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ORIGINAL ARTICLE

Evidence of aberrant lipid metabolism in hepatitis C and hepatocellular carcinoma

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Abstract

Objectives: Lipids are linked to many pathological processes including hepatic steatosis and liver malignancy. This study aimed to explore lipid metabolism in hepatitis C virus (HCV) and HCV-related hepatocellular carcinoma (HCC).

Methods: Serum lipids were measured in normal, HCV and HCV-HCC patients. Whole-genome microarray was performed to identify potential signature genes involved in lipid metabolism characterizing normal vs. HCV vs. HCV-HCC conditions.

Results: Serum cholesterol was significantly reduced in HCV and HCV-HCC patients compared with normal controls, whereas there was no difference in glucose and triglycerides. Microarray analysis identified 224 probe sets with known functional roles in lipid metabolism (ANOVA, 1.5-fold, $P \leq 0.001$). Gene-mediated fatty acid (FA) *de novo* synthesis and uptake were upregulated in HCV and this upregulation was further enhanced in HCC. Genes involved in FA oxidation were downregulated in both the HCV and HCC groups. The abnormality of cholesterol metabolism in HCV was associated with downregulation of genes involved in cholesterol biosynthesis, absorption and transportation and bile acid synthesis; this abnormality was further intensified in HCC.

Conclusions: Our data support the notion that HCV-related lipid metabolic abnormalities may contribute to hepatic steatosis and the development of cancer. Identification of these aberrations would stratify patients and improve treatment algorithms.

Keywords

hepatitis C virus (HCV), lipid metabolism, hepatic steatosis, hepatocellular carcinoma (HCC)

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. The American Cancer Society report of the incidence and prevalence of cancers within the USA states that HCC continues to show the single greatest increase in incidence compared with other cancers.[1] Hepatocellular carcinoma is unique in that it most often occurs against a background of chronic liver disease and cirrhosis. Of the many risk factors for HCC, hepatitis C virus (HCV) infection has been recognized as an important cause of its rising incidence.[2] Progress in the management of HCC is dependent on our ability to: (i) identify the highest-risk patients (those who will develop cancer) from the larger cohort of HCV patients so that treatment can be initiated early; (ii) understand the process of cancer initiation in the highest-risk patients, and (iii) develop a therapeutic strategy based on the key targets involved in tumour development.

It has been shown that hepatic steatosis, which is characterized by the accumulation of fat in the liver, is a common histological phenotype of a direct toxic effect of HCV on the hepatocyte.[3] According to previous reports, the prevalence of hepatocellular steatosis in liver biopsy specimens from patients

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with chronic hepatitis C is around 41–73%.[4] This figure is higher than in patients with chronic hepatitis B (27–51%) and autoimmune hepatitis (16–19%). It is also more than twice as high as the expected prevalence of steatosis in the general population, suggesting that HCV infection may have a direct effect on hepatic lipid metabolism. Two different forms of hepatic steatosis have been reported in patients with chronic HCV infection. Patients infected with non-genotype 3 HCV strains usually have the classical metabolic risk factors for hepatocellular steatosis. By contrast, in patients infected with HCV genotype 3, steatosis is generally induced by the virus itself through a direct cytopathic effect. Mixed forms of steatosis can also be seen in HCV genotype 3-infected patients with metabolic risk factors.

Although hepatic steatosis has been reported to be associated with more severe fibrosis and with rapid progression of disease in chronic HCV infection,[5,6] the mechanism linking HCV and steatosis and how steatosis is related to liver malignancy remain unclear. Both *in vitro* and *in vivo* studies have demonstrated that the expression of HCV core protein can induce hepatic steatosis in cell culture as well as in transgenic mice.[7,8] Hepatic steatosis was reported to predispose to lipid peroxidation and produce excess free radical activity with the potential risk of genomic mutations.[9] Hyperplasia of hepatocytes has been described in ob/ob mice with fatty liver disease, suggestive of malignant changes secondary to hepatic steatosis.[10] In addition, hepatic steatosis developed without significant inflammation and contributed to carcinogenesis in transgenic mice that expressed HCV core protein. These studies indicated that hepatic steatosis is linked to the progression of chronic hepatitis C-associated HCC. Recently, hepatic steatosis has been proposed as a risk factor for HCC in patients with chronic HCV infection. Ohata *et al.* reported that steatosis represented an independent risk factor for HCC in patients with HCV infection.[11] Hepatic steatosis was found to be associated with increased frequency of HCC in patients with HCV-related cirrhosis.[12] Further support is provided by the finding that hepatic steatosis was a useful predictor of postoperative recurrence of HCV-related HCC.[13] All of these pieces of evidence suggest that hepatic steatosis plays a role in the process of hepatocarcinogenesis.

Lipid metabolism is known to be an important process involved in hepatic steatosis. Our hypothesis upon initiating these studies was that hepatic lipid composition is important in the aetiology and pathogenesis of HCV-associated HCC. Patients with HCV-related cirrhosis are high-risk subjects for the development of HCC. Investigation of the lipid metabolism in liver tissues from high-risk patients as well as HCC patients would add new insight into HCC initiation and/or progression. The objective of this study was to explore the abnormality of lipid metabolism in HCV cirrhotic patients and HCV cirrhotic cancer patients. To this end, serum lipids were measured and microarray-based gene expression analysis using the Human Genome HG U133 Plus 2.0 Array GeneChip® from Affymetrix, Inc. (Santa Clara, CA, USA) was performed to probe >47 000 transcripts derived from ~38 500 well-characterized human genes. Real-time quantitative polymerase chain reaction (RT-qPCR) was applied to further confirm the expression of genes involved in fatty acid (FA) *de novo* synthesis, FA uptake, oxidation, antioxidant pathways, triglyceride (TG) synthesis and catalysis, and cholesterol metabolism.

