Table 6 Changes in hepatic fatty acid composition

Fatty acid	Control		p^a	Ezetimibe		p^a	p^b
	Before	After		Before	After		
C12:0 (lauric acid)	7.7±1.2	15.2±5.6	0.219	6.3±1.8	18.8±4.7	0.019	0.494
C14:0 (myristic acid)	19.9±2.5	33.0±10.1	0.228	17.6±2.2	56.6±13.0	0.014	0.148
C16:0 (palmitic acid)	185.9±23.8	303.9±118.2	0.334	169.7±22.9	583.9±176.8	0.042	0.202
C16:1n-7 (palmitoleic acid)	24.2±4.5	37.3±13.4	0.362	22.3±4.3	51.9±13.2	0.031	0.368
C17:0 (margaric acid)	4.6±0.7	3.5±0.1	0.400	5.3±0.8	16.0±4.1	0.024	0.025
C18:0 (stearic acid)	45.9±4.4	54.4±8.9	0.283	56.0±7.1	125.1±30.2	0.017	0.042
C18:1n-9 (oleic acid)	166.4±25.1	250.2±91.6	0.367	173.9±30.6	381.9±84.3	0.017	0.288
C18:2n-6 (linoleic acid)	80.4±12.3	87.9±22.5	0.556	73.9±8.5	147.3±36.1	0.035	0.066
C18:3n-6 (γ-linolenic acid)	ND	ND		ND	ND		
C18:3n-3 (α-linolenic acid)	0.6±0.4	0.0±0.0	0.171	0.6±0.4	0.0±0.0	0.178	0.981
C20:0n-6 (arachidic acid)	ND	ND		ND	ND		
C20:1n-9 (eicosenoic acid)	5.5±1.1	4.7±1.9	0.639	5.7±1.0	13.1±4.8	0.170	0.168
C20:2n-6 (eicosadienoic acid)	ND	ND		ND	ND		
C20:3n-6 (dihomo-γ-linolenic acid)	ND	ND		ND	ND		
C20:3n-9 (eicosatrienoic acid)	ND	ND		ND	ND		
C20:4n-6 (arachidonic acid)	ND	ND		ND	ND		
C20:5n-3 (eicosapentaenoic acid)	ND	ND		ND	ND		
C22:0 (behenic acid)	ND	ND		ND	ND		
C22:1n-9 (erucic acid)	14.2±2.5	11.7±2.7	0.474	16.2±2.4	19.2±1.0	0.664	0.468
C22:2n-6 (docosadienoic acid)	2.8±1.0	1.8±1.0	0.433	22.3±0.7	62.3±2.9	0.176	0.152
C22:4n-6 (docosatetraenoic acid)	ND	ND		ND	ND		
C22:5n-3 (docosapentaenoic acid)	ND	ND		ND	ND		
C22:6n-3 (docosahexaenoic acid)	13.6±3.5	7.8±3.3	0.232	14.2±3.7	48.7±19.9	0.109	0.097
C24:1 (nervonic acid)	ND	ND		ND	ND		

The data are expressed as 10^{-4} mg/mg liver, means ± SE

[a] p value for the intragroup comparison (baseline vs 6 month)

[b] p value for the intergroup comparison (changes from baseline between groups)

ND, not determined

genes involved in the L-carnitine pathway, including *CPT1A,* were coordinately downregulated. A decreased L-carnitine pathway could be associated with reduced β-oxidation of palmitic acids in mitochondria, resulting in an increase in long-chain fatty acids (lauric, myristic, palmitic, palmitoleic, margaric, stearic, oleic and linoleic acids). Unbalanced fatty acid composition could induce oxidative stress and lead to insulin resistance in the ezetimibe group. In addition, genes involved in the cholesterol and NEFA biosynthesis, including *SREBF2*, were coordinately upregulated in the ezetimibe group (Table 3), probably as a result of deceased absorption of exogenous cholesterol. Upregulation of *SREBF2* potentially represses the expression of hepatocyte nuclear factor 4, which is required for *CPT1* transcription [35]. Moreover, recent reports have demonstrated that microRNA (miR)-33, encoded by an intron of *Srebp2* [36], inhibits translation of transcripts involved in fatty acid β-oxidation, including *CPT1* [37]. miR-33 is also implicated in decreased insulin signalling by reducing insulin receptor substrate-2 [38, 39]. Hepatic gene expression profiles may, to some extent, explain hepatic fatty acid composition and impaired glycaemic control in the ezetimibe group. These novel SREBP-2-mediated pathways in the gene expression network may be relevant to a recent report that a polymorphism in the *SREBF2* predicts incidence and the severity NAFLD and the associated glucose and lipid dysmetabolism [15]. These unique hypotheses should be confirmed in future in vitro and in vivo studies.

Our study has some limitations. First, the number of patients is relatively small because the data and safety monitoring board recommended that the study intervention and enrolment be discontinued in light of the higher proportion of adverse events in the ezetimibe group than in the control group. Second, our trial was a 6 month open-label study that resulted in subtle changes in liver pathology compared with previous reports [40]. Indeed, a 6 month duration may be too short a period to expect improvement of fibrosis, which is a slowly

progressive process [40]. Third, the average serum aminotransferase levels were lower than those in previous studies [9, 10], and most of the patients had mild steatosis, fibrosis and lower NAS at baseline before ezetimibe treatment. Serum ALT levels did not decrease with ezetimibe treatment in the present study, in contrast to the significant improvement reported previously [9, 10]. And finally, secondary outcomes are always at risk of false-positive associations. Therefore, we not only presented the changes in HbA_{1c} ($p=0.001$ for ezetimibe treatment and $p=0.041$ for the intergroup difference at the end of the study), but also showed the signature of hepatic fatty acid composition and hepatic gene expression profiles that support the hypothesis that ezetimibe increases HbA_{1c} and hepatic fatty acids contents possibly through the SREBP-2–miR33 pathway. No previous studies have raised this issue, which is worth investigating. The same mechanism may underlie a statin-induced deterioration of glucose tolerance, which remains a serious concern. Furthermore, the SREBP-2–miR33 pathway may raise a concern for a safety issue of combination therapy with ezetimibe and statins because these agents may additively upregulate *SREBF2* expression [41]. Future large-scale, long-duration studies involving more severely affected patients are required to determine the definite efficacy and risks of ezetimibe in the treatment of NAFLD.

In conclusion, the present study represents the first randomised controlled clinical trial of the efficacy of ezetimibe on liver pathology, energy homeostasis, hepatic fatty acid composition and hepatic gene expression profiles in patients with NAFLD. The lipid profile and liver histology of cell ballooning and fibrosis were significantly improved by ezetimibe treatment. However, our findings suggest an increase in oxidative stress, insulin resistance and HbA_{1c} on treatment with ezetimibe, which should be taken into consideration in NAFLD patients.

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Contribution statement YT designed the study, recruited the patients, analysed the data and wrote the manuscript. TT designed the study, recruited the patients, interpreted the data and edited the manuscript. MH analysed the hepatic gene expression profiles. YK performed the statistical analyses. YZ analysed all the biopsies. KK, HM and TO recruited the patients and collected the clinical information. HS, KA and TY performed the liver biopsies and histological examinations. MN performed the DNA chip experiments. KY and EM analysed the hepatic fatty acid compositions. SK initiated and organised the study. All authors contributed to the acquisition, analysis and interpretation of data and the drafting and editing of the manuscript. All of the authors approved the final version of the manuscript.

References

1. Hamaguchi E, Takamura T, Sakurai M et al (2010) Histological course of nonalcoholic fatty liver disease in Japanese patients: tight glycemic control, rather than weight reduction, ameliorates liver fibrosis. Diabetes Care 33:284–286
2. Sakurai M, Takamura T, Ota T et al (2007) Liver steatosis, but not fibrosis, is associated with insulin resistance in nonalcoholic fatty liver disease. J Gastroenterol 42:312–317
3. Matsuzawa N, Takamura T, Kurita S et al (2007) Lipid-induced oxidative stress causes steatohepatitis in mice fed an atherogenic diet. Hepatology 46:1392–1403
4. Marí M, Caballero F, Colell A et al (2006) Mitochondrial free cholesterol loading sensitizes to TNF- and Fas-mediated steatohepatitis. Cell Metab 4:185–198
5. Nakamura S, Takamura T, Matsuzawa-Nagata N et al (2009) Palmitate induces insulin resistance in H4IIEC3 hepatocytes through reactive oxygen species produced by mitochondria. J Biol Chem 29: 14809–14818
6. Garcia-Calvo M, Lisnock J, Bull HG et al (2005) The target of ezetimibe is Niemann-Pick C1-like 1 (NPC1L1). Proc Natl Acad Sci U S A 102: 8132–8137
7. Muraoka T, Aoki K, Iwasaki T et al (2011) Ezetimibe decreases SREBP-1c expression in liver and reverses hepatic insulin resistance in mice fed a high-fat diet. Metabolism 60:617–628
8. Deushi M, Nomura M, Kawakami A et al (2007) Ezetimibe improves liver steatosis and insulin resistance in obese rat model of metabolic syndrome. FEBS Lett 581:5664–5670
9. Yoneda M, Fujita K, Nozaki Y et al (2010) Efficacy of ezetimibe for the treatment of non-alcoholic steatohepatitis: an open-label, pilot study. Hepatol Res 40:613–621
10. Park H, Shima T, Yamaguchi K et al (2011) Efficacy of long-term ezetimibe therapy in patients with nonalcoholic fatty liver disease. J Gastroenterol 46:101–107
11. Promrat K, Lutchman G, Uwaifo GI et al (2004) A pilot study of pioglitazone treatment for nonalcoholic steatohepatitis. Hepatology 39:188–196
12. Matthews DR, Hosker JP, Rudenski AS et al (1985) Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 28:412–419
13. Katz A, Nambi SS, Mather K et al (2000) Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. J Clin Endocrinol Metab 85:2402–2410
14. Musso G, Cassader M, de Michieli F, Rosina F, Orlandi F, Gambino R (2012) Nonalcoholic steatohepatitis versus steatosis: adipose tissue insulin resistance and dysfunctional response to fat ingestion predict liver injury and altered glucose and lipoprotein metabolism. Hepatology 56:933–942
15. Musso G, Cassader M, Bo S, de Michieli F, Gambino R (2013) Sterol regulatory element-binding factor 2 (SREBF-2) predicts 7-year NAFLD incidence and severity of liver disease and lipoprotein and glucose dysmetabolism. Diabetes 62:1109–1120
16. Gastaldelli A, Cusi K, Pettiti M, Hardies J, Miyazaki Y, Berria R, Buzzigoli E, Sironi AM, Cersosimo E, Ferrannini E, Defronzo RA (2007) Relationship between hepatic/visceral fat and hepatic insulin resistance in nondiabetic and type 2 diabetic subjects. Gastroenterology 133:496–506
17. Musso G, Gambino R, Cassader M (2010) Lipoprotein metabolism mediates the association of MTP polymorphism with beta-cell dysfunction in healthy subjects and in nondiabetic normolipidemic patients with nonalcoholic steatohepatitis. J Nutr Biochem 21:834–840
18. Abdul-Ghani MA, Williams K, DeFronzo RA, Stern M (2007) What is the best predictor of future type 2 diabetes? Diabetes Care 30: 1544–1548

19. Matsuda M, DeFronzo RA (1999) Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. Diabetes Care 22:1462–1470
20. Abdul-Ghani MA, Matsuda M, Balas B, DeFronzo RA (2007) Muscle and liver insulin resistance indexes derived from the oral glucose tolerance test. Diabetes Care 30:89–94
21. DeFronzo RA, Tobin JD, Andres R (1979) Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am J Physiol 237:E214–E223
22. Nagai Y, Takamura T, Nohara E et al (1999) Acute hyperinsulinemia reduces plasma concentrations of homocysteine in healthy men. Diabetes Care 22:1004
23. Brunt EM, Janney CG, Di Bisceglie AM et al (1999) Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol 94:2467–2474
24. Kleiner DE, Brunt EM, van Natta M et al (2005) Nonalcoholic Steatohepatitis Clinical Research Network. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 41:1313–1321
25. DeBose-Boyd RA, Ou J, Goldstein JL, Brown MS (2001) Expression of sterol regulatory element-binding protein 1c (SREBP-1c) mRNA in rat hepatoma cells requires endogenous LXR ligands. Proc Natl Acad Sci U S A 13:1477–1482
26. de Bari O, Neuschwander-Tetri BA, Liu M et al (2012) Ezetimibe: its novel effects on the prevention and the treatment of cholesterol gallstones and nonalcoholic fatty liver disease. J Lipids 2012:302847
27. Jia L, Ma Y, Rong S et al (2010) Niemann-Pick C1-Like 1 deletion in mice prevents high-fat diet-induced fatty liver by reducing lipogenesis. J Lipid Res 51:3135–3144
28. Musso G, Gambino R, Cassader M (2013) Cholesterol metabolism and the pathogenesis of non-alcoholic steatohepatitis. Prog Lipid Res 52:175–191
29. Teratani T, Tomita K, Suzuki T et al (2012) A high-cholesterol diet exacerbates liver fibrosis in mice via accumulation of free cholesterol in hepatic stellate cells. Gastroenterology 142:152–164
30. Altmann SW, Davis HR Jr, Zhu LJ et al (2004) Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. Science 303:1201–1204
31. Temel RE, Brown JM, Ma Y et al (2007) Hepatic Niemann-Pick C1-like 1 regulates biliary cholesterol concentration and is a target of ezetimibe. J Clin Invest 117:1968–1978
32. Joshi-Barve S, Barve SS, Amancherla K et al (2007) Palmitic acid induces production of proinflammatory cytokine interleukin-8 from hepatocytes. Hepatology 46:823–830
33. Pagliassotti MJ, Wei Y, Wang D (2007) Insulin protects liver cells from saturated fatty acid-induced apoptosis via inhibition of c-Jun NH2 terminal kinase activity. Endocrinology 148:3338–3345
34. Malhi H, Bronk SF, Werneburg NW, Gores GJ (2006) Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis. J Biol Chem 281: 12093–12101
35. Gerin I, Clerbaux LA, Haumont O et al (2010) Expression of miR-33 from an SREBP2 intron inhibits cholesterol export and fatty acid oxidation. J Biol Chem 285:33652–33661
36. Louet JF, Hayhurst G, Gonzalez FJ et al (2002) The coactivator PGC-1 is involved in the regulation of the liver carnitine palmitoyltransferase I gene expression by cAMP in combination with HNF4 alpha and cAMP-response element-binding protein (CREB). J Biol Chem 277:37991–38000
37. Xuefen X, Hailing L, Huaixin D et al (2009) Down-regulation of hepatic HNF4_ gene expression during hyperinsulinemia via SREBPs. Mol Endocrinol 23:434–443
38. Horie T, Ono K, Horiguchi M et al (2010) MicroRNA-33 encoded by an intron of sterol regulatory element-binding protein 2 (Srebp2) regulates HDL in vivo. Proc Natl Acad Sci U S A 107:17321–17326
39. Fernández-Hernando C, Moore KJ (2011) MicroRNA modulation of cholesterol homeostasis. Arterioscler Thromb Vasc Biol 31:2378–2382
40. Musso G, Cassader M, Rosina F, Orlandi F, Gambino R (2012) Impact of current treatments on liver disease, glucose metabolism and cardiovascular risk in non-alcoholic fatty liver disease (NAFLD): a systematic review and meta-analysis of randomised trials. Diabetologia 55:885–904
41. Bennett MK, Seo YK, Datta S, Shin DJ, Osborne TF (2008) Selective binding of sterol regulatory element-binding protein isoforms and co-regulatory proteins to promoters for lipid metabolic genes in liver. J Biol Chem 6:15628–15637

microRNA-122 Abundance in Hepatocellular Carcinoma and Non-Tumor Liver Tissue from Japanese Patients with Persistent HCV versus HBV Infection

