SCD, were independently associated with survival times and tumor recurrence in the test and validation sets. Combined expression of SCD and α -fetoprotein were associated with outcomes of patients with early-stage HCC. Levels of MUPA (monounsaturated palmitic acid), the product of SCD activity, were increased in aggressive HCCs; MUPA increased

© 2013 The American Gastroenterological Association. Published by Elsevier Inc. All rights reserved.

*Correspondence: Xin Wei Wang, 37 Convent Drive, Bldg. 37, Rm. 3044, Bethesda, MD 20892-4258; 301-496-2099/Fax: 301-496-0497; xw3u@nih.gov.

Disclosures: The authors have nothing to disclose.

Transcript profiling: The microarray platform and data have been submitted to the Gene Expression Omnibus (GEO) public database at NCBI following MIAME guidelines (Accession numbers: GPL4700 (platform); GSE6857 (samples)).

Author Contributions: A.B. and X.W.W. initiated and designed the study, analyzed and interpreted data, and wrote the manuscript. S.R. performed biostatistical analyses of metabolite and gene correlation. Z.Y., M.F., J.J. and X.Z. performed functional analyses. E.K. assisted with global metabolomic profiling. L-X.Q., Q-H.Y., H-L.J., J.F., H-C.S. and Z-Y.T. recruited patients, collected clinical specimens, and advised on clinical issues. All authors discussed the results and commented on the manuscript.

Writing Assistance: No writing assistance was used to compose this manuscript.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

migration and invasion of cultured HCC cells and colony formation by HCC cells. HCC cells that expressed small interfering RNA against SCD had decreased cell migration and colony formation in culture and reduced tumorigenicity in mice.

CONCLUSIONS—Using a combination of gene expression and metabolite profile analysis, we identified a lipogenic network that involves SCD and palmitate signaling and was associated with HCC progression and patient outcomes.

Keywords

HpSC-HCC; MH-HCC; fatty acid; stem cell

Cancer metabolite profiling (cancer metabolomics), the global view of the biochemical end products of cellular processes, is a promising new approach to understand the biological mechanisms underlying cancer development and progression^{1,2}. Metabolites are the best molecular indicators of cell status, since their rapid fluxes versus that of mRNA and proteins, are an extremely sensitive measure of cellular phenotype^{3,4}. Although human tumors have been extensively profiled by genomics-based studies^{5–10}, little is known about their global metabolite alterations and how these multi-level events form a network that contributes to aggressive disease and poor outcome. A systematic assessment of the pathways in which these genes and biochemical molecules interconnect may lead to a more precise set of alterations that may serve as key biomarkers or drug targets for clinical interrogation.

Hepatocellular carcinoma (HCC) represents a common and aggressive global human malignancy with extremely poor prognosis and a growing incidence in developed countries^{11,12}. HCC pathology and genetic/genomic profiles are heterogeneous, suggesting that it can initiate in different cell lineages. We recently hypothesized that the invasive characteristics of HCC may be due, in part, to the presence of hepatic cancer stem cells, which are thought to drive cancer progression through their capacity for self renewal, production of heterogeneous progeny and to limitlessly divide¹³. Indeed, our gene expression profiling studies have identified an aggressive HCC subtype expressing stem cell-like gene expression traits linked to poor prognosis, termed hepatic stem cell HCC (HpSC HCC)^{8,14,15}. HpSC-HCC differed from a mature hepatocyte subtype (MH-HCC) which expressed differentiated hepatocyte gene expression traits linked to good prognosis. Deciphering the complex molecular networks that distinguish aggressive HCC may advance our approaches to identify and therapeutically combat this aggressive population.

In this vein, we integrated metabolomics and transcriptomics of HpSC-HCC versus MH-HCC, to identify the key aberrant biochemical and molecular signaling networks related to HCC patient outcome. We found that palmitoleate, a mono-unsaturated lipid metabolite, as well as its activating enzyme, stearoyl-CoA-desaturase (SCD), play key roles in aggressive HCC. The imbalance of lipogenic components and pathways, particularly SCD, may function as key biomarkers for aggressive cancer and enable the strategic development of clinically relevant therapies.

Materials and Methods

Clinical specimens

A previously described cohort of 247 HCC patients¹⁶, obtained with informed consent from patients at the Liver Cancer Institute (LCI) and Zhongshan Hospital (Fudan University, Shanghai, China), was included. Among the LCI cohort, 60 paired tumor and nontumor samples from 30 patients were used in a training set, while the remainder of the cohort

(n=217) was used as the testing set. The study was approved by the Institutional Review Board of the LCI and NIH. A separate validation cohort of 139 patients of mixed ethnicity and etiology was used⁹. A study design diagram is shown in Supplementary Figure 1.

RNA isolation and mRNA arrays

RNA isolation was performed as previously described^{5,7}. The microarray platform and data have been submitted to the Gene Expression Omnibus (GEO) public database at NCBI following MIAME guidelines (Accession numbers: GPL4700 (platform); GSE6857 (samples)). REMARK guidelines have been followed to report the metabolite and gene markers in this study¹⁷.

Metabolomic profiling

Metabolomic profiling of 60 samples (30 paired tumor and nontumor tissues; 25mg/sample) was carried out in collaboration with Metabolon Inc.^{1, 18–20}. Liquid chromatography/mass spectrometry in positive and negative modes (LC+/LC–) and gas chromatography/mass spectrometry (GC/MS) were employed. A total of 469 metabolites were measured. No metabolites were filtered out based on a 25% missing data criteria.

