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# Proteomics Analysis of Mitochondrial Proteins Reveals Overexpression of a Mitochondrial Protein Chaperon, Prohibitin, in Cells Expressing Hepatitis C Virus Core Protein

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The hepatitis C virus (HCV) core protein is involved in viral pathogenesis such as oxidative stress induction and lipid metabolism disturbance, and is primarily located in the cytoplasm and endoplasmic reticulum in association with lipid droplets as well as in the mitochondria. To clarify the impact of the core protein on mitochondria, we analyzed the expression pattern of mitochondrial proteins in core protein-expressing cells by two-dimensional polyacrylamide gel electrophoresis. Several proteins related to the mitochondrial respiratory chain or protein chaperons were identified by mass spectrometry. Among the identified proteins with consistently different expressions, prohibitin, a mitochondrial protein chaperon, was up-regulated not only in core-expressing cells but also in full-genomic replicon cells and livers of core-gene transgenic mice. The stability of prohibitin was increased through interaction with the core protein. Further analysis demonstrated that interaction of prohibitin with mitochondrial DNA-encoded subunits of cytochrome c oxidase (COX) was disturbed by the core protein, resulting in a significant decrease in COX activity. **Conclusion:** The HCV core protein affects the steady-state levels of a subset of mitochondrial proteins including prohibitin, which may lead to an impaired function of the mitochondrial respiratory chain with the overproduction of oxidative stress. (HEPATOLOGY 2009;50:378-386.)

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Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; COX, cytochrome c oxidase; ER, endoplasmic reticulum; Ero1, ER protein endoplasmic oxidoreduction-1; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HSP, heat shock protein; IFN, interferon; MnSOD, manganese superoxide dismutase; NS, nonstructural; OST48, oligosaccharyltransferase-48; PDH, pyruvate dehydrogenase; PDI, protein disulfide isomerase; ROS, reactive oxygen species; TFA, trifluoroacetic acid.

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The hepatitis C virus (HCV) is a causative agent of chronic hepatitis, which often leads to cirrhosis and, eventually, to the development of hepatocellular carcinoma (HCC). However, the mechanism of hepatocarcinogenesis in HCV infection is not yet fully elucidated. The HCV core protein forms the viral nucleocapsid protein and has various properties that modulate cellular processes in numerous ways. The core protein binds to cellular proteins, suppresses or enhances apoptosis, and modulates the transcription of some host genes.<sup>1</sup> In addition, transgenic mice expressing the core protein develop HCC,<sup>2-4</sup> indicating a direct contribution of the core protein to the pathogenesis of hepatitis C.

The core protein is mostly localized to the endoplasmic reticulum (ER), but we and other groups have shown its localization to the mitochondria in cultured cells and transgenic mice.<sup>2,5,6</sup> In addition, the double structure of mitochondrial membranes is disrupted in hepatocytes of core-gene transgenic mice.<sup>2-4</sup> Evidence suggests that the core protein modulates some mitochondrial functions, including fatty acid  $\beta$ -oxidation, the impairment of which may induce lipid abnormalities and hepatic steatosis. In addition, the mitochondrion is an important source of reactive oxygen species (ROS). In livers of transgenic

mice harboring the core gene, increased ROS production has been observed.<sup>7-9</sup> A recent study found, by the proteomic profiling of biopsy specimens, that an impairment in key mitochondrial processes, including fatty acid oxidation and oxidative phosphorylation, and in the response to oxidative stress occurs in HCV-infected human liver with advanced fibrosis.<sup>10</sup> Therefore, it is probable that the HCV core protein affects mitochondrial functions because such pathogenesis is observed in both HCV core-transgenic mice and HCV-infected patients.<sup>11-13</sup>

The recent progress in proteomics has opened new avenues for disease-related biomarker discovery. Among proteomics approaches, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a technique for the separation and identification of proteins in a sample by displacement in two dimensions oriented at right angles to one another. This method is generally used as a component of proteomics and is the step used for the isolation of proteins for further characterization by mass spectrometry. 2D-PAGE is particularly useful when comparing two related samples such as healthy and diseased tissue. For example, proteins that are more abundant in diseased tissue may represent novel drug targets or diagnostic markers. In fact, several candidate biomarkers for many human cancers have been identified by this approach.<sup>14</sup> There are, however, tens of thousands of proteins in a cell, differing in abundance over six orders of magnitude. 2D-PAGE is not sensitive enough to detect rare proteins, and hence many proteins are not resolved. Therefore, splitting a sample into different fractions is often necessary to reduce the complexity of protein mixtures prior to 2D-PAGE. For this advantage, Lescuyer et al.<sup>15</sup> performed a 2D-PAGE of human mitochondrial proteins derived from the placenta and identified proteins mainly by peptide mass fingerprinting.

In this study, we performed a 2D-PAGE of mitochondria isolated from HepG2 cells stably expressing the HCV core protein and identified several proteins of different expressions when compared with control HepG2 cells. Among up-regulated proteins in the core-expressing cells, we focused on prohibitin, which functions as a mitochondrial protein chaperon, and found that the core protein interacts with prohibitin and represses the interaction between prohibitin and subunit proteins of cytochrome c oxidase (COX), which may lead to decreases in the expression level of the proteins and in COX activity. These results may explain the pathogenesis of liver disease in HCV infection including ROS induction.

## Materials and Methods

**Cells and Purification of Mitochondria.** Hep39 cells,<sup>16</sup> which stably express the HCV core protein, and

control HepG2 cells (Hepswx) were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and 1 mg/mL G418. Mitochondria were purified using Nycodenz (Nycomed Pharma, Zürich, Switzerland) according to the protocols reported by Okado-Matsumoto et al.<sup>17</sup> For transient transfection experiments, HepG2 cells were transfected with a core-expression plasmid using TransIT-LT1 (Mirus Bio, Madison, WI). Huh7 cells harboring HCV genotype 1b full-genomic (RCYM1)<sup>18</sup> or subgenomic replicon (5-15), and livers of 3-month-old core-gene transgenic mice<sup>2</sup> were also used for the analysis.

**2D-PAGE.** Gel electrophoresis in the first dimension was performed using an immobilized pH gradient gel (Immobiline Dry Strip gel, pH 4-7 linear, 13 cm; GE Healthcare, Uppsala, Sweden). The two-dimensional separation was performed on 12.5%, 14 × 16 cm<sup>2</sup>, SDS polyacrylamide gels. After the electrophoresis, gels were silver-stained using a silver staining kit (GE Healthcare) according to the manufacturer's protocols. The stained gels were scanned and electronic images of the gels were analyzed using ImageMaster 2D Elite software (GE Healthcare).

**In-Gel Digestion and Matrix-Assisted Laser Desorption Ionization, Time-of-Flight Mass Spectrometry (MALDI-TOF-MS).** Protein spots on the gels were excised and a "control" piece was cut from a blank region of the gel and processed in parallel with the sample. In-gel digestion with trypsin was performed as reported.<sup>19</sup> The resulting peptides were concentrated using Zip-Tip C18 (Millipore, Bedford, MA). The peptide mixtures were eluted from Zip-Tip with 75% acetonitrile in 0.1% trifluoroacetic acid (TFA). The matrix ( $\alpha$ -cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile, 0.1% TFA) was deposited on a dried sample target. Then 0.5- $\mu$ L aliquots of the analyte solution were deposited onto matrix surfaces and the solvent was allowed to evaporate at ambient temperature. The digests were analyzed with a TOF mass spectrometer, PE Biosystems Voyager DE STR MALDI (Foster City, CA).

**Database Analysis.** For protein identification the measured monoisotopic masses of the peptides were analyzed using MS-Fit provided by UCSF (<http://prospector.ucsf.edu/ucshtml3.2/msfit.htm>).

