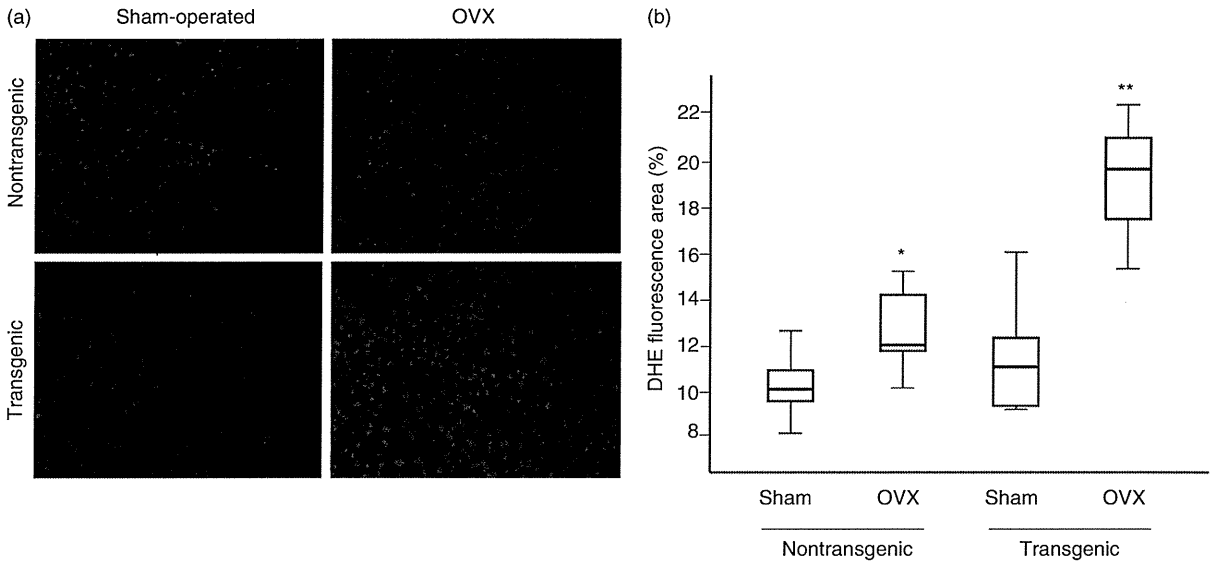

Figure 2 Reactive oxygen species (ROS) production in sham-operated and ovariectomized (OVX) FL-N/35 transgenic and non-transgenic mice. (a) Frozen liver sections from mice in each group were stained with dihydroethidium (DHE). (b) Fluorescence intensity was quantified by NIH image analysis software for three randomly selected areas of digital images for five mice in each group. The results are shown as box plot profiles. The bottom and top edges of the boxes are the 25th and 75th percentiles, respectively. Median values are shown by the line within each box. *: $P < 0.05$ versus sham-operated non-transgenic mice. **: $P < 0.05$ versus sham-operated nontransgenic mice, OVX non-transgenic mice and sham-operated transgenic mice.

oxide) production in both transgenic mice and non-transgenic mice, but the level of ROS production was greater in the OVX transgenic mice than in the OVX non-transgenic mice (Fig. 2). We next measured inflammatory cytokine levels in the liver. Ovariectomy significantly increased hepatic expression of IL-6 mRNA to the same degree in both transgenic mice and non-transgenic mice (Fig. 3). This ovariectomy-induced increase in hepatic IL-6 mRNA was consistent with the results of a previous report that OVX mice produced more hepatic

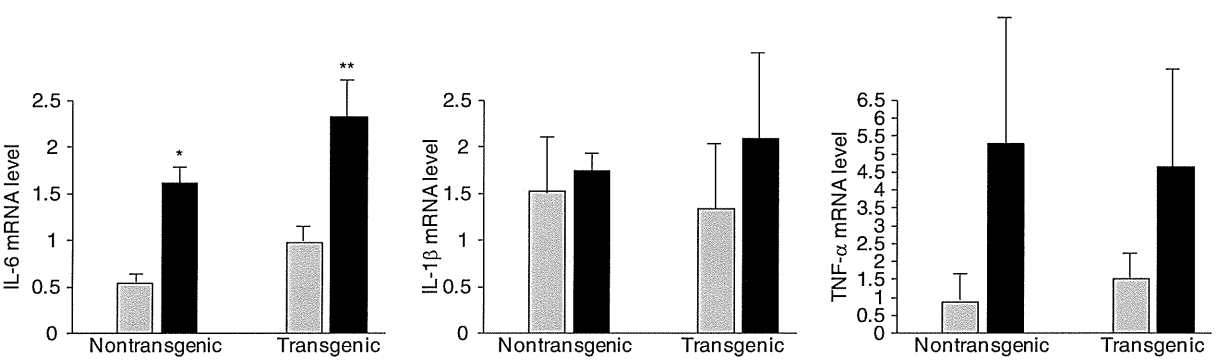

Figure 3 Expression levels of inflammatory cytokines in sham-operated and ovariectomized (OVX) FL-N/35 transgenic and non-transgenic mice. The mRNA levels of interleukin (IL)-6, IL-1β and tumor necrosis factor (TNF)-α were measured by real-time reverse transcription polymerase chain reaction for five mice in each group. The relative quantities of target mRNA in the liver were normalized with GAPDH mRNA. *$P < 0.05$ vs sham-operated non-transgenic mice. **$P < 0.05$ vs sham-operated transgenic mice. □, Sham; ■, OVX.

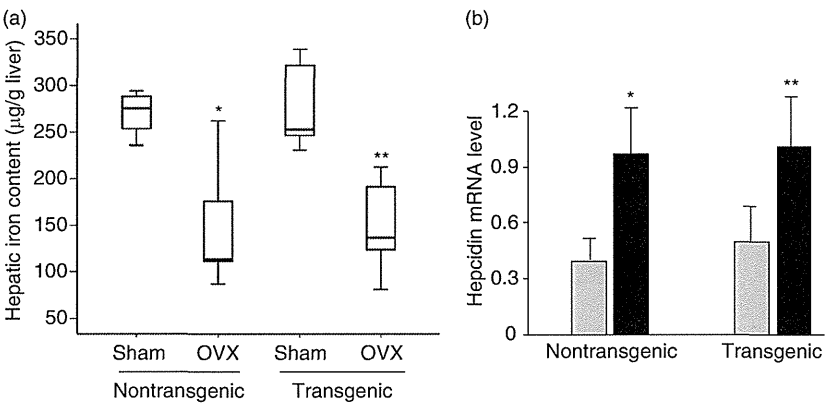

Figure 4 Hepatic iron content and hepcidin mRNA level in sham-operated and ovariectomized (OVX) FL-N/35 transgenic and non-transgenic mice. (a) Hepatic iron content in mice in each group ($n = 5$). The results are shown as box plot profiles. The bottom and top edges of the boxes are the 25th and 75th percentiles, respectively. Median values are shown by the line within each box. $^*P < 0.05$ vs sham-operated non-transgenic mice. $^{**}P < 0.05$ vs sham-operated transgenic mice. (b) The mRNA level of hepcidin was measured by real-time reverse transcription polymerase chain reaction for five mice in each group. The relative quantities of target mRNA in the liver were normalized with GAPDH mRNA. $^*P < 0.05$ vs sham-operated non-transgenic mice. $^{**}P < 0.05$ vs sham-operated transgenic mice. □, Sham; ■, OVX.

IL-6 than non-OVX mice after chemically induced liver injury.[5] There also was a trend for increase in TNF-α and IL-1β mRNA expression after ovariectomy in both the transgenic mice and non-transgenic mice, but their increases did not reach statistical significance, probably because of the large deviation (Fig. 3). These results suggested that inflammatory cytokines were unlikely to be associated with greater ROS production in OVX transgenic mice than in OVX non-transgenic mice.

Hepatic iron content and hepcidin expression level in the liver

We previously reported that male FL-N/35 transgenic mice developed hepatic iron accumulation through the reduced transcription of hepcidin,[18] a negative regulator in iron homeostasis.[21,22] Excess divalent iron can be highly toxic, mainly via the Fenton reaction producing hydroxyl radicals.[23] Therefore, we measured hepatic iron content to assess whether greater ROS production resulted from increased hepatic iron accumulation in OVX transgenic mice. Unexpectedly, ovariectomy significantly decreased hepatic iron content to the same degree in both transgenic mice and non-transgenic mice (Fig. 4a). These results are potentially explained by significantly increased transcription of hepcidin after ovariectomy (Fig. 4b). Ovariectomy-induced increase in hepatic IL-6 mRNA may in turn account for increased hepcidin transcription, because IL-6 acts to stimulate hepcidin expression through the STAT3 pathway.[24] These results suggested that hepatic iron content was not related to greater ROS production in OVX transgenic mice than in OVX non-transgenic mice.

Attenuated antioxidant potential against ovariectomy-induced ROS production in FL-N/35 transgenic mice

The increase in inflammatory cytokine production and the hepatic iron content after ovariectomy were comparable in transgenic and non-transgenic mice. Nevertheless, the serum ALT level, hepatic steatosis and ROS production were greater in OVX transgenic mice than in OVX non-transgenic mice. Therefore we measured dROM and BAP in serum to compare antioxidant potentials in OVX transgenic and OVX non-transgenic mice. We confirmed the significant negative correlation between the ratio of BAP to dROM and hepatic content of superoxide (Fig. 5). As expected, the values for dROM were higher in OVX mice than in sham-operated mice, regardless of whether they were transgenic or non-transgenic. However, a significant increase in the BAP value was found in OVX non-transgenic mice but not in OVX transgenic mice, which resulted in a lower ratio of BAP to dROM in the OVX transgenic mice than in the OVX non-transgenic mice (Table 2).

The first line of defense against ROS is the detoxifying enzymes that scavenge ROS. These include SOD and

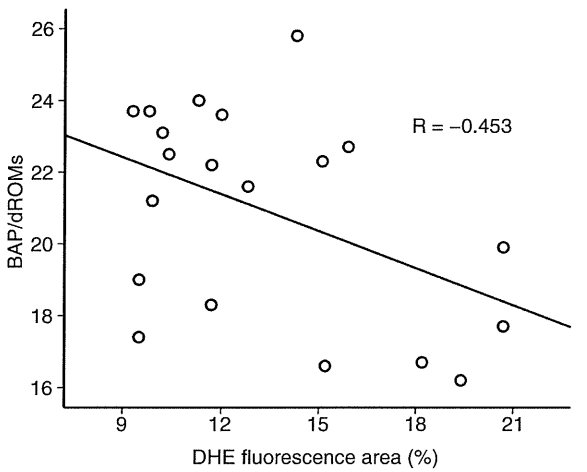

Figure 5 Negative correlation between the ratio of biological antioxidant potential (BAP) to derivatives of reactive oxygen metabolites (dROM) and hepatic content of superoxide. $R = -0.453$, $P < 0.05$. Hepatic content of superoxide was determined based on the area of dihydroethidium (DHE) fluorescence.

GPx1. Therefore we next investigated the expression levels of SOD2 and GPx1. The hepatic expression levels of SOD2 mRNA and GPx1 mRNA were significantly greater in OVX non-transgenic mice than in sham-operated non-transgenic mice, but were comparable in OVX transgenic mice and sham-operated transgenic mice (Fig. 6a). Western blot analysis of the hepatic mitochondria fractions also showed significant increases of SOD2 and GPx1 expression in OVX non-transgenic mice but not in OVX transgenic mice (Fig. 6b). These results suggested that antioxidant defense mechanisms may be induced against ovariectomy-related ROS production in non-transgenic mice but not in transgenic mice.

SIRT3 and PGC-1α expression in OVX FL-N/35 transgenic mice

Proliferator-activated receptor-γ co-activator-1α is a master regulator of mitochondrial biogenesis and respiration[25] and required for the induction of many ROS-detoxifying enzymes, including SOD2 and GPx1 upon oxidative stress.[26] SIRT3 is a member of a class III histone deacetylase and is reported to mediate PGC-1α-dependent induction of ROS-detoxifying enzymes.[27] In accordance with the changes in SOD2 and GPx1 levels after ovariectomy, the hepatic expression of SIRT3 mRNA was significantly greater in OVX non-transgenic mice than in sham-operated non-transgenic mice, but comparable in OVX transgenic mice and sham-operated transgenic mice (Fig. 7a). Western blot analysis of hepatic mitochondria showed a significant increase of SIRT3 expression in OVX non-transgenic mice but not in OVX transgenic mice (Fig. 7a).

