in the resected liver. They further discovered that CD133+ cells act as CSCs that are chemoresistant in HCC (Ma et al. 2007, 2008b). Similar findings were reported by several groups (Suetsugu et al. 2006; Yin et al. 2007), and a recent paper further suggested that an aldehyde dehydrogenase (ALDH) + cell population among the CD133+ cells may be more tumorigenic CSCs (Ma et al. 2008a).

4.3.4 CD90 (Thy-1)

CD90 is a glycosylphosphatidylinositol (GPI)-anchored protein that is particularly abundant on the surface of thymocytes and T cells; but CD90 is also expressed in various cell types including fibroblasts, endothelial cells, neurons, and hematopoietic cells (Rege and Hagood 2006). Yang et al. (2008b, 2008c) recently investigated the expression of CD90 and CD44 in CD45-depleted primary HCC cells and peripheral blood mononuclear cells and identified that CD45– CD90+ tumor cells were more tumorigenic if they expressed CD44, Oct4, Bmi1, Albumin, AFP, Wnt3a, stat3, and HIF-1α . They further demonstrated that CD90+ CD44+ cells were more aggressive than CD90+ CD44– cells, and CD44 blockage prevented tumor formation by the CD90+ cells. On the basis of these data, the authors suggested that the presence of CD45– CD90+ cells in a population could be used as a marker for human liver cancer and as a target for the diagnosis and therapy of HCC.

4.3.5 OV6

Anti-OV6 is one of the monoclonal antibodies previously developed against cells isolated from carcinogen-treated rat liver (Dunsford and Sell 1989) and is known to react with oval cells and normal bile duct epithelial cells (Van Den Heuvel et al. 2001). The antigen recognized by anti-OV6 monoclonal antibodies in human liver has not yet been determined (Strain et al. 2003). Yang et al. (2008a) recently isolated OV6+ cells from human HCC to demonstrate that OV6+ cells have CSC-like features such as high tumorigenic capability and chemoresistance (Yang et al. 2008a). The authors further showed that OV6+ cells were characterized by the activation of Wnt/β-catenin signaling and inactivation of this signaling pathway resulted in a decrease in the OV6+ cell population. These results suggest that Wnt/β-catenin signaling may be a good target for eradication of OV6+ liver CSCs.

4.3.6 EpCAM (Epithelial Cell Adhesion/Activating Molecule, CD326)

EpCAM is one of the first tumor-associated antigens identified (Herlyn et al. 1979) and has numerous synonyms including 17-1A, HEA125, MK-1, GA733-2, EGP-2, EGP34, KSA, TROP-1, ESA, and KS1/4. EpCAM is expressed in a large variety of human adenocarcinomas and squamous cell carcinomas (Went et al. 2006), but the function as well as the regulatory mechanisms of EpCAM expression remained largely unknown to date (Balzar et al. 1999). We recently showed that the expression of the EpCAM gene (TACSTD1) is activated by Wnt/β-catenin signaling in a cis-regulatory mechanism (Yamashita et al. 2007). Furthermore, a very recent paper

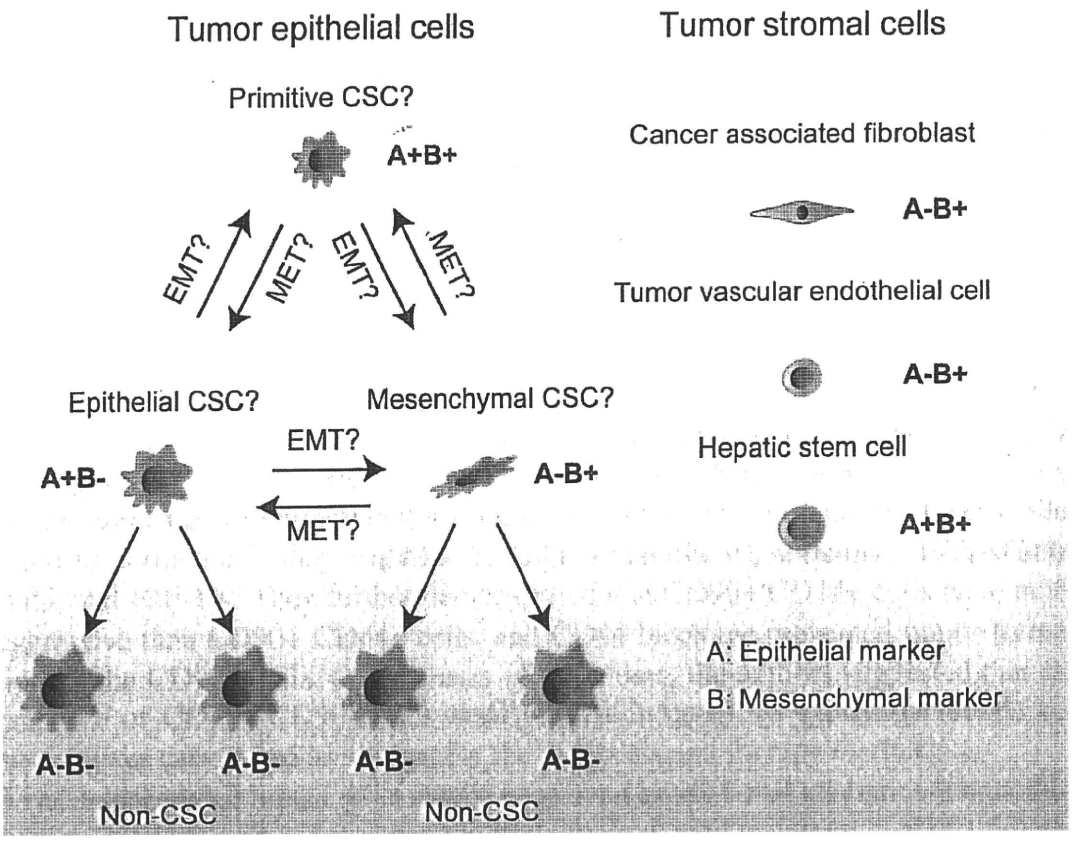
suggested that the EpCAM intracellular domain is cleaved at the cell membrane and associates with β-catenin and Lef-1 in the nucleus to activate Wnt/β-catenin signaling (Maetzel et al. 2009). These data suggest that EpCAM is not just a cell-surface molecule, but a signal transducer regulated by Wnt/β-catenin signaling in a positive-feedback manner.

EpCAM is used for the isolation of CSCs from various tumors including colonic and pancreatic cancers (Visvader and Lindeman 2008). We recently used EpCAM and AFP to identify the novel prognostic HCC subtypes related to a certain developmental stage of human liver lineages (Yamashita et al. 2008). Furthermore, we isolated EpCAM+ HCC cells from primary HCC samples and cell lines to show that EpCAM+ cells have the features of CSCs (Yamashita et al. 2009). Activation of Wnt/β-catenin signaling enriched the population of EpCAM+ CSCs, and blockage of EpCAM expression resulted in the inhibition of tumor formation by EpCAM+ cells in NOD/SCID mice. Thus, EpCAM seems a potentially useful marker and a good target for isolation and elimination of liver CSCs.