Materials and methods

Patients and liver tissue samples

In order to exclude the artefactual effects of ischaemia on the gene expression profile, all the liver tissue samples in our study were processed at the start of surgery. The normal control specimens were obtained from patients during surgery for liver metastasis of colorectal cancer or haemangioma. All the normal specimens were histologically normal. HCV cirrhotic samples and HCV cirrhotic cancer samples were obtained from patients who underwent either tumour resection or transplantation. Liver tissue was divided into two parts. One part was subjected to routine histopathological evaluation and the other was frozen immediately in liquid nitrogen and maintained at −80 °C until total RNA extraction. The sera samples were collected from patients at Indiana University Hospital between June 2001 and July 2007. Normal control sera samples were collected from patients with non-liver disease and no-cancer diagnosis. For sera from HCV cirrhotic and HCV cirrhotic cancer patients, inclusion criteria required that patients be older than 18 years, positive for anti-HCV and positive for serum HCV-RNA. Patients with underlying liver disease from any other aetiology (i.e. alcohol, hepatitis B) were excluded, as were patients treated with antiviral or immunosuppressive agents within 6 months of enrolment or patients treated with drugs known to produce hepatic steatosis, including corticosteroids, high-dose oestrogen, methotrexate and amiodarone, within 6 months of enrolment. All subjects reported no or low alcohol intake, but 25 of the normal controls, 30 HCV subjects and 22 HCV-related cancer patients had diabetes. All tissue was obtained through Indiana University Human Subjects Research Institutional Review Board (IRB) approved tissue acquisition protocols. IRB authorization was obtained to analyse and present these data.

Serum analysis

All serum samples were taken after a 6–8 h fast. Serum was separated from the blood samples by centrifugation at 1500 **g** for 15 min at room temperature. All the samples were aliquoted into siliconized microcentrifuge tubes (PGC Scientifics Corp., Frederick, MD, USA) and frozen at −80 °C until analysis. Serum glucose, TG and total cholesterol (TC) were determined by SYNCHRON® System(s) (Bristol, PA, USA) according to the manufacturer's protocol. Serum glucose and lipid evaluation statistical analyses were performed by one-way ANOVA using InStat software (GraphPad Software, Inc., San Diego, CA, USA). *P*-values of <0.05 were considered statistically significant.

Microarray procedure

Gene expression profiles of human liver tissue from normal control ($n = 9$), HCV-infected cirrhotic liver ($n = 9$) and HCV-associated

HCC tumour core tissue ($n = 10$) were compared by microarray analysis using the Affymetrix HG U133 Plus 2.0 Array GeneChip®, which contained >47 000 transcripts and variants derived from ~38 500 well-characterized human genes. All the subjects included for microarray reported no alcohol intake and did not have diabetes. Total RNA from liver tissues was prepared using the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA, USA) according to standard protocols provided by the manufacturer. The integrity of total RNA was verified with an Agilent 2100 Bioanalyser using the RNA 6000 Nano LabChip Kit (Agilent Technologies, Inc., Palo Alto, CA, USA). Standard Affymetrix protocols (GeneChip® Expression Analysis Technical Manual) were used to synthesize biotinylated cRNA, starting with 5 μg total RNA from each tissue sample, using the Affymetrix kits for cDNA synthesis, *in vitro* transcription and sample clean-up. A subsample of 15 μg of fragmented, biotinylated cRNA from each independent sample was mixed into 300 μl of hybridization cocktail, of which 200 μl were used for each hybridization. Hybridization occurred over 17 h at 42 °C. Samples were hybridized to the Affymetrix GeneChip® (HG U133 Plus 2.0 Array GeneChip®). Washing and scanning of the GeneChips® were carried out according to standard protocols.

Microarray data analysis and statistics

In this study the GeneSifter microarray data analysis system (VizX Laboratories LLC, Seattle, WA, USA; http://www.genesifter.net) was used to analyse data generated from one-way ANOVA among normal control, HCV and HCV-associated HCC tumour groups (1.5-fold; $P \leq 0.001$). This program identifies differentially expressed genes and establishes the biological significance based on Gene Ontology (GO) Consortium (http://www.Geneontology.org)[14] and Kyoto Encyclopaedia of Genes and Genomes (KEGG) public pathway resource (http://www.genome.jp/kegg/). The biological process ontologies and KEGG pathway terms associated with the differentially expressed genes were examined using a *Z*-score report. The *Z*-score was derived by 'subtracting the expected number of genes in a GO term meeting the criterion from the observed number of genes, and dividing by the standard deviation of the observed number of genes under the hypergeometric distribution'.[15] A positive *Z*-score indicates that more genes than expected fulfilled the criterion in a certain group or pathway; therefore, that group or pathway is likely to be affected.[15] The parameters used to identify differentially expressed genes using GeneSifter were: global median normalization; one-way ANOVA; quality = 1; threshold = 1.5, and log transformation. Correction for multiple testing was then performed according to Benjamini and Hochberg.[16] A false discovery rate of 5% was used as a cut-off for statistical significance.

Quantitative real-time PCR

Quantitative RT-PCR was performed to verify the differentially expressed transcripts detected by microarray as previously reported.[17] Total RNA was prepared from 20 normal controls, 20 HCV-positive cirrhotic livers and 20 HCV-HCC tumour samples.

Table 1 The assay ID for the primers used in real-time polymerase chain reaction validation

Gene	Accession no.	Probe ID	Assay ID (Applied Biosystems, Inc.)
FASN1	AI954041	212218_s_at	AIHR15B
ACACA	BE855983	212186_at	AII00BJ
HADH	BC000306	201035_s_at	AIMRUT7
PPARA	BC004162	223438_s_at	AIN0S0F
MLYCD	NM_012213	218869_at	Hs00201955_m1
SOD1	NM_000454	200642_at	Hs00916176_m1
CAT	AY028632	211922_s_at	Hs00156308_m1
CH25H	NM_003956	206932_at	Hs02379634_s1
HMGCS2	NM_005518	204607_at	Hs00985427_m1(l)
LCAT	NM_000229	204428_s_at	Hs01068069_m1(l)
RXRA	NM_002957	202449_s_at	Hs01067640_m1
AKR1D1	NM_005989	207102_at	Hs00975612_m1
ACADL	NM_001608	206069_s_at	Hs00155630_m1

cDNA was generated from 2 μg of total RNA in 20 μl of total volume using a high-capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Inc., Foster City, CA, USA). Quantitative PCR was performed using the TaqMan® Universal PCR Master Mix (Applied Biosystems, Inc.). Gene-specific primers were either purchased from Applied Biosystems, Inc. or were custom-designed using UMapIt Microarray-to-TaqMan Assay Mapping Tool software (Applied Biosystems, Inc.). The assay IDs are summarized in Table 1. Results were normalized against Ct values of 18S rRNA (Hs99999901_s1). The $2^{-\Delta\Delta Ct}$ method was used for relative quantification analysis.