Proliferator-activated receptor-γ co-activator-1α interacts with various nuclear receptors in addition to peroxisome proliferator-activated receptor-γ and is docked to the promoter of its target genes by all these nuclear receptors. Therefore, we investigated PGC-1α expression levels not only in liver homogenates but also in the nuclear fraction of mouse liver. The expression levels of PGC-1α in liver homogenates were comparable in sham-operated and OVX non-transgenic mice and in sham-operated and OVX transgenic mice. However, the expression levels of PGC-1α in the nuclear fraction of the liver significantly increased after ovariectomy in both non-transgenic and transgenic mice, and OVX transgenic mice had a lower PGC-1α expression level than OVX non-transgenic mice (Fig. 7b). These results suggested that the antioxidant potential against ovariectomy-induced ROS production may be reduced in OVX transgenic mice through lesser activation of PGC-1α than in OVX non-transgenic mice.

Table 2 Derivatives of reactive oxygen metabolites (dROM), biological antioxidant potential (BAP) and ratio of BAP to dROM

	Non-transgenic		Transgenic	
	Sham-operated	OVX	Sham-operated	OVX
dROM (U.CARR)	145.2 ± 15.1	158.7 ± 15.9*	170.8 ± 10.4	199.3 ± 21.1**
BAP (μmol/L)	3217 ± 123	3644 ± 177*	3362 ± 178	3542 ± 140
Ratio of BAP to dROM	22.3 ± 2.3	23.1 ± 2.0	20.8 ± 1.8	17.8 ± 1.9***

Data are mean ± standard deviation.
*$P < 0.05$ compared with sham-operated non-transgenic mice. **$P < 0.05$ compared with sham-operated transgenic mice. ***$P < 0.05$ compared with ovariectomized (OVX) non-transgenic mice.

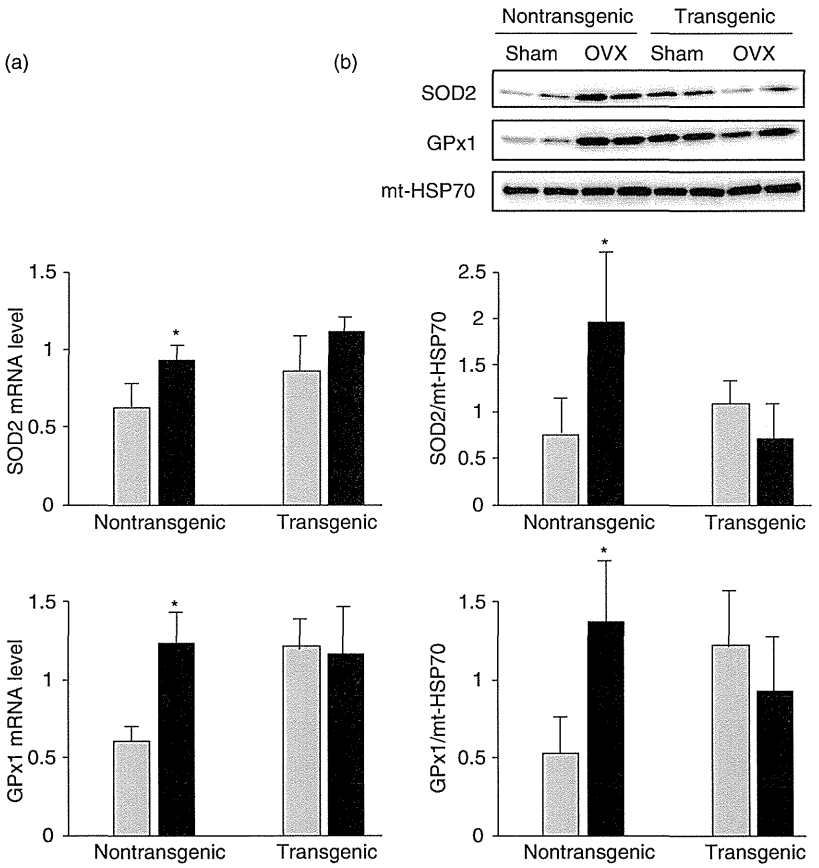

Figure 6 Expression levels of superoxide dismutase 2 (SOD2) and glutathione peroxidase 1 (GPx1) in sham-operated and ovariectomized (OVX) FL-N/35 transgenic and non-transgenic mice. (a) The mRNA levels of SOD2 and GPx1 were measured by real-time reverse transcription polymerase chain reaction for five mice in each group. The relative quantities of target mRNA in the liver were normalized with GAPDH mRNA. (b) Immunoblots for SOD2 and GPx1 were performed using mitochondrial fractions of liver lysates from five mice in each group. $^*P < 0.05$ vs sham-operated non-transgenic mice. □, Sham; ■, OVX.

Suppressed AMPK activation in OVX FL-N/35 transgenic mice

Proliferator-activated receptor-γ co-activator-1α activity is modulated through both transcriptional regulation and regulation of its activity by post-translational modifications.[28] AMPK is one of the signaling pathways regulating PGC-1α and acts both through modulation of PGC-1α transcription and by phosphorylation of the PGC-1α protein.[28] HCV has been shown to reduce the kinase activity of AMPK through Ser485/491 phosphorylation of AMPK.[29] Therefore, we examined the expression levels of AMPK to investigate the mechanisms underlying the lower PGC-1α expression in the nuclear fraction of the OVX transgenic liver. The expression levels of AMPKα, which is one of the three subunits (α, β and γ) of AMPK, were comparable in sham-operated and OVX mice and in non-transgenic and transgenic mice. However, the expression level of phosphorylated AMPKα was significantly greater in OVX non-transgenic mice than in mice in the three other groups, though it was similar in sham-operated transgenic mice and OVX transgenic mice (Fig. 7c). In addition, its levels were significantly greater in non-transgenic mice than in transgenic mice (Fig. 7c). These results suggested that AMPK was activated in OVX non-transgenic mice, but not in OVX transgenic mice, because AMPK is active only after phosphorylation of the α-subunit at a threonine residue within the kinase domain (T172) by upstream kinases.[30] Taken together, the results in the present study suggested that OVX FL-N/35 transgenic mice developed marked hepatic steatosis concomitant with increased ROS production via attenuation of antioxidant potential through inactivation of the AMPK/PGC-1α signaling pathway.

DISCUSSION

THE OVX MICE in the present study were assumed to be a standard model for evaluating the biological effect of ovariectomy because the effects of ovariectomy

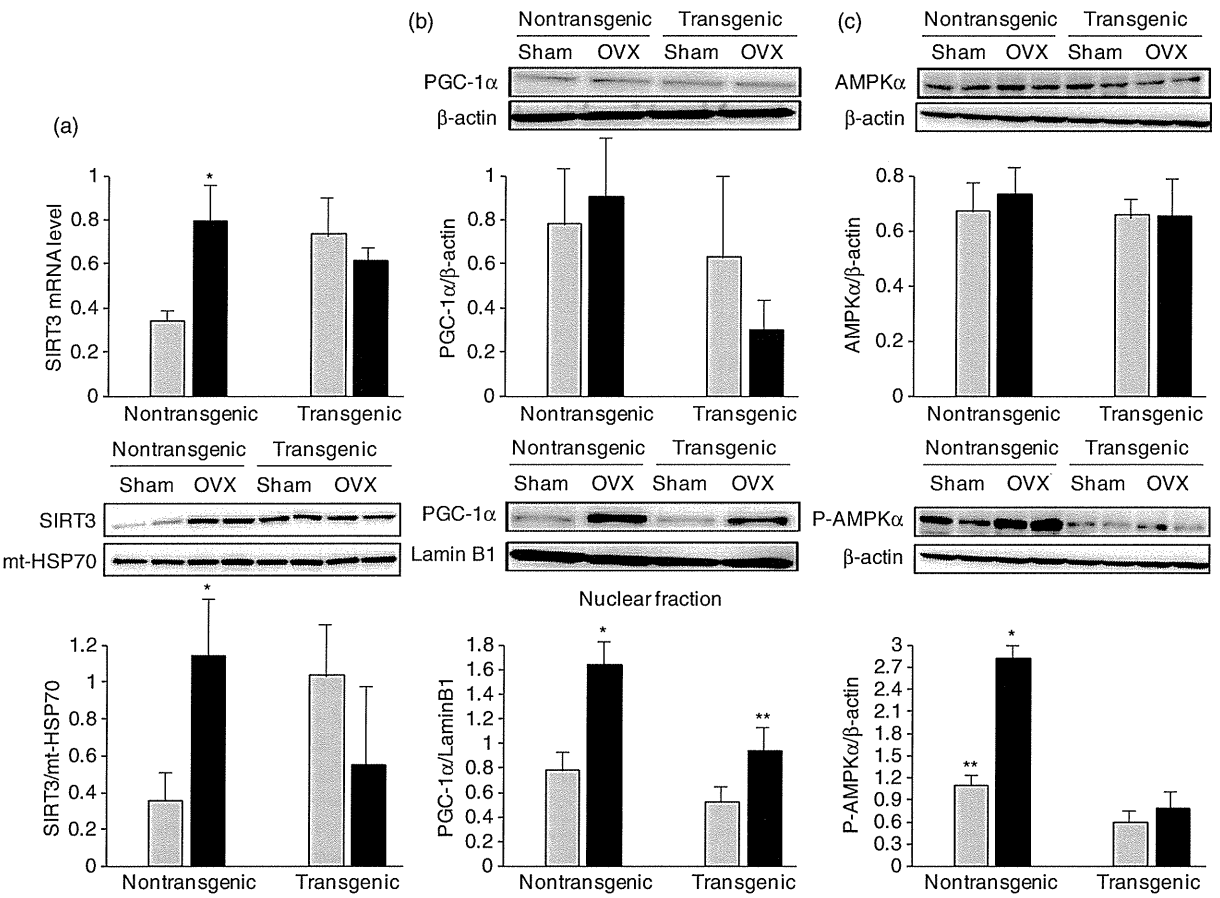

Figure 7 Expression levels of sirtuin 3 (SIRT3), peroxisome proliferator-activated receptor-γ co-activator-1α (PGC-1α), adenosine monophosphate-activated protein kinase α (AMPKα), and phosphorylated AMPKα (P-AMPKα) in sham-operated and ovariectomized (OVX) FL-N/35 transgenic and non-transgenic mice. (a) The mRNA levels of SIRT3 were measured by real-time reverse transcription polymerase chain reaction for five mice in each group. The relative quantities of target mRNA in the liver were normalized with GAPDH mRNA. Immunoblots for SIRT3 were performed using the mitochondrial fractions of liver lysates from five mice in each group. (b) Immunoblots for PGC-1α were performed using liver lysates and their nuclear fractions from five mice in each group. $*P < 0.05$ vs mice in the other three groups. $**P < 0.05$ vs sham-operated transgenic mice. (c) Immunoblots for AMPKα and P-AMPKα were performed using liver lysates from five mice in each group. $*P < 0.05$ vs mice in the other three groups. $**P < 0.05$ vs sham-operated transgenic mice. □, Sham; ■, OVX.

on dietary intake, bodyweight, uterine weight, liver weight and serum leptin levels were similar to the results from previous studies.[31–34] Ovariectomy increased ROS (superoxide) production in both transgenic liver and in non-transgenic liver, which was consistent with the ovariectomy-induced increase in NADPH oxidase activity[12] and the protective effect of estrogen against mitochondrial oxidative damage[13] found in previous studies. Of note was the much greater degree of ROS production after ovariectomy in transgenic mice than in non-transgenic mice. These results suggested that HCV protein expression has the potential to increase the sensitivity to oxidative stress in the liver. At least two possibilities may account for the increased sensitivity to oxidative stress in FL-N/35 transgenic mice. One possibility is an additive effect of HCV-induced ROS production on ovariectomy-induced oxidative stress. The HCV core protein has been shown to inhibit mitochondrial electron transport[35] and to induce ROS production.[36] In fact, basal ROS production tended to be higher in

transgenic mice than in non-transgenic mice, but was not significantly different. These results suggested that additive HCV-induced ROS production was unlikely to be the cause of the significantly increased ROS production after ovariectomy in the transgenic mice. The other possibility is HCV-associated attenuation of antioxidant potential against ovariectomy-induced oxidative stress. In this respect, OVX transgenic mice had a lower ratio of BAP to dROM than OVX non-transgenic mice and the expression of SOD2 and GPx1 in the liver was not increased. These results suggest that HCV protein attenuated antioxidant potential against ovariectomy-induced oxidative stress.