4.4 Heterogeneity of Liver Cancer Stem Cells

Although the markers listed above have been shown to be useful for the isolation of putative CSCs, it is unclear how these markers are expressed in primary HCC tissues or in HCC cell lines. It is also unclear whether the CSCs expressing these markers exist in all or are restricted to a certain subtype of HCCs. Furthermore, primary HCC tissues are composed of mixtures of mesenchymal/endothelial/inflammatory cells as well as tumor epithelial cells, and isolation of CSCs using such markers may result in the isolation of mixtures of tumor epithelial and stromal cells, a problem similar to that observed in the isolation of normal hepatic stem/progenitor cells (Fig. 16.2). Because recent findings have suggested the significance of stromal cells in tumorigenesis and metastasis of cancer (Dome et al. 2009; Karnoub et al. 2007; Mishra et al. 2008), it is possible that co-isolation of stromal cells may result in an enhanced tumorigenicity in immunodeficient mice that may not be related to the stem-like traits of tumor epithelial cells.

We recently investigated the expression of EpCAM (epithelial), CD133 (epithelial/hematopoietic/neural/endothelial), and CD90 (hematopoietic/mesenchymal/endothelial) in six HCC cell lines (Yamashita et al. 2009). Interestingly, AFP+ HCC cell lines (Hep3B, HuH1, and HuH7) have a subpopulation of EpCAM+ CD133+ cells but no CD90+ cells. In contrast, AFP– HCC cell lines (SK-Hep-1, HLE, and HLF) have a subpopulation of CD90+ cells but no EpCAM+ or CD133+ cells. Thus, AFP+ HCC cells may be more likely to have a subpopulation of epithelial CSC-markers+ cells (epithelial CSCs), whereas AFP– HCC cells may have a subpopulation of mesenchymal-markers+ cells (mesenchymal CSCs) (Fig. 16.2). These data suggest that CSC markers may not be equally expressed in all HCCs. Instead, the expression patterns of CSC markers in liver CSCs may be different in each HCC subtype, possibly due to the heterogeneity of activated signaling



and stromal cells may express the same markers currently used for the isolation of CSCs. Although CSCs are considered to be metastatic (mesenchymal CSCs?), tumorigenic (epithelial CSCs?), and drug/radiation-resistant (primitive CSCs?), these phenotypes may be distinct in each CSC subtype having distinct stem-cell markers that may be associated with EMT/MET signaling

pathways and stem-cell marker expression in normal hepatic stem/progenitor cells where these tumor-initiating cells may originate. Although primitive hepatic stem cells expressing both epithelial and mesenchymal markers (e.g., CD90+ EpCAM+) do exist in the fetal liver (Dan et al. 2006), it is unclear whether HCC subtypes containing primitive CSCs are present in primary HCC as well as in HCC cell lines that express epithelial and mesenchymal markers. Investigation and characterization of HCCs containing such primitive CSCs will be of interest in the future.

The epithelial–mesenchymal transition (EMT) and the reverse process, termed mesenchymal–epithelial transition (MET), are known to play a crucial role in embryonic development (Hugo et al. 2007). Accumulating evidence indicates that EMT also confers some important malignant traits of cancer, especially metastasis (Turley et al. 2008). CSCs are considered to be more metastatic than non-CSCs, and recently the concepts of CSC and EMT have emerged in the field of breast cancer research (Mani et al. 2008; Morel et al. 2008). In breast cancer, a population expressing low levels of CD24 (an epithelial marker) and high levels of CD44 (a

mesenchymal marker) is known to be representative of breast CSCs (Al-Hajj et al. 2003). Interestingly, activation of the EMT program by the induction of Snail or Twist genes or addition of recombinant TGF-β resulted in the enrichment of a CD24-low CD44-high population that had a high capability to form spheroids in vitro and subcutaneous tumors in vivo (Mani et al. 2008). Induction of oncogenic Ras also induced EMT and enriched the CSC population in breast cancer cells (Morel et al. 2008). In the liver, TGF-β signaling appears to induce the differentiation of hepatic stem/progenitor cells and suppress the development of HCC (Mishra et al. 2009), suggesting that it may not work in the same manner observed in breast cancers. Regardless, the association between the liver CSC phenotypes and the induction of EMT/MET programs is completely unclear and should be pursued in future studies (Fig. 16.2).

5 Conclusions

There is accumulating evidence that liver CSCs play a key role in the development and perpetuation of HCC, and the relevance of targeting CSCs has also become clear. Yet, experimental models for the treatment of HCC are still in the preliminary stages. Identification of useful CSC markers and exploration of their roles in maintaining stem-like traits are critical steps toward the clinical application of the CSC hypothesis for the improved diagnosis and the treatment of HCC.

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Original Article

ITPA gene variant protects against anemia induced by pegylated interferon-α and ribavirin therapy for Japanese patients with chronic hepatitis C

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Aim: Host genetic variants leading to inosine triphosphatase (ITPA) deficiency, a condition not thought to be clinically important, protect against hemolytic anemia in chronic hepatitis C patients receiving ribavirin. In this study, we evaluated the clinical significance of *ITPA* variants in Japanese hepatitis C patients who were treated with pegylated interferon plus ribavirin.

Methods: In this multicenter retrospective cross-sectional study, 474 hepatitis C patients were enrolled who were treated with pegylated interferon plus ribavirin in four geographically different hospitals in Japan. Patients were grouped according to hemoglobin decline of more than 3 g/dL at week 4. Two single nucleotide polymorphisms (SNP) within or adjacent to the *ITPA* gene (rs6051702, rs1127354) were genotyped.

Results: A functional SNP, rs1127354, within the *ITPA* exon was strongly associated with protection against anemia with only one (0.8%) in 129 patients with the *ITPA* minor variant A developing severe anemia ($P = 5.9 \times 10^{-20}$). For rs6051702, which had significant association in European-Americans, significant but weak association with severe hemoglobin reduction was found in Japanese ($P = 0.009$). In patients excluding genotype 1b and high viral load, those with the *ITPA* minor variant A achieved significantly higher sustained viral response rate than those with the major variant (CC) (96% vs 70%, respectively, $P = 0.0066$).

Conclusion: *ITPA* SNP, rs1127354, is confirmed to be a useful predictor of ribavirin-induced anemia in Japanese patients. Patients with the *ITPA* minor variant A (~27%) have an advantage in pegylated interferon plus ribavirin-based therapies, due to expected adherence of ribavirin doses, resulting in a higher viral clearance rate.

Key words: *c20orf194*, hemolytic anemia, hepatitis C virus, *ITPA* (inosine triphosphatase), pegylated interferon plus ribavirin therapy

INTRODUCTION

APPROXIMATELY 3% OF the worldwide population is infected with the hepatitis C virus (HCV), which represents 170 million people, with 3–4 million individuals newly infected each year. Chronic hepatitis C (CHC) has a variable course; although 20–25% of CHC patients maintain persistently normal serum aminotransferases and experience relatively slow histological progression, other patients present a more active biochemical course.[1–3] Overall, 30% of the CHC patients progress to cirrhosis in their lifetime,[3] and 3–8% of cirrhosis patients develop hepatocellular carcinoma (HCC) every year.[4–6] Among various factors, older age and hepatic steatosis are significant factors accelerating the rate of progression in CHC.[3,7–9]

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Antiviral treatment has been shown to improve liver histology and decrease incidence of HCC in CHC.[6,10] Current therapy for CHC consists of treatment with pegylated interferon (PEG IFN), which acts both as an antiviral and as an immunoregulatory cytokine, and ribavirin (RBV), an antiviral prodrug that interferes with RNA metabolism.[11,12] However, less than 50% of patients infected with HCV genotype 1 treated in this way achieve a sustained viral response (SVR) or a cure of the infection.[11,13] Older patients have showed a significantly lower SVR rate due to poor adherence resulting from adverse events and laboratory abnormalities.[14–16] In particular, hematological abnormalities and RBV-induced hemolytic anemia often necessitate dose reduction and premature withdrawal from therapy in 10–14% of patients.[11,17–20] New drugs and therapeutic approaches for CHC are actively developed and several candidates are in the early trial phase.[21,22] Given these backgrounds, effective pre-treatment screening for predictor biomarkers with the aim to evaluate possible risks over benefits from currently available treatment would allow avoiding these side-effects in patients who will not be helped by the treatment, as well as to reduce the substantial cost of the treatment.