Results

Serum lipid, glucose and body mass index

To determine any potential link between serum lipid or glucose levels and/or body mass index (BMI) on the subsequent lipid microarray profiles, we evaluated lipid, glucose and related clinical parameters in 112 patients with HCV-positive cirrhosis, 73 patients with HCV-associated cirrhosis and cancer and 103 normal controls (Table 2). No significant differences were found in serum TGs and glucose, but significant changes in serum TC (one-way ANOVA, $P = 0.0001$) and BMI (one-way ANOVA, $P = 0.0196$) were observed among the three groups. When compared with control levels, TC levels were low and BMI was high in HCV cohorts. A comparison within the patient groups showed that the level of TC in HCV alone was significantly lower than that in HCV-associated HCC, whereas the change in other parameters was not obvious.

Biological ontological pathway analysis of differentially expressed genes

In order to explore the aberrant lipid metabolism in HCV and HCV-associated cancer, microarray assay was performed with

Table 2 Serum lipid and glucose profiles for non-liver disease control, hepatitis C virus (HCV) cirrhotic and HCV hepatocellular carcinoma (HCC) patients

	Normal control	HCV cirrhosis	HCV-HCC
Sample, *n*	103	112	73
Body mass index[a,b]	26.7359 ± 0.476	28.577 ± 0.475	27.55 ± 0.51
Total cholesterol, mg/dl[c]	172.757 ± 7.363	127.536 ± 3.984	140.973 ± 4.713
Triglycerides, mg/dl	144.97 ± 11.564	150.094 ± 19.082	142.958 ± 13.197
Glucose, mg/dl	117.757 ± 6.376	121.598 ± 5.258	133.56 ± 8.545

[a]Results are mean ± standard error of the mean
[b]$P = 0.0196$ (one-way ANOVA); [c]$P = 0.0001$ (one-way ANOVA)

three groups of human liver tissue (normal control, HCV-positive cirrhotic liver, and HCV-associated cirrhosis and HCC tumour). Microarray data were analysed using ANOVA multiple group test with Benjamini–Hochberg FDR correction. Filtered for probe sets with corrected ANOVA *P*-values of ≤0.001 and fold changes of ≥1.5, 3989 genes were differentially expressed in control vs. HCV vs. HCV-HCC groups. Gene Ontology analysis showed metabolic process is one of the major categories (*Z*-score = 5.75) in biological process ontology. In this study, lipid metabolism was targeted for further investigation. The KEGG terms (http://www.genome.ad.jp/kegg)[18] revealed FA metabolism (*Z*-score = 13.68), peroxisome proliferators-activated receptor (PPAR) signalling pathway (*Z*-score = 12.75) and bile acid biosynthesis (*Z*-score = 10.35) were significantly altered.

Differential expression of genes associated with FA metabolism

Fatty acids are important energy storage molecules and serve as: (i) major constituents of all biological membrane lipids; (ii) important substrates for energy metabolism, and (iii) generators of mediators of signal transduction and transcription, and physiological regulators. Hierarchical cluster analysis revealed there are three clusters of FA metabolism-associated genes. As shown in Fig. 1, genes in the top cluster (cluster 1) were characterized by a strong increased expression in the HCV group, but slightly or no change in the HCC tumour group compared with normal controls. This cluster has three genes: prostaglandin-endoperoxide synthase 2 (PTGS2)/cyclooxygenase 2 (COX2); stearoyl-CoA desaturase 5 (SCD5), and γ-glutamyltransferase 5 (GGT5). The second cluster is characterized by a slight increase or no change in HCV patients, with the highest levels found consistently in HCC tumours. These genes are mainly related to *de novo* synthesis and uptake of FA; they are: acyl-coA synthetase long-chain family member 4 (ACSL4); acetoacetyl-CoA synthetase (AACS); elongation of very long-chain fatty acids-like 1 (ELOVL1); fatty acid synthase (FASN); glyceronephosphate O-acyltransferase (GNPAT); aldo-keto reductase family 1, member C3 (AKR1C3), and acetyl-coenzyme A carboxylase α (ACACA). Genes in the third cluster are distinguished by a gradual but large-magnitude decline in transcript levels between the HCV group and the HCC tumour group. This cluster includes genes involved in the catabolism of FA, such as enzymes for oxidation, detoxification pathways and oxidation regulation. Genes involved in the FA oxidation pathway are: acyl-coenzyme A oxidase 1, palmitoyl (ACOX1); acyl-coenzyme A oxidase 2, branched-chain (ACOX2); hydroxyacid oxidase 2 (HAO2); hydroxyacyl-coenzyme A dehydrogenase (HADH); acyl-coenzyme A dehydrogenase, C-4 to C-12 straight-chain (ACADM); acetyl-coenzyme A acyltransferase 1 (peroxisomal 3-oxoacyl-coenzyme A thiolase) (ACAA1); enoyl coenzyme A hydratase short-chain 1 mitochondrial (ECHS1); cytochrome P450, family 4, subfamily A, polypeptide 11 (CYP4A11); acyl-coenzyme A dehydrogenase, short/branched-chain (ACADSB);

