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JSH Consensus Kobe 2009: Diagnosis and Treatment of Hepatitis C

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Key words: chronic hepatitis C diagnosis guideline treatment

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Research Article **Chromatography**

Received: 31 July 2008, Revised: 7 August 2008, Accepted: 7 August 2008 Published online 27 November 2008 in Wiley Interscience

(www.interscience.wiley.com) DOI 10.1002/bmc.1139

Limitation of immunoaffinity column for the removal of abundant proteins from plasma in quantitative plasma proteomics

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ABSTRACT: In plasma proteomics, before a proteome analysis, it is essential to prepare protein samples without high-abundance proteins, including albumin, via specific preparation techniques, such as immunoaffinity capture. However, our preliminary experiments suggested that functional changes with use alter the ability of the immunoaffinity column. Thus, in this study, to evaluate the changes of the removal ability of abundant proteins from plasma by the immunoaffinity column, plasma proteome analysis was performed for the long-term test for the reproducibility of the affinity column using the fluorogenic derivatization–liquid chromatography–tandem mass spectrometry method combined with an IgY column. The specific adsorption for albumin decreased with an increase in the number of the column usage before its expiration date. Moreover, it was demonstrated that hydrophobic high molecular weight compounds in plasma adsorbed onto the column materials surface contributed to the functional changes from specific immunoaffinity adsorption into hydrophobic interaction. These results suggested that, in quantitative plasma proteomics studies, it is important to keep in mind the risk of not only the nonselective loss but also the changes in the adsorption ability of the immunoafinity column. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: plasma; proteomics; immunoaffinity column; abundant protein; FD-LC-MS/MS method

Introduction

Blood samples can be taken at a particular point in time with little burden on patients and the constituents of the blood samples could reflect a developing or existing illness because tissue-specific proteins may be released into the brood stream from the damaged or dead cells. Therefore, it is generally recognized in proteomics studies that blood samples represent the greatest potential source of information on the proteins related to human diseases. However, plasma proteome analysis aiming at quantitative protein profiling and biomarker discovery is not easily done. Since several high-abundance proteins, such as albumin, typically constitute greater than 90% of total protein mass, the detection of lower-abundance proteins which presumably are the biologically interesting population is interfered with by the dominant proteins. To address the complexity of these samples, it is essential to prepare samples via specific preparation techniques to remove high-abundance proteins from the samples before the proteome analysis (Linke *et al.*, 2007; Martosella *et al.*, 2005; Qian *et al.*, 2006; Steel *et al.*, 2003). There are a number of approaches for removing proteins based on their biochemical and biophysical features, such as molecular weight, mass, density, hydrophobicity, surface change and isoelectric point. Among these techniques, immunoaffinity capture using antibodies is rapidly becoming the prefractionation method of choice in proteomics analysis. Commercial kits using an avian immunoglobulin yolk (IgY) have recently become available due to its high avidity and lesser cross-reactivity with heterologous human proteins (Huang *et al.*, 2005; Linke *et al.*, 2007; Qian *et al.*, 2006). A number of researchers have already indicated its utility and the improvement of the detection of low-abundance proteins by the elimination of the high-abundance proteins using the IgY affinity column (Gong *et al.*, 2006; Huang *et al.*, 2005; Linke *et al.*, 2007; Liu *et al.*, 2006; Qian *et al.*, 2006).

We have recently developed a highly sensitive and quantitative proteomics method called fluorogenic derivatization–liquid chromatography–tandem mass spectrometry (FD-LC-MS/MS) (Masuda *et al.*, 2004; Toriumi and Imai, 2003). The method consists of separation of the fluorogenic derivatized proteins by high-performance liquid chromatography (HPLC), isolation of the target protein obtained by HPLC, hydrolysis and identification of the target protein by LC-MS/MS with the probability-based protein identification algorithm. This highly selective, sensitive and reproducible method enables the post-translational proteins and isoforms to be distinguished. The method was applied to the extracts of *Caenorhabditis elegans*, mousee liver and breast cancer cell lines, and revealed the proteins related to early-stage Parkinson's

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Abbreviations used: DAABD-Cl, 7-Chloro-*N*-[2-(dimethylamino)ethyl]-2,1,3-benzoxadiazole-4-sulfonamide; FD, fluorogenic derivatization; HCCA, α-cyano-4-hydroxycinnamic acid; TCEP, Tris (2-carboxyethyl) phosphine hydrochloride; TFA, trifluoroacetic acid; TOF, time-of-flight.

Contract/grant sponsor: MEXT HAITEKU (2004–2008), Grant-in-Aid for Young Scientists (Start-up).

Contract/grant sponsor: Mochida Memorial Foundation for Medical and Pharmaceutical Research.

disease (Ichibangase *et al.*, 2008), hepatocarcinogenesis (Ichibangase *et al.*, 2007) and tumor progression and metastasis (Imai *et al.*, 2008). During the course of our studies, we applied the FD-LC-MS/MS method to plasma proteomics. To detect plasma biomarkers that are probably masked by the high-abundant proteins, an IgY affinity column was utilized for the removal of the dominant proteins, such as albumin, from plasma before the fluorogenic derivatization (FD) of the plasma proteins. On the preliminary experiments, the quantitative changes of the peaks on the chromatograms obtained from the same samples were observed on every occasion of sample treatment with the affinity column. Since the detectability of the fluorogenic derivatized proteins by the HPLC-fluorescence detector is always constant, the change in the removal ability of the IgY column for the abundant proteins could be monitored during the usage of the column. Although it was reported that there was a risk of loss by inadvertent capture of low-abundance proteins (Bjorhall *et al.*, 2005; Gong *et al.*, 2006; Linke *et al.*, 2007; Plavina *et al.*, 2007; Yocum *et al.*, 2005), there are no reports of long-term tests for the reproducibility of the affinity column in quantitative proteome analysis.

In this study, to evaluate the removal ability of abundant proteins from plasma by the affinity column, we performed proteome analysis of plasma sample and protein standards by FD-LC-MS/MS combined with the IgY technique and investigated the cause of the quantitative changes of the chromatograms mentioned above.

Experimental

Materials and Methods

Reagents. 7-Chloro-*N*-[2-(dimethylamino)ethyl]-2,1,3-benzoxadiazole-4-sulfonamide (DAABD-Cl) and 6.0 M guanidine hydrochloride (pH 8.7 buffer solution) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Ethylenediamine-*N*,*N*,*N*',*N*'-tetraacetic acid sodium salt (Na_2EDTA) and 3-[(3-Cholamidopropyl) dimethyammonio]-1-propanesulfonate (CHAPS) were from Dojindo Laboratories (Kumamoto, Japan). Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) was obtained from Sigma-Aldrich (St Louis, MO, USA). Acetonitrile and trifluoroacetic acid (TFA) for the HPLC-fluorescence detection were HPLC grade and were obtained from Wako Pure Chemical Industries (Osaka, Japan). All other reagents used were of analytical grade.

Affinity Columns

The prepacked IgY-R7 Spin Column and Seppro®-IgY12 were obtained from Beckman Coluter (Fullerton, CA, USA) and GenWay Bioteck (San Diago, CA, USA), respectively. The IgY-R7 Spin Column (1.2 mL spin column) removes seven high-abundance proteins in rodent plasma (albumin, IgG, α2-antitrypsin, IgM, transferring, haptoglobin and fibrinogen) and utilizes centrifugation as the force for affinity separation. The column is said to be reusable 100 times under proper conditions. The Seppro®-IgY12 column is optimized for human plasma and removes 12 high-abundance proteins (IgA, α1-acid glycoprotein, α2-macroglobin, apolipoproteins A-I and apolipoproteins A-II besides the above seven proteins). This column is used with the high-throughput automated proteomic sample processing instrument (Magtration System SA-1; Precision System Science, Chiba, Japan) and is said to be able to be used 30 times. Both companies are corporate partners for the exclusive marketing of the IgY microbeads technology, and both column materials are the same except for recognized animal species to the IgY.

Plasma Samples

For the IgY-R7 Spin Column, plasma sample were obtained from C57BL/6N male mice (10 and 19 months; Clea Japan, Tokyo, Japan) by centrifugation at 5510 rpm for 10 min at 4°C, and frozen at −80°C until use. On the other hand, the human control plasma sample purchased from Sigma-Aldrich was used for the Seppro®-IgY12 column. The control plasma sample was passed through a 0.45 μm filter before use.

