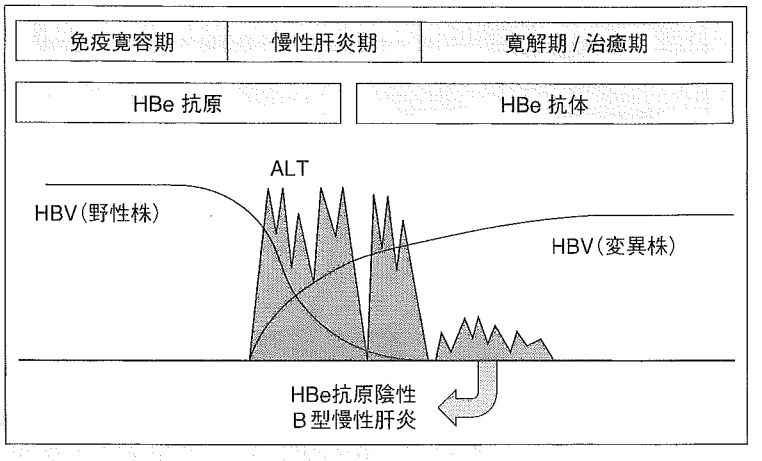
図2　HBe 抗原から HBe 抗体へのセロコンバージョン（SC）



SC は HBV（野性株）が pre-core 遺伝子あるいは core promoter 遺伝子に変異を来した HBV（変異株）となるために生じる．SC 後も HBV 増殖が持続し慢性肝炎（HBe 抗原陰性）が持続することがまれにある．

ともある．しかし，中には重症化，劇症化を来し，肝不全となる症例を認める．近年，HBe 抗原陰性でも HBV－DNA 陽性のB型慢性肝炎患者の存在が確認され，これらは HBe 抗原が産生されない pre-core 変異株や core promoter 変異株によることが報告されている[8]．HB キャリアやB型慢性肝炎からの急性増悪症例で肝不全となり死亡した症例の中には，HBV－DNA が 7.6 log copies / m*l* を超える高ウイルス症例のみならず，今述べた HBe 抗原陰性 HBe 抗体陽性の低ウイルス症例も認められる．したがって，抗原抗体系の血清マーカーのみにとらわれず，血清総ビリルビン値やプロトロンビン時間などの肝予備能を見ながら，重症化や劇症化を防ぐ必要がある．

B型慢性肝炎急性増悪の治療

急性増悪時の治療目標は ALT の正常化と重症化の防止である．基本的にはB型慢性肝炎の治療すなわち，IFN もしくはラミブジンなどの抗ウイルス薬の使用が中心となる．HB キャリアからの急性増悪例では予後の悪い亜急性型の劇症肝炎に進展する可能性が高く，肝移植も考慮しながらの診療が必要である．HBe 抗体陽性 HB キャリアに，他疾患でステロイドや免疫抑制剤を使用すると，肝炎が増悪する

表3　B型肝炎治療の新しいステージ分類

HB stage	0	I	II	III	IV	V
HBsAg	+	+	+	+	+	−**
HBeAg	+	+	+	−	−	−
HBV−DNA (copies/ml)	不問	$10^{7.6}$≦	$10^{7.6}$>	10^{5}≦	10^{5}>	不問
ALT	持続正常	持続正常以外	持続正常以外	不問	不問	不問
年齢	不問	若年 / 高年* (Ia / Ib)	若年 / 高年* (IIa / IIb)	不問	不問	不問
発癌リスク	極めて小	小 / 大	小 / 極めて大	極めて大	極めて小	極めて小
治療	不要	F_2 以上 IFN / IFN+ ラミブジン	IFN / ラミブジン	ラミブジン	不要	不要

*：若年　男性30歳未満，女性35歳未満　　高年　男性30歳以上，女性35歳以上

**：HBsAg（+）の時期が確認されていること

略語：巻末の略語集参照

ことがあり，注意が必要である．一般にウイルス量の増加が高度な症例では，肝炎も重症化する例が多いので，早期より積極的にラミブジンを使用するべきである．いずれにしても，急性感染同様に，劇症化の予知式[5][16]などを用いて，早めの治療が必要である．

最も重要なのは，治療開始のタイミングである．特にラミブジンの開始時期にはさまざまな見解がある．プロトロンビン時間が40％をきるような重症例での早期使用は当然であるが，HBキャリアからの急性増悪と慢性肝炎からの急性増悪では異なった基準を設けるべきとの意見もある[9]．すなわち，ラミブジンの有効性の検討結果から，HBキャリアからの急性増悪例ではプロトロンビン時間が60％未満，慢性肝炎からの急性増悪例では総ビリルビン値が5 mg / dl 以上で速やかにラミブジンを投与すべきと考えられる．

ラミブジン長期投与例においてみられる耐性株出現による breakthrough hepatitis が原因の急性増悪例では他の抗ウイルス薬の併用が試みられる．我が国では2004年12月より，アデホビルが保険適用となり，ラミブジン投与中にHBVの持続的な再増殖を伴う肝機能異常が確認された症例で，ラミブジンとの併用治療が可能となった．また，entecavir など，他の抗ウイルス薬の開発も進んでおり，今後

の臨床への応用が期待される．

当院の加藤ら[10]は年齢やウイルス量によって，B型肝炎治療のステージ分類（表3）を作成し，治療法の選択に使用している．HBs抗原，HBe抗原，HBV－DNA，ALT，年齢によってステージングを行い，発がんリスクを考慮して，ラミブジン，IFNなどの治療の適否を示している．しかし，これらは新たな抗ウイルス薬が使用可能になるまでの暫定的な治療選択であり，今後，選択肢が広がる可能性が十分考えられる．

おわりに

B型急性肝炎では，その多くが一過性感染で，無治療にて軽快する．まれに重症化や劇症化を引き起す．B型慢性肝炎急性増悪では，セロコンバージョンなど，一過性の悪化で改善する場合もあるが，一部に重症化や劇症化を起す症例を認める．どちらも治療の最大の目的は重症化や劇症化を未然に防ぎ，肝不全を引き起さないことである．そして，不幸にして，肝不全となったときには，肝移植を含めた集学的治療を行うことが重要である．今後，新たな抗ウイルス薬の開発により，B型肝炎に対する治療法は大きく変化する可能性が考えられる．

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加藤道夫

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B型慢性肝炎の病態をどう把握し，治療方針を立てるか？

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はじめに●

B型肝炎ウイルス(HBV)キャリアはHBe抗原陽性無症候性キャリアから慢性肝炎，肝硬変，肝細胞癌あるいは臨床的治癒とされているHBe抗体陽性無症候性キャリアまでさまざまな病態が存在する．そして，その経過もさまざまであるが，大別すると肝硬変，肝細胞癌に進行する群と臨床的治癒の状態に落ち着く群に2分される．これらHBVキャリアのそれぞれが現在どの病期にいるのか，発癌リスクはどの程度であるのか，積極的な治療の必要性はあるのか，そしてあるならどのような治療を選択すべきかという問いに対処するため，われわれはHBVキャリアのステージ分類を提唱した[1]．

HBVキャリアのステージ分類●

1995年11月以降に当院を初診したHBVキャリア207例を対象にHBVキャリアを8ステージに分類した(**表1**)．対象の性別は男性138例，女性69例で，平均年齢はそれぞれ44.3±13.4歳，42.8±15.6歳であった．

HBステージ0：HBs抗原陽性，HBe抗原陽性，ALT正常値持続のいわゆる無症候性キャリアの状態．

HBステージI：HBs抗原陽性，HBe抗原陽性，ALT異常値(持続正常以外)でHBV-DNA量が$10^{7.6}$copies/mL以上の高ウイルス群．若年例(男性：30歳未満，女性：35歳未満)をステージIa，高年例(男性：30歳以上，女性：35歳以上)をステージIbとする．

HBステージII：HBs抗原陽性，HBe抗原陽性，ALT異常値(持続正常以外)でHBV-DNA量が$10^{7.6}$copies/mL未満の低ウイルス群．若年例をステージIIa，高年例をステージIIbとする．

HBステージIII：HBs抗原陽性，HBe抗原陰性，HBV-DNA10^5copies/mL以上のプレコア変異株の増殖が持続していると考えられる群である．

HBステージIV：HBs抗原陽性，HBe抗原陰性，HBV-DNA10^5copies/mL未満のいわゆる臨床的治癒の状態である．

HBステージV：HBキャリア(HBs抗原陽性の時期が確認されている例)でHBs抗原が消失した状態である．

各ステージの例数，性別，平均年齢，ALT値，血小板数および発癌率は**表2**に示す．HBe抗原

表1　HBVキャリアのステージ分類

HBステージ	0	I	II	III	IV	V
HBsAg	＋	＋	＋	＋	＋	−**
HBeAg	＋	＋	＋	−	−	−
HBV-DNA (copies/mL)	不問	$10^{7.6}$≦	$10^{7.6}$＞	10^5≦	10^5＞	不問
ALT	持続正常	持続正常以外	持続正常以外	不問	不問	不問
年齢	不問	若年/高年* (Ia/Ib)	若年/高年* (IIa/IIb)	不問	不問	不問
発癌リスク	きわめて小	小/大	小/きわめて大	きわめて大	きわめて小	きわめて小