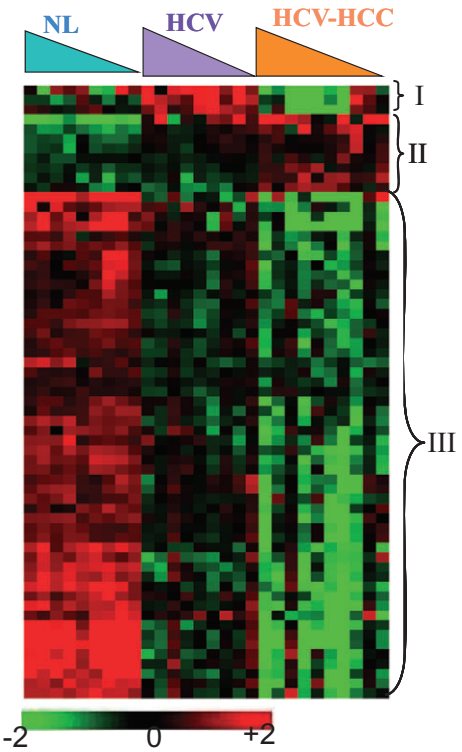

Figure 1 Hierarchical cluster analysis of fatty acid (FA) metabolism comparing gene expression among non-liver disease normal control (NL), hepatitis C virus (HCV)-related cirrhotic liver and HCV hepatocellular carcinoma (HCC) tumour samples

acyl-coenzyme A dehydrogenase, C-2 to C-3 short-chain (ACADS); acyl-coenzyme A dehydrogenase, long-chain (ACADL), and cytochrome P450, family 2, subfamily J, polypeptide 2 (CYP2J2). Genes involved in FA oxidation regulation include: peroxisome proliferator-activated receptor α (PPARA); acetyl-coenzyme A carboxylase β (ACACB); peroxisome proliferator-activated receptor γ coactivator 1 α (PPARGC1A); malonyl-CoA decarboxylase (MLYCD); solute carrier family 25 (carnitine/acylcarnitine translocase), member 20 (SLC25A20); protein kinase, AMP-activated, γ 2 non-catalytic subunit (PRKAG2); apolipoprotein A-V (APOA5), and carnitine acetyltransferase (CRAT). Genes responsible for the detoxification pathways include glycine-N-acyltransferase (GLYAT). Table 3 summarizes the differentially expressed genes associated with FA metabolism in normal control vs. HCV vs. HCV-HCC. The differentially expressed genes in FA metabolism suggest that FA *de novo* synthesis and uptake are very active in the progression of HCC, whereas the oxidation and degradation of FA are impaired.

Differential expression of genes associated with TG metabolism

Triglycerides are extremely efficient molecules for energy storage derived from the esterification of free FA. These molecules have been shown to be significantly involved in metabolic energy provision and lipoprotein metabolism. As Fig. 2 shows, the transcript level of TGs metabolism-related genes was characterized by a gradual decline in the HCV group and a substantial decline in the HCC group. Table 4 summarizes the differential expressed genes involved in TG metabolism in normal control vs. HCV vs. HCV-HCC. Together, the downregulation of microsomal TG transfer protein (MTTP), diacylglycerol O-acyltransferase homologue 2 (mouse) (DGAT2), apolipoprotein A-V (APOA5), lipase, hepatic (LIPC) and cholesteryl ester transfer protein plasma (CETP) resulted in the reduced export of lipids from liver as well as the accumulation of TGs in hepatocytes, which may cause severe lipotoxicity for liver.

Differential expression of genes associated with cholesterol metabolism and bile acid synthesis

Our study identified that genes participated in cholesterol metabolism could be divided into two groups (Fig. 3). The first group includes cholesterol 25-hydroxylase (CH25H) and very low density lipoprotein receptor (VLDLR), which were upregulated in the HCV group, but showed no change or increase in HCC tumour compared with control. Genes in the second group are characterized by downregulation in HCV patients and a subsequent strong repression in HCC patients. Levels of expression of cholesterol metabolism related genes are summarized in Table 5. Together, the cholesterol biosynthesis is inhibited by the upregulation of the negative regulator CH25H, as well as the downregulation of cholesterol biosynthesis enzyme 3-hydroxy-3-methylglutaryl-coenzyme A synthase 2 (mitochondrial) (HMGCS2). The absorbing and transportation of cholesterol are also decreased by downregulation of apolipoprotein A-I (APOA1), apolipoprotein F (APOF), CETP, lecithin-cholesterol acyltransferase (LCAT) (Niemann–Pick disease, type C1, gene)-like 1 (NPC1L1) and sortilin-related receptor, L (DLR class) A repeats-containing (SORL1). Unexpectedly, VLDLR was increased by 4.2-fold and 3.5-fold in HCV and HCV-HCC patients, respectively, compared with normal controls. Genes participating in the regulation of cholesterol metabolism were also altered. Superoxide dismutase 1 (SOD1), which is responsible for destroying free superoxide radicals, was significantly decreased in the HCV and HCV-HCC patient groups compared with the normal controls. Bile acid synthesis genes, including cytochrome P450, family 39, subfamily A, polypeptide 1 (CYP39A1), and aldo-keto reductase family 1, member D1 (δ 4-3-ketosteroid-5-β-reductase) (AKR1D1), were downregulated.

Validation of microarray data by qPCR

Twelve transcripts (FASN [AI954041), ACACA [BE855983], MLYCD [NM_012213], PPARA [BC004162], SOD1 [NM_000454], CAT [AY028632], HADH [BC000306], CH25H [NM_003956], HMGCS2 [NM_005518] RXRA [NM_002957], LCAT [NM_000229], AKR1D1 [NM_005989]), each from normal control, HCV cirrhotic liver and HCV cirrhotic HCC tumour, were selected for validating the microarray data using qPCR. A high degree of correlation between the microarray data and the data from qRT-PCR was observed ($R^2 = 0.8202$, slope = 1.359, $n = 22$) (Fig. 4).

Discussion

The results from this study indicate the following regarding HCV and HCV-HCC:

1 increased *de novo* synthesis and uptake of FA and impaired FA oxidation are associated with the pathophysiology of HCV infection;
2 increased TG synthesis results from enhanced *de novo* lipogenesis and accumulated TG storage results from reducing lipid export from liver, which may cause severe lipotoxicity for liver;
3 cholesterol metabolism is decreased by downregulation of the genes involved in cholesterol synthesis, absorption and transport and bile acid synthesis is inhibited by the reduced expression of CYP39A1 and AKR1D1, and
4 antioxidant pathways including SOD and catalase (CAT) are also decreased and thereby fail to neutralize reactive oxygen species (ROS) produced from mitochondrial, peroxisomal and microsomal oxidation.