Proliferator-activated receptor-γ co-activator-1α is required for the induction of many ROS-detoxifying enzymes upon oxidative stress.[26] SIRT3 has been shown to function as a downstream target gene of PGC-1α and mediate the PGC-1α-dependent induction of ROS-detoxifying enzymes.[27] Additionally, AMPK, which is a crucial cellular energy sensor, regulates PGC-1α activity through both modulation of PGC-1α transcription and phosphorylation of the PGC-1α protein.[28,37] Thus, AMPK/PGC-1α signaling is one of the important pathways that protect cells from oxidative stress through the induction of several key ROS-detoxifying enzymes. Recent evidence indicating that HCV replication inhibits AMPK activity[29] prompted us to investigate whether the antioxidant potential against ovariectomy-induced oxidative stress in FL-N/35 transgenic mice was attenuated through inhibition of this signaling pathway. As expected, upon ovariectomy, AMPK was activated in non-transgenic mice, but not in transgenic mice. This, in turn, led to the lower expression of PGC-1α in the nuclear fraction of the liver in OVX transgenic mice than in OVX non-transgenic mice, resulting in the absence of significant induction of SIRT3 in the mitochondrial fraction of the liver in the OVX transgenic mice. Thus, ROS production in the liver in OVX transgenic mice was increased by attenuation of the antioxidant potential through inhibition of AMPK/PGC-1α signaling. However, it remains unknown why the expression of PGC-1α in the nuclear fraction was significantly increased in OVX transgenic mice regardless of the lack of activation of AMPK. Various kinases other than AMPK and post-translational modifications other than phosphorylation have been shown to regulate PGC-1α expression.[28] Therefore further investigations are required to clarify this issue.

Of particular concern is the relevance of the present results to HCC development in patients with HCV-associated chronic liver diseases. A recent study from Japan demonstrated a higher proportion of females, especially among elderly patients with HCV-related HCC, suggesting that the sex disparity in HCC development becomes less distinct as the patient's age at HCC diagnosis increases.[6] In general, ROS production creates a pro-carcinogenic environment under which chromosomal damage is likely to occur. The present findings that OVX transgenic mice have increased hepatic ROS production compared with that in OVX non-transgenic mice may indicate one of the mechanisms by which women with HCV infection are at high risk for HCC development when some period has passed after menopause, even though we need to clinically ascertain the increased hepatic oxidative stress in HCV-infected menopausal women with HCC.

ACKNOWLEDGMENTS

THIS RESEARCH WAS supported by a Grant-in-Aid for Scientific Research (B) (no. 23390201) and a Grant-in-aid for Exploratory Research (no. 25670374) from the Japan Society for the Promotion of Science, a Health and Labor Sciences Research Grant for Research on Hepatitis from the Ministry of Health, Labor and Welfare of Japan, and Research Project Grant P2 from Kawasaki Medical School.

REFERENCES

1 Chen DS, Locarnini S, Wait S *et al.* Report from a Viral Hepatitis Policy Forum on implementing the WHO framework for global action on viral hepatitis in North Asia. *J Hepatol* 2013. doi: 10.1016/j.jhep.2013.06.029.
2 Bruno S, Silini E, Crosignani A *et al.* Hepatitis C virus genotypes and risk of hepatocellular carcinoma in cirrhosis: a prospective study. *Hepatology* 1997; **25**: 754–8.
3 Degos F, Christidis C, Ganne-Carrie N *et al.* Hepatitis C virus related cirrhosis: time to occurrence of hepatocellular carcinoma and death. *Gut* 2000; **47**: 131–6.
4 Bosch FX, Ribes J, Diaz M, Cleries R. Primary liver cancer: worldwide incidence and trends. *Gastroenterology* 2004; **127**: S5–S16.
5 Naugler WE, Sakurai T, Kim S *et al.* Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. *Science* 2007; **317**: 121–4.
6 Kumada T, Toyoda H, Kiriyama S *et al.* Characteristics of elderly hepatitis C virus-associated hepatocellular carcinoma patients. *J Gastroenterol Hepatol* 2013; **28**: 357–64.
7 Farinati F, Cardin R, De Maria N *et al.* Iron storage, lipid peroxidation and glutathione turnover in chronic anti-HCV positive hepatitis. *J Hepatol* 1995; **22**: 449–56.
8 Barbaro G, Di Lorenzo G, Asti A *et al.* Hepatocellular mitochondrial alterations in patients with chronic hepatitis C:

ultrastructural and biochemical findings. *Am J Gastroenterol* 1999; **94:** 2198–205.
9 Tanaka H, Fujita N, Sugimoto R *et al.* Hepatic oxidative DNA damage is associated with increased risk for hepatocellular carcinoma in chronic hepatitis C. *Br J Cancer* 2008; **98:** 580–6.
10 Moriya K, Nakagawa K, Santa T *et al.* Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. *Cancer Res* 2001; **61:** 4365–70.
11 Furutani T, Hino K, Okuda M *et al.* Hepatic iron overload induces hepatocellular carcinoma in transgenic mice expressing the hepatitis C virus polyprotein. *Gastroenterology* 2006; **130:** 2087–98.
12 Ji H, Zheng W, Menini S *et al.* Female protection in progressive renal disease is associated with estradiol attenuation of superoxide production. *Gend Med* 2007; **4:** 56–71.
13 Borras C, Sastre J, Garcia-Sala D, Lloret A, Pallardo FV, Vina J. Mitochondria from females exhibit higher antioxidant gene expression and lower oxidative damage than males. *Free Radic Biol Med* 2003; **34:** 546–52.
14 Beard MR, Abell G, Honda M *et al.* An infectious molecular clone of a Japanese genotype 1b hepatitis C virus. *Hepatology* 1999; **30:** 316–24.
15 Lerat H, Honda M, Beard MR *et al.* Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. *Gastroenterology* 2002; **122:** 352–65.
16 Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959; **37:** 911–7.
17 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193:** 265–75.
18 Nishina S, Hino K, Korenaga M *et al.* Hepatitis C virus-induced reactive oxygen species raise hepatic iron level in mice by reducing hepcidin transcription. *Gastroenterology* 2008; **134:** 226–38.
19 Harrison-Findik DD, Schafer D, Klein E *et al.* Alcohol metabolism-mediated oxidative stress down-regulates hepcidin transcription and leads to increased duodenal iron transporter expression. *J Biol Chem* 2006; **281:** 22974–82.
20 Cesarone MR, Belcaro G, Carratelli M *et al.* A simple test to monitor oxidative stress. *Int Angiol* 1999; **18:** 127–30.
21 Park CH, Valore EV, Waring AJ, Ganz T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *J Biol Chem* 2001; **276:** 7806–10.
22 Nemeth E, Tuttle MS, Powelson J *et al.* Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* 2004; **306:** 2090–3.
23 Fenton HJH. Oxidation of tartaric acid in presence of iron. *J Chem Soc* 1894; **65:** 899–910.
24 Pietrangelo A, Dierssen U, Valli L *et al.* STAT3 is required for IL-6-gp130-dependent activation of hepcidin in vivo. *Gastroenterology* 2007; **132:** 294–300.
25 Kelly DP, Scarpulla RC. Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. *Genes Dev* 2004; **18:** 357–68.
26 St-Pierre J, Drori S, Uldry M *et al.* Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. *Cell* 2006; **127:** 397–408.
27 Kong X, Wang R, Xue Y *et al.* Sirtuin 3, a new target of PGC-1alpha, plays an important role in the suppression of ROS and mitochondrial biogenesis. *PLoS ONE* 2010; **5:** e11707.
28 Fernandez-Marcos PJ, Auwerx J. Regulation of PGC-1alpha, a nodal regulator of mitochondrial biogenesis. *Am J Clin Nutr* 2011; **93:** 884S–90.
29 Mankouri J, Tedbury PR, Gretton S *et al.* Enhanced hepatitis C virus genome replication and lipid accumulation mediated by inhibition of AMP-activated protein kinase. *Proc Natl Acad Sci U S A* 2010; **107:** 11549–54.
30 Hawley SA, Boudeau J, Reid JL *et al.* Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. *J Biol* 2003; **2:** 28.
31 Rogers NH, Perfield JW 2nd, Strissel KJ, Obin MS, Greenberg AS. Reduced energy expenditure and increased inflammation are early events in the development of ovariectomy-induced obesity. *Endocrinology* 2009; **150:** 2161–8.
32 Pighon A, Gutkowska J, Jankowski M, Rabasa-Lhoret R, Lavoie JM. Exercise training in ovariectomized rats stimulates estrogenic-like effects on expression of genes involved in lipid accumulation and subclinical inflammation in liver. *Metabolism* 2011; **60:** 629–39.
33 Hong J, Stubbins RE, Smith RR, Harvey AE, Nunez NP. Differential susceptibility to obesity between male, female and ovariectomized female mice. *Nutr J* 2009; **8:** 11.
34 Kamada Y, Kiso S, Yoshida Y *et al.* Pitavastatin ameliorated the progression of steatohepatitis in ovariectomized mice fed a high fat and high cholesterol diet. *Hepatol Res* 2013; **43:** 401–12.
35 Korenaga M, Wang T, Li Y *et al.* Hepatitis C virus core protein inhibits mitochondrial electron transport and increases reactive oxygen species (ROS) production. *J Biol Chem* 2005; **280:** 37481–8.
36 Okuda M, Li K, Beard MR *et al.* Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology* 2002; **122:** 366–75.
37 Hardie DG, Ross FA, Hawley SA. AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat Rev Mol Cell Biol* 2012; **13:** 251–62.

INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Risk Factors for Survival and the Development of Hepatocellular Carcinoma in Patients with Primary Biliary Cirrhosis

Yasuyuki Tomiyama[1], Kazuyuki Takenaka[2], Takahiro Kodama[2], Miwa Kawanaka[3], Kyo Sasaki[1], Sohji Nishina[1], Naoko Yoshioka[1], Yuichi Hara[1] and Keisuke Hino[1]

Abstract

Objective Early diagnosis of hepatocellular carcinoma (HCC) is critical in the management of patients with primary biliary cirrhosis (PBC), since the prognosis of PBC has improved. The aim of this study was to investigate whether HCC development affects the prognosis of PBC and to identify the risk factors for HCC in Japanese patients with PBC.

Methods We compared the survival rates between patients with HCC and those without and analyzed the risk factors for HCC development in 210 patients with PBC who were followed up for a median period of 8.5 years.

Results HCC developed during follow-up in 11 patients (5.2%) and was diagnosed simultaneously at the time of diagnosis of PBC in five patients (2.4%) who were excluded from the analysis. A Kaplan-Meier analysis showed a significant difference in overall survival between the patients who did and did not develop HCC ($p<0.001$). A multivariate analysis revealed age (OR: 1.08, 95% confidence interval [CI]: 1.03-1.13, $p=0.001$), the albumin level (OR: 0.24, 95% CI: 0.10-0.56, $p=0.001$), the total bilirubin level (OR: 1.60, 95% CI: 1.09-2.36, $p=0.017$) and HCC development (OR: 2.97, 95% CI: 1.24-7.15, $p=0.015$) to be significant prognostic factors and identified only an advanced histological stage (Scheuer's classification III or IV, OR: 6.27, 95% CI: 1.80-21.83, $p=0.004$) to be a risk factor associated with HCC.

Conclusion HCC development significantly affects the survival of patients with PBC, and an advanced histological stage is the only risk factor associated with HCC development. These results highlight the important role of liver fibrosis in hepatocarcinogenesis in patients with PBC.

Key words: diabetes mellitus, liver fibrosis, prognosis, survival, ursodeoxycholic acid

(Intern Med 52: 1553-1559, 2013)
(DOI: 10.2169/internalmedicine.52.0010)

Introduction

Primary biliary cirrhosis (PBC) is a progressive cholestatic liver disease characterized by the presence of highly specific antimitochondrial antibodies, portal inflammation and lymphocyte-dominated destruction of the intralobular bile ducts that eventually leads to cirrhosis (1). The incidence of PBC has increased over recent decades, possibly attributable to augmented testing of liver biochemistry rather than a rise in disease incidence. The routine use of biochemical screening has also made it possible to diagnose PBC at an earlier stage (2). In addition to early diagnosis, the high prevalence of treatment with ursodeoxycholic acid (UDCA) makes it possible for patients with PBC to live longer. However, the natural history of PBC is still debated and depends on several variables and symptoms of liver disease (3). In general, the risk of cancer development increases as humans live longer, and the development of hepatocellular carcinoma (HCC) is no exception.