*若年：男性30歳未満，女性35歳未満
高年：男性30歳以上，女性35歳以上
**HBsAg(＋)の時期が確認されていること

1345-1551/06/¥100/頁/JCLS

◎臨床的治癒コースはステージ Ia から IIa となり，速やかにステージ IV に移行する．
◎病態進展コースはステージ Ia から Ib，IIb と進行し，
III までは到達するが IV には至らない．
◎ステージ III とステージ IV は時間的経過の差ではなくて，
病態の異なる集団である．

表 2　各 HB ステージの背景因子と発癌率

HB ステージ	0	Ⅰa	Ⅰb	Ⅱa	Ⅱb	Ⅲ	Ⅳ
例数(%)	9(4.3)	23(11.1)	44(21.3)	10(4.8)	31(15.0)	49(23.7)	41(19.8)
性別(男性/女性)	3/6	16/7	32/12	4/6	24/7	38/11**	21/20**
年齢(歳)	34.4±9.1	25.5±3.4	44.8±11.0	24.0±2.5	48.5±9.8	53.1±9.7**	45.6±15.7**
ALT(IU/L)	17.7±4.4	129.0±101.4	193.6±204.2	105.6±80.3	130.5±194.2	117.2±112.3***	41.0±39.7***
血小板数($\times 10^4$)	20.4±4.2	20.1±3.6	16.5±6.2	18.1±4.3	15.4±7.9	14.4±5.9***	19.3±7.5***
初診時発癌(−/+)	9/0	23/0	44/0	9/1	24/6	39/10	35/6
初診後発癌例	0	0	3	0	4	9	1
発癌率(%)	0	0	6.8	0	16.7	23.1*	2.9*

* $p<0.05$, ** $p<0.01$, *** $p<0.001$

陰性期のステージ III とステージ IV を比較すると，平均年齢はステージ IV が有意($p<0.01$)に若年齢であり，性別は女性は有意($p<0.01$)にステージ III 例で少数であった．また，ALT 値はステージ IV が有意($p<0.001$)に低値であった．ステージ III とステージ IV はステージ III からステージ IV へと移行するという時間的経過の差ではなくて，病態の異なる集団と考えられる．HBV キャリアの大多数が歩む臨床的治癒の状態へのコースはステージ Ia からステージ IIa となり，その後短期間ステージ III を経由した後速やかにステージ IV に移行するものと考えられる．そしてステージ IV が長期間続いた後 HBs 抗原が消失し，ステージ V となる．一方，肝硬変進展・肝癌発癌ハイリスク群はステージ Ia からステージ Ib，ステージ IIb と進行し，HBe 抗原が陰性化してステージ III までは到達するが HBV の増殖は持続し，ステージ IV に至ることはない(図 1)．臨床的治癒コースの各ステージにおける初診時の血小板数と発癌リスクは，ステージ 0，Ia，

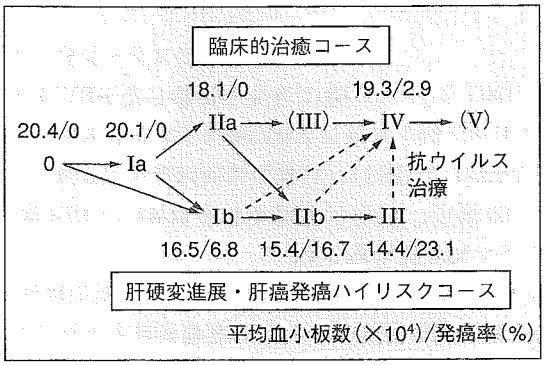

図 1　HBV キャリアの経過(臨床的治癒コースと肝硬変進展・肝癌発癌ハイリスクコース)

IIa および IV でそれぞれ 20.4 万，0%，20.1 万，0%，18.1 万，0% および 19.3 万，2.9% とほとんど変化を認めないが，肝硬変進展・肝癌発癌ハイリスクコースにあたるステージ Ib，IIb および III ではそれぞれ 16.5 万，6.8%，15.4 万，16.7% および 14.4 万，23.1% とステージの移行に従っての血小板数の低下と発癌率の増加が認められ，ステージ Ib，IIb および III のキャリアに対する抗ウイルス治療の必要性が強く示唆される．

●ステージ Ib，IIb および III は肝硬変進展・肝癌発癌のハイリスクコースである．
●ステージ III では CP，Pre C 両領域ともに変異型が有意に高率である．
●ステージ IV では両領域で野生型の残存率が高く，
ウイルス量の減少による HBe 抗原消失と考えられる．

肝癌発癌例，肝予備能低下例と HB ステージ分類●

各ステージ別の発癌率はステージ 0 0%，ステージ Ia 0%，ステージ Ib 6.8% (3/44)，ステージ IIa 0%，ステージ IIb 16.7% (4/24)，ステージ III 23.1% (9/39)，ステージ IV 2.9% (1/35) であった．ステージ Ib，ステージ IIb およびステージ III は B 型肝癌発癌のハイリスク群で積極的に抗ウイルス治療を行う必要がある．また全発癌例 (初診時有発癌例を含む) における性別および発癌確認時の年齢，ALT 値についてみると，性差は，男性 24.6% (138 例中 34 例)，女性 10.1% (69 例中 7 例) と男性で有意に発癌率が高率 ($p<0.02$) であった．発癌例の年齢分布は 50 歳代が 55.0% と最も多く，60 歳代，40 歳代がそれぞれ 17.5%，15.0% で 40 歳未満は 25 歳と 35 歳の 2 例のみであった．また，発癌確認時の ALT 値は 30IU/L 未満が 6 例 (15.0%)，40IU/L 未満 12 例 (30.0%) および 50IU/L 未満 19 例 (47.5%) と ALT 低値例が約半数を占めた．

また，初診時血小板数 10 万未満例を肝予備能低下例とすると，各ステージ別の肝予備能低下例の割合はステージ 0 0%，ステージ Ia 4.3% (1/23)，ステージ Ib 13.6% (6/44)，ステージ IIa 0%，ステージ IIb 25.8% (8/31)，ステージ III 26.5% (13/49)，ステージ IV 7.3% (3/41) であった．発癌例と同様にステージ Ib，ステージ IIb およびステージ III において肝予備能低下例が高率に認められた．

プレコア，コアプロモーター変異と HB ステージ分類●

対象 207 例中 111 例においてプレコア (PreC) およびコアプロモーター (CP) 変異について検討した．各ステージにおける野生型の割合は PreC 領域，CP 領域でそれぞれ，ステージ 0 100%，75.0%，ステージ Ia 66.7%，33.3%，ステージ Ib 65.2%，34.8%，ステージ IIa 42.9%，14.3%，ステージ IIb 53.3%，13.3%，ステージ III 3.7%，7.4%，ステージ IV 37.5%，31.8% であった．HBe 抗原陰性期でもステージ III とステージ IV では様相が異なり，PreC 領域では野生型と変異型がステージ III ではそれぞれ 1 例，15 例であるが，ステージ IV では 9 例，12 例とステージ III で変異型が有意 ($p<0.05$) に高率であった．また，CP 領域でも野生型と変異型を比較すると，ステージ III ではそれぞれ 2 例，25 例であるが，ステージ IV では 7 例，13 例とステージ III で PreC 領域と同様，変異型が有意 ($p<0.05$) に高率であった．PreC 領域と CP 領域のいずれかが野生株である率はステージ III ではわずか 11.1% (3/27) であったが，ステージ IV では 52.0% (13/25) と過半数を占めた．臨床的治癒期と考えられるステージ IV では両領域で野生型の残存率が高く，ウイルス量の減少によって HBe 抗原が消失した例が多いことを示す成績と考えられる．

HBV genotype と病態との関連●

HBV は分子進化学の発展により A 型から H 型までの 8 種の genotype に分類されている．Orito らのわが国における genotype 分布の解析[2] によると，沖縄と東北地方には genotype B が多く，それ以外の地域では genotype C が大半を占めており，わが国全体の比率としては genotype B が 12.2%，genotype C が 84.7% であった．genotype B は genotype C に比し予後良好と考えられており，PreC 領域と CP 領域の変異の有無についての検討でも，変異型は genotype B の 16% に比し genotype C では 58% と genotype C で有意に高率と報告されている[3]．当院で無作為に抽

●ステージ Ib では若年齢を過ぎても HBV-DNA 量高値が持続し，抗ウイルス薬治療が必要である．
●ステージ IIb 全例とステージ III の ALT 異常男性例は抗ウイルス治療の絶対適応である．
●ステージ III の発癌数は全ステージ中最大で，ALT の正異に関係なく発癌例がみられる．

出したB型慢性肝疾患60例中56例(93.3%)はgenotype Cであり，その他はgenotype A，B，FおよびB+Cが1例ずつであった．大阪でもB型慢性肝疾患の大半はgenotype Cであり，前述のPreC，CP変異とステージ分類との関係もgenotype Cのキャリアにおいての成績と考えられるが，genotype Bのキャリアでは変異型が有意に低値とのことで，HBe抗原陰性期でのステージIIIの比率がきわめて低率ではないかと推察される．

