Table 1　Factors associated with sustained virological response to 48-week peginterferonribavirin combination therapy in patients infected with HCV genotype 1b, identified by multivariate analysis (n=114) [11]

Factor	Category	Risk ratio (95% confidence interval)	*P*
Amino acid substitution in core region	1: double wild	1	
	2: non-double wild	0.102 (0.022-0.474)	0.004
LDL cholesterol (mg/dL)	1: < 86	1	
	2: ≥ 86	12.87 (2.177-76.09)	0.005
Gender	1: male	1	
	2: female	0.091 (0.017-0.486)	0.005
ICG R15 (%)	1: < 10	1	
	2: ≥ 10	0.107 (0.017-0.678)	0.018
γ-GTP	1: < 109	1	
	2: ≥ 109	0.096 (0.0011-0.819)	0.032
Ribavirin dose (mg/kg)	1: < 11.0	1	
	2: ≥ 11.0	5.173 (1.152-23.22)	0.032

問に対して，今回のアンサーパッドの集計では 74% の賛同が得られた．

Consensus Statement 2:

肝発癌や抗ウイルス療法の治療効果と関連する宿主側因子として肝組織の線維化の程度（staging）が重要であるが，stagingの評価には肝生検が推奨される．(Level 1, Grade C)

2. ウイルス変異と病態

C 型肝炎の診断には HCV RNA の測定とともに，ウイルス量，型（genotype）の測定が重要である．さらに HCV RNA 遺伝子の変異について新たな知見が得られている．これらの因子は C 型肝炎に対する IFN 療法（RBV の併用療法を含む）の治療効果の予測に非常に重要である．ウイルス量の測定法は，2000 年以降アンプリコア HCV モニター法が用いられてきたが，2007 年末から高感度かつ広範囲の測定レンジをもつ real-time PCR 法を用いた測定が可能となっている．このようなウイルス量とウイルスの型（genotype または serotype）の測定は IFN 治療の効果予測や治療中の抗ウイルス効果をみるなど臨床的な有用性が高い[8]．

ウイルスの遺伝子変異は，主として genotype 1b 型のウイルスで多く検討されている．IFN 単独投与における NS5A aa2209-2248 (interferon sensitivity determining region；ISDR) 領域のアミノ酸変異数が治療効果に関係することが明らかになった．HCV-J のアミノ酸配列と比較して ISDR のアミノ酸変異数が多い場合，IFN 単独療法での SVR 率が高いことが報告されている[9]．さらに現在治療の主体である，PEG-IFN と RBV 併用療法（48 週間）においても ISDR の変異数は効果予測に重要である[10]．

Table 2　Effect of the IFN treatment on the annual incidence of hepatocellular carcinoma in each fibrosis staging

	Control	IFN-treated All	SVR	non-SVR
Patient's number	490	2400	789	1658
Staging				
F1	0.45%	0.08%	0.11%	0.07%
F2	1.99%	0.54%	0.10%	0.78%
F3	5.34%	1.95%	1.29%	2.20%
F4	7.88%	4.16%	0.49%	5.32%

Data were adopted from IHIT study[16]

Consensus Statement 3:

ISDR の変異は，IFN 単独または RBV との併用療法における SVR に関係するので，治療前に測定すべきである．(Level 2a, Grade B)

さらに，HCV Core 領域のアミノ酸置換の有無（70 番目と 91 番目の変異）が PEG-IFN と RBV 併用療法の

治療効果に関係することが報告された（Table 1）[11]．米国の報告でもCore領域の70番目のアミノ酸置換が抗ウイルス作用に関係することが示された[12]．

Consensus Statement 4:

Core領域の70番目，91番目のアミノ酸置換は，IFN・RBV併用療法におけるSVR，NVRに関係するため，治療前に測定すべきである．（Level 2a，Grade B）

またNS5A領域のaa2334-2379（IFN/ribavirin resistance determining region，IRRDR）のアミノ酸変異数がPEG-IFN・RBV併用療法の治療効果に関係するという報告もある[13]．さらに新規治療薬であるプロテアーゼ阻害剤では，NS3領域の遺伝子変異が耐性に関係すると報告されている．一方，発癌との関係では，Core領域のアミノ酸置換の有無やNS3蛋白の二次構造が関係するという報告もなされているが，これらの点に関しては，さらなる検討が必要である．

3．自然経過とIFN治療適応（高齢者，PNALTを含む）

C型急性肝炎の60～80％が慢性化するとされているが，輸血後肝炎以外では感染時期が特定できないことが多く，また，無症状で緩徐な経過をたどることが多いためC型慢性肝炎の自然史には不明な点が多い．比較的若くHCVに感染した者を追跡した欧米の報告では，HCV感染が感染者全体の生命予後に与える影響は少なく，20年近く経過した症例でも多くは肝線維化の進展も軽度にとどまるとしている[14]．この成績は，輸血後肝炎患者においては平均20年～30年の経過で肝硬変へ進展し，平均30年～40年の経過で肝癌を併発するというわが国の報告とは進展速度が大きく乖離する[15]．一方，C型慢性肝炎の肝線維化の進展度と肝癌の発生との間の密接な関連性は多くの論文で示されており，わが国における肝硬変の年率発癌率は5～8％に至る（Table 2）[16]．このため，以下のコンセンサスが得られた．

Consensus Statement 5:

わが国の肝硬変患者の年率発癌率は欧米より高く，5～8％であることを考慮して治療適応を選択すべきである．（Level 2b/3，Grade B）

C型慢性肝炎患者の線維化の進展速度は症例によりまちまちであるが，Poynard Tら[17]は無治療のC型慢性肝炎平均の年率肝線維化進展率が0.133（stage）であると報告し，Shiratori Yら[18]も同様に0.10（stage）であるとしている．ALT持続正常のC型慢性肝炎患者では線維化の進展はさらに緩徐で，5年後の肝組織の線維化に著変なかったとする報告や，年率肝線維化進展率が平均0.05（stage）であったとする報告がある[19]．最近では，アルコール多飲以外にも，肝組織への鉄の過剰沈着，肝脂肪化，インスリン抵抗性がC型慢性肝炎の肝線維化を促進する因子であり，生活習慣の改善が重要であるとされている．