Statistical analyses

BRB ArrayTools V3.8 was used to search for differentially expressed metabolites or genes or survival risk prediction^{5,7,16}. Unsupervised hierarchical clustering analysis was performed using GENESIS V1.7.6 developed by Alexander Sturn (IBMT-TUG, Graz, Austria). Principal components analysis was performed using Partek V6.5. Interconnected metabolite-gene pairs (Spearman correlation) or gene surrogates of metabolites were identified using in-house R codes (R V2.1.20). Kaplan-Meier survival analysis was used to compare patient survival based on prediction results, using PrismGraph V5.0 and the statistical p-value was generated by the Cox-Mantel log-rank test. Pathway analysis was performed using Ingenuity Pathway Analysis V8.6. Cox proportional hazards regression was performed using STATA 11.0. All p-values are two-sided and the statistical significance was defined as $p < 0.05$ unless otherwise noted.

Reagents

Palmitic acid and palmitoleic acid were purchased from Cayman Chemical. Metformin was purchased from Sigma. SCD inhibitor (CGX0168) was a generous gift from Complegen Inc. SCD, AMPK, AMPK-p, FAS and SREBP1 antibodies were purchased from Cell Signaling.

Cell Culture, Migration, Invasion, Colony Formation, Cell Cycle, Apoptosis and Immunohistochemistry Assays

Huh7 and HepG2 cells were cultured in DMEM (Gibco) supplemented with 10% FCS, penicillin, streptomycin and L-glutamine. Cells were treated with 100uM MUPA²¹ for 72hrs. 24 hrs prior to cell collection, 10mM metformin was added to appropriate wells. Protein analysis was performed according to the manufacturer's recommendation with modifications (see supplemental text). Proteins were separated and detected as previously described⁷. Migration and invasion assays were performed according to the manufacturer's recommendation (BD Biocoat Matrigel and Control chambers; BD Biosciences)^{7, 22}. For histological analysis, paraffin-embedded fresh frozen tumor specimens or explanted xenograft mouse tumors were stained for SCD 1:200 using Envision+ kit (DAKO). For cell cycle analysis, after 48hr of SCD siRNA transfection, cells were fixed and assessed for DNA content by flow cytometry (BD FACSCaliber). For apoptosis assays, after 48hr control of SCD siRNA transfection, Huh7 cells were incubated with 7-AAD and APC Annexin V (BD Pharmingen) followed by FACS.

Animal studies

Seven-week-old female athymic nude mice (CrI:NU-Foxn1nu) were purchased from Charles River Laboratories (Willmington, MA). All research involving animals complied with protocols approved by the National Cancer Institute-Bethesda Animal Care and Use Committee. Each treatment group consisted of 5 animals. Huh7 cells (0.5×10^6) suspended in 200uL of a 1:1 mixture of media and Matrigel (BD Biosciences) were subcutaneously injected into both flanks of the animals. Thus, 10 tumor sites were monitored for each treatment group. Subcutaneous nodules with a diameter of at least 0.2cm that persisted for at least 3 days were considered tumor growth. The size of subcutaneous tumors was measured by caliper. Tumor size and incidence were recorded bi-weekly.

Results

Metabolic profiling of aggressive HCC

To profile the metabolome and transcriptome of HCC cells, we partitioned a cohort of 386 HCC cases into a training set (n=30), a test set (n=217) and an independent validation set (n=139) (Supplementary Figure 1 and Supplementary Table 1). The training set consists of well-defined and matched HCC cases from EpCAM⁺AFP⁺ HpSC-HCC and EpCAM⁺AFP⁻ MH-HCC subtypes representing extreme patient outcomes⁸. We performed untargeted metabolomic profiling among 30 paired tumor and nontumor tissue specimens of the training set (Supplementary Figure1). Unsupervised principal components analysis of all measurable metabolites indicated that these biochemical end products could discriminate tumor from nontumor tissue (Figure 1A). To identify biochemical pathways functionally linked to aggressive HCC, we restricted our search to metabolites that were associated with tumor tissues, tumor subtypes and patient survival using class comparison and class prediction algorithms with 10-fold cross validation, an approach used successfully in the past^{5,7}. We first identified 253 metabolites that could significantly discriminate HCC tissues from their paired nontumor tissues (Supplementary Table 2). Among these metabolites, 48 could differentiate HpSC and MH-HCC subtypes in tumor samples (77% prediction accuracy; multivariate permuted $p < 0.05$; sensitivity = 0.72; specificity = 0.83) (Supplementary Table 3). However, no metabolite was found to significantly differentiate HpSC and MH-HCC subtypes in paired nontumor tissues using the same class prediction algorithms (43% accuracy; multivariate permuted $p < 0.7$; sensitivity = 0.40; specificity = 0.47). Therefore, the majority of measureable metabolic alterations seem to occur in tumor tissues. Furthermore, in a univariate cox regression analysis, we found that 28 of the 48 metabolites were significantly associated with overall survival (Figure 1B; Supplementary Table 4).

Integration of metabolomics and transcriptomics

Following transcriptomic profiling of the 60 samples used for metabolomics, we found 169 genes that could significantly discriminate HpSC from MH tumors (Supplementary Figure1). Since the 28 metabolites and 169 genes, independently identified above, are linked to aggressive HCC, we reasoned that a majority of these genomic products are likely interconnected within the same altered molecular pathways. To identify such molecular nodes, we first searched for significantly correlated metabolites and genes within the same samples ($p < 0.05$). Next, since each of the 28 metabolites could be associated with one or many of the 169 genes and vice versa, we performed a correlation analysis with randomization to determine the principal metabolite-gene pairs. We found that the experimental and randomized data were different and that 15 metabolites were most correlated with the 169 tumor and stem-like genes (Figure2A upper left panel). The reverse approach indicated that 121 genes were most correlated with the 15 tumor and stem-like metabolites (Figure2A upper right panel). This integrative approach therefore revealed significantly enriched

interconnected gene-metabolite pairs, which may comprise an aggressive HCC signaling network.