Carolyn Spaniel[1,2], Masao Honda[3], Sara R. Selitsky[1,4], Daisuke Yamane[1], Tetsuro Shimakami[3], Shuichi Kaneko[3], Robert E. Lanford[5], Stanley M. Lemon[1*]

1 Departments of Medicine and Microbiology & Immunology and the Lineberger Comprehensive Cancer Center, the University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, United States of America, **2** Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas, United States of America, **3** Department of Gastroenterology, Kanazawa University Graduate School of Medicine, Takara-Machi, Kanazawa, Japan, **4** Department of Genetics, the University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, United States of America, **5** Department of Virology and Immunology, Texas Biomedical Research Institute, San Antonio, Texas, United States of America

Abstract

Mechanisms of hepatic carcinogenesis in chronic hepatitis B and hepatitis C are incompletely defined but often assumed to be similar and related to immune-mediated inflammation. Despite this, several studies hint at differences in expression of miR-122, a liver-specific microRNA with tumor suppressor properties, in hepatocellular carcinoma (HCC) associated with hepatitis B virus (HBV) versus hepatitis C virus (HCV) infection. Differences in the expression of miR-122 in these cancers would be of interest, as miR-122 is an essential host factor for HCV but not HBV replication. To determine whether the abundance of miR-122 in cancer tissue is influenced by the nature of the underlying virus infection, we measured miR-122 by qRT-PCR in paired tumor and non-tumor tissues from cohorts of HBV- and HCV-infected Japanese patients. miR-122 abundance was significantly reduced from normal in HBV-associated HCC, but not in liver cancer associated with HCV infection. This difference was independent of the degree of differentiation of the liver cancer. Surprisingly, we also found significant differences in miR-122 expression in non-tumor tissue, with miR-122 abundance reduced from normal in HCV- but not HBV-infected liver. Similar differences were observed in HCV- vs. HBV-infected chimpanzees. Among HCV-infected Japanese subjects, reductions in miR-122 abundance in non-tumor tissue were associated with a single nucleotide polymorphism near the IL28B gene that predicts poor response to interferon-based therapy (TG vs. TT genotype at rs8099917), and correlated negatively with the abundance of multiple interferon-stimulated gene transcripts. Reduced levels of miR-122 in chronic hepatitis C thus appear to be associated with endogenous interferon responses to the virus, while differences in miR-122 expression in HCV- versus HBV-associated HCC likely reflect virus-specific mechanisms contributing to carcinogenesis. The continued expression of miR-122 in HCV-associated HCC may signify an important role for HCV replication late in the progression to malignancy.

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* E-mail: smlemon@med.unc.edu

Introduction

Globally, liver cancer is the fifth and seventh most common malignancy in men and women, respectively, and the third most deadly [1]. Most (85-95%) of these cancers are hepatocellular carcinoma (HCC) [2], and many are associated with persistent intrahepatic infections with hepatitis C virus (HCV) or hepatitis B virus (HBV) [2,3]. Although the total cancer death rate decreased within the United States by over 1.5% between 2001-2007, deaths due to liver cancer increased by 50% among males and by 29% in females [4]. These changes in the incidence of HCC are largely due to increases in HCV-associated malignancy. Similarly, while HBV infection historically has been the major risk factor underlying development of HCC in Asia, in Japan it has been supplanted in recent decades by HCV infection [5].

The exact mechanisms underlying HCV- and HBV-associated malignancy are unknown [6,7]. Chronic infections with either virus may result in cirrhosis, which alone is a major risk factor for liver cancer [2]. However, there may also be

virus-specific mechanisms at work. While immune-mediated mechanisms are both necessary and sufficient for the development of HBV-related cancer in murine models, liver cancer arises in the absence of inflammation in HCV-transgenic mice [8,9]. Moreover, some HCV proteins may interact with host tumor suppressors and possibly impair cellular responses to DNA damage [10]. If virus-specific mechanisms of oncogenesis are important in the development of HCC, it is reasonable to anticipate that the pathways leading to HCV- and HBV-associated cancer might differ, possibly leaving distinguishing genetic or epigenetic marks in the tumors that arise. If so, understanding these differences would be important for biomarker discovery, and potentially design of preventative and therapeutic interventions.

Here, we describe a study that was aimed at determining whether the abundance of microRNA-122 (miR-122) is different in liver cancer arising in patients with chronic HCV infection compared to cancers arising in the context of chronic HBV infection. Mature microRNAs (miRNAs) are 20-23 nucleotides in length and encoded either by microRNA genes or from within conventional protein-coding genes. They act generally by binding to specific sites within the 3' untranslated region (3' UTR) of cellular mRNAs, to which they recruit RNA-induced silencing complexes (RISC) that repress translation and destabilize the mRNA [11–13]. miR-122 is a liver-specific miRNA that accounts for the majority of miRNAs in hepatocytes [14]. It regulates a large number of genes within the liver [15], and has several tumor suppressor-like properties [16,17]. Importantly, miR-122 is a crucial host factor for HCV replication, binding to the 5' untranslated RNA segment of the viral genome, physically stabilizing it, and promoting viral protein expression [18–20].

Because of its liver-specific nature and tumor suppressor-like qualities [16,17], it is of interest to know whether miR-122 expression is altered in liver cancers. Prior studies investigating miR-122 expression in liver cancers have produced conflicting results, particularly as related to the underlying viral causes of cancer. Two early studies suggest that miR-122 abundance is generally reduced in HCC [21,22]. However, Hou et al. [23] reported that miR-122 expression was maintained in both HBV- and HCV-associated cancer, while Varnholt et al. [24] reported that miR-122 levels were increased significantly in HCV-associated cancers when compared to non-cancerous tissue. Coulouarn et al. [25] reported higher miR-122 expression levels in HCV- versus HBV-associated cancers. To some extent, these conflicting results may reflect different patient populations, or possibly methodologic differences, not only in the measurement of miR-122 abundance but also in how miR-122 abundance was compared across tissue samples.

In an effort to resolve this controversy, we conducted a comprehensive analysis of miR-122 expression in liver cancers arising in a genetically homogenous group of Japanese patients. Using a highly accurate, miR-122-specific quantitative reverse-transcription, polymerase chain reaction (qRT-PCR) assay, and paying particular attention to how miR-122 measurements are compared between tissue samples, we show that miR-122 expression is significantly reduced in HBV-associated HCC but not in most HCV-associated cancers. We also demonstrate that miR-122 abundance is reduced in non-tumor HCV-infected liver in association with increased expression of interferon (IFN)-stimulated genes (ISGs).

Materials and Methods

Ethics statement

Liver tissue was obtained from Japanese patients undergoing surgical resection of liver cancer (primary or metastatic) at the Liver Center of Kanazawa University Hospital (Kanazawa, Japan). All subjects provided written informed consent for participation in the study, and tissue acquisition procedures were approved by the ethics committee of Kanazawa University under a protocol entitled "Gene expression analysis of peripheral blood cells and liver in patients with liver and gastrointestinal cancers". Archived liver tissue and serum samples were collected prior to December 15, 2011 from chimpanzees housed and cared for at the Southwest National Primate Research Center (SNPRC) of the Texas Biomedical Research Institute in accordance with the Guide for the Care and Use of Laboratory Animals. All protocols were approved by the Institutional Animal Care and Use Committee. SNPRC is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International and operates in accordance with the NIH and U.S. Department of Agriculture guidelines and the Animal Welfare Act.

Human subjects and tissue samples

Paired samples of HCC and non-tumor liver tissue were obtained from Japanese patients undergoing surgical resection of HCC at the Liver Center of Kanazawa University Hospital (Kanazawa, Japan). Non-infected 'normal' liver tissue was similarly collected from patients undergoing resection of metastases of non-hepatic primary cancers. Patients were categorized as HCV-infected by the presence of HCV RNA (COBAS Ampli-Prep/COBAS TaqMan System) and absence of hepatitis B surface antigen (HBsAg) in serum or plasma at the time of surgery, while HBV infection was defined by the presence of HBsAg and absence of anti-HCV antibodies. HCC was categorized according to the degree of cellular differentiation, while fibrosis and inflammation in non-tumor tissue from HBV- and HCV-infected patients were compared after scoring each [26,27]. The IL28B genotype of study subjects with HCV infection was defined at the rs8099917 locus as described previously [28].

Chimpanzee care and sample collection

We studied archived liver tissue and serum samples collected prior to December 15, 2011 from chimpanzees housed and cared for at the Southwest National Primate Research Center (SNPRC) of the Texas Biomedical Research Institute. At the time samples were obtained, animals considered to be non-infected ('normal') were negative for HBV and HCV markers; HBV infection was defined as the presence of serum HBsAg, and HCV infection by the presence of HCV RNA detectable in sera by RT-PCR.

Small RNA quantitation in human samples

Human tissue samples were stored in liquid nitrogen until processed for RNA extraction. Approximately 1 mg of tissue was ground using a tissue homogenizer and total RNA isolated using the mirVana miRNA isolation kit (Ambion). Liver RNA samples were subsequently stored at -80°C or on dry ice during shipment. The quality of the isolated RNA (RIN score) was assessed using an Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit, Agilent Technologies) [29]. Quantification of miR-122, miR-191, Let-7a, miR-24, and the small nuclear RNA (snRNA) U6 was carried out by quantitative reverse-transcription, polymerase chain reaction (qRT-PCR) in a two-step process. RNA (12.5 ng) was reversed transcribed in a 10 µl reaction mix using reagents provided with the Universal cDNA Synthesis kit (Exiqon) and the manufacturer's recommended procedure. Quantitative PCR was carried out subsequently with the SYBR Green Master Mix Kit (Exiqon), mixed locked-nucleic acid primer sets specific for each miRNA or snRNA (Exiqon), and the CFX96 PCR System (Bio-Rad). Results are presented as relative copy number normalized to total RNA. Alternatively, absolute miR-122 copy numbers were estimated using serial dilutions of single-stranded synthetic miR-122 (Dharmacon) as a standard.

miR-122 and HCV RNA quantitation in chimpanzee samples

Total RNA was extracted from serum and liver using RNA Bee (Leedo Medical Labs, Houston, TX), chloroform extraction and isopropanol precipitation. Detection of miR-122 was performed using primers and probes for miR-122 included in the ABI TaqMan assay (Cat No. 4373151) and the ABI TaqMan microRNA Reverse Transcription Kit (Cat No. 4366596). The RT reaction was performed with 5 ng of total cell RNA, and the PCR amplification was performed with one-tenth of the resulting cDNA. The RT reaction was performed at 16°C for 30 min, followed by 42°C for 30 min, and 85°C for 5 min. The TaqMan Universal PCR Master Mix with no AmpErase UNG was used for PCR amplification with reaction conditions of 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. A standard curve was generated using a synthetic RNA equivalent to mature miR-122. HCV viral RNA levels in the serum and liver were determined using a real-time, quantitative RT-PCR (TaqMan) assay detecting sequences in the viral 5' noncoding RNA using an ABI 7500 sequence detector (PE Biosystems, Foster City, CA) as previously described [30]. Synthetic HCV RNA was used to generate a standard curve for determination of genome equivalents. The forward primer was from nucleotide 149 to 167 (5'-tgcggaaccggtgagtaca-3'), the reverse primer was from nucleotide 210 to 191 (5'-cgggtttatccaagaaagga-3') and the probe was from nucleotide 189 to 169 (5'-ccggtcgtcctggcaattccg-3') in the 5' NCR of HCV.

Affymetrix array analysis

Human RNA samples were subjected to high-density oligonucleotide microarray analysis as described previously [28]. In brief, cDNA amplified using the WT-Ovation Pico RNA Amplification System (NuGen, San Carlos, CA, USA) was used for fragmentation and biotin labeling with the FL-Ovation cDNA Biotin Module V2 (NuGen). Biotin-labeled cDNA suspended in hybridization cocktail (NuGen) was hybridized to Affymetrix U133 Plus 2.0 GeneChips, followed by labeling with streptavidin-phycoerythrin. Probe hybridization was determined using a GeneChip Scanner 3000 (Affymetrix) and analyzed using GeneChip Operating Software 1.4 (Affymetrix).

Statistical analysis

Statistical analyses were carried out using Prism V software (Graphpad Software, Inc). The paired t test was used for comparison of results arising from groups of paired tissue specimens (HCC versus non-tumor tissue), while the unpaired t test or Mann-Whitney test was used for comparisons between groups of unrelated tissues (e.g., HBV versus HCV infection). Nonparametric analysis of the correlation between miR-122 and ISG expression levels was done by the Spearman method. Other statistical tests were as described in the text.