HBステージ分類と抗ウイルス治療の必要性●

ステージIaはステージ0の無症候性キャリアが肝炎期に移行した状態のすべてのHBキャリアが通過する高ウイルスのステージであり，発癌リスクがきわめてまれで通常は抗ウイルス治療の必要はない．しかし，組織学的に線維化ステージがF2以上に進行している例は早期に肝硬変に進展する可能性があり，抗ウイルス治療の適応と考えられる．ALT値が高値を持続する例は通常HBV-DNA量が減少しステージIIaとなるが，ステージIIaからは若年発症のB型肝癌例があり，ALT値持続高値例は抗ウイルス治療の適応となる．Ia，IIaとも薬剤としては若年で免疫応答が良好であるのでインターフェロン(IFN)が第一選択となると考える．ステージIbは若年齢を過ぎてもHBV-DNA量の高値が持続する群で，発癌リスクはステージIIbよりは低頻度であるがリスク大で抗ウイルス治療の必要がある．Suzukiら[4]は多変量解析によって，高ウイルス群であることがYMDD変異株出現に最も寄与する因子であることを報告しており，ラミブジン(ゼフィックス®)単独での治療効果の持続は困難で，エンテカビルなどの抗ウイルス効果の強い薬剤あるいは併用治療が適応になると考えられる．ステージIIbは発癌リスクがきわめて大で抗ウイルス治療の絶対適応である．薬剤はラミブジンなどの核酸アナログ単独あるいはIFN，HBワクチンとの併用の選択が考えられる．ステージIIIの発癌数は全ステージ中最大でALT値の正異に関係なく発癌例がみられる．受診キャリア中の頻度も最大で，全例に対して治療が必要かどうかは今後の検討課題と考えられるが，少なくともALT値異常の特に男性例は絶対適応であろう．薬剤は高年例が大半を占め，ラミブジンの治療効果が良好でYMDD変異株の出現も低率であるため，現在のところラミブジンが第一選択であり，YMDD変異株出現例にはアデホビル(ヘプセラ®)などの他の核酸アナログの併用あるいは切り替えで対応できると考えられる．ステージIVはいわゆる臨床的治癒といわれる病態で，抗ウイルス治療の最終目標である．まれに発癌例を認めるが，治療の対象にはならない．ステージVも非B非C肝癌におけるオカルトB型肝癌の問題も残るが抗ウイルス治療の対象にはならないと考えられる．

おわりに●

B型肝癌発癌抑止のためには，HBVキャリアがどの病期にいるかを診断することが肝要である．われわれが提唱したこのHBステージ分類はその診断に有用と考える．治療適応例には早期に適切な抗ウイルス治療を開始し，発癌例を1名でも減少させたいと考えている．

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Significance of liver negative-strand HCV RNA quantitation in chronic hepatitis C

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***Background/Aims*: Liver negative-strand hepatitis C virus (HCV) RNA is the most direct indicator of active viral replication but has only been examined in a few semiquantitative studies.**

***Methods*: Positive- and negative-strand HCV RNA in the right (R) and left (L) liver lobes was quantified by rTth-based strand-specific real-time polymerase chain reaction for 48 chronic hepatitis C patients.**

***Results*: Close correlations between lobes were seen for positive- and negative-strand amounts ($r=0.950$; $P<0.001$ and $r=0.920$; $P<0.001$, respectively). The ratio of negative to positive strands (median, 0.14 for R and 0.13 for L) varied by 2 log directly in relation to HCV replication assessed by liver negative strands but had no relation to liver positive strands and circulating HCV. Only negative-strand quantitation was inversely correlated with age ($r=-0.322$; $P=0.026$ for R and $r=-0.340$; $P=0.018$ for L), while liver tissues with hepatitis B virus DNA contained larger amounts of each strand. In 27 patients treated with enhanced interferon monotherapy, the amounts of liver negative strands (<4 log copies/100 ng RNA) were the only independent predictor of a sustained virologic response.**

***Conclusions*: Negative-strand quantitation is uniform in the liver and bears distinct relevance to the disease.**

***Keywords*: Negative-strand HCV RNA; HCV replication; Chronic hepatitis C**

1. Introduction

Hepatitis C virus (HCV) replication, like that of other single-strand, positive-sense RNA viruses, is presumably preceded by the synthesis of negative-strand RNA. Thus, the amounts of negative-strand RNA-replicative intermediates in liver tissues should serve as a more reliable marker of active viral replication than positive-strand HCV RNA in the liver or in circulation. Serum HCV loads are affected by replication within the liver and extrahepatic sites and by immunologic clearance of the virus. The detection of liver positive-strand (genomic) HCV RNA can simply imply contamination by such circulating virions. Thus far, only a few semiquantitative studies have been done on the clinical relevance of liver negative-strand HCV [1–4], and controversy remains. Patients with chronic hepatitis C can show uneven distribution of liver injury, but intrahepatic variation of HCV replication also remains to be clarified. To further address these issues, we quantitatively analyzed positive- and negative-strand HCV RNA in each liver lobe by strand-specific real-time polymerase chain reaction (PCR) using rTth.

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2. Patients and methods

2.1. Patients

Forty-eight patients with chronic hepatitis C underwent laparoscopic liver biopsies. All patients were positive for serum HCV RNA (Amplicor HCV Test, Roche Diagnostics K.K., Tokyo, Japan). No confounding etiology of liver disease was found in any patient. They were negative for hepatitis B surface antigen in the serum. The group was comprised of 25 men and 23 women ranging in age from 33 to 70 years (median, 57 years). Sixteen (33%) patients had a history of blood transfusion 8–52 years (median, 36 years) earlier. Biopsies were performed using 13-gauge Tru-Cut needles (Hakko Medical Co., Ltd, Nagano, Japan), and liver tissues sufficient for histologic and virologic evaluation were obtained from the anterior segment of the right lobe and the lateral segment of the left lobe. Specimens 15 mm long and 2 mm wide were embedded in paraffin for histopathological study. The remaining portions were immediately frozen and then stored at −80 °C until PCR testing. With one patient, the specimen from the left lobe was subjected to only virologic evaluation due to its limited size. Paired serum samples were obtained from all patients at laparoscopy and stored at −80 °C without thawing until virologic tests. Of the 48 patients, 27 (Table 1) were treated with enhanced interferon (IFN) monotherapy. After laparoscopy, 3 MU of IFN-β (Feron, Toray Co., Tokyo, Japan) was administered twice a day for 2 weeks followed by 9 MU of IFN-α (Sumiferon, Sumitomo Pharm. Co., Osaka, Japan) daily for 2 weeks and thrice weekly for 20 weeks. The study was approved by the local research ethics committee in accordance with the 1975 Declaration of Helsinki, and all patients provided written informed consent.

2.2. Virologic testing

Circulating HCV genomic RNA was quantified by a PCR assay (Amplicor HCV Monitor Test version 2.0, Roche Diagnostics K.K.). HCV RNA of ≧6.4 log copies/mL was measured after serum dilution. HCV genotypes were determined by a PCR genotyping system [5].

2.3. Positive- and negative-strand HCV RNA quantitation by rTth-based strand-specific real-time reverse-transcription polymerase chain reaction (RT-PCR)

Strand-specific TaqMan RT-PCR was designed to quantify the 5′ untranslated region of the HCV genome using a thermostable enzyme, rTth (Applied Biosystems, Foster City, CA). Total hepatic RNA, 100 ng, was added to an RT reaction mixture containing 2 μL of 10× RT buffer (Applied Biosystems), 20 nmol of $MnCl_2$, 5 U of rTth, 24 U of RNasin (Promega, Madison, WI), 4 nmol of each dNTP, and 10 pmol of sense primer HCV-20F (5′-CGACACTCCACCATGAATCACT-3′) for the negative-strand assay or antisense primer HCV-114R (5′-GAGGCTG-CACGACACTCATACT-3′) for the positive-strand assay. The RT reaction was performed in a final volume of 20 μL at 70 °C for 60 min. The reaction

Table 1
Baseline patient characteristics before IFN therapy

		n
Age	<50	11 (41%)
	≥50	16 (59%)
Sex	Male	16 (59%)
	Female	11 (41%)
Transfusion history	+	9 (33%)
	−	18 (67%)
ALT	<2×ULN	17 (63%)
	≥2×ULN	10 (37%)
Liver histology		
Grading score[a]	<7	17 (63%)
	≥7	10 (37%)
Staging score[a]	<4	19 (70%)
	≥4	8 (30%)
Between-lobe grade discrepancy	+	10 (38%)
	−	16 (62%)
Between-lobe stage discrepancy	+	9 (35%)
	−	17 (65%)
HCV genotype	1	21 (78%)
	2	6 (22%)
Serum HCV RNA	<5.4 log copy/mL	6 (22%)
	≥5.4 log copy/mL	21 (78%)
+Strand[a]	<5 log copy/100 ng RNA	6 (22%)
	≥5 log copy/100 ng RNA	21 (78%)
−Strand[a]	<4 log copy/100 ng RNA	9 (33%)
	≥4 log copy/100 ng RNA	18 (67%)
−/+ Strand ratio[a]	<0.1	14 (52%)
	≥0.1	13 (48%)
Between-lobe+strand discrepancy	+	4 (15%)
	−	23 (85%)
HBV antibody (anti-HBc and/or anti-HBs)	+	7 (26%)
	−	20 (74%)
Liver HBV DNA	+	2 (7%)
	−	25 (93%)

