Nicoliniら[95]は，同様のエピルビシン使用DEBを肝移植前の肝細胞癌患者8例に投与し，抗癌剤非使用塞栓（Embosphere™）例8例と比較した．治療は栄養血管が残存する限り2カ月1回行い，CTは3カ月毎に評価した．DEB群ではCRが77%の病変に得られたが，単純塞栓群では27.2%のみであった．組織学的壊死率も有意（p＝0.043）にDC Bead群で高く，重大な有害事象もなかったと，DC Beadを評価している．

Poggiら[96]は，白金製剤Oxaliplatin使用のDrug-eluting microsphere（HepaSphere™）を用いて，薬物動態のpreliminaryな結果を報告している．Oxaliplatinはmicrosphereに完全に結合し，腫瘍部位には周囲肝の約20倍の組織濃度になることが判明したとしている．Grossoら[97]は，ドキソルビシンまたはエピルビシンとHepaSphereを平均径42mmの肝細胞癌治療に用いた50例のpreliminaryなデータを示している．1カ月後の奏効率はCRが48%，PRが36%，SDが16%であったが，6カ月後でもCRが51.6%，PRが25.8%であると，反復治療の効果が報告されている．

2. 動注化学療法の進歩

門脈浸潤を伴ったりTACE不応・TACE不能となったりした進行肝細胞癌に対する治療は，最新の治療ガイドラインではソラフェニブが推奨されている．わが国では現在でも，これら進行肝癌症例に5-FU＋インターフェロン，5-FU＋シスプラチンなどの持続動注化学療法が選択されることが多い．

これまではほとんどわが国のデータしか報告されておらず，持続動注化学療法は世界のコンセンサスにはなっていないが，2009年以後韓国から2報の研究が出された．Wooら[98]は，多施設無作為化比較試験で，5-FU＋シスプラチン持続動注療法の際の薬剤を低用量群（それぞれ170mg/m^2を1～5日目，7mg/m^2を1～5日目）と高用量群（それぞれ500mg/m^2を1～3日目，60mg/m^2を2日目に）に分けて効果を比較した．何れのレジメンも効果的だったが，高用量群では奏効率16.7%，生存期間中央値193日で，やや治療効果が良好であった．Eunら[99]は，52例に5-FU＋シスプラチン持続動注療法（FP療法）を行ったが，このうち31例にはインターフェロンαを加え，3者治療（FPI）とした．FP療法，FPI療法での奏効率は57.1%，19.4%で，有意差はなかったがFP療法の方が高率であり，インターフェロンを加える3者療法は奏効率・生存率にベネフィットはなかった．

Nagamatsuら[100]は，低用量FP療法で，リピオドールをエマルジョン化して用いることの意義を論じた．通常の5-FU＋シスプラチン持続動注療法で，シスプラチンをリピオドールとエマルジョンにして投与した51例では，CRが10例，PRが34例に得られ，奏効率が86.3%に達した．リピオドールを使用した低用量FP療法は，切除不能・門脈浸潤合併肝癌の有力な治療法であるとしている．

Hirookaら[101]は，門脈浸潤を伴う進行肝癌に対して，動注化学療法（HAI）を行う前に減量治療としてラジオ波焼灼療法を行うことの意味をhistorical controlを用いたretrospective研究で行った．RFA後にHAIを行った20例では30%にCR，55%にPRが得られたが，通常のHAIのみの群ではCRは0%，PRが33.3%と低かった．2年生存率はそれぞれ78.8%, 16.9%で($p<0.0001$)，HAI前RFA治療の意義が大きかったとしている．

D. 肝癌に対する分子標的治療薬

1. ソラフェニブ（Sorafenib: ネクサバール™）

Llovetら[102]は，多国籍多施設の無作為化比較試験（SHARP study）で，進行肝癌に対する分

子標的薬ソラフェニブの有効性を報告した．肝切除・局所治療・TACE非適応の602例の二重盲検試験で，ソラフェニブ400mgを1日2回投与群と無投与群に割り付けて生存期間をみたところ，無治療群の50%生存期間は7.9カ月に対してソラフェニブ群では10.7カ月であり，リスク比0.69（p<0.001）で有意に生存期間延長がみられた．画像診断的な腫瘍進展までの期間（中央値）は，無治療群2.8カ月に対してソラフェニブ群5.5カ月と有意に（p<0.001）腫瘍増殖抑制効果がみられたとしている．副作用は下痢・手足皮膚反応・低リン血症などがみられたが，耐えられる範囲であり，総合的にはこれら進行肝癌症例での第一選択治療とした．

SHARP Studyは，主として欧米人に対して行われた臨床試験で，C型肝炎感染例が多く，やや高齢者が多い背景であった．これに対し，同様な基準で進行肝癌を対象として，アジア人症例に対してソラフェニブの第III相無作為化比較試験が行われた[103]．B型肝炎・若年者が多く，またSHARP studyよりもさらに進行した症例が多い背景であったが，ソラフェニブは全生存期間を4.2カ月から6.5カ月に延長し，死亡ハザードを0.68に低下させた．また腫瘍進展までの期間を1.4カ月から2.8カ月に延長し，そのハザード比0.57も含め，治療効果のインパクトはSHARP studyとほぼ同じものとなった．2010年にはこれら無作為化比較試験を含めたメタアナリシス[104]が行われ，ソラフェニブ治療は，ソラフェニブを使用しない化学療法や無治療対象群より生存期間を有意に延ばすと報告されている．

早々とソラフェニブ治療の適応拡大に関する論文が出始めている．ソラフェニブの治療は，肝機能の良い肝癌進行例が対象であるが，Wörnsら[105]はChild Bの15例，Child Cの4例に使用した経験を示し，Grade3/4の肝障害が頻発(23%)するが注意深い観察を行えば使用可能であると報告している．韓国のKimら[106]は，5-FU＋シスプラチンによる化学療法無効の肝癌24例にソラフェニブを使用した．抗腫瘍効果では，奏効例はみられなかったがSDが14例（58.3%）にみられたとして，「ある程度の効果」を示した．ヨーロッパからはドキソルビシン併用ソラフェニブ療法の第I相試験[107]が16例で行われ，病変制御率69%と，ドキソルビシン単独療法よりも勝っていたと報告している．さらに，TACEにソラフェニブを併用する第III相多施設無作為化比較試験が予定されているという論文[108]も公開された．ソラフェニブの治療効果を向上させる目的で，テガフールMetronomic治療を併用することの意義[109]も第II相臨床試験の結果として報告された．