Results

miR-122 abundance in HCV- versus HBV-associated liver cancer

We measured miR-122 abundance in paired tumor and non-tumor tissues collected from 26 patients undergoing surgical resection of HCC: 16 with concomitant chronic HCV infection, and 10 infected with HBV. The age, gender, histological classification of HCC, and fibrosis score of non-tumor tissues are shown in Figure 1 (see also Table 1). Subjects infected with HCV (predominantly genotype 1b) were approximately one decade older than those with HBV infection (66.6 ± 8.0 s.d. versus 54.3 ± 9.1 s.d. years, p=0.001, Figure 1A), consistent with previous studies indicating that HCC is generally diagnosed at an earlier age in HBV-infected Japanese patients [31]. There were no significant differences in the histological classification of HCC or scores for fibrosis or inflammatory activity in non-tumor tissues between the two groups (Figure 1B and C, and Table 1). There were more females among those with HCV infection (10 male and 6 female) than HBV (9 male and 1 female), but this difference did not achieve statistical significance (Chi square test with Yate's correction).

qRT-PCR revealed significant differences in the abundance of miR-122 in both tumor and non-tumor tissue samples when the HBV- and HCV-infected groups were compared (Figure 2). miR-122 abundance (miR-122 copy number per µg total RNA) was significantly lower in HCC tissue from HBV-infected versus HCV-infected subjects (p=0.009 by two-sided t test). In contrast, the miR-122 abundance in non-tumor tissue from HBV-infected patients was significantly greater than that in the HCV-infected patients (p=0.0005 by two-sided t test). The mean miR-122 abundance in HCC tissue was less than half that in non-tumor tissue in HBV-infected patients (p=0.003 by two-sided, paired t test). Strikingly, this relationship was reversed in the HCV-infected patients, in whom miR-122 abundance in HCC tissue was almost twice that in the non-tumor tissue (p=0.008 by two-sided paired t test). There was no significant difference in the abundance of miR-122 in the non-tumor tissue from HBV-infected patients and HCV-associated

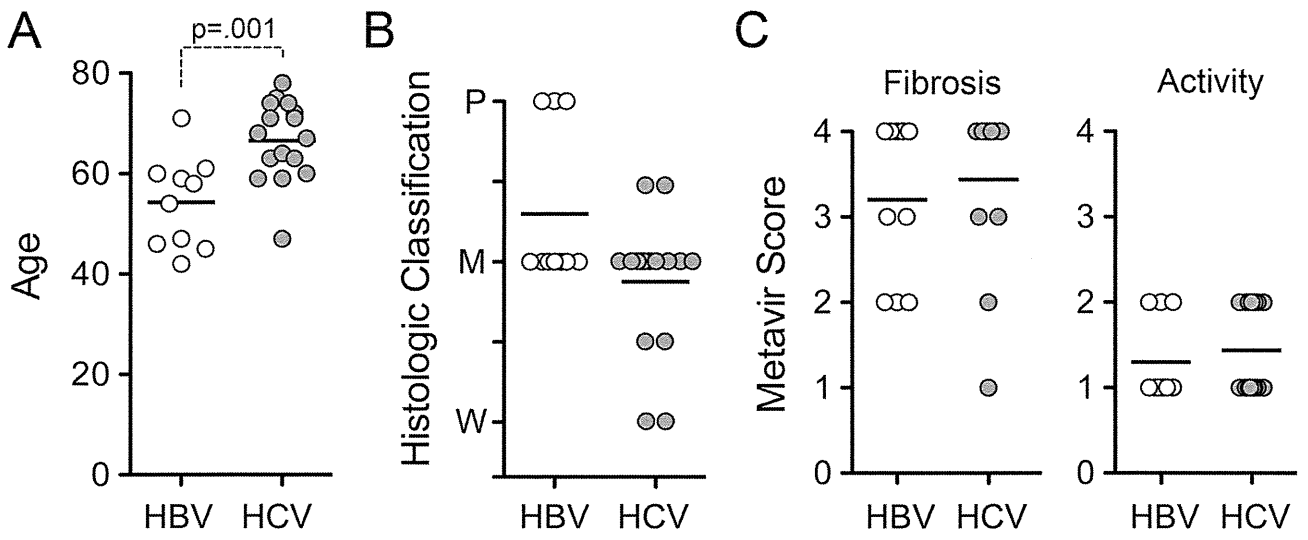

Figure 1. Age, histological classification of tumors, and scoring of non-tumor tissue for inflammation and fibrosis. (**A**) Age of subjects from whom HBV- and HCV-associated HCC and paired non-tumor samples were obtained. (**B**) Histological classification of tumors: W = well differentiated, M = moderately differentiated, P = poorly differentiated. (**C**) Individual scores for fibrosis and inflammatory activity in non-tumor tissue. Bars represent mean values. See also Table 1.

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HCC. miR-122 abundance varied quite widely in liver tissue collected from non-infected individuals undergoing resection of metastatic tumors. Despite this, miR-122 abundance was significantly less in HBV-associated cancer tissue and non-tumor HCV-infected tissue than in the non-infected tissues (p=0.016 and 0.013, respectively).

To account for potential differences in degradation of the RNA or efficiency of reverse transcription between tissue samples, we assessed the abundance of several other small RNAs against which we could normalize the abundance of miR-122. U6, a noncoding snRNA component of the spliceosome, is commonly used to normalize miRNA abundance. However, we observed substantial differences in U6 abundance in these tissues, suggesting that U6 would be a poor normalizer (Figure 3A). Substantially less variation was observed in the abundance of the miRNAs, miR-24 or Let-7a (Figure 3B and C), for which the standard deviation of the critical threshold [25] in the PCR assay was 0.79 and 1.27, respectively, compared to 1.34 for U6. Notably, we observed no difference in the abundance of Let-7a in HBV-associated cancer and non-tumor tissues (p=0.52 by two-sided, paired t test), despite a prior report suggesting that Let-7a expression is regulated by the HBx protein and increased in abundance in HBV-associated HCC [32]. In addition, although miR-24 negatively regulates the expression of hepatocyte nuclear factor 4-alpha (HNF4-alpha) and thus might be up-regulated in some liver cancers [33], we did not observe this. A strong positive correlation was evident between the abundance of miR-24 and Let-7a (Figure 3E, Spearman r_s=0.7959, p<0.001 by two-tailed t test), suggesting that these miRNAs might belong to a common regulatory network and that either could be used to normalize miR-122 abundance. In contrast, there was no correlation between miR-24 and either U6 or miR-122 abundance (Figure 3D and F), which indicates that U6 and miR-122 are regulated independently of miR-24. Importantly, when the miR-122 abundance was normalized to miR-24 levels, miR-122 expression remained significantly depressed in HBV-associated HCC when compared either with paired non-tumor tissue, or HCC tissue from HCV-infected subjects (p<0.001 and p=0.002, respectively, Figure 2B). In replicate assays, the abundance of miR-122 in non-tumor HCV-infected tissue also remained significantly lower than either non-infected or HBV-infected liver tissues (Figure 2B). Similar associations were found when miR-122 abundance was normalized to Let-7a (data not shown).

To assess further the possibility of bias in these results due to differences in the quality of the RNA samples, we compared the RNA integrity number (RIN) [29] of each sample with the abundance of each of the small RNAs detected. Interestingly, while the quantity of U6 snRNA detected correlated positively with the RIN score (Spearman r_s = 0.5216, two-tailed p = 0.0001) (Figure S1A in Supporting Information), this was not the case with miR-24 or Let-7a (r_s = -0.124 and -0.045, respectively). RIN scores also did not vary significantly between tumor and non-tumor tissue-derived RNA samples, or RNA from HBV- vs. HCV-infected tissue. Thus, although the quality of the RNA samples was generally high (mean RIN = 8.0 ± 0.17 s.e.m.), it was an important factor in determining the abundance of U6 but not either of these miRNAs. These data suggest that U6 is less stable than the miRNAs and provide additional support for the use of miR-24 (or Let-7a) as a standard against which to normalize miR-122 abundance (see Discussion). Nonetheless, when miR-122 results were normalized to U6 abundance, the correlations described above

Table 1. Characteristics of Study Subjects.

	HCV (n = 16)	HBV (n=10)	Non-infected (n=9)
Mean Age (years)	66.6 ± 8.0 s.d.	54.3 ± 9.1	60.1 ± 14.3
Gender (M/F)	10M/6F	9M / 1F	5M / 4F
HCV Genotype			
1a	0	n/a	n/a
1b	14		
2	2		
3	0		
Fibrosis Stage	n (%)	n (%)	n (%)
0	0 (0)	0 (0)	9 (100)
1	1 (6)	0 (0)	0 (0)
2	1 (6)	3 (30)	0 (0)
3	4 (25)	2 (20)	0 (0)
4	10 (63)	5 (50)	0 (0)
Inflammation	n (%)	n (%)	n (%)
0	0 (0)	0 (0)	9 (100)
1	9 (56)	7 (70)	0 (0)
2	7 (44)	3 (30)	0 (0)
3	0 (0)	0 (0)	0 (0)
4	0 (0)	0 (0)	0 (0)
HCC Histologic Differentiation	n (%)	n (%)	
Well	2 (13)	0 (0)	n/a
Moderate-Well	2 (13)	0 (0)	
Moderate	10 (63)	7 (30)	
Poor-Moderate	2 (13)	0	
Poor	0 (0)	3 (30)	
IL28B genotype (rs8099917)	n (%)		
TT	9 (56)	n.d.	n.d.
TG	7 (44)		
GG	0 (0)		

n/a = "not applicable"; n.d. = "not done"

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between miR-122 abundance, in both tumor and non-tumor tissues, and the type of virus infection remained strongly statistically significant (Figure S1B in Supporting Information). The mean miR-122 abundance was substantially lower in HBV-associated HCC tissue than in HBV-infected non-tumor tissue ($p = 0.003$ by paired t-test), while this relationship was reversed in HCV-infected liver ($p = 0.001$). miR-122 abundance was also significantly lower in non-tumor tissue from HCV-infected subjects than HBV-infected subjects ($p < 0.001$).

To exclude the possibility of bias due to the trend toward a less differentiated histologic classification among HBV-associated cancers (Figure 1B), we limited the comparison of miR-122 abundance to those HCC tissues that were scored as moderately differentiated and their corresponding paired non-tumor samples. While this reduced the number of subjects available for analysis, miR-122 abundance remained significantly lower in HBV- versus HCV-associated cancer tissue: $p=0.007$ when compared on the basis of miR-122 copy number/mg RNA (Figure 2C) vs. $p=0.033$ when normalized to miR-24 (Figure 2D). Thus differences in miR-122 abundance in HCC associated with HBV vs. HCV infection are independent of the degree of histologic differentiation of the cancer.

Collectively, these results provide strong evidence that miR-122 expression is reduced in HCC associated with HBV infection, but not in most HCV-associated liver cancers.

Reduced miR-122 abundance is associated with interferon responses in HCV-infected liver

The data shown in Figure 2 indicate that miR-122 is frequently reduced in abundance in non-tumor, HCV-infected liver tissue, but not in liver infected with HBV. To determine whether similar HCV-induced suppression of miR-122 expression occurs in chimpanzees (*Pan troglodytes*), the only animal species other than humans that is permissive for HCV infection, we measured miR-122 abundance in liver tissues

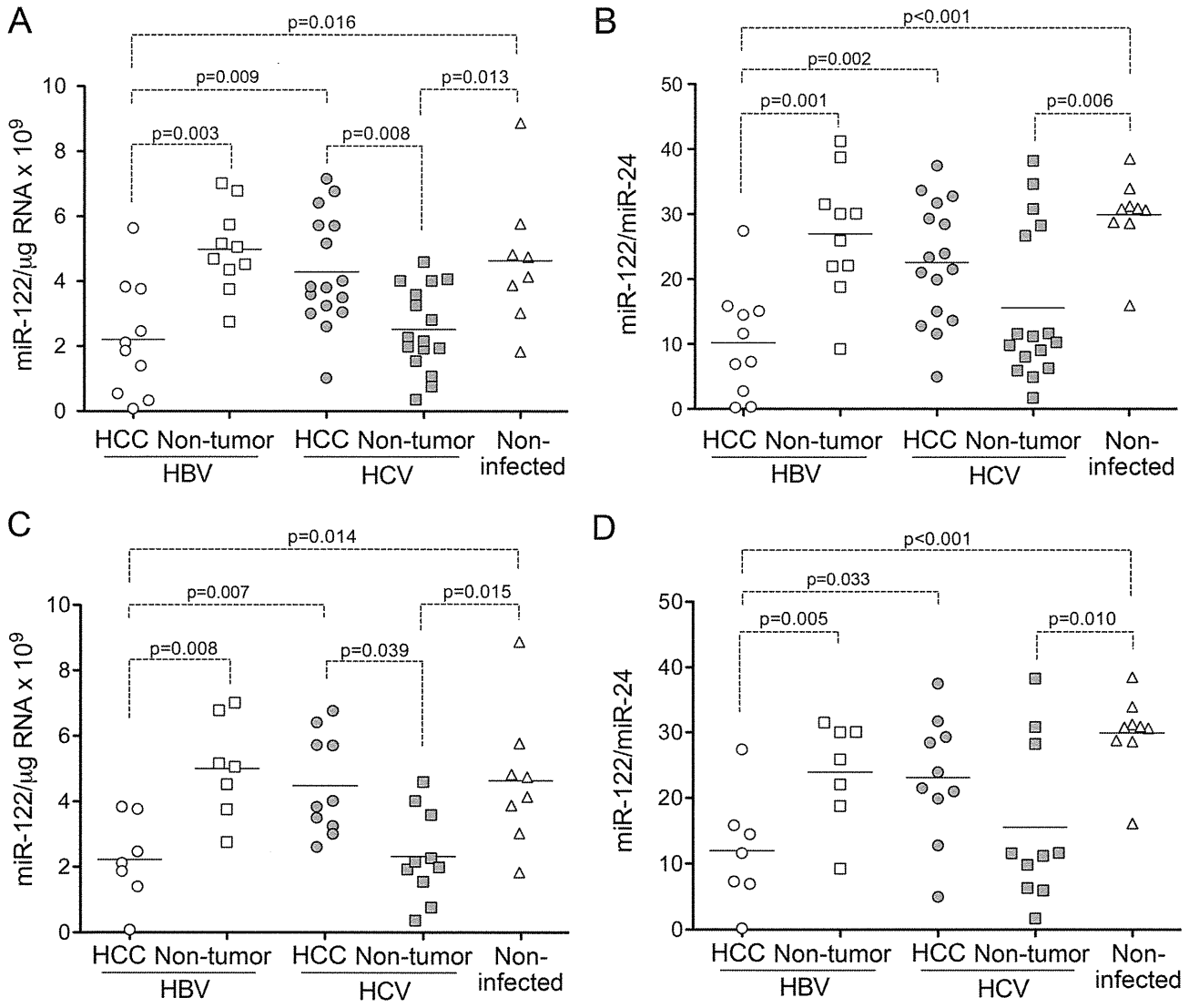

Figure 2. miR-122 expression in paired HCC and non-tumor liver tissue from patients with chronic HBV and HCV infection and control, non-infected liver tissue. (**A**) miR-122 abundance quantified by qRT-PCR in paired tumor and non-tumor tissues and non-infected ('normal') liver from patients undergoing resection of metastatic tumors, normalized to total RNA. (**B**) Relative miR-122 abundance normalized to miR-24 abundance in the same tissues. (**C**) miR-122 abundance in HCC classified histologically as "moderately differentiated", paired non-tumor tissue from the same patients, and non-infected ('normal') liver. (**D**) miR-122 abundance in the subset of tissues shown in panel C, normalized to miR-24 abundance. The statistical significance of differences between paired observations was estimated using the paired t test, while differences between non-paired observations were analyzed by the Mann-Whitney test.