**Immunoblotting and Immunoprecipitation.** Purified mitochondria were lysed and sonicated in RIPA buffer, then centrifuged at 16,000 rpm for 10 minutes. Protein concentration was determined using a BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL). The samples were separated by sodium dodecyl sulfate (SDS)-PAGE and electrotransferred onto a polyvinylidene fluoride membrane (Immobilon; Millipore, Japan), then blocked with BlockAce (Snow Brand, To-

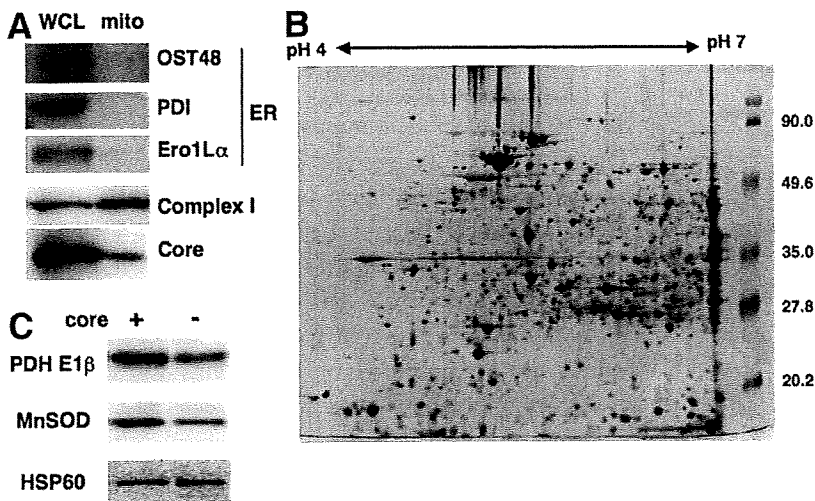


Fig. 1. 2D-PAGE of mitochondria purified from core-expressing cells. (A) Whole-cell lysates (WCL) and purified mitochondria (mito) derived from core-expressing cells were subjected to SDS-PAGE and immunoblotted with anti-core, anti-subunit of complex I (mitochondrial protein), or anti-OST48, PDI, Ero1L $\alpha$  (ER proteins) antibodies. (B) Purified mitochondria of core-expressing cells were subjected to 2D-PAGE and the gel was stained with silver. The numbers shown on the right are molecular weights. (C) Purified mitochondria of core-expressing and control cells were subjected to SDS-PAGE and blotted with an anti-E1 $\beta$  subunit of PDH (PDH E1 $\beta$ ), anti-MnSOD, or anti-HSP60 antibody.

kyo, Japan). The membrane was subsequently incubated with specific primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies and visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Antibodies against the core protein (Anogen, Mississauga, Canada), manganese superoxide dismutase (MnSOD) (BD Biosciences, San Jose, CA), prohibitin (Neomarkers, Fremont, CA), oligosaccharyltransferase-48 (OST48), heat shock protein (HSP) 60 (Santa-Cruz Biotechnology, Santa Cruz, CA), pyruvate dehydrogenase (PDH), ubiquinol-cytochrome c oxidoreductase, COX (Molecular Probes, Eugene, OR), protein disulfide isomerase (PDI), ER protein endoplasmic oxidoreduction-1 (Ero1)-L $\alpha$ , and I $\kappa$ B $\alpha$  (Cell Signaling Technology, Danvers, MA), were used as primary antibodies. For immunoprecipitation experiments, cells were lysed in NET-N buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) and the lysates were incubated with anti-prohibitin overnight followed by the addition of protein Sepharose 4B (GE Healthcare), then washed with the same buffer five times. Immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting with specific antibodies.

**Determination of COX Activity.** COX activity was determined with a MitoProfile Rapid Microplate Assay Kit (MitoSciences, Eugene, OR) using 10  $\mu$ g of purified mitochondria. The assay was performed three times independently.

**Statistical Analysis.** Results are expressed as means  $\pm$  SE. The significance of the difference in means was determined by Student's *t* test or Mann-Whitney's *U* test.

## Results

**Presence of HCV Core Protein in Purified Mitochondria.** Increasing evidence suggests that the HCV

core protein is localized to mitochondria as well as to ER and the nucleus. Therefore, we first investigated whether the core protein is expressed in the mitochondria of core-expressing (Hep39) cells used in this study. We used NycoDenz discontinuous gradients to extract mitochondria as described.<sup>17</sup> In the mitochondria derived from core-expressing HepG2 cells, the core protein was detected by immunoblotting, whereas ER resident proteins such as an ER-specific type I transmembrane protein OST48, ER-resident molecular chaperon PDI, and ER membrane-associated N-glycoprotein Ero1-L $\alpha$ , were not (Fig. 1A). In this fraction, reduced nicotinamide adenine dinucleotide (NADH)-ubiquinone oxidoreductase, complex I of mitochondrial oxidative phosphorylation system, was more strongly expressed than that in the whole cell. These results indicate that the purified mitochondria fraction was free of ER, and that a portion of the core protein was localized to the mitochondria in core-expressing cells.

### Proteomics Analysis of Mitochondria by 2D-PAGE.

For proteomics analysis, purified mitochondrial proteins derived from core-expressing cells were subjected to 2D-PAGE followed by silver-staining of the gel. In this study we analyzed only acidic proteins using IPG strips covering pH 4 to pH 7 because the analysis of acidic proteins by 2D-PAGE is relatively easy. The mitochondrial fraction was also extracted from Heps wx, a control cell line resistant to G418 but does not express the core protein, then similarly subjected to 2D-PAGE and used for comparing the expression pattern. We repeated the above procedure (purification of mitochondria, 2D-PAGE, and silver-staining) five times, and confirmed a similar expression pattern in core-expressing cells. The representative gel image is shown in Fig. 1B. ImageMaster 2D Elite software detected about 1100 spots on the silver-stained acidic gel, i.e., at pH 4-7 and Mrs of 20-100 kDa. The number of

**Table 1. Proteins of Differential Expression in Mitochondria of Core-Expressing Cells**

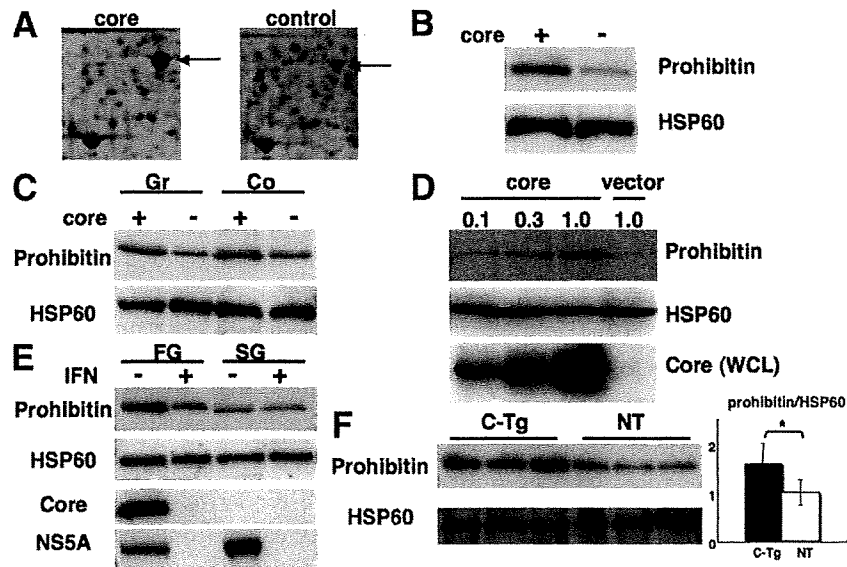
Protein Name	Fold Change (Mean $\pm$ SD)
<b>Increased</b>	
Succinyl-CoA:ketoacid CoA transferase	10.43 $\pm$ 1.29
NADH-specific isocitrate dehydrogenase a subunit precursor	9.64 $\pm$ 4.66
Unknown	8.65 $\pm$ 2.40
GrpE-like protein co-chaperon	5.71 $\pm$ 0.49
Leucine aminopeptidase	4.26 $\pm$ 1.14
Pyruvate dehydrogenase E1 component b subunit	3.79 $\pm$ 1.34
CGO15alt2	3.18 $\pm$ 0.80
HSP70	3.11 $\pm$ 1.39
Prohibitin	2.60 $\pm$ 0.24
3-Hydroxyisobutyrate dehydrogenase	2.47 $\pm$ 0.77
HSPC108	2.46 $\pm$ 0.69
MnSOD	2.35 $\pm$ 0.65
Ubiquinol-cytochrome c oxidoreductase core I protein	2.00 $\pm$ 0.23
<b>Decreased</b>	
Aldehyde dehydrogenase 2	0.12 $\pm$ 0.02
Aldehyde dehydrogenase 5 precursor	0.25 $\pm$ 0.03
ATP synthase a subunit isoform 1	0.50 $\pm$ 0.09
<b>Reference protein</b>	
HSP60	1.02 $\pm$ 0.02

protein spots was smaller than those reported in a recent study investigating the human placental mitochondrial proteome.<sup>15</sup>

We then compared the intensity of the spots between core-expressing and control cells. Analysis of repeated experiments by Student's *t* test revealed 13 increased and three decreased spots in intensity in core-expressing cells. These spots were excised and digested with trypsin, then proteins were identified by mass spectrometry. The names of the identified proteins are listed in Table 1. Among them were proteins related to mitochondrial respiratory chain, protein chaperons, and lipid metabolism. Because antibodies to some of these proteins are commercially available, expression levels of the proteins were examined by immunoblotting. The expression levels of the PDH-E1 $\beta$  subunit and MnSOD, which were identified as increased proteins, were higher in core-expressing cells than in control cells (Fig. 1C), whereas that of HSP60, which was identified as having a similar expression, was unchanged.