The completion of the Human Genome Project has led to the advent of a new era of scientific research, including a revolutionary approach: the genome-wide association study (GWAS). Several recent studies have demonstrated remarkable associations between single nucleotide polymorphisms (SNP) near or within the region of the *IL28B* gene, which codes for IFN-λ3.[23–28] Another recent study indicated that genetic variants leading to inosine triphosphatase (ITPA) deficiency, a condition not thought to be clinically important, protect against hemolytic anemia in CHC patients receiving RBV.[29] The results obtained in one GWAS study need to be evaluated and confirmed in the context of different geographical and racial populations, and independent cohorts. Here, we describe clinical evaluation of two SNP within or adjacent to the *ITPA* gene (6051702 and rs1127354), that was recently highlighted by the GWAS of HCV treatment-induced anemia.[29]

METHODS

Patients

IN THIS RETROSPECTIVE cross-sectional case–control study, 474 patients with chronic HCV infection treated at Tokyo Medical and Dental University Hospital, Nagoya City University Hospital, Yamanashi University Hospital, Nagasaki Medical Center and Hyogo University of Health Science Hospital in Japan were enrolled from April 2007 to April 2009. Each patient was treated with PEG IFN-α-2b (1.5 μg/kg s.c. once a week) or PEG IFN-α-2a (180 μg/kg once a week) plus RBV (600–1000 mg daily depending on bodyweight). The treatment duration was set at a standard 48 weeks for genotype 1b high viral load (≥5 log copies/mL) patients and 24 weeks for genotype 1 low viral load (≤5 log copies/mL) and genotypes 2 and 3 patients. On-treatment dose reduction and discontinuation of PEG IFN or RBV were decided based on the recommendations of package inserts or clinical situations in individual patients to avoid possible side-effects. The rates of PEG IFN and RBV administration achieved were calculated as percentages of actual total dose administrated of a standard total dose of 24 weeks, according to bodyweight before therapy. Hepatitis B surface antigen (HBsAg) positive and/or anti-HIV positive individuals were excluded from this study. Hemoglobin (Hb) values were measured at baseline and every week until 8 weeks. We considered Hb decline at week 4 to be a clinically important time point, as previously reported.[29] The threshold of Hb reduction of more than 3 g/dL was chosen as a clinically significant Hb decline according to the previous reports.[29–31]

Informed consent was obtained from each patient who participated in the study. The study protocol conformed to the relevant ethical guidelines as reflected in a priori approval by the ethics committees of all the participating universities and hospitals.

Patient evaluation

The following factors were analyzed to determine whether they were related to the efficacy of combination therapy: age, sex, previous IFN therapy, grade of inflammation and stage of fibrosis on liver biopsy, pre-treatment biochemical parameters, such as white blood cells, neutrophils, Hb, platelet count, alanine transaminase (ALT) level, serum HCV RNA level (log IU/mL). Liver biopsy specimens were evaluated blindly, to determine the grade of inflammation and stage of fibrosis, by an independent interpreter who was not aware of the clinical data. Activity of inflammation was graded on a scale of 0–3: A0, showing no activity; A1, showing mild activity; A2, showing moderate activity; and A3, showing severe activity. Fibrosis was staged on a scale of 0–4: F0, showing no fibrosis; F1, showing moderate fibrosis; F2, showing moderate fibrosis with

few septa; F3, showing severe fibrosis with numerous septa without cirrhosis; and F4, showing cirrhosis.

SNP genotyping

Human genomic DNA was extracted from whole blood of each patient. Genetic polymorphisms, rs1127354 in *ITPA*, rs6051702 in *C20orf194*, and rs8099917 around the *IL28B* gene were determined by real-time detection polymerase chain reaction with a TaqMan probe or DigiTag2 assay typing one tag SNP located within each locus.[23] Another functional SNP, rs727010 within the *ITPA* gene, was excluded due to no variants in the Asian genetic population as reported in the International HapMap Project database. Our preliminary genotyping of a 100-patient population did not find variants in that SNP.

Outcomes

The primary end-point was Hb decline and dose reduction of PEG IFN or RBV in week 4, the secondary end-point was SVR. An SVR was defined as serum HCV RNA undetectable at 24 weeks after the end of treatment. A transient viral response (TVR) meant that HCV RNA became undetectable during treatment but reappeared at the end of follow up. A null response (NR) was defined as persistently positive HCV RNA throughout the treatment. Adverse events and drug adherence were recorded.

Statistical analyses

The association between individual ITPA SNP and the incidence of significant Hb decline was tested by a basic allelic test and calculated using the χ^2-test. Multivariate logistic regression analysis with stepwise forward selection was performed with *P*-values of less than 0.05 as the criteria for model inclusion. These statistical analyses were conducted by using SPSS software package ver. 18J (Chicago, IL, USA) or Microsoft Excel Mac 2008 (Redmond, WA, USA). Discrete variables were evaluated by Fisher's exact probability test. The *P*-values were calculated by two-tailed Student's *t*-tests for continuous data and χ^2-test for categorical data, and those of less than 0.05 were considered statistically significant.

RESULTS

THE CLINICAL CHARACTERISTICS of the 474 patients are summarized in Table 1. First, we compared baseline clinical and host genetic characteristics of patient groups according to the SNP within the *ITPA* gene, rs1127354, between major homozygote (CC) and a group of heterozygote (CA) and minor homozygote (AA) (Table 2). There were no significant differences in age, sex, blood cell counts, ALT levels, serum viral loads, frequencies of core 70/91 mutations[32,33] and the numbers of NS5A interferon sensitivity determining region (ISDR) mutations[34,35] between the two groups. The SNP in the *ITPA* gene did not show significant linkage between the SNP around the *IL28B* gene, rs8099917, which is strongly associated with IFN treatment responses.[23,25] In contrast, the SNP in the *ITPA* gene showed significant linkage with the SNP in *C20orf194*, rs6051702 ($P = 7.1 \times 10^{-14}$).[29]

Next, we analyzed two SNP, rs6051702 in *C20orf194* and rs1127354 in *ITPA* loci, respectively, for their association with significant Hb decline at 4 weeks of PEG IFN plus RBV treatment using a basic allelic model that compares frequencies of alleles in cases versus controls. The SNP, rs6051702, which showed the strongest association in the European-American population,[29] was