Treatment of Mouse Plasma with the IgY-R7 Spin Column

Mouse plasma treated with the spin column was carried out according to the manufacturer-instructed column usage and loading capacity [10 μL plasma diluted with dilution/washing buffer: 10 mM Tris–HCl, 150 mM NaCl, pH 7.4 (TBS)]. Three buffers (dilution/washing buffer; stripping buffer: 100 mM glycine, pH 2.5; neutralization buffer: 100 mM Tris–HCl, pH 8.0) were used under the separation scheme that consisted of sample loading–washing–eluting–neutralization followed by a re-equilibration scheme for a total cycle time of 40 min. To increase the recovery of the non-specific proteins, the resulting flow-though fraction and the washing fractions were collected and concentrated to 10 μL with 3.0 kDa molecular weight cutoff device according to the manufacturer's instructions (Microcon YM-3; Millipore, Billerica, MA, USA).

Treatment of Protein Standards and Control Human Plasma Sample with the Seppro®-IgY12 Column

The molecular weight standards, consisting of phosphorylase B, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and lysozyme for electrophoresis (Table 1; 12 mg/mL protein amount; low range; Bio-Rad, Herccules, CA, USA), were employed in sample processing without dilution to evaluate the recovery of non-specific proteins from the column. The injected amount of the protein standards was 2.4 mg protein per injection to the affinity column. Since, in the instructions, 15 μL of plasma (generally corresponding to 70–80 mg proteins/mL) was diluted to 500 μL and loaded to the affinity column, the injected amount of the standards was compatible. According to the manufacturer's instructions, the protein standards were set in the sample holding and then the flow-though fraction was obtained.

The control human plasma sample was also treated according to the manual. Briefly, 15 μL of plasma sample was diluted with the dilution/washing buffer to 500 μL, and the sample was set in the sample holding, as in the case of the protein standards. The

Table 1. Protein standards and the number of labeled region with DAABD-Cl

Protein	Source	MW (kDa)	No. of rabeled region
Phosphorylase B	Rabbit muscle	97.4	10
Serum albumin	Bovine	66.2	35
Ovalbumin	Hen egg white	45	5
Carbonic anhydrase	Bovine	31	2
Trypsin inhibitor	Soybean	21.5	5
Lysozyme	Hen egg white	14.4	6

resulting flow-though fraction of the plasma was concentrated to 15 μL with a Microcon YM-3 device. A series cycle including the sample loading–washing–eluting–neutralization finished in 65 min.

FD-LC-MS/MS Conditions

Each FD condition was optimized for the protein standards and for plasma samples in order to obtain the highest peak on the chromatograms. For the protein standards, a 2.5 μL aliquot of the flow-though fraction was mixed with 30 μL of a mixture of 0.83 mM TCEP, 3.3 mM Na_2EDTA and 16.6 mM CHAPS in the pH 8.7 buffer solution, 12.5 μL of the buffer solution and 5.0 μL of 8.0 mM DAABD-Cl in acetonitrile. The mixture was reacted at 50°C for 5.0 min, and the reaction was stopped with 1.5 μL of 20% TFA. A 20 μL aliquot of the above reaction mixture was injected to the HPLC system (Hitachi L-2000 series; Hitachi Instruments, Tokyo, Japan) using a column of Intrada WP-RP (30 nm pore size, 250 × 4.6 mm i.d., Imtakt Co, Kyoto, Japan) at 60°C with a flow rate of 0.55 mL/min. The eluent (A) and eluent (B) were water–acetonitrile–TFA (90:10:0.15, v/v/v) and water–acetonitrile–TFA (30:70:0.05, v/v/v), respectively. The gradient condition was established from 5 to 100% eluent (B) over a period of 60 min. For mouse and control plasma samples, a 6.0 μL aliquot of the flow-though fraction was mixed with 30 μL of the above mixture of TCEP, Na_2EDTA and CHAPS, 10 μL of the buffer solution and 4.0 μL of 825 mM DAABD-Cl in dioxane. After the FD reaction (50°C for 5.0 min), the reaction was stopped with 2.0 μL of 20% TFA. An aliquot (10 μL) of the reaction mixture was injected, and the longer column (Intrada WP-RP 30 nm pore size, 500 × 4.6 mm i.d., Imtakt Co) together with a precolumn (Intrada WP-RP 30 nm pore size, 5.0 × 2.0 mm i.d., Imtakt Co) at 60°C was adopted with a flow rate of 0.55 mL/min on the HPLC system. The mobile phases consisted of water–acetonitrile–TFA (A) 90:10:0.15 and (B) 30:70:0.05. Mobile phase (C) was the same as (A), except with 0.05% TFA. The gradient condition is described in Fig. 1. Fluorescence detection was carried out at 395 and 505 nm for the excitation and emission wavelengths, respectively. The peak height of each protein peak obtained from the HPLC chromatograms was calculated by HITACHI EZChrom Elite™ Chromatography Data System (Hitachi Instruments) and the identification of the standard proteins was accomplished according to the previous report (Ichibangase *et al.*, 2007).

Scanning Electron Microscopy and Matrix-assisted Laser Desorption/Ionization MS Analysis

Scanning electron microscopy (SEM) and matrix-assisted laser desorption/ionization (MALDI) MS analyses were conducted in Jeol Datum (Tokyo, Japan) and Bruker Daltonics Japan (Kanagawa, Japan), respectively.

For SEM analysis, the column materials were diluted with phosphate buffer (300 mOsm) and fixed with fixative (2.5% glutaraldehyde in PBS, pH 7.0) for 10 min. The fixed sample was captured on the filter (SEM-Pore: 0.6 μm i.d.; 10 μm) and washed with the buffer. After the osmium-fix (2% OsO_4) and a brief rinse with the fixative solutions, specimens were dehydrated in a series of graded ethanol (30–100%). The immersed specimens in ethanol were replaced with isoamyl phenylacetate and subjected to critical point drying. The dried samples were coated with osmium using a plasma coater (OPC80N, Jeol). Images were acquired using a Jeol JSM-7401F in normal SEM mode. For the low power microscope images for the whole picture of a material, a lower electron image (LEI) was applied.

For MALDI MS analysis, the column materials were washed and spotted on a plate. α-Cyano-4-hydroxycinnamic acid (HCCA) was used as matrix. MALDI mass spectra were acquired with time-of-flight (TOF) MS (autoflex III, Bruker Daltonics) in positive linear mode.

Results and Discussion

Quantitative Functional Changes of the IgY-R7 Spin Column after a Number of Treatments with Mouse Plasma

For the detection of low-abundance proteins in mouse plasma, the removal of high-abundance proteins from mouse plasma with the affinity column was performed prior to the FD-LC-MS/MS proteome analysis. Typical chromatograms are shown in Fig. 1(A), obtained from the same mouse plasma sample treated with 80 and 86 cycles of the same spin column, respectively. All peak heights obtained from the 86 cycles of the spin column were clearly higher compared with those obtained from the 80 cycles. Although a difference between column lots might exist, there were also significant differences in the removability of the affinity column between the second (column lot no. 2) and the 44th cycles (column lot no. 1) of the treated spin column [Fig. 1(B)]. The relative standard deviation (RSD) of the protein peaks was calculated between-day ($n = 3$) using samples provided by the same treatment number of the column. The RSD values were less than 21.6%, obtained from the peak in Fig. 1(A), suggesting that the detectability of the fluorogenic derivatized proteins by HPLC is constant. Therefore, it was considered that the quantitative changes of the peaks on the chromatograms might result from the changes in the affinity column by the sample treatment.

Evaluation of Seppro®-IgY12 Column for the Adsorption of Protein Standards using an Automatic Instrument

To eliminate a manual usage error from the sample processing, a high-throughput automated instrument, SA-1, for the removal of high-abundance proteins from human plasma samples with a Seppro®-IgY12 column was investigated. For the evaluation of the exact adsorption ratio of specific and non-specific proteins to the affinity column, the affinity column was periodically treated with protein standards after treatment with a control human plasma sample some dozen times. Since the other investigator has reported the non-specific adsorption of the protein by concentration methods such as a centrifugal filter to be about 15% (Linke *et al.*, 2007), the flow-through fraction of the injected protein standards from the affinity column was subjected directly to the FD-LC-MS/MS analysis without a protein concentration step in this study.