#### *Up-regulation of Prohibitin by the Core Protein.*

Among the identified proteins, we focused on prohibitin, an up-regulated protein in mitochondria of core-expressing cells (Fig. 2A). Prohibitin is a mitochondrial protein associated with cell proliferation.<sup>20</sup> It also works as a chaperon of mitochondrial proteins.<sup>21,22</sup> We confirmed an increased prohibitin expression level in core-expressing cells



**Fig. 2.** Up-regulation of prohibitin in core-expressing cells. (A) Protein spot corresponding to prohibitin (arrow) in 2D-PAGE. (B) Purified mitochondria from core-expressing or control cells were subjected to SDS-PAGE and immunoblotted with anti-prohibitin or anti-HSP60 antibody. (C) Mitochondria were purified from growing (Gr) or confluent (Co) cells in 100-mm dishes and subjected to SDS-PAGE, then immunoblotted with an anti-prohibitin or anti-HSP60 antibody. (D) HepG2 cells in six-well plates were transfected with different amounts ( $\mu$ g) of core-expressing plasmid and mitochondrial proteins were analyzed by immunoblotting with anti-prohibitin or anti-HSP60 antibody. The expression levels of the core protein in whole-cell lysates (WCL) were also determined. (E) Cells harboring HCV replicon were untreated or treated with IFN and expression levels of prohibitin in mitochondria were determined. Expression of HCV core and NS5A proteins was also examined. FG, full-genomic replicon cells; SG, subgenomic replicon cells. (F) Expression levels of prohibitin in mitochondria were determined in liver tissues HCV core-gene transgenic and nontransgenic mice. Prohibitin/HSP60 expression levels were determined by densitometry. C-Tg, core-gene transgenic mouse; NT, nontransgenic littermate ( $n = 3$ ) \* $P < 0.05$ .

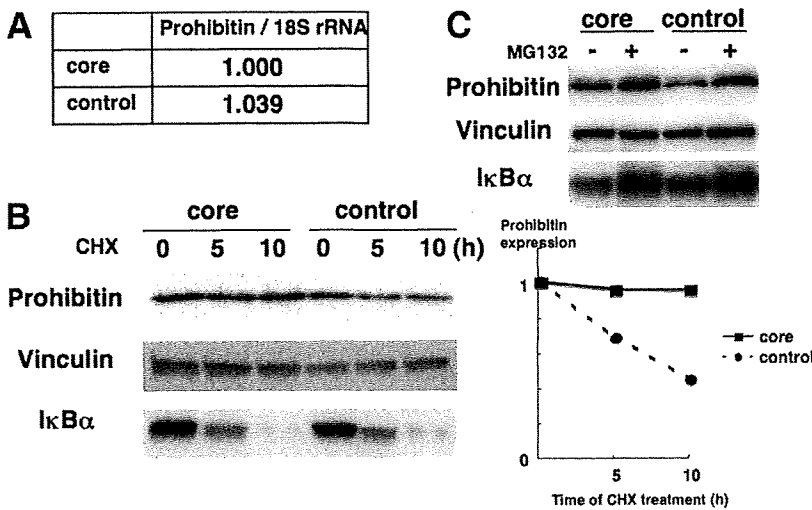


Fig. 3. Increased protein stability of prohibitin in core-expressing cells. (A) RNA was extracted from core-expressing and control cells, and the amount of specific mRNA was determined by real-time PCR with specific primers/probe against prohibitin. The amount of prohibitin mRNA was standardized by that of 18S ribosomal RNA (18S rRNA). (B) Cells were incubated with 100 ng/mL cycloheximide and harvested at the timepoints indicated above the lanes (numbers are hours of cycloheximide treatment). Whole-cell lysates were subjected to SDS-PAGE and immunoblotted with anti-prohibitin, anti-I $\kappa$ B $\alpha$ , or anti-vinculin (as an internal standard) antibody. The intensity of each band was measured by densitometry, and expression levels (prohibitin/vinculin) are shown in the right panel. (C) Cells were harvested after incubation with 20  $\mu$ M MG132 for 8 hours and subjected to immunoblotting with anti-prohibitin, anti-I $\kappa$ B $\alpha$ , or anti-vinculin antibody.

by immunoblotting (Fig. 2B). Because prohibitin is associated with cell proliferation, it is possible that prohibitin expression changed according to the cell proliferative status. As shown in Fig. 2C, core-expressing cells had high prohibitin expression levels in the cells in both confluent growth and growing statuses compared with control cells. We also determined the expression levels in cells synchronized with aphidicolin followed by l-mimosine treatment and found an increased expression level in core-expressing cells (data not shown). To exclude the possibility that the increased prohibitin expression level is due to the expansion of limited cell clones, not specific to the core protein expression, we examined prohibitin expression in cells transiently expressing the core protein and found that prohibitin expression level increased dose-dependently in core-expressing cells (Fig. 2D). We also examined the prohibitin expression levels in Huh7 cells harboring full- or subgenomic HCV replicon. For this purpose, we used interferon (IFN)-treated replicon cells (cured cells) as a control. Core and nonstructural (NS)5A proteins were not detected after treatment of full-genomic replicon cells with IFN, suggesting a successful elimination of replicon. Prohibitin expression levels in cells with full-genomic replicon were increased compared with those in IFN-treated cured cells, whereas levels of prohibitin expression were low in subgenomic replicon cells regardless of IFN-treatment (Fig. 2E). In addition, prohibitin expression levels were also increased in livers of 3-month-old transgenic mice expressing the core protein compared with those in nontransgenic littermates (Fig. 2F).

We next sought to determine the mechanism of the increased steady-state level of prohibitin in core-expressing cells. To determine prohibitin messenger RNA (mRNA) expression, we performed a real-time polymerase chain reaction (PCR) using specific primers/probe.

No difference in prohibitin mRNA was observed between core-expressing and control cells (Fig. 3A). We next determined the stability of prohibitin in these cells. By treating the cells with cycloheximide, the expression levels of prohibitin gradually decreased in control cells (Fig. 3B). On the other hand, in core-expressing cells prohibitin was hardly degraded by cycloheximide treatment for 10 hours, whereas I $\kappa$ B $\alpha$  was equally degraded in both cells. This result suggests that prohibitin was stabilized in the presence of the core protein. Because prohibitin has been shown to be degraded by proteasome,<sup>23</sup> we examined expression levels of prohibitin in the presence of proteasome inhibitor MG132. By treatment with MG132, prohibitin expression was increased to the similar level in core-expressing and control cells. These results suggest that the core protein may inhibit proteasomal degradation of prohibitin by some mechanism, including the prevention of degradation by interaction with the core protein. Then, core-expressing cells were lysed and subjected to immunoprecipitation with an anti-prohibitin antibody. As shown in Fig. 4, the core protein was coimmunoprecipitated with an anti-prohibitin antibody. To exclude a non-specific interaction with the antibody or Sepharose beads, cells expressing a small amount of prohibitin by transfection with small interfering RNA (siRNA) against prohibitin were also examined. In these cells the amount of the coimmunoprecipitated core protein decreased. In addition, the core protein was not coimmunoprecipitated by control immunoglobulin G (IgG), indicating a specific interaction of prohibitin with the core protein. These results suggest that prohibitin expression increased in core-expressing cells owing to the increased stability presumably by interaction with the core protein.

**Impaired Chaperon Function of Prohibitin in Core-Expressing Cells.** We next examined the effect of

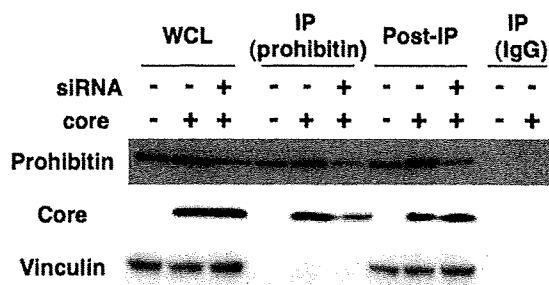


Fig. 4. Interaction of the core protein with prohibitin. Core-expressing and control cells were transfected with or without siRNA against the prohibitin gene, then harvested and lysed in NET-N buffer 3 days after transfection. Whole-cell lysates (WCL) were immunoprecipitated (IP) with an anti-prohibitin antibody or control IgG and immunoblotted with anti-prohibitin or anti-core antibody. Supernatants after the immunoprecipitation were harvested and similarly immunoblotted (Post-IP).

the interaction of prohibitin with the core protein on the function of prohibitin. Prohibitin works as a chaperon of mitochondrial proteins. Nijtmans et al.<sup>21</sup> demonstrated that prohibitin exerts a chaperon function particularly for the stabilization of mitochondrial DNA-encoded proteins. COX is a mitochondrial respiratory complex IV formed by 14 subunits, 10 of which are encoded by nuclear DNA and the rest by mitochondrial DNA.<sup>24</sup> We examined the interaction of prohibitin with subunit II of COX encoded by mitochondrial DNA. As shown in Fig. 5A, the level of COX II coimmunoprecipitated with an anti-prohibitin antibody was decreased in core-expressing cells, although the amount of immunoprecipitated prohibitin was higher than that in control cells. On the other hand, the subunit IV of COX encoded by nuclear DNA was similarly coimmunoprecipitated between core-expressing and control cells. When prohibitin expression was decreased by siRNA transfection, coimmunoprecipitation of COX subunits was similarly decreased with the amount of immunoprecipitation of prohibitin itself being low. We next determined expression levels of COX subunits in the mitochondria in these cells. Expression levels of mitochondrial DNA-encoded subunits I and II in core-expressing cells were decreased, whereas the levels of nuclear DNA-encoded subunits IV and VIb were similar to those in control cells. When transfected with prohibitin-siRNA, expression levels of all of the COX subunits examined were decreased in both core-expressing and control cells, suggesting that protein levels of these subunits are dependent on prohibitin (Fig. 5B, see Supporting Fig. 1 for densitometry). Similar data were observed when blots for COX II and IV were developed together in the same membrane (Supporting Fig. 2). We also determined COX activity in these cells and found that core-expressing cells had a significantly decreased COX activity (about 70% of that in control cells, Fig. 5C). These results

suggest that interaction of prohibitin with the core protein is associated with an impaired function of prohibitin as a mitochondrial chaperon, which may trigger disordered assembly and function of mitochondrial respiratory complexes.