以前より血清ALT値の高い肝硬変では発癌率が高かったが，ALT値が40 IU/*l*以下のC型慢性肝炎でも血清ALT値と発癌率が関連することが示された．実際の臨床の場では，C型慢性肝炎患者の血清ALT値は30 IU/*l*以下に治療の目標値を設定すべきである．

Consensus Statement 6:

肝発癌予防のためにはALT値を30 IU以下に保つべきである．（Level 2a，Grade A）

また，わが国でC型慢性肝炎患者に対するIFN治療が始まって20年以上が経過し多くの患者が著効を得ているが，著効後にも肝癌が発症することが知られ，治療前の肝組織の線維化進展例，高齢者，男性に肝癌併発のリスクが高いことが報告されている．Burno Sら[20]は著効を示した肝硬変症例の年率発癌率は非著効例の3分の1ではあるが，依然，0.66％であることを示した．

Consensus Statement 7:

C型慢性肝炎や肝硬変患者では定期的な肝癌のスクリーニング検査を行うべきである．IFN治療で著効が得られても，特に肝線維化進展例，高齢，男性患者では肝発癌のリスクが高く，定期的な画像診断・腫瘍マーカーによる検査が引き続き必要である．（Level 2b，Grade A）

C型慢性肝炎に対する抗ウイルス療法ではPeg-IFN・RBV併用療法が第一選択の治療法であるが，両薬剤には多くの副作用がある．特に高齢者ではグレード3以上の副作用の発生率が高く，両薬剤の減量を余儀なくされることも少なくない．しかし，IFN治療の年齢制限については，上限なし35％，75歳まで64％という意見であり，わが国では高齢者にも積極的にIFN治療を導入していることが明らかとなった．AASLDのガイドラインでは，治療適応は病態の重症度，副作用のリスク，完治の可能性，生命予後への影響，患者の治療への意欲などを総合的に捉え，個別化して判断すべきであるとしている[1]．さらに，Zeuzem Sら[21]が遺伝子型1型のALT持続正常のC型慢性肝炎患者に対するPeg-IFN・RBV併用療法の著効率が40％であることを報告

して以来，わが国でも ALT 正常の C 型慢性肝炎患者に対する治療が広く行われるようになり，「肝癌抑制を目指した ALT 正常の C 型慢性肝炎患者に対する治療ガイドライン」が示されている（厚生労働省肝炎等克服緊急対策研究事業熊田班）．同班の共同研究において，ALT 正常例であっても血小板数が 15 万以下では，組織学的に繊維化が進展している症例が多いことが明らかにされた（Fig. 2）[22]．今回のパネルディスカッションにおいても，IFN の投与対象として下記のコンセンサスが得られた．

Consensus Statement 8:

IFN 治療は肝組織の Grade/Stage をふまえ，心身の状態，完全著効や生命予後改善の可能性，重篤な副作用を惹起する可能性を個別に評価して考慮する．特に高齢者においても，肝疾患が生命予後を規定する場合には，安全性に十分配慮し IFN 治療を考慮すべきである．（Level 6/3，Grade A）

Consensus Statement 9:

HCV RNA 陽性で治療禁忌に該当しない成人は，原則として IFN 治療適応がある．肝発癌抑制を目指した場合，ALT 30 IU/l 以下かつ血小板数 15 万/μl 未満であれば IFN 治療の適応であり，経過観察中に ALT 31 IU/l 以上となった症例も治療を考慮すべきである．ALT 30 IU/l 以下かつ血小板数 15 万/μl 以上では原則経過観察であるが，治療の希望が強い場合，年齢，ウイルス量と遺伝子型，肝線維化の進展度，合併症の有無，副作用の素因を総合的に評価し，治癒の可能性と治療のリスクをふまえて治療を考慮すべきである．（Level 3, Grade B）

2）治　療

1. PEG-IFN・RBV 併用療法

わが国では，PEG-IFN・RBV 併用療法に対して 2 つの全国臨床試験が行われた[23,24]．その結果，本療法は低ウイルス量の初回治療症例を除く C 型慢性肝炎の標準治療となっている．これらの試験では，genotype 1b かつ高ウイルス量症例におけるウイルス学的著効（SVR）を期待しがたい因子として，高齢者，女性，線維化進行例，前インターフェロン治療無効例，投与期間 80% 以下が，挙げられている．これらの解析結果で欧米と特に異なる点は，女性が男性より SVR 率が低いことである．しかし，わが国における市販後の複数の臨床研究ではこれを支持するデータが多く認められ[25,26]，次の

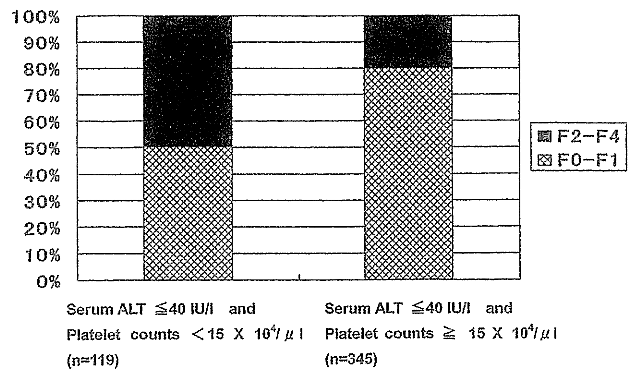

Fig. 2 The relation between platelet counts and the histological findings in the patients with normal ALT.
Forty hundred and sixty four HCV carriers with normal serum ALT ($\leqq$ 40 IU/l) were classified according to the platelet counts. Around 20% of patients with ALT $\leqq$ 40 IU/l and platelet counts $\geqq$ 15 $\times$ 10^4/μl (n=345) were in stage F2-4, whereas approximately 50% of patients with ALT $\leqq$ 40 IU/l and platelet count $<$ 15 $\times$ 10^4/μl (n=119) were in stage F2-4[22].