These transcriptional changes are associated with increased levels of hepatic lipid and decreased levels of cholesterol. To our knowledge, this is one of the few studies to investigate the molecular causes of the abnormal lipid metabolism in human hepatitis C-related cirrhosis and hepatitis C-associated cancer by using whole-genome microarrays. Our data suggest that aberrant FA *de novo* synthesis and impaired degradation of lipid contribute to the

Table 3 Differential expression of fatty acid metabolism-related genes

Accession no.	Probe ID	Gene symbol	Gene name	Fold change	
				HCV	HCV-HCC
Fatty acid biosynthesis					
NM_000963	204748_at	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	3.14	–
AL571375	224901_at	SCD5	Stearoyl-CoA desaturase 5	4.32	–
NM_022977	202422_s_at	ACSL4	Acyl-CoA synthetase long-chain family member 4	9.19	15.42
NM_023928	218434_s_at	AACS	Acetoacetyl-CoA synthetase	2.29	3.44
H93026	57163_at	ELOVL1	Elongation of very long-chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 1	1.81	2.29
AB018580	209160_at	AKR1C3	Aldo-keto reductase family 1, member C3 (3-α hydroxysteroid dehydrogenase, type II)	–	2.79
BC003005	200627_at	PTGES3	Prostaglandin E synthase 3 (cytosolic)	–	1.60
NM_014236	201956_s_at	GNPAT	Glyceronephosphate O-acyltransferase	–	1.98
AI954041	212218_s_at	FASN	Fatty acid synthase	–	2.70
BE855983	212186_at	ACACA	Acetyl-coenzyme A carboxylase α	–	1.97
Fatty acid oxidation					
T62985	213501_at	ACOX1	Acyl-coenzyme A oxidase 1, palmitoyl	–1.92	–2.20
BF435852	227962_at	ACOX1	Acyl-coenzyme A oxidase 1, palmitoyl	–2.01	–3.80
NM_003500	205364_at	ACOX2	Acyl-coenzyme A oxidase 2, branched-chain	–2.64	–3.92
R06750	238160_at	ACOT12	Acyl-coA thioesterase 12	–1.57	–8.14
AI860341	214274_s_at	ACAA1	Acetyl-coenzyme A acyltransferase 1 (peroxisomal 3-oxoacyl-coenzyme A thiolase)	–1.83	–4.30
NM_001607	202025_x_at	ACAA1	Acetyl-coenzyme A acyltransferase 1 (peroxisomal 3-oxoacyl-coenzyme A thiolase)	–2.32	–5.51
NM_000017	202366_at	ACADS	Acyl-coenzyme A dehydrogenase, C-2 to C-3 short-chain (ACADS)	–2.60	–3.29
NM_001609	205355_at	ACADSB	Acyl-coenzyme A dehydrogenase, short/branched-chain (ACADSB)	–3.44	–6.46
BE897866	226030_at	ACADSB	Acyl-coenzyme A dehydrogenase, short/branched-chain (ACADSB)	–4.03	–6.28
NM_000016	202502_at	ACADM	Acyl-coenzyme A dehydrogenase, C-4 to C-12 straight-chain	–1.86	–2.52
NM_001608	206069_s_at	ACADL	Acyl-coenzyme A dehydrogenase, long-chain	–3.16	–7.42
D16350	210377_at	ACSM3	Acyl-coA synthetase medium-chain family member 3	–5.48	–19.19
NM_005622	205942_s_at	ACSM3	Acyl-coA synthetase medium-chain family member 3	–6.55	–27.51
NM_001995	207275_s_at	ACSL1	Acyl-coA synthetase long-chain family member 1	–1.72	–3.13
AF237813	209460_at	ABAT	4-aminobutyrate aminotransferase	–1.77	–3.12
NM_000663	206527_at	ABAT	4-aminobutyrate aminotransferase	–3.12	–3.91
NM_001701	206913_at	BAAT	Bile acid coenzyme A: amino acid N-acyltransferase (glycine N-choloyltransferase)	–2.12	–2.74
NM_020139	218285_s_at	BDH2	3-hydroxybutyrate dehydrogenase, type 2	–	–2.62
AW136198	235155_at	BDH2	3-hydroxybutyrate dehydrogenase, type 2	–1.71	–4.56
NM_015974	220753_s_at	CRYL1	Crystallin, lambda 1	–1.90	–2.38
NM_017545	220224_at	HAO1	Hydroxyacid oxidase (glycolate oxidase) 1(HAO1)	–1.82	–4.82
NM_016527	220801_s_at	HAO2	Hydroxyacid oxidase 2 (long-chain)	–5.99	–18.55

Table 3 *Continued*

Accession no.	Probe ID	Gene symbol	Gene name	Fold change	
				HCV	HCV-HCC
AF001903	211569_s_at	HADH	Hydroxyacyl-coenzyme A dehydrogenase	–1.78	–2.43
NM_005327	201036_s_at	HADH	Hydroxyacyl-coenzyme A dehydrogenase	–1.74	–2.13
BC000306	201035_s_at	HADH	Hydroxyacyl-coenzyme A dehydrogenase	–2.13	–2.63
BC001627	223211_at	HACL1	2-hydroxyacyl-CoA lyase 1	–1.70	–2.07
D26480	210452_x_at	CYP4F2	Cytochrome P450, family 4, subfamily F, polypeptide 2	–1.55	–3.84
D12620	206514_s_at	CYP4F3	Cytochrome P450, family 4, subfamily F, polypeptide 3	–1.63	–4.68
BC041158	1554837_a_at	CYP4A11	Cytochrome P450, family 4, subfamily A, polypeptide 11	–3.80	–7.93
NM_000778	207407_x_at	CYP4A11	Cytochrome P450, family 4, subfamily A, polypeptide 11	–2.49	–4.86
D13705	211231_x_at	CYP4A11	Cytochrome P450, family 4, subfamily A, polypeptide 11	–4.02	–9.37
NM_000775	205073_at	CYP2J2	Cytochrome P450, family 2, subfamily J, polypeptide 2	–2.08	–6.89
NM_000236	206606_at	LIPC	Lipase, hepatic	–5.07	–10.47
NM_004092	201135_at	ECHS1	Enoyl coenzyme A hydratase, short-chain, 1, mitochondrial	–1.50	–1.78
NM_018281	218552_at	ECHDC2	Enoyl coenzyme A hydratase domain containing 2	–1.93	–3.31
AF233336	209368_at	EPHX2	Epoxide hydrolase 2, cytoplasmic	–2.88	–8.36
NM_012254	219733_s_at	SLC27A5	Solute carrier family 27 (fatty acid transporter), member 5	–2.71	–9.21
Fatty acid oxidation regulation					
AF202889	224243_at	APOA5	Apolipoprotein A-V	–2.78	–8.39
AF202890	224244_s_at	APOA5	Apolipoprotein A-V	–2.95	–11.06
R99037	214584_x_at	ACACB	Acetyl-coenzyme A carboxylase β	–2.23	–2.84
NM_000755	205843_x_at	CRAT	Carnitine acetyltransferase	–1.66	–1.53
NM_012213	218869_at	MLYCD	Malonyl-CoA decarboxylase	–1.72	–3.28
BC004162	223438_s_at	PPARA	Peroxisome proliferator-activated receptor α	–2.04	–3.60
NM_013261	219195_at	PPARGC1A	Peroxisome proliferator-activated receptor γ, coactivator 1 α	–1.93	–6.53
AF087875	222582_at	PRKAG2	Protein kinase, AMP-activated, γ 2 non-catalytic subunit	–2.13	–3.10
NM_016203	218292_s_at	PRKAG2	Protein kinase, AMP-activated, γ 2 non-catalytic subunit	–1.92	–2.99
AJ249976	233748_x_at	PRKAG2	Protein kinase, AMP-activated, γ 2 non-catalytic subunit	–1.70	–2.75
BC001689	203658_at	SLC25A20	Solute carrier family 25 (carnitine/ acylcarnitine translocase), member 20	–1.72	–2.79
Detoxification and antioxidation pathway					
NM_004121	205582_s_at	GGT5	γ-glutamyltransferase 5	2.18	–
NM_005838	206930_at	GLYAT	Glycine-N-acyltransferase	–4.84	–13.25
AI793201	231683_at	GLYAT	Glycine-N-acyltransferase	–4.87	–14.21
AW024233	222083_at	GLYAT	Glycine-N-acyltransferase	–4.02	–20.22
AY028632	211922_s_at	CAT	Catalase	–2.56	–3.94