高価で副作用が少なくない薬剤であり，治療効果予測や効果判定法に関する見解もでている．Vincenziら[110]は，早期に皮膚副作用が出現するとソラフェニブの治療効果予測ができると報告した．日本人よりも皮膚副作用の出にくい欧米人での検討ではあるが，Grade 1以上の皮膚障害のあった29例では腫瘍制御率は48.3%であったのに対し，副作用のなかった例では19.4%と有意に低かった．また，Time-to-progressionも8.1カ月vs 4.0カ月と有意差が認められた．Yauら[111]はアジア人51例での第II相試験での検討を行い，B型肝炎が背景の肝癌でも同様な効果を示すが，肺転移症例では効果不良であるとした．治療効果判定の問題では，Maksimovicら[112]は，ソラフェニブにより肝癌の血流が低下し壊死になる一方，出血も頻繁に起こるため，画像診断・機能診断には注意を要すると述べている．これら出血所見はたいてい壊死所見よりも早く起こるという．

2. その他の分子標的治療薬

肝細胞癌に対する新規の分子標的薬は，開発段階で第I相試験以後のものがすでに30種類以上あり，続々と市場に現れる可能性がある．

治療効果と副作用の問題から，肝細胞癌に対してのスニチニブ（スーテント™）は開発が中止となったが，スイスのKoeberleら[113]は，進行肝癌症例における多施設第II相試験の結果を報告している．45例エントリーし，エンドポイントである12週間後の無進行生存率は33%と，「ある程度の効果」は得られたとまとめた．ラパチニブ（タイケルブ™）の第II相試験が進行肝癌26例で行われた[114]．EGFRとHER2/NEUの阻害剤であるラパチニブでは，奏効例はなく，3カ月以上のSDが23%にみられたのみで，病変非進行期間は1.9カ月と，やや劣る結果であった．

米国のThomasら[115]は，進行肝癌に対してベバシズマブ（アバスチン™）とエルロチニブ（タルセバ™）の併用投与を行う第II相試験の結果を報告した．16週時点での生存と病変非進行生存をエンドポイントとして，40症例の治療が行われた．奏効率（PR例）が25%，16週での病変非進行生存は62.5%，生存期間中央値は68週と極めて良好であったが，Grade 3以上の副作用は多く，強い倦怠感20%，高血圧15%，下痢10%，トランスアミナーゼ上昇10%，消化管出血12.5%，創感染5%，血小板減少2.5%のほか，蛋白尿・高ビリルビン血症・背部痛・高カリウム血症・食欲低下が各1例にみられた．肝癌に対する分子標的薬としてはかつてない効果を示しているが，副作用が忍容範囲かどうかが問題と考えられる．台湾のHsuら[116]は，ベバシズマブ（アバスチン™）とカペシタビン併用を第一選択とする進行肝癌治療の成績を発表した．45症例に治療が行われ，9%に奏効が得られ，52%に病変制御が可能であった．病変非進行生存の中央値2.9カ月，全生存期間の中央値が5.9カ月と「ほどほどの」結果であった．

Pinterら[117]は，進行肝癌にサリドマイドを投与する第I相・第II相試験の結果を報告した．28例が登録され，2例がSDとなったが，他の26例はPDであった．また，全生存期間の中央値は5.1カ月と，治療効果は不良であった．