[a] Mean values of right and left liver lobes.

was then treated with 5 μL of 10× chelating buffer (Applied Biosystems), 75 nmol of $MgCl_2$, 10 nmol of each dNTP, 10 pmol of antisense primer HCV-114R for the negative-strand assay or sense primer HCV-20F for the positive-strand assay, and 5 pmol of TaqMan probe HCV-P43 (5′FAM-CCCTGTGAGGAACTACTGTCTTCAC-GCAGATAMRA3′). The final volume was adjusted to 50 μL. The samples were promptly set in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) and incubated at 70 °C for 2 min and then at 94 °C for 2 min. Real-time PCR amplification and data analysis were subsequently performed for 45 cycles (94 °C for 20 s and 62 °C for 1 min). Copy numbers of the 95-base target sequence were determined using the standard curve based on measurements of serial 10-fold dilutions of synthetic positive- and negative-strand HCV RNA. The sensitivity was 2 log copies/reaction for the positive-strand assay and 3 log copies/reaction for the negative-strand assay. The dynamic ranges were 2–7 log copies/reaction and 3–7 log copies/reaction, respectively. In each assay, false detection of an incorrect strand occurred when the amount of incorrect strand added reached 7 log copies. The positive- and negative-strand quantitation before normalization was ≦6.4 and ≦5.9 log copies/100 ng liver RNA, respectively, in this study. Thus, the strand-specificity was unlikely to be affected by an excess of incorrect strands. Self-priming or endogenous priming was ruled out by the lack of amplification product following RT-PCR of total hepatic RNA without primers in the RT mixture. All assays were done in duplicate, and the mean values were obtained. Hepatic RNA samples from the same liver were always measured in the same run.

The HCV-specific primers and probe used are conserved among genotypes. To verify that HCV genotypes 1b, 2a and 2b could be quantified with similar efficiency, high-concentration serum samples of each genotype were obtained from eight patients and diluted to 4.4 log copies/reaction by Amplicor HCV Monitor version 2.0, which is known to equally amplify all genotypes. The positive-strand HCV quantitation by the TaqMan RT-PCR was the same for genotypes 1b (5.3±0.7), 2a (5.5±0.4) and 2b (4.9±0.5 log copies/reaction) ($P=0.141$ by one-way analysis of variance).

2.4. Normalization of hepatic HCV RNA amounts and criteria for between-lobe discrepancies

GAPDH mRNA in total hepatic RNA, 100 ng, and control total RNA (Raji cell line), 100 ng, was also quantified by real-time RT-PCR, and copy numbers were determined using the standard curve (Human GAPD Endogenous Control, Applied Biosystems). Hepatic HCV RNA and GAPDH mRNA quantitation, which were performed in separate tubes, showed a linear relationship with the amounts of target RNA (Fig. 1). The HCV RNA copy number was divided by the ratio of the sample GAPDH mRNA amounts to the TaqMan control value. Thus, normalized hepatic HCV RNA amounts were obtained and used for data analysis. In preliminary experiments, assay variance for the $\log_{10}$ transformed HCV RNA quantitation before normalization was evaluated based on five measurements of 10 liver samples (intra-assay coefficients of variation (CVs)=0.88–2.85% and inter-assay CVs=1.19–6.91% for the positive-strand assay; intra-assay CVs=2.27–9.72% and inter-assay CVs=1.52–18.11% for the negative-strand assay). Assay variance was greater for the negative-strand assay, which may be attributable to interfering factor(s) such as a large amount of positive strands in the RT reaction. The mean SDs of intra-assay variance were 0.106 and 0.081 for <5 and ≧5 log copies, respectively, in the positive-strand assay, whereas they were 0.374, 0.256 and 0.158 for <4, 4–5 and ≧5 log copies, respectively, in the negative-strand assay. The HCV RNA quantitation was assumed to vary within twice these SDs. Between-lobe HCV RNA differences were considered significant when the normalized HCV RNA amounts differed by more than the estimated variance for normalized values. All discrepancies were confirmed by repeating the assays.

2.5. Detection of liver hepatitis B virus (HBV) DNA by nested PCR

Total hepatic DNA, 100 ng, was subjected to nested PCR to amplify HBV DNA. The primers were set in the surface region (outer sense 5′-TCGTGTTACAGGCGGGGTTT-3′; outer antisense 5′-CGAACCACT-GAACAAATGGC-3′; inner sense 5′-CAAGGTATGTTGCCCGTTTG-3′; inner antisense 5′-GGCACTAGTAAACTGAGCCA-3′) and the X region (outer sense 5′-GCATGGAGACCACCGTGAA-3′; outer antisense 5′-CAGACCAATTTATGCCTACAG-3′; inner sense 5′-TACATAAGAG-GACTCTTGGACT-3′; inner antisense 5′-CAGACCAATTTATGCCTA-CAG-3′). PCR products (233 and 151 bp, respectively) were visualized by 3% agarose electrophoresis and ethidium bromide staining. All assays were done in duplicate. The sensitivity was 1 copy/100 ng liver DNA for each primer set. To avoid contamination in all PCR assays, the contamination avoidance measures [6] were strictly applied throughout the study, and positive and negative controls were used.

2.6. Histologic evaluation

After routine staining with hematoxylin–eosin, all liver biopsy specimens were examined by the same experienced pathologist without knowledge of their source. Biopsy specimens were semiquantitatively

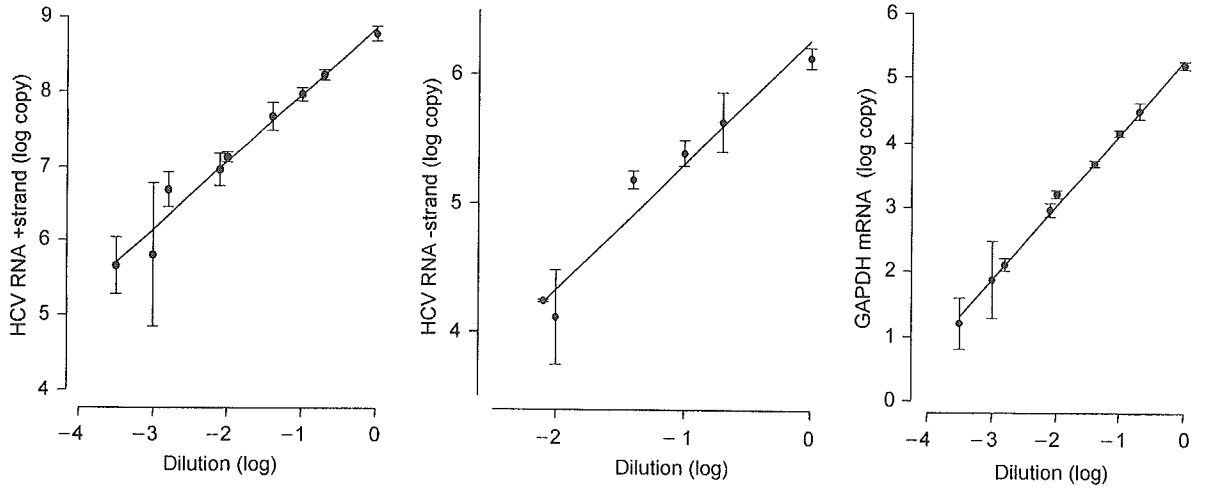

Fig. 1. Changes in hepatic HCV RNA and GAPDH mRNA quantitation in separate tubes according to the amounts of target RNA. Serial dilutions of total hepatic RNA, 100 ng, were subjected to real-time RT-PCR, and copy numbers were determined using the standard curve. The copy number (log) showed a linear relationship ($P<0.001$) with the amounts of target RNA expressed as dilution (log) ($y=8.86+0.90x$ [$n=9$, $r=0.985$] for positive-strand HCV RNA, $y=6.27+0.96x$ [$n=6$, $r=0.976$] for negative-strand HCV RNA and $y=5.27+1.13x$ [$n=9$, $r=0.998$] for GAPDH mRNA). Data are the mean±2SD of triplicate measurements. Pearson's correlation test was performed to examine the relationship.

evaluated using the modified histologic activity index described by Ishak et al. [7].

2.7. Statistical analysis

Viral load was $\log_{10}$ transformed to obtain a more symmetrical distribution without outliers. An arbitrary value of 0 log copy/100 ng liver RNA was attributed to the liver tissues negative by PCR. Data on continuous variables were presented as mean ± SD unless otherwise stated. Statistical analysis for group comparisons was performed using the Wilcoxon nonparametric test. Correlations between the variables were calculated using Spearman rank order correlations. To assess variables potentially related to virologic and histologic between-lobe discrepancies and responses to IFN, stepwise multivariate logistic regression models were used. All analyses were done with SAS (version 8.02) (SAS Institute, Inc., Cary, NC). A P value of less than 0.05 (2-tailed) was considered to indicate significance.