[1]Department of Hepatology and Pancreatology, Kawasaki Medical School, Japan, [2]Department of Gastroenterology and Hepatology, Yamaguchi Grand Medical Center, Japan and [3]Department of General Internal Medicine 2, Kawasaki Hospital, Kawasaki Medical School, Japan
Received for publication December 27, 2012; Accepted for publication February 25, 2013
Correspondence to Dr. Keisuke Hino, khino@med.kawasaki-m.ac.jp

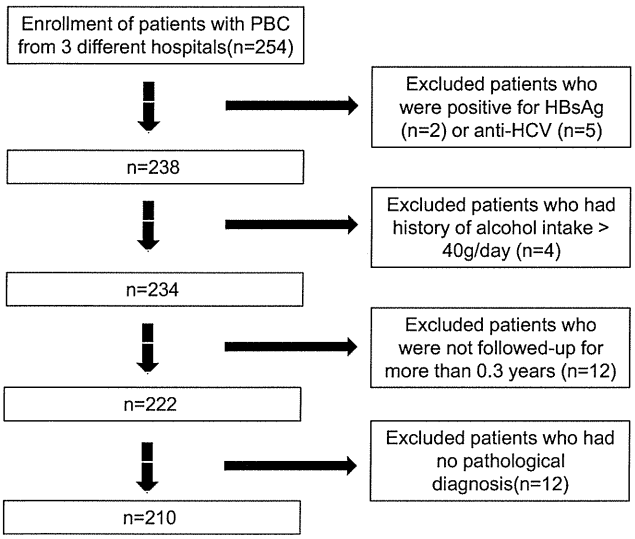

Figure 1. Numbers of patients included and excluded in the retrospective cohorts.

Patients with PBC are considered to be at low risk for the development of HCC (4), although several reports have revealed that PBC is associated with an increased risk of HCC (5-8). Additionally, whether the development of HCC affects the overall survival of patients with PBC remains controversial (5, 7, 9, 10). This matter and the identification of risk factors for HCC development are of importance for improving the prognosis of patients with PBC. There are, however, few studies reporting the risk factors for HCC among patients with PBC (5-7, 9, 11). The age at diagnosis (7, 11), male sex (5, 7, 10, 11), a history of blood transfusions (7, 11), cigarette smoking (6), portal hypertension (11), a more advanced histologic stage of disease (9, 10) and superinfection with hepatitis C virus (HCV) (6) have been reported to be risk factors for HCC in patients with PBC. Such risk factors remain a matter of debate due to recent changes in the nutritional environment and/or lifestyle. For instance, previous studies did not include obesity or diabetes mellitus as clinical parameters for assessing risk factors related to the development of HCC in patients with PBC, even though these parameters have been recognized to be risk factors for the development of HCC (12, 13).

The aim of this study was to determine whether HCC development affects the prognosis of PBC and to identify the risk factors for HCC development in Japanese patients with PBC based on recent clinical evidence.

Materials and Methods

This study was conducted among three series of patients from three different liver disease centers who were treated for a diagnosis of PBC between May 1984 and May 2010. A total of 245 patients were recruited, including 116 patients from Kawasaki Medical School affiliated hospital, 72 patients from Yamaguchi Grand Medical Center and 57 patients from Kawasaki Hospital. The diagnosis of PBC was established when the patient fulfilled at least one of the following criteria defined by the Intractable Hepato-Biliary Disease Study Group of Japan: (i) laboratory abnormalities consistent with chronic cholestatic liver disease and the presence of chronic nonsuppurative destructive cholangitis, (ii) positive antimitochondrial antibodies and a liver histology compatible with PBC, (iii) a medical history and laboratory abnormalities consistent with chronic cholestatic liver disease and positive antimitochondrial antibodies. The histological stage was classified according to Scheuer's classification (14). The following 35 patients were excluded from the analysis due to confounding risk factors for HCC or incomplete clinical parameters: hepatitis B surface antigen (HBsAg) positivity in two patients, anti-HCV antibody (anti-HCV) positivity in five patients, a history of excessive alcohol consumption (>40 g/day) in four patients, a lack of follow-up for more than 0.3 years in 12 patients and a lack of pathological diagnosis in 12 patients (Fig. 1).

The diagnosis of diabetes mellitus was made based on a history of antidiabetic medication use, including oral hypoglycemic agents and insulin, since the diagnostic criteria for diabetes mellitus proposed by the Japan Diabetes Society were not applied to all patients due to the lack of several biochemical parameters. The diagnosis of portal hypertension was made based on the presence of esophageal or gastric varices, ascites or splenomegaly. The clinical characteristics at diagnosis of PBC are shown in Table 1. The patients were regularly assessed with biochemical tests every one to four months and followed for a median period of 8.5 (range, 0.3-25.8) years. To determine the presence of HCC, abdominal ultrasonography was performed in all patients without HCC at intervals of four to 12 months. HCC was diagnosed based on the findings of abdominal ultrasonography and confirmed based on the findings of computed tomography (CT), magnetic resonance imaging, hepatic arteri-

Table 1. Clinical Characteristics of Patients at Diagnosis of PBC

Characteristic	Mean ± SD or frequency (number of analyzed patients)
Age (years)	58 ± 11 (210)
Gender (Male/Female)	31/179
Body mass index (kg/m^2)	22.4 ± 3.1 (196)
Blood transfusion (+/-/unknown)	13/192/5
Diabetes mellitus (+/-)	19/191
Portal hypertension (+/-)	39/171
Platelet count (× 10^4/μL)	21.9 ± 8.2 (210)
ALT (IU/L)	58 ± 53 (210)
AST (IU/L)	56 ± 40 (207)
Alkaline phosphatase (IU/L)	523 ± 434 (207)
γ-GT (IU/L)	230 ± 233 (207)
Total bilirubin (mg/dL)	0.9 ± 0.9 (210)
Albumin (g/dL)	3.9 ± 0.5 (209)
Prothrombin time (%)	82.9 ± 16.4 (209)
IgM (mg/dL)	410 ± 279 (203)
Anti-HBc (+/-/unknown)	13/40/157
Histological stage (I or II/III or IV)	169/41
Treatment with UDCA (+/-)	189/21

ALT: alanine aminotransferase, AST: aspartate aminotransferase, γ-GT: γ-glutamyltransferase, Anti-HBc: anti-hepatitis B core antibody, UDCA: ursodeoxycholic acid

ography and/or a fine-needle aspiration liver biopsy.

Overall survival was defined as the period from the day of PBC diagnosis until death, liver transplantation or the last medical examination and compared between the patients who did and did not develop HCC. Laboratory parameters measured at the time of PBC diagnosis were compared to those measured at the time of HCC diagnosis in patients who developed HCC during the follow-up period. The study protocol conformed to the 1975 Declaration of Helsinki Declaration and was approved by the ethics committees of the involved institutions.

Statistical analysis

Baseline continuous variables are expressed as the mean ± SD. Comparisons between groups were made using the Mann-Whitney test for continuous variables and the χ^2 test with Yates correction or Fisher's exact test for categorical variables. Cumulative survival was calculated using the Kaplan-Meier method, and the differences among the groups were analyzed with the log-rank test. Univariate and multivariate analyses of predictors of survival were performed using the Cox proportional hazards model. A multivariate analysis of predictors for the development of HCC was performed using the logistic regression test. A p value of less than 0.05 was considered to be significant. All analyses described above were performed using the SPSS software package (version 11, SPSS Inc., Chicago, IL).

Results

Development of HCC in patients with PBC

Sixteen (7.6%) of the 210 patients with PBC developed HCC. A diagnosis of HCC was made in 11 patients (5.2%) during follow-up, whereas HCC and PBC were almost simultaneously diagnosed in the remaining five patients (2.4%) who had diabetes mellitus and advanced histological stages, except for a man aged 84 who was potentially at high risk for HCC due to his age. These five patients (three men and two women) were thereafter excluded from the analysis. The clinical and histological features of the 11 patients who developed HCC during follow-up are summarized in Table 2. The HCC incidence according to gender was 3.6% (1/28) in men and 5.6% (10/177) in women. The mean interval between the diagnosis of PBC and the development of HCC was 11.4±5.7 years. Antibodies to hepatitis B core antigens (anti-HBc) were positive in two patients (18.2%), negative in five patients (45.4%) and unknown in four patients (36.4%), respectively. Six patients (54.5%) and one patient (9.1%) had advanced histological stages (III) and diabetes mellitus at the diagnosis of PBC, respectively. It should be noted that the follow-up period until the development of HCC was significantly longer in the patients with mild histological stages (I or II) (14.8±4.1 years) than in those with advanced histological stages (III) (8.5±5.5 years), suggesting a potential risk for HCC development after a long period of time even in patients with a mild histological stage of PBC. Six patients had solitary tumors whose size was less than 30 mm, except for one. These tumors were treated with local ablation, such as percutaneous microwave coagulation therapy, percutaneous ethanol injection or radiofrequency ablation, transarterial chemoembolization (TACE), combination of local ablation and TACE or liver transplantation, in all patients, except one (case 9 in Table 2) who could not be treated due to rupture of HCC.

To determine whether HCC develops as liver damage progresses, laboratory parameters (platelet count, alanine aminotransferase [ALT], aspartate aminotransferase [AST], alkaline phosphatase, γ-glutamyltransferase, total bilirubin, albumin and prothrombin time) were compared between the two time points of PBC diagnosis and HCC diagnosis in the 11 patients. The serum albumin level, prothrombin activity and platelet count were significantly lower at the time of HCC diagnosis than at the time of PBC diagnosis, suggesting that the development of HCC largely depends on the progression of liver disease (Fig. 2). Patients with PBC who developed HCC had significantly lower cumulative survival rates than those who did not. The 5-, 10-, 15- and 20-year cumulative survival rates of the PBC patients with HCC and those without HCC were 91/98%, 61/87%, 51/83% and 10/80%, respectively ($p<0.001$, Fig. 3).

Factors associated with survival in patients with PBC

We next investigated the factors associated with survival in patients with PBC in order to determine whether HCC development actually affects the prognosis. In addition to the baseline characteristics of the patients at the time of PBC diagnosis, treatment with UDCA and the development of HCC were incorporated into the parameters used to ana-

Table 2. Characteristics of 11 Patients with PBC who Developed HCC

Case	Age at PBC/HCC diagnosis	Sex	BMI	DM	Anti-HBc	Histological stage at§	HCC				Cause of death
							Solitary or multiple	Maximum size (mm)‡	Vascular invasion	Therapy¶	
1	64/67	M	25.8	-	+	III	Solitary	10	-	PMCT	Sepsis
2	66/75	F	26.9	-	-	III	Multiple	45	-	PEI + TACE	Hepatic failure
3	65/74	F	23.2	-	+	III	Multiple	32	-	TACE	Hepatic failure
4	59/70	F	23.3	+	-	III	Multiple	20	-	REI + TACE	HCC
5	61/75	F	17.8	-	-	II	Multiple	30	-	TACE	Hepatic failure
6	38/55	F	19.5	-	-	I	Solitary	30	-	PEI + TACE	Hepatic failure
7	38/55	F	20.8	-	NA†	I	Multiple	15	-	TACE	Hepatic failure
8	58/76	F	23.1	-	-	I	Solitary	30	-	TACE	Hepatic failure
9	64/66	F	22.2	-	NA	III	Solitary	100	+	-	HCC rupture
10	41/49	F	27.7	-	NA	I	Solitary	10	-	Transplantation	Alive
11	48/65	F	24.6	-	NA	III	Solitary	27	-	TACE	Alive

BMI: body mass index, DM: diabetes mellitus, Anti-HBc: anti hepatitis B core antibody, †NA: unknown, §at the diagnosis of PBC, ‡tumor sizes in cases 2, 3, 4, 5 and 7 represent the largest ones among multiple tumors. ¶PMCT: percutaneous microwave coagulation therapy, PEI: percutaneous ethanol injection, TACE: transarterial chemoembolization, RFA: radiofrequency ablation