Table 1 Baseline characteristics of participating patients

Total number	474
Age (years)	57.2 ± 10.0
Sex (male/female)	264/210
Bodyweight (kg)	61.1 ± 10.8
HCV genotypes	
1b/2a/2b/3a	416/31/26/1
1b, high viral load/others	387/87
NS5A-ISDR mutations (genotype 1b, $n = 334$, 0–1/≥2)	285/49
Core mutations (genotype 1b, $n = 379$)	
C70 (wild/mutant)	240/139
C91 (wild/mutant)	234/145
Histology at biopsy ($n = 278$)	
Grade of inflammation (A0/1/2/3)	4/97/154/23
Stage of fibrosis (F0/1/2/3/4)	8/102/74/74/20
White blood cells (/μL)†	5707 ± 1495
Neutrophils (/μL)†	2568 ± 1013
Hemoglobin (g/dL)†	14.2 ± 1.4
Platelet count ($\times 10^{-3}$/μL)†	158 ± 58
ALT (IU/L)†	89 ± 66
Serum HCV RNA (log [IU/mL])‡	6.0 ± 0.9
PEG IFN (PEG IFN-α-2a/PEG IFN-α-2b)	40/434
Hb decline at week 4 (g/dl)	2.4 ± 1.4
Severe anemia, Hb <10 g/dL at week 4	56/474 (11.8%)

†Data are expressed as mean ± standard deviation.
‡Data are shown as median (range) values.
High viral load: HCV RNA ≥ 5 log IU/mL.
ALT, alanine transaminase; Hb, hemoglobin; HCV, hepatitis C virus; IFN, interferon; ISDR, interferon sensitivity determining region; PEG, pegylated.

Table 2 Clinical and host genetic characteristics of patients according to *IPTA* gene variants

	ITPA SNP, rs1127354		
	CC ($n = 345$)	CA + AA ($n = 129$)	*P*-value
Age (years)†	57.2 ± 10.0	57.1 ± 10.1	0.87
Sex (male/female)	192/153	72/57	0.97
White blood cells (/μL)‡	5312 ± 1537	4995 ± 1388	0.92
Neutrophils (/μL)‡	1696 ± 1415	1803 ± 1516	0.49
Hemoglobin (g/dL)‡	14.2 ± 1.4	14.1 ± 1.4	0.52
Platelet count ($\times 10^{-3}$/μL)‡	157 ± 54	159 ± 70	0.81
ALT (IU/mL)‡	91 ± 70	83 ± 55	0.46
Serum HCV RNA (log copies /mL)†	6.1 ± 0.7	5.8 ± 1.1	0.093
NS5A-ISDR mutations (genotype 1b, $n = 334$, 0–1/≥2)	213/31	72/18	0.095
Core mutations (genotype 1b, $n = 389$)			
aa. 70 (wild/mutant)	179/96	61/43	0.25
aa. 91 (wild/mutant)	172/103	62/42	0.60
IL28B, rs8099917 (TT/TG/GG)	233/94/3	96/29/1	0.23
C20orf194, rs6051702 (AA/AC/CC)	254/85/6	47/72/10	$\mathbf{7.1 \times 10^{-14}}$*

*P-values were calculated by student's t-test or by χ^2 analysis.
†Data are show as median (range) values.
‡Data are expressed as mean ± standard deviation.
IL28B SNP, major allele-T and minor allele-G.
C20orf194 SNP, major allele-A and minor allele-C.
ALT, alanine transaminase; HCV, hepatitis C virus; SNP, single nucleotide polymorphisms.

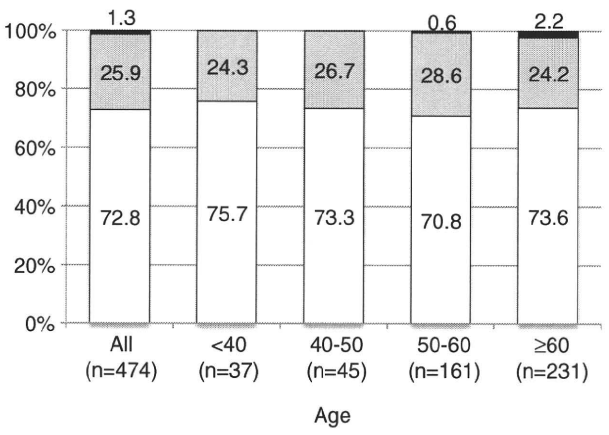

Figure 1 *ITPA* variant, rs1127354, known to be responsible for inosine triphosphatase deficiency and its age-related differences. *ITPA*, inosine triphosphatase. The numbers in parentheses denote numbers of patients.

associated with the Hb decline significantly but with smaller effect size (odds ratio [OR] = 1.40, $P = 9.0 \times 10^{-3}$, Table 3). Notably, another SNP in the *ITPA* gene, rs1127354, showed overwhelming association with the Hb decline (OR = 62.8, $P = 5.9 \times 10^{-20}$). The prevalence of *ITPA* variants is shown in Figure 1. Percentages of *ITPA*, rs1127354, major homozygote (CC), heterozygote (CA) and minor homozygote (AA) were 72.8%, 25.9% and 1.3%, respectively. There was no difference in the frequency of the *ITPA* variants throughout ages and sexes (Fig. 1).

To asses the clinical relevance of these SNP, we analyzed the proportion of patients suffering clinically significant anemia, which we defined as a decline in Hb levels of more than 3 g/dL or Hb levels of less than 10 g/dL, which is the threshold at which RBV dose reduction is recommended. As depicted in Figure 2, in *ITPA*-CC patients, Hb loss of more than 3 g/dL devel-

Table 3 Association of *C20orf194* and *IPTA* gene variants with treatment-induced Hb decline

Gene	SNP	Allele (major/minor)	MAF (%)	OR	*P*-value*
C20orf194	rs6051702	A/C	19.9	1.40	9.0×10^{-3}
ITPA	rs1127354	C/A	14.2	62.8	5.9×10^{-20}

*The SNP-phenotype associations were analyzed using a basic allelic test.
P-values were calculated by χ^2 analysis.
Hb, hemoglobin; MAF, minor allele frequency; OR, odds ratio; SNP, single nucleotide polymorphisms.

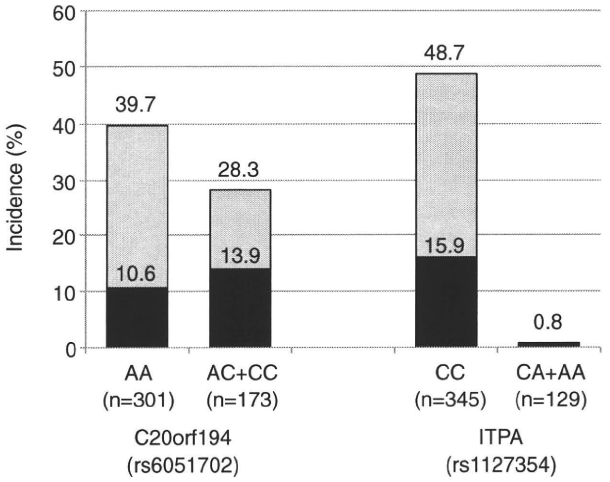

Figure 2 Effects of *c20orf194* and *ITPA* single nucleotide polymorphisms (SNP) on clinically significant anemia induced by pegylated interferon plus ribavirin treatment. Percentages of patients with hemoglobin (Hb) decline of >3 g/dL or Hb levels of >10 g/dL at week 4 of treatment are shown for each SNP in two genes, *c20orf94* (rs6051702) and *ITPA* (rs1127354).