Genes surrogates of fatty-acid related metabolites are associated with outcome

Hierarchical clustering of the correlative values between the 15 metabolites and their 121 gene surrogates revealed two distinct groups of metabolites (Figure 2A lower panel). Interestingly, metabolites in cluster-1 were mainly fatty acid-related, whose identity was validated by MS/MS (Supplementary Fig 2), while cluster-2 contained a mixture of metabolites associated with various pathways. To assess the functional significance of the cluster-1 fatty acid-related metabolites, we globally and unbiasedly searched for their gene surrogates by utilizing the gene expression data of the test set (Supplementary Figure 1). We found 273 gene surrogates for the fatty acid-related metabolite cluster which could predict HpSC and MH subtypes in the test set (86% accuracy; multivariate permuted $p < 0.01$; sensitivity = 0.72; specificity = 0.91). The 273 gene signature was also significantly ($p = 0.0001$) associated with overall survival in the test set (Supplementary Figure 1, Figure 2B left panel and Supplementary Table 5), and in an etiologically and ethnically diverse HCC cohort in a separate validation set ($p = 0.0019$) (Supplementary Figure 1 and Figure 2B right panel). Furthermore, univariate Cox analysis showed that the 273 gene predictor was an independent predictor of patient survival in multivariate Cox regression analysis of the test (HR = 1.8, 95% CI = 1.08–3.00, $p = 0.025$) and validation set (HR = 2.3, 95% CI = 1.39–3.82, $p = 0.001$) (Table 1 and Supplemental Table 6). Additional multivariate models including CLIP or TNM staging showed similar results (data not shown). We also found that the 273 gene signature was significantly associated with disease-free survival ($p = 0.0407$), recurrence ($p = 0.0584$) and with early recurrence ($p = 0.0010$), typified by tumor metastasis, but not late recurrence ($p = 0.1075$) (Supplementary Figures 3A, 3B, 3C and 3D, respectively). Therefore, the fatty acid surrogate genes are associated with stem-like HCC and metastasis-related prognosis.

SCD as a biomarker for aggressive HCC

We next assessed the biological networks impacted by the 273 gene surrogates and found that many of these genes were related to PI3K signaling, a critical pathway in cancer cells (Figure 2C) ²³. Intriguingly, SCD, the singular enzyme responsible for the conversion of saturated palmitic acid (SPA) to its monounsaturated form, palmitoleic acid (palmitoleate; monounsaturated palmitic acid, MUPA) ²⁴, was among the 273 geneset. Interestingly, two of the metabolic products of SPA were present among the cluster-1 fatty acid-related metabolites and elevated in HpSC tumors, namely MUPA and 15-methylpalmitate (Figure 2A lower panel). In fact, SCD expression was highly and significantly correlated with the expression of these SPA products in HCC tissues (Figure 3A). Since the end-products of SCD activity seemed to be elevated in aggressive HCC, we next analyzed the expression level of SCD in HpSC and MH tumors and paired nontumors. Analysis of the microarray data showed that SCD was significantly elevated in HpSC tumors vs nontumors and in HpSC tumors vs MH tumors (Supplementary Figure 4A). Similar alterations in the levels of FAS and SREBP1, two enzymes involved in palmitate synthesis, were also observed in these samples (Supplemental Figures 4B and 4C). In addition, representative tumor specimens showed concordant SCD gene and protein expression as measured by IHC (Supplemental Figure 4D). Moreover, patients with high levels of SCD in their tumors had worse survival compared to those with low levels of SCD in both the test and validation cohorts (Figures 3B and 3C). We also found that SCD expression in tumors was significantly associated with disease-free survival (LCI cohort: $p = 0.0485$, LEC cohort: $p = 0.0047$) (Figures 3B and 3C). However, the significance of SCD was reduced when time to recurrence was used as an endpoint (Supplementary Figures 5A, 5B and 4C). Similar to the lipid gene surrogate data, the level of SCD in nontumor tissue was not associated with

overall or disease-free survival (Supplementary Figs 5D and 5E). We also examined the potential contribution of the liver microenvironment, by assessing the level of liver enzymes, alanine transaminase and aspartate transaminase (ALT and AST), among the HpSC and MH groups and their correlation with SCD metabolites. We found that ALT was significantly elevated in MH HCC and although not significant, a positive correlation could be observed between MUPA/SPA and ALT (Supplemental Figure 6A and 6C). No measureable alterations were observed with AST (Supplemental Figure 6B and 6D). We also explored the relation between SCD expression and BMI, glucose levels or steatosis among our cohort, but no significant relation was observed (data not shown). Taken together, SCD activity in tumor cells, but not adjacent nontumor cells, seems to play a significant role in aggressive HCC and prognosis by regulating the balance between SPA and MUPA.

