1．樹状細胞によるC型肝炎における肝発癌予防

林　紀夫*　巽　智秀*　考藤　達哉*　竹原　徹朗*

はじめに

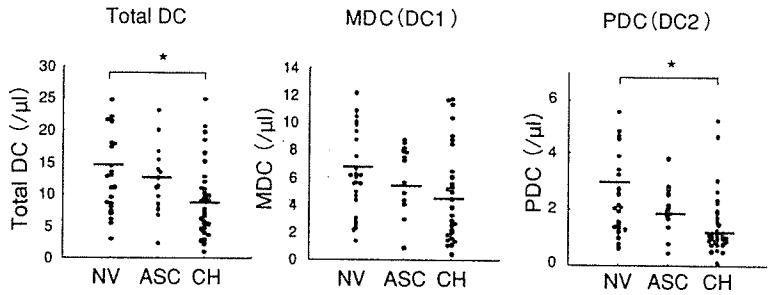
わが国において肝癌による死亡率が急増しており，肝癌の約8割はC型肝炎による肝硬変を基盤に発生する．肝癌死を減らすためには慢性肝炎から肝硬変への進展を抑制することが肝要であり，そのためにはC型肝炎ウイルス（HCV）の排除を目指す必要がある．HCV排除には免疫系の賦活が重要であるが，生体において樹状細胞（DC）やマクロファージといった抗原提示細胞は，免疫細胞の活性化や機能調節を行うことで，ウイルスに対する初期および獲得免疫応答の制御を行っている．本研究ではC型肝炎におけるDC機能の評価とDCを用いた肝発癌予防への知見について述べたい．

Ⅰ．C型肝炎におけるDC機能

DCは生体において最も強力な抗原提示細胞であり，ナイーブT細胞を刺激できる唯一の細胞である．DCは生体内の局在によってその分化・成熟度が異なる．骨髄から血液中に動員されたDC前駆細胞は，生体内の各臓器に広く分布し，ウイルス，細菌感染などの刺激を受けたDCは，分化成熟シグナルが入る．その結果，成熟したDCは二次リンパ組織に移動しそこでT細胞，NK細胞，NKT細胞などの免疫担当細胞との相互作用により免疫応答を誘導すると考えられている．ヒトDCではその系統によりミエロイドDC（Myeloid DC; MDC）とプラズマサイトイドDC（Plasmacytoid DC; PDC）のサブセットに分類される．MDCはLPSやCD40リガンドによる刺激を受けるとIL-12やTNF-αなどのサイトカインを産生し，PDCはウイルス感染に際し多量のⅠ型インターフェロン（IFN）を産生する．われわれはC型慢性肝炎末梢血単球から誘導したDCは，アロT細胞刺激能やIL-12産生能が非感染者に比べて低下していることを既に報告している[1]．

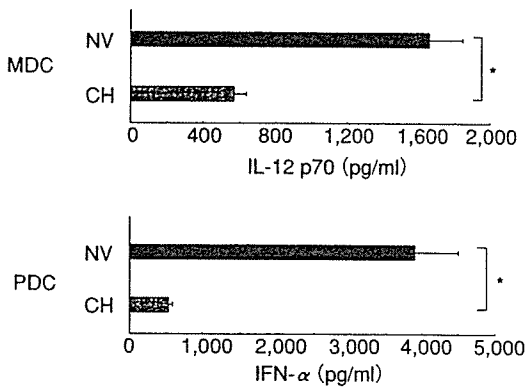
しかし，末梢血単球から*in vitro*で誘導したDCによる解析ではC型肝炎における実際の生体の状況を反映しているのかという疑問が生じることから，末梢血中に存在するMDC，PDCを直接分離し機能解析を行った[2]．まず末梢血中のDCサブセットの頻度･数を検討した．末梢血単核球の中でLineageマーカー（CD3, CD14, CD16, CD19, CD20, CD56）が陰性でHLA-DR陽性の細胞をDCとし，さらにCD11cとCD123の染色性でMDCとPDCを同定した．末梢血単核球での頻度から各DCサブセットの絶対数を計算し，HCV感染群と非感染群とで比較した．またHCV感染者をALT正常キャリア群（ASC群）とALT異常を伴う肝炎群（CH群）と分けて比較した（図1）．DC総数，MDC, PDCいずれもCH群では非感染群より低下していたが，ASC群は非感染群と同等であった．また，MDCからのIL-12産生能，PDCからの

*大阪大学大学院医学系研究科消化器内科学



*:p < 0.01, NV: normal volunteer ASC: Asymptomatic carrier CH: chronic hepatitis

図1 C型慢性肝炎患者におけるDC数



*:p < 0.05, NV: normal volunteer CH: chronic hepatitis

図2 DCサブセットのサイトカイン産生能の比較

IFN-α産生能を比較すると，いずれも非感染群に比較してCH群では低下していた(図2)．HCVの排除にはHCV特異的CTLの誘導が必要であるが，CTLの誘導にはTh1有意の免疫環境が重要であることが明らかになっている．MDCとナイーブCD4T細胞を共培養し，その後のT細胞のサイトカイン産生能を検討すると，非感染者のMDCはIFN-γを産生するTh1細胞を強く誘導したのに対し，C型肝炎患者のMDCはTh1誘導能が非感染者に比べ著しく低下していた．一方，PDCとナイーブCD4T細胞を共培養すると，C型肝炎患者PDCは非感染者DCに比較してIL-10産生細胞を多く誘導することが明らかになり，C型肝炎患者ではCTL誘導がされにくく，このことがHCV持続感染の機序の一端であると考えられた．今後DCサブセットの機能をさらに解析し，C型肝炎患者DCの機能低下，IFN-αへの反応性の低下のメカニズムを明らかにすることで，DCを標的としたC型肝炎に対する免疫療法が可能になると考えている．

Ⅱ．C型肝炎におけるNK細胞とNK細胞レセプターの解析

NK細胞はもともとトランスフォームした細胞に対する非特異的な細胞障害活性をもとに定義された細胞集団である．NK細胞による細胞障害機序についてはMHC class Ⅰ発現を低下あるいは欠損した細胞を傷害するという事象が知られていた．その分子機構に関しては近年NK細胞に発現している抑制性レセプター群が次々に同定され，これらが正常細胞に構成的に発現しているMHC class Ⅰを認識することにより，一般にNK活性は抑制されているが，腫瘍細胞のようにMHC class Ⅰの発現が低下した細胞では，その抑制が解除され細胞障害活性を示すと考えられている．KIRやCD94/NKG2Aは，ヒトにおける代表的な抑制性レセプターである．さらにより近年になってNK細胞には活性化レセプターNKG2Dの存在が明らかになった(図3)．NKG2Dはほぼすべての NK細胞に発現していることと，そのリガンドがMICA/Bであることが同定されたことで特に注目されている．MICAおよびMICBはともに43 kDaの蛋白で，細胞膜上に発現する糖タンパク質である．MICA/Bは消化管上皮細胞や一部の胸腺細胞

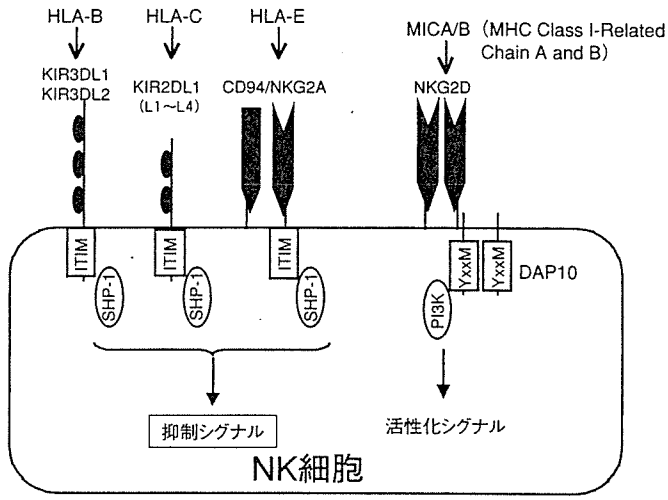
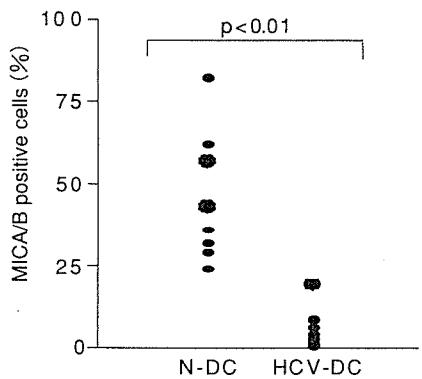


図3　NK細胞の活性化メカニズム



N-DC: DC derived from normal volunteer
HCV-DC: DC derived from patients infected with Hepatitis C virus