HCV, hepatitis C virus; HCC, hepatocellular carcinoma

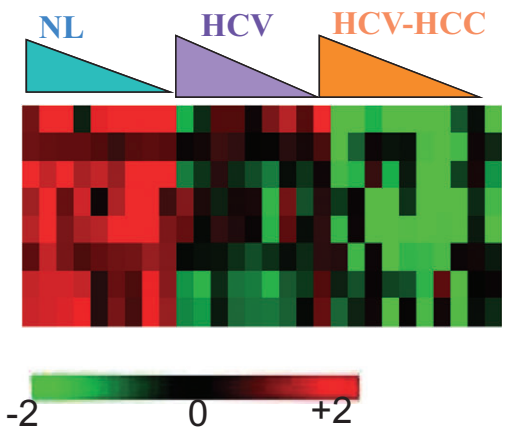

Figure 2 Hierarchical cluster analysis of triglycerol metabolism comparing gene expression among non-liver disease normal control (NL), hepatitis C virus (HCV)-related cirrhotic liver and HCV hepatocellular carcinoma (HCC) tumour samples

development of HCV-related steatosis, and the abnormal lipid and cholesterol metabolism involved in the pathophysiology of HCV may also contribute to hepatocarcinogenesis.

Hepatitis C virus infection has been shown to interfere with lipid metabolism by increasing FA neosynthesis, impairing FA oxidation, increasing TG accumulation, and decreasing cholesterol metabolism activity.[19–23] In the current study, we demonstrate that enhanced *de novo* FA synthesis in HCV cirrhotic patients is congruent with an upregulation of AACS, ACSL4, ELOVL1 and PRGES3, and this process is further enhanced with the additional elevated expression of FASN and ACACA in HCV cirrhotic HCC patients. The expression of FASN is extremely low in normal cells and tissues, whereas it is significantly upregulated or activated in many cancer types. For example, extremely high levels of FASN are expressed in premalignant, invasive and metastatic lesions of many human epithelial cancers.[24–27] Because the over-expression or increased activity of FASN represents one of the most frequent phenotypic alterations in cancer cells, FASN may actively contribute to the development, maintenance and/or promotion of the malignant phenotype. Consistent with the reported data, our study found FASN expression was significantly increased by more than two-fold in HCV-HCC tumour patients compared with normal controls. Interestingly, FASN has also been shown to be an HCV-inducible gene which directly links viral infection to the lipid metabolic disorder.[28] However, the expression of FASN in HCV-positive cirrhotic patients did not show any significant change in this study. Most genetic alterations overlapped between the HCV and HCV-HCC groups compared with normal tissue and far fewer differences were observed between the two diseased groups. The differential expression of FASN in HCV and HCV-HCC patients highlights a potential to identify hepatitis C patients at highest risk for cancer. FASN is more likely to function as a metabolic intermediate of oncogenesis linking energy, anabolism and malignant transformation in HCV-associated cancer development.

ACACA is an enzyme involved in the initial phase of TG and FA synthesis and the rate-limiting enzyme for the long-chain FA synthesis that catalyses the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA (also a FASN substrate). ACACA has been shown to be over-expressed at both the mRNA and protein levels, not only in advanced breast carcinomas, but also in preneoplastic lesions associated with increased risk for the development of infiltrating breast cancer.[29] Knockdown of ACACA by RNAi impaired tumour cell proliferation and caused cell death.[30,31] Here, we showed that the expression of ACACA did not change in HCV cirrhotic cohorts compared with normal controls, which is consistent with a previous report from Yasui *et al.*[32] However, it displayed a 1.97-fold increase in HCC tumour samples. Further investigation about whether ACACA is a predictor of HCC in patients with HCV-related cirrhosis and how ACACA functions in HCV-associated HCC initiation or progression will add insight into the malignancy mechanism of HCV-associated hepatic steatosis. Sterol regulatory element-binding protein-1 (SREBP-1) has been described as a transcription factor that is selectively involved in the activation of genes associated with FA metabolism and *de novo* lipogenesis. Unexpectedly, the expression of SREBP-1 was not significantly changed in either HCV cirrhotic or HCV-HCC patients.