3. Results

3.1. Amounts of positive- and negative-strand HCV RNA in right (R) and left (L) liver lobes

Normalized positive-strand HCV loads in the right liver lobe (median, 5.9; range, 2.5–8.5 log copies/100 ng liver RNA) showed a correlation with those in the left liver lobe (median, 6.0; range, negative to 6.8 log copies/100 ng liver RNA) ($r=0.950$; $P<0.001$) (Fig. 2A). Six (13%) of the 48 patients had a between-lobe discrepancy of 0.3–2.2 log. The discrepancy was related to gender (6 [26%] of 23 women vs. none of 25 men) (odds ratio 10.9 [95% CI 1.3–90.9], $P=0.027$). Fig. 2B shows a correlation between normalized negative-strand HCV loads in the right lobe (median, 4.9; range, negative to 7.2 log copies/100 ng liver RNA) and the left lobe (median, 5.0; range, negative to 6.3 log copies/100 ng liver RNA) ($r=0.920$; $P<0.001$). A discrepancy of 2.0 log was seen in one (2%) patient (Table 2).

In 38 patients with detectable levels of positive and negative strands in each liver lobe, the ratio of negative- to positive-strand HCV (median, 0.14; range, 0.01–0.81 for R and median, 0.13; range, 0.03–0.45 for L) increased according to negative-strand liver HCV ($r=0.282$; $P=0.086$ for R and $r=0.441$; $P=0.006$ for L) (Fig. 3). The ratio showed no correlation with positive-strand liver HCV ($r=-0.192$; $P=0.248$ for R and $r=-0.097$; $P=0.564$ for L) and circulating HCV ($r=0.154$; $P=0.355$ for R and $r=0.106$; $P=0.527$ for L). Serum HCV RNA loads ranged between 3.1 and 7.6 log copies/mL (median, 6.1), and displayed a relation to the positive-strand liver HCV quantitation ($r=0.604$; $P<0.001$ for R and $r=0.634$; $P<0.001$ for L) and the negative-strand liver HCV quantitation ($r=0.632$; $P<0.001$ for R and $r=0.609$, $P<0.001$ for L).

3.2. Determinants of positive- and negative-strand HCV RNA amounts in the liver

The amounts of positive- and negative-strand HCV in each liver lobe were correlated with patient characteristics including age, gender, mode of infection, duration of infection estimated from years after blood transfusion, serum alanine aminotransferase (ALT) levels, histologic grade and stage, HCV genotypes and detection of HBV DNA in the corresponding liver lobe. An inverse correlation was found between the negative-strand liver HCV

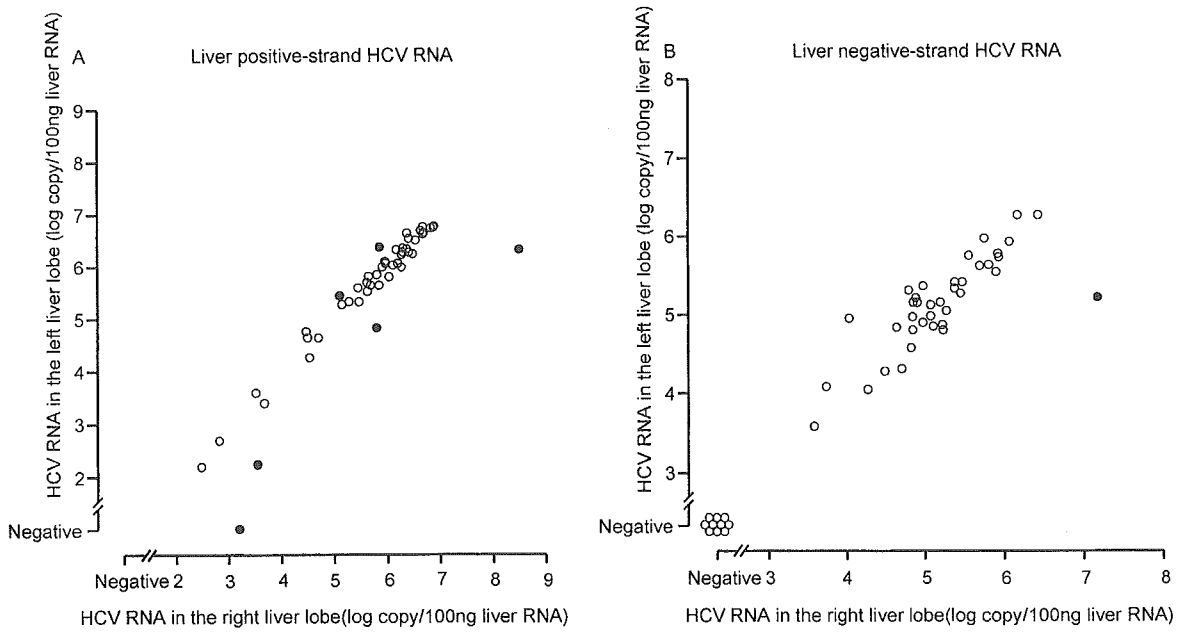

Fig. 2. Correlations between positive-strand HCV RNA levels of the right and left liver lobes ($r=0.950$; $P<0.001$) (A) and between negative-strand HCV RNA levels of each liver lobe ($r=0.920$; $P<0.001$) (B) in the 48 chronic hepatitis C patients. Between-lobe discrepancy of the viral loads was found in six (13%) patients for positive-strand HCV RNA and one (2%) patient for negative-strand HCV RNA (closed circles).

Table 2
Normalized positive- and negative-strand HCV RNA amounts in the right and left liver lobes in 48 chronic hepatitis C patients

Patient	Age (years)	Positive-strand HCV RNA[a]		Negative-strand HCV RNA[a]		HBV DNA[b]	
		Right	Left	Right	Left	Right	Left
1	62	5.96	6.08	5.23	4.80	–	–
2	47	6.88	6.73	6.18	6.26	–	–
3	66	4.48	4.74	3.59	3.58	–	–
4	52	6.68	6.60	5.90	5.53	–	–
5	68	6.38	6.62	5.28	5.04	–	–
6	49	6.04	5.79	4.98	4.89	–	–
7	64	6.11	6.00	4.99	5.36	–	–
8	49	6.54	6.48	5.94	5.72	–	–
9	54	8.51	6.30[c]	7.18	5.20[c]	–	–
10	46	6.30	6.26	5.23	4.86	–	–
11	67	4.49	4.63	4.28	4.04	–	–
12	57	5.80	5.83	5.08	4.97	–	–
13	53	4.53	4.25	–	–	–	–
14	48	5.69	5.62	4.83	4.57	–	–
15	48	5.97	6.04	4.91	5.15	–	–
16	57	5.48	5.30	4.71	4.30	–	–
17	33	6.89	6.76	6.08	5.92	–	+
18	57	5.65	5.79	4.64	4.83	–	–
19	69	3.68	3.38	–	–	–	–
20	64	6.41	6.26	5.11	4.84	–	–
21	59	6.20	6.04	5.38	5.40	–	–
22	56	5.86	6.36[c]	5.76	5.97	–	–
23	67	5.45	5.57	4.86	5.15	–	–
24	38	6.28	6.20	5.81	5.63	–	–
25	48	2.49	2.18	–	–	–	–
26	60	2.83	2.68	–	–	–	–
27	48	5.28	5.30	4.04	4.95	–	–
28	68	5.80	4.80[c]	–	–	–	–
29	44	5.62	5.68	4.85	4.96	–	–
30	43	3.20	–[c]	–	–	–	–
31	58	6.70	6.64	5.46	5.26	–	–
32	56	6.63	6.67	6.43	6.26	–	–
33	45	6.28	5.97	5.20	5.15	–	–
34	50	6.36	6.32	5.93	5.77	+	+
35	63	5.11	5.43[c]	4.49	4.28	–	–
36	70	4.71	4.62	–	–	–	–
37	52	6.83	6.72	4.80	5.30	–	–
38	41	3.56	2.23[c]	–	–	–	–
39	51	6.41	6.52	5.70	5.62	+	+
40	67	5.64	5.51	–	–	–	–
41	61	3.52	3.57	–	–	–	–
42	52	5.91	5.97	5.08	5.11	–	–
43	59	6.18	6.30	5.38	5.32	–	–
44	57	6.48	6.23	5.48	5.40	–	–
45	67	5.15	5.26	3.75	4.08	–	–
46	58	6.68	6.73	5.56	5.75	+	+
47	62	6.30	6.34	4.89	5.20	–	–
48	66	5.85	5.63	4.85	4.79	–	–

[a] Hepatic HCV RNA amounts were normalized to GAPDH mRNA amounts and expressed as log copy/100 ng liver RNA.
[b] Liver HBV DNA was detected by nested PCR using two sets of primers in the surface and X regions, respectively.
[c] Between-lobe differences in the normalized HCV RNA amounts were considered significant according to the intra-assay variance-based criteria.

quantitation and age ($r=-0.322$; $P=0.026$ for R and $r=-0.340$; $P=0.018$ for L). The positive-strand liver HCV quantitation, however, had no relation to age ($r=-0.237$; $P=0.104$ for R and $r=-0.216$; $P=0.140$ for L) (Fig. 4). The amounts of positive- and negative-strand liver HCV did not differ between 38 patients with HCV genotype 1b and 10 patients with genotype 2 (seven with genotype 2a and three with genotype 2b), but were affected by concomitant liver HBV. By using X primers, HBV DNA was detected in both liver lobes in three patients and only in the left lobe in another patient. None of the patients tested positive for liver HBV DNA using surface primers. The four