consensus statement が採用された．

Consensus Statement 10:

PEG-IFN・RBV 併用療法において，ウイルス学的非著効に至りやすい症例の特徴は，60 歳以上の高齢者，とくに高齢女性，線維化進行例，過去の IFN 単独治療無効例，投与期間 80% 以下の症例，などが挙げられる．（Level 2a，Grade B）

治療中の HCV RNA の消失時期と SVR との間には密接な関連がある．全国臨床試験の成績では[23,24]，アンプリコア法で測定した HCV RNA が投与開始後 4 週で消失した症例の SVR 率は 100%～76%，5 週以降 12 週までに消失した症例でも 73%～71% と高率であった．しかし，13 週以降 24 週までに消失した症例では 36%～29% で 48 週治療では再燃が増え，24 週以降に陰性化した症例からは 1 例も SVR が得られなかった．従って，治療中の HCV RNA 陰性化時期および減少率は治療効果の予測に有用であり，HCV RNA 測定時期は，4 週，12 週，24 週が推奨される．

HCV RNA（アンプリコア法）が 12 週で 2 log 以上の低下または 24 週で陰性化が得られなければ，SVR は得られない．従って，欧米の practice guideline では，このような症例に対して治療中止が推奨されている．しかし，わが国における 52 例の後ろ向き検討では，再燃

例，無効例における治療終了後6カ月のALT正常化率はそれぞれ56%（5/9），62%（8/13）で，1例を除いて全例で治療終了2年後までの長期のbiochemical responseが得られた[27]．従って，欧米と異なりわが国においては以下のコンセンサスが得られた．

Consensus Statement 11:

Genotype 1型において，HCV RNAが12週で2 log以上の低下または24週で陰性化が得られなければ，48週間の標準治療ではウイルス学的著効は得られない（Level 1，Grade A）．しかし，24週時HCV RNAが陰性化しなくても長期のbiochemical responseが得られることがあり，ALTが正常化していれば治療を継続する意義がある．（Level 4，Grade C）

Genotype 1に対する72週投与の有用性については，これまで5つランダム化比較試験が報告されている[28)~32)]．いずれもIFNの治療法や無作為化する対象症例が異なるため，これらの結果を画一的に評価することはできない．しかし，サブ解析をするとHCV RNAが13～24週に陰性化する，いわゆるlate virological responderでは72週投与の有用性が示されている．

わが国においても，genotype 1b高ウイルス量113例において，48週投与群とHCV RNAが陰性化してから44週間延長投与する群で無作為比較試験が行われており，SVR率は通常投与群で36%，延長投与で53%であり，特にHCV RNAが16～24週に陰性化した症例で延長投与のSVR率が有意に高かった（9%　vs. 78%，p=0.005）[33]．また，Akutaらは，年齢，性別，HCV RNA陰性化時期を合わせたcase-control studyを行い，48週投与（n=130）のウイルス学的著効率が33%であったのに対し，72週投与（n=65）では62%と高率であり，特に70番91番コア変異例とISDR野生例で72週投与の有用性があったと報告している[34]．従って，Genotype 1型において，HCV RNA（アンプリコア法）が12以降24週までに陰性化する症例では72週延長投与を推奨する．

最近では，HCV RNAの陰性化の判定には，従来法より感度の高いリアルタイムPCR法を用いている．リアルタイムPCRを用いた72週投与の有用性については十分なエビデンスはないが，36週までにHCV RNAが陰性化した症例からでも72週投与でSVRが得られている．従って，次のconsensus statementが採用された．

Consensus Statement 12:

リアルタイムPCR法を用いた場合，36週までに陰性化すれば72週投与でウイルス学的著効率の向上が得られる．（Level 2b，Grade C）

一方，Genotype 2/3型における短期投与の有用性については，これまで6つのランダム化比較試験が報告されている[35)~40)]．しかし，その有用性については一致した見解が得られていない．最近Mangiaらは，12週短期投与における再燃に関与する因子を解析し，年齢45歳以上，血小板数14万/μL未満，BMI 30 kg/m^{2}以上が関連すると報告した[41]．すなわち，これらの再燃因子を有する症例では短期投与は行うべきではなく，高齢者や線維化進展例の多いわが国では，一般的に短期投与を推奨されない．

欧米では，個々の治療効果の規定因子はHCV陰性化時期などの治療に対するウイルスの反応性に帰納するとの考え方が支配的である．従って，欧米のpractice guidelineでは治療中のウイルスの反応性のみによる画一的な推奨が行われている．しかしわが国では，再燃因子の有無によりたとえ同様の治療中の反応性が得られたとしても最終治療効果は異なることが示唆されている．例えば，Akutaらはviral kineticsに関与する因子を検討し，同じEVRが得られても女性と高度線維化例ではSVR率が低いことを報告している[42]．従って，PEG-IFN・RBV併用療法では，治療中のウイルス反応性を考慮して治療期間を設定すべきであるが，再燃リスクを有する症例の多いわが国では，治療期間の最適化には年齢や性別などの再燃因子を考慮すべきである．

Helibling は代償性肝硬変124例をRBV 1000/1200 mg（標準投与量）群と600/800 mg（低用量）群に無作為に割付しPEG-IFNと併用療法を行い，有用性を検討した[43]．その結果SVR率は標準投与量群で52%，低用量群で38%と前者で良好であった．重篤な副作用はそれぞれ14%，18%で，薬剤減量を必要とした症例は78%，57%であった．SVRに寄与する因子はgenotype 2/3と血小板150×10^9/L以上であった．従って，わが国においても代償性肝硬変に対して，PEG-IFN/RBV併用療法は可能であるが，副作用出現に対して注意が必要である．（Level 1，Grade A）

過去の通常型のIFN（RBV併用例を含む）に対して無効または再燃した症例に対する，PEG-IFN・RBV併用療法の有用性を検討したランダム化比較試験はこれまで7つある[44)~50)]．これらのSVR率は6%から45%