## Discussion

In the present study we analyzed expression levels of mitochondrial proteins in HepG2 cells expressing the HCV core protein and identified a set of proteins with different expressions. Some of those proteins were related to the mitochondrial respiratory chain (Table 1). Because the core protein was shown to be associated with the induction of oxidative stress,<sup>7-9</sup> the core protein may modulate the expression and function of proteins forming mitochondrial respiratory complexes, which naturally

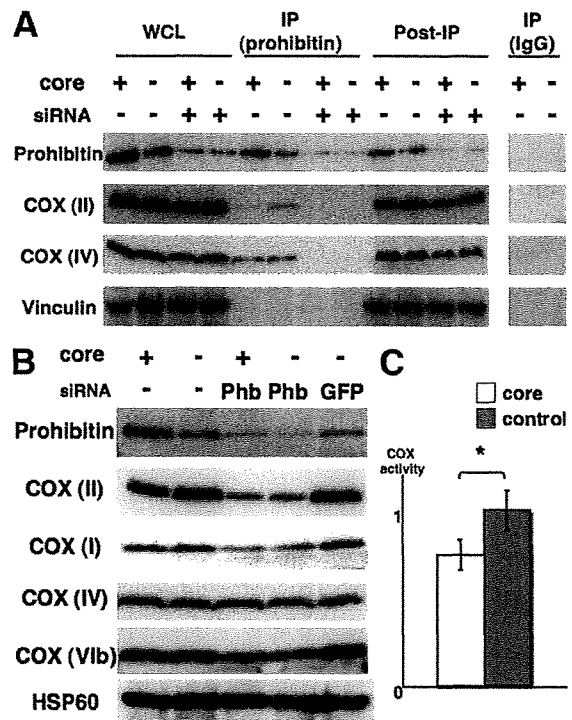


Fig. 5. Effects of core-prohibitin interaction on interaction/expression of COX subunit proteins and COX activity. (A) Whole-cell lysates (WCL) of core-expressing and control cells were subjected to immunoprecipitation with an anti-prohibitin antibody or control IgG, and the interaction of prohibitin with COX subunits was determined by immunoblotting of immunoprecipitated proteins (IP). Supernatants after the immunoprecipitation were harvested and similarly immunoblotted (Post-IP). (B) Cells were transfected with or without siRNA against the prohibitin (Phb) or GFP gene and harvested 3 days after transfection for purification of mitochondria. Purified mitochondria were subjected to SDS-PAGE and immunoblotted with several anti-COX subunits antibodies. The expression levels of HSP60 were also examined as an internal control. (C) COX activity was determined by measuring cytochrome c oxidation. The activity was normalized by taking the average rate of control cells as 1. Data shown are means  $\pm$  SE (n = 5). \*P < 0.05.

leads to ROS accumulation. In addition, MnSOD, which plays a key role in protecting cells from oxidative damage, was up-regulated in core-expressing cells, reflecting ROS increase in the cells. Several protein chaperons such as HSP70 and GrpE-like protein co-chaperon were also identified as up-regulated proteins. Because these proteins are known to be important in the mitochondrial protein-import mechanisms, the modulated expression of these proteins may be associated with the different expressions of the identified mitochondrial proteins.

Prohibitin, a mitochondrial protein chaperon, was identified as an up-regulated protein in core-expressing cells. Prohibitin is a ubiquitously expressed and highly conserved protein that was originally determined to play a predominant role in inhibiting cell-cycle progression and cellular proliferation by attenuating DNA synthesis.<sup>20,25</sup> Prohibitin is present in the nucleus and interacts with transcription factors that are important in cell cycle progression. In core-expressing cells used in this study, prohibitin was also detected in the nucleus and its expression level was also higher than that in control Hepswx cells or HepG2 cells (data not shown). The growth rate of core-expressing cells, however, was similar to that of control cells (data not shown). The physiological significance of the high expression level of prohibitin in the nucleus remains to be determined, but it may be related to enhanced apoptosis by Fas ligand, as shown by Ruggieri et al.,<sup>16</sup> because prohibitin interacts with E2F, Rb, and p53 and modulates the transcription activity of these factors and induces apoptosis.<sup>26,27</sup>

Mitochondrial prohibitin acts as a protein chaperon by stabilizing newly synthesized mitochondrial translation products through direct interaction.<sup>21</sup> We examined the interaction between prohibitin and mitochondrially encoded subunit II of COX and found a suppressed interaction between these proteins in core-expressing cells. In addition, there are several studies that showed the association of prohibitin with the assembly of mitochondrial respiratory complex I as well as complex IV (COX).<sup>21,28</sup> Complex I also consists of both nuclear- and mitochondrial-DNA-encoded subunits; therefore, it is probable that the assembly and function of complex I are impaired by the core protein. We attempted to examine the interaction of prohibitin with the mitochondrial DNA-encoded subunit of complex I, but commercially available antibodies against this subunit could not detect the protein itself by immunoblotting (data not shown). With respect to the complex I function, we found a decreased complex I activity in core-expressing cells (H. Miyoshi et al., manuscript in preparation). Other groups have also shown that complex I activity is decreased in the liver of transgenic mice harboring HCV core and envelope genes<sup>9</sup>

as well as in cultured cells.<sup>29</sup> From these findings, the interaction between prohibitin and the core protein may impair the function of complex I as well as complex IV, leading to an increase in ROS production. In fact, the suppression of the prohibitin function is shown to result in an increased production of ROS,<sup>30</sup> a phenomenon observed in core-expressing cells used in this study (Miyoshi et al., in prep.) as well as in the liver of core-gene transgenic mice.<sup>7,8</sup> Interestingly, Berger and Yaffe<sup>31</sup> showed that loss of function of prohibitin leads to an altered mitochondrial morphology, that is, the loss of the normal reticular morphology and organized mitochondrial distribution. In hepatocytes from the core-gene transgenic mice, we observed a change in morphology of mitochondria, a disappearance of the double structure of mitochondrial membranes.<sup>2</sup> These changes in mitochondrial morphology are somewhat different, but the dysfunction of prohibitin may be responsible for the morphological abnormality of mitochondria observed in the core-gene transgenic mice.

We concluded that prohibitin overexpression is due to increased stability induced by the interaction with the core protein. In this study we showed that prohibitin might be degraded by proteasome, although we could not detect ubiquitinated forms of prohibitin. If the degradation is mediated by ubiquitin as reported,<sup>23</sup> it is possible that the interaction with the core protein interferes with ubiquitin-binding and protects prohibitin from degradation by proteasome. Some posttranslational protein modifications such as phosphorylation are other possible factors for the stabilization, because prohibitin can be serine-phosphorylated<sup>32</sup>; however, in our examination no serine/threonine/tyrosine phosphorylation of prohibitin was detected in core-expressing cells (data not shown). Thus far, there are no studies showing that prohibitin stabilization leads to a suppressed function as a mitochondrial chaperon. Therefore, this finding is novel and noteworthy because the prohibitin expression level has been considered to be proportional to the chaperon function. Prohibitin is highly expressed in several human tumors.<sup>33,34</sup> In addition, a 2D-PAGE of the hepatoma cell line HCC-M identified prohibitin as a positively regulated protein.<sup>35</sup> In these studies, the mechanism of prohibitin overexpression was not elucidated, but considering that prohibitin is associated with the inhibition of cell proliferation, the function of prohibitin is suppressed by stabilization by some molecules in the tumor, similar to the mechanism we suggest in the current study.

In addition to HepG2 cells constitutively expressing the core protein, increased prohibitin expression levels were also found in livers of core-gene transgenic mice.



The difference in expression levels between the transgenic mice and nontransgenic littermates, however, was a little bit smaller than that in the studies of HepG2 cells. This may be due to the low expression level of the core protein in the transgenic mice compared with that in core-expressing HepG2 cells because the expression level of prohibitin was proportionally increased to that of the core protein as shown in this study (Fig. 2D). Otherwise, there might be some *in vivo* mechanism for suppressing prohibitin expression in mice.