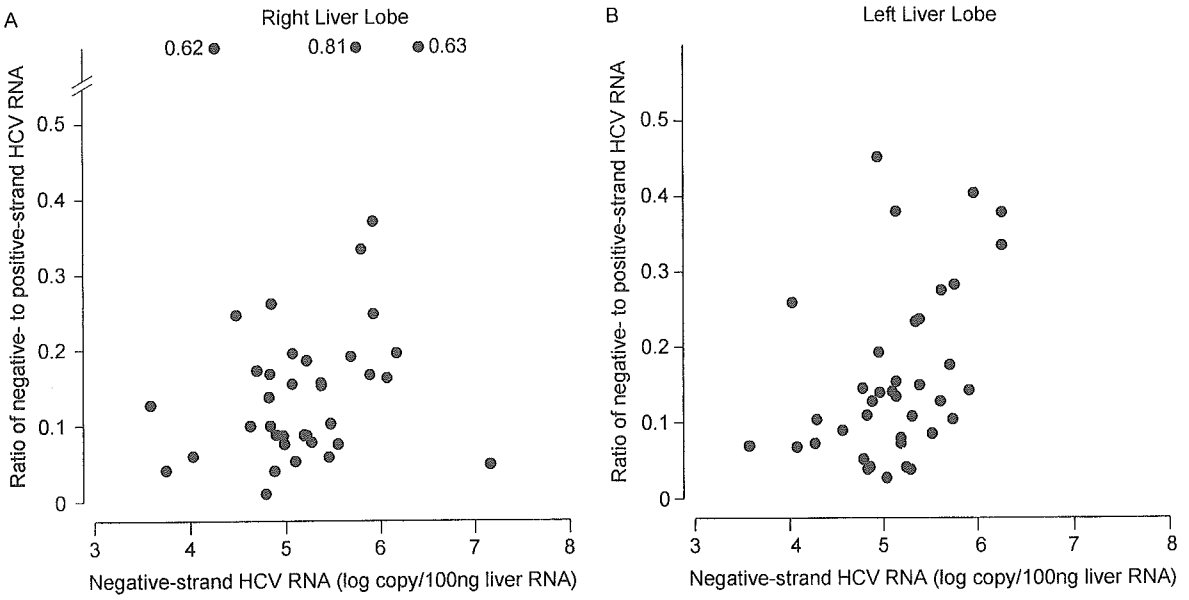

Fig. 3. Direct variation of ratios of negative- to positive-strand HCV RNA in liver tissues in relation to HCV replication, assessed by negative-strand HCV RNA in the right liver lobe ($r=0.282$; $P=0.086$) (A) and the left liver lobe ($r=0.441$; $P=0.006$) (B).

HBV DNA-positive liver tissue samples from the left lobe contained larger amounts of positive and negative strands than the 44 HBV DNA-negative tissues (6.6±0.2 vs. 5.4±1.4; $P=0.007$ and 5.8±0.1 vs. 3.9±2.2 log copies/100 ng liver RNA; $P=0.006$, respectively). For the right liver lobe, the positive- and negative-strand liver HCV quantitation also tended to be high in the three HBV DNA-positive liver tissues (6.5±0.2 vs. 5.6±1.2; $P=0.081$ and 5.7±0.2 vs. 4.0±2.3 log copies/100 ng liver RNA; $P=0.049$, respectively). None of the patient characteristics examined showed a relationship to the ratio of negative- to positive-strand HCV and serum HCV RNA load.

3.3. Histologic variation between right and left liver lobes

The total necroinflammatory grade ranged between 2 and 10 (median 7) in each liver lobe ($P=0.295$ by signed rank test). The fibrosis stage ranged from 1 to 6 (median 4) in the right lobe and from 2 to 6 (median 3) in the left lobe ($P=0.614$). Fig. 5 shows the histologic between-lobe variation among the 47 patients studied. Eleven (23%) patients showed differences of the necroinflammatory grade defined as a difference of ≧2 points, and 19 (40%) patients of the fibrosis stage defined as difference of ≧1 point. The between-lobe variation in the HCV quantitation had no impact on the histologic variation. The mean grading score of the right and left liver lobes was <7 in 10 (91%) out of the 11 patients with a grade difference compared with 16 (44%) out of the 36 patients without it (odds ratio 6.5 [95% CI 1.3–33.3], $P=0.025$). The difference in the fibrosis stage, however, had no relation to any of the patient characteristics examined.

3.4. Factors influencing the efficacy of IFN treatment

Eighteen (67%) out of the 27 patients were negative for serum HCV RNA at the end of treatment, and eight (30%) patients displayed sustained HCV clearance over 6 months posttreatment. The end-of-treatment virologic response was independently associated with an absence of between-lobe discrepancy of the necroinflammatory grade (odds ratio 0.2 [95% CI 0–0.9], $P=0.042$). However, the amounts of negative-strand HCV RNA in the liver were identified as the only independent predictor of a sustained virologic response. The mean negative-strand quantitation of the right and left liver lobes was <4 log copies/100 ng liver RNA in all sustained virologic responders (SVRs) compared with 1 (5%) of the 19 non-SVRs (odds ratio 85.4 [95% CI 5.4–999], $P=0.002$).

4. Discussion

Little has been known about the clinical significance of quantifying negative-strand RNA-replicative intermediates in the liver. The present study analyzed the ratio of liver negative- to positive-strand RNA. This ratio is the most reliable parameter since it does not depend on genotypes or normalization to the cellular GAPDH mRNA quantitation. For each liver lobe, the median ratio of 0.1 was similar to that found with cell-based HCV replicon systems [8]. Importantly, it was disclosed that the ratio was not constant but varied by 2 log values in relation to the intrahepatic HCV-replicative status. These observations suggest that the negative-strand quantitation is not merely a reflection of

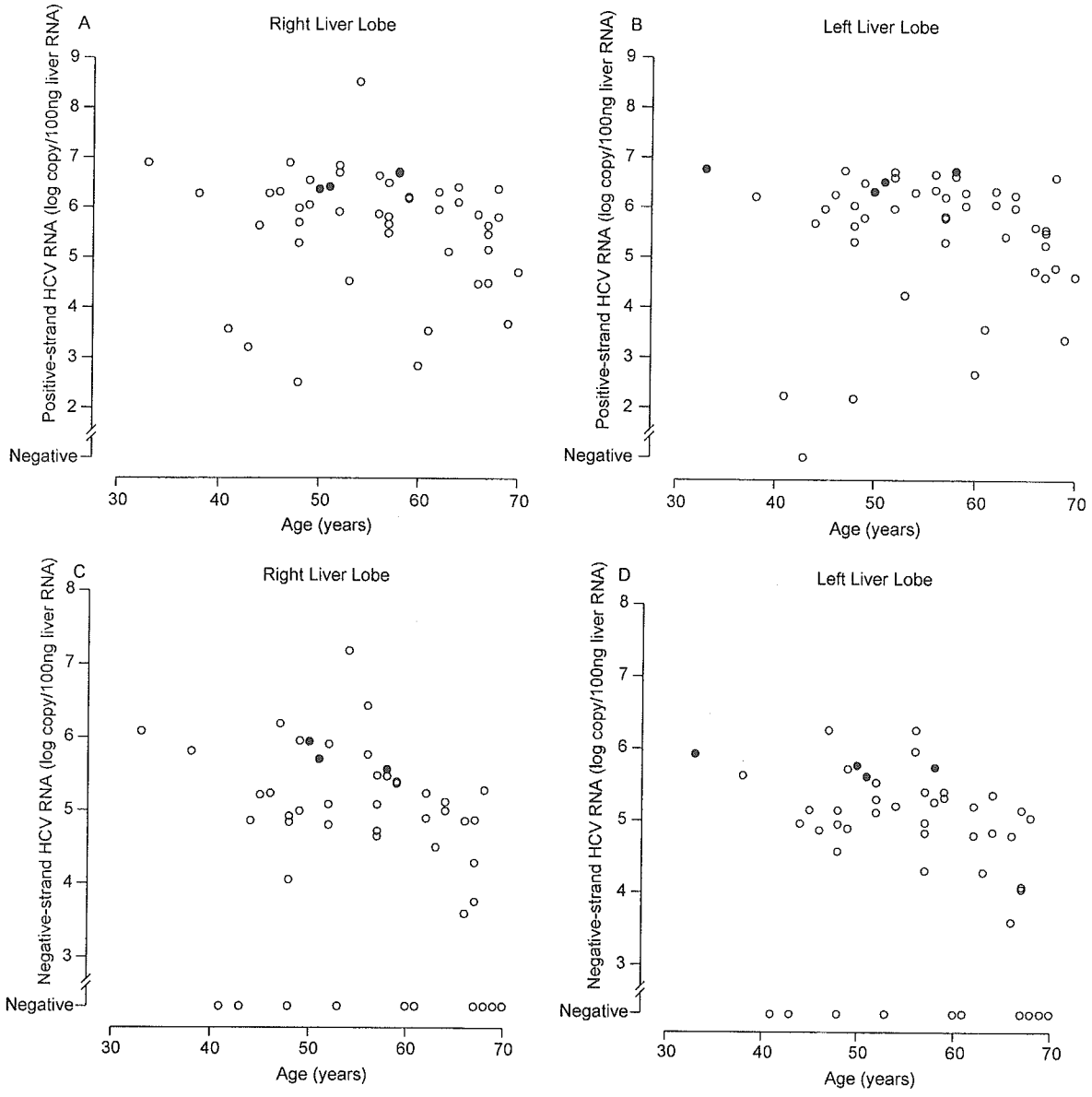

Fig. 4. Liver HCV RNA levels in relation to age and occult HBV infection in 48 chronic hepatitis C patients. No relationship was evident between age and positive-strand HCV RNA levels of the right liver lobe ($r=-0.237$; $P=0.104$) (A) and the left liver lobe ($r=-0.216$; $P=0.140$) (B), whereas inverse correlations were found between age and negative-strand HCV RNA levels of the right liver lobe ($r=-0.322$; $P=0.026$) (C) and the left liver lobe ($r=-0.340$; $P=0.018$) (D). HBV DNA-positive liver tissues (closed circles) contained higher levels of positive-strand HCV RNA ($P=0.081$ for the right liver lobe and $P=0.007$ for the left liver lobe) and negative-strand HCV RNA ($P=0.049$ for the right liver lobe and $P=0.006$ for the left liver lobe).

liver positive strands but should serve as a distinct HCV replicative marker.