と様々であるが，とくに過去の治療における再燃例では，再治療効果が高い．一方，AASLD の practice guideline[1]では，過去に十分な PEG-IFN・RBV 併用療法が行われた再燃・無効例に対しては，PEG-IFN・RBV 併用療法の再投与は推奨されていない．しかし最近，過去の PEG-IFN・RBV 併用療法の再燃・無効例であっても，PEG-IFN・RBV の再投与により 12 週で HCV RNA の陰性化が達成されれば，48〜72 週投与により 48%〜68% の SVR 率が得られたとの報告がある[45)〜52)]．再治療により 12 週陰性化が得られる症例を治療前に同定することは現時点では困難であることや，プロテアーゼ阻害薬の開発状況を考慮すると依然議論の余地はあるが，以下の consensus statement が採用された．

Consensus Statement 13:

過去の IFN 療法の再燃・無効例であっても，PEG-IFN・RBV 併用療法の再治療で，HCV RNA の 12 週陰性化が達成されれば，ウイルス学的著効が期待できる．(Level 2b, Grade B)

2. 従来型 IFN あるいは PEG-IFN 単独療法の位置づけ

我が国では，1992 年に C 型慢性肝炎に対する IFN 単独治療が開始され，その臨床的効果が多数報告されている．しかし現在では，1b・高ウイルス量例に対する標準治療は PEG-IFN・RBV 併用療法となり，HCV 排除を目的とする単独療法の適応は，一部の患者に限定されている．IFN あるいは PEG-IFN 単独治療の対象として，1. 低ウイルス症例，2. リバビリン併用困難例(慢性腎不全など)，3. 急性肝炎例，4. 肝癌根治例，5. 維持療法（線維化進展例や抗ウイルス療法抵抗例）が挙げられている[53)]．

アンサーパッドにおいて，「初回治療例において，低ウイルス量の患者にふさわしい治療は？」と質問したところ，PEG-IFN または IFN 単独療法は 60% に支持されたが，最初から IFN・RBV の併用療法を行うとする意見が 37% に見られた．欧米のガイドラインでは PEG-IFN・RBV 併用療法のみを推奨しているが，我が国では初回の低ウイルス量例には PEG-IFN あるいは IFN 単独療法も一定の評価を受けていることが示された．

急性 HCV 感染は，70% 程度が慢性感染に移行するため，治療介入が必要である．急性 C 型肝炎患者で持続感染への移行が疑われる症例では，発症後 12 週から 24 週以内に IFN6MIU 週 3 回あるいは PEG-IFN 週 1 回 12〜24 週間の単独治療が推奨される．しかし，リバビリンの併用が治療効果を向上した成績がなく，単独療法で十分な有効性が期待できる[54)]．

わが国では IFN の発がん抑制効果について多くの臨床的検討が行われてきた[55)〜57)]．肝硬変例を対象とするメタアナリシス解析では IFN 投与により発癌率が低下することが確認されている．また線維化進展例（F3-F4）においても IFN 投与にて HCV が排除されると発癌率が低下し予後改善効果があることも報告されている[16)]．我が国の成績では，60 歳以上の患者への少量長期 IFN 単独治療は，ALT および AFP の低下を誘導し非治療群と比較して肝発癌を抑制することが示されている[56)]．さらに，我が国を含めて複数の施設から肝癌根治療例に対する IFN の発癌抑制効果が報告されている[57)]．特に，IFN 投与群では二次再発，三次再発が低下することも注目すべき効果である[58)]．一方，欧米で実施された前向きランダム化試験（HALT-C）では PEG-IFN・RBV 無反応例に対する PEG-IFN 少量長期投与が肝疾患の進展を阻止しなかったことが示された[59)]．すなわち IFN 維持療法に関する欧米と我が国の臨床成績が相反する結果が示された．この理由として，我が国の治療対象が高齢であり，かつ肝発癌率が高いことが治療介入による効果の差になっていることが推測され，今後明らかにすべき課題である．

今回，SVR が期待できない場合でも，「IFN 長期投与は ALT 値の低下が見られれば，肝発癌抑制や生命予後の改善効果を期待できるか？」という質問に対し，89% の同意が得られた．

Consensus Statement 14:

肝癌根治例では生命予後延長効果を期待した IFN 投与を推奨する．(Level 1, Grade A)

Consensus Statement 15:

SVR が期待できない場合でも，IFN 長期投与は ALT 値の低下が見られれば，肝発癌抑制や生命予後の改善効果が期待できる．(Level 2a, Grade B)

3. 治療方針のコンセンサス

C 型肝炎に対する治療方針として，C 型肝炎の治療目標，抗ウイルス療法の治療適応，Peg-IFN・RBV 併用療法(薬剤投与量との関係)，ウイルス排除不能例・肝硬変例の治療，新規抗ウイルス剤の各項目について，下記のようにまとめた．