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B. 動脈塞栓術，ほか

29 A-P シャント・側副血行路を有する肝細胞癌に対する TACE

適応

通常の肝細胞癌に対する TACE の適応に準ずる。

禁忌

通常の肝細胞癌に対する TACE の禁忌に準ずるが，手技中にカテーテルが目的位置まで挿入できない場合には，TACE を断念する。

術前準備

❶腫瘍の位置から可能性のある肝外側副血行路を予想し(図 1)，dynamic CT で起始部を同定しておく。

❷選択する血管によっては 150 cm 長のマイクロカテーテルが必要となる。

❸細径のバルーンカテーテルや金属コイルが必要になることがある。

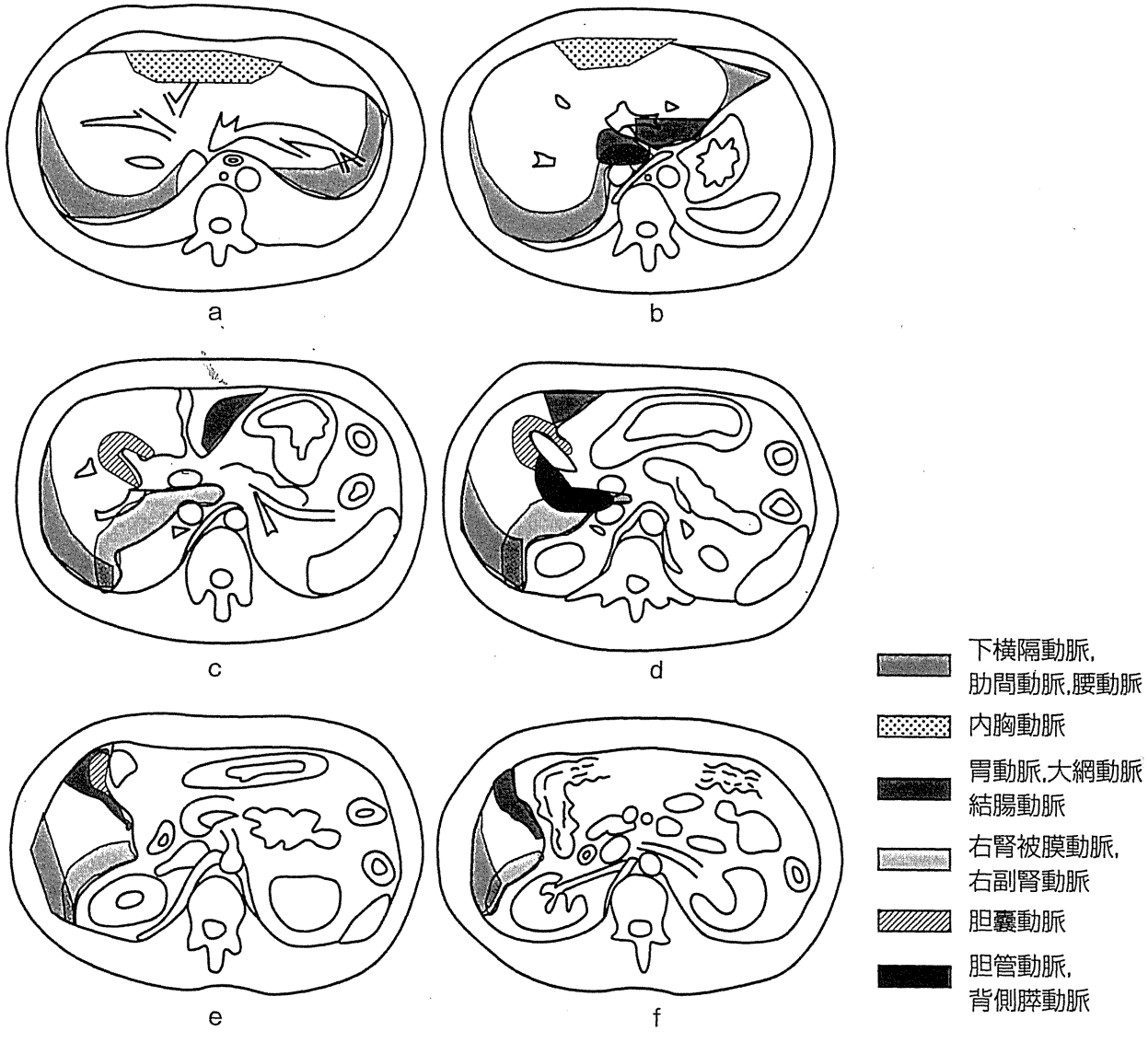

図 1　肝外側副血行路の分布域

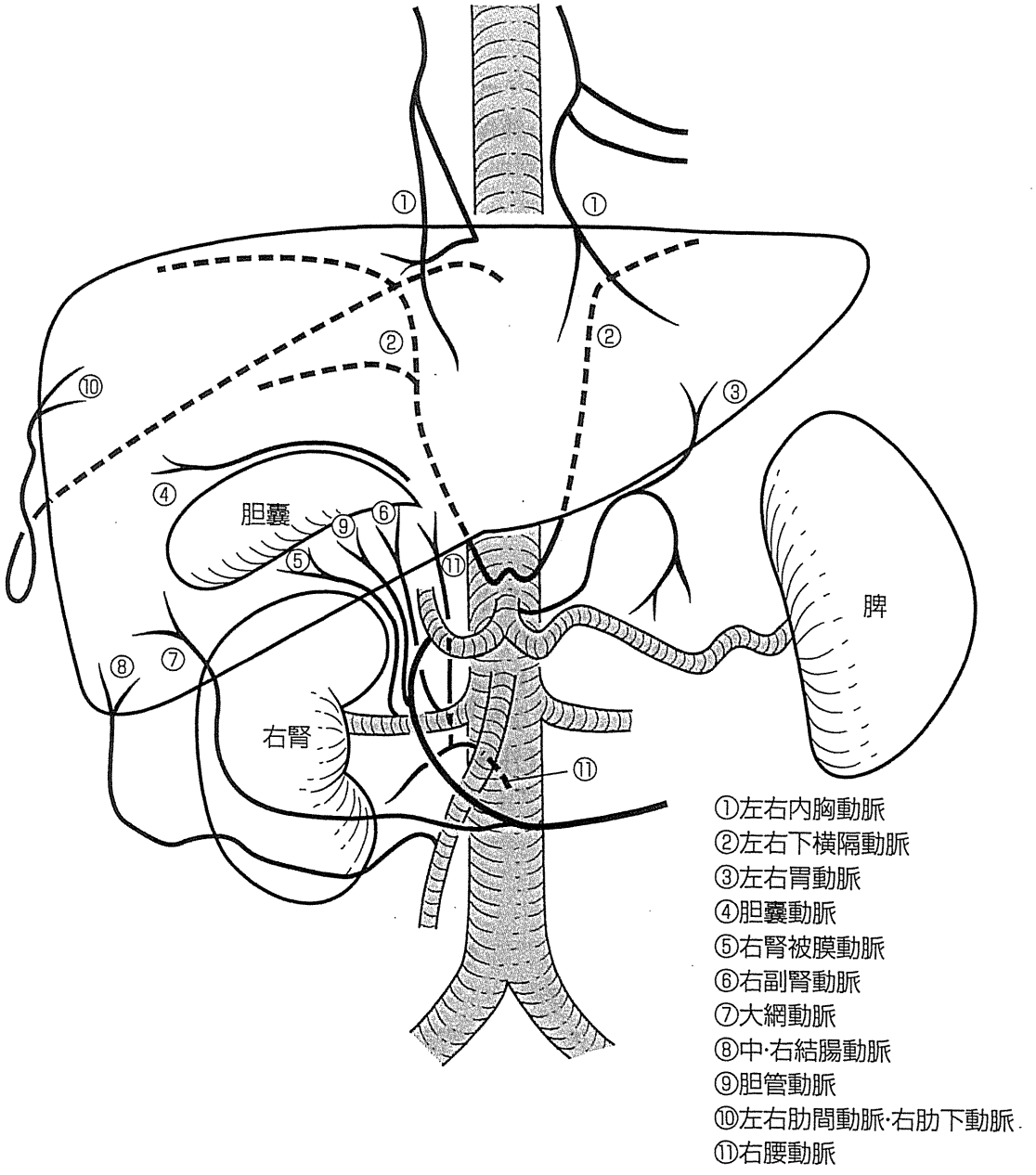

図 2　代表的な肝外側副血行路

手技

1 A-P シャントを有する肝細胞癌に対する TACE

❶門脈描出が軽度な場合は，通常のリピオドール TACE を行う。

❷門脈描出が高度で，シャントの責任血管が腫瘍の栄養血管と異なる，あるいは主要栄養血管でない場合には，責任血管を金属コイルやゼラチンスポンジで塞栓したのちに，通常のリピオドール TACE を行う。

❸門脈描出が高度で，シャントの責任血管が腫瘍の主要栄養血管である場合には，抗癌薬(エピルビシンなど)を浸み込ませたゼラチンスポンジで塞栓する。

❹ ❸の場合，バルーンカテーテルで責任血管の血流を停滞させたり，血管収縮薬(エピネフリン)を投与し血流を停滞させた状態で，リピオドール TACE を行う方法もある。

2 肝外側副血行路経由の TACE(図 2)

❶腫瘍の局在から関与する可能性のある血管を選択し造影を行う。腹腔動脈，上腸間膜動脈，腎動脈，大動脈から分岐する

手技のポイント

- 肝外側副血行路からの腫瘍への供血は，繰り返すTACEにより肝動脈が損傷されている例で認められることが多いが，肝外に突出する腫瘍では初回治療時に認められることがある[1,3]。また，手術や経皮的局所療法後の癒着も側副血行路の発達を促進する。
- 腹腔動脈の起始部や大動脈から急峻に分岐する下横隔動脈などは，通常の二重管法では選択できないこともあり，親カテーテルに側孔や側溝を作製する方法が有用である[4,5]。
- 下横隔動脈の起始部が閉塞している場合は，背側膵動脈，副腎動脈，左胃動脈などの複数の血管から再建されることが多く(retroperitoneal fine network)，そのなかでカテーテル挿入が容易な血管からアプローチする[6]。
- 肝外側副血行路は大切なアプローチルートであり，安易に金属コイルで塞栓しない。
- 内胸動脈，肋間動脈，腰動脈では，筋肉や皮膚を栄養する血管へリピオドール抗癌薬混合液を注入してはならない。肋間動脈から肝に分布する枝は，肋骨肋軟骨移行部で急峻に頭側に向かうため，そこをこえるまでマイクロカテーテルを進める[3]。また肋間動脈や腰動脈では，Adamkiewicz動脈の位置を確認するために，マイクロカテーテルでの選択を行う前に，複数本の肋間動脈や腰静脈(少なくとも塞栓する上下の血管)を撮影しておく[2]。
- 側副血行路間には吻合が豊富に存在するため，手技中は塞栓物質の動態を慎重に観察する[7]。
- 無漿膜野の腫瘍が再発した場合，最初は右下横隔動脈や右腎被膜動脈などから主に栄養されるが，TACEと再発を繰り返すうちに小さな腎被膜動脈，副腎動脈，肋間動脈，腰動脈，背側膵動脈などから栄養されるようになる(肝外側副路のマーチ)[8]。