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collected previously from 45 HCV-infected chimpanzees, and compared this to that present in 10 HBV-infected animals, and 6 that were not infected with either virus. These results showed that miR-122 expression was significantly reduced in HCV-infected liver compared to both HBV-infected ($p<0.0001$) and normal, non-infected ($p=0.007$) chimpanzee liver (Figure 4A). A strong, negative correlation (Spearman $r_s= -0.63$, $p<0.0001$) existed between hepatic miR-122 expression levels and HCV RNA copy numbers in serum (Figure 4B). The mean miR-122 abundance was lower in HBV-infected liver than in uninfected chimpanzee liver (Figure 4A), but the difference did not achieve statistical significance ($p=0.059$ by two-tailed t test). Thus, intra-hepatic miR-122 abundance is reduced in HCV-infected chimpanzees as well as humans. This is consistent with earlier studies that have found reduced intrahepatic expression of miR-122 in patients with advanced chronic hepatitis C [34–36].

Sarasin-Filipowicz et al. [36] reported previously that miR-122 levels were reduced in liver from HCV-infected

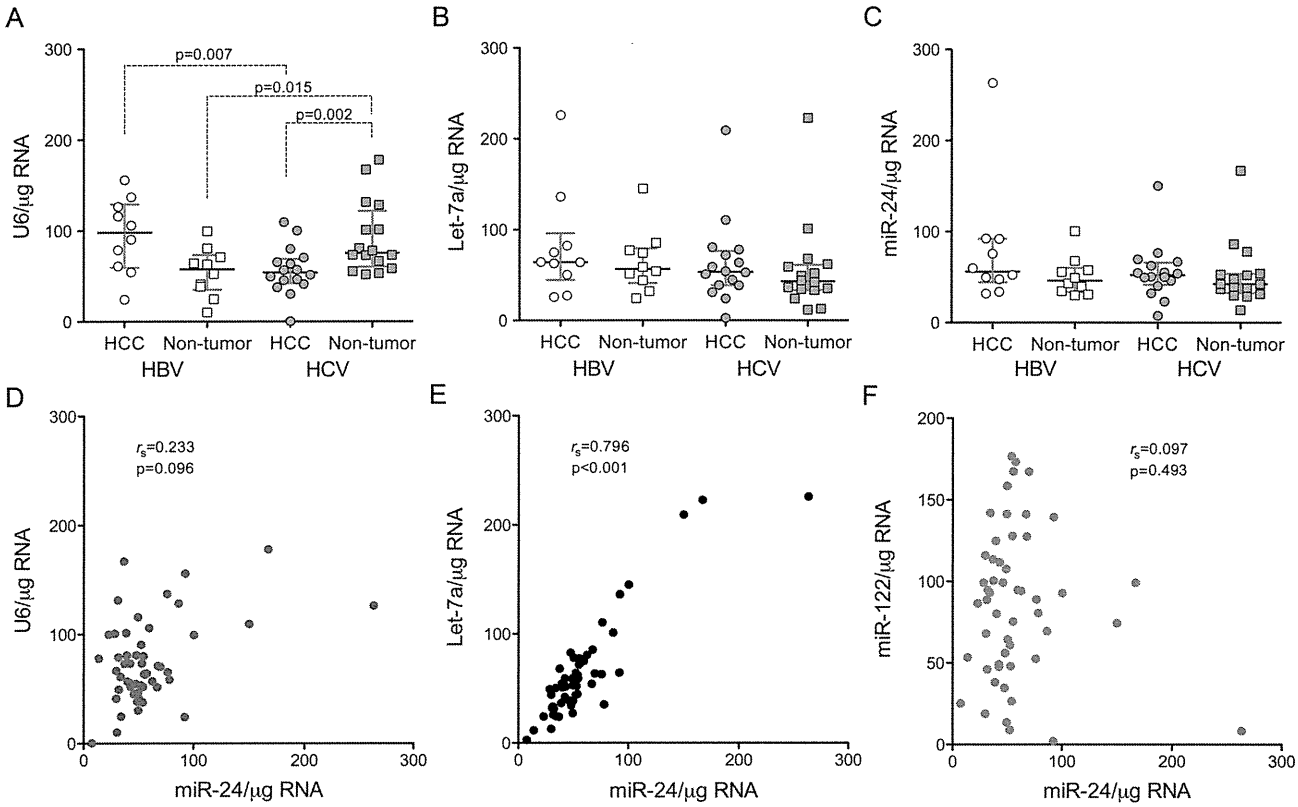

Figure 3. Comparison of small RNAs as normalizers for assessing miR-122 abundance. Shown in the panels at the top are the relative abundance of (**A**) U6 snRNA, (**B**) Let-7a, and (**C**) miR-24 miRNAs in paired tumor and non-tumor tissues from subjects with HBV or HCV infection, normalized to total RNA. Bars represent median and quartiles for each group. Statistical comparisons between groups were made with paired or unpaired t tests, and are shown only if p<.05. In the lower set of panels, (**D**) U6, (**E**) Let-7a, and (**F**) miR-122 abundance are plotted as a function of miR-24 abundance. r_s = Spearman rank-order correlation coefficient.

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patients who responded poorly to treatment with pegylated IFN-α and ribavirin (Peg-IFN/RBV). Consistent with this, we observed a negative correlation between miR-122 abundance in non-tumor tissue from HCV-infected human subjects and the GT versus TT genotype at the rs8099917 locus in the IL28B gene (p=0.011, Figure 5A) that is predictive of a poor response to Peg-IFN/RBV therapy [37]. HCV-infected patients with the TT genotype are prone to a greater inflammatory response than those with TG or GG [38]. Thus, differences in IL28B genotype may have contributed to a correlation we observed between miR-122 abundance and A1 versus A2 Metavir activity scores (Figure 5B, 6 of 7 subjects with an A2 Metavir score had the TT genotype). Importantly, the association between IL28B genotype and miR-122 abundance was observed only in non-tumor liver from HCV-infected patients, and not in paired HCC tissue (Figure 5A).

Patients who are non-responsive to Peg-IFN/RBV, or who have IL28B genotypes predictive of a poor response to Peg-IFN/RBV therapy, are likely to have increased pre-treatment intra-hepatic ISG transcript levels compared to those who respond well to treatment [39–41]. We thus asked whether a correlation existed between miR-122 abundance and levels of selected ISG transcripts in HCV-infected non-tumor tissue determined by Affymetrix 133U Plus 2.0 GeneChip assay. For this analysis, we selected ISGs that were shown previously to be correlated with treatment response [39] (Figure 5C). We also included Mx1 and OAS1, both well-characterized ISGs. Overall, the Affymetrix signals for these genes showed a strong trend toward negative correlations with miR-122 abundance. Fourteen of 24 ISGs demonstrated a Spearman rank-order coefficient, $r_s \leq -0.300$; this negative correlation was significant ($p<0.05$) for 7 of the ISGs by one-tailed t test (Figure 3C). These data are consistent with the notion that reduced miR-122 abundance is associated with strong intrahepatic IFN-mediated responses to the virus.

miR-191 abundance is increased in HBV-associated HCC

Since Elyakim et al. [42] reported recently that miR-191 was increased in HCC arising in a study population comprised mostly of HBV-infected subjects, we also quantified miR-191 expression levels in the human tissue samples. We confirmed

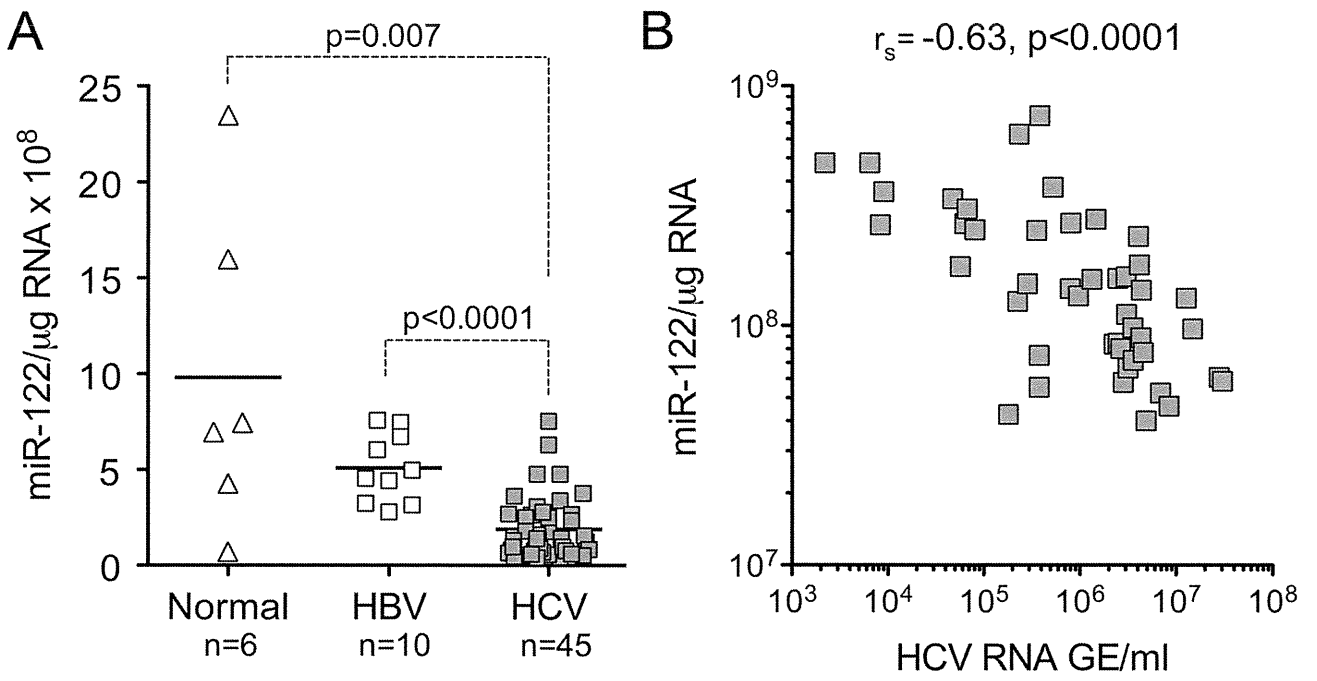

Figure 4. miR-122 expression in chimpanzee liver tissue. (**A**) Hepatic miR-122 abundance in liver biopsies from chimpanzees infected with HBV or HCV, or not infected with either virus ('normal'). Statistical significance was assessed by non-paired two-sided t test. Bars represent mean values. (**B**) Liver miR-122 expression plotted against serum HCV RNA abundance from acutely HCV-infected chimpanzees. r_s = Spearman rank-order correlation coefficient.

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miR-191 levels were modestly increased in HBV-associated HCC compared to non-tumor HBV-infected tissue when normalized to total RNA (p=0.049 by two-sided, paired t test, Figure 6). This trend remained significant only by one-sided t test when the miR-191 abundance was normalized to miR-24 abundance (p=0.045), and was absent when miR-191 levels were normalized to U6 snRNA. miR-191 abundance in non-tumor, HBV-infected tissue was similar to that in both tumor and non-tumor liver from HCV-infected subjects (Figure 6).

Discussion/Conclusions

miR-122 is a critical regulator of hepatic gene expression and an essential host factor for HCV replication [15,18,43]. It also has important tumor suppressor properties [16,44], and recent reports indicate that loss of its expression promotes carcinogenesis in knockout mice [45,46]. While its abundance is often reduced in HCC [21,22], two previous studies suggest that miR-122 expression may be preserved in liver cancer arising in patients with HCV infection [24,25]. We confirm this, showing in a genetically and geographically homogeneous population of patients, and normalizing results either to total RNA or to levels of miR-24, that miR-122 abundance is significantly reduced from normal in HBV-associated HCC but not in liver cancer associated with HCV infection (Figure 2A and B). This difference in miR-122 expression is independent of the histologic classification of the tumors (Figure 2C and D), as well as the degree of fibrosis or inflammation in paired non-tumor tissue from the same patients. Conversely, we show that miR-191 tends to be increased in abundance in HBV-associated cancer, but not HCV-associated HCC (Figure 6). These observations have important implications for the pathogenetic mechanisms involved in viral carcinogenesis within the liver. While HCC may arise as a result of factors common to both HBV and HCV infection (such as chronic inflammation, oxidative stress, and progressive fibrosis leading to cirrhosis), distinctive molecular signatures associated with HBV- versus HCV-associated cancer suggest there are fundamental differences in the ways these two viruses cause cancer.