治療目標

治療目標の第一は，HCV RNA 排除による肝炎治癒であり，IFN 治療によってウイルス排除が得られた場合

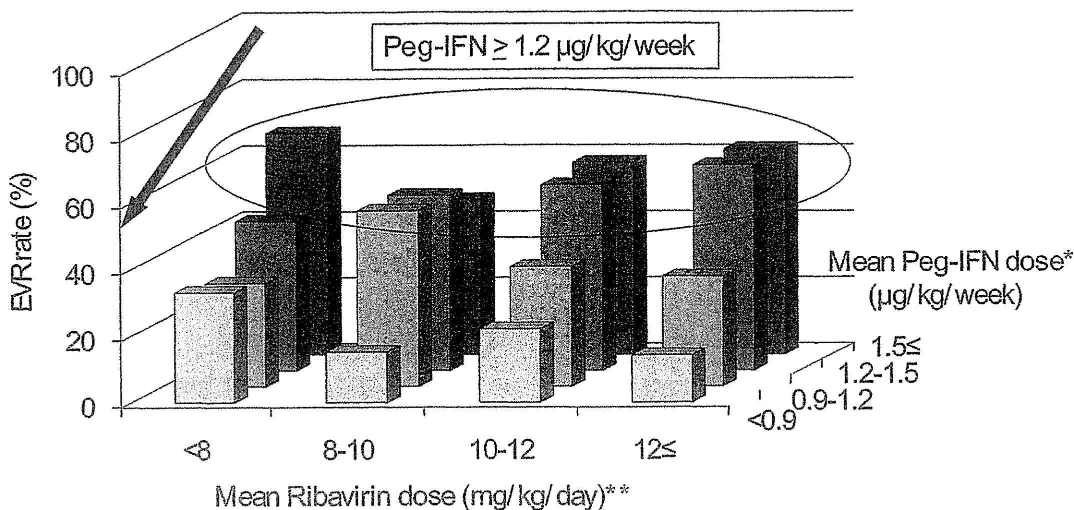

Fig. 3 C-EVR rate according to PEG-IFN alfa-2b and ribavirin doses during 12 weeks after start of therapy. *, $p<0.0001$; Peg-IFN **, $p=0.34$; Ribavirin (Mantel-Haentzel chi-square test). The c-EVR rates were 54% and 56% for patients who received more than 1.5 μg/kg/week and 1.2-1.5 μg/kg/week of Peg-IFN alfa-2b and declined to an average rate of 38% in patients given 0.9-1.2 μg/kg/week of Peg-IFN alfa-2b, to an average rate of 22% in patients given less than 0.9 μg/kg/week of Peg-IFN alfa-2b.[61]

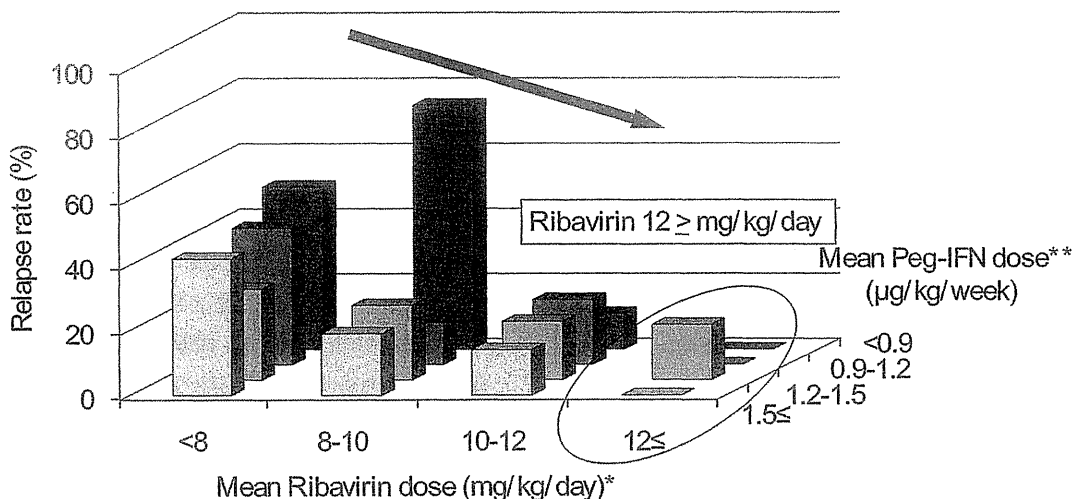

Fig. 4 Relapse rate according to Peg-IFN alfa-2b and ribavirin doses during treatment of patients who completed treatment. *, $p=0.0001$; Ribavirin **, $p=0.15$; Peg-IFN (Mantel-Haentzel chi-square test). The relapse rate was 60% in patients receiving less than 6 mg/kg/day of ribavirin, and declined to 41% at 6-8 mg/kg/day, 27% at 8-10 mg/kg/day, 22% at 10-12 mg/kg/day and 11% in patients given ≥ 12 mg/kg/day.[62]

には，肝発癌抑止効果や生命予後改善が得られる．現時点で最も治療効果の高い抗ウイルス療法はPeg-IFN・RBV併用療法である．第二の目標として，ウイルス排除ができない場合には肝病変進展予防あるいは肝発癌予防を目指すことが重要である．

治療適応

治療適応については，C型肝疾患が患者の予後を規定し，薬剤の副作用への対処が可能であると考えられる症例を抗ウイルス療法の対象とする．このうち，良好な治療効果が予測される症例が，"良い適応"症例である．さらに，SVRの可能性が低い高齢者や線維化進展例でも，合併疾患がなければ抗ウイルス療法を提示す

べきであるということに88％の同意が得られた．

Consensus Statement 16:

C 型肝疾患が患者の予後を規定し，薬剤の副作用への対処が可能であると考えられる症例は抗ウイルス療法の対象とする．（Level 6，Grade B/C）

たとえ，SVR の可能性が低い症例でも合併疾患がなければ抗ウイルス療法を提示すべきである．

Peg-IFN・RBV 併用療法（薬剤投与量との関係）

Genotype1 型における EVR（治療開始 12 週の HCV RNA 陰性化）の達成には，Peg-IFN 投与量が用量依存性に関与する（Fig. 3）．Peg-IFNα2a 投与量 80％ 以上[60] あるいは Peg-IFNα2b 平均投与量 1.2 μg/kg/週以上[61]を目標とし，極力，減量投与開始は避ける（Level 2b/3, Grade B）．また，Genotype1 型のウイルス陰性化例における治療後再燃には，RBV が用量依存性に関与し，予定投与量の 80％ 以上あるいは平均投与量 10 mg/kg/日（可能であれば 12 mg/kg/日）以上を目標とする[62]（Level 2b/3，Grade B）（Fig. 4）．一方，Genotype2/3 型における減量（PegIFNα2a 135 μg/週　あるいは PegIFNα2b 1.0 μg/kg/週，RBV 400 mg/day）については，治療効果に有意な影響を及ぼさないものと考えられる（Level 2a，Grade B）[63)～65]．