In this study, COX subunit IV as well as II were found to interact with prohibitin (Fig. 5A). Although there are no studies demonstrating that prohibitin also works as chaperon for nuclear DNA-encoded mitochondrial proteins as far as we investigated, knockdown of prohibitin expression by siRNA led to decreases in expression levels of both nuclear (COX IV, VIb) and mitochondrial (COX I, II) DNA-encoded subunits in mitochondria (Fig. 5B and Supporting Figs. 1 and 2). We showed that COX IV interacts with prohibitin (Fig. 4), suggesting that prohibitin also works for stable expression of nuclear DNA-encoded COX IV. Degrees of decrease in COX IV and VIb expression, however, were smaller than those in I and II. Prohibitin might contribute to stabilization of COX IV and VIb by mechanism(s) other than chaperon function. Steglich et al.<sup>36</sup> showed that prohibitin regulates protein degradation by the m-AAA protease in mitochondria. Recently, Da Cruz et al.<sup>37</sup> showed that SLP-2, a member of the stomatin gene family, interacts with prohibitin and regulates the expression of mitochondrial proteins such as COX IV and ND6 of complex I encoded by nuclear DNA by AAA proteases. In view of these findings, COX IV and VIb expression in mitochondria is dependent on prohibitin but other factors may also be involved in the attainment of stable expression of these subunits. The expression levels of COX II and IV in the whole-cell lysates were not so drastic among cell samples (Fig. 5A) compared to those in the mitochondria (Fig. 5B). The reason is not clear, but it is possible that redundant proteins such as improperly folded proteins by lack of chaperons were included in the whole-cell lysates.

In summary, we analyzed mitochondrial proteins in core-expressing HepG2 cells by proteomics analysis and identified prohibitin as an up-regulated protein. The dysfunction of prohibitin induced by the core protein may lead to ROS overproduction in the mitochondrion, which plays a key role in the pathogenesis of chronic hepatitis C. The restoration of prohibitin function might be a therapeutic option for correcting the dysregulated assembly and dysfunction of mitochondrial respiratory chain complexes.

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Original Article

# Chronic hepatitis C in patients co-infected with human immunodeficiency virus in Japan: a retrospective multicenter analysis

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**Aim:** A nationwide survey in Japan revealed that nearly one-fifth of human immunodeficiency virus (HIV)-positive patients are co-infected with hepatitis C virus (HCV). We conducted a study to further analyze the features of liver disease in HIV-HCV co-infected patients.

**Methods:** We analyzed 297 patients from eight hospitals belonging to the HIV/AIDS Network of Japan.

**Results:** HCV genotypes 1, 2, 3, 4 and mixed genotypes were detected in 55.2, 13.7, 18.9, 0.9 and 11.3% of patients, respectively, in contrast to the fact that only genotypes 1 and 2 are detected in HCV mono-infected patients in Japan. This is compatible with the transmission of HCV through imported blood products contaminated by HCV. Sixteen of 297 HIV-HCV co-infected patients had advanced liver disease accompanied by ascites, hepatic encephalopathy or hepatocellular carcinoma. The average age of such patients was  $41.1 \pm 14.0$  years,

which was much younger than that of HCV mono-infected patients with the same complications. The progression speed of liver disease estimated from the changes in the levels of serum albumin, bilirubin, or platelet was slower in patients who achieved sustained virological response with interferon treatment than in those who did not receive it. The overall sustained virological response rate to interferon treatment was 43.3%.

**Conclusions:** Our findings suggest that liver disease is more advanced in HIV-HCV co-infected patients than in HCV mono-infected patients, and interferon treatment may retard the progression of liver disease in such patients.

**Key words:** acquired immunodeficiency syndrome, chronic liver disease, genotype, interferon therapy

## INTRODUCTION

THE PROGNOSIS OF human immunodeficiency virus (HIV) infection has markedly improved since the introduction of hyperactive anti-retroviral therapy (HAART).<sup>1,2</sup> Opportunistic infection has been pre-

vented or properly managed, resulting in lower mortality rates. Liver disease, in particular related to hepatitis C virus (HCV) infection, has now become the main cause of mortality among HIV-infected patients on HAART in Western countries.<sup>3,4</sup> A national survey among Japanese HIV-infected patients with coagulation disorders has shown that the mortality rate related to HCV-related liver disease after 1997 was twofold that before 1997.<sup>5</sup> In Japan, therefore, HCV infection may also be a major cause of death in HIV-HCV co-infected patients. However, there has been no extensive analysis of liver disease in HIV-HCV co-infected patients in Japan.

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Interferon (IFN) treatment in combination with ribavirin administration, which is now the first choice for HCV mono-infected patients,<sup>6</sup> is also a standard treatment for chronic hepatitis in HIV–HCV co-infected patients. Eradication of HCV is assumed to improve liver function, and normalization of serum aminotransferase (ALT) levels by IFN treatment may retard the progression of liver disease in HIV–HCV co-infected patients, even if they are on HAART. However, in general, the response rate to IFN treatment is lower in HIV–HCV co-infected patients than in HCV mono-infected patients.<sup>7</sup> The effects of IFN treatment on liver function and prognosis in HIV–HCV co-infected patients in Japan are yet undefined.

In 2004, we conducted a nationwide survey to determine the prevalence of HCV infection in HIV-infected patients by distributing a questionnaire to the hospitals in the HIV/AIDS Network of Japan, which revealed that 935 (19.2%) of 4877 HIV-positive patients were also positive for anti-HCV antibody.<sup>8</sup> In this study, we analyzed the progression of liver diseases and the impact of IFN treatment on the parameters of liver function in HIV–HCV co-infected patients in a multicenter retrospective study.

## METHODS

### Registry of patients with HIV–HCV co-infection

THE QUESTIONNAIRE REGARDING the current state of HIV–HCV co-infection was sent to the 366 hospitals in the HIV/AIDS Network of Japan in 2004, sponsored by the Japanese Ministry of Health, Labour and Welfare. One hundred seventy-six hospitals (48.1%) responded. The results, already published,<sup>8</sup> showed that HIV–HCV co-infected patients are concentrated in particular hospitals in big cities around Japan. Among these hospitals, we chose three hospitals in the Tokyo metropolitan area, and one each in the Hokkaido, Chubu, Osaka, Chugoku and Kyushu areas. These eight hospitals belong to the HIV/AIDS Network and had more HIV–HCV co-infected patients than other hospitals.

In the study, the following information was obtained from the hospitals regarding each HIV–HCV co-infected patient who visited the hospitals at least once between January and December in 2004: (1) age and sex of HIV-positive patients with anti-HCV; (2) possible transmission routes of HIV; (3) history of habitual alcohol intake; (4) date of the first and last visits; (5) counts of

white blood cells, CD4-positive lymphocytes and platelets at the first and last visits; (6) levels of serum albumin and bilirubin at the first and last visits; (7) levels of HIV-RNA and HCV-RNA at the first and last visits; (8) history of IFN treatment with or without ribavirin; (9) history of HAART; and (10) history of jaundice, ascites, hepatic encephalopathy and hepatocellular carcinoma (HCC). The study sheets were completed by the physicians in charge and sent to the Department of Internal Medicine, University of Tokyo.

### Ethical issues

The protocol of the current survey was approved by the ethical committee of each institution, and written informed consent was obtained from each patient.

### Statistical analysis

The collected data were analyzed using Mann–Whitney's *U*-test whenever appropriate. *P*-values less than 0.05 were regarded as statistically significant.

## RESULTS

### Clinical backgrounds of registered patients

FROM THE EIGHT hospitals, 297 patients were registered. The number, age, sex, estimated transmission routes and history of habitual alcohol intake are shown in Table 1. Two hundred and ninety (97.6%) were male patients. The mean age of the patients was  $37.9 \pm 10.3$ .

HCV genotype was determined in 212 patients. One hundred seventeen (55.2%) patients were infected by genotype 1 HCV. Infection by genotypes 2, 3 or 4 HCV was found in 29 (13.7%), 40 (18.9%) and 2 (0.9%) patients, respectively. Twenty-four (11.3%) patients were infected by HCV of mixed genotypes. In the remaining 85 patients, the genotype was indeterminable or undetermined. The mean ages of patients infected by different HCV genotypes were similar (Table 1).

In 259 (87.2%) of 297 registered patients, HIV was most probably transmitted through the administration of blood products. Other transmission routes were sexual contacts among men who have sex with men (MSM) (4.0%), heterosexual contacts (3.0%) and intravenous drug use (IDU) (0.3%). Habitual alcohol consumption was noted in only one patient with genotype 1 HCV (0.6%).