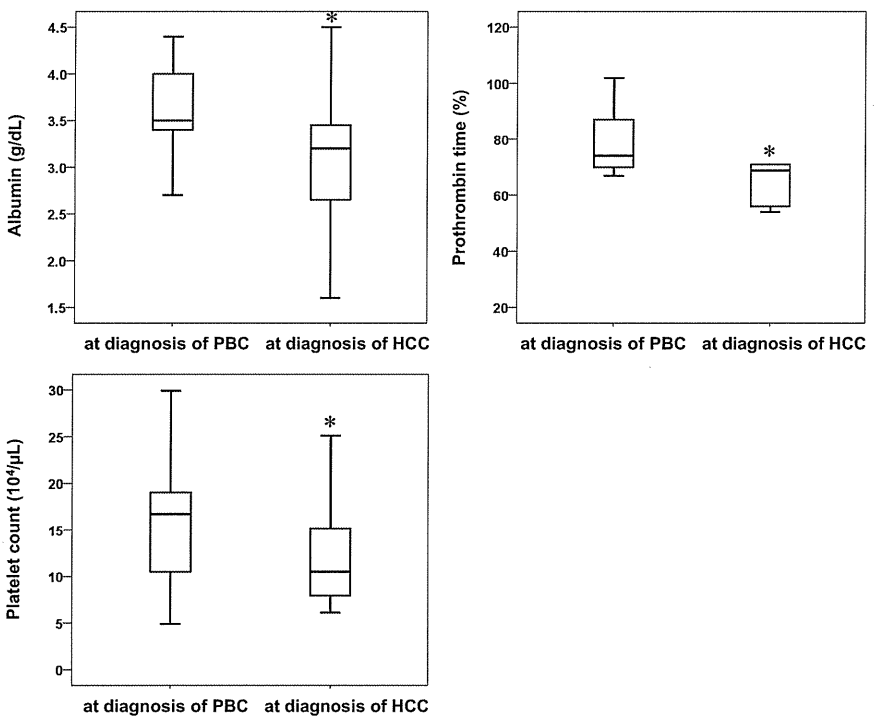

Figure 2. Serum albumin levels, platelet counts and prothrombin activity at diagnosis of primary biliary cirrhosis (PBC) and hepatocellular carcinoma (HCC) in the patients with PBC who developed HCC. The results are shown as box plot profiles. The bottom and top edges of the boxes are the 25th and 75th percentiles, respectively. *: $p<0.05$

lyze the factors associated with the survival of patients with PBC. However, we did not incorporate the response to treatment with UDCA into these parameters due to a lack of established biochemical criteria for the response to UDCA allowing for prediction of the prognosis. Treatment with UDCA (600 mg daily) was started within six months from diagnosis of PBC in 186 patients (91%). The reason why UDCA was not administered in the remaining 19 patients was unknown. The duration of UDCA treatment was almost the same as the follow-up period, since almost all of the patients continued taking UDCA due to a lack of moderate to severe adverse effects.

A univariate analysis identified age, portal hypertension, platelet count, AST, the total bilirubin and albumin levels, prothrombin activity, advanced histological stage (Scheuer criteria III or IV) and HCC development to be significant predictors of survival in patients with PBC (Table 3). Among these predictors, a multivariate analysis revealed age (OR: 1.08, 95% CI: 1.03-1.13, p=0.001), the total bilirubin (OR: 1.60, 95% CI: 1.09-2.36, p=0.017) and albumin levels (OR: 0.24, 95% CI: 0.10-0.56, p=0.001) and HCC development (OR: 2.97, 95% CI: 1.24-7.15, p=0.015) to be significant factors associated with survival in patients with PBC (Table 3).

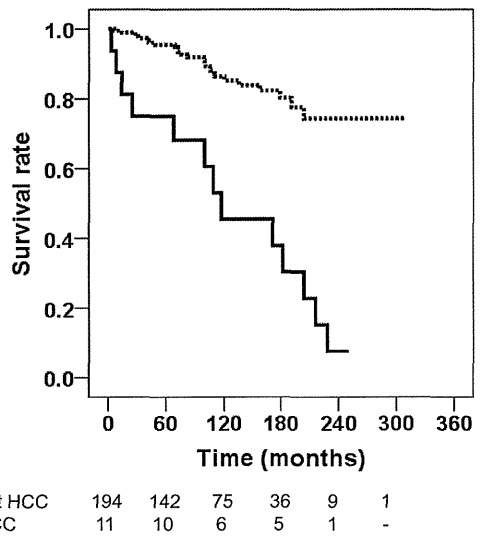

Figure 3. Cumulative survival curves for the patients with primary biliary cirrhosis who developed hepatocellular carcinoma (HCC) and those who did not. The solid and broken lines indicate patients with HCC and those without HCC, respectively. Log-rank test p<0.001

Factors associated with the development of HCC in patients with PBC

As the development of HCC has been demonstrated to be significantly associated with survival in patients with PBC, it is important to determine screening targets for HCC to improve the prognosis for patients with PBC. In this study, the patients with PBC who developed HCC had lower platelet counts (p=0.008), lower albumin levels ((p=0.02) and more advanced histological stages of disease (Scheuer criteria III or IV) (p=0.005) at the time of PBC diagnosis than those who did not (Table 4). A multivariate analysis identified only an advanced histological stage (Scheuer criteria III or IV) (OR: 6.27, 95% CI: 1.80-21.83, p=0.004) as being a predictor of the development of HCC in patients with PBC (Table 5).

Discussion

The frequency of HCC development in patients with PBC is estimated to be around 3% (0.7-3.6%) according to recent and relatively large cohort studies conducted in European countries, the United States and Japan, although this frequency increases as the histological stage progresses (5-10, 15, 16). In this study 5.2% of the patients with PBC developed HCC during follow-up and 2.4% of the patients had HCC simultaneously at the time of PBC diagnosis. The incidence of HCC was slightly higher in the present study than that reported in previous studies; however, it is unknown whether this difference is significant. The higher incidence of HCC observed in the present study may be explained by the relatively longer period of follow-up in a restricted number of institutions. Such situations potentially include less drop out patients during the follow-up period.

HCC was demonstrated to develop as liver damage progressed in the patients with PBC, as indicated by significant decreases in the serum albumin levels, prothrombin activity and platelet counts at the time of HCC development. These results are consistent with a previous observation of a Japanese study group in which all patients had progressed to an advanced histological stage (Scheuer criteria III or IV) by the time of HCC development (7). We also found that HCC development is a significant risk factor for survival in patients with PBC. Because the PBC patients had already progressed to an advanced histological stage at the time of HCC diagnosis, these results appear to be reasonable and are consistent with those of a study from Spain and Italy (9) and a recent Japanese report based on a nationwide survey (10). In fact, the six PBC patients with HCC (6/9, 67%) evaluated in the present study died of hepatic failure (Table 2). In some of the patients with HCC, it was difficult to determine whether the cause of death was hepatic failure or HCC. Therefore, deaths resulting from progressive hepatic failure related to portal venous invasion or rupture of HCC were defined as deaths caused by HCC. Deaths resulting from progressive hepatic failure during the course of treatment for HCC without portal venous invasion were defined as deaths caused by hepatic failure.

In contrast to PBC, HCC development (n=19) was not found to be a significant risk factor for survival in patients with alcoholic liver cirrhosis (n=103) who were followed during almost the same period as those followed in this study and who developed hepatic failure within a relatively short period of time unless they stopped drinking alcohol (unpublished data). The long-term clinical course of PBC may also account for the association between HCC development and a poor prognosis in patients with PBC. In addition to HCC development, age, the albumin level and the total bilirubin level at onset of PBC were selected as significant prognostic factors in patients with PBC in the present study. These results are in part consistent and in part inconsistent with those of previous studies (7, 17), most likely due to the different backgrounds of the patients studied.

That the majority (90%) of patients were treated with UDCA may account for why we did not find any differences in survival between the treated patients and the untreated patients. However, a limitation of this study is that the response to treatment with UDCA could not be incorporated into the analytic factors for survival. The response to UDCA has been reported to be associated with a better prognosis among PBC patients with moderately advanced disease compared with patients with mild disease (18), who accounted for the majority (80%) of the patients in the present study. Recent biochemical criteria for predicting the outcomes of patients with early PBC at low risk for long-term development of liver cirrhosis (19) may be useful for assessing whether the response to UDCA treatment affected the

Table 3. Univariate and Multivariate Analyses of Predictors for Survival in Patients with PBC

Variables	Univariate			Multivariate		
	Hazard ratio	95% CI	p value	Hazard ratio	95% CI	p value
Age (years)	1.09	1.04-1.14	<0.001	1.08	1.03-1.13	0.001
Male	1. 65	0.63-4.34	0.31			
Body mass index (kg/m^2)	0.92	0.81-1.05	0.24			
Blood transfusion	1.19	0.28-5.03	0.62			
Diabetes mellitus	3.70	1.56-7.14	0.009			
Portal hypertension	3.34	1.93-7.04	0.002			
Platelet count (× 10^4/μL)	0.92	0.87-0.97	0.003			
ALT (IU/L)	1.00	0.99-1.00	0.64			
AST (IU/L)	1.01	1.00-1.01	0.04			
Alkaline phosphatase (IU/L)	1.00	1.00-1.00	0.24			
γ-GT (IU/L)	1.00	1.00-1.00	0.87			
Total bilirubin (mg/dL)	2.03	1.50-2.76	<0.001	1.60	1.09-2.36	0.017
Albumin (g/dL)	0.13	0.06-0.27	<0.001	0.24	0.10-0.56	0.001
Prothrombin time (%)	0.94	0.91-0.96	<0.001			
IgM (mg/dL)	1.00	1.00-1.00	0.12			
Anti-HBc	0.83	0.23-3.00	0.27			
Histological stage III or IV	5.10	2.40-10.66	<0.001			
Treatment with UDCA	3.35	0.45-24.78	0.24			
Development of HCC	5.15	2.31-11.49	<0.001	2.97	1.24-7.15	0.015

ALT: alanine aminotransferase, AST: aspartate aminotransferase, γ-GT: γ-glutamyltransferase, Anti-HBc: anti hepatitis B core antibody, UDCA: ursodeoxycholic acid, HCC: hepatocellular carcinoma

Table 4. Comparison of Clinical Characteristics at Diagnosis of PBC between Patients who Developed HCC and Those who Did Not

Variables	Patients with HCC	Patients without HCC	p value
Number of patients	11	194	
Age (years)	58 ± 11	58 ± 10	0.91
Gender (Male/Female)	1/10	27/167	0.54
Body mass index (kg/m^2)	23.6 ± 2.7	22.3 ± 3.1	0.10
Blood transfusion (+/-)	1/10	11/178	0.50
Diabetes mellitus (+/-)	1/10	14/180	0.58
Portal hypertension (+/-)	4/7	33/161	0.12
Platelet count (× 10^4/μL)	16.0 ± 7.5	22.5 ± 8.0	0.008
ALT (IU/L)	47 ± 29	59 ± 55	0.66
AST (IU/L)	60 ± 36	55 ± 40	0.40
Alkaline phosphatase (IU/L)	468 ± 338	518 ± 432	0.72
γ-GT (IU/L)	127 ± 86	236 ± 238	0.08
Total bilirubin (mg/dL)	1.6 ± 1.7	0.9 ± 0.6	0.11
Albumin (g/dL)	3.6 ± 0.6	4.0 ± 0.5	0.02
Prothrombin time (%)	76.8 ± 14.0	83.6 ± 16.5	0.30
IgM (mg/dL)	479 ± 393	405 ± 273	0.91
Anti-HBc (+/-/unknown)	2/6/3	11/30/153	0.65
Histological stage (I or II/III or IV)	5/6	163/31	0.005
Treatment with UDCA (+/-)	11/0	175/19	0.33

ALT: alanine aminotransferase, AST: aspartate aminotransferase, γ-GT: γ-glutamyltransferase, Anti-HBc: anti hepatitis B core antibody, UDCA: ursodeoxycholic acid

Table 5. Multivariate Analysis of Predictors for Development of HCC in Patients with PBC

Variables	Odds ratio	95% CI	p value
Histological stage III or IV	6.27	1.80-21.83	0.004

survival of patients with PBC in this study.

Diabetes mellitus was demonstrated to increase the risk of HCC in a large cohort of patients without concomitant liver disease (12) and in a cohort of patients with hepatitis C, hepatitis B or alcoholic cirrhosis (20, 21). However, these previous studies did not include diabetes mellitus as a clinical parameter for assessing risk factors related to the development of HCC in patients with PBC (5-7, 9-11). We did not find any associations between diabetes mellitus and the development of HCC, although the proportion of patients with diabetes mellitus (9%) in this study was relatively small. Although a link between insulin resistance and metabolic hepatocarcinogenesis has been reported (22), the contribution of diabetes mellitus to the development of HCC in patients with chronic liver diseases may vary according to the etiology of liver disease.