We next performed Cox regression analysis to address whether SCD was confounded by underlying clinical parameters (Table 2). In univariate Cox analysis, SCD was significantly associated with overall survival (HR = 1.78; 95% CI = 1.18–2.69; $p=0.0006$), while a multivariate analysis revealed that SCD is an independent predictor of patient survival in the test (HR = 1.73, 95% CI = 1.09–2.73, $p = 0.019$) and validation sets (HR= 2.36, 95%CI= 1.38–4.06), $p=0.002$) (Supplementary Table 7). Additional multivariate models including CLIP or TNM staging showed similar results (data not shown). A comparison of the relative prediction capacity of SCD, the 273 lipogenic genes or a previously published 70 gene predictor of HCC subtype⁸ showed that the lipogenic-related biomarkers were more significantly associated with patient outcome (Supplementary Figure 7A). Next, we determined whether prognostic prediction could be improved by combining SCD with AFP, the only clinically available HCC prognostic marker, or with BCLC staging, since it was an independent predictor of HCC survival. Although low and high risk patients were each classified into the same outcome groups by SCD and AFP, there was a subset of patients misclassified by both methods (discordant cases) who have poorer outcome than low risk patients (Figure 3D). Similar to results with AFP, we found that SCD could further discriminate patients classified as BCLC Stage A (early stage patients with small tumors who are suitable for curative treatment), but not stage B/C (Figure 3E and Supplemental Figure 7B). Similar results were observed for the 273 gene surrogates (Supplemental Figures 7C and 7D). Therefore, the addition of an SCD predictive component to established clinical parameters may improve clinical risk prediction, and determine treatment regimens, particularly for early stage patients.

SCD affects aggressive phenotypes and tumor formation

Our results thus far indicate that SCD is a biomarker for HCC with poor outcome. In this vein, we set out to determine how SCD and its related fatty acids, MUPA and SPA, functionally contribute to aggressive HCC and how modulation of SCD activity may ameliorate this effect. We first screened a panel of HCC cell lines to determine their endogenous SCD expression (Supplementary Figure 8A). We found that EpCAM⁺AFP⁺ HCC cells with stem-like HCC gene expression traits such as HepG2, Hep3B, Huh1 and Huh7¹⁵, expressed measureable levels of SCD protein, while EpCAM⁻AFP⁻ HCC cells such as MHCC97 and SK-Hep1 did not. Next, we determined the role of SCD activity on aggressive cell features, such as colony formation, cell migration and cell invasion²⁵. Treatment of Huh7 cells with MUPA, the biological end product of SCD, resulted in a significant elevation of cell migration and invasion, whereas treatment with SPA, the enzymatic substrate of SCD, significantly reduced these properties (Figure 4A and Figure 4B). A similar effect was observed in HepG2 cells (data not shown).

Next, we modulated the level of SCD using metformin, an anti-diabetic drug with anti-cancer value, that can reduce SCD activity to modulate fatty acid production^{26,27}. In both

Huh7 and HepG2 cells, treatment with metformin significantly reduced SCD with a concomitant increase in AMPK phosphorylation (Supplementary Figure 8B upper panels). A reduction of FAS was also observed in Huh7 cells upon metformin treatment, however the level of SREBP1 did not seem to be affected (Supplementary Figure 8B lower panel). In the presence of metformin, Huh7 and HepG2 cell migration and invasion was reduced (Figure 4B and Supplementary Figure 8C). Moreover, metformin could abrogate the elevation of these cell phenotypes observed in the presence of MUPA, the metabolic product of SCD (Figures 4C and Supplementary Figure 8C). Similar effects were observed with CGX0168, a specific small molecule inhibitor of SCD²⁸ (Supplementary Figure 8D and data not shown). In parallel, MUPA was capable of increasing colony formation of EPCAM⁺AFP⁺ HCC cells, while metformin treatment produced a lower number of colonies when compared to cells expressing normal SCD levels (Supplementary Figure 8E). Similar to its effect on migration, the elevation of colony numbers observed in the presence of MUPA could be reverted in the presence of metformin (Supplementary Figure 8E). Next, to examine the affect of SCD level on tumor formation, we subcutaneously injected Huh7 cells treated with SCD siRNA or control siRNA into nude mice. Reduction of SCD via SCD siRNA (Supplementary Figure 9A) led to a decrease in cell migration and colony formation (Supplemental Figure 9B along with tumor incidence and tumor volume (Figure 4D). Explanted tumors from control and SCD siRNA xenografts did not show a measurable difference in SCD expression, suggesting that the tumor cells that escaped from an inhibitory effect of the SCD siRNA were able to form tumors (Supplemental Figure 9C). The reduction of SCD did not seem to affect cell cycle, however an increase in apoptosis was observed, likely due to accumulation of SPA (Supplemental Figures 9D and 9E). Thus, tumorigenic cell features associated with elevated levels of saturated fatty acid could be reduced by inhibition of SCD.

Discussion

While several studies have explored potential biomarker roles of metabolites or genes in HCC^{8,9,29–32}, our study has provided a comprehensive global metabolomic profile of HCC integrated with its transcriptome. Our integrative approach allowed us to uncover a novel signaling pathway, involving the regulation of palmitic acid saturation status, which may affect HCC aggressiveness. This method revealed that a lipogenic signature related to HCC tumors, particularly SCD, a modulator of fatty acid flux, has prognostic value in two independent HCC cohorts irrespective of known clinical factors. As such, SCD and its related metabolites may be valuable HCC biomarkers and prognostic indicators for molecular re-staging. Our data suggest that a crucial imbalance between saturated and unsaturated fatty acid occurs in HCC with poor prognosis, with a shift toward unsaturation. Reshifting this balance towards saturation may be a therapeutic strategy to revert aggressive phenotypes.

The need for novel biomarkers or those that can improve upon known diagnostic or prognostic measures for HCC is vital. Our integrative bioinformatic analyses showed that SCD is independently associated with HCC outcome. In fact, an improved prediction can be achieved by combining SCD with clinically relevant serum AFP or tumor staging. This suggests that further stratification of patient groups is possible even within patients who are predicted to have a relatively better outcome based on current clinical tests.