At first, to eliminate the non-specific adsorption to the affinity column, the protein standards mixture was divided into two parts and one was subjected into the untreated affinity column, and the flow-though fraction was derivatized with the fluorogenic reagent, DAABD-Cl, and separated by the HPLC system (4.8 μg protein/HPLC injection). Another part of the protein standards mixture was diluted and derivatized with DAABD-Cl, and injected onto the HPLC system (4.8 μg protein/HPLC injection). The obtained chromatograms are depicted in Fig. 2. Each protein peak was collected, digested in peptide mixtures, and identified by applying the peptides to HPLC-MS/MS with a

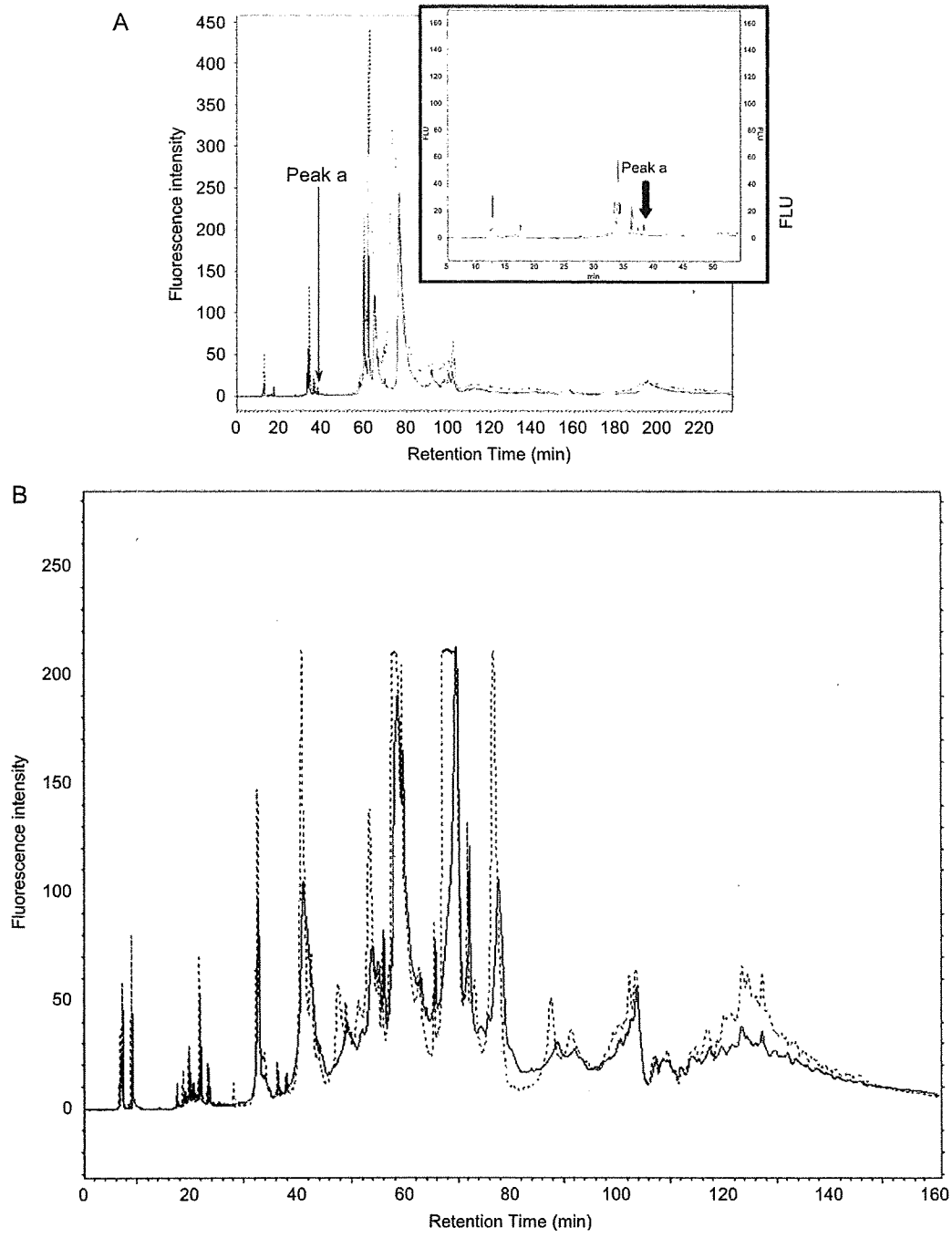

Figure 1. Chromatograms of mouse plasma samples treated with IgY-R7 Spin Columns. (A) The mouse plasma (19 months, C57BL/6N) treated 80 times (solid line) and 86 times (dotted line) in the same spin column. The gradient conditions were as follows: time (min), 0 → 10 → 20 → 44 → 48 → 68 → 80 → 90 → 108 → 120 → 130 → 160 → 180 → 230; B (%): 5 → 5 → 30 → 30 → 35 → 38 → 39.2 → 39.2 → 42 → 43 → 44 → 45 → 47 → 58; C (%): 0 → 0 → 0 → 0 → 0 → 0 → 0 → 60.8 → 58 → 57 → 56 → 55 → 53 → 42. (B) The mouse plasma samples (10 months, C57BL/6N) were treated with the second cycle (column lot no. 2; solid line) and the 44th cycle (column lot no. 1; dotted line) of each spin column. The gradient conditions were as follows: time (min), 0 → 5 → 10 → 22 → 24 → 34 → 54 → 60 → 60.1 → 80 → 130 → 140 → 150 → 160 → 170; B(%): 5 → 5 → 30 → 30 → 35 → 38 → 42 → 43 → 43 → 47 → 58 → 60 → 60 → 75 → 78; C(%): 0 → 0 → 0 → 0 → 0 → 0 → 0 → 0 → 57 → 53 → 42 → 40 → 40 → 25 → 22.

database-searching algorism. Table 2 shows the adsorption ratio of the proteins to the untreated column and the identified protein names. The protein names of peak 1 and 2 could not be identified since these peaks were peptides coexisting in the protein standards. Also, carbonic anhydrase and ovalbumin could not be detected. Since carbonic anhydrase has only two cycteine residue for labeling with DAABD-Cl, its detection might be difficult. The reason for the undetected ovalbumin was not clear. Since this study was aimed at investigating the changes in the adsorption of the specific and non-specific proteins using the column, this issue was not examined further. Consequently, although the affinity column was able to efficiently remove bovine serum

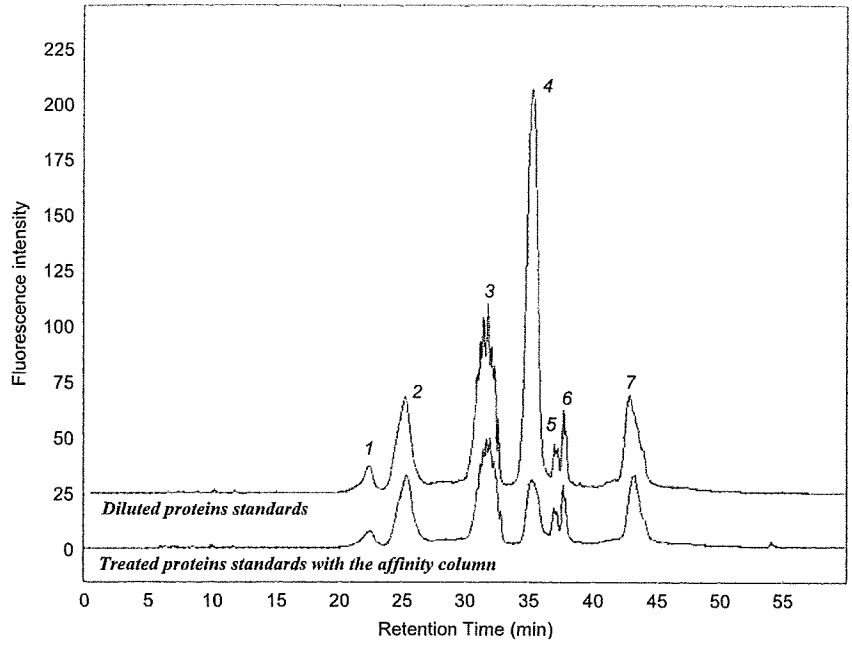

Figure 2. Chromatograms of the protein standards which were treated with Seppro®-IgY12 column and diluted to the same protein amount (4.8 μg/HPLC injection) as the amount for the column treatment. The peak numbers correspond to Table 2.

Table 2. Adsorption ratio to the untreated Seppro®-IgY12 column and protein names obtained by FD-LC-MS/MS method

Peak no.	Adsorption ratio to the untreated Seppro®-IgY12	Protein name
1	38.5%	Peptide
2	30.6%	Peptide
[a]3	45.9%	Lysozyme
4	83.9%	Bovine serum albumin (BSA)
[b]5	18.3%	Trypsin inhibitor
6	28.5%	Trypsin inhibitor
7	30.4%	Phosphorylase B

[a]3, [b]5: Most highest peak

albumin (BSA; 83.9%) as compared with the other proteins in the standards, non-specific binding to the column materials or to carrier proteins such as albumin itself was observed in the 18.3–45.9% range and could result in the loss of presumed biomarkers.