ウイルス排除不能例・肝硬変例の治療

IFN 療法を積極的に行うことが治療の第一選択であるが，IFN 非適応例や無効例に対しては肝庇護療法や瀉血療法の重要性は認識されており，77％ の同意が得られた．

Consensus Statement 17:

IFN 非適応例や IFN で ALT 値や AFP の改善が得られない症例には，肝庇護剤による治療を行い，効果不十分な場合は，瀉血療法を併用する．（Level 3/6，Grade B/C）

次に，代償性肝硬変では，IFN を主体とした治療で HCV RNA 排除を目指し，非代償性肝硬変では，肝予備能の改善や発癌予防を目標とした治療を行う．代償性肝硬変で発癌予防を目指す場合には，AST・ALT 値，AFP 値の改善を目標とし，IFN のみでなく肝庇護剤，瀉血療法，分岐鎖アミノ酸製剤を単独あるいは組み合わせて治療することが望ましい．AASLD guideline[1] では，“C 型慢性肝疾患に対して抗ウイルス療法が奏功しない場合，肝移植を考慮する”という方針のみにとどまるのに対し，肝不全への進展，発癌予防に向けたわが国独特の肝庇護療法に対して，91％ の同意が得られた．

Consensus Statement 18:

代償性肝硬変で発癌予防を目指す場合には，AST・ALT 値，AFP 値の改善を目標とし，IFN のみでなく肝庇護剤，瀉血療法，分岐鎖アミノ酸製剤を単独あるいは組み合わせて治療する．

新規抗ウイルス剤

新規抗ウイルス剤として，プロテアーゼ阻害剤，ポリメラーゼ阻害剤などが有効であり，PEG-IFN/RBV との併用で著効率が向上する（Level 1b，B）．特に，プロテアーゼ阻害剤である Telaprevir（VX950）に Peg-IFN・RBV を加えた 3 者併用療法では，新規症例で 6～7 割[66]，PEG-IFN・RBV 併用療法の再燃例で約 7 割，無効例でも約 4 割に SVR を認めている．

今後の治療方針として，より早期のウイルス排除が期待される線維化進展例や高齢者では PEG-IFN・RBV 併用療法を行うが，それ以外の症例では新規治療を考慮に入れた治療選択が必要となる．

おわりに

わが国の C 型肝炎は，欧米に比し高齢で組織進展例が多い．このため，年率発がん率が高く，IFN 治療に対しても有効率が低く副作用の発現が多い．このような患者背景に即したわが国独自のエビデンスの確立が求められるが，現状では信頼度の高い情報が集積されていないものも多い．今回，アンサーパッドを用いて聴取したわが国の肝臓専門医の意見を基に，これらの多くをコンセンサスステートメントとしてまとめた．肝臓専門医の共感が得られた提言ではあるが，国際的に承認されるためには全国的な多施設研究による今後の検証が必要である．その一方で，わが国の患者の現状は欧米の患者の未来像を示している可能性が高いことから，日本の実情に即したガイドラインを海外にむけて発信し批判の俎上に載せることは，日本肝臓学会が果たすべき重要な役割と考える．このため，本論文に Informative statement や Recommendation を追記して Hepatology Res. にも掲載する．

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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

Kanzo 2009; 50: 665—677

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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.

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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

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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

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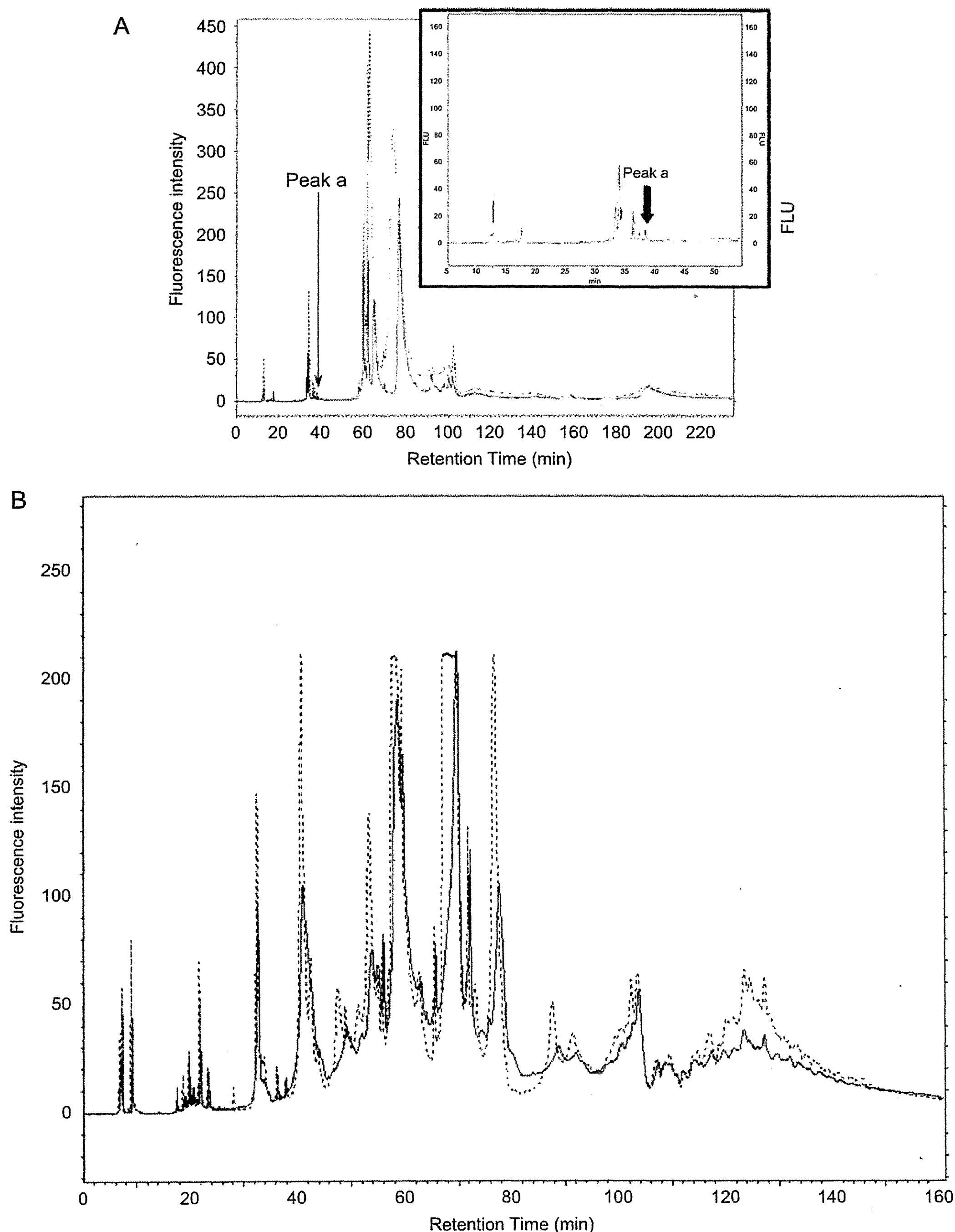