Chronic hepatitis C is known as a disease with uneven distribution of lesions in the whole liver [9]. Previous studies have shown a correlation between positive-strand HCV RNA levels of the right and left liver lobes [9,10]. The present study demonstrated a close correlation between lobes not only for positive strands but also for negative strands. Thus, HCV replication within the liver was shown to be uniform, and a single biopsy seemed generally representative of the whole liver. Although the between-lobe variation of HCV RNA loads should be interpreted with caution when the difference is small, it was only found in women, raising a possibility that sex hormone(s) and sex-linked genetic factor(s) are involved in the heterogeneity of HCV replication. In the present study, the amounts of positive- and negative-strand HCV and the ratio of negative to positive strands showed no correlation with the necroinflammatory grade and the fibrosis stage. However, we must stress the possibility that the HCV replication level, especially that assessed by negative strands, may have some relevance to histologic features such as steatosis [4].

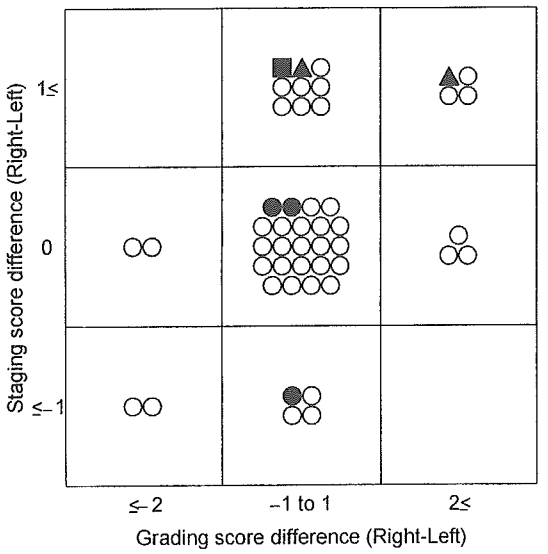

Fig. 5. Histologic and virologic discrepancies between the right and left liver lobes in chronic hepatitis C patients. ○, no between-lobe HCV RNA discrepancy; ●, larger positive-strand amounts in the right lobe; ■, larger positive- and negative-strand amounts in the right lobe; ▲, larger positive-strand amounts in the left lobe.

Factors affecting HCV replication within the liver have been the subject of controversial discussions from the standpoint of the liver and circulating positive strands. Based on the negative-strand level, HCV replication in each liver lobe was shown to be inversely correlated with age. The efficiency of negative-strand RNA synthesis can be influenced by various host factors at multiple levels [11]. The data obtained raise the possibility that some age-related factor(s) may be involved in the regulation of HCV replication within the liver. The present study further showed that liver tissues with concomitant occult HBV contained larger amounts of negative- and positive-strand HCV RNA. Among HCV patients, those carrying occult HBV can manifest severer liver disease and display a poor response to IFN [12]. Occult HBV may also have relevance for hepatocarcinogenesis [13], although the mechanism remains to be clarified. Although further studies are necessary, the data obtained raise the possibility that occult HBV exerts virulence partly by enhancing HCV replication.

As for IFN-based therapy, only limited data are available on the significance of the liver negative-strand HCV RNA quantitation. In a previous semiquantitative study, the negative-strand levels were not related to the outcomes of short-term IFN-α therapy (3 MU thrice weekly for 10 weeks) [2]. Our patients were treated with 6-month enhanced IFN monotherapy [14]. A sustained virologic response was only associated with small amounts of liver negative-strand HCV RNA (<4 log copies/100 ng liver RNA). Based on these preliminary data, further studies are warranted in populations treated with the currently standard regimen of peginterferon and ribavirin.

In conclusion, our findings combined indicate that liver negative-strand HCV RNA quantitation offers clinically relevant information distinct from that available from positive strands within the liver and in the circulation.

Acknowledgements

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Persistence of Acute Infection With Hepatitis B Virus Genotype A and Treatment in Japan

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Among the 97 adult patients with acute hepatitis B who were admitted to the Toranomon Hospital in Metropolitan Tokyo during 28 years from 1976 to 2003, 31 (32%) were infected with hepatitis B virus (HBV) genotype A, nine (9%) with genotype B, 44 (45%) with genotype C, one (1%) each with genotypes E and F. HBV in the remaining 11 (11%) patients were untypeable. All the 31 patients with acute hepatitis B caused by HBV genotype A infection were male with a median age of 31 years, and 16 (52%) contracted infection through extramarital sexual contacts. The baseline HBV DNA level was higher in the seven (23%) patients in whom infection with HBV genotype A persisted than the remaining 24 (77%) with spontaneous resolution (median: >8.7 vs. 6.0 log genome equivalents/ml, $P=0.004$). Persistent infection was more frequent in patients with maximum alanine aminotransferase <500 IU/L than ≥500 IU/L (83% [5/6] vs. 4% [1/25], $P=0.0001$). Of the six patients with persistent HBV genotype A infection who received interferon and/or lamivuidine for treatment of chronic active hepatitis, three (50%) responded with the loss of hepatitis B e antigen (HBeAg); hepatitis B surface antigen (HBsAg) was cleared from serum in one patient who received interferon and lamivudine in sequence. HBV genotype A persisted along with HBeAg in the remaining three patients given antiviral therapy as well as another who was not treated. In conclusion, infection with HBV genotype A prevails in patients with acute hepatitis B in Japan where genotypes B and C are common, is often contracted sexually (16/31 [52%]) and tends to persist (7/31 [23%]). Infection was cleared in only one of the six (17%) patients who received antiviral therapy. ***J. Med. Virol. 76:33–39, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: acute hepatitis; chronic hepatitis; genotypes; hepatitis B virus; interferon; lamivudine

INTRODUCTION

Approximately 350 million people are infected persistently with hepatitis B virus (HBV) throughout the world [Lee, 1997], and most reside in Asia and Africa. There are eight genotypes of HBV, defined by a sequence divergence in the entire genome exceeding 8%, and are designated by capital alphabet letters from A to H in the order of discovery [Okamoto et al., 1988; Norder et al., 1992; Stuyver et al., 2000; Arauz-Ruiz et al., 2002]. Genotypes of HBV have distinct geographical distributions [Magnius and Norder, 1995; Lindh et al., 1997; Miyakawa and Mizokami, 2003], and are associated with the severity of liver disease and responses to antiviral treatment [Chu and Lok, 2002; Kao, 2002; Miyakawa and Mizokami, 2003].

In countries highly endemic for HBV, such as China and Africa, the carrier state is established mainly through horizontal transmission during infancy [Botha et al., 1984; Yao, 1996]. In Europe and the United States where the prevalence of HBV is low, by contrast, persistent infection occurs predominantly by infection in

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adults. Japan is exceptional in that perinatal transmission from infected mothers had been the main route of persistent HBV infection [Okada et al., 1976], before the national immunoprophylaxis program for babies born to carrier mothers was launched in 1986 [Noto et al., 2003].

In Japan, genotypes B and C prevail and together they account for more than 95% of persistent HBV infection [Orito et al., 2001; Kobayashi et al., 2002, 2003]. Acute infection with HBV genotype A keeps increasing, however, particularly in men with extramarital sexual contacts [Kobayashi et al., 2002, 2003, 2004; Ogawa et al., 2002]. Infection with HBV genotype A in the adult has a high propensity to become chronic, which would contribute to the persistent carrier state in Western countries where the perinatal or childhood transmission of HBV is rare [Heijtink et al., 1999; Lindh et al., 2000].

During 28 years from 1976 to 2003, 31 patients were diagnosed with acute hepatitis B who were infected with HBV genotype A at the Toranomon Hospital in the Metropolitan Tokyo. The infection persisted in seven (23%) patients accompanied by biopsy-proven chronic hepatitis B. Their clinical course were followed with a special reference to the response to antiviral treatment.