We did not identify male sex to be a significant factor associated with HCC development in patients with PBC, which is inconsistent with the results of some previous studies (5, 7, 10, 11). In particular, the discrepancy between the present study and a recent Japanese nationwide study (10) may be explained by at least three factors. First, the patient sample size was much smaller in this study than in the nationwide survey. Second, the present study included more patients with an advanced histological stage of disease (Scheuer criteria III or IV) (18%) than the nationwide survey (12%), although other clinical parameters, such as age, gender and the frequency of treatment with UDCA, were similar between the two studies. Third, the exclusion criterion regarding a history of excessive alcohol consumption (>40 g/day) employed in this study may be associated with this discrepancy since the nationwide survey did not clearly

exclude patients with a history of excessive alcohol consumption. As shown in a previous study (9), we also found only an advanced histological stage (Scheuer's classification III or IV) to be a risk factor associated with the development of HCC in patients with PBC. Considering that cholangiocytes, not hepatocytes, are primarily affected and liver fibrosis progresses as the histological stage advances in PBC, these results indeed highlight the important role of liver fibrosis in hepatocarcinogenesis in patients with PBC. In addition, it should be noted that the follow-up period until the development of HCC was significantly longer in the patients with a mild histological stage (I or II) than in those with an advanced histological stage (III or IV). These results suggest a potential risk for HCC development after a long period of time, even in PBC patients with a mild histological stage of disease at the time of diagnosis.

In conclusion, the development of HCC has been demonstrated to affect the survival of Japanese patients with PBC. Considering that the prognosis of PBC has improved in general as a result of early diagnosis and the use of UDCA, the early diagnosis of HCC is also crucial for obtaining a better prognosis for patients with PBC. Therefore, strict surveillance for HCC is necessary among patients with PBC who are in the advanced stage of disease.

The authors state that they have no Conflict of Interest (COI).

This study was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology (No. 23390201) and in part by the Ministry of Health, Labor and Welfare, Japan.

References

1. Kaplan MM, Gershwin ME. Primary biliary cirrhosis. N Engl J Med **353**: 1261-1273, 2005.
2. Selmi C, Bowlus CL, Gershwin ME, Coppel RL. Primary biliary cirrhosis. Lancet **377**: 1600-1609, 2011.
3. Kaplan MM. Primary biliary cirrhosis. N Engl J Med **335**: 1570-1580, 1996.
4. Lööf L, Adami HO, Sparen P, et al. Cancer risk in primary biliary cirrhosis: a population-based study from Sweden. Hepatology **20**: 101-104, 1994.
5. Jones DE, Metcalf JV, Collier JD, Bassendine MF, James OF. Hepatocellular carcinoma in primary biliary cirrhosis and its impact on outcomes. Hepatology **26**: 1138-1142, 1997.
6. Floreani A, Baragiotta A, Baldo V, Menegon T, Farinati F, Naccarato R. Hepatic and extrahepatic malignancies in primary biliary cirrhosis. Hepatology **29**: 1425-1428, 1999.
7. Shibuya A, Tanaka K, Miyakawa H, et al. Hepatocellular carcinoma and survival in patients with primary biliary cirrhosis. Hepatology **35**: 1172-1178, 2002.
8. Caballeria L, Pares A, Castells A, Gines A, Bru C, Rodes J. Hepatocellular carcinoma in primary biliary cirrhosis: similar incidence to that in hepatitis C virus-related cirrhosis. Am J Gastroenterol **96**: 1160-1163, 2001.
9. Cavazza A, Caballeria L, Floreani A, et al. Incidence, risk factors, and survival of hepatocellular carcinoma in primary biliary cirrhosis: comparative analysis from two centers. Hepatology **50**: 1162-1168, 2009.
10. Harada K, Hirohara J, Ueno Y, et al. Incidence of and risk factors for hepatocellular carcinoma in primary biliary cirrhosis: National data from Japan. Hepatology (in press).
11. Suzuki A, Lymp J, Donlinger J, Mendes F, Angulo P, Lindor K. Clinical predictors for hepatocellular carcinoma in patients with primary biliary cirrhosis. Clin Gastroenterol Hepatol **5**: 259-264, 2007.
12. El-Serag HB, Tran T, Everhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. Gastroenterology **126**: 460-468, 2004.
13. Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. N Engl J Med **348**: 1625-1638, 2003.
14. Scheuer P. Primary biliary cirrhosis. Proc R Soc Med **60**: 1257-1260, 1967.
15. Nijhawan PK, Therneau TM, Dickson ER, Boynton J, Lindor KD. Incidence of cancer in primary biliary cirrhosis: the Mayo experience. Hepatology **29**: 1396-1398, 1999.
16. Howel D, Metcalf JV, Gray J, Newman WL, Jones DE, James OF. Cancer risk in primary biliary cirrhosis: a study in northern England. Gut **45**: 756-760, 1999.
17. Floreani A, Caroli D, Variola A, et al. A 35-year follow-up of a large cohort of patients with primary biliary cirrhosis seen at a single centre. Liver Int **31**: 361-368, 2011.
18. Kuiper EM, Hansen BE, de Vries RA, et al. Improved prognosis of patients with primary biliary cirrhosis that have a biochemical response to ursodeoxycholic acid. Gastroenterology **136**: 1281-1287, 2009.
19. Corpechot C, Chazouilleres O, Poupon R. Early primary biliary cirrhosis: biochemical response to treatment and prediction of long-term outcome. J Hepatol **55**: 1361-1367, 2011.
20. N'Kontchou G, Paries J, Htar MT, et al. Risk factors for hepatocellular carcinoma in patients with alcoholic or viral C cirrhosis. Clin Gastroenterol Hepatol **4**: 1062-1068, 2006.
21. El-Serag HB, Richardson PA, Everhart JE. The role of diabetes in hepatocellular carcinoma: a case-control study among United States Veterans. Am J Gastroenterol **96**: 2462-2467, 2001.
22. Hines IN, Hartwell HJ, Feng Y, et al. Insulin resistance and metabolic hepatocarcinogenesis with parent-of-origin effects in A×B mice. Am J Pathol **179**: 2855-2865, 2011.

OPEN ACCESS Freely available online

PLOS | ONE

Identification of a Functional Variant in the *MICA* Promoter Which Regulates *MICA* Expression and Increases HCV-Related Hepatocellular Carcinoma Risk

Paulisally Hau Yi Lo[1], Yuji Urabe[1,2], Vinod Kumar[1], Chizu Tanikawa[1], Kazuhiko Koike[3], Naoya Kato[4], Daiki Miki[2,5], Kazuaki Chayama[2,5], Michiaki Kubo[5], Yusuke Nakamura[1,6], Koichi Matsuda[1]*

1 Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo, Japan, **2** Departments of Medical and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan, **3** Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan, **4** Unit of Disease Control Genome Medicine, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan, **5** Center for Genomic Medicine, The Institute of Physical and Chemical Research (RIKEN), Kanagawa, Japan, **6** Departments of Medicine and Surgery, and Center for Personalized Therapeutics, The University of Chicago, Chicago, Illinois, United States of America

Abstract

Hepatitis C virus (HCV) infection is the major cause of hepatocellular carcinoma (HCC) in Japan. We previously identified the association of SNP rs2596542 in the 5′ flanking region of the *MHC class I polypeptide-related sequence A* (*MICA*) gene with the risk of HCV-induced HCC. In the current study, we performed detailed functional analysis of 12 candidate SNPs in the promoter region and found that a SNP rs2596538 located at 2.8 kb upstream of the *MICA* gene affected the binding of a nuclear protein(s) to the genomic segment including this SNP. By electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assay, we identified that transcription factor Specificity Protein 1 (SP1) can bind to the protective G allele, but not to the risk A allele. In addition, reporter construct containing the G allele was found to exhibit higher transcriptional activity than that containing the A allele. Moreover, SNP rs2596538 showed stronger association with HCV-induced HCC ($P = 1.82 \times 10^{-5}$ and OR = 1.34) than the previously identified SNP rs2596542. We also found significantly higher serum level of soluble MICA (sMICA) in HCV-induced HCC patients carrying the G allele than those carrying the A allele ($P = 0.00616$). In summary, we have identified a functional SNP that is associated with the expression of MICA and the risk for HCV-induced HCC.

Citation: Lo PHY, Urabe Y, Kumar V, Tanikawa C, Koike K, et al. (2013) Identification of a Functional Variant in the *MICA* Promoter Which Regulates *MICA* Expression and Increases HCV-Related Hepatocellular Carcinoma Risk. PLoS ONE 8(4): e61279. doi:10.1371/journal.pone.0061279

Editor: Erica Villa, University of Modena & Reggio Emilia, Italy

Received September 24, 2012; **Accepted** March 11, 2013; **Published** April 11, 2013

Funding: This work was supported by the Ministry of Education, Culture, Sports, Science and Technology of the Japanese government, the Ministry of Health, Labour, and Welfare of the Japanese government. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: koichima@ims.u-tokyo.ac.jp

Introduction

Hepatocellular carcinoma (HCC) is one of the common cancers in the world. It is well-known to be associated with the chronic infection of Hepatitis B (HBV) and Hepatitis C (HCV) viruses. In Japan, nearly 70% of HCC patients are infected with HCV [1]. The annual rate of developing HCC among patients with HCV-related liver cirrhosis in Japan is estimated to be about 4–8 percent [2]. Recent analyses have identified various genetic factors that are related with viral induced liver diseases [3–5]. In our previous two-stage genome-wide association study (GWAS) using a total number of 1,394 cases and 5,486 controls, a SNP rs2596542 located on chromosome 6p21.33 was shown to be significantly associated with HCV-induced HCC ($P = 4.21 \times 10^{-13}$ and OR = 1.39) [6]. This SNP is located within the class I major histocompatibility complex (MHC) region and is at about 4.8 kb upstream of *MHC class I polypeptide-related sequence A* (*MICA*) gene. We also identified that the risk A allele of SNP rs2596542 was strongly associated with the low expression of soluble MICA (sMICA) in the serum of HCV-related HCC patients [6].

MICA is a membrane protein which is up-regulated in various tumor cells and also induced in response to various cellular stresses such as infection, hypoxia, and heat shock [7]. It is an important component of the innate immune response, as MICA can bind to the NKG2D receptor and subsequently activate natural killer (NK) cells, CD8+ cells, and γδ T cells [8,9]. Moreover, membrane MICA can be shed by metalloproteinases, including MMP9, ADAM10, and ADAM17, and secreted into serum as a soluble form [10,11]. Since these metalloproteinases are often activated in HCC, the expressions of both membrane-bound MICA and sMICA are increased [12,13]. SNP rs2596542 was found to be associated with the progression from chronic hepatitis C (CHC) to HCC and also with serum sMICA level. Hence, both rs2596542 and sMICA would be possible prognostic biomarkers for CHC patients. However, their underlying molecular mechanisms were not fully elucidated so far.

We hypothesize that *MICA* variations could affect sMICA level by either one or both of the following two possible mechanisms: (1) the genetic variation(s) in the coding region affecting the protein stability and (2) the transcriptional regulation. Previously, variable

numbers of tandem repeats (VNTRs) in exon 5 of *MICA* were identified to affect MICA subcellular localization and serum MICA level [14]. The exon 5 of *MICA* encodes the transmembrane domain and the insertion of an extra G nucleotide in the domain would result in a premature stop codon that would generate MICA protein without a transmembrane domain and subsequently affect sMICA level [14]. However, our previous results indicated that MICA VNTR was not significantly associated with the sMICA level or HCC risk [6]. Therefore, in the current study, we have tried to investigate whether the *MICA* variations would affect the *MICA* transcription in the liver cancer cells. Through the functional analysis of genetic variations in the *MICA* promoter region, we here report a causative SNP rs2596538 that increases the binding affinity of the transcription factor Specificity Protein 1 (SP1) and the risk of progression of the disease.

Materials and Methods

Samples and genotyping

DNA samples for direct sequencing (50 HCV-related HCC cases), imputation analysis (721 HCV-related HCC cases and 5,486 HCV-negative controls), and serum samples for sMICA ELISA (246 HCV-related HCC) were obtained from BioBank Japan [15,16]. Genotyping of SNPs from 1,394 HCC patients and measurement of sMICA expression by ELISA were performed in the previous study [6]. Genotyping of SNP rs2596542 in 1,043 CHC was performed previously in RIKEN using Illumina HumanHap610-Quad BeadChip [17]. All CHC subjects had abnormal levels of serum alanine transaminase for more than 6 months and were positive for both HCV antibody and serum HCV RNA. The SNP rs2596542 in liver cirrhosis samples without hepatocellular carcinoma from BioBank Japan (n = 420) and the University of Tokyo (n = 166) were genotyped using Illumina HumanHap610-Quad BeadChip or invader assay [18]. All subjects were either subjected to liver biopsy or diagnosed by non-invasive methods including hepatic imaging, biochemical data, and the presence/absence of clinical manifestations of portal hypertension [18]. The samples used in the current project were listed in Table S1. Case samples with HBV co-infection were excluded from this study. The subjects with cancers, chronic hepatitis B, diabetes or tuberculosis were excluded from non-HCV controls. All subjects were Japanese origin and provided written informed consent. This research project was approved by the ethical committees of the University of Tokyo and RIKEN.