図4　HCV感染者におけるIFN α 刺激後樹状細胞のMICA/Bの発現

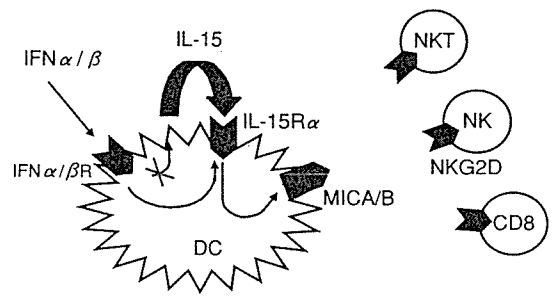


図5　IFNによるMICA/B発現におけるIL-15の役割

を除いては正常細胞には発現しておらず，ストレスを受けた細胞やトランスフォームした上皮細胞で発現が誘導されると考えられている．

われわれはC型肝炎患者末梢血単核球よりDCを誘導し，IFN-α で24時間刺激後，DC上のMICA/Bの発現を解析した[3]（図4）．C型肝炎患者DC上のMICA/Bの発現は，健常者DCに比べて有意に低下していた．IFN-α 刺激DCとNK細胞を24時間混合培養ののちに，NK活性を比較すると健常者のDCではNK活性が増加するが，HCV感染者DCではNK活性が全く認められなかったことから，C型肝炎患者DC上のMICA/Bの発現低下がこれに起因する可能性を明らかにした．DC機能は種々のサイトカインによって制御されている．DCにおけるMICA/Bの発現誘導を多くのサイトカインを用いて検討したが，健常者においてはIFN-α/β 以外にIL-15に同様の作用が認められた．しかしながらHCV感染者DCではⅠ型IFN刺激ではMICA/Bの発現は誘導されなかったが，IL-15では発現誘導が起こり，Ⅰ型IFNの下流のシグナルとしてIL-15が重要な役割を果たしていることを明らかにした（図5）．一方，C型肝炎患者NK細胞におけるNK細胞レセプターの発現の解析を行った[4]．KIRファミリーのKIR2DL1/DS1，KIR2DL2.3/2DS2，KIR2DS4，KIR3DL1いずれも健常者との有意差を認めなかった．これに対しレクチンタイプの抑制性レセプターであるCD94お

よびNKG2AはC型肝炎患者NK細胞にて発現が有意に亢進していた．同じくレクチンタイプの活性化レセプターNKG2Dは両群間で発現に差を認めなかった．C型肝炎患者，健常者における肝細胞に対するNK細胞認識機構の相違にCD94/NKG2A抑制性レセプターが果たす役割を明らかにするために，NK細胞と肝細胞の共培養時に抗NKG2A抗体を添加し，NK活性を検討した．C型肝炎患者NK細胞は，健常者NK細胞に比して，肝癌細胞に対する細胞障害活性が低いのみならず，培養上清中のIL-10やTGF-βの産生が増大していた．これらは抗NKG2A抗体の添加によって健常人NK細胞との差がなくなったことから，C型肝炎ではCD94/NKG2A発現亢進により，リガンド陽性肝細胞を認識し，NK細胞障害活性の低下，抑制性サイトカインの産生亢進を惹起することを明らかにした．

Ⅲ．C型肝炎における免疫ネットワーク

DCや傷害を受けた上皮細胞に発現するMICA/BはNKG2Dを介してNK細胞あるいはNKT細胞を活性化する．また，MICA/BはCD8陽性T細胞に対して副刺激活性がある．DCおよび一部の上皮細胞（肝細胞など）に発現しているCD1dはNKT細胞を活性化する．NKT細胞の活性化は，詳細なメカニズムは不明であるがNK細胞の活性化を誘導する．このようなDC，NKT細胞，NK細胞の活性化はその後のT細胞の応答性に影響を与える．従来獲得免疫応答の形成に関しては，抗原提示細胞による抗原の捕捉と提示を中心に解析されてきたが，獲得免疫の強さとTh1/Th2バランスは初期先天免疫応答により，よりダイナミックな調整を受けていることが明らかになっている（図6）．

Ⅳ．DCによる肝癌治療

日本における死因の第1位は癌による死亡であるが，その中でも肝癌，大腸癌による死亡は今後増加が予想されている．現在までこれら消化器癌に対して手術療法，放射線療法，化学療法などさまざまな治療法が集学的に行われ，その予後は改善してきているが，依然再発症例も多く，新たな癌治療法の確立は急務である．免疫学の進歩によって次世代の治療法として免疫療法が期待されている．現在では一部の癌抗原が同定され，癌抗原由来のペプチドを用いた癌治療が可能となってきている．また生体で最も強力な抗原提示細胞である樹状細胞（DC）を用いた癌免疫療法が検討され，現在多くの癌において臨床試験が開始されているが，今のところ期待されたほどの結果は報告されていない．この原因として臨床応用の対象となる進行癌では全身の免疫反応がTh2系あるいはTr/Th3系のような抑制された状況にあることや，進行癌ではDC機能が，健常人に比して有意に低下していることが一因であることが示されている．

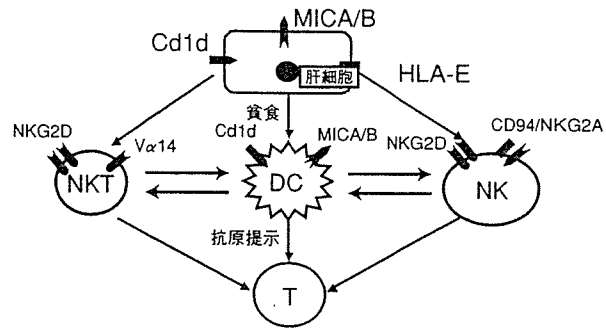


図6 免疫応答のネットワーク

われわれはTh1系サイトカインであるIL-12に注目し，IL-12投与とDCによる癌ワクチンの併用効果を検討した．マウス肝癌細胞をマウス皮下に接種後，肝癌細胞lysateをパルスしたDCによるワクチンと，IL-12による腹腔内投与を行い，その抗腫瘍効果を検討した（図7）．マウス皮下肝癌腫瘍の形成はDCのみでも抑制されたが，IL-12の併用によってより強い抗腫瘍効果を示し，治療を受けたマウス脾リンパ球の肝癌細胞に対する細胞傷害活性は，DCワクチンとIL-12の併用にてより増大することを明らかにした[5]．以上の結果はDCワクチンのより効果的な臨床応用を示唆する結果である．

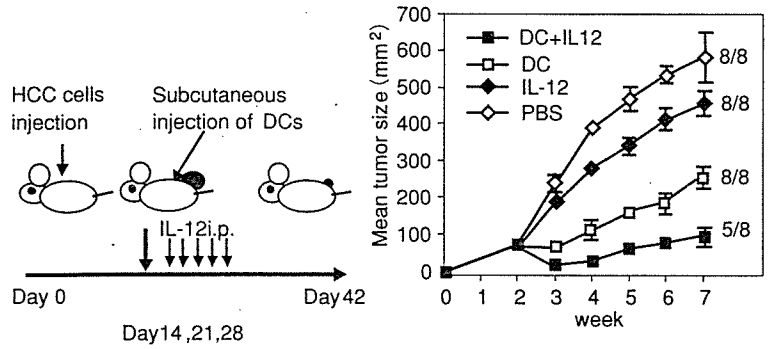


図7　肝癌lysateをパルスしたDCによる癌ワクチン

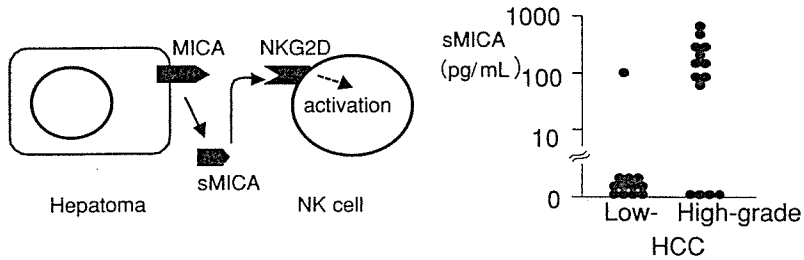


図8　進展した肝細胞癌ではsMICAが血清中に分泌され，これによりNK細胞のNKG2Dがinternalizationし，NK細胞の活性が抑制される．

Ⅴ．可溶性MICAによるNK細胞活性化の抑制

DC上のMICA分子はNK細胞活性化に重要な役割を果たしているが，最近血清中に可溶性MICAが存在することが明らかになり，これがNK細胞上のNKG2Dと結合し，NK細胞の活性化を抑制することが示された．われわれは血清中可溶性MICAを肝細胞癌患者で測定した．可溶性MICAは肝細胞癌患者，特に進行がん患者において増大しており，進行がん組織より産生されることが示唆された(図8)．さらに可溶性MICAの増大はNK細胞によるDC活性化を抑制することを明らかにした[6]．これらのことは可溶性MICAが肝癌の進行によって担癌患者の免疫環境に抑制的に働くことを示唆するものであった．

ま と め

C型肝炎でのDC機能解析，NK細胞活性化機構，肝癌のDCによる癌免疫治療の基礎的検討におけるわれわれの知見に関して述べてきた．従来免疫応答は抗原提示細胞を中心とした獲得免疫応答の形成を中心に解析されてきたが，十分な獲得免疫を得るためには自然免疫系の活性化が必須であることが明らかになってきており，今後は自然免疫系の解析とともに，自然免疫と獲得免疫へのネットワークをより詳細に解析することが必要となってきている．C型肝炎においても，その病態を明らかにするために，より詳細な解析が望まれており，将来的にはより効果的な治療法の開発につながると期待している．