Figure 1. Chromatograms of mouse plasma samples treated with IgY-R7 Spin Columns. (A) The mouse plasma (19 months, C57BL/6N) treated 80 times (solid line) and 86 times (dotted line) in the same spin column. The gradient conditions were as follows: time (min), 0 → 10 → 20 → 44 → 48 → 68 → 80 → 90 → 108 → 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 cysteine 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

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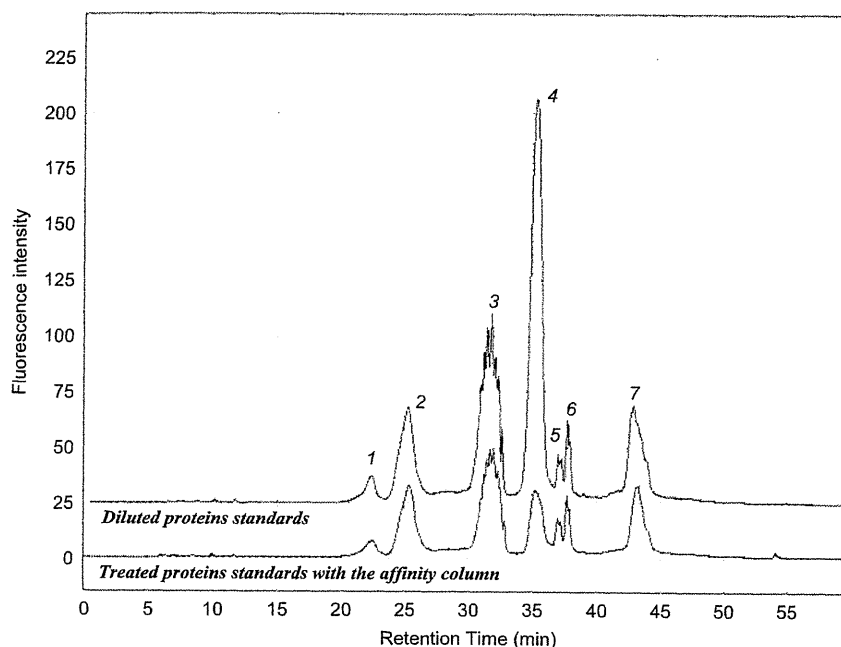

Figure 2. Chromatograms of the protein standards which were treated with Seppro®-IgY12 column and diluted to the same protein amount (4.8 µg/ HPLC injection) as the amount for the column treatment. The peak numbers correspond to Table 2.

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

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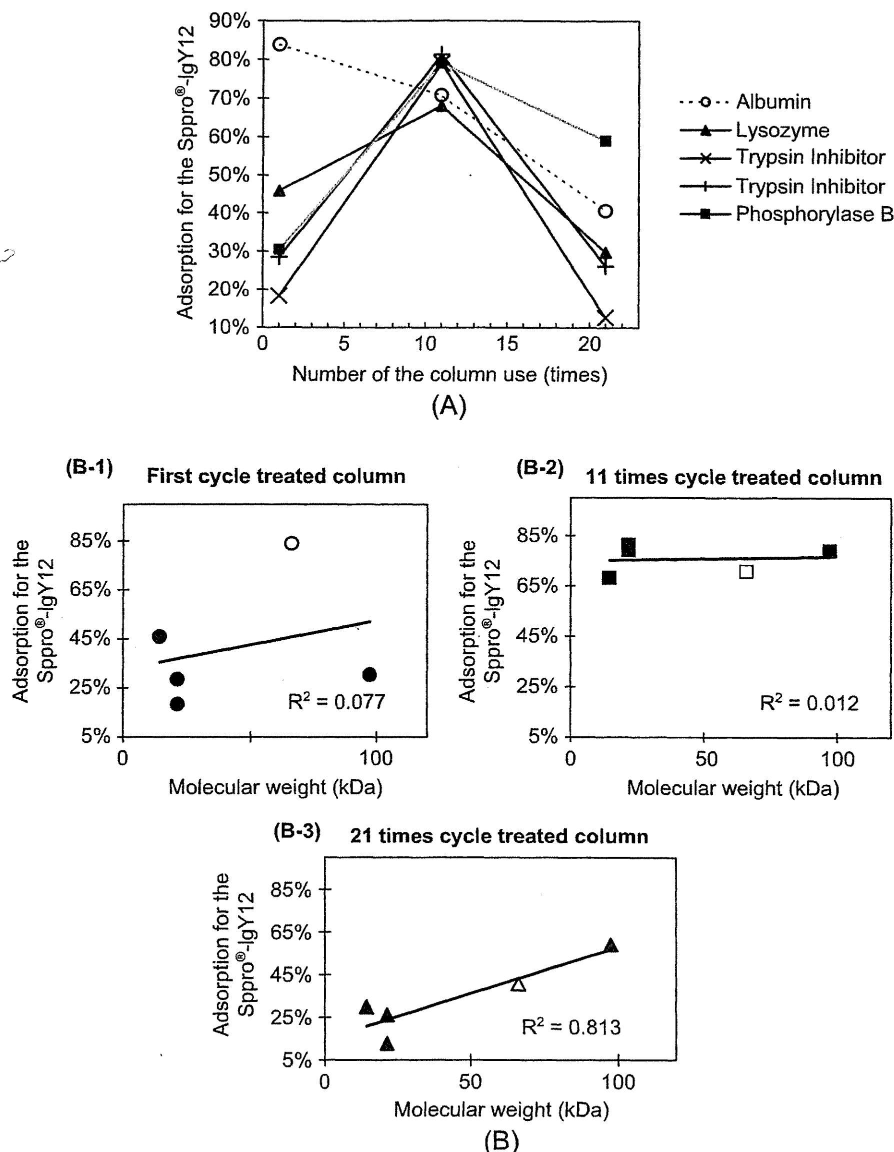