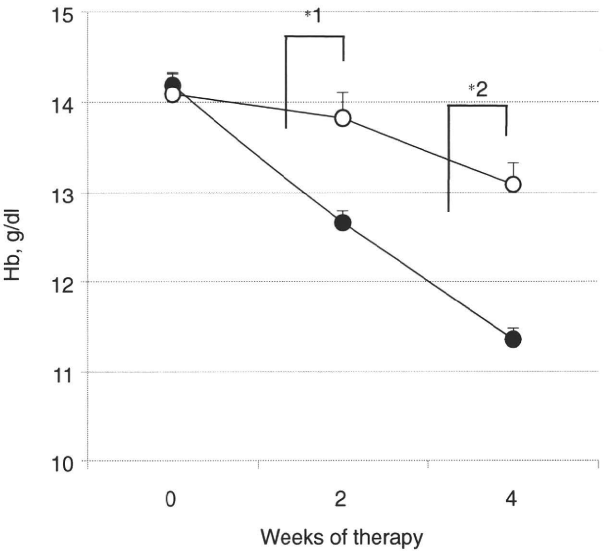

Figure 3 Time-dependent hemoglobin (Hb) decline in *ITPA* major and minor variants. Error bars indicate mean + standard error. Asterisks 1 and 2 indicate statistical significance of $P = 6.6 \times 10^{-13}$ and $P = 3.0 \times 10^{-29}$, respectively.

oped in 48.7% at week 4, and 15.9% of patients achieved Hb levels of less than 10 g/dL. In contrast, only one patient (0.8%) with *ITPA*-CA/AA developed anemia. These differences in the incidence of the treatment-induced Hb decline were consistent throughout ages. The time-dependent Hb decline in patients with *ITPA*-CC and *ITPA*-CA/AA is shown in Figure 3. In patients with *ITPA*-CC, mean Hb drop was 2.9 ± 1.3 g/dL, which was significantly higher than that of patients with *ITPA*-CA/AA (1.1 ± 0.7 g/dL). These results demonstrate that the *ITPA* minor variant A has a protective phenotype for the treatment-induced anemia. The positive predictive value of the *ITPA*-major (CC) for the development of severe anemia was 48.7%, while the negative predictive value of *ITPA*-hetero/minor (CA/AA) was 99.2%. In accordance with the incidence of anemia, there was significant difference in the incidence of RBV dose reduction. At week 4 of treatment, RBV doses were reduced in 27.9% of *ITPA*-CC patients while in only 14.4% of *ITPA*-CA/AA patients ($P = 0.012$, Fig. 4). Similarly to RBV, PEG IFN dose reduction was apparently higher in *ITPA*-CC patients though it did not reach statistical significance.

Knowing that significantly less frequent drug reduction occurred in patients with the *ITPA*-minor variant A, we next investigated if the *ITPA* gene variants affected final treatment outcomes. The treatment outcomes were available in 339 patients with genotype 1b and high viral load

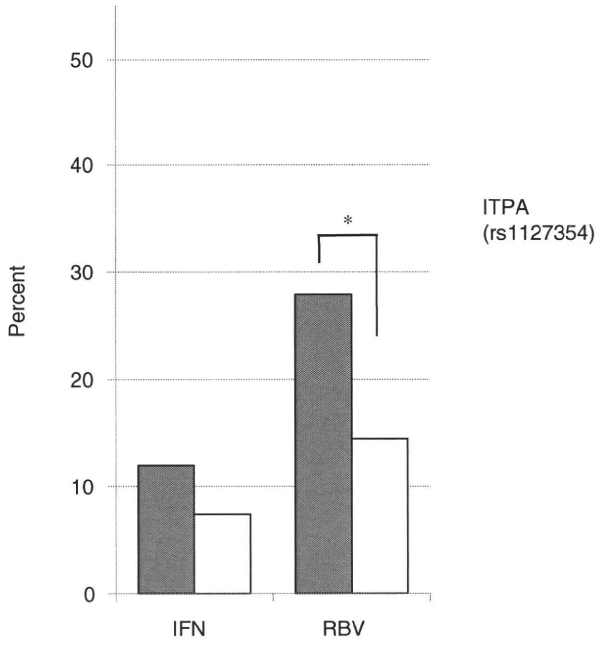

Figure 4 Percentages of patients requiring pegylated interferon (IFN) or ribavirin (RBV) dose reduction at week 4 in *ITPA* major and minor variants. *Y*-axis indicates percents of patients who required dose reduction. *$P = 0.012$.

Table 4 Sustained viral response rates of each group according to *IPTA* gene variants

IPTA SNP, rs1127354	Genotype 1b, high viral load		Others	
	CC	CA + AA	CC	CA + AA
SVR	92 (37.1%)	39 (42.9%)	41 (70%)	25 (96%)
TVR	90 (36.3%)	34 (37.3%)	15 (25%)	1 (4%)
NR	66 (26.6%)	18 (19.8%)	3 (5%)	0 (0%)
Total	248	91	59	26
P-value	0.33		**0.0066**	

High viral load; serum HCV RNA ≥ 5 logIU/mL.
"Others" include genotypes genotype 1b, serum HCV RNA <5 logIU/ml, genotypes 2a, 2b and 3a.
P-values were calculated by χ^2-test analyses of SVR versus TVR plus NR.
HCV, hepatitis C virus; SVR, sustained viral response; TVR, transient viral response; NR, null response.

(HCV RNA ≥5.0 log IU/ml) and 85 others, which included genotype 1b, low viral load and genotype 2a, 2b and 3a patients (Table 4). In patients with genotype 1b and high viral load, there was no significant difference in SVR rates between *ITPA*-CC and *ITPA*-CA/AA patients (37.1% and 42.9%, respectively). In contrast, there was a striking difference in SVR rates between *ITPA*-CC and *ITPA*-CA/AA in the other IFN-sensitive group (non-1b or low viral load); the SVR rate was 70% in *ITPA*-CC patients, while 96% of *ITPA*-CA/AA patients achieved SVR ($P = 0.0066$). These results indicate that the *ITPA* minor variant A is significantly associated with SVR in the IFN-sensitive group excluding genotype 1b and high viral load. Using those subpopulations of patients, we conducted a statistical analysis for association of several host and viral parameters with SVR. As shown in Table 5, univariate analysis identified four significant parameters including age, platelet count, stages of fibrosis and the ITPA SNP, rs1123354. Multivariate logistic regression analysis identified that only age and the ITPA SNP were significantly associated with SVR.

DISCUSSION

RECENT GWAS ON HCV infection have identified two important host genetic polymorphisms. One is the SNP in the *IL28B* gene, which is strongly associated with response to therapy of chronic genotype 1 HCV infection,[23–28] and another is the SNP in the *ITPA* gene, which precisely predicts RBV treatment-associated anemia in the European-American population.[29] In our present study, a functional SNP in the *ITPA* locus, rs1127354, is strongly associated with protection against anemia among 474 Japanese patients ($P = 5.9 \times 10^{-20}$, Table 3). Only one of 129 patients (0.8%) who carry the rs1127354 minor allele A had severe anemia (Figs 2,3). These data are consistent with the previous study in the US population[29] as well as a recent Japanese study by

Table 5 Univariate and multivariate logistic regression analyses of host and viral characteristics of patients excluding genotype 1b high virus load based on therapeutic responses ($n = 85$)

Variable	*P*-value (univariate)	*P*-value (multivariate)	OR	95% CI
Age	**0.017**	**0.047**	0.916	0.840–0.999
Sex (male vs female)	0.19	–		
Baseline Hb level	0.17	–		
Baseline platelet count	**0.019**	0.307	1.092	0.923–1.292
Stage of fibrosis (F0–2 vs 3–4)	**0.0020**	0.083	4.221	0.827–21.531
PEG IFN adherence (≥80% vs <80%)	0.67	–		
RBV adherence (≥80% vs <80%)	0.30	–		
ITPA SNP rs1127354 (CC vs CA + AA)	**0.0066**	**0.023**	12.680	1.386–116.042
IL28B SNP rs8099917 (TT vs TG + GG)	0.29	–		

CI, confidence interval; Hb, hemoglobin; IFN, interferon; ITPA, inosine triphosphatase; OR, odds ratio; PEG, pegylated; RBV, ribavirin; SNP, single nucleotide polymorphisms.