文　献

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2. C型慢性肝疾患における抗ウイルス療法と肝庇護療法による肝発癌の予防

山田剛太郎* 戸川 三省* 新山 豪一* 川中 美和*

はじめに

C型慢性肝疾患における抗ウイルス療法としてインターフェロン(IFN)治療が保険承認されて10数年が経過した．しかしながら，2001年までの10年間はIFN単独療法のみであったためgenotype 1b型の高ウイルス量の症例では著効例(SVR)は10％以下と少なく，大半が無効例(NR)のまま経過した．一方，過去10数年間のうちにC型慢性肝炎の患者は急速に高齢化し，NR例の多くは60歳以上となっている．

1999年の第21回犬山シンポジウムで演者らは「C型慢性肝炎におけるIFN治療の長期予後と効果不十分例の対策」について発表した[1]．1989年4月～1994年3月の間にIFN治療を実施し，治療効果が不十分な症例に対してその後5年間追跡できた症例をもとに，肝庇護療法によるALTの改善と肝癌発生の抑制について検討した．その際の結論はNR, PRの症例ではその後の肝庇護療法が重要で，特に線維化ステージF3症例ではALTを80 IU以下に維持することにより，発癌率を低下させ，長期的な予後の改善が期待できるものと推測した．Tarao[2]は当時，すでに肝庇護療法によるALTの抑制が肝発癌の予防に有用であることを論文発表していたが，まさに彼らの成績を支持するデータであった．

*川崎医科大学附属川崎病院肝臓消化器病センター内科

2001年12月以降にはIFN・Ribavirin(Rib)の併用療法，2002年2月からはIFNの長期投与，さらにPEG-IFNが認可され，従来のIFN単独療法でSVRとならなかった症例についても肝発癌のhigh risk症例を中心に発癌抑制を期待してIFNの再投与を実施している．

前回の発表から5年が経過したが，その間に追跡症例からも発癌症例が急増し，一部はALTの落ち着いた症例からも発癌が認められている．今回改めて，IFN治療効果が不十分であった症例について10年間の長期予後を観察できた症例をもとに肝庇護療法とIFNの再投与が肝発癌の予防に役立っているかどうかについて検討した．

Ⅰ．対象と方法

対象はC型慢性肝炎にて1989年4月～1995年3月にIFN治療を実施した症例で，治療効果不十分のため，不完全著効(ICR)，有効(PR)，無効(NR)と判定された症例で，その後10年間，治療経過が観察できた症例である．全例，IFN治療前に肝生検を実施し，肝組織診断は改めて新犬山分類のstagingとgradingで診断した．

1995年4月～2000年3月までの前半の5年間を観察できた症例は127例，さらに2000年4月～2004年3月までの後半の5年間を観察できた症例は107例である．

10年間の経過観察は治療として肝庇護療法(SNMC, UDCA)ないしはIFNの再投与の有無，

表1　背景因子：肝発癌例と非発癌例の比較

	total	HCC (+)	HCC (−)	P-value
N	107	39	68	
年齢	59.9 ± 9.7	63.8 ± 6.5	57.6 ± 10.6	<0.01
性別（M/F）	48/59	19/20	29/39	NS
飲酒歴（有/無）	14/92	7/32	7/60	NS
輸血歴（有/無）	37/69	16/23	21/46	NS
家族歴（有/無）	15/91	3/36	12/55	NS
Fibrosis (F1/F2/F3/F4)	23/21/50/13	1/2/26/10	22/19/24/3	<0.0001
Activity (A1/A2/A3)	29/46/27	3/19/14	26/27/13	0.0012
血小板数	14.3 ± 5.3	11.2 ± 4.1	16.1 ± 5.1	<0.01
Serotype (1型/2型/不明)	86/13/8	31/4/4	55/9/4	NS
治療効果 (ICR/PR/NR)	19/36/52	2/12/25	17/24/27	0.0076

臨床経過としてALTの変動，HCV-RNAの陰性化，肝発癌の有無について検討した．

ALT (IU) の変動は前回の方法に準じ[1]，ALTの年平均値を求め，高度上昇 (H)，中程度上昇 (M)，軽度上昇 (L) に分け，さらに肝発癌例では発癌までの期間，非発癌例では過去10年間，Hが主体ならALT高値型，Mが主体ならALT中値型，Lが主体ならALT低値型に分類した．

Ⅱ．成　績

1．背景因子

107例の観察開始時 (1995年4月) の平均年齢は59.9歳と高齢で，男女比は48/59と女性が多い．肝生検の線維化診断はF3が50例と最も多い．Serotypeは1型が86例と大半を占め，しかも100 KIU以上の高値例が多い．1994年までのIFNの治療効果はICR/PR/NR=18/36/52とNR, PR例が多い．10年間の経過観察中に39例が肝発癌をきたしたが，発癌例と非発癌例の比較では年齢，肝生検の線維化stageと炎症のactivity，血小板数，IFN治療効果で差を認めた (表1)．しかし，1995～1999年の5年間に発癌をきたした14例と2000～2004年の5年間に発癌した25例の背景因子には有意の差は認められなかった．

10年間の累積肝発癌率はF1～2では極めて低いのに対して，F3では高く，特に後半の5年間では発癌率が急速に高まっている (図1)．

2．10年間のALTの変動型と肝発癌

IFN治療後，ALTの中程度ないしは高度上昇例に対してはほとんど全例で肝臓用剤 (SNMC, UDCA) による治療を実施し，さらに32例ではIFNの再投与を実施した．これら治療によって2例でHCV-RNA陰性化し，41％がALT低値型を示したが，残りの症例は，ALT高値型 (22％) ないしはALT中値型 (37％) であった．肝線維化ステージごとにALT型別に肝発癌率を1995～1999年と2000～2004年に分けて検討した．F3症例では1995～1999年の5年間の肝癌発生率は前回の検討と同様にALT高値型では33％程度に比して，中，低値型では極めて少なかった．一方，2000～2004年の5年間ではALT高値型のみでなく，中値型で59％，低値型も30％と多数の癌発生が観察された (表2)．ロジステック回帰モデルを用いた肝発癌に寄与する因子の検討でも1995～2004年の10年間ではALTの変動型はなお，有意な因子であったが，後半5年間 (2000～2004年)

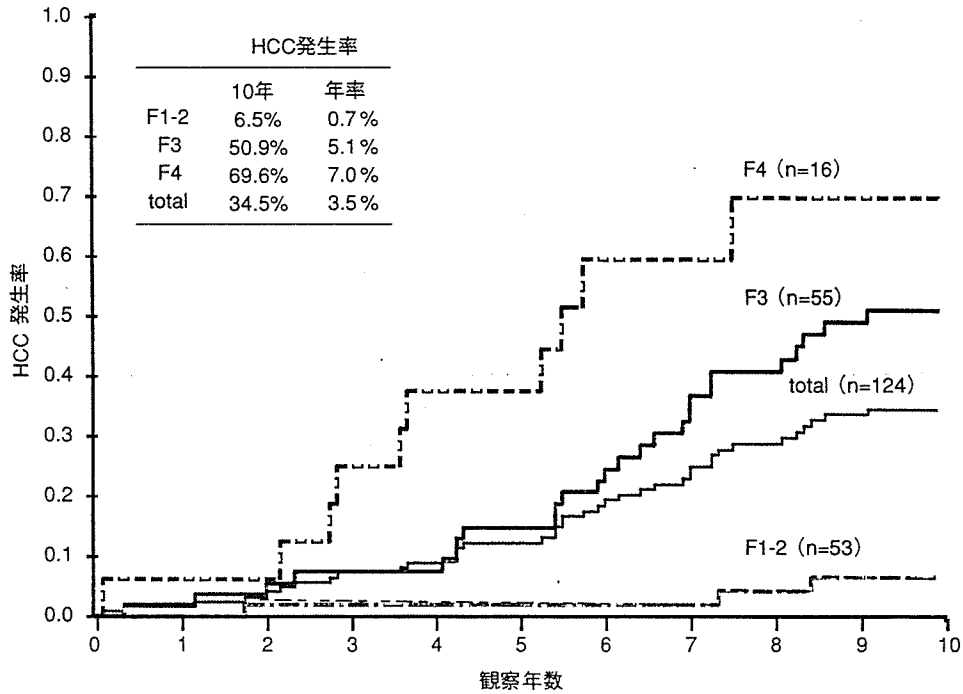

図1　肝線維化Stage別累積肝発癌率（Kaplan-Meier法）

表2　F3症例におけるALT変動型別のHCC発生率
—1995～1999年と2000～2004年の比較—

	L	20/21 (95.2%)	0/21 (0%)	6/20 (30.0%)
F3	M	22/22 (100%)	3/22 (13.6%)	10/17 (58.8%)
	H	12/12 (100%)	4/12 (33.3%)	3/ 6 (50.0%)

では有意差は失われた.