Our study highlights the challenges inherent in comparing miRNA abundance in different clinical samples. In addition to potential differences in the proportion of cells present within a biopsy that are of hepatocellular origin vs. derived from other cell lineages, a constant concern is the quality of the RNA. While our initial analysis, like many studies, compared miR-122 copy numbers based on the quantity of total RNA subjected to RT-PCR, this approach can be biased by differences in the quality of the RNA and degree of RNA degradation. Although our RNA samples were of generally high quality (see Figure S1A in Supporting Information), we determined miR-24, Let-7a, and U6 snRNA copy numbers and evaluated each as a standard against which miR-122 abundance could be normalized to account for potential differences in RNA integrity (Figure 3). Median miR-24 and Let-7a copy numbers did not

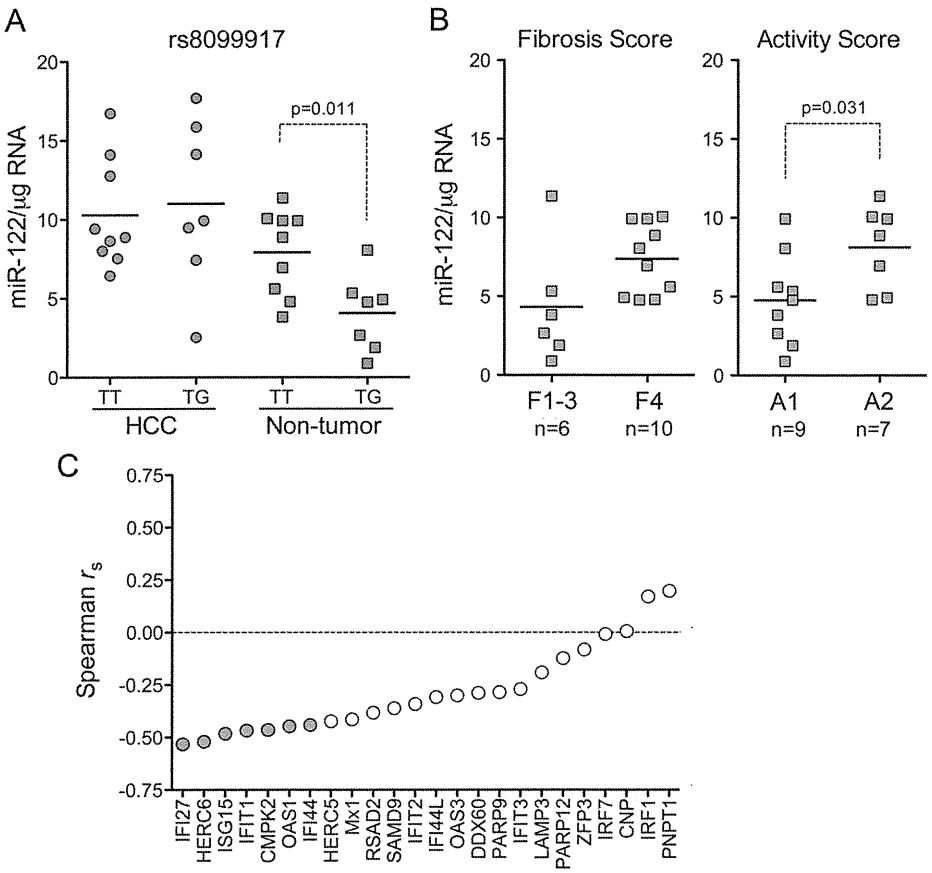

Figure 5. miR-122 expression, IL28B genotype, Metavir scores and ISG transcript levels in HCV-infected human liver. (**A**) miR-122 expression in HCC and paired non-tumor samples from subjects with HCV infection, grouped according to rs8099917 genotype (TT or GT). (**B**) miR-122 expression levels in non-tumor tissue from HCV-infected subjects categorized according to Metavir score for (left) fibrosis and (right) inflammatory activity. (**C**) Correlation between miR-122 abundance and expression levels of selected ISGs determined by Affymetrix U133 Plus 2.0 Array analysis. With the exception of OAS1 and Mx1, intrahepatic transcript levels of these ISGs have been shown previously to be predictive of Peg-IFN/RBV treatment outcome [31]. "r_s" = Spearman rank-order correlation coefficient. Filled symbols indicate a statistically significant negative correlation ($p<0.05$ by one-sided t test).

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vary significantly between tumor and non-tumor tissue samples from HBV- and HCV-infected subjects (one-way ANOVA), suggesting that the expression of these miRNAs is relatively constant in liver and that either could serve as a standard for normalizing miR-122 abundance. In contrast, median U6 copy numbers varied significantly between these tissue groups ($p = 0.004$ by one-way ANOVA with Kruskal-Wallis test) and, more importantly, were strongly correlated negatively with the RIN score, a measure of RNA integrity [29] (Figure S1A in Supporting Information). There was no correlation between the RIN score and miR-24 or Let-7a abundance, suggesting that U6 snRNA may be less stable and more prone to degradation than the miRNAs. This may be due to the greater length of U6 (106 nts vs. ~20-23 nts for miRNAs), or the absence of terminal modifications that may influence the stability of miRNAs [47]. Consistent with this, Let-7a was found to have greater biological stability and to be superior to U6 for normalization of miRNA abundance in previous studies of rat hepatocyte RNA [48]. Nonetheless, even though these data argue against the use of U6 as a standard for normalizing miR-122 copy numbers, we found the abundance of miR-122 was significantly reduced in HCC associated with HBV but not HCV infection, and that miR-122 abundance was significantly depressed in non-tumor tissue infected with HCV but not HBV, using any of these small RNAs, including U6, to normalize the miR-122 results.

While it remains unclear exactly how miR-122 contributes to the HCV lifecycle, it is known to promote viral replication independently of its regulation of hepatic genes [49]. It binds to two sites near the 5' end of the viral genome [18], recruiting argonaute 2 (EIF2C2) and physically stabilizing the RNA by protecting it from 5' exonucleolytic Xrn1-mediated decay [19,20]. However, miR-122 has other, positive effects on HCV replication beyond its ability to physically stabilize the viral

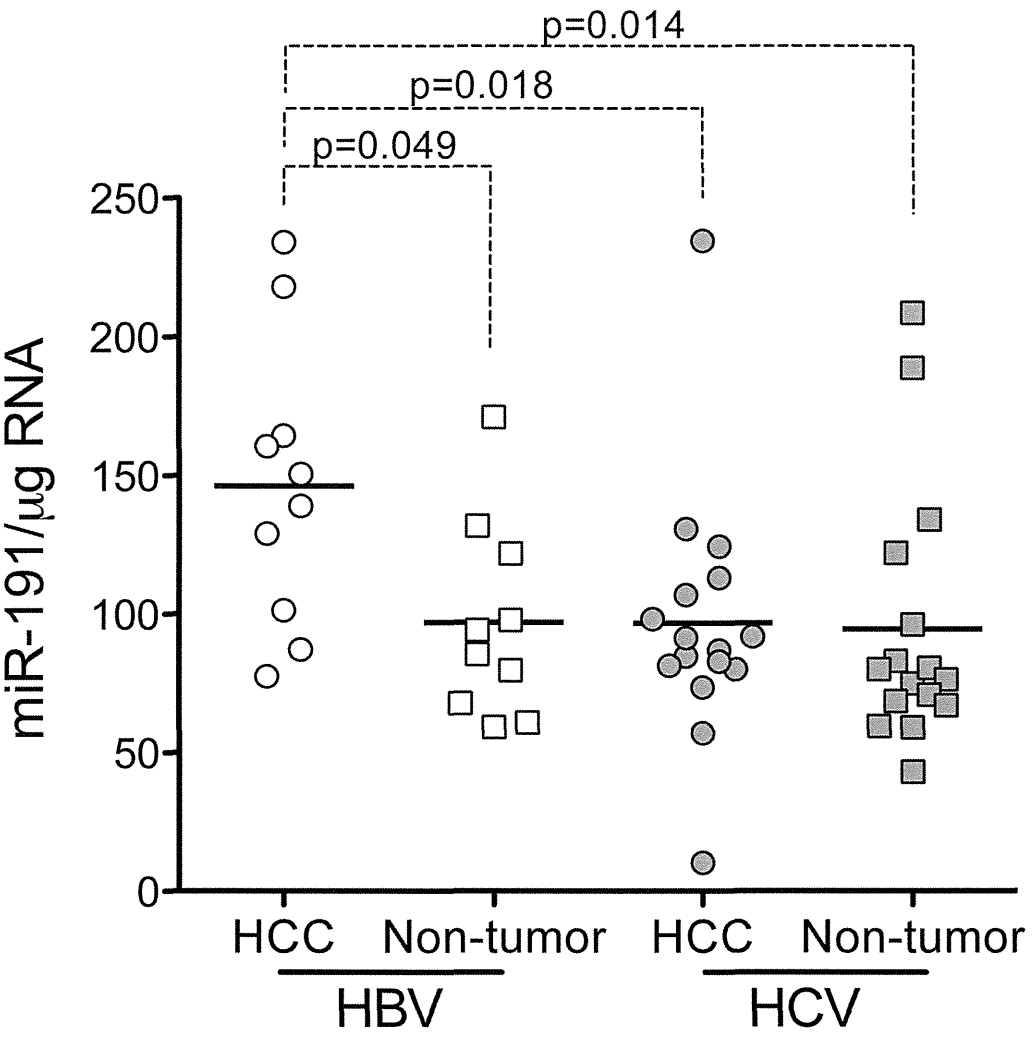

Figure 6. Relative abundance of miR-191 in paired HCC and non-tumor tissue from subjects with HBV or HCV infection. Relative miR-191 abundance in paired tumor and non-tumor samples from HBV- and HCV-infected subjects normalized to total RNA. Statistical significance was assessed in two-sided paired t tests for comparisons between tumor and non-tumor tissue, or two-sided unpaired t test for comparison between infection groups.

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genome [20,50]. It is essential for HCV replication, and its therapeutic silencing with an antisense oligonucleotide has potent antiviral effects [15,51]. No other RNA virus is known to rely so completely on a cellular miRNA for its replication cycle. Thus, the continued expression of miR-122 in HCV-associated HCC could reflect close linkage between carcinogenesis and HCV replication and viral protein expression. We speculate that miR-122 expression is preserved in HCV-associated HCC (in contrast to HBV-associated cancer) because HCV-encoded proteins help to drive a multi-stage process of carcinogenesis within infected cells. This may result from the ability of the virus to directly disable DNA damage responses or other cellular tumor suppressor functions, and to contribute directly to malignant conversion of hepatocytes as reviewed elsewhere [10,52]. Early loss of miR-122 during the progression to cancer would eliminate virus replication, protecting the cell from further effects of viral protein expression. In contrast, in HBV-infected cells, a loss of miR-122 expression could both accelerate tumorigenesis and enhance replication, as miR-122 appears to restrict, rather than promote, HBV replication [53–56]. Although speculative, this hypothesis raises the interesting possibility that HCV-associated cancers arise within the small minority of hepatocytes infected with the virus, and not the much larger number of uninfected bystander cells [52,57].

Epigenetic mechanisms are likely to contribute to the differential expression of miR-122 and miR-191 in HCC. The miR-122 promoter is hyper-methylated in the HBV-associated HCC-derived cell line, Hep3B [58]. It remains to be seen whether differences exist in methylation of the promoter in vivo in HBV- versus HCV-associated cancers, but bacterial artificial

chromosome array-based methylated CpG island amplification (BAMCA) studies indicate significant differences in the methylation patterns present in HBV- and HCV-associated HCC [59]. The HBx protein expressed by HBV may influence cellular methyltransferase activity, and could possibly contribute to altered methylation patterns [60]. An additional possibility is that epigenetic differences in HBV- and HCV-associated HCC could reflect different cell types from which the cancer originates, as HBV may be capable of infecting hepatocyte progenitors [61].

Our results also show that miR-122 expression is reduced in non-tumor liver tissue from HCV-infected persons. In contrast, contrary to a recent report by Wang et al. [54], we found that miR-122 is expressed at normal levels in non-tumor HBV-infected liver (Figure 2). Several lines of evidence suggest that this difference may reflect a more active IFN response in HCV- versus HBV- infected livers. In vitro studies suggest that IFN-β inhibits miR-122 expression [36,62], and HCV stimulates a more robust intrahepatic innate immune response than HBV [63,64]. Consistent with this, our results reveal a correlation between miR-122 abundance in non-tumor tissues and IL28B genotype, defined by a single nucleotide polymorphism (rs8099917) associated with response to Peg-IFN/RBV as well as endogenous pre-treatment ISG expression levels (Figure 5A) [40,41]. We also found an inverse relationship between the abundance of several ISG transcripts and miR-122 (Figure 5C). Interestingly, this relationship was not observed in tumor tissues from these patients, suggesting that genetic or epigenetic changes alter miR-122 regulation in HCC tissue, or that the cancer cells are refractory to stimulation by type 1 IFNs.

Consistent with our findings in HCV-infected patients, we also observed a reduction in miR-122 abundance in liver tissue from HCV-infected chimpanzees (Figure 4A), and an inverse correlation between the abundance of HCV RNA in the liver and serum HCV RNA levels (Figure 4B). Although Sarasin-Filopowicz et al. [36] demonstrated a trend toward lower miR-122 abundance in liver tissues with high viral RNA copy numbers, this did not achieve statistical significance and no correlation was evident between serum RNA levels and miR-122 abundance in the patients studied by this group. It is not clear why such a relationship exists in chimpanzees but not infected humans. One possibility is that it might be related to the fact that chimpanzees generally have very robust intrahepatic innate immune responses to HCV, with uniformly high levels of intrahepatic ISG expression [65]. The uniformly high intrahepatic innate immune response in chimpanzees contrasts with extensive variation in the intensity of ISG responses in HCV-infected humans [39], possibly allowing for a negative correlation between serum viral RNA level and miR-122 abundance to become manifest.