Imputation study

The imputation study was performed by using a hidden Markov model programmed in MACH [19] and haplotype information from 1000 genomes database [20]. The imputation results were confirmed by direct DNA sequencing in 50 randomly selected samples.

Cell culture

Human liver cancer cell lines HLE and HepG2 were purchased from JHSF (Osaka, Japan) and ATCC. These cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum. Cells were cultured at 37°C with 5% CO_2.

EMSA

HLE cells were grown in 15 cm culture plate until they reached 95% confluency. The plate was then sealed with parafilm and immersed in a water bath at 42.5°C for 1.5 hours [21]. Nuclear extracts from these cells were prepared according to the standard protocol [22]. EMSA was carried out using DIG Gel Shift Kit, 2nd Generation (Roche) according to the manufacturer's instructions. The sequences of the 12 probes were listed in the Table S2. In brief, 30 fmol of labeled probes were hybridized with 5 μg nuclear extract for 15 minutes at room temperature. The mixtures were then loaded into a 6% TBE gel, separated by electrophoresis at 4°C and transferred onto a nylon membrane. The membrane was then hybridized with anti-digoxigenin-AP antibody and developed by CSPD solution. For competition study, nuclear extracts were incubated with non-labeled oligonucleotides first before adding labeled probe. For supershift assay, SP1 antibody (SC-59X, Santa Cruz Biotechnology) was added into the nuclear extract and incubated on ice for 30 minutes first before adding labeled probe. The mixtures were then separated by electrophoresis using 4% TBE gel. All EMSAs were repeated twice for reconfirmation of the results.

ChIP

The HLE cells (G allele homozygote) and HepG2 cells (heterozygote) were used in the ChIP assay. The plasmid pCAGGS-SP1 was transfected into both cells by using FuGENE6 Transfection Reagent (Roche). The ChIP assays were carried out using Chromatin Immunoprecipitation Assay Kit (Millipore) according to the manufacturer's protocol. In brief, the cells were treated with formaldehyde to crosslink DNA-protein complexes at 48 hours post-transfection. DNA-protein complexes were then sheared by sonication and immunoprecipitated by rabbit polyclonal anti-SP1 antibody (SC-59X, Santa Cruz Biotechnology). The resulting DNAs were analyzed by PCR (Table S2). In order to determine the binding specificity of SP1 to the SNP rs2596538 allele, the PCR products from HepG2 cells were further subcloned into pCR 2.1 vector and sequenced to assess G to A ratio in both input DNA and immunoprecipitant.

Dual luciferase reporter assay

Three copies of 31 bp DNA fragments equivalent to the EMSA oligonucleotides of SNP rs2596538 were cloned into pGL3-promoter vector (Promega). The plasmids were co-transfected with pCAGGS-SP1 and pRL-TK plasmids (Promega) into HLE cells by FuGENE6 Transfection Reagent (Roche). The pCAGGS-SP1 plasmid provided the expression of transcription factor SP1, and pRL-TK plasmid served as internal control for transfection efficiency [23]. The cells were lysed at 48 hours post-transfection, and relative luciferase activities were measured by Dual Luciferase Assay System (Toyo B-Net).

Western blotting

Cancer cell lysates were prepared by using pre-chilled RIPA buffer, and 25 μg of each lysate was loaded into the gel and separated by SDS-PAGE. Western blotting was performed according to the standard protocol. Rabbit anti-MICA antibody (ab63709, abcam: 1/1000) and rabbit anti-SP1 antibody (17-601, Upstate Biotechnology: 1/500) were used in the experiment.

Statistical analysis

The case-control association was analyzed by Student's *t*-test and Fisher's exact test as appropriate. The association of allele dependent sMICA expression was studied by Kruskal-Wallis test using R statistical environment version 2.8.1. The LD and coefficients (D' and r^2) were calculated by Haploview version 4.2 [24].

Table 1. Association of rs2596542 with the progression from CHC to LC and HCC.

	Case MAF	Control MAF	P*	OR	95% C.I.
LC vs CHC	0.3797	0.3442	0.04842	1.166	1.01–1.35
HCC vs LC	0.4012	0.3797	0.20296	1.094	0.95–1.26

MAF, minor allele frequency; OR, odds ratio for minor allele. C.I., confidence interval. SNP rs2596542 was analyzed in 1,043 chronic hepatitis C (CHC), 586 liver cirrhosis without hepatocellular carcinoma (LC) and 1,394 HCV-induce hepaticellular carcinoma patients (HCC). *calculated by Armitage trend test.
doi:10.1371/journal.pone.0061279.t001

Results

Analyses of SNP rs2596542 in HCV-infected patients at different disease stages

Since the development of HCC consists of multiple steps, we investigated the role of SNP rs2596542 with disease progression. SNP rs2596542 was genotyped in patients at three different disease categories of CHC (chronic hepatitis C) without liver cirrhosis (LC) or HCC, LC without HCC, and HCC. The statistical analysis indicated that SNP rs2596542 was significantly associated with disease progression from CHC to LC with P-value of 0.048 and odds ratio of 1.17 (Table 1). The risk allele frequency among HCC patients (40.1%) was higher than that among LC patients (38.0%), but the association was not statistically significant (P-value of 0.203 and odds ratio of 1.09). These results suggested the involvement of *MICA* with both liver fibrosis and hepatocellular carcinogenesis.

HCV-HCC risk is not associated with MICA copy number variation

A previous report has indicated the deletion of the entire *MICA* locus in 3.2% of Japanese population [25] and this deletion was shown to be associated with the risk of nasopharyngeal carcinoma (NPC), especially in male [26]. To identify the functional SNP that may affect *MICA* mRNA expression, we analyzed the relation between the *MICA* copy number variation (CNV) and the HCC susceptibility. We quantified this CNV by real-time PCR in 375 HCV-related HCC patients and 350 HCV-negative controls. As shown in Table S3, we found no difference in the copy numbers between HCC cases and controls, indicating that this CNV is unlikely to be causative genetic variation for the risk of HCC.

Direct sequencing of 5′ flanking region of *MICA*

We then focused on the variations in the 5′ flanking region of the *MICA* gene which may be associated with its promoter activity. We had conducted direct DNA sequencing of the 5-kb promoter region which included the marker SNP rs2596542 using genomic DNAs of 50 HCC subjects and identified 11 SNPs showing strong linkage disequilibrium with the marker SNP rs2596542 ($D'>0.953$ and $r^2>0.832$) (Fig. S1, Table 2).

Allele specific binding of nuclear protein to genomic region including SNP rs2596538

To investigate whether these genetic variations would affect the binding affinity of some transcription factors, we had conducted the electrophoretic mobility shift assay (EMSA) using the nuclear extract of HLE human hepatocellular carcinoma cells. Since MICA is a stress-inducible protein [21], we first treated the cells with heat shock treatment at 42°C for 90 minutes and confirmed significant induction of MICA expression as shown in Fig. 1a. Then we performed EMSA using 24 labeled-oligonucleotides corresponding to each allele of the 12 candidates' SNPs. The results of EMSA demonstrated that an oligonucleotide corresponding to a G allele of SNP rs2596538 exhibited stronger binding affinity to a nuclear protein(s) than that to an A allele (Fig. 1b). We then confirmed the specific binding of nuclear proteins to the G allele by competitor assay using non-labeled oligonucleotides (Fig. 1c). The self (G allele) oligonucleotides inhibited the formation of DNA-protein complex in a dose-dependent manner, but the non-self (A allele) oligonucleotides showed no inhibition effect. Taken together, some nuclear protein(s) in hepatocellular carcinoma cells would interact with a DNA fragment including the G allele of SNP rs2596538.

Table 2. Linkage disequilibrium between 11 candidate SNPs and SNP rs2596542.

SNP ID	Relative position[a]	A1	A1 frequency	D'	r^2
rs2596542	−4815	A	0.36		
rs2428475	−4788	G	0.36	1	1
rs28366144	−4586	T	0.36	1	1
rs2428474	−4387	G	0.39	1	0.88
rs2251731	−4045	A	0.39	1	0.88
rs2844526	−3703	C	0.38	1	0.918
rs2596541	−3572	A	0.38	1	0.918
rs2523453	−3285	G	0.38	1	0.918
rs2544525	−3259	C	0.38	1	0.918
rs2523452	−2870	G	0.34	0.953	0.832
rs2596538	−2778	A	0.34	0.953	0.832
rs2844522	−2710	C	0.34	0.953	0.832

Note: Direct DNA sequence of 5-kb promoter region of MICA from 50 HCV-HCC subjects. D' and r^2 were calculated by comparing the genotypes of these SNPs to the marker SNP rs2596542 by Haploview. A1, minor allele; [a]Relative position to exon 1 of the *MICA* gene.
doi:10.1371/journal.pone.0061279.t002

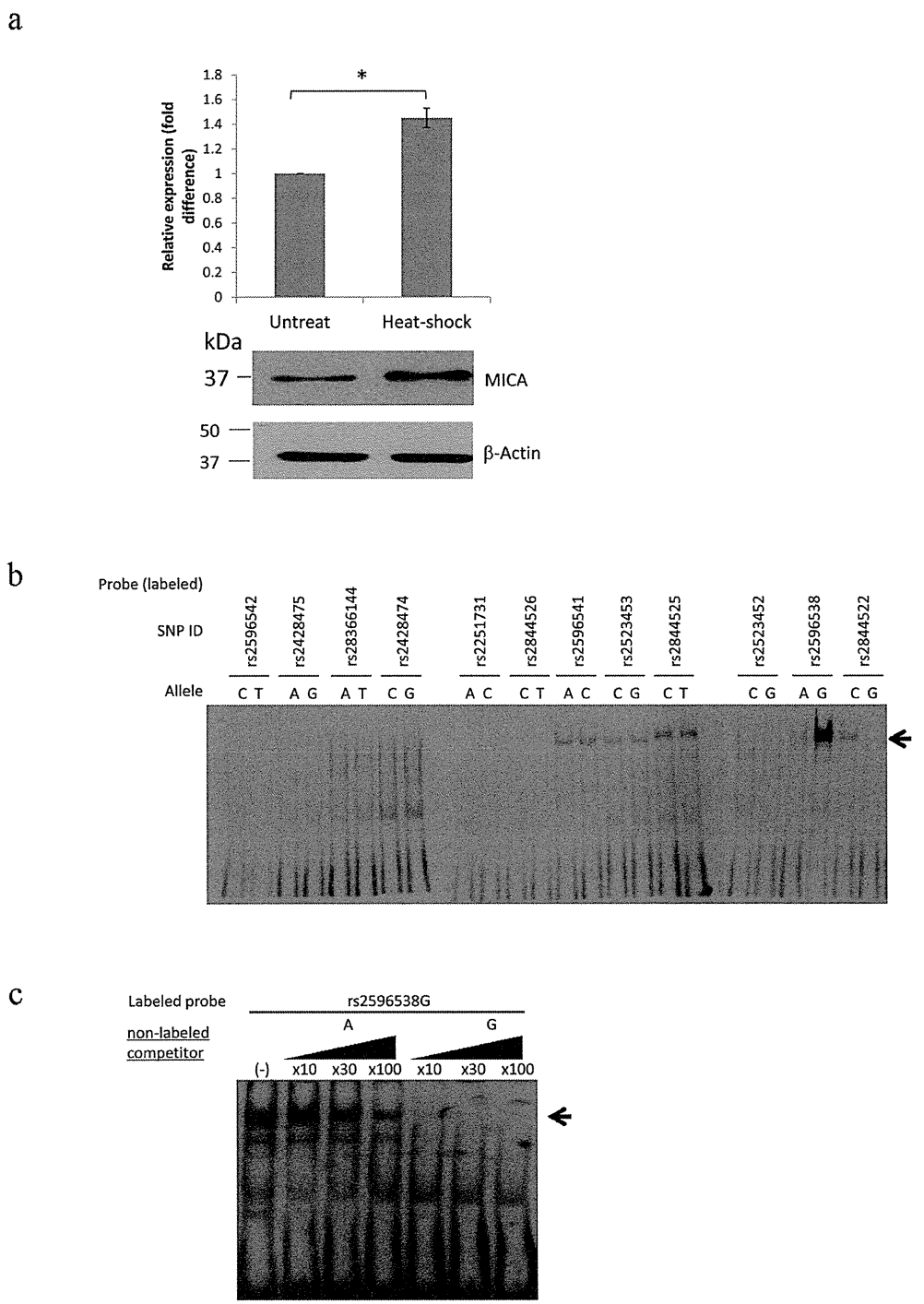

Figure 1. SNP rs2596538 affects the binding affinity of nuclear proteins. (**A**) Real-time quantitative PCR (upper) and Western blotting (lower) of MICA before and after heat shock treatment in HLE cells. *B2M* and β-actin are served as internal and protein loading control. (**B**) EMSA using 31 bp labeled probes flanking each SNP located within the 4.8 kb region upstream of *MICA* transcription start site. A black arrow indicates the shifted band specific to G allele of SNP rs2596538. (**C**) EMSA using the labeled G allele of SNP rs2596538 and nuclear extract from heat treated HLE cells. Non-labeled A or G allele of SNP rs2596538 at different concentrations are used as competitors. Pointed arrow indicates shifted band. *$P<0.05$ by Student's *t*-test.
doi:10.1371/journal.pone.0061279.g001