下横隔動脈，腎被膜動脈，副腎動脈，肋間動脈，腰動脈の選択には主にシェファードフックカテーテルを使用するが，選択できない場合はコブラカテーテルを用いる。内胸動脈はヘッドハンターカテーテルからマイクロカテーテルを進めて選択するが，直接選択する場合はインターナルマンマリーカテーテルが有用である。

❷DSAで腫瘍濃染が確認されたら，できる限り栄養血管にまでマイクロカテーテルを進める。腫瘍の栄養血管は細いことが多く，2Fr以下のマイクロカテーテルが有用である。また，内胸動脈でのカテーテル操作はヘパリン化(3,000単位静注)したのちに行う。

❸栄養血管にカテーテルが挿入できたらTACEを施行するが，リピオドールや抗癌薬の使用量は肝動脈からの使用量の半量程度を目安とする[1]。

❹内胸動脈，肋間動脈，腰動脈では，皮膚や筋肉の栄養血管が外せない場合はTACEを断念し，必要に応じて大きめのゼラチンスポンジのみで塞栓する[1,2]。

❺胆嚢動脈，消化管の動脈，背側膵動脈では，胆嚢や消化管壁，膵実質の染まりが描出されなくなる位置までカテーテルを進めることができない場合は塞栓を断念する[1]。

❻IVR-CTやコーンビームCTを使用することで，合併症が軽減する。

成績

1 A-Pシャントを有する肝細胞癌に対するTACE

❶リピオドールTACEが施行できれば，通常の腫瘍の場合とほぼ同等の治療効果が得られる。

❷ゼラチンスポンジのみで塞栓した場合はやや治療効果は劣るが，再発時にはA-Pシャントが消失・軽減し，通常のリピオドールTACEが施行可能になることがある。

2 肝外側副血行路経由のTACE

❶下横隔動脈　下横隔動脈は，40%は腹腔

動脈，39％は大動脈，15％は腎動脈，4％は左胃動脈，2％は肝動脈から分岐する[9]。動脈硬化や弓状靱帯の圧排により起始部が閉塞した際には，種々の後腹膜の血管から再建される[6]。右下横隔動脈からは下大静脈を栄養する枝が，左下横隔動脈からは胃枝が分岐するため注意を要する。筆者らの検討では，右下横隔動脈からの腫瘍への供血の頻度は，肝外側副血行路からの供血が認められた全症例の83％，左下横隔動脈では12％であり，そのうちリピオドール TACE が施行可能であったものはそれぞれ96％，100％であった[1]。

❷**胆嚢動脈**　胆嚢動脈の深在枝は肝動脈右前下区域枝(A5)と吻合し，胆嚢動脈からもしばしばA5の1枝が分岐する[1]。胆嚢床部を主に栄養するが，肝動脈損傷が著しい場合は，深部の腫瘍も栄養する。また，胆嚢床に突出する腫瘍は初回より胆嚢動脈から栄養されることがある。発現頻度は24％で，リピオドール TACE の成功率は70％であった[1]。

❸**腎動脈，腎被膜動脈，副腎動脈**　腎被膜動脈，副腎動脈などは複数本存在し，また腎動脈から被膜を貫通する小枝も腫瘍の栄養血管となる[8]。右腎被膜動脈や副腎動脈は，肋間動脈，背側膵動脈，尾状葉枝などと吻合する[7]。発現頻度は19％で，リピオドール TACE の成功率は100％であった[1]。

❹**内胸動脈**　内胸動脈の各分枝からの腫瘍への供血頻度は，横隔枝47％，筋横隔動脈32％，上腹壁動脈12％，前肋間動脈5％，剣状突起動脈3％，心膜横隔動脈1％と報告されている[10]。右下横隔動脈からの TACE 後の再発病変で関与することが多い。発現頻度は右内胸動脈では8％，左内胸動脈では1％で，リピオドール TACE の成功率はいずれも100％であった[1]。

❺**肋間動脈，肋下動脈，腰動脈**　下横隔動脈，内胸動脈，腎被膜動脈，腰動脈，肋間動脈の間には豊富な吻合が存在し[7]，肋間動脈，肋下動脈，腰動脈からの供血は，下横隔動脈や腎被膜動脈からの TACE 後に顕著化することが多い。腫瘍への供血頻度は T10 ＞ T9 ＞ T11 ＞ T12 ≧ L1 ＞ T8 ≧ L2 の順であり[1]，肋間動脈からの栄養血管は肋骨肋軟骨移行部レベルで急峻に分岐し[3]，肋下動脈からの栄養血管は筋枝から分岐，腰動脈からの栄養血管は背側枝と筋枝の分岐部の近傍から分岐することが多い[2]。Adamkiewicz 動脈への塞栓物質の流入が危惧される場合，TACE は禁忌である。発現頻度は右肋間・肋下動脈では10％，腰動脈では2％で，リピオドール TACE の成功率はそれぞれ53％，91％であった[1,2]。

❻**大網動脈，左右胃動脈，中・右結腸動脈，背側膵動脈，胆管動脈**　大網動脈からの供血は，破裂例や腹膜播種病変を有する例でも認められる。右胃大網動脈から分岐する大網動脈が主に関与するが，左胃大網動脈からのものも稀に関与する。大網動脈と結腸動脈の間には吻合が存在するため，塞栓時には注意を要する[7]。胆管動脈は後上膵胃十二指腸動脈から急峻に分岐し，右肝動脈や胆嚢動脈と吻合する。出現頻度は大網動脈では13％で，ほかはいずれも4％以下で，リピオドール TACE の成功率は大網動脈で74％，それ以外では63～75％であった[1]。

術後管理

通常のリピオドール TACE に準ずる。

合併症

1 A-P シャントを有する肝細胞癌に対する TACE

シャントを介して門脈内に多量のリピオドールが流入すれば，広範な肝実質障害が生じる。

❷肝外側副血行路経由のTACE

❶下横隔動脈　胸水，肺底部無気肺，横隔膜運動不良，脳梗塞(下横隔動脈と肺静脈の吻合が存在するとリピオドールが体循環に流入し生じる)