Finally, our results indicate that miR-191 expression may be increased in HBV-associated HCC (Figure 6). This supports a previous study in which miR-191 abundance was increased in HCC of mixed origin, but predominantly associated with HBV infection [42]. miR-191 antagonism has been shown to have anti-tumor potential in studies of Hep3B and SNU423 cells [42], which are both derived from HBV-associated cancers. Our data suggest that elevations of miR-191 are confined to HBV-associated liver cancer (Figure 6), and suggest that virus-specific differences in miRNA signatures may be important in understanding the origins of liver cancer. While these differences may be predictive of response to specific therapeutic interventions, they are unlikely to be of sufficient magnitude or specificity to guide therapy in individual patients.

Supporting Information

Figure S1. U6 snRNA copy number as a standard for normalization of miR-122 abundance. (**A**) U6 copy number (relative copy number per μg RNA) plotted as a function of the RNA integrity number (RIN score, on a scale of 1 to 10) determined as described in Methods in the main text. A strong negative correlation exists between U6 copy number and the RIN score: Spearman r_s = 0.5216, two-tailed p = 0.0001). (**B**) miR-122 abundance in HCC and non-tumor tissues from HBV- and HCV-infected subjects, normalized to U6 snRNA copy number. Statistical significance was assessed using paired and unpaired t tests, as described in the main text.
(TIF)

Author Contributions

Conceived and designed the experiments: CS MH SRS REL SML. Performed the experiments: CS SRS DY TS. Analyzed the data: CS MH SRS DY REL SML. Contributed reagents/materials/analysis tools: MH SK REL. Wrote the manuscript: CS SML.

References

1. Ferlay JSH, Bray F, Forman D, Mathers C, Parkin DM (2010). ancer Incidence Mortal Worldwide IARC CancerBase No. 10
2. Wang XGJ, Thorgeirsson S, editors (2011) Molecular Genetics of Liver Neoplasia. New York: Springer Verlag Science. pp. 51-73.
3. Lok AS, Everhart JE, Wright EC, Di Bisceglie AM, Kim HY et al. (2011) Maintenance peginterferon therapy and other factors associated with hepatocellular carcinoma in patients with advanced hepatitis C. Gastroenterology 140: 840-849. doi:10.1053/j.gastro.2010.11.050. PubMed: 21129375.
4. White D, El-Serag H (2011) Epidemiology of Hepatocellular Carcinoma. In: X Wang[!(surname)!]S Thorgeirsson. Molecular Genetics of Liver Neoplasia. New York, NY: Springer Verlag Science. pp. 51-73.
5. Kiyosawa K, Umemura T, Ichijo T, Matsumoto A, Yoshizawa K et al. (2004) Hepatocellular carcinoma: recent trends in Japan. Gastroenterology 127: S17-S26. doi:10.1053/j.gastro.2004.03.068. PubMed: 15508082.
6. McGivern DR, Lemon SM (2011) Virus-specific mechanisms of carcinogenesis in hepatitis C virus associated liver cancer. Oncogene 30: 1969-1983. doi:10.1038/onc.2010.594. PubMed: 21258404.
7. Tsai WL, Chung RT (2010) Viral hepatocarcinogenesis. Oncogene 29: 2309-2324. doi:10.1038/onc.2010.36. PubMed: 20228847.
8. Nakamoto Y, Guidotti LG, Kuhlen CV, Fowler P, Chisari FV (1998) Immune pathogenesis of hepatocellular carcinoma. J Exp Med 188: 341-350. doi:10.1084/jem.188.2.341. PubMed: 9670046.
9. Lerat H, Honda M, Beard MR, Loesch K, Sun J et al. (2002) Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. Gastroenterology 122: 352-365. doi:10.1053/gast.2002.31001. PubMed: 11832450.

10. McGivern DR, Lemon SM (2009) Tumor suppressors, chromosomal instability, and hepatitis C virus-associated liver cancer. Annu Rev Pathol 4: 399-415. doi:10.1146/annurev.pathol.4.110807.092202. PubMed: 18928409.
11. Wilson RC, Doudna JA (2013) Molecular mechanisms of RNA interference. Annu Rev Biophys 42: 217-239. doi:10.1146/annurev-biophys-083012-130404. PubMed: 23654304.
12. Djuranovic S, Nahvi A, Green R (2011) A parsimonious model for gene regulation by miRNAs. Science 331: 550-553. doi:10.1126/science.1191138. PubMed: 21292970.
13. Meijer HA, Kong YW, Lu WT, Wilczynska A, Spriggs RV et al. (2013) Translational repression and eIF4A2 activity are critical for microRNA-mediated gene regulation. Science 340: 82-85. doi:10.1126/science.1231197. PubMed: 23559250.
14. Chang J, Nicolas E, Marks D, Sander C, Lerro A et al. (2004) miR-122, a mammalian liver-specific microRNA, is processed from hcr mRNA and may downregulate the high affinity cationic amino acid transporter CAT-1. RNA Biol 1: 106-113. doi:10.4161/rna.1.2.1066. PubMed: 17179747.
15. Lanford RE, Hildebrandt-Eriksen ES, Petri A, Persson R, Lindow M et al. (2010) Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science 327: 198-201. doi:10.1126/science.1178178. PubMed: 19965718.
16. Bai S, Nasser MW, Wang B, Hsu SH, Datta J et al. (2009) MicroRNA-122 inhibits tumorigenic properties of hepatocellular carcinoma cells and sensitizes these cells to sorafenib. J Biol Chem 284: 32015-32027. doi:10.1074/jbc.M109.016774. PubMed: 19726678.
17. Lewis AP, Jopling CL (2010) Regulation and biological function of the liver-specific miR-122. Biochem Soc Trans 38: 1553-1557. doi:10.1042/BST0381553. PubMed: 21118125.
18. Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P (2005) Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. Science 309: 1577-1581. doi:10.1126/science.1113329. PubMed: 16141076.
19. Shimakami T, Yamane D, Jangra RK, Kempf BJ, Spaniel C et al. (2012) Stabilization of hepatitis C virus RNA by an Ago2-miR-122 complex. Proc Natl Acad Sci U S A 109: 941-946. doi:10.1073/pnas.1112263109. PubMed: 22215596.
20. Li Y, Masaki T, Yamane D, McGivern DR, Lemon SM (2013) Competing and noncompeting activities of miR-122 and the 5' exonuclease Xrn1 in regulation of hepatitis C virus replication. Proc Natl Acad Sci U S A 110: 1881-1886. doi:10.1073/pnas.1213515110. PubMed: 23248316.
21. Kutay H, Bai S, Datta J, Motiwala T, Pogribny I et al. (2006) Downregulation of miR-122 in the rodent and human hepatocellular carcinomas. J Cell Biochem 99: 671-678. doi:10.1002/jcb.20982. PubMed: 16924677.
22. Gramantieri L, Ferracin M, Fornari F, Veronese A, Sabbioni S et al. (2007) Cyclin G1 is a target of miR-122a, a microRNA frequently down-regulated in human hepatocellular carcinoma. Cancer Res 67: 6092-6099. doi:10.1158/0008-5472.CAN-06-4607. PubMed: 17616664.
23. Hou J, Lin L, Zhou W, Wang Z, Ding G et al. (2011) Identification of miRNomes in human liver and hepatocellular carcinoma reveals miR-199a/b-3p as therapeutic target for hepatocellular carcinoma. Cancer Cell 19: 232-243. doi:10.1016/j.ccr.2011.01.001. PubMed: 21316602.
24. Varnholt H, Drebber U, Schulze F, Wedemeyer I, Schirmacher P et al. (2008) MicroRNA gene expression profile of hepatitis C virus-associated hepatocellular carcinoma. Hepatology 47: 1223-1232. PubMed: 18307259.
25. Coulouarn C, Factor VM, Andersen JB, Durkin ME, Thorgeirsson SS (2009) Loss of miR-122 expression in liver cancer correlates with suppression of the hepatic phenotype and gain of metastatic properties. Oncogene 28: 3526-3536. doi:10.1038/onc.2009.211. PubMed: 19617899.
26. Bedossa P, Poynard T (1996) An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. Hepatology 24: 289-293. doi:10.1002/hep.510240201. PubMed: 8690394.
27. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ (1994) Classification of Chronic Hepatitis: Diagnosis, Grading and Staging. Hepatology 19: 1513-1520. doi:10.1002/hep.1840190629. PubMed: 8188183.
28. Honda M, Sakai A, Yamashita T, Nakamoto Y, Mizukoshi E et al. (2010) Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C. Gastroenterology 139: 499-509. doi:10.1053/j.gastro.2010.04.049. PubMed: 20434452.
29. Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M et al. (2006) The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol 7: 3. doi:10.1186/1471-2199-7-3. PubMed: 16448564.
30. Lanford RE, Guerra B, Lee H, Averett DR, Pfeiffer B et al. (2003) Antiviral effect and virus-host interactions in response to alpha interferon, gamma interferon, poly(i)-poly(c), tumor necrosis factor alpha, and ribavirin in hepatitis C virus subgenomic replicons. J Virol 77: 1092-1104. doi:10.1128/JVI.77.2.1092-1104.2003. PubMed: 12502825.
31. Nagaoki Y, Hyogo H, Aikata H, Tanaka M, Naeshiro N et al. (2012) Recent trend of clinical features in patients with hepatocellular carcinoma. Hepatol Res 42: 368-375. doi:10.1111/j.1872-034X.2011.00929.x. PubMed: 22151896.
32. Wang Y, Lu Y, Toh ST, Sung WK, Tan P et al. (2010) Lethal-7 is down-regulated by the hepatitis B virus x protein and targets signal transducer and activator of transcription 3. J Hepatol 53: 57-66. doi:10.1016/j.jhep.2009.12.043. PubMed: 20447714.
33. Hatziapostolou M, Polytarchou C, Aggelidou E, Drakaki A, Poultsides GA et al. (2011) An HNF4alpha-miRNA inflammatory feedback circuit regulates hepatocellular oncogenesis. Cell 147: 1233-1247. doi:10.1016/j.cell.2011.10.043. PubMed: 22153071.
34. Trebicka J, Anadol E, Elfimova N, Strack I, Roggendorf M et al. (2013) Hepatic and serum levels of miR-122 after chronic HCV-induced fibrosis. J Hepatol 58: 234-239. doi:10.1016/S0168-8278(13)60572-3. PubMed: 23085648.
35. Morita K, Taketomi A, Shirabe K, Umeda K, Kayashima H et al. (2011) Clinical significance and potential of hepatic microRNA-122 expression in hepatitis C. Liver Int 31: 474-484. doi:10.1111/j.1478-3231.2010.02433.x. PubMed: 21199296.
36. Sarasin-Filipowicz M, Krol J, Markiewicz I, Heim MH, Filipowicz W (2009) Decreased levels of microRNA miR-122 in individuals with hepatitis C responding poorly to interferon therapy. Nat Med 15: 31-33. doi:10.1038/nm.1902. PubMed: 19122656.
37. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K et al. (2009) Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41: 1105-1109. doi:10.1038/ng.449. PubMed: 19749757.
38. Yoshizawa H (2002) Hepatocellular carcinoma associated with hepatitis C virus infection in Japan: projection to other countries in the foreseeable future. Oncology 62 Suppl 1: 8-17. doi:10.1159/000048270. PubMed: 11868791.
39. Sarasin-Filipowicz M, Oakeley EJ, Duong FH, Christen V, Terracciano L et al. (2008) Interferon signaling and treatment outcome in chronic hepatitis C. Proc Natl Acad Sci U S A 105: 7034-7039. doi:10.1073/pnas.0707882105. PubMed: 18467494.
40. Urban TJ, Thompson AJ, Bradrick SS, Fellay J, Schuppan D et al. (2010) IL28B genotype is associated with differential expression of intrahepatic interferon-stimulated genes in patients with chronic hepatitis C. Hepatology 52: 1888-1896. doi:10.1002/hep.23912. PubMed: 20931559.
41. Abe H, Hayes CN, Ochi H, Maekawa T, Tsuge M et al. (2011) IL28 variation affects expression of interferon stimulated genes and peg-interferon and ribavirin therapy. J Hepatol 54: 1094-1101. doi:10.1002/hep.24499. PubMed: 21145800.
42. Elyakim E, Sitbon E, Faerman A, Tabak S, Montia E et al. (2010) hsa-miR-191 is a candidate oncogene target for hepatocellular carcinoma therapy. Cancer Res 70: 8077-8087. doi:10.1158/0008-5472.CAN-10-1313. PubMed: 20924108.
43. Jangra RK, Yi M, Lemon SM (2010) miR-122 regulation of hepatitis C virus translation and infectious virus production. J Virol 84: 6615-6625. doi:10.1128/JVI.00417-10. PubMed: 20427538.
44. Fornari F, Gramantieri L, Giovannini C, Veronese A, Ferracin M et al. (2009) MiR-122/cyclin G1 interaction modulates p53 activity and affects doxorubicin sensitivity of human hepatocarcinoma cells. Cancer Res 69: 5761-5767. doi:10.1158/0008-5472.CAN-08-4797. PubMed: 19584283.
45. Tsai WC, Hsu SD, Hsu CS, Lai TC, Chen SJ et al. (2012) MicroRNA-122 plays a critical role in liver homeostasis and hepatocarcinogenesis. J Clin Invest 122: 2884-2897. doi:10.1172/JCI63455. PubMed: 22820290.
46. Hsu SH, Wang B, Kota J, Yu J, Costinean S et al. (2012) Essential metabolic, anti-inflammatory, and anti-tumorigenic functions of miR-122 in liver. J Clin Invest 122: 2871-2883. doi:10.1172/JCI63539. PubMed: 22820288.
47. Burns DM, D'Ambrogio A, Nottrott S, Richter JD (2011) CPEB and two poly(A) polymerases control miR-122 stability and p53 mRNA translation. Nature 473: 105-108. doi:10.1038/nature09908. PubMed: 21478871.