F3症例のうち，この10年間にIFN再投与を受けた症例での肝発癌率は16.7%(2/12)と，再投与を受けていない症例での発癌率54.8%(17/31)に比較して有意に少ない(表3).

Ⅲ. 考察およびまとめ

C型慢性肝炎の抗ウイルス療法もPEG-IFNの単独療法，さらにPEG-IFNとRibの併用療法による保険診療が認可され，症例に応じたテーラーメードな治療が可能な時代を迎えている．しかし，従来のIFN療法で無効であった多くの1b型の高

表3　F3症例のIFN再投与例と非再投与例におけるHCC発生率（2000～2004年）

ALT	IFN再投与率	HCC発生率 IFN再投与(+)	HCC発生率 IFN再投与(−)
L	3/20	1/ 3 (33.3%)	5/17 (29.4%)
M	6/17	1/ 6 (16.7%)	9/11 (81.8%)
H	3/6	0/ 3 (0%)	3/ 3 (100%)
Total		2/12 (16.6%)	17/31 (54.8%)

ウイルス量患者は肝庇護療法を主体に治療してきたが，現在では65歳以上の高齢者が多く，併用

療法の恩恵を受けることの難しい症例が増加している．

1999年にIFN治療後5年を経過した症例をもとに，肝庇護療法による肝発癌抑制効果を検討し，発癌リスクの高いF3症例では肝庇護療法によりALTを80 IU以下に維持することによって長期予後の改善が期待されることを報告した．しかしながら，今回，これらF3症例のさらに5年間の追跡調査では，ALT中値型のみでなく，年平均ALT 40 IU以下の低値型でも30％に発癌が認められた．

したがって，線維化の進展したF3症例では肝庇護療法によって炎症を抑制し，肝発癌を遅らせることはできるが，長期的には肝発癌を阻止することは難しいものと推測された．一方，IFNの再投与例では肝発癌の頻度は明らかに少なく，発癌抑制効果が期待されるが，全体の症例数が少なく，また，再投与例の多くは長期投与が認可された2002年以降の投与であり，今後さらに長期予後を観察する必要がある．高齢化した肝発癌ハイリスク群に対する発癌抑制療法として肝庇護療法に加えて，新たな治療法の確立が待たれる．

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Influence of hepatitis B virus genotypes on the response to antiviral therapies

Norio Akuta* and Hiromitsu Kumada

Department of Gastroenterology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo, 105-0001, Japan

Hepatitis B virus (HBV) has been classified into eight genotypes (A–H) based on genome sequence divergence. Genotypes of HBV have distinct geographical distributions, and two genotypes account for most HBV worldwide. Hepatitis B e antigen expression lasts longer and liver disease is more severe with graver outcomes in carriers of genotype C than B in Asia. Accumulating lines of evidence indicate a better response to interferon and lamivudine in patients with chronic hepatitis B who are infected with genotype B rather than C. The therapeutic response may differ, however, in patients infected with HBV of the same genotype. For example, the response to lamivudine is poorer in patients infected with subtype Ba, which contains a recombination with genotype C, than in those with subtype Bj without such a recombination. Influence of genotypes on therapeutic response needs to be examined in patients infected with the other genotypes, particularly in those with genotype A or D infection.

Keywords: chronic hepatitis, genotypes, hepatitis B e antigen, hepatitis B virus, interferon, lamivudine

Introduction

Worldwide, 350 million people are estimated to be persistently infected with hepatitis B virus (HBV),[1] and three-quarters of these people reside in Asia. The morbidity and mortality of persistent HBV infection are a major public health concern. More than one million deaths every year are due to end-stage HBV liver disease, such as decompensated liver cirrhosis and hepatocellular carcinoma (HCC). The carrier state of HBV is established mainly through mother-to-baby infection in Japan, where the prevalence of hepatitis B surface antigen carriage (HBsAg) in the general population used to be less than 2%, while horizontal transmission during infancy plays an additional role in the other countries in Asia and accounts for most persistent infections in Africa where HBsAg prevailed in >8% of the general population during the past. Although much less efficient, horizontal transmission can lead to persistent HBV infection in Western countries through sexual contact and illicit intravenous drug use. Individuals with persistent HBV infection need to be identified early and receive efficient antiviral therapy in order to prevent the development of serious liver disease.

Genotypes of HBV

The response to antiviral therapy in HBV infection is influenced by many host and viral factors. Recently, HBV genotypes have attracted increasing attention since they influence the activity and outcome of HBV-associated chronic liver disease, as well as the response to antiviral therapies. In 1988, four genotypes of HBV (A–D) were proposed based on sequence divergence of >8% in the entire HBV genome, which consists of approximately 3200 base pairs.[2] Later, an additional four genotypes (E–H) were identified by the same criteria.[3–5] HBV genotypes have distinct geographical distributions,[6] and the full picture awaits further epidemiological surveys in many as yet unexamined countries. Overall, genotype A prevails in Northwest Europe, sub-Saharan Africa, India and the United States, B and C are frequent in Southeast Asia, Japan and Oceania, and D is common in the Mediterranean countries. Genotype E is restricted to Africa, and F is found mainly in Central and South America. The distribution of genotypes G and H is yet to be determined. Since persistent HBV infection is frequent in Asia, genotypes B and C prevailing there have been studied most extensively with their clinical and therapeutic differences unfolding rapidly. Differences between genotypes A and D prevailing in Western countries, India and the United States, also, are increasingly coming to the fore.

Clinical manifestations of persistent HBV infection with distinct genotypes

Prospective, case-controlled and cross-sectional studies predominantly but not entirely indicate that the severity and outcome of chronic hepatitis B are more serious in patients infected with

*Corresponding author. Tel: +81-44-877-5111; Fax: +81-44-860-1623; E-mail: akuta-gi@umin.ac.jp

genotype C compared with B.[7-10] Liver cirrhosis and HCC are more frequent in carriers of genotype C than B.[7,11-13] Very recently, chronic liver disease was detected more frequently in Japanese individuals infected with genotype C than D [221/350 (63%) compared with 6/38 (16%), $P<0.001$].[14]

The synthesis of hepatitis B e antigen (HBeAg) is regulated at both translational and transcriptional levels.[15] Mutations to create stop codons in the precore region, typified by the G-to-A mutation at nucleotide (nt) 1896 (G1896A), shut down the translation of HBeAg completely. The double mutation in the core promoter, A-to-T at nt 1762 and G-to-A at nt 1764 (A1762T/G1764A), interferes with the proper transcription of HBeAg precursor, thereby downregulating the synthesis of HBeAg. The precore stop codon mutation (G1896A) is detected more frequently in persons infected with genotype B than C; it is inhibited in those with genotype A, because it destabilizes the e encapsidation signal.[16] In remarkable contrast, the core promoter double mutation (A1762T/G1764A) is more common in those with genotype C than B. Overall, the ramifications of this are that the seroconversion to the loss of HBeAg takes longer in individuals infected with genotype C than B, and is accompanied by the development of severe liver disease.

Sugauchi *et al.*[17] reported two subtypes of genotype B, one of which possesses a recombination with genotype C over the precore region plus core gene (subtype Ba) while the other does not (subtype Bj). The distribution of subtype Bj is restricted to Japan (hence the 'j' for Japan), in contrast to subtype Ba found in all Asian countries other than Japan ('a' for Asia, therefore). Since HBeAg and the double mutation in the core promoter (A1762T/G1764A) are significantly more frequent in carriers of subtype Ba than Bj,[18] subtypes of genotype B may influence the clinical outcome and the response to antiviral therapies for chronic hepatitis B.

To a lesser extent, clinical differences between genotype A and D infections have been reported from Europe, where these genotypes are frequent. HBV infection is contracted in adulthood in these countries, principally through sexual contacts and illicit drug use, and HBV infection is more likely to persist in persons infected with genotype A rather than D or the other genotypes.[19] These findings stand at variance with those of Sanchez-Tapias *et al.*[20] who found sustained biochemical remission and clearance of HBV DNA to be more frequent in infection with genotype A than genotype D (log-rank, 14.2, $P=0.002$) or genotype F (log-rank, 4.2, $P=0.03$); the rate of HBsAg clearance was also found to be higher in genotype A compared with D infection (log-rank, 4.06, $P=0.03$). Likewise in a comparison between 60 and 63 patients in India infected with HBV genotype A or D, respectively, genotype D was significantly associated with severe liver disease (61% compared with 30%, $P<0.05$) and tended to be more frequent in those with HCC below 40 years of age (63% compared with 44%, $P=0.06$).[21] Clinical differences amongst HBV genotypes manifest themselves in the distribution of acute and chronic liver disease in those who visit hospitals. In our Toranomon Hospital in metropolitan Tokyo, 57 adult patients with acute hepatitis B and 1077 with chronic hepatitis B were admitted during the same period.[22] The distribution of genotypes were: genotype A (acute, 22.8% versus chronic, 1.9%; $P<0.00001$); B (14.0% versus 9.4%); C (43.9% versus 87.7%, $P=0.004$); D (1.8% versus 0.2%); F (1.8% versus 0.2%); and untypeable (15.8% versus 0.6%, $P=0.001$).