Ochi *et al.*[31] Our data were similar to these two reports; rs1127354 was the most significant SNP that was associated with RBV-induced anemia in Asian genetic populations. Additionally, we have demonstrated that the incidence of early dose reduction was significantly higher in *ITPA*-major (CC) patients as expected (Fig. 4) and, more importantly, that a significantly higher SVR rate was achieved in *ITPA*-hetero/minor (CA/AA) patients with HCV non-1b or low viral load strains (70% vs 96%, $P = 0.0066$, Table 4). Taken together, our results demonstrate that the *ITPA* minor variant A is not only a protective allele of PEG IFN and RBV treatment-associated anemia in the Japanese population, but also a significant predictor of SVR in certain HCV strains that show good response to IFN.

An SNP in *C20orf194*, rs6051702, which showed significant association with Hb reduction in European-Americans ($P = 1.1 \times 10^{-45}$),[29] was also significant in our study of a Japanese population, but with smaller effect size on Hb reduction ($P = 0.014$). The discrepancy may be due to the low levels of the linkage disequilibrium (LD) with the functional SNP in the *ITPA* gene in the Japanese/Asian population as compared with the high LD in white subjects. Indeed, it is reported that the predictive values of the *C20orf194* SNP varied between different races including African-Americans ($P = 0.19$) and Hispanics ($P = 9.5 \times 10^{-3}$).[29]

Ochi *et al.* sequenced the Japanese patient genome including ITPA and DDRGK1 loci, which are located adjacently on chromosome 20. They identified 83 SNP with major allele frequency of more than 0.05, of which four SNP including rs1127354 were significantly associated with RBV-induced anemia and which were in almost absolute LD with each other.[31] Their report indicates that the ITPA SNP, rs1127354, which we genotyped in the present study, represent a dominant variant of ITPA deficiency that protects against RBV-induced anemia in Japanese/Asian genetic populations. In our study, however, 51.3% of the ITPA-major (CC) patients did not develop significant Hb decline (Fig. 2). This finding suggests that there are other low-frequency ITPA variants or SNP in other enzymes that are involved in erythrocyte purine nucleoside metabolism.

The response to PEG IFN plus RBV treatment is affected by several viral and host factors such as age, sex,[36,37] NS5A-ISDR[38] and core region.[32,33] To maintain good adherence to drugs, especially RBV, it is important to achieve good treatment responses. Increased RBV exposure during the treatment phase was associated with an increased likelihood of SVR in the US[39] and Japanese studies.[40] Because patients with *ITPA* minor variant A are refractory to RBV-induced anemia, they are advantaged in maintaining good adherence to RBV and may be given even higher doses of RBV, resulting in a higher SVR rate. However, a study by Fellay *et al.* and a very recent replication study by Thompsom *et al.*[30] did not observe any significant association between the *ITPA* minor variants and early or late anti-HCV treatment outcomes.[29] A possible explanation for the discrepancy is that older and histologically more advanced patients were predominant in our study. Mean age in the US study was 47.5 years while 57.2 years in our present study. The percentage of advanced fibrosis (F3 or F4) was 12.0% in the US study while 34% in our study. It is well known that the incidence of drug dose reduction or discontinuation could increase according to old age as well as advanced stages, that may compromise final treatment outcomes.[14,15] Importantly, we have additionally demonstrated that in patients with other than genotype 1b HCV, ITPA minor variant A was significantly associated with better SVR rates in univariate and multivariate analyses. Because the typical PEG IFN plus RBV treatment period is shorter (24 weeks) in genotype 1 low viral load and genotype 2 patients than in genotype 1 high viral load (48 weeks), early dose reduction of RBV may be more critical to the final treatment outcome.

Ribavirin is a synthetic guanosine analog, and has actions *in vitro* against a wide range of RNA and DNA viruses.[41] Possible antiviral mechanisms of ribavirin include immune modulation by switching the T-cell phenotype from type 2 to type 1,[42] anti-proliferative effect by inhibition of cellular GTP synthesis,[41] and direct inhibition of virus replication.[43] Although monotherapy with RBV clinically showed minimal effect on the viral load and almost no effect on the viral clearance,[44–47] combinatory use of RBV with IFN elicits strong synergistic effects against HCV *in vitro*[48] and *in vivo*.[49,50]

Ribavirin is directly toxic to erythrocytes and is associated with hemolysis, which is usually reversible and dose-related.[49,50] RBV is incorporated into erythrocytes where it undergoes phosphorylation to its pharmacologically active forms through adenosine kinase. The RBV-phosphate conjugates are unable to cross the erythrocyte cell membrane and are thus accumulated intracellularly and cleared slowly from red cells with a half-life of approximately 40 days.[51] Inosine triphosphatase (ITP) deficiency or low activity variants, in turn, lead to an accumulation of ITP in red blood cells and may compete with RBV triphosphate, and may protect from RBV-induced hemolysis.[52,53]

There are several STAT-C agents (specifically targeted antiviral therapies for hepatitis C) being tested for

clinical efficacy against hepatitis C.[21,22] Most experts believe that when new drugs are approved to treat hepatitis C they will be used in combination with PEG IFN and RBV. Moreover, recent clinical trials including NS3 protease inhibitors have shown that PEG IFN plus RBV would be necessary to achieve optimal treatment responses.[18,19,54] Our present results may give a valuable pharmacogenetic diagnostic tool for the tailoring of RBV dosage to minimize drug-induced adverse events and for further optimization of the clinical anti-HCV chemotherapeutics.

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Cell Metabolism
Article

A Liver-Derived Secretory Protein, Selenoprotein P, Causes Insulin Resistance

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SUMMARY

The liver may regulate glucose homeostasis by modulating the sensitivity/resistance of peripheral tissues to insulin, by way of the production of secretory proteins, termed hepatokines. Here, we demonstrate that selenoprotein P (SeP), a liver-derived secretory protein, causes insulin resistance. Using serial analysis of gene expression (SAGE) and DNA chip methods, we found that hepatic SeP mRNA levels correlated with insulin resistance in humans. Administration of purified SeP impaired insulin signaling and dysregulated glucose metabolism in both hepatocytes and myocytes. Conversely, both genetic deletion and RNA interference-mediated knockdown of SeP improved systemic insulin sensitivity and glucose tolerance in mice. The metabolic actions of SeP were mediated, at least partly, by inactivation of adenosine monophosphate-activated protein kinase (AMPK). In summary, these results demonstrate a role of SeP in the regulation of glucose metabolism and insulin sensitivity and suggest that SeP may be a therapeutic target for type 2 diabetes.