48. Lardizábal MN, Nocito AL, Daniele SM, Ornella LA, Palatnik JF et al. (2012) Reference genes for real-time PCR quantification of microRNAs and messenger RNAs in rat models of hepatotoxicity. PLOS ONE 7: e36323. doi:10.1371/journal.pone.0036323. PubMed: 22563491.
49. Norman KL, Sarnow P (2010) Modulation of hepatitis C virus RNA abundance and the isoprenoid biosynthesis pathway by microRNA miR-122 involves distinct mechanisms. J Virol 84: 666-670. doi: 10.1128/JVI.01156-09. PubMed: 19846523.
50. Li Y, Masaki T, Lemon SM (2013) miR-122 and the Hepatitis C RNA genome: More than just stability. RNA Biol 10: 919–24. PubMed: 23770926.
51. Janssen HL, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M et al. (2013) Treatment of HCV Infection by Targeting MicroRNA. N Engl J Med, 368: 1685–94. PubMed: 23534542.
52. McGivern DR, Lemon SM (2011) Virus-specific mechanisms of carcinogenesis in hepatitis C virus associated liver cancer. Oncogene 30: 1969-1983. doi:10.1038/onc.2010.594. PubMed: 21258404.
53. Chen Y, Shen A, Rider PJ, Yu Y, Wu K et al. (2011) A liver-specific microRNA binds to a highly conserved RNA sequence of hepatitis B virus and negatively regulates viral gene expression and replication. FASEB J 25: 4511-4521. doi:10.1096/fj.11-187781. PubMed: 21903935.
54. Wang S, Qiu L, Yan X, Jin W, Wang Y et al. (2012) Loss of microRNA 122 expression in patients with hepatitis B enhances hepatitis B virus replication through cyclin G(1) -modulated P53 activity. Hepatology 55: 730-741. doi:10.1002/hep.24809. PubMed: 22105316.
55. Liu WH, Yeh SH, Chen PJ (2011) Role of microRNAs in hepatitis B virus replication and pathogenesis. Biochim Biophys Acta 1809: 678-685. doi:10.1016/j.bbagrm.2011.04.008. PubMed: 21565290.
56. Qiu L, Fan H, Jin W, Zhao B, Wang Y et al. (2010) miR-122-induced down-regulation of HO-1 negatively affects miR-122-mediated suppression of HBV. Biochem Biophys Res Commun 398: 771-777. doi:10.1016/j.bbrc.2010.07.021. PubMed: 20633528.
57. Liang Y, Shilagard T, Xiao SY, Snyder N, Lau D et al. (2009) Visualizing hepatitis C virus infections in human liver by two-photon microscopy. Gastroenterology 137: 1448-1458. doi:10.1053/j.gastro.2009.07.050. PubMed: 19632233.
58. Jung CJ, Iyengar S, Blahnik KR, Ajuha TP, Jiang JX et al. (2011) Epigenetic modulation of miR-122 facilitates human embryonic stem cell self-renewal and hepatocellular carcinoma proliferation. PLOS ONE 6: e27740. doi:10.1371/journal.pone.0027740. PubMed: 22140464.
59. Arai E, Ushijima S, Gotoh M, Ojima H, Kosuge T et al. (2009) Genome-wide DNA methylation profiles in liver tissue at the precancerous stage and in hepatocellular carcinoma. Int J Cancer 125: 2854-2862. doi: 10.1002/ijc.24708. PubMed: 19569176.
60. Jung JK, Park SH, Jang KL (2010) Hepatitis B virus X protein overcomes the growth-inhibitory potential of retinoic acid by downregulating retinoic acid receptor-beta2 expression via DNA methylation. J Gen Virol 91: 493-500. doi:10.1099/vir.0.015149-0. PubMed: 19828754.
61. Hsia CC, Thorgeirsson SS, Tabor E (1994) Expression of hepatitis B surface and core antigens and transforming growth factor-alpha in "oval cells" of the liver in patients with hepatocellular carcinoma. J Med Virol 43: 216-221. doi:10.1002/jmv.1890430304. PubMed: 7523580.
62. Pedersen IM, Cheng G, Wieland S, Volinia S, Croce CM et al. (2007) Interferon modulation of cellular microRNAs as an antiviral mechanism. Nature 449: 919-922. doi:10.1038/nature06205. PubMed: 17943132.
63. Wieland S, Thimme R, Purcell RH, Chisari FV (2004) Genomic analysis of the host response to hepatitis B virus infection. Proc Natl Acad Sci U S A 101: 6669-6674. doi:10.1073/pnas.0401771101. PubMed: 15100412.
64. Bigger CB, Guerra B, Brasky KM, Hubbard G, Beard MR et al. (2004) Intrahepatic gene expression during chronic hepatitis C virus infection in chimpanzees. J Virol 78: 13779-13792. doi:10.1128/JVI.78.24.13779-13792.2004. PubMed: 15564486.
65. Lanford RE, Guerra B, Bigger CB, Lee H, Chavez D et al. (2007) Lack of response to exogenous interferon-alpha in the liver of chimpanzees chronically infected with hepatitis C virus. Hepatology 46: 999-1008. doi:10.1002/hep.21776. PubMed: 17668868.

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ER stress induced impaired TLR signaling and macrophage differentiation of human monocytes

Takuya Komura [a,1], Yoshio Sakai [b,1], Masao Honda [a], Toshinari Takamura [a], Takashi Wada [b], Shuichi Kaneko [a,*]

[a] Disease Control and Homeostasis, Kanazawa University, 13-1, Takaramachi, Kanazawa 920-8641, Japan
[b] Department of Laboratory Medicine, Kanazawa University, 13-1, Takaramachi, Kanazawa 920-8641, Japan

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ABSTRACT

Endoplasmic reticulum (ER) stress causes impairment of the intracellular protein synthesis machinery, affecting various organ functions and homeostasis systems, including immunity. We found that ER stress induced by the N-linked glycosylation inhibitor, tunicamycin, caused susceptibility to apoptosis in the human monocytic cell line, THP-1 cells. Importantly, prior to tunicamycin-induced apoptosis, the proinflammatory response to toll-like receptor (TLR) 4 ligand lipopolysaccharide (LPS) stimulation was attenuated with respect to the expression of the proinflammatory cytokines. This impaired expression of proinflammatory cytokines was a consequence of the inhibition of NF-κB activation. Moreover, tunicamycin-induced ER stress disturbed the differentiation of THP-1 cells into macrophages induced by phorbol-12-myristate-13-acetate treatment. We also confirmed that ER stress affected the response of primary human monocytes to TLR ligand and their ability to differentiate into macrophages. These data suggest that ER stress imposes an important pathological insult to the immune system, affecting the crucial functions of monocytes.

1. Introduction

Homeostasis of physiological activities and structural components of the body requires properly functioning cellular machinery, including the components involved in protein synthesis. The endoplasmic reticulum (ER) plays a central role in the synthesis, maturation and assembly of secretary and structural proteins [1], and under some conditions, ER stress can disrupt ER function [2]. ER stress affects physiological cellular activities, disturbing cell-type specific functions or cell viability and inducing apoptosis [3]. ER stress has been observed in a variety of diseases, such as cancer, metabolic diseases, atherosclerosis and neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease [4–7]; however, it is not clear whether ER stress is the cause or consequence of these diseases.

Composed of many cell types, including monocytes, macrophages, dendritic cells (DCs) and lymphocytes, the immune system contributes to homeostasis by protecting the host from exogenous pathogens or harmful unexpected endogenous events, such as cancer. Monocytes are critical immune cells that express pattern-recognition molecules, toll-like receptors (TLRs), important for innate immunity against various pathogens [8,9]. Monocytes are also the progenitors for differentiation into macrophages or DCs, both of which are pivotal regulators of immune reaction [10].

Previously, we observed that human monocytes in patients with diabetes were under ER stress, were functionally impaired regarding TLR signaling and were vulnerable to apoptosis [11]. These observations suggested that ER stress is involved in the impairment of monocytes, thereby compromising host immunity.

In this study, we further examined how ER stress affects monocytes. When primary human monocytes and THP-1 human monocytic cell line cells were stimulated with toll-like receptor (TLR) 4 ligand lipopolysaccharide (LPS), we observed that monocytes were attenuated in their capability to secrete proinflammatory cytokines. Moreover, when cells were under ER stress induced by the N-linked glycosylation inhibitor, tunicamycin, decreased activation of NF-κB was observed. In addition to this functional impairment of cytokine expression, ER stress also impaired monocyte differentiation into macrophages. Interestingly, the TLR4 signaling in differentiated macrophages was also impaired under ER stress. These results demonstrate that ER stress is an important pathological condition that broadly affects monocyte-lineage cells, influencing the innate immune system function of monocytes and macrophages as well as the differentiation capability of progenitor cells for antigen presentation.

* Corresponding author. Address: Disease Control and Homeostasis, Graduate School of Medical Science, Kanazawa, University, Kanazawa 920-8641, Japan. Fax: +81 76 234 4250.
E-mail address: skaneko@m-kanazawa.jp (S. Kaneko).
[1] These authors contributed equally to this work.

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2. Materials and methods

2.1. Human monocytes

THP-1 cells, a human monocytic cell line, were obtained from American Type Culture Collection (Manassas, VA). THP-1 cells were cultured in RPMI1640 culture medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen). Human primary monocytes were obtained from healthy volunteers as follows: peripheral blood mononuclear cells (PBMCs) were freshly isolated from heparinized venous blood using Ficoll–Hypaque (Sigma–Aldrich, St. Louis, MO). $CD14^+$ monocyte subpopulations were isolated using a magnetic cell sorting system in accordance with the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany).

2.2. Induction of macrophage-differentiation

For macrophage differentiation, THP-1 cells were cultured in medium supplemented with Phorbol-12-myristate-13-acetate (PMA) (50 ng/ml) (Sigma–Aldrich) for 72 h. Primary human monocytes were cultured in medium supplemented with GM-CSF (100 ng/ml) (Sigma–Aldrich) for 4 days. Macrophage differentiation was assessed by morphology under microscopic examination and expression of the macrophage-related surface markers, CD11b and CD68, as analyzed by flow cytometry analysis and quantitative real-time detection PCR (RTD-PCR).

2.3. ER stress induction

To induce ER stress, THP-1 cells were treated with 1 or 5 μg/ml of tunicamycin (Sigma–Aldrich) and 3 μM thapsigargin (Sigma–Aldrich) for 12 h. The treated cells were assessed for apoptosis and cytokine expression in response to LPS stimulation.

2.4. Flow cytometry analysis

Flow cytometry analysis was performed as previously reported. For the surface molecule expression, cells were incubated with phycoerythrin (PE)-labeled anti-TLR4 (eBioscience, San Diego, CA), anti- CD11b and CD68 (BD Biosciences, San Jose, CA) in PBS containing 2% bovine serum albumin (BSA) (Sigma–Aldrich). For assessment of apoptosis, cells were incubated with fluorescein isothiocyanate (FITC)-labeled anti-CD14 antibody (BD Bioscience) and with PE-labeled Annexin-V and 7-amino-actinomycin D (7-AAD, Apoptosis Detection Kit I, BD Biosciences). Apoptotic cells were determined by flow cytometry for the fraction of cells labeled with Annexin-V that were 7-AAD negative using a FACSCalibur™ flow cytometer (BD Biosciences). Data were analyzed using CELLQuest™ Software (BD Biosciences). At least 10,000 cells per sample were analyzed.

2.5. RTD-PCR

RTD-PCR was performed as previously described [12]. Briefly, total RNA obtained from cells using a MicroRNA isolation kit (Stratagene, La Jolla, CA) was reverse-transcribed using 1 μg oligo (dT) primer and Super Script II reverse transcriptase (Invitrogen) in accordance with the manufacturers' protocol. The relative quantities of mRNA expression were analyzed by RTD-PCR using an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). Primer pairs and probes for BCL-2, BCL-XL, TLR4, MyD88, TNF-α, IL-1β, C/EBP homologous protein (CHOP), immunoglobulin heavy chain binding protein (BiP), CD11b, CD68, and β-actin were obtained from the TaqMan assay reagents library. Gene expression levels were calculated with the $2^{-\triangle\triangle Ct}$ method using β-actin as the internal control.

2.6. TLR ligand stimulation

LPS (1 μg/ml) from *E. coli* (Sigma–Aldrich) was added to conditioned THP-1 cells (3×10^5 cells) or PMA-induced differentiated cells in AIM-V culture medium (Invitrogen). After 3 h incubation, the gene expression of TNF-α and IL-1β was analyzed by RTD-PCR. In addition, the concentration of TNF-α and IL-1β in the culture medium supernatants after 12 h LPS stimulation was measured using an ELISA kit (eBioscience) in accordance with the manufacturer's protocol. With regard to primary human monocytes (5×10^5 cells), the gene expression of TNF-α and IL-1β was analyzed by RTD-PCR after 4 h LPS stimulation. The concentration of TNF-α and IL-1β in the culture medium supernatants after 12 h LPS stimulation was measured using an ELISA kit.

2.7. Measurement of caspase-3 activity

THP-1 cells (3×10^5) were harvested and treated with tunicamycin (5 μg/ml) in RPMI1640 culture medium. After 48 or 72 h incubation, THP-1 cells were lysed, and the DEVD-cleaving activity of active caspase-3 in the lysate was measured using labeled Asp-Glu-Val-Asp-p-nitroanilide (DEVD-pNA) as the substrate and the Caspase-3 Colorimetric Assay Kit (Promega, Madison, WI) in accordance with manufacturer's protocol. The *p*NA light emission was quantified using a microtiter plate reader at a wavelength of 405 nm.

2.8. Western blot

Conditioned THP-1cells were washed twice with PBS and lysed in 100 μl of lysis buffer (10 mM Tris–HCl (pH 7.4), 1% SDS). A total of 10 μg of total proteins were loaded per well and separated on a 7% SDS–PAGE by electrophoresis, followed by transfer to a nitrocellulose membrane. The transferred nitrocellulose membranes were probed with anti-TLR4, anti-MyD88 or anti-β-actin antibody (Cell Signaling Technology, Danvers, MA) at a concentration of 1:500. The secondary goat-anti-rabbit antibody conjugated with horseradish peroxidase (HRP) was used at a concentration of 1:1000 (Cell Signaling Technology). The membrane was visualized by chemiluminescence using the ECL kit (Amersham Biosciences, Piscataway, NJ). Each expression level was assessed and compared to β-actin expression using densitometry.