Influence of HBV genotype on the response to antiviral therapy

Until lamivudine was developed for clinical use, interferon had remained the sole practical antiviral for chronic hepatitis B, ever since initial clinical trials by Hoofnagle and colleagues[23,24] in the mid-1980s. The response to interferon, judged by the loss of HBeAg from serum, is achieved in at most 20% of treated patients.[25] Moreover, Asian patients who have acquired the HBV carrier state at birth or in early infancy respond to interferon more poorly than Caucasian patients who contracted it in adulthood.[26] To make matters even worse, patients with chronic hepatitis B positive for anti-HBe antibodies are much less responsive to interferon than those with serum HBeAg.[27] Limited experience indicates that HBV genotypes make a difference in the response to interferon in patients with chronic hepatitis B.

Zhang *et al.* compared the response between 10 patients with genotype A infection and 21 patients with genotype D or E infection.[28] Since all patients they studied were positive for anti-HBe antibodies, the negative influence of genotype A on seroconversion to anti-HBe was excluded. They found the response to interferon was higher in patients infected with genotype A compared with D or E (70% versus 40%, $P=0.001$).[28] Likewise, Kao *et al.*[29] reported the response to interferon to be higher in patients infected with genotype B rather than C [13/32 (41%) versus 4/26 (15%), $P=0.045$]. More recently, Wai *et al.*[30] compared the response between patients randomized to interferon or placebo. They found the response was better in patients with genotype B than C infection who were allocated to interferon treatment [12/31 (39%) versus 7/42 (17%), $P=0.034$]; the response rate did not differ in those who received placebo.

Lamivudine [(−)-β-L-2′,3′-dideoxy-3′-thiacytidine] is a nucleoside analogue with a potent antiviral activity. Since its approval in 1998, lamivudine has gained wide popularity for the treatment of chronic hepatitis B due to high a efficacy with minimal untoward effects.[31-35] We believe in continued lamivudine treatment for patients with or without serum HBeAg,[36-39] and have accumulated experience with 286 patients including seven who have received lamivudine for 7 years or longer.[40] HBV genotype also makes a difference in the response to lamivudine in patients with chronic hepatitis B. Among the 16 patients who had received lamivudine for 3 years or longer, the virological response with the loss of HBV DNA detectable by non-amplified method was achieved in two of the three (67%) patients infected with genotype B and in seven of the 13 (54%) patients infected with genotype C.

Kao *et al.*[41] reported on the response to lamivudine in patients treated for 6–30 months infected with genotype B compared with C [3/13 (23%) versus 2/18 (11%), no significant differences]. They found resistance to lamivudine in two (15%) patients with genotype B and in four (22%) with genotype C. Chien *et al.*[42] reported that the sustained response to lamivudine was much higher in patients infected with genotype B compared with genotype C [38/62 (61%) versus 5/20 (20%), $P=0.009$]. We monitored 213 patients on continued lamivudine treatment for drug-resistant HBV variants for mutations in the tyrosine-methionine-aspartate-aspartate (YMDD) motif in the viral DNA polymerase/reverse transcriptase.[43] The emergence of YMDD mutants was no different amongst patients infected with genotype A, B or C. However, YMDD mutants developed significantly more frequently in patients infected with subtype Ba

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than Bj during 2 years on lamivudine [3/4 (75%) versus 1/14 (7%), $P<0.05$]. Severe acute exacerbation of hepatitis occurred in four of the 185 (2%) patients with genotype C along with the emergence of YMDD mutants, but in none of the 28 patients with the other genotypes. In patients with chronic hepatitis B in Germany, risk of lamivudine resistance was significantly higher in carriers of HBsAg of serotype adw than ayw [7/13 (54%) versus 1/13 (8%), $P=0.03$];[44] serotype adw corresponded to genotype A and ayw to D.[45]

Conclusion

HBV genotypes influence the severity of liver disease and response to interferon and lamivudine. They are also expected to influence the response to adefovir dipivoxil, which has recently been approved for treatment of chronic hepatitis B,[46] as well as the emergence of resistant mutants;[47] although as yet no differences have been observed in the response to adefovir dipivoxil in relation to HBV genotypes.[48] Should poor responses to a given antiviral be predicted in patients infected with HBV of certain genotypes, they can be directed to the other therapeutic options to spare the cost and burden of treatment. In evaluating the association of HBV genotypes with the response to antiviral therapies, however, it needs to be taken into account that patients visiting hospitals are biased for severe liver disease. Moreover, once full-blown disease develops, it would become refractory to any antiviral treatments. Thus, genotype differences may be attenuated in patients with severe liver disease seen in hospitals,[49,50] probably due to exclusion of patients with less severe disease who can still benefit from treatments. This view would be supported by different distributions of genotypes B (12% and 54%, respectively) and C (84% and 47%) between patients visiting hospitals and individuals found with HBV infection at routine check-ups in the same district of Japan.[51,52] Therefore, in evaluating the influence of HBV genotypes on response to antiviral therapies, one has to keep in mind not only patients with liver disease who visit hospitals, but also those who have not and who may benefit from early treatment.

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HEPATOLOGY

Significance of hepatitis B virus DNA clearance and early prediction of hepatocellular carcinogenesis in patients with cirrhosis undergoing interferon therapy: Long-term follow up of a pilot study

KENJI IKEDA, MASAHIRO KOBAYASHI, SATOSHI SAITOH, TAKASHI SOMEYA, TETSUYA HOSAKA, NORIO AKUTA, YOSHIYUKI SUZUKI, FUMITAKA SUZUKI, AKIHITO TSUBOTA, YASUJI ARASE AND HIROMITSU KUMADA

Department of Gastroenterology, Toranomon Hospital and Okinaka Memorial Institute for Medical Research, Tokyo, Japan

Abstract

Background and Aim: Because the anti-carcinogenic effect and mechanism of interferon (IFN) in patients with hepatitis B virus (HBV)-related cirrhosis have not been elucidated, quantitative analysis of HBV-DNA concentration was carried out sequentially.

Method: Of 60 consecutive patients with cirrhosis who began IFN therapy between 1986 and 1990, 57 patients were completely observed for the appearance of hepatocellular carcinoma (HCC). All patients underwent intermittent administration of IFN for a median period of 18 months. HBV-DNA was quantified using transcription mediated amplification and hybridization protection assay. A HBV-DNA count <3.7 log-genome equivalent (LGE)/mL (equivalent to $10^{3.7}$ or 5000 copies/mL) was considered to be a negative value.

Results: Of 25 patients who had HBV-DNA loss after IFN therapy, nine lost HBV-DNA during the therapy and 16 lost HBV-DNA after cessation of the therapy. The other nine patients showed a transient loss of HBV-DNA, and the remaining 23 retained persistently positive HBV-DNA during and after therapy. Although HCC developed in two (8.0%) of the 25 patients with HBV-DNA loss, carcinogenesis was found in 11 (34.4%) of 32 patients without HBV-DNA loss (Fisher's exact test, $P = 0.026$). In the two exceptional patients, HCC was detected at 1.2 and 3.6 years after loss of HBV-DNA, respectively. When the HBV-DNA concentration decreased by 2 LGE/mL (decrease to 1/100) at 6 months after initiation of interferon, HBV-DNA became negative eventually in 15 (60.0%) of 25 patients.

Conclusion: A significant decrease or loss of serum HBV-DNA prevents development of HCC, and sequential analysis of HBV-DNA could be very useful in both the prediction and the early detection of small HCC.

Key words: cancer prevention, carcinogenesis, DNA, hepatitis B virus, hepatocellular carcinoma, interferon, liver cirrhosis.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a leading cause of death in many parts of sub-Saharan Africa and Asia.[1,2] It is also one of the most common neoplasms in Japan. Abundant epidemiological and molecular biological evidence shows that the hepatitis B virus (HBV) is an important factor in the development of HCC,[3–6] but the precise role of HBV-DNA viruses in the oncogenesis of HCC is still unknown. Although increasing evidence indicates that the HBV plays an important role in the development of HCC, particularly after the discovery of integrated forms of HBV,[7,8] current serological and virological markers are still insufficient for establishing this relationship. Because a really curative therapy is not available for HCC at present, the accurate prediction

Correspondence: Dr Kenji Ikeda, Department of Gastroenterology, Toranomon Hospital, Toranomon 2-2-2, Minato-ku, Tokyo 105-8470, Japan. Email: ikedakenji@tora.e-mail.ne.jp

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and early detection of HBV-related HCC is essential in the current situation. Needless to say, a cohort of patients with HBV-related cirrhosis has a significantly high risk for the development of HCC,[6,9] but the degree of risk of carcinogenesis in an individual patient cannot be predicted as yet. Hepatocellular carcinogenesis in patients with HBV infection may be associated with persistence of aminotransferase, concentration of HBV-DNA, or merely the severity of the liver disease.