❷胆嚢動脈　胆嚢炎・胆嚢梗塞

❸腎動脈，腎被膜動脈，副腎動脈　腎梗塞，膵炎・膵壊死(副腎動脈と背側膵動脈との間には吻合が存在)，副腎壊死は生じにくい。

❹内胸動脈　皮膚炎・皮膚潰瘍・皮膚壊死

❺肋間動脈，肋下動脈，腰動脈　皮膚炎・皮膚潰瘍・皮膚壊死，脊髄梗塞

❻大網動脈，左右胃動脈，中・右結腸動脈，背側膵動脈，胆管動脈　腸管虚血・潰瘍・腸管壊死，膵炎・膵壊死，胆管梗塞・胆管狭窄，大網壊死は生じにくい。

文献

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ORIGINAL ARTICLE

Characterization of naturally occurring protease inhibitor-resistance mutations in genotype 1b hepatitis C virus patients

Hiroko Shindo · Shinya Maekawa · Kazuki Komase · Ryota Sueki · Mika Miura · Makoto Kadokura · Kuniaki Shindo · Fumitake Amemiya · Takatoshi Kitamura · Yasuhiro Nakayama · Taisuke Inoue · Minoru Sakamoto · Shun-ichi Okada · Yasuhiro Asahina · Namiki Izumi · Masao Honda · Shuichi Kaneko · Nobuyuki Enomoto

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Abstract
Background and aims Protease inhibitor (PI)-resistant hepatitis C virus (HCV) variants may be present in substantial numbers in PI-untreated patients according to recent reports. However, influence of these viruses in the clinical course of chronic hepatitis C has not been well characterized.
Methods The dominant HCV nonstructural 3 (NS3) amino acid sequences were determined in 261 HCV genotype 1b-infected Japanese patients before pegylated interferon plus ribavirin (PEG-IFN/RBV) therapy, and investigated the patients' clinical characteristics as well as treatment responses including sustained virological response (SVR) rate. HCV-NS3 sequences were also determined in 39 non-SVR patients after completion of the therapy.
Results Four single mutations (T54S, Q80K, I153V, and D168E) known to confer PI resistance were found in 35 of 261 patients (13.4%), and double mutations (I153V plus T54S/D168E) were found in 6 patients (2.3%). Responses to PEG-IFN/RBV therapy did not differ between patients with and without PI-resistance mutations (mutation group, SVR 48%; wild-type group, SVR 40%; $P = 0.38$). On the other hand, two mutations appeared in two non-SVR patients after PEG-IFN/RBV therapy (I153V and E168D, 5.1%).
Conclusions PI-resistance-associated NS3 mutations exist in a substantial proportion of untreated HCV-1b-infected patients. The impact of these mutations in the treatment of PIs is unclear, but clinicians should pay attention to avoid further development of PI resistance.

Keywords HCV · Protease inhibitor · Naturally occurring viral resistance mutations

Introduction

Hepatitis C virus (HCV) infects more than 170 million persons worldwide and thus represents a global health problem. At least 130 million infected individuals are chronic carriers of HCV and are at significant risk of developing liver cirrhosis and hepatocellular carcinoma [1]. The current standard treatment with pegylated interferon plus ribavirin (PEG-IFN/RBV) is complicated by frequent adverse reactions, and a sustained virologic response (SVR) can be achieved only in 50% of patients infected with the most prevalent genotype 1 [2]. In Japan, since 70% of patients are infected with intractable genotype 1b HCV, more effective treatments are urgently required.

A promising approach is the development of specifically targeted antiviral therapies for hepatitis C (STAT-C). HCV-specific protease inhibitors (PIs) target an essential step in HCV replication by blocking the nonstructural 3/4A (NS3/4A) protease-dependent cleavage of the HCV polyprotein

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H. Shindo · S. Maekawa (✉) · K. Komase · R. Sueki · M. Miura · M. Kadokura · K. Shindo · F. Amemiya · T. Kitamura · Y. Nakayama · T. Inoue · M. Sakamoto · S. Okada · N. Enomoto
First Department of Internal Medicine, University of Yamanashi, 1110, Shimokato, Chuo, Yamanashi 409-3898, Japan
e-mail: maekawa@yamanashi.ac.jp

Y. Asahina · N. Izumi
Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan

M. Honda · S. Kaneko
Department of Gastroenterology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan

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[1]. Among these NS3/4A PIs, telaprevir, boceprevir, SCH446211, danoprevir (ITMN-191), nalaprevir (SCH900 518), and TMC435 are now under clinical trials [1, 3–7]. In PROVE1 and PROVE2 studies [3, 4] undertaken in North America and Europe, the SVR rate was favorable (67 and 69%, respectively) in a triple therapy regimen including telaprevir. In addition, some studies have suggested that shortening of treatment duration may be possible for patients who achieve a rapid virologic response (RVR) [8, 9].

However the sole use of STAT-C drugs, such as PIs, promotes production and selection of drug-resistant variants in patients experiencing viral rebound during treatment [3, 10, 11] as well as in HCV replicon experiments [11, 12]. Therefore, these drugs should be used in combination with the PEG-IFN/RBV to prevent the appearance of drug-resistant variants. However, Kuntzen et al. [13] demonstrated the presence of these drug-resistant variants in high frequencies (8.6–16.2%) by population-based sequencing in patients not treated with the drugs [1, 13]. Gaudieri et al. [14] have suggested that regions of NS3 protease and NS5B polymerase are likely to be under HLA immune pressure and therapeutic selection, and that drug-resistant variants may occur naturally to escape the immune system. These observations seem quite astonishing and troubling, since a substantial number of patients may not respond to the new therapies such as STAT-C drugs.

In the present study, to assess the prevalence of NS3 mutations conferring PI resistance in HCV genotype 1b-infected Japanese patients who had not been previously treated with PIs, as well as to assess the influence of those mutations in response to PEG-IFN/RBV therapy, the dominant HCV-NS3 sequences were determined in 261 HCV-1b patients before starting the PEG-IFN/RBV therapy.

Methods

Patients

Serum samples were acquired from 261 HCV genotype 1b-infected adult Japanese patients before combination therapy with PEG-IFN (PEGINTRON®, Schering-Plough, Tokyo, Japan) plus RBV (REBETOL®, Schering-Plough) between 2004 and 2008 at the University of Yamanashi, Musashino Red Cross Hospital and Kanazawa University. The therapy was administered according to the standard PEG-IFN/RBV treatment protocol established for Japanese patients by a hepatitis study group of the Ministry of Health, Labor, and Welfare, Japan. Specifically, the patients were subcutaneously administered PEG-IFNα-2b, 1.5 μg/kg body weight, once weekly and RBV 600–800 mg daily per os for 48 weeks. These patients were not infected with human immunodeficiency virus (HIV). The study was approved by the ethics committees of all participating universities and the hospital, and the protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Institutional Review Board at Massachusetts General Hospital. Written informed consent was obtained from each study participant.