Next, the time series changes of the specific and non-specific adsorption of proteins to the column were investigated. The protein standards mixture was treated with the column periodically after 10 and 20 cycles of treatment of the control plasma sample. The relation of the changes of the protein standards adsorption to the number of uses of the affinity column is shown in Fig. 3(A). The specific adsorption of BSA decreased with an increase in the number of times the column was used. However, the non-specific adsorption for lysozyme, trypsin inhibitor and phosphorylase B reached a maximum at 11 cycles and decreased at 21 cycles. Since the affinity column was optimized for human plasma, the absorption of BSA for the column might be weaker than for plasma albumin. However, the adsorption of albumin in control plasma also decreased with an increase in the number of times the column was used (data not shown). Moreover, since the slopes of decrease differed among the protein standards, the correlation of each adsorption with molecular weight of each protein was calculated [Fig. 3(B)]. The open dots show the value of BSA in Fig. 3(B). The correlation coefficient value was the closest to 1.00 ($R^2 = 0.813$) for 21 cycles [Fig. 3(B-3)], demonstrating that the adsorption ability of the column does not depend any longer on the affinity of the antibody but on the hydrophobicity of the protein. In contrast, the correlation of the values obtained from the first cycle [Fig. 3(B-1); $R^2 = 0.077$] was not fairly observed between the adsorption ability and the hydrophobicity, and the value for BSA was apart from those for other proteins. Therefore, BSA was specifically removed as compared with the other protein standards by the immunoaffinity adsorption. Also, as shown in Fig. 3(B-2), the result obtained from 11 cycles ($R^2 = 0.012$) demonstrated that all proteins bound to the surface of the affinity column materials equally. Therefore, the present data demonstrates that the quantitative changes of the adsorption for the affinity column appear before the limited use of the column (30 times in the manufacturer's instructions).

Understanding the State of the Plasma-treated Column Materials

To understand the state of the column materials of the Seppro®-IgY12 column, the untreated and 11- and 21-times-treated column materials were subjected to electron microscopy and MALDI-TOF-MS analysis.

As shown in Fig. 4, the SEM images show an obvious difference between the untreated and treated materials. The attachment of the unknown bio-molecules to the materials surface appeared and increased with an increase in the number of treatments.

Next, in order to characterize the attached compounds, the same materials were subjected to MALDI-TOF-MS analysis. Since

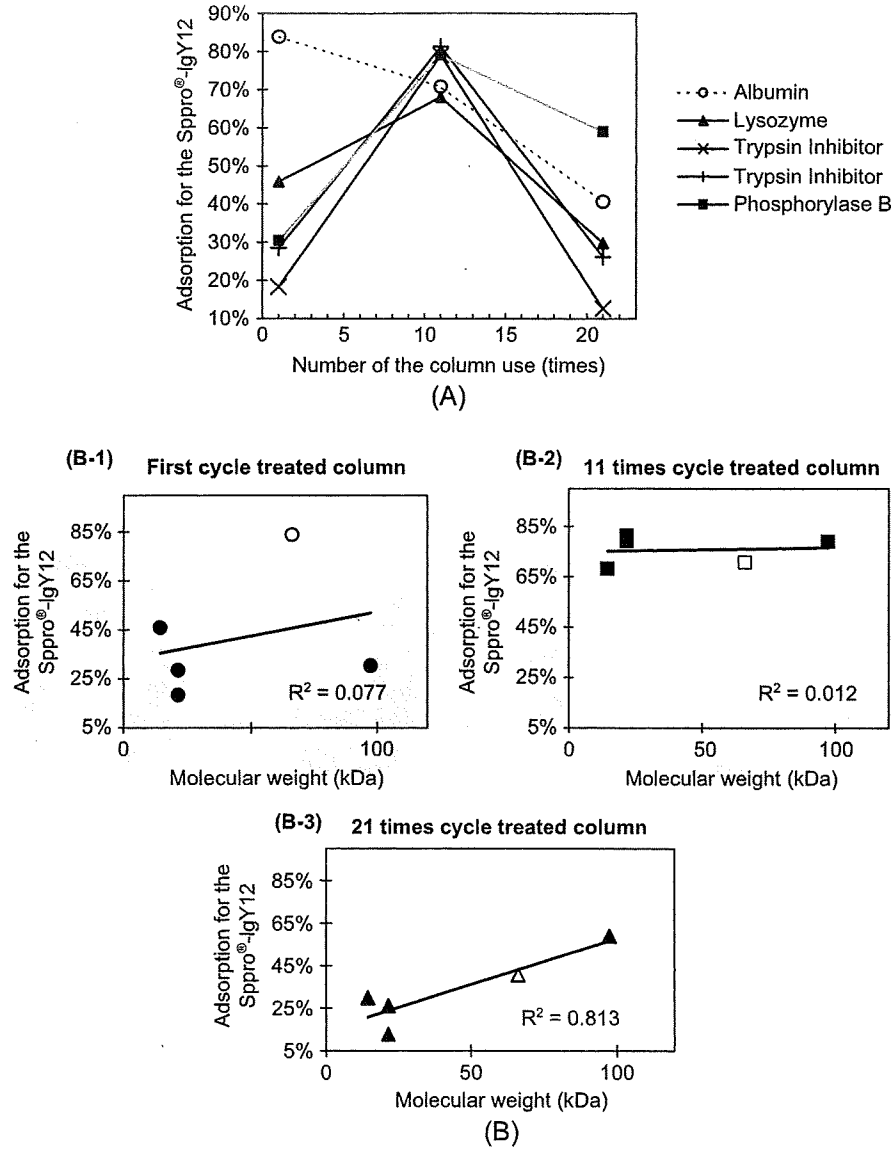

Figure 3. Changes of the adsorption ratio of protein standards for Seppro®-IgY12 column. (A) Relation of the adsorption ratio to the number of times the column was used. (B) Correlation of the adsorption ratio for the column with molecular weight of each protein standard using the column treater one (B-1), 11 (B-2) and 21 (B-3). The open dots show the value for BSA.

direct laser irradiation of the materials could affect the instrument, the positions to be irradiated were the points of the existence of many matrices on a few column materials. Although several peaks existed of less than 70,000 *m/z* in each mass spectrum, the higher molecular weight peaks (7266, 9689, 14,532 and 29,041 *m/z*) appeared in the treated but not in the untreated materials (Fig. 5). After the materials were washed with acetonitrile, the higher molecular weight peaks in the treated materials disappeared (data not shown). Therefore, the compounds attached to the material surface should be hydrophobic high-molecular-weight compounds existing in human plasma.

Two analyses of the column materials surface demonstrated that the hydrophobic high-molecular-weight compounds in plasma adsorbed onto the surface of the affinity column materials and contributed to the changes in the adsorption ability of plasma protein from immunoaffinity into hydrophobic interactions. Howevere, further studies are needed to characterize the exact details of the compounds.

Conclusions

To investigate the ability to remove abundant proteins from plasma by immnoaffinity using the IgY column, FD-LC-MS/MS method was applied to the long-term test of the reproducibility of the column. It was demonstrated that the immunoaffinity column was effective in removing BSA from the protein standards mixture, but, in addition, removing other proteins in the 18.3–45.0% range. The results suggested that the proteins of possible biomarkers could be lost and their quantification made difficult. Moreover, the specific adsorption of BSA in the protein standards mixture and of albumin in the control human plasma samples decreased with an increase in the number of times the column was used with both samples before its use expired. To examine the cause of the functional changes of the immunoaffinity, the correlations between the adsorption ratio for the affinity column and molecular weight of the adsorbed proteins were calculated, and the column materials surface was also investigated by SEM and