Our results indicate that SCD is a biomarker for HCC, however, there are some limitations to our study. The role of the microenvironment, in particular, is important to consider, despite the lack of significant metabolic changes observed among the nontumor specimens of this study. Since the majority of the cases assessed for metabolite alterations were cirrhotic, the presence and role of the biochemical changes in the underlying liver remains to

be studied. In addition, since SCD was predictive of outcome in patients of mixed ethnicities and etiologies, its potential utility may not be restricted to patients with underlying hepatitis B viral infection. In fact, further assessment shows the potential prognostic capacity of SCD in non-hepatitis B patients of the LEC cohort. The utility of SCD in additional ethnic and etiological cohorts requires future exploration.

Our current findings corroborate and extend upon earlier studies of SCD in cancer. For example, an unbalanced level of saturated and unsaturated fatty acid has previously been associated with lung cancer risk³³ and case-control studies have demonstrated a decreased risk of breast cancer in women with low SCD³⁴. In liver-related studies, SCD has been associated with genetic predisposition to hepatocarcinogenesis in mice and rats³⁵ while suppression of SCD could reduce proliferation in HCC cell lines in an Akt-dependent fashion³⁶. These studies suggest that SCD is an important signaling molecule in cancer, however no information on aberrant tissue expression or activity of SCD in cancer patients has previously been shown. Here, we have demonstrated the capacity to measure SCD level in tissue specimens based on gene expression as well as immunohistochemistry. Thus, our study identifies SCD as a biomarker for HCC aggressiveness in a patient population based on its expression in tissue biospecimens and provides insight into how modulation of its activity could be pursued to clinically address aggressive HCC.

Although the mechanism underlying SCD's role in HCC aggressiveness is unclear, our data suggests that its regulation of SPA level and activity may contribute to disease progression. One plausible mechanism may be to reduce SPA bioavailability through conversion to MUPA and/or modification to 15-methylpalmitate. Methylpalmitate is known to prevent kupffer cell activation, affect cytokine expression and inhibit liver fibrosis in animal models, which functionally opposes SPA³⁷. Thus, methyl forms of SPA may affect the liver microenvironment to promote HCC aggressiveness. These hypotheses along with a potential cellular mechanism to induce 15-methylpalmitate warrant further investigation. Other approaches to modulate fatty acid saturation status may be through direct or indirect inhibition of SCD itself. Recent progress in the development of pharmacological agents that target SCD activity³⁸ is encouraging and may be novel therapies for aggressive HCC.

Metabolic syndrome, including obesity, diabetes and fatty liver disease are linked to HCC risk and progression^{39,40}, however the mechanisms underlying these events remain unclear. Accumulating evidence suggests that alterations of lipid metabolism in cancer cells affects insulin uptake and glucose utilization, leading to an enhanced capacity for cell proliferation and nutrient usage under what would normally be considered unviable conditions⁴¹. MUPA, in particular, functions as an insulin-sensitizing lipokine, extending tissue-specific metabolic alterations to systemic metabolism. The activity of saturated and unsaturated fatty acids in cancer cells may not only have an impact on their viability, but may also dramatically alter insulin/glucose homeostasis, thus affecting the onset and progression of metabolic syndrome. In addition, the incorporation of fatty acids into various lipid classes may affect cellular compartments and matrices, thereby creating a microenvironment suitable for cancer progression. A detailed understanding of these mechanisms could be exploited to target components of the palmitic acid pathway, such as SCD, that regulate lipid metabolite balance in aggressive HCC and provide an avenue for clinical intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Paul Lee at Harbor UCLA Medical Center for advice on SCD inhibitors, John Swindle and Pauline Gee at Complegen Inc for providing CGX0168, and Metabolon Inc. for performing global metabolic profiling. We also thank Snorri Thorgeirsson for providing array and clinical data as an independent validation cohort and Curtis Harris for critical comments.

Grant support: This work was supported in part by grants (Z01-BC 010313 and Z01-BC010876) from the Intramural Research Program of the Center for Cancer Research, the US National Cancer Institute.

Abbreviations

HCC	hepatocellular carcinoma
SCD	stearoyl-CoA-desaturase
MUPA	mono-unsaturated palmitic acid
HpSC-HCC	hepatic stem cell HCC
MH-HCC	mature hepatocyte subtype
EpCAM	epithelial cell adhesion molecule
AFP	alphafetoprotein

References

Author names in bold designate shared co-first authorship

1. Sreekumar A, Poisson LM, Rajendiran TM, et al. Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. *Nature*. 2009; 457:910–914. [PubMed: 19212411]
2. Akavia UD, Litvin O, Kim J, et al. An integrated approach to uncover drivers of cancer. *Cell*. 2010; 143:1005–1017. [PubMed: 21129771]
3. Nielsen J, Oliver S. The next wave in metabolome analysis. *Trends Biotechnol*. 2005; 23:544–546. [PubMed: 16154652]
4. Heinemann M, Zenobi R. Single cell metabolomics. *Curr Opin Biotechnol*. 2011; 22:26–31. [PubMed: 20934866]
5. Budhu A, Forgues M, Ye QH, et al. Prediction of venous metastases, recurrence and prognosis in hepatocellular carcinoma based on a unique immune response signature of the liver microenvironment. *Cancer Cell*. 2006; 10:99–111. [PubMed: 16904609]
6. Jia HL, Ye QH, Qin LX, et al. Gene expression profiling reveals potential biomarkers of human hepatocellular carcinoma. *Clin Cancer Res*. 2007; 13:1133–1139. [PubMed: 17317821]
7. Ye QH, Qin LX, Forgues M, et al. Predicting hepatitis B virus-positive metastatic hepatocellular carcinomas using gene expression profiling and supervised machine learning. *Nat Med*. 2003; 9:416–423. [PubMed: 12640447]
8. Yamashita T, Forgues M, Wang W, et al. EpCAM and alpha-fetoprotein expression defines novel prognostic subtypes of hepatocellular carcinoma. *Cancer Res*. 2008; 68:1451–1461. [PubMed: 18316609]
9. Lee JS, Heo J, Libbrecht L, et al. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. *Nat Med*. 2006; 12:410–416. [PubMed: 16532004]
10. Hoshida Y, Villanueva A, Llovet JM. Molecular profiling to predict hepatocellular carcinoma outcome. *Expert Rev Gastroenterol Hepatol*. 2009; 3:101–103. [PubMed: 19351279]
11. Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. *Nat Genet*. 2002; 31:339–346. [PubMed: 12149612]
12. Parkin DM, Bray F, Ferlay J, et al. Global cancer statistics, 2002. *CA Cancer J Clin*. 2005; 55:74–108. [PubMed: 15761078]