Fatty acid oxidation occurs in three subcellular organelles, with β-oxidation confined to mitochondria and peroxisomes and CYP4A-catalysed ω-oxidation occurring in the endoplasmic reticulum.[33] In this study, we found genes involved in FA β-oxidation were downregulated: the expression of PPARA, HADH and ACOX1 was substantially inhibited in both HCV cirrhotic and HCC patients. Carnitine palmitoyl transferase-1 (CPT-1), the rate-limiting enzyme of mitochondrial β-oxidation, is the main catabolic pathway of FA and of the acyl CoA oxidase (AOX). Interestingly, we did not observe the differential expression of CPT-1 among these three groups of samples. Obviously, the decreased expression of PPARA, ACADS, ACADL, HADHA and ACOX1 may lead to steatosis in HCV cirrhotic patients through downregulation of the β-oxidation of FA. We also noticed that genes involved in antioxidation and detoxification were downregulated. The decreased expression of GLYAT, GGT5 and CAT indicates a failure of neutralizing ROS produced from FA oxidation and a reduction in hepatoprotection. The impaired FA oxidation is most likely exacerbated in HCV-associated cancer patients in response to an amplified expression of those genes. Therefore, HCV infection resulted in an impairment of fat homeostasis, insufficient FA oxidation and excess ROS. This detrimental alteration may also contribute to the initiation and progression of HCC.

Triglycerides are efficient molecules for energy storage and play a fundamental role in several different aspects of lipid metabolism and energy supply. Previous studies reporting serum/plasma TG levels in HCC patients have been inconsistent, ranging from

Table 4 Differential expression of triglyceride metabolism-related genes

Accession no.	Probe ID	Gene symbol	Gene name	Fold change	
				HCV	HCV-HCC
AI623321	205675_at	MTTP	Microsomal triglyceride transfer protein	–1.50	–4.20
AF097831	217289_s_at	–	Glucose-6-phosphatase, catalytic subunit	–2.48	–3.42
AW469523	226064_s_at	DGAT2	Diacylglycerol O-acyltransferase homologue 2 (mouse)	–4.30	–3.57
AB048286	224327_s_at	DGAT2	Diacylglycerol O-acyltransferase homologue 2 (mouse)	–3.90	–4.38
AF202889	224243_at	APOA5	Apolipoprotein A-V	–2.78	–8.39
AF202890	224244_s_at	APOA5	Apolipoprotein A-V	–8.39	–11.06
NM_000236	206606_at	LIPC	Lipase, hepatic	–5.07	–10.56
NM_000078	206210_s_at	CETP	Cholesteryl ester transfer protein, plasma	–2.81	–24.15

HCV, hepatitis C virus; HCC, hepatocellular carcinoma

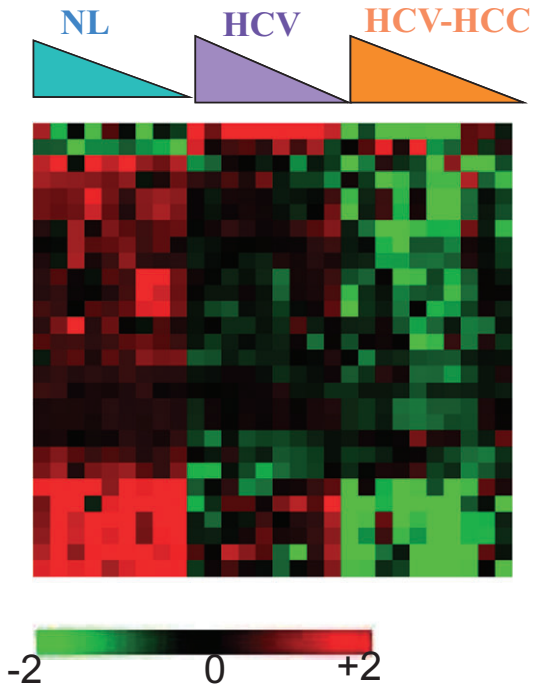

Figure 3 Hierarchical cluster analysis of cholesterol and bile acid metabolism comparing gene expression among non-liver disease normal control (NL), hepatitis C virus (HCV)-related cirrhotic liver and HCV hepatocellular carcinoma (HCC) tumour samples

decreased to increased to no significant change.[34–36] In our study, FA *de novo* synthesis was extremely increased and FA oxidation was substantially inhibited. Genes involved in lipid export from liver such as MTTP, DGAT2, APOA5 and CETP, were significantly downregulated. However, serum lipid profile analysis showed that the level of serum TGs did not show any significant change in either HCV cirrhotic or HCV cirrhotic cancer patients compared with controls. This may have resulted from the induced lipogenesis and impaired TGs secretion by HCV core protein.[37] Serum TGs level may not be a reliable marker for reflecting the serum lipid profile of HCV and HCV-associated cancer patients.

Cholesterol is not only an essential molecule for building cell membranes, but also the precursor to several essential hormones, bile acids and oxysterols. In HCC and chronic liver diseases, the synthesis and metabolism of cholesterol are impaired.[34,35,38] In our study in HCV cirrhotic and HCV cirrhotic HCC patients, we found that both groups displayed lower serum cholesterol levels than the normal controls, but the cholesterol level in cancer patients showed a slighter increase than that in HCV cirrhotic non-cancer cohorts. Consistently, our microarray analysis identified that cholesterol 25-hydroxylase (CH25H), which is an important negative regulator for cholesterol synthesis, was significantly upregulated in HCV cirrhotic samples, but did not show significant change in HCC. No difference was observed in the gene expression of hepatic 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), the rate-limiting step in cholesterol biosynthesis, cholesterol 7-hydroxylase (CYP7A1), the rate-limiting enzyme in the synthesis of bile acid from cholesterol, liver X receptors (LXRs), or maintenance of cholesterol homeostasis through regulating Cyp7A1 and ATP-binding cassette transporter-A1 (ABCA1). SREBP-2, which is best known for the upregulation of genes involved in cholesterol biosynthesis and uptake, did not display any significant change in patient groups compared with controls. Quantitative RT-PCR confirmed the non-differential expression of these genes (data not shown). Low-density lipoprotein receptor (LDLR), which plays a critical role in regulating the amount of cholesterol in the blood, was also not detected in our list (ANOVA, $P < 0.001$). However, we found downregulation of LDLR in both HCV and HCV-HCC with a P-value > 0.001 but <0.05. Interestingly, the expression of VLDLR increased by 4.2-fold and 3.5-fold in HCV cirrhotic and HCV-HCC patients, respectively. One possible explanation is that the enhanced expression of VLDR compensates for the increased accumulation of lipids in the liver. Saitoa *et al.* demonstrated that the ablation of cholesterol biosynthesis in neural stem cells increases their expression of vascular endothelial growth factor (VEGF) and angiogenesis but causes neuron apoptosis.[39] Whether the impaired endogenous cholesterol biosynthesis is related to hepatic angiogenesis and/or hepatocyte regeneration requires further investigation.