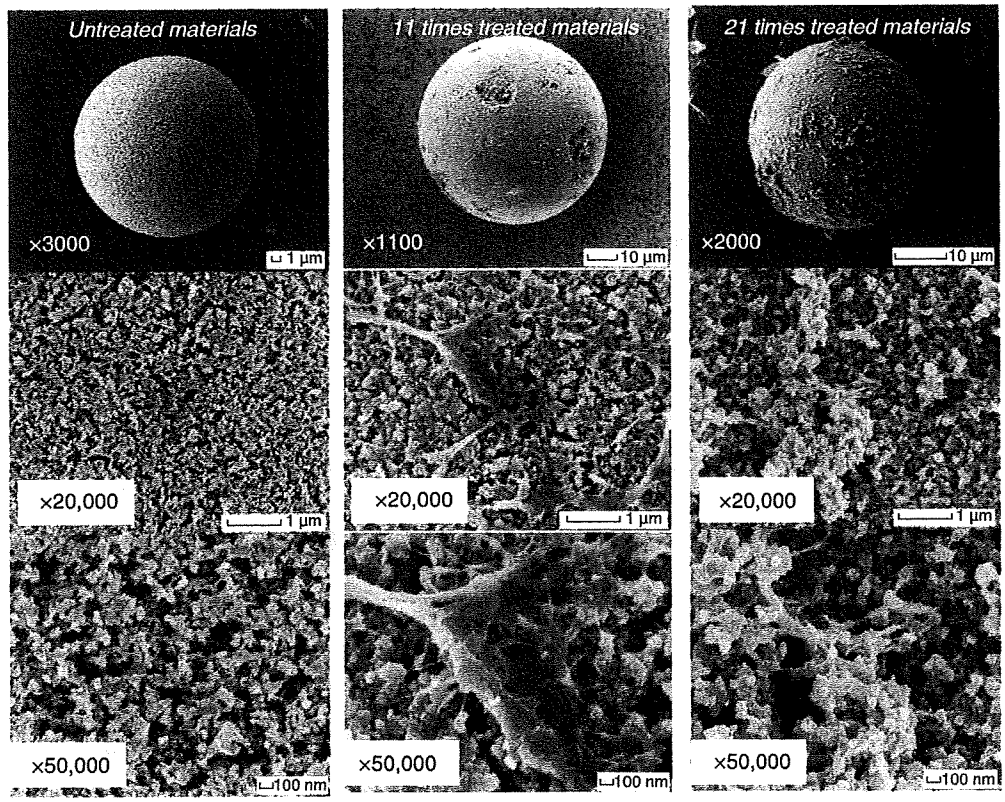

Figure 4. SEM images of the untreated and 11- and 21-times-treated column material surfaces. Magnification in SEM was controlled in a range of × 1100–3000 to show the whole picture of the material.

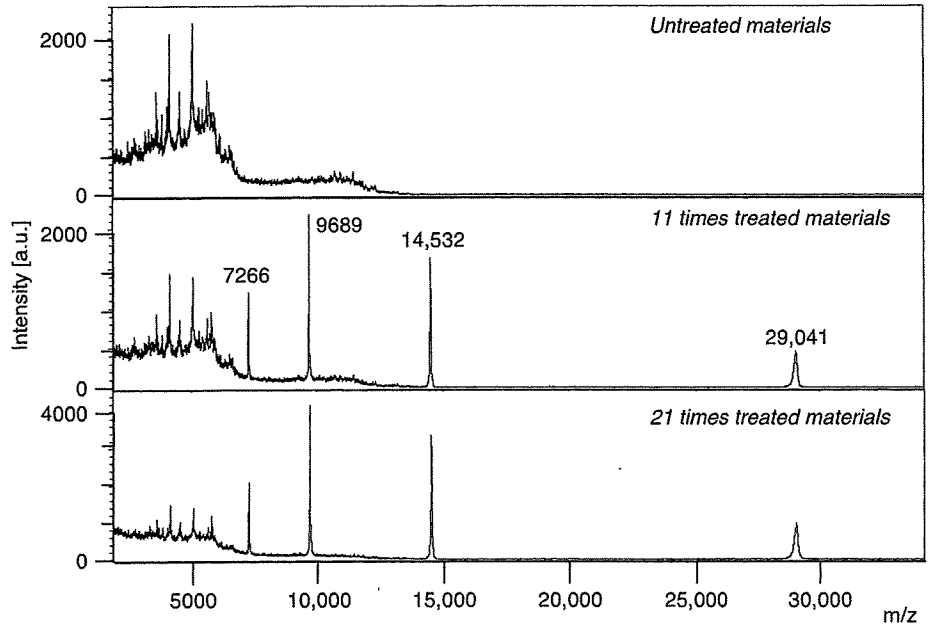

Figure 5. MALDI-TOF-mass spectrum of the untreated and 11- and 21-times-treated column material surface.

MALDI MS analysis. These data demonstrated the attachment of the hydrophobic high-molecular-weight compounds in plasma to the surface, suggesting that on every sample treatment with the affinity column, the adsorption ability of plasma protein changed into hydrophobic interactions. Further studies to characterize the attached compounds are required, and the elucidation of the compounds might lead to the improvement of the affinity column technique and contribute to progress in quantitative plasma proteomics.

Reproducibility is prerequisite for accurate quantitative proteome analysis of clinical samples for biomarker identification and quantification. For this purpose, it is generally essential to

prepare protein samples without high-abundance proteins via specific pre-fractionation techniques to enhance the detection of low-abundance proteins in plasma, and thus, immunoaffinity separation is now chosen as a reliable pre-fractionation method. However, this study indicated that, in quantitative plasma proteomics studies, it is important to keep in mind the risk of not only nonselective loss but also functional changes of the adsorption ability for the immunoaffinity column.

Acknowledgements

We would like to thank Precision System Science Co. Ltd for providing the high-throughput automated proteomic sample processing instrument, Magtration System SA-1, and Bruker Daltonics Japan for performing MALDI MS analysis of the affinity column materials. Part of this work was supported by a MEXT HAITEKU (2004–2008), Grant-in-Aid for Young Scientists (Start-up) and the Mochida Memorial Foundation for Medical and Pharmaceutical Research.

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J Infect Chemother (2009) 15:6–12
DOI 10.1007/s10156-008-0661-9

ORIGINAL ARTICLE

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Molecular characterizations of carbapenem and ciprofloxacin resistance in clinical isolates of *Pseudomonas putida*

Received: October 24, 2008 / Accepted: December 12, 2008

Abstract To analyze the genetic mechanisms of carbapenem and ciprofloxacin resistance in clinical isolates of *Pseudomonas putida*, 27 clinical isolates (comprising 11 carbapenem- and ciprofloxacin-resistant strains, 13 carbapenem-resistant and ciprofloxacin-susceptible strains, and 3 carbapenem- and ciprofloxacin-susceptible strains) were collected from different patients. Carbapenem resistance was examined by polymerase chain reaction (PCR) and DNA sequencing for metallo-β-lactamase (MBL) and integrase genes (*IntI*-1 and *IntI*-3), and by reverse transcriptase–PCR (RT-PCR) for expression of the porin gene (*oprD*). Ciprofloxacin resistance was characterized by PCR and DNA sequencing for mutations in the quinolone-resistance determining regions of the *gyrA* and *parC* genes. The bla_{IMP-1} MBL and *intI-1* and/or *intI-3* genes were detected in all carbapenem-resistant strains, and decreased expression of the *oprD* gene as compared to carbapenem-susceptible strains was observed in several strains. All the 11 strains with ciprofloxacin minimal inhibitory concentrations (MICs) of ≥64 mg/l had substitution in GyrA (Thr83Ile), and one (ciprofloxacin MIC of 512 mg/l) of these strains also had substitution in ParC (Ser87Leu). Overproduction of the efflux pump was observed in 10 of the 11 ciprofloxacin-resistant strains. We concluded that the production of IMP-1 type MBL was the most critical factor in developing high-level resistance to carbapenems, and mutations in the target proteins and overproduction of the efflux pump synergistically contribute to the acquisition of high-level resistance to ciprofloxacin in clinical isolates of *P. putida*.

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Key words *Pseudomonas putida* · DNA gyrase · Topoisomerase IV · Efflux pump · OprD

Introduction

Pseudomonas putida, which is often isolated from immunocompromised patients, is the causative pathogen of diseases such as urinary tract infections, and is also one of the important causes of nosocomial infections.[1] Although many strains of *P. putida* are usually sensitive to antimicrobial agents such as carbapenems and fluoroquinolones,[2] in recent years the emergence of resistance to them has been seen in clinical isolates of *P. putida*, and this has been a growing concern.[3–5]

Previous studies have demonstrated that the IMP- or VIM-type metallo-β-lactamase (MBL) is present in carbapenem-resistant *P. putida*,[3,4,6,7] and both types are often encoded by mobile gene cassettes inserted into integrons[6] which are located on plasmids in some cases. In this country, it has been reported that *P. putida* strains with MBL genes move together with class 1 and/or class 3 integrons.[7] Furthermore, a lack of outer membrane protein (OprD) expression, or its down-regulation, have been shown to be factors contributing to carbapenem resistance.[8,9]

In the case of fluoroquinolone resistance, however, the most widely documented mechanisms involve mutations in the quinolone-resistance determining regions (QRDRs) of DNA gyrase and topoisomerase IV. The foregoing factors are also thought to be critical to fluoroquinolone resistance in *Pseudomonas aeruginosa*.[10]

In Gram-negative organisms, overexpression of the efflux pump is another fluoroquinolone-resistance-causing factor.[11] There are thought to be efflux pumps specific to some β-lactams and other antibiotics.[12] Drug resistance due to efflux pumps has been reported in many bacterial species, with great variety in the protein structure of pumps and the types of drug to which there is resistance. In the case of *P. aeruginosa*, the up-regulation of resistance-nodulation-

cell division (RND) efflux pumps, such as MexAB-OprM, can contribute to multiple-drug resistance.[13] Although efflux pumps have been shown to exist in *P. putida*, their role in clinical isolates of this species has been unclear due to a lack of data.