### Outcomes of IFN treatment in HIV–HCV co-infected patients

Serum HCV-RNA levels were available both at the first visit and registry to the study (i.e. the end of observa-

**Table 1** Demography, transmission route and HCV genotypes in HIV-HCV co-infected patients

HCV genotype (%)	Number (%)	HCV sub-genotypes	Viral load† (High: Low)	Age	Sex (Male: Female)	Transmission route				
						Transfusion	MSM	Hetero-sexual	IDU	Others
1	117 (55.2)	1a 31, 1b 43, 1a+1b 31, undetermined 12	31:11	38.3 ± 10.4	114:3	102	7	1	0	7
2	29 (13.7)	2a 16, 2b 11, undetermined 2	5:5	39.8 ± 9.5	29:0	24	1	1	0	3
3	40 (18.9)	3a 40	12:2	36.1 ± 8.9	40:0	38	0	0	0	2
4	2 (0.9)	4a 2	2:0	38.5 ± 2.1	2:0	2	0	0	0	0
Mixed	24 (11.3)	2a+3a 6, 1b+3a 3, others 15	11:0	38.7 ± 8.7	24:0	24	0	0	0	0
Others	85	Undetermined 85	6:1	36.2 ± 11.5	81:4	69	4	7	1	4
Total	297		67:19	37.9 ± 10.3	290:7	259 (87.2%)	12 (4.0%)	9 (3.0%)	1 (0.3%)	16 (5.5%)

†Viral loads are available in only a subset of patients. High viral load: more than 1 Mcq/mL by branched DNA-probe assay or more than 100 KIU/mL by Amplicor monitor assay.

HCV, hepatitis C virus; HIV, human immunodeficiency virus; IDU, intravenous drug users; MSM, men who have sex with men.

tion) in 158 patients. Of these 158, 60 patients (38.0%) received IFN treatment for HCV, and 35 of these 60 patients did it in combination with ribavirin. Those who did not complete the scheduled treatment were excluded from the current analysis.

As shown in Table 2, 26 (43.3%), 11 (18.4%) and 23 (38.3%) of the treated patients achieved sustained virological response (SVR), end-of-treatment virological response (ETR) and no virological response (NR), respectively. The SVR rate in patients with each genotype is shown in Table 2. The SVR rate in the patients who underwent IFN treatment in combination with ribavirin was 31.4% in total. The SVR rate in patients with each genotype who underwent IFN/ribavirin combination therapy is shown in Table 2.

All of the 26 patients who achieved SVR remained negative for serum HCV-RNA in the further follow-up periods. In contrast, none of the patients with ETR or NR became negative for serum HCV-RNA in the follow-up periods. In five patients who did not receive IFN treatment, HCV-RNA was negative at the end of the observation period, although it was positive at least twice before the registry. The profiles of the five patients are shown in Table 3.

**Changes in liver function and associated complications (Table 4)**

As mentioned above, the data on liver function and serum HCV-RNA positivity were available both at the first visit and registry (end of observation) in 158 of the 297 registered patients. The mean observation period was 9.5 ± 5.0 and 8.2 ± 8.2 years in the IFN-treated and IFN-untreated patients, respectively. Unfortunately, few, if any, patients underwent liver biopsy, because most HIV-HCV co-infected patients had coagulation disorders.

The annual change in the serum albumin concentration was +0.05 ± 0.42 g/dL in the IFN-treated patients, and -0.80 ± 0.82 g/dL in the non-IFN-treated patients. The annual change in the serum bilirubin concentration was +0.08 ± 0.38 mg/dL in the IFN-treated patients, while it was +0.15 ± 0.15 mg/dL in the non-IFN-treated patients. Among the IFN-treated patients, the serum bilirubin concentration decreased by 0.02 ± 0.08 mg/dL in the patients who achieved SVR, which was significantly larger than that in the non-IFN-treated patients at the end of the observation (*P* < 0.05). The annual changes in platelet counts were +0.06 ± 1.13 (×10<sup>4</sup>/μl) in the IFN-treated patients and -0.94 ± 0.95 (×10<sup>4</sup>/μl) in the non-IFN-treated patients. The change in platelet

Table 2 Virological response to interferon treatment in HIV-HCV co-infected patients

Genotype	Viral load (High : Low)†	Response			Total
		SVR	ETR	NR	
(a) Response to interferon treatment in total (with or without ribavirin)					
1	9:6	7 (33.3%)	1	13	21
2	5:3	4 (40.0%)	2	4	10
3	5:1	5 (62.5%)	1	2	8
4	1:0	0	1	0	1
Mixed	5:1	2 (33.3%)	3	1	6
Others	6:2	8 (57.1%)	3	3	14
Total	31:13	26 (43.4%)	11	23	60
(b) Response to ribavirin/interferon combination therapy including peginterferon					
1	8:2	2 (15.3%)	0	11	13
2	1:2	1 (25.0%)	0	3	4
3	4:1	4 (66.7%)	1	1	6
4	1:0	0	1	0	1
Mixed	4:1	1 (20.0%)	3	1	5
Others	3:0	3 (50.0%)	1	2	6
Total	21:6	11 (31.4%)	6	18	35

†Viral loads are available in only a subset of patients. High viral load: more than 1 Meq/mL by Branched DNA-probe assay or more than 100 KIU/mL by Amplicor monitor assay.

ETR, end of treatment virological response; NR, no virological response; SVR, sustained virological response.

counts in the patients who achieved SVR was significantly larger than that in the non-IFN-treated patients ( $P < 0.05$ , Table 4).

No symptoms of hepatic failure (ascites or hepatic encephalopathy) were observed in the 60 IFN-treated patients while they were observed in six of the 98 non-IFN-treated patients. HCC was found in one IFN-treated patient after SVR, while it was found in two non-IFN-treated patients (Table 4).

### Impact of HAART on liver function and associated complications (Table 5)

Information on HAART was available in 292 patients. The mean observation periods were  $8.4 \pm 4.2$  years in 234 patients on HAART, and  $9.8 \pm 6.0$  years in 58 patients not on HAART. Changes in the levels of albumin, bilirubin or platelet were similar between the two groups (statistically not significant). The morbidities of hepatic decompensation symptoms (ascites and hepatic encephalopathy) and HCC were not significantly different between the two groups. In total, nine patients had hepatic decompensation and seven had HCC, and the average age of such patients was  $41.1 \pm 14.0$  years, which was much younger than that of HCV mono-infected patients with the same complications.<sup>9</sup>

### DISCUSSION

IN THE CURRENT study, the features of liver disease in HIV-HCV co-infected patients in Japan were analyzed. The determination of HCV genotypes revealed that genotype 3 or 4, which is rarely seen in HCV mono-infected patients in Japan,<sup>10</sup> was found in a substantial fraction of HIV-infected patients. In addition, some of these patients were infected with HCV of mixed genotypes. These results are compatible with the fact that HCV is transmitted through imported blood products that were contaminated by HCV, as is the case with HIV infection.<sup>11</sup> Infection by HCV of mixed genotypes may reflect frequent administrations of blood products of different lots.

We evaluated the response rate to IFN treatment in HIV-HCV co-infected patients in Japan. Because the IFN treatment protocol varied between facilities, it was not easy to evaluate the effects of the treatments including IFN in this cohort. However, the regimen of ribavirin/IFN combination therapy was similar between the hospitals: the treatment period was 24 weeks in patients with HCV genotypes 2 and 3, and 48 weeks in those with HCV of other genotypes when either pegylated or standard IFN in combination with ribavirin was used.<sup>12</sup> Therefore, it may be possible to estimate the effect

**Table 3** Clinical backgrounds of patients who spontaneously cleared HCV in HIV-infected patients

Patient no.	Age	Sex	Transmission route	Observation period (years)	HCV-RNA (KIU/mL)	HCV genotype	HIV-RNA ( $\times 10^3$ /mL)	WBC ( $\mu$ L)	CD4+ T cells ( $\mu$ L)	Platelets ( $\times 10^3$ /mL)	ALT (U/l)	HAART
1	33	M	Transfusion	8.8	290	ND	200 000	4500	5	26.3	21	Yes
2	31	M	MSM	2.3	Positive†	ND	13 000	5760	931	22.7	29	Yes
3	27	M	Transfusion	9.3	>850	3a	180 000	4000	51	10.1	84	Yes
4	53	M	Transfusion	4.5	Positive†	1a	20 000	4800	296	35.4	24	No
5	22	M	Transfusion	7.8	220	ND	990	5500	125	33.1	44	Yes

†Positive: HCV-RNA was positive by qualitative PCR, but was not quantitatively determined.  
 ALT, aminotransferase; HAART, highly active anti-retroviral therapy; HCV, hepatitis C virus; HIV, human immunodeficiency virus; MSM, men who have sex with men; ND, not determined; WBC, white blood cells.