Interferon (IFN) has been reported to be effective in patients with HBV-related chronic hepatitis, which, on early control studies,[10–12] decreases serum HBV-DNA concentration and improves biochemical data and subsequently suppresses disease progression to cirrhosis.[13,14] Although the various effects of IFN in HBV infection have been well investigated from the virological, biochemical, and medico-economical viewpoints,[15–17] the influence of IFN on the long-term outcome for liver cirrhosis and on hepatocellular carcinogenesis is still controversial.[18–23] In order to clarify the mechanism of the anticarcinogenic activity of IFN, if any, we analyzed HBV-DNA concentration serially in a cohort of 60 patients with cirrhosis.

The purposes of this study are: (i) to elucidate the relation of hepatocellular carcinogenesis to longitudinal clinical courses of consecutive cirrhotic patients with IFN therapy; and (ii) to investigate a prediction of cancer preventative activity by early HBV-DNA elimination.

METHODS

Patients

Of 189 patients who were diagnosed as having HBV-related cirrhosis using peritoneoscopy and/or liver biopsy from 1983 to 1990 in our hospital, a total of 60 patients underwent IFN therapy from 1986 to 1990. Because three patients were lost to follow up, the remaining 57 patients (95.0%) were analyzed for virological outcome, carcinogenesis, and eventual prognosis: the reason for the dropout from the observation in the three patients was simply relocating house.

Table 1 shows the demography and laboratory data of the consecutive 57 patients who began IFN therapy from 1986 to 1990. There were 45 men and 12 women, with an age range from 19 to 60 years and a median of 41 years. Median values of bilirubin and albumin were 0.9 mg/dL and 4.1 g/dL, respectively. All the patients had a high HBV-DNA concentration of 3.7 log-genome equivalent (LGE)/mL or more at the time of IFN therapy.

Table 1 Demography and laboratory data of 57 patients with hepatitis B virus-related cirrhosis undergoing interferon therapy

Demography	
Men : women	45:12
Age (median, range)	41 (19–60)
Decompensated cirrhosis	3 (5.3%)
Past alcohol consumption of 500 kg or more	3 (5.3%)
Laboratory data (median, range)	
Bilirubin (mg/dL)	0.9 (0.4–2.6)
Albumin (g/dL)	4.1 (3.0–4.9)
Aspartic transaminase (IU/L)	65 (16–404)
Alanine transaminase (IU/L)	74(12–586)
Platelet count ($\times 10^3/mm^3$)	125 (68–332)
Antibodies to hepatitis C virus positive	0
Hepatitis B e antigen positive	41 (71.9%)
Hepatitis B virus DNA (LGE/mL)	7.2 (3.9–> 8.7)
Observation period (year)	13.6 (6.5–16.1)

LGE/mL, log-genome equivalent, expressed as 10^n copy/mL.

Interferon treatment

IFN-α was administered in 35 patients (61.4%) and IFN-β in the remaining 22 patients (38.6%). The daily quantity of IFN was three million units in 22 (38.6%) and six million units in 35 (61.4%), twice a week administration was carried out in 54 (94.7%) and three times a week administration in three (5.3%). All patients received intermittent IFN therapy for a median of 18 months (range, 2–132 months), but the duration of the IFN therapy was arbitrary in this pilot study. Although the daily dose of IFN and the duration of the therapy varied in this study, 52 (91.2%) of the 57 patients received IFN for 6 months or longer.

Follow up of patients and diagnosis of HCC

Follow up of the patients was made on a monthly basis after diagnosis of liver cirrhosis using monitoring virological, hematological, and biochemical data, including α-fetoprotein. All results for these laboratory tests, including HBV markers, were obtained throughout the observation period in each patient. Patients were classified into four groups according to patterns of serial concentration of HBV-DNA: type A, disappearance of HBV-DNA during and after IFN therapy; type B, loss of HBV-DNA after cessation of IFN administration; type C, transient loss of HBV-DNA only during IFN administration; type D, persistently positive HBV-DNA during and after the therapy. Clinical courses of alanine aminotransferase (ALT) fluctuation were also classified into four groups according to normalization of the ALT value.

Imaging diagnosis was made two or more times per year for each patient using computed tomography (CT), ultrasonography (US) or magnetic resonance imaging (MRI). HCC was diagnosed using typical hypervascular characteristics on angiography in addition to certain features of CT, US and MRI. Pathological confirmation of surgically resected specimens was carried out in six (46.2%) of 13 patients with HCC development.

Assays of HBV markers

Serum hepatitis B surface antigen was measured using radioimmunoassay (Dainabot, Tokyo, Japan) and reversed passive hemagglutination (Institute of Immunology, Tokyo, Japan) using commercial assay kits. hepatitis B e antigen (HBeAg) and antibody to HBeAg were determined using ELISA (Institute of Immunology) with commercial kits. Anti-hepatitis C virus antibody (third-generation anti-HCV) was assessed using ELISA kits (Dainabot).

HBV-DNA was assayed using frozen sera stored at −80°C, and quantified using transcription-mediated amplification and hybridization protection assay (Chugai Diagnostics Science, Tokyo, Japan), as described by Kamisango *et al.*[24] A HBV-DNA value of <3.7 LGE/mL (equivalent to $10^{3.7}$ copies/mL or 5000 copies/mL) was considered to be a low value. For all serial sera from the diagnosis of cirrhosis to the end of the observation period in each patient, the DNA quantification was simultaneously carried out using identical measurement kits.

Statistical analysis

Standard statistical measures and procedures were used. The Mann–Whitney *U*-test and χ^2 tests were employed for the examination of background characteristics between the groups with and without HBV-DNA elimination. Fisher's exact test was also used to analyze the relation of HBV markers to carcinogenesis. Rates of cumulative HBV-DNA disappearance, carcinogenesis and survival were calculated using Kaplan–Meier analysis,[25] and the differences between the analyzed groups were assessed using a log-rank test. A *P*-value of <0.05 using a two-tailed test was considered to be significant. Data analysis was carried out using the computer program SPSS version 11.[26]

RESULTS

HBV-DNA in clinical courses

HBV-DNA was positive in all patients at the initiation of IFN therapy (3.9–8.7 LGE/mL). HBV-DNA became negative (<3.7 LGE/mL) in 25 of 57 patients (43.9%) during the observation period, with a median of 13.6 years. The remaining 32 patients did not show a sustained negative HBV-DNA after the therapy, although nine patients did show transient negative values for a limited period during the therapy.

Clinical courses of HBV-DNA were classified into the four categories mentioned above. Nine patients (15.8%) lost HBV-DNA during and after IFN therapy (type A), 16 patients (28.1%) lost HBV-DNA after cessation of the therapy (type B). The other nine patients (15.8%) showed a transient loss of HBV-DNA (type C), and the remaining 23 (40.4%) retained persistently positive HBV-DNA (type D).

The cumulative rate of HBV-DNA disappearance was calculated using Kaplan–Meier analysis (Fig. 1).

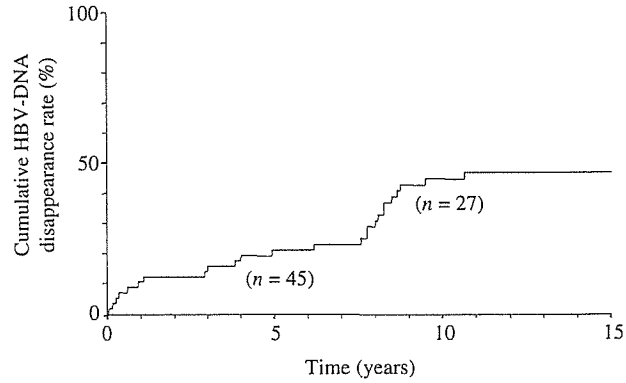

Figure 1 Cumulative hepatitis B virus (HBV)-DNA disappearance rate in the 57 cirrhotic patients with interferon therapy.

DNA became negative in 10.5% at the end of the first year after initiation of IFN therapy, in 12.3% at the third year, 21.0% at the fifth year, 43.7% at the tenth year, and 46.7% at the fifteenth year, respectively.

Hepatocellular carcinogenesis and serial concentration of HBV-DNA

A total of 13 patients developed HCC during the observation period.

The relationship between carcinogenesis and serial concentration of HBV-DNA was analyzed (Fig. 2). None of the nine patients in the type A group developed HCC. Two (12.5%) of 16 patients in the type B group developed HCC: HCC were detected 1.2 years after the disappearance of HBV-DNA in one patient, and 3.6 years after the disappearance of HBV-DNA in the other patient. Three (33.3%) of nine patients in the type C group showed carcinogenesis, and eight (34.8%) of 23 patients in the type D group developed HCC during the observation. Hepatocellular carcinogenesis was significantly associated with persistent positive HBV-DNA after initiation of IFN (2/25 *vs* 11/32; $P = 0.019$ using the $\chi 2$ test, $P = 0.026$ using Fisher's exact test).