INTRODUCTION

Insulin resistance is an underlying feature of people with type 2 diabetes and metabolic syndrome (Saltiel and Kahn, 2001), but is also associated with risk for cardiovascular diseases (Després et al., 1996) and contributes to the clinical manifestations of nonalcoholic steatohepatitis (Ota et al., 2007). In an insulin-resistant state, impaired insulin action promotes hepatic glucose production and reduces glucose uptake by peripheral tissues, resulting in hyperglycemia. The molecular mechanisms underlying insulin resistance are not fully understood, but are now known to be influenced by the secretion of tissue-derived factors, traditionally considered separate from the endocrine system. Recent work in obesity research, for example, has demonstrated that adipose tissues secrete a variety of proteins, known as adipocytokines (Friedman and Halaas, 1998; Maeda et al., 1996; Scherer et al., 1995; Steppan et al., 2001; Yang et al., 2005), which can either enhance or impair insulin sensitivity, thereby contributing to the development of insulin resistance.

SeP (in humans encoded by the *SEPP1* gene) is a secretory protein primarily produced by the liver (Burk and Hill, 2005; Carlson et al., 2004). It contains ten selenocysteine residues and functions as a selenium supply protein (Saito and Takahashi, 2002). However, the role of SeP in the regulation of glucose metabolism and insulin sensitivity has not yet been established. Furthermore, the clinical significance of SeP in human diseases has not been well defined, although studies of SeP knockout mice showed SeP deficiency to be associated with neurological injury and low fertility (Hill et al., 2003; Schomburg et al., 2003).

The liver plays a central role in glucose homeostasis and is also the site for the production of various secretory proteins. For example, recent work in our laboratory has revealed that genes encoding secretory proteins are abundantly expressed in the livers of people with type 2 diabetes (Misu et al., 2007). Moreover, genes encoding angiogenic factors, fibrogenic factors, and redox-associated factors were differentially expressed in the livers of people with type 2 diabetes (Takamura et al., 2004; Takeshita et al., 2006), possibly contributing to the pathophysiology of

type 2 diabetes and its clinical manifestations. On the basis of these findings, we hypothesize that, analogous to adipose tissues, the liver may also contribute to the development of type 2 diabetes and insulin resistance, through the production of secretory proteins, termed hepatokines.

RESULTS

Identification of a Hepatic Secretory Protein Involved in Insulin Resistance

To identify hepatic secretory proteins involved in insulin resistance, we performed liver biopsies in humans and conducted a comprehensive analysis of gene expression profiles, using two distinct methods. First, we obtained human liver samples from five patients with type 2 diabetes and five nondiabetic subjects who underwent surgical procedures for malignant tumors, and we subjected them to serial analysis of gene expression (SAGE) (Velculescu et al., 1995). Consequently, we identified 117 genes encoding putative secretory proteins with expression levels in people with type 2 diabetes, 1.5-fold or greater higher than those in normal subjects. Next, we obtained ultrasonography-guided percutaneous needle liver biopsies from ten people with type 2 diabetes and seven normal subjects (Table S1 available online), and we subjected them to DNA chip analysis to identify genes whose hepatic expression was significantly correlated with insulin resistance (Table S2). We performed glucose clamp experiments on these human subjects and measured the metabolic clearance rate (MCR) of glucose (glucose infusion rate divided by the steady-state plasma glucose concentration) as a measure of systemic insulin sensitivity. As a result, we found that *SEPP1* expression levels were upregulated 8-fold in people with type 2 diabetes compared with normal subjects, as determined by SAGE (Table S2). Additionally, there was a negative correlation between hepatic *SEPP1* messenger RNA (mRNA) levels and the MCR of glucose, indicating that elevated hepatic *SEPP1* mRNA levels were associated with insulin resistance (Figure 1A). As a corollary, we found a positive correlation between the levels of hepatic *SEPP1* mRNA and postloaded or fasting plasma glucose (Figures 1B and 1C).

Elevation of SeP in Type 2 Diabetes

To characterize the role of SeP in the development of insulin resistance, we measured serum SeP levels in human samples (Table S3), using enzyme-linked immunosorbent assays (ELISA), as described previously (Saito et al., 2001). Consistent with elevated hepatic *SEPP1* mRNA levels, we found a significant positive correlation between serum SeP levels and both fasting plasma glucose and hemoglobin A_{1c} (HbA_{1c}) levels (Figures 1D and 1E). HbA_{1c} is a clinical marker of protein glycation due to hyperglycemia, and elevated HbA_{1c} levels generally reflect poor glucose control over a 2–3 month period. Additionally, serum levels of SeP were significantly elevated in people with type 2 diabetes compared with normal subjects (Figure 1F and Table S4). Similar to data derived from clinical specimens, in rodent models of type 2 diabetes, including OLETF rats and KKAy mice, hepatic *Sepp1* mRNA and serum SeP levels were elevated (Figures 1G–1J and Table S5).

SeP Expression in Hepatocytes Is Regulated by Glucose, Palmitate, and Insulin

To clarify the pathophysiology contributing to the hepatic expression of SeP in type 2 diabetes, we investigated the effects of nutrient supply on *Sepp1* mRNA expression in cultured hepatocytes. We found that the addition of glucose or palmitate upregulated *Sepp1* expression, whereas insulin downregulated it in a dose- and time-dependent manner (Figures 2A, 2C, 2E, and 2F). Similar effects on SeP protein levels were observed in primary mouse hepatocytes (Figures 2B, 2D, and 2G). Consistent with the negative regulation of *Sepp1* by insulin in hepatocytes, *Sepp1* mRNA levels were elevated in the livers of fasting C57BL6J mice, compared with those that had been fed (Figure 2H). Thus, multiple lines of evidence suggest that elevated SeP is associated with the development of insulin resistance.

SeP Impairs Insulin Signaling and Dysregulates Glucose Metabolism In Vitro

Because there is no existing cell culture or animal model in which SeP is overexpressed, we purified SeP from human plasma using chromatographic methods (Saito et al., 1999; Saito and Takahashi, 2002) to examine the effects of SeP on insulin-mediated signal transduction. Treatment of primary hepatocytes with purified SeP induced a reduction in insulin-stimulated phosphorylation of insulin receptor (IR), and Akt (Figures 3A and 3B). SeP exerts its actions through an increase in cellular glutathione peroxidase (Saito and Takahashi, 2002). Coadministration of BSO, a glutathione synthesis inhibitor, rescued cells from the inhibitory effects of SeP (Figure 3C). Moreover, SeP increased phosphorylation of IRS1 at Ser307, the downregulator of tyrosine phosphorylation of IRS (Figure S1A). Similar effects of SeP were also observed in C2C12 myocytes (Figure S1B). Next, we assessed whether SeP dysregulated cellular glucose metabolism. In H4IIHEC hepatocytes, treatment with SeP upregulated mRNA expression of *Pck1* and *G6pc*, key gluconeogenic enzymes, resulting in a 30% increase in glucose release in the presence of insulin (Figures 3D–3F). Treatment with SeP alone had no effects on the levels of mRNAs encoding gluconeogenic enzymes or on glucose production in the absence of insulin, suggesting that SeP modulates insulin signaling. Additionally, treatment with SeP induced a reduction in insulin-stimulated glucose uptake in C2C12 myocytes (Figure 3G). These in vitro experiments indicate that, at physiological concentrations, SeP impairs insulin signal transduction and dysregulated cellular glucose metabolism.