**Table 4** Changes in clinical parameters and IFN treatment in HIV-HCV co-infected patients

Outcome of IFN treatment	Number	Observation period (years)	$\Delta$ Albumin†	$\Delta$ Bilirubin‡	$\Delta$ Platelets§	Ascites/encephalopathy	HCC
IFN-treated patients	60	9.5 $\pm$ 5.0	0.05 $\pm$ 0.42	0.08 $\pm$ 0.38*	0.06 $\pm$ 1.13	0	1
SVR	26	9.1 $\pm$ 4.4	0.13 $\pm$ 0.59	(-) 0.02 $\pm$ 0.08*	0.14 $\pm$ 0.76*	0	1
ETR	11	14.6 $\pm$ 7.0	(-) 0.07 $\pm$ 0.14	0.51 $\pm$ 1.04	0.07 $\pm$ 1.50	0	0
NR	23	7.4 $\pm$ 2.0	0.01 $\pm$ 0.30	0.09 $\pm$ 0.30	(-) 0.18 $\pm$ 0.32	0	0
Non-IFN-treated patients	98	8.2 $\pm$ 8.2	(-) 0.80 $\pm$ 0.82	0.15 $\pm$ 0.15	(-) 0.94 $\pm$ 0.95	6	2
All	158	8.7 $\pm$ 4.7	(-) 0.45 $\pm$ 2.93	0.13 $\pm$ 0.52	(-) 0.59 $\pm$ 3.78	6	3

\*P < 0.05 versus patients without IFN treatment.

† $\Delta$ Albumin: changes in albumin concentration (g/dL)/observation period (years).

‡ $\Delta$ Bilirubin: changes in bilirubin concentration (mg/dL)/observation period (years).

§ $\Delta$ Platelet: changes in platelet count ( $\times 10^3$ / $\mu$ L)/observation period (years).

ETR, end of treatment virological response; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFN, interferon; NR, no virological response; SVR, sustained virological response.

Table 5 Changes in clinical parameters and HAART in HIV–HCV co-infected patients

	Number	Age	Sex (M : F)	Observation period (years)	$\Delta$ Albumin†	$\Delta$ Bilirubin‡	$\Delta$ Platelets§	IFN	Ascites/encephalopathy	HCC
HAART (+)	234	37.8 ± 10.4	227:7	8.4 ± 4.2	(–) 0.002 ± 0.18	0.13 ± 0.53	(–) 0.40 ± 3.71	143 (61.1%)	6	5
HAART (–)	58	38.1 ± 10.5	58:0	9.8 ± 6.0	(–) 0.14 ± 0.18	0.03 ± 0.25	(–) 1.40 ± 3.30	30 (51.7%)	3	2

† $\Delta$ Albumin: changes in albumin concentration (g/dL)/observation period (years).‡ $\Delta$ Bilirubin: changes in bilirubin concentration (mg/dL)/observation period (years).§ $\Delta$ Platelet: changes in platelet count ( $\times 10^4$ /L)/observation period (years).

HAART, highly active anti-retroviral therapy; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIV, human immunodeficiency virus.

of ribavirin/IFN combination therapy in HIV–HCV co-infected patients in this study.

The response rate to ribavirin/IFN combination therapy was 31.4% in total, and 15.3% in patients with HCV genotype 1, which are comparable rates to those achieved in previous studies on HIV–HCV co-infected patients in Western countries.<sup>7</sup> The low response rate in HIV–HCV co-infected patients compared with HCV mono-infected patients<sup>12</sup> may be attributed to several factors: impaired immune response, high HCV loads and viral quasi-species caused by frequent chances of transmission. Of these, high viral loads may be essential, because Table 2 shows that patients with genotype 1 HCV achieved SVR even by IFN monotherapy if their viral loads were low. In the era of IFN monotherapy, patients with favorable conditions were treated first of all: pretreatment viral loads in patients who received IFN monotherapy were lower than those who received PEG-IFN–ribavirin combination therapy. This may be the reason why the efficacy of PEG-IFN–ribavirin combination therapy was lower than that with IFN monotherapy in this study.

The serum bilirubin concentrations and platelet counts were improved in the patients who achieved SVR by IFN treatment. Although the response rate to IFN treatment is lower in HIV–HCV co-infected patients than in HCV mono-infected patients, the overall benefit of IFN treatment on liver function may be similarly expected in the patients who achieved SVR. HAART showed no impact on the liver function in HIV–HCV co-infected patients. Improvement of liver function can be expected only in IFN-treated patients, although there is a possibility that only patients with preserved liver function were able to receive IFN treatment. Given that liver disease is the major life-threatening factor in HIV-infected patients, IFN treatment should be considered in the early stage of HIV–HCV co-infection.

It should be noted that nine patients had hepatic decompensation and seven had HCC, and the average age of such patients was much younger than that of HCV mono-infected patients with the same complications.<sup>9</sup> This finding is compatible with reports from Western countries showing a faster progression of fibrosis<sup>13</sup> and earlier development of HCC.<sup>14</sup> A possibly interesting finding is that five patients (approximately 3% of patients whose serum HCV-RNA level was serially determined) cleared HCV-RNA from the serum without IFN treatment. Previous reports showed that some HIV-infected patients could spontaneously clear HCV-RNA.<sup>15–17</sup> The clearance of HCV among patients with chronic HCV infection is rare, although it has been



reported in Japan.<sup>18</sup> Three of the five patients had high HCV loads and low CD4<sup>+</sup> T-lymphocyte counts, which are generally thought to be unfavorable for spontaneous HCV clearance. A difference in immune status of HIV-infected patients from HCV mono-infected patients may be involved in such an observation, although further studies are awaited.

In summary, our study demonstrated that approximately 20% of HIV-infected patients are co-infected with HCV. Some of the HIV-HCV co-infected patients had advanced liver disease such as ascites, encephalopathy or HCC at a younger age than HCV mono-infected patients, suggesting that the progression of liver disease may be more rapid in HIV-HCV co-infected patients than in HCV-mono-infected ones. Treatments with regimens including IFN, which may improve liver function and decrease liver-related death, should be considered in HIV-HCV co-infected patients.

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# Association between Changes in Obesity Parameters and Incidence of Chronic Kidney Disease in Japanese Individuals

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## Key Words

Chronic kidney disease · Body mass index · Waist circumference · Health screening

## Abstract

Obesity increases the risk for chronic kidney disease (CKD). By analyzing data on individuals who underwent general health screening in two consecutive years, we investigated whether changes in body mass index (BMI) or waist circumference (WC) were associated with the appearance or disappearance of the CKD components; micro-/macroalbuminuria ( $\geq 30$  mg urinary albumin per gram creatinine) and a low estimated glomerular filtration rate (eGFR;  $< 60$  ml/min/1.73 m<sup>2</sup>). Logistic regression analysis showed that in men with micro-/macroalbuminuria at the first visit, a BMI reduction of  $\geq 0.42$  or a WC reduction of  $\geq 3.0$  cm over the 1-year period resulted in a significantly reduced incident of micro-/macroalbuminuria at the second visit. On the other hand, a BMI gain of  $\geq 0.33$  over 1 year in men without micro-/macroalbuminuria and a low eGFR at the first visit significantly increased the incident of micro-/macroalbuminuria and a low eGFR, respectively, at the second visit. These findings indicate that lowering the obesity indexes in men with micro-/macroalbuminuria reduced the incidence of this condition at the 1-year follow-up and that, on the con-

trary, an increase in BMI in men without micro-/macroalbuminuria and a low eGFR at the first examination increased the risk of these conditions during the 1-year follow-up period.

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## Introduction

Chronic kidney disease (CKD), now recognized as a potential risk factor for cardiovascular disease as well as for end-stage renal disease [1], is a worldwide public health problem [2]. Several cross-sectional and longitudinal epidemiological studies showed that obesity may increase the prevalence and incidence of CKD [3–8] and end-stage renal disease [9], although there might be differences according to gender and ethnicity [9–11]. However, fewer studies have investigated whether changes in obesity indexes, such as body weight, body mass index (BMI), and waist circumference (WC), are associated with changes in CKD status [12–14]. In the current study, we retrospectively analyzed data on individuals who underwent general health screening at our institute for 2 consecutive years and investigated whether changes in obesity indexes were associated with changes in CKD status in these Japanese individuals.

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## Subjects and Methods

### Study Population

The study was approved by The Ethical Committee of Mitsui Memorial Hospital. At our institution, 3,312 (1,203 women, 2,109 men) individuals underwent general health screening including that on urinary albumin excretion between October 2005 and October 2006 (first visit) and also in the subsequent year (second visit). Among the 3,312 individuals, data on 2,861 (1,114 women, 1,747 men) who reported not taking anti-hypertensive drugs at both visits were used for the present study. The mean  $\pm$  SD of the interval between the two visits of the enrolled individuals by the study subjects was  $355 \pm 52$  days. Individuals who were taking antihypertensive medications were excluded from the analysis because certain depressor drugs may affect renal function and the extent of proteinuria [15, 16] and because the database did not include information on the class of drugs used. At the time of the health examination, recommendations may have given to overweight or obese subjects to reduce body weight. However, in analyzing data for this study, there was no intention to examine which strategies for weight control, if any, would have an impact on the status of CKD during the follow-up.

In Japan, regular health check-ups for employees are legally mandated; thus, the majority of these subjects did not have serious health problems. In addition, all or most of the costs of the screening are usually paid by the company to which they belong or by each subject. In addition, there are several courses in the health screening program; however, which to choose is up to each individual, but not to physicians or company one belongs to. Therefore, the study population is not considered to be enriched for certain diseased condition.