Therefore we conducted the present study with the aim of supplementing our knowledge in this regard, since more data is needed to assess the risks stemming from the increasing antibiotic resistance of *P. putida*.[14–16] In this study, we examined the genetic mechanisms of carbapenem and fluoroquinolone resistance in clinical isolates of *P. putida* obtained from the University of Tokyo Hospital.

Materials and methods

Bacterial strains

The bacterial strains used in this study were 27 clinical isolates of *P. putida* (consisting of 11 carbapenem- and ciprofloxacin (CIP)-resistant strains, 13 carbapenem-resistant and CIP-susceptible strains, and 3 carbapenem- and CIP-susceptible strains) recovered from different patients at the University of Tokyo Hospital, from November 2002 through to September 2005. All isolates were identified by the Vitek I system (bioMerieux Japan, Tokyo), the gelatin hydrolysis test, and the acylamidase test. Bacteria were stored at –80°C in trypticase soy broth (TSB, Becton Dickinson, Franklin Lakes, NJ, USA) containing 20% glycerol. Subsequently, bacteria were inoculated on Trypticase Soy Agar (Becton Dickinson) and incubated at 37°C overnight.

Antimicrobial agents

The antimicrobial agents used for susceptibility testing were as follows: imipenem (IPM, Banyu Pharmaceutical, Tokyo, Japan); meropenem (MEM, Dainippon Sumitomo Pharmaceutical, Osaka, Japan); doripenem (DRM, Shionogi Pharmaceutical, Osaka, Japan); tetracycline (TET, Pola Pharma, Tokyo, Japan); chloramphenicol (CHL, Sankyo, Tokyo, Japan); ciprofloxacin and amikacin (CIP, AMK, Meiji Seika Kaisha, Tokyo, Japan); ceftazidime (CAZ, Nippon–Glaxo-SmithKline, Tokyo, Japan); aztreonam (ATM, Eisai, Tokyo, Japan); piperacillin (PIPC, Toyama Chemical, Tokyo, Japan).

Susceptibility testing

Susceptibility testing was carried out using the broth microdilution method as described by the Clinical and Laboratory Standards Institute (CLSI)[17] with Mueller Hinton broth (MHB, Becton Dickinson). Quality control for the minimal inhibitory concentrations (MICs) was performed using the following reference strains: *Staphylococcus aureus* ATCC 21293, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853. Susceptibility break-points were defined according to CLSI recommendations.

Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) was carried out after digestion of the bacterial DNA with 20 U of the restriction endonuclease *Spe*I (New England Biolabs, Ipswich, MA, USA) in 1% pulsed field certified agarose (Bio-Rad, Tokyo, Japan) in 0.5×tris-borate+EDTA (TBE) buffer with a CHEF-DR II apparatus (Bio-Rad) for the analysis of genomic DNA macrorestriction patterns. BioNumerics software (version 4.0, Applied Maths, Kortrijk, Belgium) was used to analyze the DNA restriction patterns and determine their similarity, based on calculation of the Dice similarity coefficient and using the unweighted pair-group method using arithmetic averages (UPGMA) algorithm. Isolates were considered to be a cluster if the similarity value was more than 0.80.

Detection of MBL and integrase genes

Detection of MBL and integrase genes was performed by polymerase chain reaction (PCR) amplification. Genomic DNA of *P. putida* isolates was extracted as previously described.[4] PCR amplification for MBL (bla_{IMP-1}, bla_{IMP-2}, bla_{VIM-1}, and bla_{VIM-2}) and integrase (*intI-1* and *intI-3*) genes was performed with specific primer sets according to a method described previously (Table 1),[7] and was carried out using Premix *Taq* enzyme (Takara Bio, Shiga, Japan) according to the manufacturer's instructions.

Analysis of *oprD* gene expression by RT–PCR

The transcription of the porin gene (*oprD*) was analyzed with reverse transcriptase–PCR (RT–PCR). Mid-logarithmic phase cultures in TSB were pelleted by centrifugation at 13 000 g, and RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Total RNA, 1 μg, was reverse transcribed into single-stranded cDNA using a SuperScript III First-Strand Synthesis Supermix Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. PCR amplification of cDNA was performed using *oprD* gene-specific primer sets designed using the primer3 program available at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. The number of PCR cycles used came within the linearity range for PCR amplification, and constitutive expression of 16S rRNA assessed from the same cDNA preparation was used as a standard. Ten-microliter samples of each PCR product were separated by electrophoresis in 2.0% agarose and visualized by ethidium bromide staining. This experiment was repeated at least twice. The bands of the *oprD* gene were quantitated using image-scanning software (Scion Image, Scion Corporation, MD, USA) and results were standardized with the 16S rRNA band density.

Table 1. Primer used for sequencing and RT–PCR

Study and gene	Primer name	5′-sequence-3′	Reference or GenBank accession no.
PCR of MBL and integrase genes			
bla_{IMP-1}	F1	5′-ACCGCAGCAGAGTCTTTGCC-3′	7
	R1	5′-ACAACCAGTTTTGCCTTACC-3′	
bla_{IMP-2}	F2	5′-GTTTTATGTGTATGCTTCC-3′	7
	R2	5′-AGCCTGTTCCCATGTAC-3′	
bla_{VIM-1}	F3	5′-AGTGGTGAGTATCCGACAG-3′	7
	R3	5′-ATGAAAGTGCGTGGAGAC-3′	
bla_{VIM-2}	F4	5′-ATGTTCAAACTTTTGAGTAAG-3′	7
	R4	5′-CTACTCAACGACTGAGCG-3′	
intI-1	F6	5′-GCATCCTCGGTTTTCTGG-3′	7
	R6	5′-GGTGTGGCGGGCTTCGTG-3′	
intI-3	F8	5′-ATCTGCCAAACCTGACTG-3′	7
	R8	5′-CGAATGCCCCAACAACTC-3′	
PCR and sequencing of QRDRs of *gyrA*, *gyrB*, *parC* and *parE* genes			
gyrA	gyrA-F	5′-CGGTGACGTGATCGGTAAGT-3′	NP_743923.1
	gyrA-R	5′-GAAGTGCGCATGACCAAACT-3′	
gyrB	gyrB-F	5′-TACCTGGTGGAGGGTGACTC-3′	NP_742183.1
	gyrB-R	5′-AACGCAGTTTGTCGATGTTG-3′	
parC	parC-F	5′-CGAACAGGCCTACCTCAACT-3′	NP_747015
	parC-R	5′-ATGATCCGAAGTCGTTCGC-3′	
parE	parE-F	5′-TCAAGGCCAGCAAGAAGGT-3′	NP_747018
	parE-R	5′-CTTCGTCGAGGGCGTAGTAG-3′	
RT-PCR of *oprD* gene expression			
oprD	oprD-F	5′-ACAATCAAGGCCTGGTGTTC-3′	NP_743366.1
	oprD-R	5′-GACGTCACGTTCCCATTCTT-3′	
16S rRNA	16S-F	5′-AAGCAACGCGAAGAACCTTA-3′	AE015451
	16S-R	5′-CGGACTACGATCGGTTTTGT-3′	

PCR and sequencing of QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* genes

Chromosomal DNA was extracted from *P. putida* isolates as previously described.[16] The QRDRs of the *gyrA*, *gyrB*, *parC*, and *parE* genes were amplified using primers designed using the primer3 program (Table 1). Amplification was carried out using Premix *Taq* enzyme (Takara Bio) as recommended by the manufacturer with an annealing temperature of 57°C. PCR products were sequenced using a BigDye Terminator v1.1 Cycle Sequence Kit (Applied Biosystems, Foster City, CA, USA) and an automated DNA sequencing system (ABI PRISM 3130xl, Applied Biosystems). A similarity search for the amino acid sequences deduced against DDBJ/EMBL/GenBank sequence databases was conducted using the BLAST program at the DNA Databank of Japan (Shizuoka, Japan).