13. Reya T, Morrison SJ, Clarke MF, et al. Stem cells, cancer, and cancer stem cells. *Nature*. 2001; 414:105–111. [PubMed: 11689955]
14. Ji J, Yamashita T, Budhu A, et al. Identification of microRNA-181 by genome-wide screening as a critical player in EpCAM-positive hepatic cancer stem cells. *Hepatology*. 2009; 50:472–480. [PubMed: 19585654]
15. Yamashita T, Ji J, Budhu A, et al. EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features. *Gastroenterology*. 2009; 136:1012–1024. [PubMed: 19150350]
16. Roessler S, Jia HL, Budhu A, et al. A unique metastasis gene signature enables prediction of tumor relapse in early-stage hepatocellular carcinoma patients. *Cancer Research*. 2010; 70:10202–10212. [PubMed: 21159642]
17. McShane LM, Altman DG, Sauerbrei W, et al. Reporting recommendations for tumor marker prognostic studies (REMARK). *J Natl Cancer Inst*. 2005; 97:1180–1184. [PubMed: 16106022]
18. Lawton KA, Berger A, Mitchell M, et al. Analysis of the adult human plasma metabolome. *Pharmacogenomics*. 2008; 9:383–397. [PubMed: 18384253]
19. DeHaven CD, Evans AM, Dai H, et al. Organization of GC/MS and LC/MS metabolomics data into chemical libraries. *J Cheminform*. 2010; 2:9. [PubMed: 20955607]
20. Evans AM, DeHaven CD, Barrett T, et al. Integrated, nontargeted ultrahigh performance liquid chromatography/electrospray ionization tandem mass spectrometry platform for the identification and relative quantification of the small-molecule complement of biological systems. *Anal Chem*. 2009; 81:6656–6667. [PubMed: 19624122]
21. Welters HJ, Diakogiannaki E, Mordue JM, et al. Differential protective effects of palmitoleic acid and cAMP on caspase activation and cell viability in pancreatic beta-cells exposed to palmitate. *Apoptosis*. 2006; 11:1231–1238. [PubMed: 16703263]
22. Ji J, Zhao L, Budhu A, et al. Let-7g targets collagen type I alpha2 and inhibits cell migration in hepatocellular carcinoma. *J Hepatol*. 2010; 52:690–697. [PubMed: 20338660]
23. Hill R, Wu H. PTEN, stem cells, and cancer stem cells. *J Biol Chem*. 2009; 284:11755–11759. [PubMed: 19117948]
24. Ntambi JM, Miyazaki M. Regulation of stearoyl-CoA desaturases and role in metabolism. *Prog Lipid Res*. 2004; 43:91–104. [PubMed: 14654089]
25. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000; 100:57–70. [PubMed: 10647931]
26. Kim E, Liu NC, Yu IC, et al. Metformin inhibits nuclear receptor TR4-mediated hepatic stearoyl-CoA desaturase 1 gene expression with altered insulin sensitivity. *Diabetes*. 2011; 60:1493–1503. [PubMed: 21478464]
27. Martin-Castillo B, Vazquez-Martin A, Oliveras-Ferraro C, et al. Metformin and cancer: doses, mechanisms and the dandelion and hormetic phenomena. *Cell Cycle*. 2010; 9:1057–1064. [PubMed: 20305377]
28. Yee JK, Mao CS, Hummel HS, et al. Compartmentalization of stearoyl-coenzyme A desaturase 1 activity in HepG2 cells. *J Lipid Res*. 2008; 49:2124–2134. [PubMed: 18599738]
29. Laurent-Puig P, Legoix P, Bluteau O, et al. Genetic alterations associated with hepatocellular carcinomas define distinct pathways of hepatocarcinogenesis. *Gastroenterology*. 2001; 120:1763–1773. [PubMed: 11375957]
30. Wurmbach E, Chen YB, Khitrov G, et al. Genome-wide molecular profiles of HCV-induced dysplasia and hepatocellular carcinoma. *Hepatology*. 2007; 45:938–947. [PubMed: 17393520]
31. Chen T, Xie G, Wang X, et al. Serum and urine metabolite profiling reveals potential biomarkers of human hepatocellular carcinoma. *Mol Cell Proteomics*. 2011; 10:M110.
32. Wu H, Xue R, Dong L, et al. Metabolomic profiling of human urine in hepatocellular carcinoma patients using gas chromatography/mass spectrometry. *Anal Chim Acta*. 2009; 648:98–104. [PubMed: 19616694]
33. Igal RA. Stearoyl-CoA desaturase-1: a novel key player in the mechanisms of cell proliferation, programmed cell death and transformation to cancer. *Carcinogenesis*. 2010; 31:1509–1515. [PubMed: 20595235]