2.9. Microscopic observation of immunofluorescent cells

For assessment of NF-κB nuclear translocation, THP-1 cells were treated with tunicamycin for 12 h, followed by stimulation with LPS (1 μg/ml) for 40 min. Treated cells were plated at a density of 1×10^5 cells per 22-mm glass coverslip. Cells were fixed for 30 min in cold 4% paraformaldehyde, permeabilized for 2 min at 25 °C in 0.25% Triton X-100 and incubated with PBS supplemented with 10% BSA. Fixed and permeabilized cells were incubated with PBS with 10% BSA containing anti-NF-κB p65 (Cell Signaling Technology). After being washed three times in PBS, coverslips were incubated with the anti-rabbit IgG conjugated with FITC (Jackson Immunoresearch, West Grove, PA). Nuclei were stained with 4, 6-diamino-2-phenylindole (Vector Laboratories, Burlingame, CA). Fluorescent cells were examined with a laser-scanning confocal microscope (Radiance 2100; Bio-Rad, Hercules, CA).

2.10. NF-κB ELISA

Quantitative analysis of NF-κB p65 activation was performed using the CASE™ activation of signaling ELISA kit for NF-κB p65 S536 (SABiosciences Corporation, MD) according to the manufacturer's instructions with slight modifications. Briefly, 1.5×10^4 - cells were seeded and incubated on the 96-well plate overnight. Cells were fixed, washed and blocked to avoid non-specific antibody binding. Cells were incubated with anti-pan-NF-κB p65 S536 antibody or anti-phospho-NF-κB specific antibody, washed, and incubated with the secondary antibody. After washing cells, colorimetric detection was performed using a microtiter plate reader at a wavelength of 450 nm.

2.11. Statistical analysis

Data are expressed as means ± SEM. The Mann–Whitney *U* test was applied to assess the significant difference between the two groups. Statistical significance was determined as $P < 0.05$.

3. Results

3.1. Attenuation of TLR signaling prior to apoptosis in human monocytes under ER stress

We examined how ER stress affects the viability of THP-1 cells by assessing the frequency of apoptotic THP-1 cells when treated with tunicamycin (5 μg/ml). Apoptotic cells were defined as cells positive for Annexin-V and negative for 7-AAD by flow cytometry. As shown in Fig. 1A and B, a low frequency of apoptotic cells was observed at 12 h. After 24 h, an increased frequency of apoptosis was observed among THP-1 cells treated with tunicamycin compared to untreated cells. When cells were treated with tunicamycin for 48 h and 72 h, the activity of the pro-apoptotic protease, caspase-3, was significantly increased (Fig. 1C). Furthermore, the expression of the anti-apoptotic genes, BCL-2 and BCL-XL was substantially lower in tunicamycin-treated THP-1 cells compared with untreated cells (Fig. 1D). When cells were treated with tunicamycin, the activity of the pro-apoptotic protease caspase-3 was significantly increased after 48 h (Fig. 1C), though not at 12 h (data not shown). We also observed similar results using 1 μg/ml tunicamycin treatment, but alternations were less compared with 5 μg/ml tunicamycin treatment (data not shown). These results suggest that ER stress induced conventional apoptosis in the human monocytic cell line.

Monocytes typically express pattern-recognition molecules such as TLRs that are important for innate immunity against various pathogens. TLR4 is the receptor for the LPS ligand, and plays a role in host defense against gram-negative infection [13]. To determine whether THP-1 cells were functionally affected under ER stress induced by tunicamycin treatment, we assessed the responsiveness of THP-1 cells to LPS stimulation *in vitro*.

We examined whether the expression of TLR4 and MyD88, a crucial adaptor molecule for TLR signaling, was affected in THP-1 cells under ER stress induced by tunicamycin treatment at 12 h. We confirmed that tunicamycin treatment induced ER stress on THP-1 cells at 12 h, observing that the expression levels of pivotal genes related to ER stress, CHOP and BiP, were up-regulated (Fig. 2A). Whereas expression of TLR4 and MyD88 was not affected

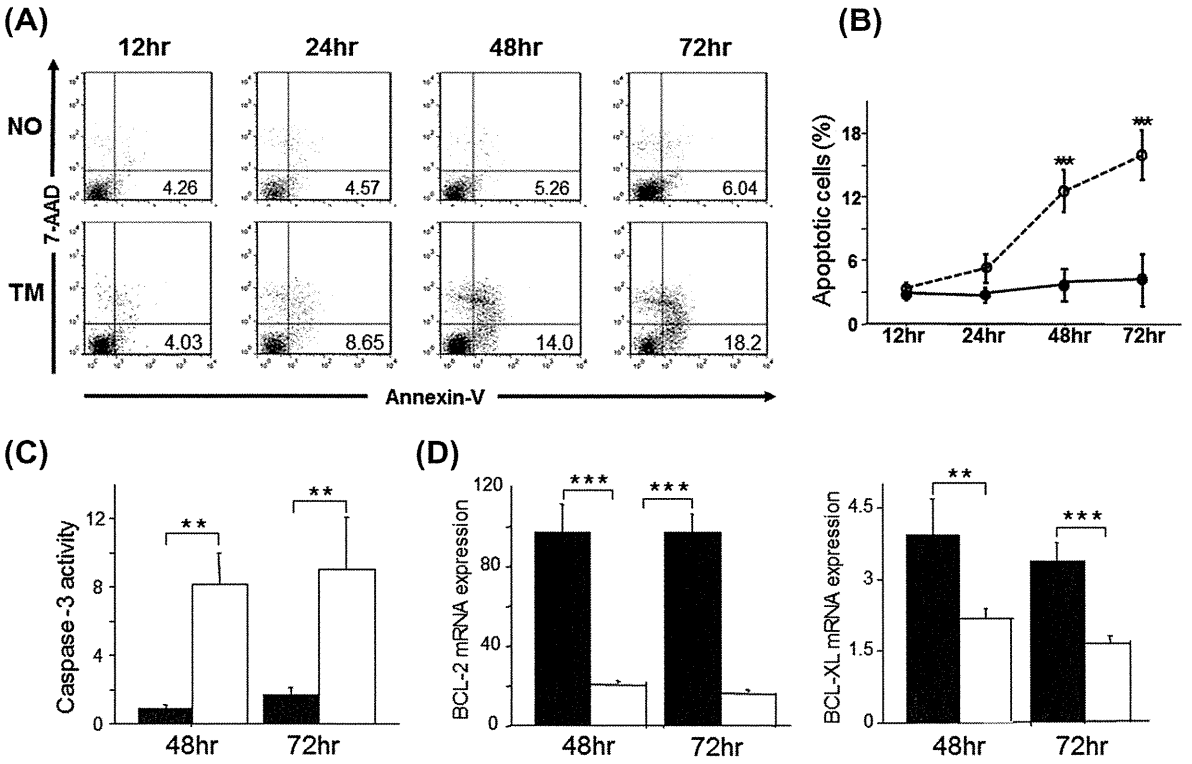

Fig. 1. ER stress increased the susceptibility of THP-1 cells to apoptosis. (A–B) THP-1 cells were incubated in culture medium supplemented with tunicamycin (5 μg/ml). The frequency of apoptotic cells was analyzed by flow cytometry at 12 h, 24 h 48 h and 72 h. More apoptotic cells were observed among the THP-1 cells treated with tunicamycin for more than 48 h incubation compared to the untreated THP-1 cells. (A) A representative scatter gram of Annexin-V and 7-AAD for THP-1 cells treated with tunicamycin. The numbers in each quadrant indicate the percentage of apoptotic cells. (B) The average number of apoptotic cells was calculated in triplicate for each condition. (C) Caspse-3 activity in THP-1 cells treated with tunicamycin was significantly increased at 48 h and 72 h incubation. (D) The BCL-2 and BCL-XL expressions in THP-1 cells incubated with tunicamycin for 48 h and 72 h were significantly down-regulated. Filled circle, no treatment; open circle, treatment with tunicamycin (5 μg/ml). TM, tunicamycin. Data are expressed as means ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$.

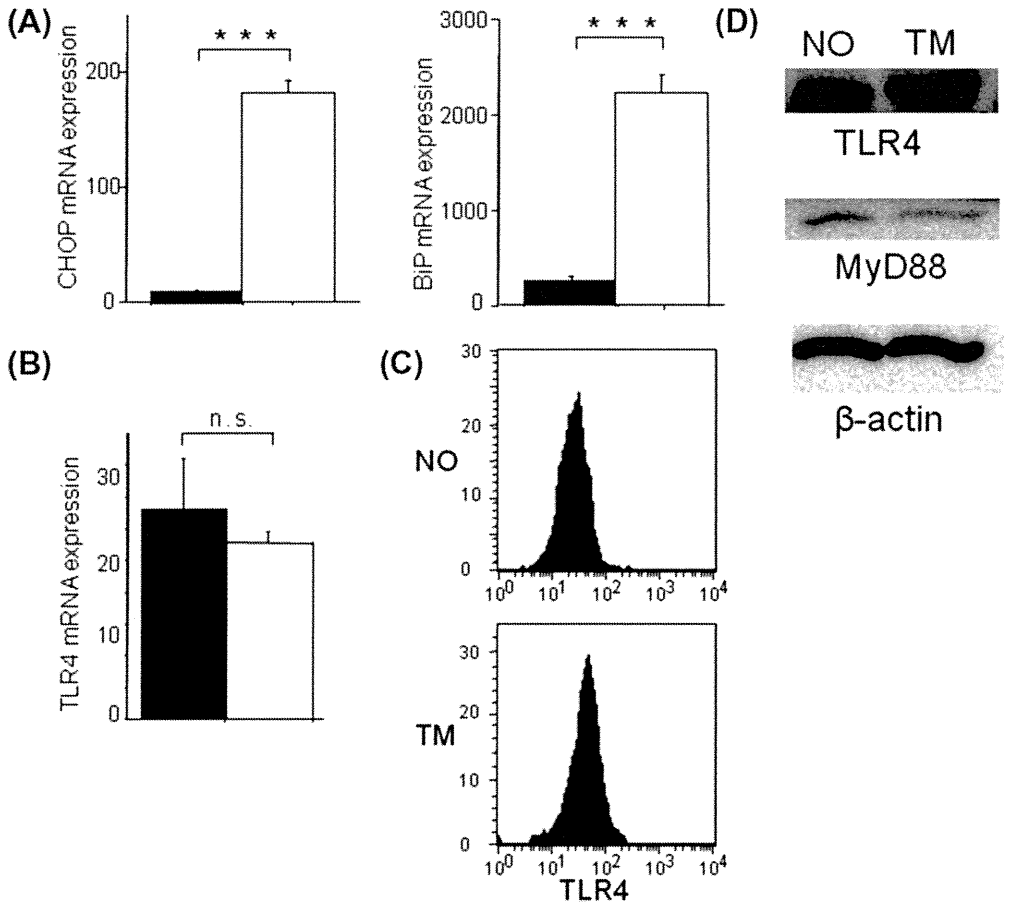

Fig. 2. Tunicamycin treatment induced ER-stress related molecules, but did not affect the expression of TLR4 and its accessory molecule MyD88 in THP-1 cells. (A) The transcriptional expression levels of the ER stress markers CHOP and BiP in THP-1 cells incubated with tunicamycin for 12 h were significantly up-regulated (RTD-PCR). Data are expressed as means ± SD of four independent experiments. (B–C) The expression of TLR4 was not affected by 12 h incubation with tunicamycin, as assessed by RTD-PCR (B), flow cytometry (C) and Western blot (D) Similarly, MyD88 expression was not affected (D). Filled bars, no treatment; open bars, treatment with tunicamycin (5 μg/ml). $^{***}P < 0.001$.

with 12 h incubation with tunicamycin (Fig. 2B–D), the gene expression of the proinflammatory cytokines, TNF-α and IL-1β, was down-regulated in the tunicamycin-treated THP-1 cells that were stimulated with LPS for 3 h (Fig. 3A). Concomitantly, the concentration of TNF-α and IL-1β in culture medium was also decreased after 12 h stimulation with LPS (Fig. 3B), indicating that the production of these cytokines from THP-1 cells was diminished. We also observed similar results using a lower concentration of tunicamycin (1 μg/ml), along with another ER stress inducer, thapsigargin, which is an endoplasmic reticulum Ca^{2+} ATPase inhibitor (Supplemental Fig. 1). Both 12 h tunicamycin (1 μg/ml) and 12 h thapsigargin (3 μM) treatments induced ER stress, but did not induce apoptosis, alteration of caspase-3 activity, or expression of anti-apoptotic genes (data not shown).Furthermore, we confirmed a similar effect of ER stress attenuation of TLR4 signaling in primary human monocytes (Fig. 3C and D). The gene expression of IL-1β was significantly down-regulated in tunicamycin-treated primary human monocytes that were stimulated with LPS for 4 h (Fig. 3C), and the gene expression of TNF-α was slightly down-regulated. The concentration of TNF-α and IL-1β in culture medium was also decreased after 12 h stimulation with LPS (Fig. 3D). These results demonstrate that TLR signaling in human monocytes under tunicamycin-induced ER stress was functionally impaired with regard to the TLR4 stimulation-signaling to produce proinflammatory cytokines.

3.2. ER stress inhibited the activation of NF-κB in THP-1 cells stimulated with LPS

To further assess how ER stress affects the expression of proinflammatory cytokines, we examined the expression of NF-κB, a pivotal transcriptional factor of proinflammatory cytokines [14,15]. THP-1 cells treated with tunicamycin were stimulated with LPS, and the activation of NF-κB was assessed every 40 min up to 120 min. Before LPS stimulation, no significant difference in NF-κB activation was observed, with or without treatment of tunicamycin in THP-1 cells (Fig. 4A). However, after LPS stimulation, NF-κB activation was not induced in THP-1 cells under ER stress compared with untreated THP-1 cells (Fig. 4A). Concomitantly, the translocation of NF-κB into the nucleus induced by LPS stimulation for 40 min was not observed in untreated THP-1 cells when cells were under ER stress (Fig. 4B). Furthermore, we examined the translocation of NF-κB induced by TNF-α stimulation in THP-1 cells under ER stress to clarify whether other stimulation also affects the activation of NF-κB under ER stress. We did not observed significant translocation of NF-κB following TNF-α stimulation, as compared with LPS stimulation (Supplemental Fig. 2), suggesting that the effect on NF-κB mobilisation under ER stress was caused only by TLR signaling. These results demonstrate that activation of NF-κB in response to the LPS stimulation was impaired in THP-1 cells under ER stress.