SeP Impairs Insulin Signaling and Disrupts Glucose Homeostasis In Vivo

To examine the physiological effects of SeP in vivo, we treated female C57BL/6J mice with two intraperitoneal injections of purified human SeP (1 mg/kg body weight), 12 and 2 hr before the experiments. Injection of purified human SeP protein resulted in serum levels of 0.5–1.5 μg/mL (data not shown). These levels correspond to the incremental change of SeP serum levels in people with normal glucose tolerance to those with type 2 diabetes (Saito et al., 2001). Glucose and insulin tolerance tests revealed that treatment of mice with purified SeP induced glucose intolerance and insulin resistance (Figures 3H and 3I). Blood insulin levels were significantly elevated in

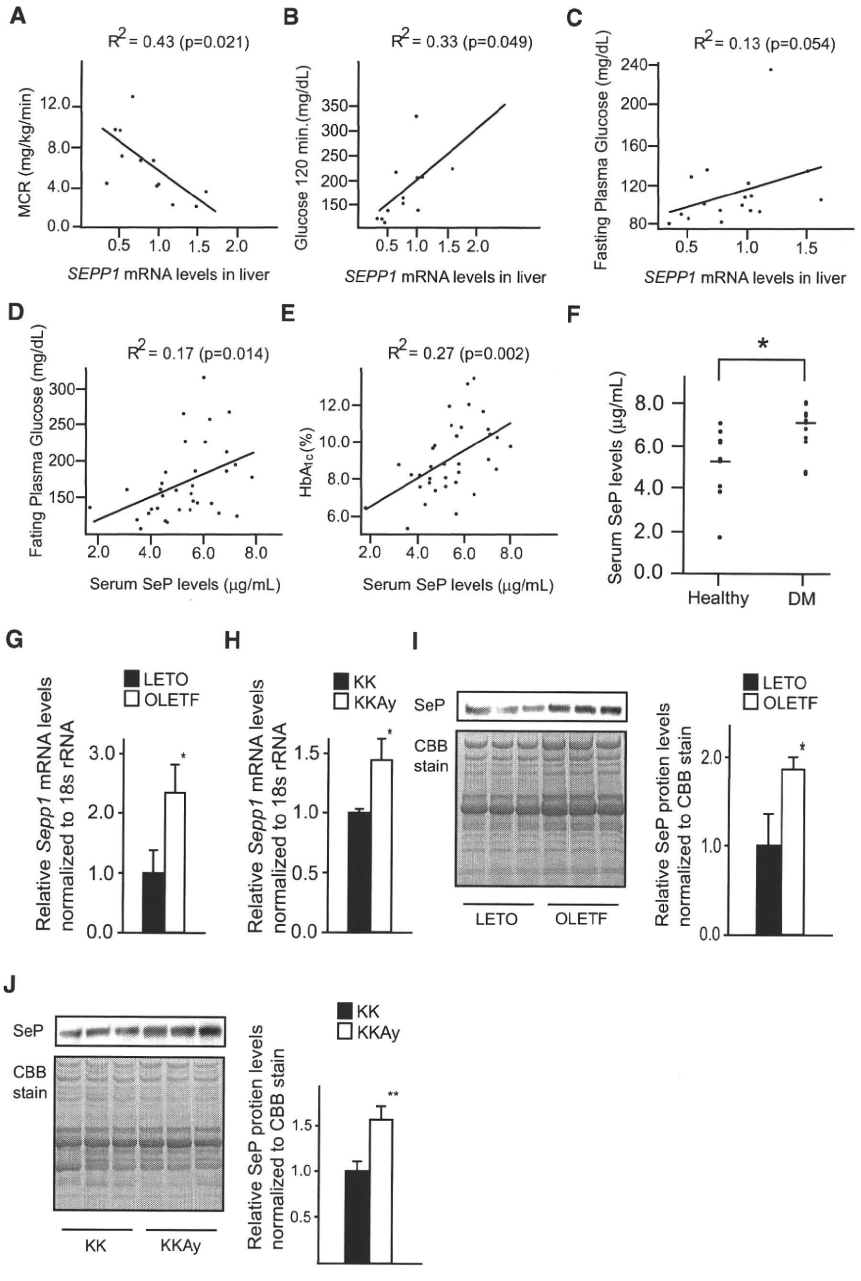

Figure 1. Elevation of Serum SeP Levels and Hepatic *Sepp1* Expression in Type 2 Diabetes

(A–C) Individual correlations between hepatic *SEPP1* mRNA levels and metabolic clearance rate (MCR) of glucose (A), postloaded plasma glucose levels (B), and fasting plasma glucose levels (C) in humans (n = 12–17). MCR equals the glucose infusion rate divided by the steady-state plasma glucose concentration, and is a measure of systemic insulin sensitivity. MCR values were determined by glucose clamp. *SEPP1* mRNA levels were quantified with DNA chips.

(D and E) Correlations between serum levels of SeP and fasting plasma glucose levels (D) and HbA_{1c} (E) in people with type 2 diabetes (n = 35).

(F) Serum levels of SeP in people with type 2 diabetes and healthy subjects (n = 9–12). Age and body weight were not significantly different between the two groups. Data represents the means ± SEM from two groups. *p < 0.05.

(G and H) Hepatic *Sepp1* mRNA levels in an animal model of type 2 diabetes (n = 5–6).

(I and J) Serum SeP levels in an animal model of type 2 diabetes. SeP was detected by western blotting. Coomassie brilliant blue (CBB)-stained gel is used as a control for protein loading. Graphs display the results of densitometric quantification, normalized to CBB-stained proteins (n = 5).

Data represent the mean ± SEM from five to six mice per group. *p < 0.05, **p < 0.01. See also Tables S1–S5.

SeP-injected mice, although those of glucagon and GLP-1 were unaffected during a glucose tolerance test (Figure S1C). Western blot analysis showed a reduction in insulin-induced serine phosphorylation of Akt in both liver and skeletal muscle of SeP-injected mice (Figures 3J and 3K). Hyperinsulinemic-euglycemic clamp studies showed that treatment with SeP significantly increased endogenous glucose production and decreased peripheral glucose disposal (Figure S1D and Figures 3L and 3M). Additionally, serum levels of injected human SeP protein negatively correlated with rates of peripheral glucose disposal (Figure S1E). These data indicate that SeP impairs insulin signaling in the liver and skeletal muscle and induces glucose intolerance in vivo.

Knockdown of *Sepp1* in Liver Improves Glucose Intolerance and Insulin Resistance in Mice with Type 2 Diabetes

To determine whether knockdown of endogenous *Sepp1* enhances insulin signaling, we transfected H4IIEC hepatocytes with *Sepp1*-specific small interfering RNA (siRNA), and we observed a reduction in endogenous *Sepp1* mRNA and SeP protein levels (Figures 4A and 4B). Insulin-stimulated serine phosphorylation of Akt was enhanced in these treated cells (Figure 4C). Similarly, delivery of *Sepp1*-specific siRNAs into KKAy mice via a hydrodynamic transfection method (McCaffrey et al., 2002; Zender et al., 2003) resulted in a 30% reduction in SeP protein levels in the liver and blood (Figures 4D–4G and Figure S2). Knockdown of *Sepp1* improved both glucose intolerance (Figures 4H and 4I) and insulin resistance (Figures 4J and 4K) in KKAy mice.

SeP-Deficient Mice Show Improved Glucose Tolerance and Enhanced Insulin Signaling in Liver and Muscle

We further confirmed the long-term effects of lowered SeP using *Sepp1* knockout mice (Hill et al., 2003). SeP knockout mice were viable and displayed normal body weights when maintained on a selenium-sufficient diet. Body weight, food intake, and O_2 consumption were unaffected by SeP knockout (Figures S3A