Table 5 Differential expression of cholesterol and bile acid metabolism-related genes

Accession no.	Probe ID	Gene symbol	Gene name	Fold change	
				HCV	HCV-HCC
Cholesterol biosynthesis					
NM_005518	204607_at	HMGCS2	3-hydroxy-3-methylglutaryl-coenzyme A synthase 2 (mitochondrial)	–1.98	–2.32
N58493	213789_at	EBP	Emopamil binding protein (sterol isomerase)	–2.34	–1.89
Cholesterol transport, absorption					
L22431	209822_s_at	VLDLR	Very low density lipoprotein receptor	4.25	3.56
X02162	217073_x_at	APOA1	Apolipoprotein A-I	–	–1.74
NM_000039	204450_x_at	APOA1	Apolipoprotein A-I	–1.50	–1.77
AF123759	219340_s_at	CLN8	Ceroid-lipofuscinosis, neuronal 8 (epilepsy, progressive with mental retardation)	–1.50	–2.15
AF123758	223912_s_at	CLN8	Ceroid-lipofuscinosis, neuronal 8 (epilepsy, progressive with mental retardation)	–1.50	–2.23
AF123757	222874_s_at	CLN8	Ceroid-lipofuscinosis, neuronal 8 (epilepsy, progressive with mental retardation)	–	–3.16
W93695	229958_at	CLN8	Ceroid-lipofuscinosis, neuronal 8 (epilepsy, progressive with mental retardation)	–1.83	–3.52
NM_001638	207262_at	APOF	Apolipoprotein F	–17.20	–2.90
NM_000229	204428_s_at	LCAT	Lecithin-cholesterol acyltransferase	–3.44	–13.50
NM_000078	206210_s_at	CETP	Cholesteryl ester transfer protein, plasma	–2.81	–23.97
AF192523	224305_s_at	NPC1L1	NPC1 (Niemann–Pick disease, type C1, gene)-like 1	–1.98	–2.26
NM_003105	203509_at	SORL1	Sortilin-related receptor, L(DLR class) A repeats-containing	–1.96	–4.43
AV728268	212560_at	SORL1	Sortilin-related receptor, L(DLR class) A repeats-containing	–2.11	–4.68
Cholesterol metabolism regulation					
NM_003956	206932_at	CH25H	Cholesterol 25-hydroxylase	8.72	–
NM_006556	203515_s_at	PMVK	Phosphomevalonate kinase	–1.73	
NM_002957	202449_s_at	RXRA	Retinoid X receptor, α	–2.06	–2.39
BE675800	202426_s_at	RXRA	Retinoid X receptor, α	–3.44	–2.46
AJ249976	233748_x_at	PRKAG2	Protein kinase, AMP-activated, γ 2 non-catalytic subunit	–1.70	–2.75
NM_016203	218292_s_at	PRKAG2	Protein kinase, AMP-activated, γ 2 non-catalytic subunit	–1.92	–2.99
AF087875	222582_at	PRKAG2	Protein kinase, AMP-activated, γ 2 non-catalytic subunit	–2.13	–3.10
BF063728	227731_at	CNBP	CCHC-type zinc finger, nucleic acid binding protein	–1.21	–1.47
Bile acid biosynthesis					
AI796334	244407_at	CYP39A1	Cytochrome P450, family 39, subfamily A, polypeptide 1	–4.01	–6.21
NM_016593	220432_s_at	CYP39A1	Cytochrome P450, family 39, subfamily A, polypeptide 1	–3.17	–17.16
BC010358	1553977_a_at	CYP39A1	Cytochrome P450, family 39, subfamily A, polypeptide 1	–3.55	–16.25
NM_005989	207102_at	AKR1D1	Aldo-keto reductase family 1, member D1 (δ 4-3-ketosteroid-5-β-reductase)	–4.69	–15.56
Antioxidant pathways					
NM_000454	200642_at	SOD1	Superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1 (adult))	–1.50	–1.85

HCV, hepatitis C virus; HCC, hepatocellular carcinoma

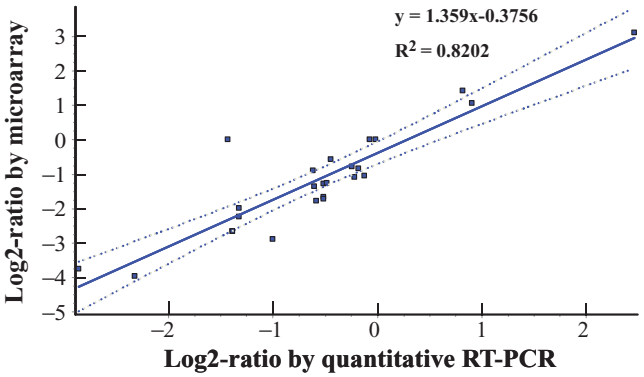

Figure 4 Comparison of quantitative real-time polymerase chain reaction (RT-PCR) data with microarray data. Log2 ratios were calculated from expression values measured by microarray (y-axis) vs. the corresponding log2 ratios derived from quantitative RT-PCR values (x-axis) (n = 12). RNA was isolated from the livers of 20 non-liver disease normal controls, 20 hepatitis C virus (HCV) and 20 HCV hepatocellular carcinoma patients. Correlation coefficient (r) = 0.9056. R^2 = 0.8202

Conclusions

Although a small number of samples were used in this study, our data warrant further investigation into HCV-related lipid metabolic abnormalities. Our results support the notion that HCV-related lipid metabolic abnormalities may contribute to hepatic steatosis and the development of cancer. The identification of these aberrations would stratify patients and improve treatment algorithms.

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Conflicts of interest

None declared.

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