Amplification and sequencing of full-length HCV genomes

Viral loads were determined using the Amplicor HCV RNA kit, version 2.0 (Roche Diagnostics, Tokyo, Japan) or the Cobas TaqMan test (Roche Diagnostics). HCV RNA was extracted from pretreatment serum samples by the AGPC method using Isogen (Wako, Osaka, Japan) according to the manufacturer's protocol. Complementary DNA was synthesised using Superscript II (Invitrogen, Tokyo, Japan) and random primers (Invitrogen), and then amplified by two-step nested PCR using the primers listed in Supplementary Table 1. All samples were initially denatured at 95°C for 7 min, followed by 40 cycles of amplification with denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 45 s using the BD Advantage™ 2 PCR Enzyme system (BD Biosciences Clontech, CA, USA). PCR amplicons were directly sequenced using BigDye Terminator version 3.1 (ABI, Tokyo, Japan) and universal M13 forward/reverse primers using an ABI prism 3130 sequencer (ABI).

Sequence alignment and analysis

Sequences were determined in both directions, particularly for the ambiguous stretches, were assembled using the Vector NTI software (Invitrogen), and base-calling errors were corrected following the inspection of chromatograms. If mixed bases were detected as two different chromatogram peaks at the same residue, only the dominant base was called after evaluation of all overlapping fragments. A consensus sequence was generated from the alignment on the basis of the most common amino acid at each site.

Determination of PI resistance mutations

Multiple viral NS3 mutations were observed in amino acid positions reported to confer PI resistance among 261 patients: V36, Q41, F43, T54, V55, Q80, R109, I153, R155, A156, D168, V170, and M175. NS3 amino acid mutations with proven PI resistance in previously published studies (Table 1) were designated as resistance proven mutations (e.g., V36M/A). Mutations in the PI-resistance site not known to confer drug resistance were designated resistance unproven mutations (e.g., V36I). Patients were allocated to two groups according to the presence of PI-resistance

mutations (including resistance unproven mutations), and clinical characteristics including HCV RNA levels and responses to PEG-IFN/RBV therapy were compared. To assess the influence of PEG-IFN/RBV therapy on NS3 mutational status, posttreatment HCV-NS3 sequences in 39 of 58 non-SVR patients were also examined.

Statistical analysis

Statistical differences in the data, including all available patients' demographic, biochemic, hematologic, and virologic data such as sequence variation factors, were determined among the various groups by Student's *t* test or Mann–Whitney *U* test for numerical variables and Fisher's exact probability test for categorical variables.

Results

Prevalence of dominant PI-resistance-associated nonstructural 3 mutations in untreated patients

Figure 1 shows the frequency of substitutions in 261 patients for each of 181 NS3 protease amino acid residues compared to the consensus sequence. A total of 41 resistance proven mutations were detected in 35 (13.4%) patients: T54S (14 patients, 5.4%), Q80K (1 patient, 0.4%), I153V (22 patients, 8.4%), D168E (4 patients, 1.5%), T54S plus I153V double mutation (4 patients, 1.5%), and I153V plus D168E double mutation (2 patients, 0.8%). The mutation number increased to 54 in 47 (18.0%) patients when resistance unproven mutations were included: V36I (2 patients, 0.8%), I153L (11 patients, 4.2%), and I153V plus V36I double mutation (2 patients, 1.5%). Double mutations were found in 7 patients (2.7%) (Table 1). Q80L was observed in 47 (18%) patients but these were excluded from consideration because a previous study demonstrated that this mutation does not confer resistance [15]. All mutations observed in this study would confer low- to moderate-level PI resistance according to previous studies [6, 15–19]. No mutations conferring high-level resistance such as R155 or A156 [11, 17, 19–22] were observed.

Clinical characteristics of patients with PI-resistance mutations

Table 2 presents the characteristics of patients classified according to the presence of PI-resistance mutations

Table 1 Prevalence of PI-resistance-associated NS3 mutations

Drug-resistance mutations described in the literature				Detected resistance mutations
NS3 residue	Resistance mutations	Drugs	References	Genotype 1b ($N = 261$), (%)
V36	A, M, L, G, C	Telaprevir, Boceprevir	[1, 3, 4, 10, 11, 19, 31, 37]	I × 2 (0.8)
Q41	R	ITMN-191, Boceprevir	[19]	
F43	S, C	ITMN-191, Boceprevir, Telaprevir, TMC435	[15, 19]	
T54	A, S	Telaprevir, Boceprevir, SCH900518	[1, 3, 10, 11, 19, 20, 31, 38]	**S × 14 (5.4)**
V55	A	Boceprevir	[1]	
Q80	R, K	TMC435	[6, 15]	**K × 1 (0.4)**
R109	K	SCH446211	[17]	
I153	V	SCH446211	[17]	**V × 22 (8.4)**, L ×11 (4.2)
R155	K, T, I, M, G, L, S, Q	Telaprevir, Boceprevir, ITMN-191, BILN2061, TMC435	[1, 3, 4, 6, 10, 11, 15, 19, 20]	
A156	S, T, V, I, G	Telaprevir, Boceprevir, ITMN-191, BILN2061, SCH446211, TMC435, SCH900518	[1, 3, 4, 10, 11, 15, 17, 19, 20, 38]	
D168	A, V, E, N, T, H	BILN2061, ITMN-191, TMC435	[6, 15, 20]	**E × 4 (1.5)**
V170	A	Telaprevir, Boceprevir	[1, 19, 20]	
M175	L	Boceprevir	[39]	
Total number (%) of patients with resistance proven mutations				35 (13.4)
Total number (%) of patients with resistance proven and unproven mutations				47 (18.0)

Amino acid mutations conferring PI resistance in the literatures and those observed in PI-treatment-naive patients in this study are indicated. Bold indicates resistance proven mutations, and the others indicate resistance unproven mutations

Double mutations found were as follows: V36I and I153V × 1, T54S and I153V × 4, I153V and D168E × 2

(including resistance unproven mutations). Age, sex ratio, body mass index, alanine aminotransferase (ALT) levels, serum albumin, platelet count, and fibrosis stage did not differ between the NS3 mutation and wild-type groups. No significant difference was observed between the two groups in the parameters of PEG-IFN/RBV treatment response, HCV sequence variations in interferon sensitivity determining region (ISDR), Core 70, interferon plus ribavirin resistance-determining region (IRRDR), or interleukin 28B (IL28B) single nucleotide polymorphism (SNP) (rs8099917; T/G and G/G vs. T/T) [23–30]. These clinical variables were also compared between the mutation group defined as resistance proven mutations and the wild-type group, but no notable differences were observed.

Unimpaired in vivo fitness of viral strains with resistance mutations

Because most PI-resistance mutations described till date have been associated with reduced replicative capacity of varying degrees [1, 10, 11, 13, 17, 20–22, 31, 32], we examined viral replication levels in patients with drug-resistance mutations (Fig. 2). The estimated P value indicated no significant difference between the mutation (median 1,500 KIU/ml) and wild-type (median 1,800 KIU/ml) groups ($P = 0.69$). The results indicate that drug-resistant HCVs were not necessarily impaired in their ability to replicate in vivo. However, patients with double mutations ($N = 7$) tended to have low viral loads (median 1,200 KIU/ml) ($P = 0.09$).