34. Chajes V, Hulten K, Van Kappel AL, et al. Fatty-acid composition in serum phospholipids and risk of breast cancer: an incident case-control study in Sweden. *Int J Cancer*. 1999; 83:585–590. [PubMed: 10521790]
35. Falvella FS, Pascale RM, Gariboldi M, et al. Stearoyl-CoA desaturase 1 (Scd1) gene overexpression is associated with genetic predisposition to hepatocarcinogenesis in mice and rats. *Carcinogenesis*. 2002; 23:1933–1936. [PubMed: 12419843]
36. Calvisi DF, Wang C, Ho C, et al. Increased lipogenesis, induced by AKT-mTORC1-RPS6 signaling, promotes development of human hepatocellular carcinoma. *Gastroenterology*. 2011; 140:1071–1083. [PubMed: 21147110]
37. Rodriguez-Rivera A, Galicia-Moreno M, Reyes-Gordillo K, et al. Methyl palmitate prevents CCl(4)-induced liver fibrosis. *J Appl Toxicol*. 2008; 28:1021–1026. [PubMed: 18626905]
38. Liu G. Stearoyl-CoA desaturase inhibitors: update on patented compounds. *Expert Opin Ther Pat*. 2009; 19:1169–1191. [PubMed: 19691439]
39. Kant P, Hull MA. Excess body weight and obesity--the link with gastrointestinal and hepatobiliary cancer. *Nat Rev Gastroenterol Hepatol*. 2011; 8:224–238. [PubMed: 21386810]
40. de Haas EC, Oosting SF, Lefrandt JD, et al. The metabolic syndrome in cancer survivors. *Lancet Oncol*. 2010; 11:193–203. [PubMed: 20152771]
41. Cao H, Gerhold K, Mayers JR, et al. Identification of a lipokine, a lipid hormone linking adipose tissue to systemic metabolism. *Cell*. 2008; 134:933–944. [PubMed: 18805087]

A.

B.

FIGURE 1. Significant differentially expressed metabolites associated with HCC tumors and subtypes

A, Principal components analysis of all metabolites (n=469) measured in biospecimens is shown comparing tumor (red) versus nontumor (blue) tissues. Each axes represents one of three principal components. **B**, A S-plot of the normalized metabolite expression (tumor vs nontumor) in log2 scale of the 28 tumor, subtype and survival-related metabolites is shown. Each point represents one metabolite in one sample, colored by tissue type (HpSC: orange; MH: blue). Metabolites with a Y prefix are currently unknown.

FIGURE 2. Gene surrogates of the 28-metabolite signature are associated with survival and the PI3K network

A, upper left panel: A plot of the correlation between the 28 metabolites with 169 genes is shown. The orange curve represents the plot of correlation between the 28 metabolites and 169 randomly chosen genes. Upper right panel: A plot of the correlation between the 15 metabolites with 169 genes. The orange curve represents the plot of correlation between the 169 genes and 15 randomly chosen metabolites. For both upper panels, the experimental data is shown in the blue curve. The standard deviation is shown for the randomization analysis. The red-dashed line represents the point of largest difference between the experimental and randomized data. lower panel: Hierarchical clustering of 15 metabolites and 169 genes whose expression was significantly ($p < 0.05$) altered in tumor tissues of HpSC HCC. Each row represents an individual metabolite and each column represents an

individual gene. Genes were ordered by centered correlation and complete linkage according to their correlation coefficient. Pseudocolors indicate positive (orange) or negative (blue) correlation values or missing values (grey), respectively. The scale represents the correlation values from 1 to -1 in log 2 scale. Metabolites with a Y prefix are currently unknown. **B**, left panel: Survival risk prediction of the 217 test cases of the LCI cohort was performed using BRB ArrayTools restricted to the 273 geneset with 2 risk groups (high vs low), 2 principal components, and 1000 permutations of the significance of the log rank test. A Kaplan-Meier overall survival analysis curve is shown for high and low risk survival groups with the log rank p value. right panel: Survival Risk Prediction of 139 cases of the LEC validation cohort was performed using BRB ArrayTools restricted to the 273 geneset with 2 risk groups (high vs low), 2 principal components, and 1000 permutations of the significance of the log rank test. A Kaplan-Meier overall survival analysis curve is shown for high and low risk survival groups with the log rank p value. **C**, The PI3K network is altered in HpSC HCC. The blue colored genes represent those genes among the 273 gene surrogates that were imported to Ingenuity Pathway Analysis.

FIGURE 3. SCD expression is associated with fatty-acid metabolites and with patient outcome
A, Correlation analysis of SCD and palmitoleate or 15-methylpalmitate is shown. The Spearman r value and p -value are presented. **B**, A Kaplan-Meier overall survival analysis curve (upper panel) or disease-free analysis curve (lower panel) is shown for high and low risk survival groups among the LCI cohort with the log rank p value based on SCD categorized as high or low according to its median expression among tumor specimens. **C**, A Kaplan-Meier overall survival analysis curve (upper panel) or disease-free analysis curve (lower panel) is shown for high and low risk survival groups among the LEC cohort with the log rank p value based on SCD categorized as high or low according to its median expression among tumor specimens. **D**, Kaplan-Meier curves show overall survival of the LCI cohort subgrouped by SCD and AFP. Disc: discordant risk assessments; high SCD

expression and low risk predicted by AFP (<300 ng/mL) or vice-versa. **E**, Kaplan-Meier curves show overall survival of BCLC Stage A patients among the LCI cohort subgrouped by SCD.