### Laboratory Analysis

Blood samples were taken from the subjects after an overnight fast. Serum levels of total cholesterol (TC), HDL-cholesterol (HDL-C), and triglycerides (TG) were determined enzymatically. Serum uric acid was measured by the uricase-peroxidase method, hemoglobin A<sub>1C</sub> was determined using the latex agglutination immunoassay, and creatinine was determined by the enzymatic method. Plasma glucose was measured by the hexokinase method and serum insulin was measured by enzyme immunoassay.

Creatinine and urine albumin were measured by TBA-200FR (Toshiba Medical Systems, Tochigi, Japan) and by Accute (Toshiba Medical Systems), respectively, using commercially available kits, Accuras Auto CRE (Shino-test, Tokyo, Japan) and IATRO U-ALB by turbidimetric immunoassay (Mitsubishi Kagaku Iatron, Tokyo, Japan), respectively. Serum creatinine was calibrated using the following formula: serum creatinine (Jaffe method) =  $0.2 + \text{serum creatinine (enzyme method)}$ . Glomerular filtration rate (GFR) was estimated by equations of the simplified version of Modification of Diet in Renal Disease (MDRD) [17], where 0.881 is a coefficient for eGFR specific to the Japanese population [18]: estimated GFR (eGFR) for Japanese =  $186.3 \times (\text{serum creatinine})^{-1.154} \times (\text{age})^{-0.203} \times 0.881 \times 0.742$  (for females). eGFR values  $<60$  ml/min/1.73 m<sup>2</sup> were classified as low [19]. For the diagnosis of micro-/macroalbuminuria, spot urine samples were collected and analyzed; micro-/macroalbuminuria was defined to be present when the urinary albumin excretion ratio (UAER), expressed as milligrams per gram creatinine, was  $\geq 30$  mg/g. Normoalbuminuria, microalbuminuria, and macroalbuminuria

were defined as a UAER of  $<30$ ,  $30$ – $299$ , and  $\geq 300$  mg/g, respectively. Micro-/macroalbuminuria and a low eGFR were considered to be the components of CKD [19]. The difference in BMI and WC between the two visits was designated as  $\Delta$ BMI and  $\Delta$ WC, respectively.

### Statistical Analysis

Skewed variables (TG, UAER) are presented as median values (interquartile range). Other data are expressed as the mean  $\pm$  SD unless stated otherwise. Analyses of variance, the Mann-Whitney U test,  $\chi^2$  tests, and logistic regression analysis were conducted as appropriate to assess the statistical significance of differences between groups using computer software, Dr. SPSS II (Chicago, Ill., USA). A value of  $p < 0.05$  was taken to be statistically significant.

## Results

### Baseline Characteristics

The mean  $\pm$  SD age of the individuals enrolled was  $52.0 \pm 10.1$  years at the first visit (table 1). Of the 88 females and 149 males with micro-/macroalbuminuria, 83 and 134, respectively, had microalbuminuria and 5 and 15, respectively, had macroalbuminuria.

### Changes in BMI and WC Values between the Two Visits

The mean BMI at the second visit was slightly lower than that at the first visit ( $p < 0.001$ , by paired t test) in men, but did not differ significantly in women. The mean WC at the second visit was slightly larger than that at the first visit ( $p < 0.001$ , by paired t test) in women and smaller ( $p < 0.001$ , by paired t test) in men. In this study, we calculated quartiles of WC or BMI by taking into the entire population. Ranges for each quartile for  $\Delta$ BMI and  $\Delta$ WC are shown in table 2. About half of the subjects had a decreased WC value at the second visit. The correlation coefficient between the first-visit BMI and  $\Delta$ BMI was  $-0.09$  ( $p = 0.010$ ) in women and  $-0.09$  ( $p = 0.002$ ) in men, and that between the first-visit WC and  $\Delta$ WC was  $-0.31$  in women ( $p < 0.001$ ) and  $-0.28$  ( $p < 0.001$ ) in men.

### Changes in the Prevalence of Micro-/Macroalbuminuria and a Low eGFR between the Two Visits

Figure 1 shows the number of subjects with micro-/macroalbuminuria and a low eGFR at the first and second visits. Of those with micro-/macroalbuminuria at the first visit, 34% did not have micro-/macroalbuminuria at the second visit, but 4% of subjects who did not have micro-/macroalbuminuria at the first visit had de-

**Table 1.** Clinical characteristics and laboratory data

Variables	Women (n = 1,114)		Men (n = 1,747)	
	Visit 1	Visit 2	Visit 1	Visit 2
Age, years	51.3 ± 9.9	52.3 ± 9.9	52.5 ± 10.1	53.4 ± 10.1
Height, cm	157.1 ± 5.7	157.1 ± 7.8	169.7 ± 5.9	169.7 ± 5.9
Weight, kg	52.3 ± 7.7	52.3 ± 7.8	67.8 ± 9.2	67.6 ± 9.3
BMI	21.2 ± 2.9	21.2 ± 2.9	23.5 ± 2.7	23.5 ± 2.8
ΔBMI	–	0.0 ± 0.7	–	–0.1 ± 0.7
WC, cm	76.2 ± 8.6	76.9 ± 8.9	85.3 ± 7.5	85.0 ± 7.4
ΔWC, cm	–	0.7 ± 6.0	–	–0.3 ± 3.8
Systolic BP, mm Hg	116 ± 18	115 ± 18	124 ± 17	124 ± 18
Diastolic BP, mm Hg	72 ± 11	72 ± 11	79 ± 11	79 ± 11
Total cholesterol, mg/dl	217 ± 36	215 ± 34	210 ± 32	207 ± 31
LDL-cholesterol, mg/dl	130 ± 30	127 ± 30	128 ± 32	126 ± 31
HDL-cholesterol, mg/dl	69 ± 14	68 ± 14	56 ± 14	56 ± 13
TG, mg/dl	84 ± 46	84 ± 42	127 ± 80	126 ± 101
TG, median (interquartile range)	74 (55–99)	74 (54–101)	107 (77–152)	102 (74–144)
Uric acid, mg/dl	4.5 ± 0.9	4.5 ± 0.9	6.1 ± 1.2	6.0 ± 1.2
Fasting glucose, mg/dl	90 ± 17	91 ± 14	98 ± 21	99 ± 20
Hemoglobin A <sub>1C</sub> , %	5.2 ± 0.6	5.2 ± 0.5	5.4 ± 0.8	5.5 ± 0.7
Antidiabetic medication, n (%)	5 (0.4)	9 (0.8)	46 (2.6)	58 (3.3)
Blood urea nitrogen, mg/dl	13.4 ± 3.2	13.6 ± 3.3	14.4 ± 3.5	14.5 ± 3.5
Serum creatinine, mg/dl	0.63 ± 0.09	0.62 ± 0.09	0.86 ± 0.28	0.84 ± 0.30
UAER, median (interquartile range)	7.5 (5.1–12.2)	7.9 (5.4–13.1)	5.2 (3.7–10.0)	5.6 (3.9–10.7)
UAER ≥30 mg/g Cr, n (%)	88 (7.9)	91 (8.2)	149 (8.5)	165 (9.4)
eGFR, ml/min/1.73 m <sup>2</sup>	69.5 ± 9.3	70.1 ± 9.2	70.9 ± 10.0	71.8 ± 10.2
Low eGFR, n (%)	155 (13.9)	138 (12.4)	212 (13.1)	201 (11.5)
Current smoker	99 (8.9)	93 (8.3)	581 (33.3)	542 (31.0)

Data are means ± SD, median (interquartile range), n, or percentage. BMI = Body mass index; WC = waist circumference; BP = blood pressure; TG = triglycerides; UAER = urinary albumin excretion rate; eGFR = estimated glomerular filtration rate.

**Table 2.** Range for each quartile of ΔBMI and ΔWC

	Q1	Q2	Q3	Q4
ΔBMI	–5.33/–0.42 (–0.75)	–0.41/–0.04 (–0.21)	–0.04/0.32 (0.13)	0.33/3.67 (0.62)
ΔWC, cm	–21.0/–3.0 (–5.0)	–2.9/–0.1 (–1.5)	0.0/2.7 (1.0)	2.8/23.0 (5.0)

BMI = Body mass index; WC = waist circumference. Medians are given in parentheses.

veloped micro-/macroalbuminuria at the second visit (fig. 1a, b). Of individuals who had a low eGFR at the first visit, 28% did not have a low eGFR at the second visit, but alternatively, 3% of individuals who did not have a low eGFR at the first visit had a low eGFR at the second visit.

#### Association between Changes in BMI or WC and Albuminuric Status

Next, we investigated whether decreases in BMI and WC values were associated with changes in CKD status (fig. 2a, b). Logistic regression analysis adjusted for age, systolic blood pressure, HDL- and LDL-cholesterol, fast-