Effects of efflux pump inhibitor

Susceptibility testing of IPM and CIP by microbroth dilution was performed in the presence and absence of the efflux pump inhibitor (EPI), L-phenylalanine-arginine-N-naphthylamide (PAβN, Sigma-Aldrich, St. Louis, MI, USA).[18] The EPI was incorporated in MHB at a concentration of 25 mg/l. When comparing MICs in the presence and absence of the EPI, a > 2-fold decrease in MICs for each antibiotic was indicated as a significant change. This experiment was repeated at least twice.

Nucleotide sequence accession number

The partial DNA sequence of the *parC* gene in Pp12 isolates has been assigned to the DDBJ/EMBL/GenBank database under the accession number AB361291.

Results

Susceptibility testing

The results of susceptibility testing are given in Table 2. All of the 24 carbapenem-resistant strains were also resistant or intermediate-resistant to the antibiotics CAZ, ATM, and CHL. Several strains showed resistance to PIPC, AMK, and TET. Three carbapenem- and CIP-susceptible strains were susceptible to the antibiotics IPM, MEM, DRM, CIP, CAZ, AMK, and TET.

PFGE

The genetic backgrounds of 27 *P. putida* strains were analyzed by PFGE. Two carbapenem- and CIP-resistant strains (Pp7 and Pp17) showed gel patterns with a similarity of higher than 0.80 (80%). However, the other strains showed different PFGE patterns with a low similarity (data not shown).

Table 2. Profiles of *Pseudomonas putida* stains in this study

Strain	Type of genes		mRNA expression[a]		Mutations[b]		MICs (mg/L)									
	β-Lactamase	Integrase	*OprD*	Gene	GyrA	ParC	IPM	MEM	DRM	CIP	CAZ	ATM	PIPC	AMK	TET	CHL
Carbapenem and ciprofloxacin-resistant strains (n=11)																
Pp1	bla_{IMP-1}	*intl-1*	2.26		Thr83Ile	–	512	256	>256	64	>64	>64	64	>128	128	256
Pp7	bla_{IMP-1}	*intl-1, intl-3*	0.12		Thr83Ile	–	128	16	128	128	>64	32	64	>128	4	128
Pp8	bla_{IMP-1}	*intl-1, intl-3*	0.11		Thr83Ile	–	128	256	128	128	>64	32	64	>128	8	>512
Pp9	bla_{IMP-1}	*intl-1, intl-3*	0.09		Thr83Ile	–	128	32	256	128	>64	16	64	>128	4	512
Pp12	bla_{IMP-1}	*intl-1*	0.53		Thr83Ile	Ser87Leu	128	64	>256	512	>64	32	32	64	64	512
Pp14	bla_{IMP-1}	*intl-1, intl-3*	0.12		Thr83Ile	–	128	256	256	128	>64	>64	128	>128	4	512
Pp16	bla_{IMP-1}	*intl-1, intl-3*	0.49		Thr83Ile	–	64	64	128	64	>64	32	32	>128	2	512
Pp17	bla_{IMP-1}	*intl-1, intl-3*	0.08		Thr83Ile	–	64	32	128	64	>64	16	16	>128	4	128
Pp18	bla_{IMP-1}	*intl-1*	0.04		Thr83Ile	–	64	128	256	512	>64	>64	>256	16	32	512
Pp22	bla_{IMP-1}	*intl-1, intl-3*	0.10		Thr83Ile	–	32	256	64	128	>64	32	32	>128	8	512
Pp24	bla_{IMP-1}	*intl-1, intl-3*	0.44		Thr83Ile	–	16	128	128	64	>64	32	32	>128	2	512
Carbapenem-resistant and ciprofloxacin-susceptible strains (n=13)																
Pp2	bla_{IMP-1}	*intl-1*	0.23		–	–	512	256	>256	<=0.5	>64	>64	64	32	32	512
Pp3	bla_{IMP-1}	*intl-1*	1.77		–	–	256	64	256	<=0.5	>64	32	32	<=2	2	128
Pp4	bla_{IMP-1}	*intl-1*	0.39		–	–	256	32	>256	<=0.5	>64	>64	16	16	4	128
Pp5	bla_{IMP-1}	*intl-1*	1.26		–	–	256	32	>256	<=0.5	>64	32	32	32	2	256
Pp6	bla_{IMP-1}	*intl-1*	0.07		–	–	128	128	256	<=0.5	>64	>64	32	<=2	16	512
Pp10	bla_{IMP-1}	*intl-1*	0.86		–	–	128	64	256	<=0.5	>64	32	16	64	4	128
Pp11	bla_{IMP-1}	*intl-1*	0.10		–	–	128	32	256	<=0.5	>64	32	32	>128	8	>512
Pp13	bla_{IMP-1}	*intl-1*	0.34		–	–	128	64	>256	<=0.5	>64	>64	>256	>128	64	>512
Pp15	bla_{IMP-1}	*intl-1*	0.36		–	–	128	64	256	<=0.5	>64	16	16	16	8	256
Pp19	bla_{IMP-1}	*intl-1*	0.11		–	–	64	128	256	<=0.5	>64	32	16	>128	8	>512
Pp20	bla_{IMP-1}	*intl-1*	0.44		–	–	64	32	256	<=0.5	>64	>64	32	64	8	256
Pp21	bla_{IMP-1}	*intl-1*	0.32		–	–	64	32	256	<=0.5	>64	32	16	16	8	256
Pp23	bla_{IMP-1}	*intl-1*	0.52		–	–	32	128	64	<=0.5	>64	16	64	4	2	256
Carbapenem and ciprofloxacin-susceptible strains																
Pp1487	ND	ND	0.84		–	–	2	4	2	<=0.5	<=1	16	16	<=2	0.5	8
Pp928	ND	ND	0.90		–	–	1	4	2	<=0.5	<=1	16	8	<=2	0.5	32
Pp1035	ND	ND	1.00		–	–	1	1	<=0.5	<=0.5	<=1	32	<=4	<=2	1	4

IPM, imipenem; MEM, meropenem; DRM, doripenem; CIP, ciprofloxacin; CAZ, ceftazidime; AMK, amikacin; ATM, aztreonam; PIPC, piperacillin; TET, tetracycline; CHL, chloramphenicol; ND, not detected

[a]The bands of the *oprD* gene by RT–PCR were quantitated using image-scanning software, and the results were standardized with the 16S rRNA band density

[b]Amino acid mutations were identified compared with the sequences of *P. putida* KT2440. –, no amino acid mutations

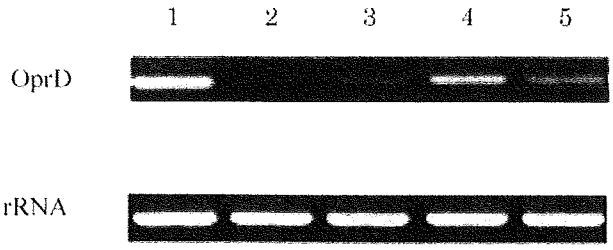

Fig. 1. Expression of *oprD* gene and 16S rRNA in clinical isolates of *P. putida* was analyzed with RT–PCR. Lanes 1 and 2, carbapenem- and ciprofloxacin-resistant strains (Pp1, 7; IMP MIC of 512, 128 mg/l); lanes 3 and 4, carbapenem-resistant and ciprofloxacin-susceptible strains (Pp2, 3; IMP MIC of 512, 256 mg/l); lane 5, carbapenem- and CIP-susceptible strains (Pp1035; IMP MIC of 1 mg/l)

MBL and integron genes

Irrespective of the levels of carbapenem resistance, all of the 24 carbapenem-resistant strains possessed bla_{IMP-1} (Table 2). The MBL genes bla_{IMP-2}, bla_{VIM-1}, and bla_{VIM-2} were not amplified by PCR. All carbapenem-resistant strains possessed *intI-1* integrase genes, and 8 carbapenem- and CIP-resistant strains had both *intI-1* and *intI-3* genes.

Analysis of *oprD* gene expression by RT–PCR

Semiquantitative analysis of *opr*D gene expression was conducted by RT–PCR. Representative electrophoresis of RT–PCR product of the *oprD* gene and 16S rRNA is shown in Fig. 1. The expression of the *oprD* gene in isolates Pp2 and Pp7 (IPM MIC of 512 and 128 mg/l, respectively) was decreased compared with that of the isolate Pp1035 (IPM MIC of 1 mg/l). On the other hand, those in isolates Pp1 and Pp3 (IPM MIC of 512 and 256 mg/l, respectively) was increased compared with Pp1035. The band density of the *oprD* gene indicated that 21 carbapenem-resistant strains were considered to have decreased expression of this gene (Table 2). The Pearson correlation coefficient (r value) was 0.43 for *oprD* expression levels compared with MICs for IPM, for which there was a low correlation.