FIGURE 4. Abrogation of SCD reduces cell migration, invasion, colony formation and tumor formation

A, Huh7 cells were treated with 100uM SPA or 100uM MUPA for 3 days, incubated for 22 hrs in boyden chambers and those migrating and invading were quantified. Representative images are shown on the left. A model depicting SPA conversion to MUPA by SCD is shown at the top left panel. **B,** Corresponding quantitation of Huh7 migration and invasion data is presented as the mean $\pm$ SD of triplicate experiments with p-value relative to DMSO. **C,** Huh7 cells were treated with metformin (10mM) for 2 days or 100uM MUPA for 3 days or in combination, incubated for 22 hrs in boyden chambers followed by quantitation of migration and invasion. Data is presented as the mean $\pm$ SD of triplicate experiments with p-value (METf (metformin) or MUPA vs. DMSO or metformin vs MUPA+Metformin). **D,** Tumor incidence of Huh7 cells transfected with SCD siRNA or control siRNA after

subcutaneous injection into both flanks of immunocompromised mice (n=5). Percent tumor incidence is shown with the log-rank p-value (upper panel). Growth curve of tumor xenografts of Huh7 cells transfected with SCD siRNA or control siRNA is shown in the lower panel. Data represent averages $\pm$ standard error of the mean. *P<0.05 or ** p<0.001 by 2-sided Student's t-test.

Table 1

Univariate and Multivariate Cox regression analysis of the 273 gene signature and clinical factors associated with overall survival of the LCI test cohort (n=217)

Clinical variable	Number of cases ^a	Hazard ratio (95% CI) ^b	P value ^c
Univariate analysis^d			
273 Gene Predictor (high vs low risk)	106/111	2.33 (1.49–3.66)	<0.0001
Gender (male vs female)	185/27	1.78 (0.82–3.87)	0.144
Age (>=50 y vs < 50 y)	119/93	0.75 (0.49–1.16)	0.197
AFP (>300 ng/mL vs <= 300 ng/mL)	114/94	1.67 (1.08–2.58)	0.021
ALT (>50 U/L vs <= 50 U/L)	86/126	1.14 (0.74–1.76)	0.552
Cirrhosis (yes vs no)	194/18	4.77 (1.17–19.4)	0.029
Tumor size (>3cm vs <=3cm)	139/72	2.24 (1.35–3.71)	0.002
Multinodular (yes vs no)	43/169	1.45 (0.88–2.38)	0.14
Encapsulation (no vs yes)	122/76	1.53 (0.95–2.49)	0.083
Microscopic vascular invasion (yes vs no)	18/190	2.37 (1.26–4.49)	0.008
HBV (AVR-CC vs CC) ^e	53/138	1.39 (0.85–2.26)	0.187
BCLC staging (B-C vs A-O)	45/158	3.63 (2.27–5.83)	<0.0001
CLIP staging (1–5 vs 0)	110/93	2.15 (1.32–3.49)	0.002
TNM staging (II–III vs I)	111/90	3.01 (1.79–5.08)	<0.0001
Multivariate analysis^f			
273 Gene Predictor (high vs low risk)		1.80 (1.08–3.00)	0.025
AFP (>300 ng/mL vs <= 300 ng/mL)		1.07 (0.65–1.76)	0.789
BCLC staging (B-C vs A-O)		3.15 (1.94–5.12)	<0.0001

^a Analysis was performed on the LCI test set (n=217); For some cases, some clinical variable data was not available

^b 95% CI: 95% confidence interval

^c P-value, bold values indicate significant P values

^d Univariate analysis, Cox proportional hazards regression

^e AVR-CC, active viral replication; CC, chronic carrier

^f Multivariate analysis, Cox proportional hazards regression adjusting for AFP and BCLC staging

Table 2

Univariate and Multivariate Cox regression analysis of SCD and clinical factors associated with overall survival of the LCI test cohort (n=217)

Clinical variable	Number of cases ^a	Hazard ratio (95% CI) ^b	P value ^c
Univariate analysis^d			
SCD Predictor (high vs low risk)	107/105	1.78 (1.18–2.69)	0.006
Gender (male vs female)	185/27	1.88 (0.90–3.83)	0.093
Age (>=50 y vs < 50 y)	119/93	0.80 (0.53–1.18)	0.263
AFP (>300 ng/mL vs <= 300 ng/mL)	114/94	1.69 (1.13–2.53)	0.011
ALT (>50 U/L vs <= 50 U/L)	86/126	1.20 (0.80–1.79)	0.369
Cirrhosis (yes vs no)	194/18	5.09 (1.26–20.7)	0.023
Tumor size (>3cm vs <=3cm)	139/72	2.30 (1.40–3.76)	0.001
Multinodular (yes vs no)	43/169	1.65 (1.06–2.57)	0.025
Encapsulation (no vs yes)	122/76	1.67 (1.05–2.65)	0.029
Microscopic vascular invasion (yes vs no)	18/190	2.48 (1.35–4.55)	0.003
HBV (AVR-CC vs CC)	53/138	1.36 (0.85–2.16)	0.196
BCLC staging (B-C vs A-O)	45/158	3.70 (2.38–5.72)	<0.0001
CLIP staging (1–5 vs 0)	110/93	2.19 (1.38–3.48)	0.001
TNM staging (II–III vs I)	111/90	3.04 (1.85–4.99)	<0.0001
Multivariate analysis^f			
SCD Predictor (high vs low risk)		1.73 (1.09–2.73)	0.019
AFP (>300 ng/mL vs <= 300 ng/mL)		1.14 (0.72–1.80)	0.564
BCLC staging (B-C vs A-O)		3.38 (2.15–5.30)	<0.0001

^a Analysis was performed on the LCI test set (n=217); For some cases, some clinical variable data was not available

^b 95% CI: 95% confidence interval

^c P-value, bold values indicate significant P values

^d Univariate analysis, Cox proportional hazards regression

^e AVR-CC, active viral replication; CC, chronic carrier

^f Multivariate analysis, Cox proportional hazards regression adjusting for AFP and BCLC staging