MATERIALS AND METHODS

Patients With Acute Hepatitis B

During 28 years from August 1976 through September 2003, 97 patients were diagnosed with acute hepatitis B at the Department of Gastroenterology at the Toranomon Hospital in Metropolitan Tokyo. Genotypes of HBV were A in 31 (32%) of them, B in nine (9%), C in 44 (45%), E and F in one (1%) each, while they were untypeable in the remaining 11 (11%). The 31 patients with acute hepatitis B caused by infection with HBV genotype A were followed clinically and examined virologically. Seven of them (23%) developed persistent HBV infection accompanied by chronic hepatitis B, and six received antiviral therapy and evaluated for the response. All the patients possessed IgM antibody to hepatitis B core (anti-HBc) in high titers, but they were negative for antibody to hepatitis delta virus, IgM antibody against hepatitis A virus or antibody to hepatitis C virus. A number of these patients have been reported previously with respect to clinical features [Kobayashi et al., 2003], perpetuation of acute infection [Kobayashi et al., 2002] and shifts of HBV genotypes with time [Kobayashi et al., 2004]. The study protocol conformed to the 1975 Declaration of Helsinki, and was approved by the Ethic Committee of Toranomon Hospital. An informed consent for this study was obtained from each patient.

Serological Markers of HBV Infection

Hepatitis B surface antigen (HBsAg) was determined by hemagglutination (MyCell, Institute of Immunology Co., Ltd., Tokyo, Japan) and hepatitis B e antigen (HBeAg) by enzyme-linked immunosorbent assay (ELISA) with commercial kits (ELISA, F-HBe; Kokusai Diagnostic, Kobe, Japan). Anti-HBc of IgM class was determined by radioimmunoassay (HBc-antiM RIA, Dinabot, IL). HBV DNA was determined by transcription-mediated amplification and hybridization assay (TMA; Chugai Diagnostics, Tokyo, Japan) and the results were expressed as log genome equivalents (LGE) per milliliter of serum, over a detection range from 3.7 to 8.6 LGE/ml.

Genotypes of HBV

The six major genotypes (A–F) were determined by ELISA by the combination of epitopes on preS2-region products by monoclonal antibodies which is specific for each of them [Usuda et al., 1999, 2000] by commercial assay kits (HBV GENOTYPE EIA; Institute of Immunology, Co., Ltd., Tokyo, Japan). Genotype G was determined by the preS2 serotype for genotype D and HBsAg serotype adw [Kato et al., 2001]; the combination is specific for this genotype.

Genetic Subgroups of Genotype A

Subgroups of genotype A designated Ae prevalent in Europe and Aa frequent in Africa, as well as Asia [Sugauchi et al., 2004] that correspond to subgroup A′ reported originally by Bowyer et al. [1997], were determined by the nucleotide (nt) sequence in the S gene specific for each of them [Sugauchi et al., 2003]. Briefly, nucleic acids were extracted from serum and a sequence of the large S gene was amplified by polymerase chain reaction (PCR) with nested primers. The first-round PCR was carried out with BGF1 (sense, 5′-CTG TGG AAG GCT GGC ATT CT-3′ [nt 2,757–2,776]) and BGR2 (antisense, 5′-GGC AGG ATA GCC GCA TTG TG-3′ [nt 1,050–1,079]) primers, and the second-round PCR with PLF5Bm (sense, 5′-TGT GGA TCC TGC ACC GAA CAT GGA GAA-3′ [nt 136–162]) and BR112 (antisense, 5′-TTC CGT CGA CAT ATC CCA TGA AGT TAA GGG A-3′ [nt 865–895]) as well as BGF5 (sense, 5′-TGC GGG TCA CCA TAT TCT TG-3′ [nt 2,811–2,830]) and BGR6 (antisense, 5′-AGA AGT CCA CCA CGA GTC TA-3′ [nt 249–268]) for 35 cycles each (94°C, 1 min [5 min in the first cycle]; 53°C, 2 min; and 72°C, 3 min [7 min in the last cycle]). The amplification products were run on gel electrophoresis and stained with BIG Dye (Applied Biosystems, CA). The they were purified by Qiquick PC purification kit (Qiagen, Hilden, Germany), and sequenced in AGI Prism 310 Genetic Analyzer (Applied Biosystems).

Antiviral and Other Treatment

Patients in whom HBV infection persisted and with chronic hepatitis diagnosed by liver biopsy, received interferon (IFN) or lamivudine, or both in sequence. Natural IFN-α (Smiferon; Sumitomo Pharmaceutical Co., Ltd., Tokyo, Japan) or IFN-β (Feron, Toray Co., Ltd., Tokyo, Japan) in a dose of 3 or 6 mega units (MU) was injected subcutaneously two or three times in week (tiw) for up to 1 year with or without induction by 6 MU

daily for 8 weeks. Lamivudine (Glaxo-Welcome, Greenford, UK) was given orally at a daily dose of 100 mg until HBeAg was lost from serum in responders, and continued indefinitely in non-responders who failed to achieve HBeAg seroconversion. The response to antiviral treatment was defined by the loss of HBeAg from serum accompanied by normalization of ALT levels and clearance of HBV DNA determined by the TMA method with the detection limit of 3.7 LGE/ml.

Some patients had received glycyrrhizin either intravenously (Stronger Neo-Minophagen C [SNMC]; Minophagen Pharmaceutical Co., Ltd., Tokyo, Japan) or orally (GLYCYRON Tab; Minophagen Pharmaceutical Co., Ltd.), with or without oral ursodeoxycholic acid (UDCA; Mitsubishi Welpharmar Co., Ltd., Tokyo, Japan), before they were referred to the Toranomon Hospital.

Statistical Analysis

Categorical variables were compared between groups by the χ^2-test or Fisher's exact test, and non-categorical variables by the Mann–Whitney U-test.

RESULTS

Patients With Acute Hepatitis B Infected With HBV Genotype A

Infection resolved spontaneously in 24 of the 31 patients with acute hepatitis B who were infected with HBV genotype A, while it persisted in the remaining seven (23%) patients none of whom carried human immunodeficiency virus type 1. The persistence of acute infection tended to be more frequent in patients infected with HBV genotype A than those with genotype B (1/9 [11%]), or C (3/42 [7%]); one each patient infected with genotypes E and F cleared infection.

Table I compares demographic, clinical, and virological characteristics between patients in whom HBV infection did and did not persist. All the seven patients with chronic HBV infection were men, and tested negative for HBsAg in serum before they developed acute hepatitis; HBsAg persisted in them during 6 months or longer after they first tested positive for it. Of the 31 patients with acute infection with HBV genotype A, 16 (52%) confided having had extramarital sexual contacts. Homosexual activities were experienced somewhat more frequently in patients with than without persistent HBV infection (4/7 [57%] vs. 5/24 [22%]).

The maximum median ALT level was significantly lower (234 vs. 1,836 IU/L, $P = 0.0001$), while median HBV DNA level was significantly higher (median: >8.7 vs. 6.0 LGE/ml, $P = 0.004$) in patients in whom HBV genotype A infection persisted than in those who cleared it (Table I). None of the eight patients with HBV DNA <5 LGE/ml developed persistent infection. Infection persisted significantly more often in patients with the maximum ALT <500 IU/L than ≥500 IU/L (5/6 [83%] vs. 1/25 [4%], $P = 0.0001$).

Genetic subgroup of genotype A was Ae (the original European type) in all the 20 patients for whom subgrouping was possible. None of them were infected with HBV of subgroup Aa (Asian/African type corresponding to A′ of Bowyer et al. [1997]). Of the seven patients in whom infection persisted, five were infected with HBV of subgroup Ae; subgrouping was not feasible in the remaining two (Cases 2 and 4 in Table II).

Clinical Courses of the Seven Patients in Whom Infection With HBV Genotype A Persisted

Of the seven patients in whom infection with HBV genotype A persisted, six received treatment with IFN and/or lamivudine after transfer to Tornomon Hospital (Table II). They all had chronic hepatitis in the first liver biopsy; it was undertaken before treatment in five (71%). Three of them (50%) responded to treatment with the clearance of HBeAg from serum, normalization of ALT levels and loss of HBV DNA determined by the TMA method with the detection limit of 3.7 LGE/ml. In the remaining four patients, including the single one (Case 7) who did not receive antiviral treatment due to the absence of active hepatitis, infection with HBV genotype A persisted along with HBeAg and fluctuating ALT levels in serum.

Figure 1 depicts clinical courses of the three patients who responded to antiviral treatments. Case 1 received

TABLE I. Baseline Characteristics of Patients With Acute Hepatitis Induced by HBV Genotype A in Whom Hepatitis Persisted or Resolved

Features	Persisted (n = 7)	Resolved (n = 24)	Differences
Male	7 (100%)	24 (100%)	NS[c]
Age (years)[a]	26 (21–54)	33 (25–56)	NS
Sexual transmission	5 (71%)	11 (48%)	NS
Homosexual	4 (57%)	5 (22%)	
Heterosexual	1 (13%)	6 (26%)	
Maximum ALT (IU/L)[a]	234 (143–774)	1,836 (46–3,300)	$P = 0.0001$
HBsAg titer (2^N)[b]	11 (11 to ≥13)	11 (8 to ≥13)	NS
HBeAg-positive	7 (100%)	23 (96%)	NS
HBV DNA (LGE/ml)	>8.7 (6.3 to >8.7)	6.0 (<2.6 to >8.7)	$P = 0.004$

[a]Median values are shown with the range in parentheses.
[b]Determined by the hemagglutination assay on serial twofold dilutions of serum.
[c]Not significant.