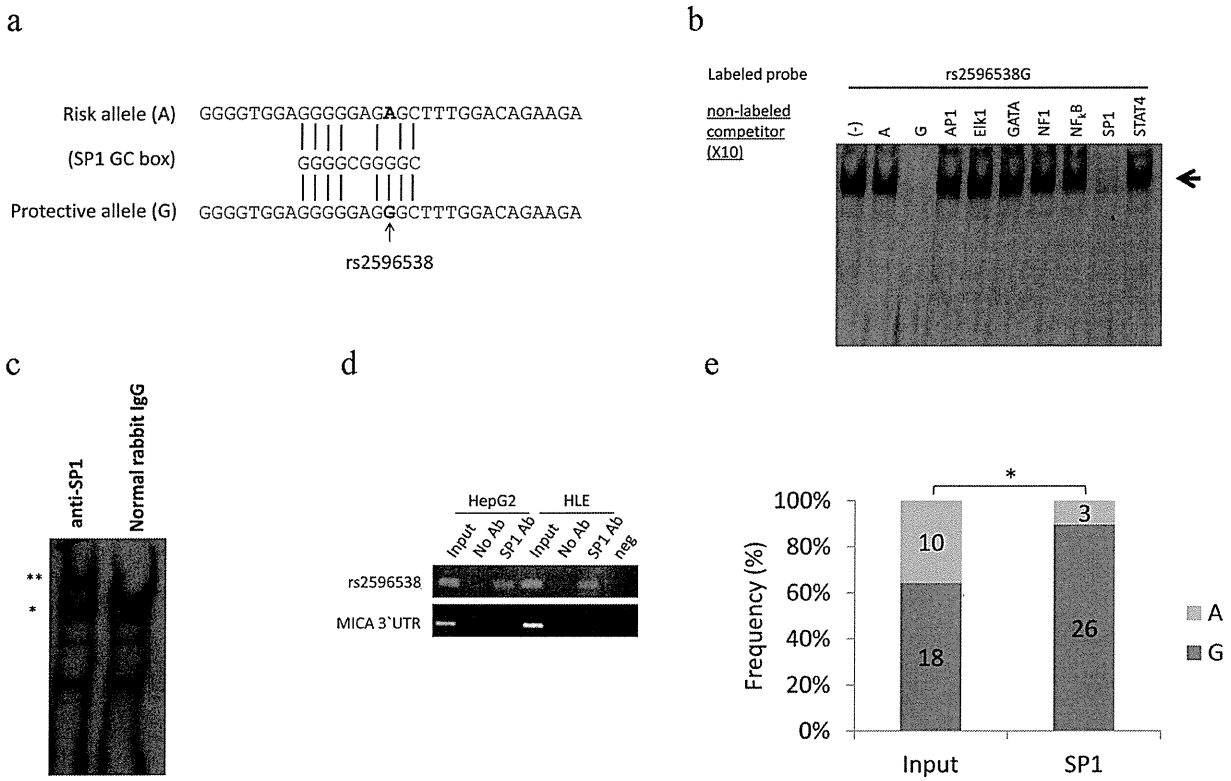

Figure 2. Binding of transcription factor SP1 to G allele of SNP rs2596538. (**A**) Multiple alignment of a GC box and DNA sequence of A or G probe of SNP rs2596538 used in EMSA. (**B**) EMSA using the labeled G allele of SNP rs2596538 and nuclear extract from heat treated HLE cells. Non-labeled consensus oligonucleotides of seven transcription factors are used as competitors. Pointed arrow indicates shifted band. (**C**) EMSA using the labeled G allele of SNP rs2596538 and nuclear extract from heat shock treated HLE cells in the presence of anti-SP1 antibody or normal rabbit IgG. Asterisks on the left side indicate the shifted (*) and super-shifted bands (**). Normal rabbit IgG serves as a negative control. (**D**) ChIP assay using HepG2 and HLE cell lines were ectopically expressed with SP1 protein. DNA-protein complex was immunoprecipitated with anti-SP1 antibody followed by PCR amplification using a primer pair flanking SNP rs2596538. DNAs precipitated without antibody are served as a negative control. PCR primers flanking the 3' UTR region of *MICA* are served as a negative control. (**E**) Genotype distribution at SNP rs2596538 in PCR fragment amplified from the input genomic DNA and DNA-protein complex immunopurified from HepG2 cells by using anit-SP1 antibody. *$P<0.05$ by Student's *t*-test.
doi:10.1371/journal.pone.0061279.g002

SNP rs2596538 regulates the binding of SP1

Since *in silico* analysis identified a putative GC box in a protective G allele but not in a risk A allele (Fig. 2a), the transcription factor SP1 might preferentially bind to the G allele. Base on this information, we further performed competitor assay using non-labeled oligonucleotides (Table S2) and found that among seven tested oligonucleotides, only SP1-consensus oligonucleotides could effectively inhibit the binding of the nuclear protein(s) to the labeled G allele (Fig. 2b). In addition, we identified that the addition of anti-SP1 antibody caused a supershift of a band corresponding to the DNA-protein complex while control IgG did not cause the band shift (Fig. 2c). This result clearly indicated that the SP1 protein is very likely to be a component of the DNA-protein complex.

Furthermore, we performed chromatin immunoprecipitation (ChIP) assay to confirm the binding of SP1 to this genomic region *in vivo*. We had used two cell lines with different genetic backgrounds at SNP rs2596538 locus: HLE cells carrying the only G allele, while HepG2 cells harboring both A and G alleles. After the introduction of SP1 expression vector (pCAGGS-SP1) into these cell lines, the cell extracts were subjected to ChIP assay using anti-SP1 antibody (Fig. 2d). Subsequent PCR experiments indicated that SP1 bound to a genomic fragment containing the G allele of SNP rs2596538 *in vivo*, while 3' UTR region of *MICA* (negative control) was not immunoprecipitated with anti-SP1 antibody. To further evaluate the binding ability of SP1 to each allele *in vivo*, we sub-cloned the DNA fragment that amplified from genomic DNA of HepG2 cells before and after immunoprecipitation by anti-SP1 antibody. The subsequent sequencing results showed that 26 out of 29 tested clones contained the G allele, demonstrating the preferential binding of SP1 to the G allele (Fig. 2e).

SP1 over-expression preferentially up-regulates MICA expression at G allele

To further investigate the physiological role of the interaction between SP1 and this genomic region, we performed reporter gene assay. Three copies of 31-bp DNA fragments flanking the candidate functional SNP rs2596538 were subcloned into the multiple cloning sites of the pGL3 promoter vector. The relative luciferase activity of the plasmid including the G allele was significantly higher than that including the A allele (Fig. 3a). Furthermore, over-expression of SP1 in the cells could significantly enhance the luciferase activity of the G-allele vector, while the enhancement of the A-allele vector was relatively modest (Fig. 3a).

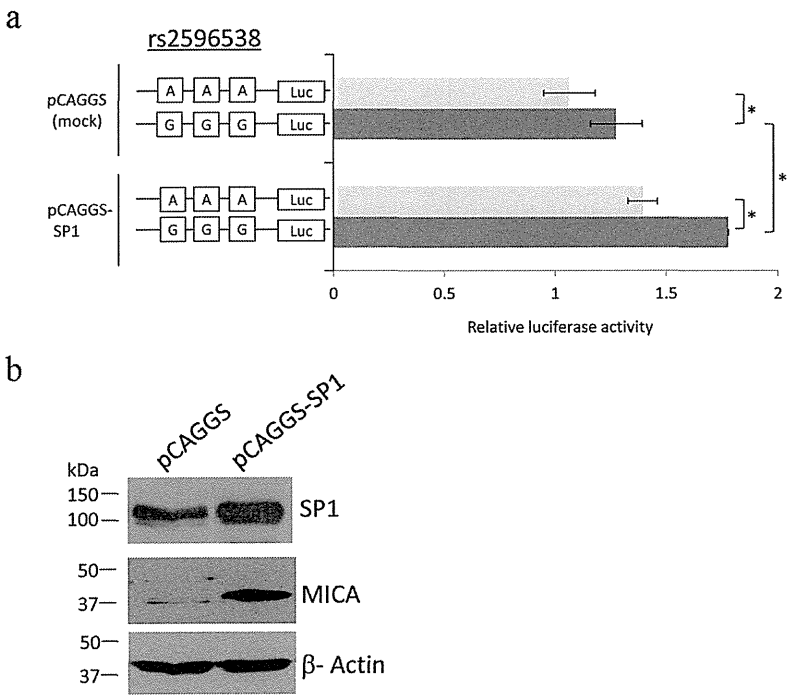

Figure 3. Transcriptional regulation of MICA by SP1 through genomic region including SNP rs2596538. (**A**) Reporter assay using constructs including 3 copies of 31 bp DNA fragment flanking SNP rs2596538. Reporter constructs are transfected into HLE cells with pRL-TK and pCAGGS or pCAGGS-SP1 vector. The value of relative luciferase activity was calculated as the firefly luciferase intensity divided by the renilla luciferase intensity. The data represent the mean ± SD value of 4 independent studies. (*$P<0.05$, Student's *t*-test) (**B**) MICA expression in HLE cells after transfection with pCAGGS or pCAGGS-SP1 vector. β-actin is served as a protein loading control.
doi:10.1371/journal.pone.0061279.g003

We also evaluated the effect of ectopically expressed SP1 on the MICA expression in HLE cells. Western-blot analysis showed that MICA protein expression was significantly increased after the SP1 over-expression (Fig. 3b). These results provided a strong evidence that the G allele has higher transcriptional potential that can be inducible by SP1.

Association of SNP rs2596538 with HCC risk and sMICA level in HCV-induced HCC patients

To further investigate the role of SNP rs2596538 in human carcinogenesis, we investigated the association of SNP rs2596538 with HCV-induced HCC in 721 HCV-HCC cases and 5,486 HCV-negative controls that had been genotyped using Illumina HumanHap610-Quad Genotyping BeadChip in our previous study [6]. We performed imputation analysis by using haplotype data from 1000 genome database [20] and found that an A allele of SNP rs2596538 was considered to be a risk allele for HCV-related HCC (Table 3, odds ratio = 1.343, $P = 1.82\times10^{-5}$). The functional SNP rs2596538 exhibited a stronger association with the HCC risk than the marker SNP rs2596542 (2.46×10^{-5}). We also analyzed the relationship between the SNP rs2596538 and the sMICA level among 246 HCV-induced HCC patients and found a significant association with the P-value of 0.00616 (Fig. 4). These results were concordant with our functional analyses in which the G allele exhibited a higher affinity to SP1 and revealed a higher transcriptional activity.

Table 3. Association of SNP rs2596542 and SNP rs2596538 with HCV-induced HCC.

SNP ID	Relative position[a]	A1	OR	*P* value
rs2596542	−4815	A	1.339	2.46×10^{-5}
rs2596538	−2778	A	1.343	1.82×10^{-5}

Note: Genotype data of 721 HCV-HCC cases and 5,486 HCV-negative controls were imputed using 1000 genomes as reference. A1, risk allele; OR, odds ratio for the risk allele calculated by considering the protective allele as a reference.
[a]Relative position to exon 1 of the *MICA* gene.
doi:10.1371/journal.pone.0061279.t003

Discussion

Approximately 160 million people (2.35% of the worldwide population) are estimated to have HCV infection [27]. Since HCV carriers have an increased risk to develop liver cirrhosis and subsequent HCC [28,29], the prediction of cancer risk is especially important for CHC patients. In our previous study, we have identified that SNP rs2596542 located in the upstream of *MICA* gene was significantly associated with the risk of HCC development among CHC patients as well as the serum level of sMICA [6]. In this study, we found that the genetic variant at SNP rs2596538 strongly affected the binding affinity of SP1. Over-expression of SP1 remarkably induced *MICA* expression in cells carrying the G allele that has a higher affinity to the SP1 binding. These findings are concordant with higher serum sMICA level among HCC patients with the G allele at SNP rs2596538. SP1 is a