Figure 3. Changes of the adsorption ratio of protein standards for Seppro®-IgY12 column. (A) Relation of the adsorption ratio to the number of times the column was used. (B) Correlation of the adsorption ratio for the column with molecular weight of each protein standard using the column treater one (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

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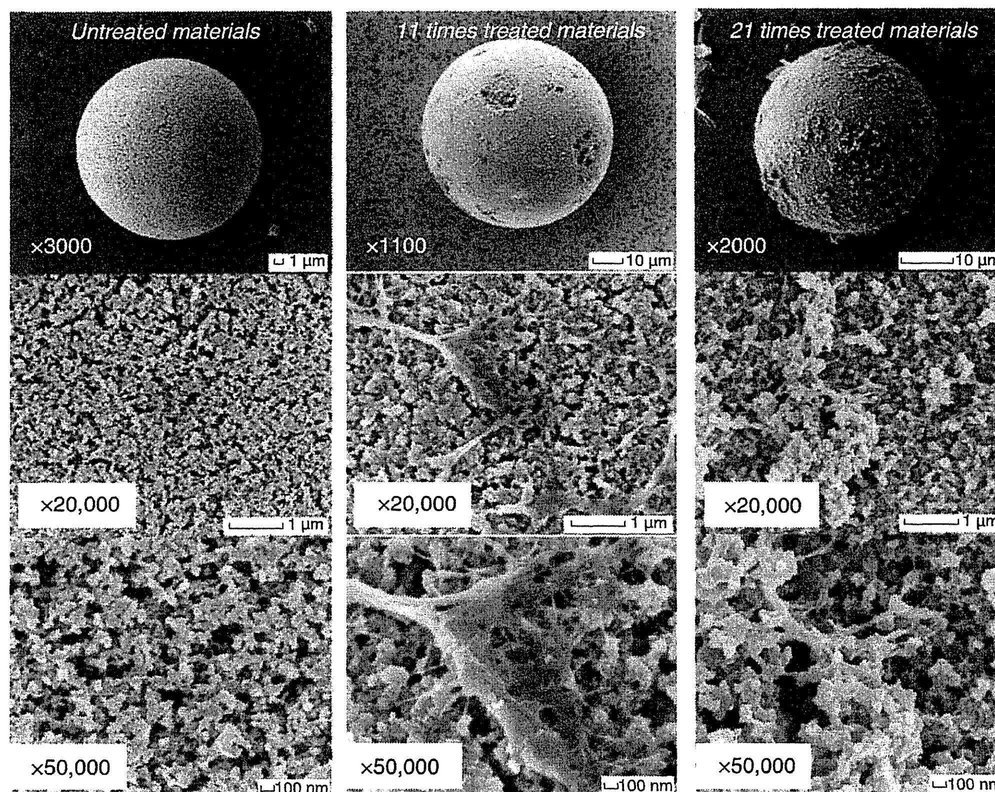

Figure 4. SEM images of the untreated and 11- and 21-times-treated column material surfaces. Magnification in SEM was controlled in a range of × 1100–3000 to show the whole picture of the material.

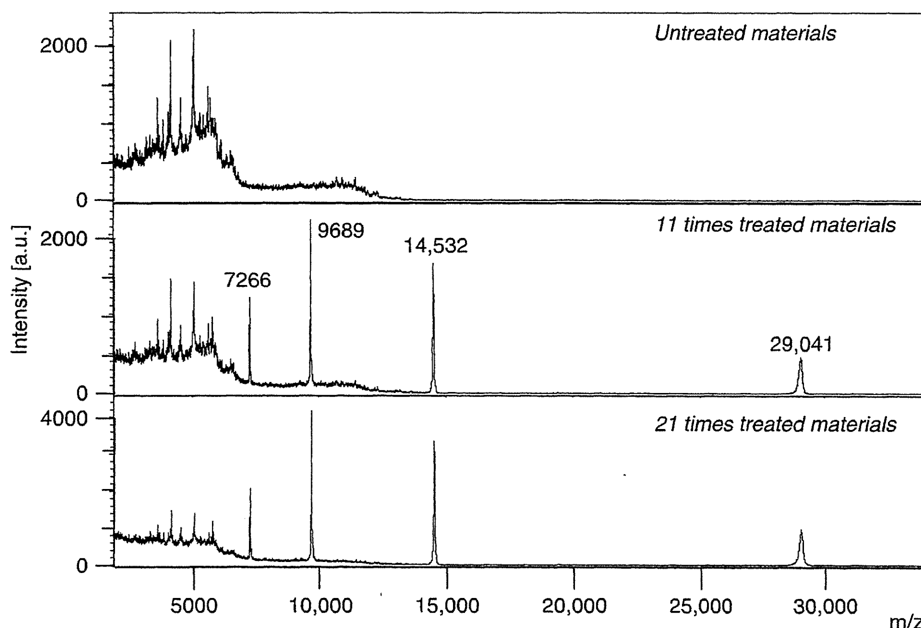

Figure 5. MALDI-TOF-mass spectrum of the untreated and 11- and 21-times-treated column material surface.

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

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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

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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*.

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-

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