Mutations in QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* genes

The nucleotide sequences and derived amino acid sequences in the QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* genes for each *P. putida* strain were compared with those of the control strain *P. putida* KT2440[19] (Table 2). In all of the 11 CIP-resistant strains (CIP MICs of > 64 mg/l), Thr83Ile substitutions were detected in GyrA. One strain (Pp12 having CIP MIC of 512 mg/l) had double substitutions of Thr83Ile in GyrA and Ser87Leu in ParC. None of the isolates had substitution in GyrB and ParE.

Effects of efflux pump inhibitor

The change in MIC for IPM and CIP was examined with and without the addition of EPI at a final concentration of 25 mg/l (Table 3). All of the 11 carbapenem- and CIP-resistant strains were affected by the EPI, and in 10 of them, there was a > 2-fold decrease in CIP MICs in the presence of the EPI compared with those in the absence of the EPI. In 3 strains (Pp5, Pp12, and Pp16) of the 24 carbapenem-resistant strains, there was a > 2-fold decrease in IMP MICs with the EPI as compared with those without it.

Table 3. Effects of efflux pump inhibitors in clinical isolates of *Pseudomonas putida*

Strain	MIC (mg/L)[a]			
	Ciprofloxacin		Imipenem	
	Alone	+EPI[b]	Alone	+EPI
Carbapenem and ciprofloxacin-resistant strains (n=11)				
Pp1	64	**16**	512	256
Pp7	128	**32**	128	128
Pp8	128	**32**	128	64
Pp9	128	**32**	128	64
Pp12	512	**128**	128	**8**
Pp14	128	**16**	128	64
Pp16	64	**16**	64	**16**
Pp17	64	**8**	64	32
Pp18	512	**32**	64	128
Pp22	128	64	32	32
Pp24	64	**8**	16	16
Carbapenem-resistant and ciprofloxacin-susceptible strains (n=13)				
Pp2	<=0.5	<=0.5	512	256
Pp3	<=0.5	<=0.5	256	128
Pp4	<=0.5	<=0.5	256	128
Pp5	<=0.5	<=0.5	256	**64**
Pp6	<=0.5	<=0.5	128	512
Pp10	<=0.5	<=0.5	128	256
Pp11	<=0.5	<=0.5	128	64
Pp13	<=0.5	<=0.5	128	128
Pp15	<=0.5	<=0.5	128	128
Pp19	<=0.5	<=0.5	64	64
Pp20	<=0.5	<=0.5	64	32
Pp21	<=0.5	<=0.5	64	32
Pp23	<=0.5	<=0.5	32	32
Carbapenem and ciprofloxacin-susceptible strains				
Pp1487	<=0.5	<=0.5	2	1
Pp928	<=0.5	<=0.5	1	<=0.5
Pp1035	<=0.5	<=0.5	1	<=0.5

[a] >2-fold MIC changes are in bold face
[b] Final concentration of additional EPI was 25 mg/l

Discussion

In this study, all the 24 carbapenem-resistant strains produced IMP-1 type MBL. Moreover, 16 of these strains contained the *intI-1* gene and 8 strains contained both the *intI-1* and *intI-3* integrons. These results suggested that *intI-1* was possibly linked with bla_{IMP-1}, but it remains to be discovered whether *intI-3* was closely linked with bla_{IMP-1}. These findings were in agreement with the report of Shibata et al.[7] The class 1 integron is the most common type detected among Gram-negative organisms in Japan. There were a few reports of Gram-negative bacteria which possess class 3 integrons, such as *Serratia marcescens* (Genbank accession no. AB070224) and *Klebsiella pneumoniae* (Genbank accession no. AY219651). Furthermore, the strains that possess

both class 1 and class 3 integrons have rarely been reported in clinical isolates of *P. putida*,[7] and their functional association is not clear.

The PFGE patterns of Pp7 and Pp17 strains suggested that these *P. putida* strains shared a common lineage. As for the other strains, since there was no great degree of similarity among them, there were unlikely to be a common source of infection.

The OprD protein allows the entry of carbapenems, and its reduced expression is frequently noted in carbapenem-resistant isolates of *P. aeruginosa* and *P. putida*.[5] We measured the expression of the *oprD* gene by semiquantitative RT–PCR. The expression was decreased in 21 carbapenem-resistant strains, which included 18 strains with relative *oprD* expression of 50%, or less than those of Pp1035. Although we did not confirm the role of OprD by inactivating the *oprD* gene via genetic means, these results suggested that the OprD expression might contribute to the carbapenem resistance.

Previous studies have demonstrated that multiple mutations in the QRDRs of DNA gyrase and topoisomerase IV are required for high-level fluoroquinolone resistance in *P. aeruginosa* and *P. putida*.[5,10] In this study, all isolates having CIP MICs of > 32 mg/l had a single substitution (Thr83Ile) in GyrA, and one strain (Pp12 having a CIP MIC of 512 mg/l) had double substitutions in GyrA (Thr83Ile) and ParC (Ser87Leu). As we describe the effects of the EPI later, CIP resistance was not fully affected by the EPI in the strain Pp12 (Table 3). It was thought that these double mutations mainly contributed to this resistance. This was the first time that a substitution of Ser87Leu in ParC has been detected in *P. putida*. In the present study, we did not find a clear relationship between the degree of CIP resistance and the number of substitutions in GyrA and ParC in *P. putida*. However, such a relationship might have been found if a larger number of isolates had been examined.

Several antibiotic efflux systems contributing to multidrug resistance (including resistance to carbapenems and fluoroquinolones) have been characterized in *P. aeruginosa*.[8,12] Although the existence of efflux pumps in *P. putida* has been confirmed,[14,15] their role in clinical isolates of *P. putida* remains unclear. In this regard, we found that IPM MICs decreased > 2-fold more with the EPI than without it in only 3 of the 24 carbapenem-resistant strains. Furthermore, we performed a similar experiment against MEM using PAβN or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, Sigma-Aldrich), but there was not a > 2-fold decrease of MICs with EPI (data not shown). This suggests that while overproduction of the efflux pump may in part contribute to the acquisition of resistance to carbapenems, production of MBL plays a major role in resistance to carbapenems in clinical isolates of *P. putida* in this study. Although a report has demonstrated that increasing efflux pump activity as a mechanism giving IPM resistance may be more important and/or occur frequently in *Acinetobacter baumannii*,[20] we could not clarify the reason why IPM MICs decreased with EPI in this study.

Efflux systems are also believed to be involved in reducing susceptibility to fluoroquinolones. Recently, DNA gyrase and topoisomerase IV mutations and efflux pumps were found to act synergistically in relation to the MICs of fluoroquinolones for *E. coli, Campylobacter* species, and *P. mirabilis*.[19,21,22] We found that all the 11 CIP-resistant strains were affected by it, and in 10 of these the presence of the EPI reduced MIC levels by more than 2-fold as compared with those in the absence of the EPI, although the level of the MICs with the EPI was still above the breakpoint of resistance. In this experiment, we increased the EPI concentration to 30 mg/l to see if the dose of the EPI affected the results, but this had very little effect (data not shown).

In contrast to the strain Pp12 described earlier, the strain Pp18, which had the same CIP MIC (512 mg/l) as the strain Pp12 but a single substitution (Thr83Ile) in GyrA, the CIP MIC was greatly reduced to 32 mg/l in the presence of the EPI. Therefore, it was thought that this resistance was mainly due to the efflux pump systems. These data indicate that mutations in DNA gyrase (and topoisomerase IV) and overproduction of efflux systems synergistically contributed to the acquisition of a high level of CIP resistance in our clinical isolates of *P. putida*.

Overall, our results showed that the production of IMP-1 MBL was largely responsible for developing a high level of resistance to carbapenems. In the case of CIP, our results indicate that mutation in DNA gyrase (and topoisomerase IV) and overproduction of the efflux pump synergistically contributed to a high level of resistance in the clinical isolates of *P. putida*.

Acknowledgment We thank Sankichi Horiuchi for valuable comments on this study. We did not receive any financial support from third parties.

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Hepatology Research 2009; 39: 657–663 doi: 10.1111/j.1872-034X.2009.00517.x

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 ± 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).[1,2] Opportunistic infection has been prevented 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.[3,4] 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.[5] 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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Received 20 January 2009; revised 9 February 2009; accepted 10 February 2009.

Interferon (IFN) treatment in combination with ribavirin administration, which is now the first choice for HCV mono-infected patients,[6] 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.[7] 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.[8] 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,[8] 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 ± 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 Meq/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 ($\times 10^4/\mu l$) in the IFN-treated patients and −0.94 ± 0.95 ($\times 10^4/\mu l$) in the non-IFN-treated patients. The change in platelet