Cumulative carcinogenesis rates were analyzed according to the ultimate course of the serial assay of HBV-DNA (Fig. 3). Fifth-year hepatocellular carcinogenesis rates were 0% in patients with HBV-DNA loss, and 9.4% in patients without HBV-DNA elimination; 10-year rates were 8.0% and 22.5%; and 15-year rates were 8% and 44.0%, respectively. The carcinogenesis rate in patients with HBV-DNA elimination was significantly lower than in those without DNA elimination ($P = 0.011$, using a log-rank test).

Hepatocellular carcinogenesis and HBeAg and aminotransferase

The relationship between carcinogenesis and HBeAg positivity during the clinical course was assessed.

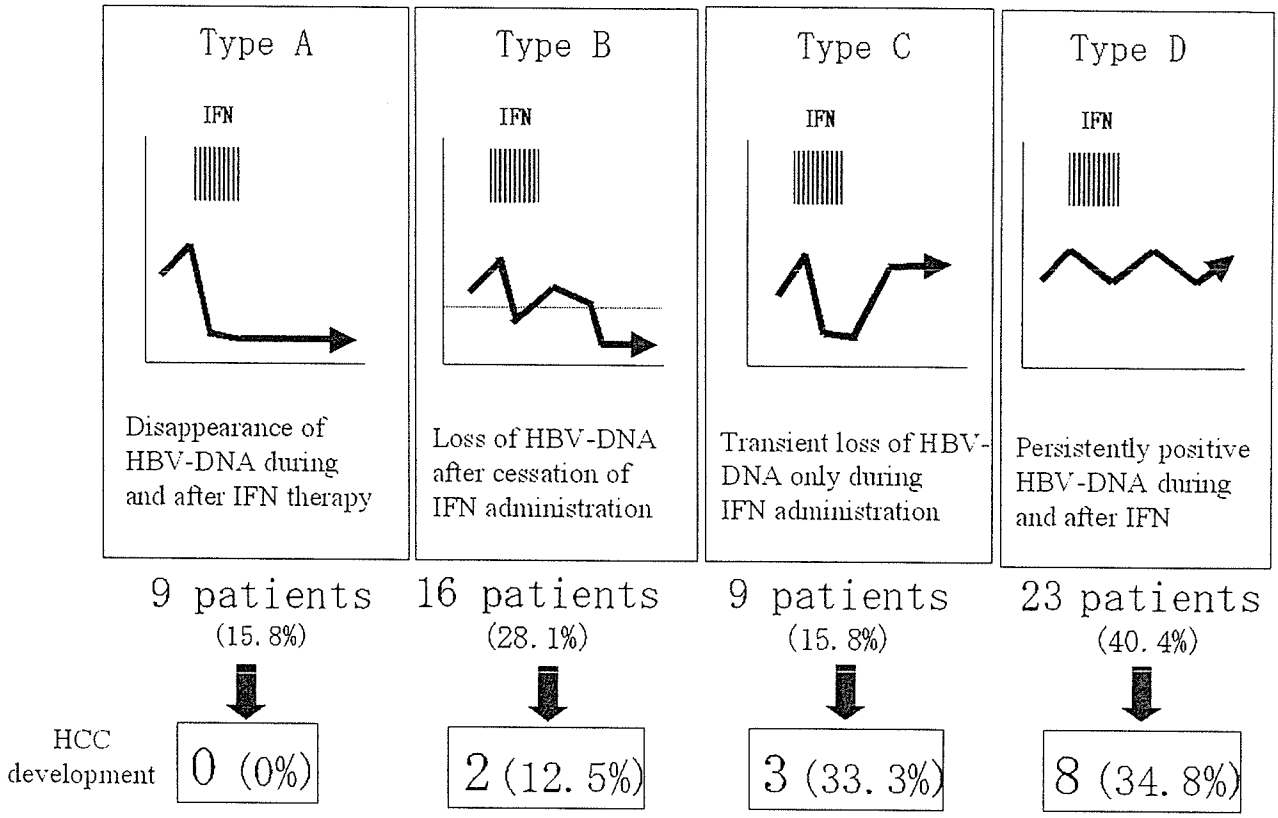

Figure 2 Relation between types of serial hepatitis B virus (HBV)-DNA concentration and carcinogenesis. HCC, hepatocellular carcinoma; IFN, interferon.

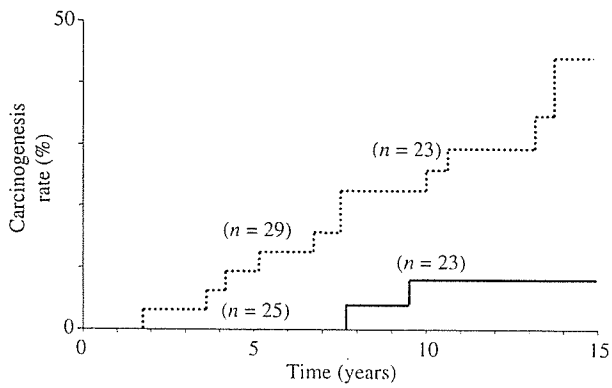

Figure 3 Cumulative hepatocellular carcinogenesis rates in patients (—; $n = 25$) with and (---; $n = 32$) without eventual hepatitis B virus (HBV)-DNA clearance.

HBeAg was positive in 41 patients (71.9%) and negative in 16 (28.1%) at the initiation of IFN therapy. Twenty-eight (68.3%) of the 41 patients showed continuous loss of HBeAg after IFN therapy. HCC developed in four (25.0%) of the 16 patients without HBeAg from the beginning, four (14.3%) of the 28 patients with HBeAg clearance, and five (38.5%) of 13 patients with persistent HBeAg positivity. HBeAg clearance did not significantly decrease the incidence of carcinogenesis risk ($P = 0.12$ using the χ^2 test with Yates' correction).

The relationship between carcinogenesis and a longitudinal course of ALT after IFN therapy was also analyzed. Four (18.2%) of 22 patients with normalization of ALT after IFN therapy developed HCC; nine (25.8%) of 35 patients with persistently abnormal ALT levels developed HCC. The serial values of ALT were not significantly associated with carcinogenesis risk ($P = 0.075$ using the χ^2 test with Yates' correction).

The cumulative HBeAg disappearance rate, HBV-DNA disappearance rate, and ALT normalization rate were calculated in those patients with positive HBeAg at the beginning of IFN treatment (Fig. 4). The HBeAg disappearance rate and DNA disappearance rates were 55.4% and 14.6% at the end of the fifth year, and 55.4% and 40.1% at the tenth year, respectively. The ALT normalization rate at the fifth year was 25.4% and the tenth year rate was 41.2%. Although the incidence of virological and biochemical improvement gradually increased after therapy, the rates evidently differed between virological and biochemical responses.

Influence of the length of interferon therapy on HBV-DNA loss

The influence of the length of the therapy on virological response was assessed.

Although 25 (43.8%) of 57 patients cleared HBV-DNA on overall analysis, 21 (46.6%) of 45 patients who received IFN for more than 6 months and 20 (50%) of 40 patients who received IFN for more than 12 months lost HBV-DNA. Similarly, the HBV-DNA disappearance rate slightly increased correlating with the length of IFN administration: 55.5% in patients who were treated for more than 18 months, 56.0% with more than 24 months' treatment, 64.7% in more than 36 months' treatment, 58.3% in more than 48 months' treatment, and 71.4% in more than 60 months' treatment (Fig. 5). The longer the IFN therapy was carried out, the higher the rate of HBV-DNA disappearance.

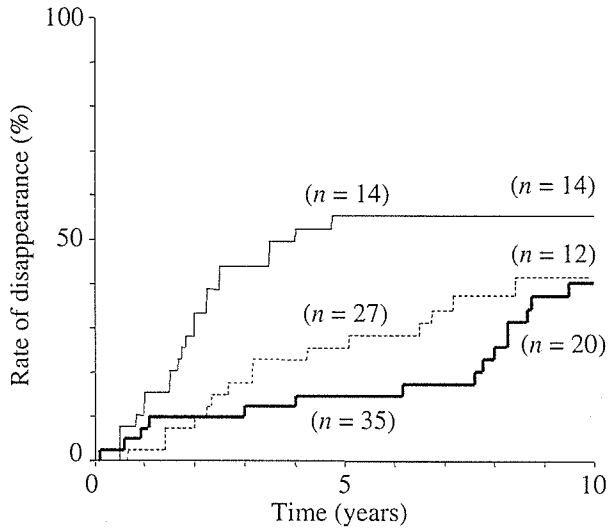

Figure 4 Cumulative (—) hepatitis B e antigen (HBeAg) disappearance rate, (—) hepatitis B virus (HBV)-DNA disappearance rate, and (---) alanine transaminase normalization rate in 41 patients with positive HBeAg at the initiation of interferon therapy.