Resistance mutations and virologic response to PEG-IFN/RBV therapy

To determine the difference in virologic response to PEG-IFN/RBV therapy according to the PI mutation, frequency of HCV RNA levels below detection at 4 weeks (rapid viral response, RVR) and 12 weeks (complete early viral response, cEVR), and SVR rate (%) were investigated in each group. The frequency of HCV RNA levels below detection at 4 and 12 weeks was 14 and 50%, respectively, in the mutation group, and was 11 and 46%, respectively, in the wild-type group. The SVR rate was 48 and 40% in the mutation and wild-type groups, respectively ($P = 0.38$). No significant difference was observed between the two groups in any of the indexes investigated (Table 2). The time-dependent viral clearance rate during PEG-IFN/RBV therapy was estimated in 133 patients including 25 patients (19%) with PI-resistance mutations available for the analysis. Kaplan–Meier analysis demonstrated that HCV clearance did not differ between the two groups with and without resistance mutations (log-rank test, $P = 0.30$) (Fig. 3).

Changes in nonstructural 3 amino acid sequence diversity during PEG-IFN/RBV therapy

Full-length NS3 protease sequences were determined in 39 non-SVR patients after PEG-IFN/RBV therapy. A single amino acid change at resistance-associated sites in two patients was observed. In one patient, isoleucine (Ile) at position 153 changed to valine (Val), and glutamic acid (Glu) changed to aspartic acid (Asp) at position 168 in the second (Fig. 4). At the nucleotide level, ATC (Ile) changed to GTC (Val) in I153V, and GAA (Glu) changed to GAC (Asp) in E168D. Both mutations were caused by one nucleotide exchange. No other changes were observed in the other 37 patients.

Discussion

Here we report that in 18% (47/261) HCV genotype 1b-infected patients who had not been previously treated with NS3 PIs, the viral genome contained dominant amino acid mutations within the NS3 PI-resistance sites. Even after confining the data to established PI-resistance mutations, the mutation rate was still significant in 13.4% (35/261). No clinical differences were observed between patients

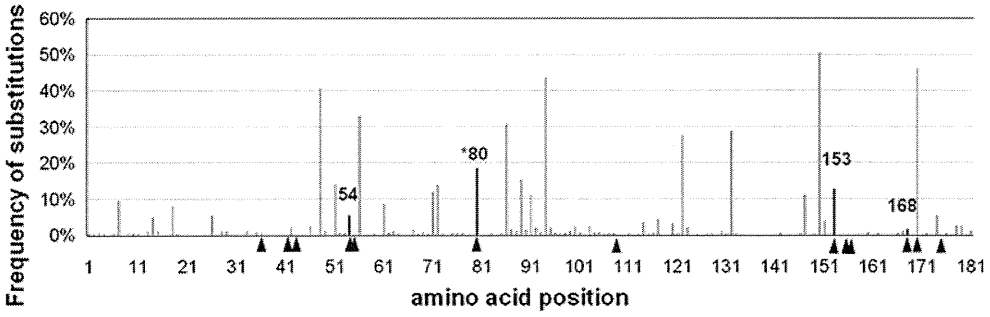

Fig. 1 Frequency of polymorphic mutations for each of the 181 NS3 protease amino acid residues in 261 patients. *Arrowheads* indicate the sites reported to confer PI resistance. *Dark bars* denote the amino acid variations at the resistant sites in this study. **80*, we detected one resistant mutation (Q80K) and 47 (18%) non-resistant variations (Q80L) at the 80th residue

Table 2 Characteristics of patients with or without HCV genomes harboring drug-resistance mutations

Characteristics	Mutation type ($N = 47$)	Wild-type ($N = 214$)	P value
Patients' characteristics			
Age, median (range)	59 (46–72)	57 (19–77)	0.17
Male, no. (%)	26 (55)	112 (52)	0.70
BMI, median (range)	23.2 (15.5–31.9)	22.8 (16.1–31.9)	0.41
ALT IU/ml	81.3 ± 72.6[a]	74.8 ± 51.9	0.93
Serum albumin g/dl	4.00 ± 0.37	4.01 ± 0.36	0.81
Platelet count × $10^4/\mu l$	15.8 ± 4.3	14.5 ± 4.8	0.18
HCV RNA KIU/ml, median (range)	1,500 (58–6,310)	1800 (28–15,849)	0.69
Fibrosis, no. (%)			0.97
F0	0 (0)	7 (3)	
F1	23 (50)	89 (42)	
F2	9 (20)	52 (24)	
F3	9 (20)	40 (19)	
F4	5 (11)	26 (12)	
IFN pre-treatment no. (%)	15/40 (38)[b]	66/172 (38)	1.00
IL28B (rs8099917) T/G or G/G no. (%)	6/20 (30)	19/67 (28)	1.00
Response to PEG-IFN/RBV therapy			
SVR total cases no. (%)	22/46 (48)	83/210 (40)	0.38
RVR in total cases no. (%)	6/44 (14)	22/195 (11)	0.83
cEVR in total cases no.(%)	22/44 (50)	92/200 (46)	0.75
SVR 48w treatment no. (%)	16/29 (55)	55/130 (42)	0.29
End of treatment response no. (%)	26/41 (63)	123/202 (61)	0.91
HCV genome sequence variation			
ISDR mutation ≤1 no. (%)	32/46 (70)	167/210 (80)	0.21
Core70 R no. (%)	26/44 (59)	136/210 (65)	0.56
IRRDR mutation >3 no. (%)	25/38 (66)	107/190 (56)	0.34

[a] Mean ± SD

[b] Number/total number (%)

harboring viruses with and without these mutations. Moreover, no differences were observed in the responses of either group to PEG-IFN/RBV therapy.

Recent studies reported that significant number of patients who were never treated with PI possess viral sequences with PI-resistance-associated NS3 mutations. In these studies, the prevalence of PI-resistance mutations was determined to be 8.6–16.2% [13, 14], in HCV genotype 1- and 3-infected patients in European–American populations. These patients were often coinfected with HIV. Analysis of the public HCV databases (EuHCVdb and Los Alamos) also reported the presence of naturally occurring PI-resistance-associated NS3 mutations in worldwide isolates [33]. However, in vivo and in vitro studies demonstrated that most of the mutations observed conferred only low- to moderate-level PI resistance [7, 13, 14, 34, 35]. Regarding viral fitness, PI-resistant HCVs show lower fitness at varying degrees as revealed by in vitro studies [1, 10, 11, 17, 20–22, 31, 32], but HCV RNA levels in a clinical study did not differ significantly. The response to PEG-IFN/RBV therapy was almost comparable to that in HCV-infected patients without PI-resistance mutations either in HCV replicon experiments or in a clinical study of small number of treated patients [34].