Prediction of future HBV-DNA elimination

We assessed the relation between an early HBV-DNA response and a future HBV-DNA loss. When the HBV-DNA concentration decreased by ≥2 LGE/mL (decrease to 1/100) during the first 6 months, 15 (60.0%) of 25 patients eventually lost HBV-DNA. In contrast, when the HBV-DNA decrease was <2 LGE/mL during the period, HBV-DNA loss was found in 10 (31.3%) of 32 patients ($P = 0.036$, χ^2 test). Similarly, future HBV-DNA loss was estimated from a decrease in concentration of HBV-DNA at the end of 12 months: HBV-DNA eventually became negative in 15 (62.5%) of 24 patients with a larger DNA decrease of ≥2 LGE/mL at the end of 12 months, eventual DNA loss was found in only 10 (30.3%) of 33 patients with a smaller DNA decrease by <2 LGE/mL. The 12-month decrease of HBV-DNA was significantly associated with future DNA loss ($P = 0.030$, χ^2 test).

The early response of HBV-DNA and the length of IFN therapy were analyzed together for the prediction of eventual HBV-DNA loss. Of 25 patients with a HBV-DNA decrease of ≥2 LGE/mL during the initial 6 months, two (33.3%) of six patients with short IFN therapy of ≤6 months showed a HBV-DNA loss, but 13 (68.4%) of 19 patients with long-term IFN therapy of >6 months lost HBV-DNA. Of 32 patients with a HBV-DNA decrease of <2 LGE/mL in the first 6 months, one (20.0%) of five patients with short IFN therapy showed HBV-DNA loss, but nine (33.3%) of 27 patients with long-term IFN administration lost HBV-DNA. Therefore, according to the early HBV-DNA response and the duration of the therapy, the rate of sustained HBV-DNA decrease to <3.7 LGE/mL varied, with a range of 20.0–68.4%.

Prognosis after IFN therapy

A total of eight patients (14.0%) died in the period of observation: six from development of HCC and the

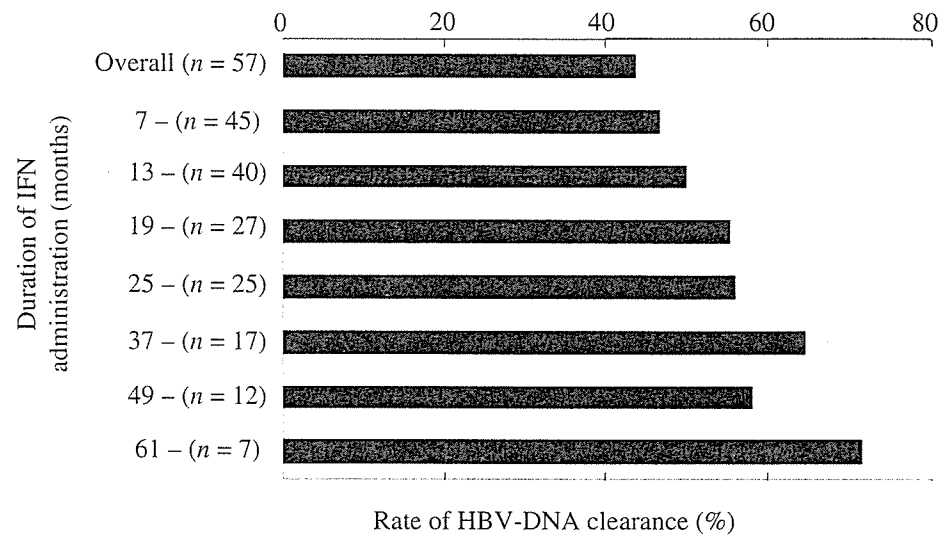

Figure 5 Influence of the length of interferon (IFN) therapy on hepatitis B virus (HBV)-DNA clearance.

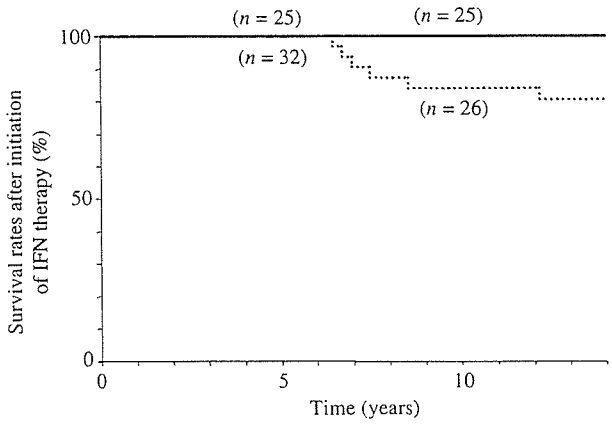

Figure 6 Cumulative survival rates after the initiation of interferon (IFN) therapy in patients (—; $n = 25$) with and (---; $n = 32$) without eventual hepatitis B virus DNA clearance.

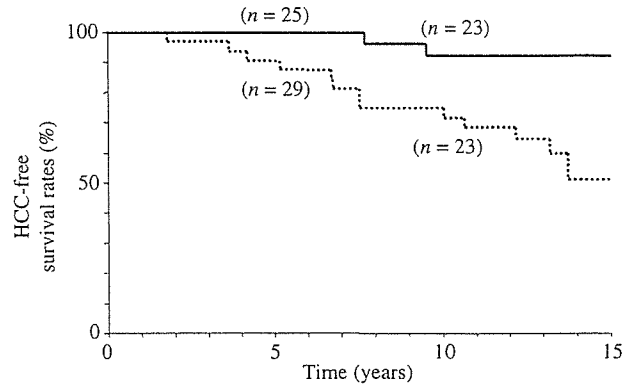

Figure 7 Hepatocellular carcinoma (HCC)-free survival rates in patients (—; $n = 25$) with and (---; $n = 32$) without eventual hepatitis B virus DNA clearance.

other two from liver failure due to aggravation of cirrhosis.

Of 13 patients with HCC development, two patients with HBV-DNA loss have not shown any tumor recurrence after surgical resection, and both patients are alive at the end of the observation. In contrast, nine (81.8%) of 11 patients with persistently high HBV-DNA developed HCC recurrence after therapy, and six (54.5%) of the patients died during the observation period. All six patients died from the development of HCC and none from aggravation of cirrhosis or extrahepatic disease.

Of 44 patients without HCC development until the end of the observation period, none of 23 patients with HBV-DNA loss died, but two (9.5%) of 21 patients with persistently positive HBV-DNA have died from liver failure.

Survival rates were compared between those patients with and without HBV-DNA loss (Fig. 6). Fifth-year survival rates in patients with and without HBV-DNA loss were 100% and 100%, seventh year rates were 100% and 90.5%, tenth year rates were 100% and 84.1%, and twelfth year rates were 100% and 80.6%, respectively. The cumulative survival rate in patients with HBV-DNA loss was significantly higher than that in patients without HBV-DNA clearance ($P = 0.0030$, log-rank test).

The HCC-free survival rates were also assessed in the two patient groups (Fig. 7). Fifth-year HCC-free survival rates in patients with and without HBV-DNA loss were 100% and 90.6%, seventh year rates were 100% and 81.3%, tenth year rates were 92% and 74.8%, and fifteenth year rates were 92% and 51.2%, respectively. The HCC-free survival rate in patients with HBV-DNA loss was significantly higher than that in patients without HBV-DNA clearance ($P = 0.0036$, log-rank test).

DISCUSSION

Until recently, several authors mentioned the anti-carcinogenic activity of IFN in patients with HBV-related cirrhosis. Oon[18] and Ikeda *et al.*[21] have shown that IFN significantly decreased carcinogenesis in patients undergoing IFN therapy with a relative risk of 0.03 and 0.39, respectively. Lin *et al.* also demonstrated an anti-tumor activity of IFN, with a relative risk of 0.11 in a randomized controlled trial for patients with chronic hepatitis and cirrhosis.[23] Mazzella *et al.*,[19] Fattovich *et al.*[20] and the International Interferon-alpha Hepatocellular Carcinoma Study Group in Europe[22] demonstrated a low relative risk for carcinogenesis in patients with IFN therapy, but none could show a statistically significant difference. Aside from the slightly inconsistent results after IFN therapy for cirrhosis, we tried to elucidate the relationship between virological response and HCC development, using a cohort of consecutive patients with cirrhosis who underwent IFN therapy more than 10 years ago. Considering that the disease activity and carcinogenic potency can change significantly in the course of HBV-related liver disease, a longitudinal analysis was carried out for the study of the clinical process and the mechanism of anti-tumor activity of IFN in HBV-positive cirrhosis patients.

In this clinical study, sequential trends of HBV concentration were significantly associated with hepatocellular carcinogenesis, as was found in natural clinical courses of patients without IFN.[27] Although only two of 25 patients who developed HCC showed a disappearance of HBV-DNA during or after IFN therapy, 11 of 32 patients who showed carcinogenesis could not eliminate HBV-DNA using treatment with IFN ($P = 0.019$). A point in common found in the two exceptional patients with HCC development after elimination of HBV-DNA was that the HCC were detected immediately after a significant decrease in the HBV-DNA level after using IFN in the clinical courses: 1.2 years and 3.6 years after in each patient. We can reasonably consider that the discovered HCC in the patients already existed at an indiscernible size at the time of HBV-DNA elimination, and that the minimal HCC automatically grew gradually for the following few years after the decrease in HBV-DNA levels occurred. Even including these two patients with HCC development, the risk of hepatocellular carcinogenesis was significantly associ-