The prevalence of 13.4% for PI-resistance-proven patients observed in the present study was almost comparable to the results of previous studies. Although HIV is known to increase HCV replication in coinfection with HCV [36], and HIV patients are often treated with the HIV-specific PIs, the HIV infection might not affect the natural occurrence of HCV-specific PI-resistance mutations since our studied patients were all proven to be free from coinfection with HIV infection. As shown in Table 1 and Fig. 1, I153 V (22/261, 8.4%), T54S (14/261, 5.4%), and D168E (4/261, 1.5%) were among the most prevalent PI-resistance-proven mutations in the present study. The most frequent mutation detected in our study I153V was reported to appear secondarily to the occurrence of R109K mutations in a HCV replicon system [17]. Although the role of this mutation is not understood, the I153V mutation on its own conferred SCH446211 resistance to the HCV replicon to a lesser degree [17]. Interestingly, I153V was often found in double mutations in our study, as shown in Fig. 2. This suggests analogy between in vitro and in vivo data. T54S and D168E, the other frequent mutations, have been also reported to occur as single dominant mutations in previous in vitro or in vivo studies in HCV genotype 1

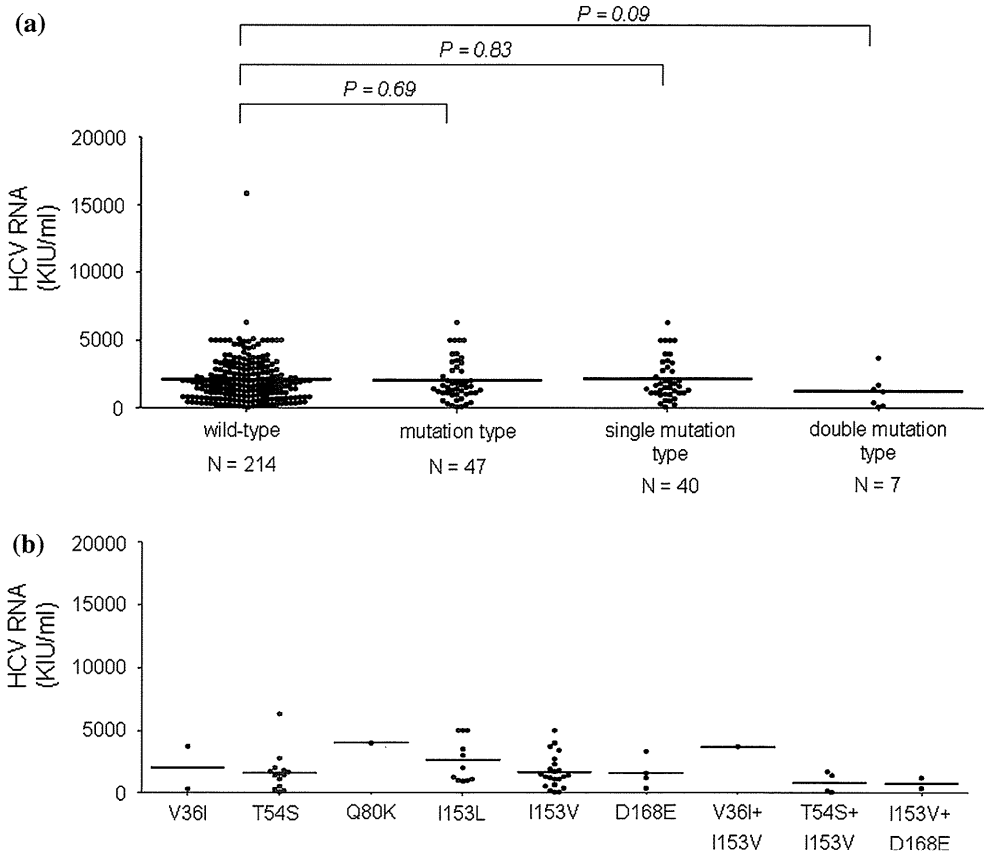

Fig. 2 In vivo fitness of HCV with PI-resistance-associated NS3 mutations. HCV RNA levels were compared between patients with and without NS3 PI-resistance-associated mutations (**a**) and between patients with each resistance mutation (**b**). The estimated *P* value (Mann–Whitney *U* test) indicates no significant difference between the wild-type and other groups (wild-type vs. mutation type, wild-type vs. single mutation type, and wild-type vs. double mutation type). (Wild-type, $N = 214$; mutation type, $N = 47$; single mutation type, $N = 40$; double mutation type, $N = 7$; V36I, $N = 2$; T54S, $N = 14$; Q80K, $N = 1$; I153L, $N = 11$; I153V, $N = 22$; D168E, $N = 4$; E176A, $N = 1$; V36I + I153V, $N = 1$; T54S + I153V, $N = 4$, and I153V + D168E, $N = 2$)

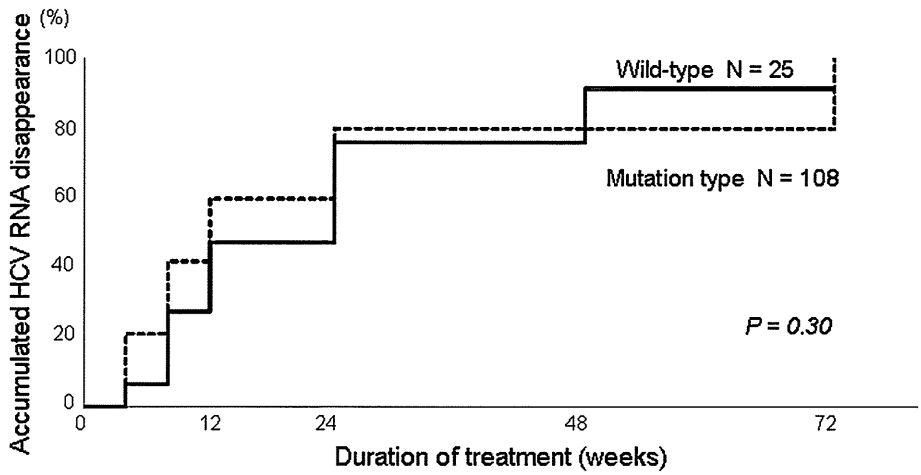

Fig. 3 Comparison of virologic response to PEG-IFN/RBV therapy between HCV-infected patients with and without PI-resistance-associated NS3 mutations. Time-dependent HCV clearance rate analysis was based on serum HCV RNA positivity during PEG-IFN/RBV therapy for HCV isolates with resistance mutations or wild-type sequences. A total of 133 patients for whom the limit of viral genome detection could be determined were analyzed. Among this group, NS3 mutations were detected in 25 patients (19%). The estimated *P* value (log-rank test) shows no significant difference between the two groups ($P = 0.30$)

infections showing moderate degrees of resistance [16, 18, 19].

Most PI-resistance mutations described to date have been associated with varying degrees of reduced replicative capacity [10, 11, 17, 20–22, 31, 32]. In the present study, HCV RNA levels of those patients with low- to moderate-level resistance mutations were similar to those in patients in the wild-type groups, suggesting that in vitro viral fitness