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英文要旨
Present status of artificial liver support for acute liver failure in Japan

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We examined the present status of artificial liver support (ALS) for acute liver failure (ALF) in Japan, by sending the questionnaires about the number of ALF cases, the methods of ALS and the recovery rate of consciousness. ALS comprising plasma exchange and hemodiafiltration (HDF) has been performed in more than 80% of 125 hospitals, but high flow volume HDF (high flow continuous HDF or on-line HDF) in 23 hospitals only. Totally, 50% of patients with fulminant hepatitis and late-onset hepatic failure achieved restoration of consciousness, which was lower than that reported by high flow volume HDF. On-line HDF which reduces the cost and simplifies the procedure is expected to be the main ALS in patients with ALF in the future.
Key words: acute liver failure, artificial liver support, on-line hemodiafiltration

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Original Article

Possible widespread presence of hepatitis A virus subgenotype IIIA in Japan: Recent trend of hepatitis A causing acute liver failure

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Aim: Recently, the number of acute hepatitis A cases has decreased in Japan. However, six patients with acute liver failure caused by hepatitis A virus (HAV) have been admitted to Chiba University Hospital, Japan, in the last 18 months, between 2010 and June 2011. The aim of this study is to characterize the recent HAV genotypes from an urban hospital in Japan and to compare the clinical differences.

Methods: Hepatitis A virus RNA was detected by strand-specific reverse transcription. Then, HAV VP1/2A regions were amplified by nested polymerase chain reaction (PCR).

Sequences were directly determined and phylogenetic trees were constructed for determining HAV subgenotypes.

Results: Analysis of these HAV genomes revealed that 4 and 2 belonged to subgenotypes IA and IIIA, respectively.

Conclusions: Fujiwara *et al.* reported a frequency of HAV subgenotype IIIA of only 2.1% in Japan. We conclude that HAV subgenotype IIIA might be widespread in our country.

Key words: acute liver failure, hepatitis A virus, Japan, subgenotype IIIA

INTRODUCTION

HEPATITIS A VIRUS (HAV) is a member of the genus *Hepatovirus* in the *Picornaviridae* family. HAV is a positive-stranded RNA virus with an approximately 7.5 kb genome, is usually spread via the fecal-oral route, causes acute hepatitis, and occasionally leads to acute liver failure with fatal outcome in unvaccinated individuals.[1,2] There is only one serotype of HAV, but based on sequences of the VP1/2A genomic region, at least six genotypes (I to VI) exist.[3] Three (I, II and III) of the genotypes are of human origin.

Several studies on HAV genotypes in Japan were reported.[3–6] In 1992, Robertson *et al.*[3] reported the existence of two predominant subgenotypes, IA and IIIB. In 2003, Fujiwara *et al.*[4] determined that 44 of 47 acute hepatitis A cases belonged to subgenotype IA, two to IB, and one to IIIA. In 2006, Takahashi *et al.*[5] also reported that 57 of 58 sequences belonged to IA and only one to IIIA. Toyoda *et al.*[6] reported that all 61 isolates they determined between 1992 and 2003 belonged to subgenotype IA. These reports revealed that the HAV subgenotype IA was endemic to Japan.[4–6]

Recent studies on HAV genotypes from South Korea have shown a distinct pattern change in circulating HAV genotypes over the past 10 years.[7] Until early 2000, almost all isolates tested had been identified as subgenotype IA.[8] A more recent study showed that subgenotype IIIA has been predominant since 2008.[7] In addition, a rise in the frequency of hepatitis A outbreaks has recently been observed in South Korea, our immediate neighbor, although the number of hepatitis A

cases in Japan has been progressively decreasing during the last several years.[9] The two countries have some cultural similarities. There is no universal hepatitis A vaccination program in either country, whereas Korea, but not Japan, has such a program against hepatitis B. We also reported that HAV 5′NTR subgenotype IA from Korea had high homology to Japanese sequences.[9] These circumstances have raised concerns about a possible HAV epidemic in Japan. The aim of this study is to characterize the recent HAV genotypes from an urban hospital in Japan and to compare the clinical differences.

METHODS

Patients

SERA WERE COLLECTED from immunoglobulin M (IgM) antibodies to HAV (IgM-HA) positive patients upon admission to Chiba University Medical School Hospital, Chiba, Japan. HAV infection was defined by positive reactions for IgM-HA and serum HAV RNA by polymerase chain reaction (PCR) with primers from the highly conserved 5′ non-translated region (5′NTR).[9] These patients presented with acute liver failure without encephalopathy on admission between 2010 and June 2011 (Table 1). This study was approved by the ethics committee of Chiba University, Japan (permission number 1160), the ethics committee of the National Institute of Infectious Diseases Japan (permission number 305), and complied with the Helsinki Declaration.

RNA extraction and detection of HAV RNA by PCR

RNA was extracted from 100 μL of serum samples according to the guanidium thiocyanate method and subjected to RT-PCR for the VP1/2A region of the HAV genome.[3] Complementary DNA was synthesized with HAV-3273 (5′-CCA AGA AAC CTT CAT TAT TTC ATG-3′), then amplified with HAV-3273 and HAV-2799 (5′-ATT CAG ATT AGA CTG CCT TGG TA-3′) for 40 cycles at 94°C, 50°C, and 72°C. Then, the first PCR product was further amplified with inner primer pairs HAV-2907 (5′-GCA AAT TAC AAT CAT TCT GAT GA-3′) and HAV-3162 (5′-CTT CYT GAG CAT ACT TKA RTC TTT G-3′) in the same manner. Amplified products were separated by agarose gel electrophoresis and stained with ethidium bromide.

Sequencing of the VP1/2A region

Sequences were directly determined as previously described.[9]

Phylogenetic analysis

A phylogenetic tree was constructed by using GENETYX, version 10 (Genetyx, Tokyo, Japan) based on the nucleotide sequences of the amplified VP1/2A region. The GenBank accession numbers for the nucleotide sequences of HAV isolates are AB643799 – AB643804. HAV complete genome sequences were retrieved from the DDBJ/EMBL/GenBank genetic database and used as references in this study.

RESULTS

SIX PATIENTS WITH acute liver failure caused by HAV were admitted during an 18-month period between 2010 and June 2011 (Table 1). All patients had >38.5°C fever on admission. All patients presented with acute liver failure with coagulopathy but without encephalopathy (non-fulminant cases) (Fig. 1). Patient no. 2 was a hepatitis B virus carrier. All patients recovered

Table 1 Profiles of six acute liver failure patients infected with hepatitis A virus in Japan

Patient no.	Age (years)/sex/ nationality	Month of onset	Nadir PT (%/INR)	Peak ALT (IU/L)	Peak total bilirubin (mg/dL)	Presumed route of transmission	Isolate name/ subgenotype
1	69/F/JPN	2010 Mar	23/2.88	7731	8.5	Raw scallop	Ch24/IIIA
2	46/M/JPN	2010 Apr	25/2.71	3388	12.6	Unknown	Ch23/IA
3	59/M/JPN	2010 Jun	35/2.01	5693	22.8	Raw oyster	Ch26/IA
4	30/F/KOR	2010 Jul	36/1.98	6958	5.0	Raw oyster	Ch25/IIIA
5	54/M/JPN	2011 Jan	20/3.20	2979	10.1	Sushi	Ch27/IA
6	37/M/JPN	2011 Jan	34/2.11	9826	3.9	Sushi	Ch29/IA

ALT, alanine transaminase; F, female; G, subgenotype; INR, international normalized ratio; JPN, Japan; KOR, South Korea; M, male; PT, prothrombin time.
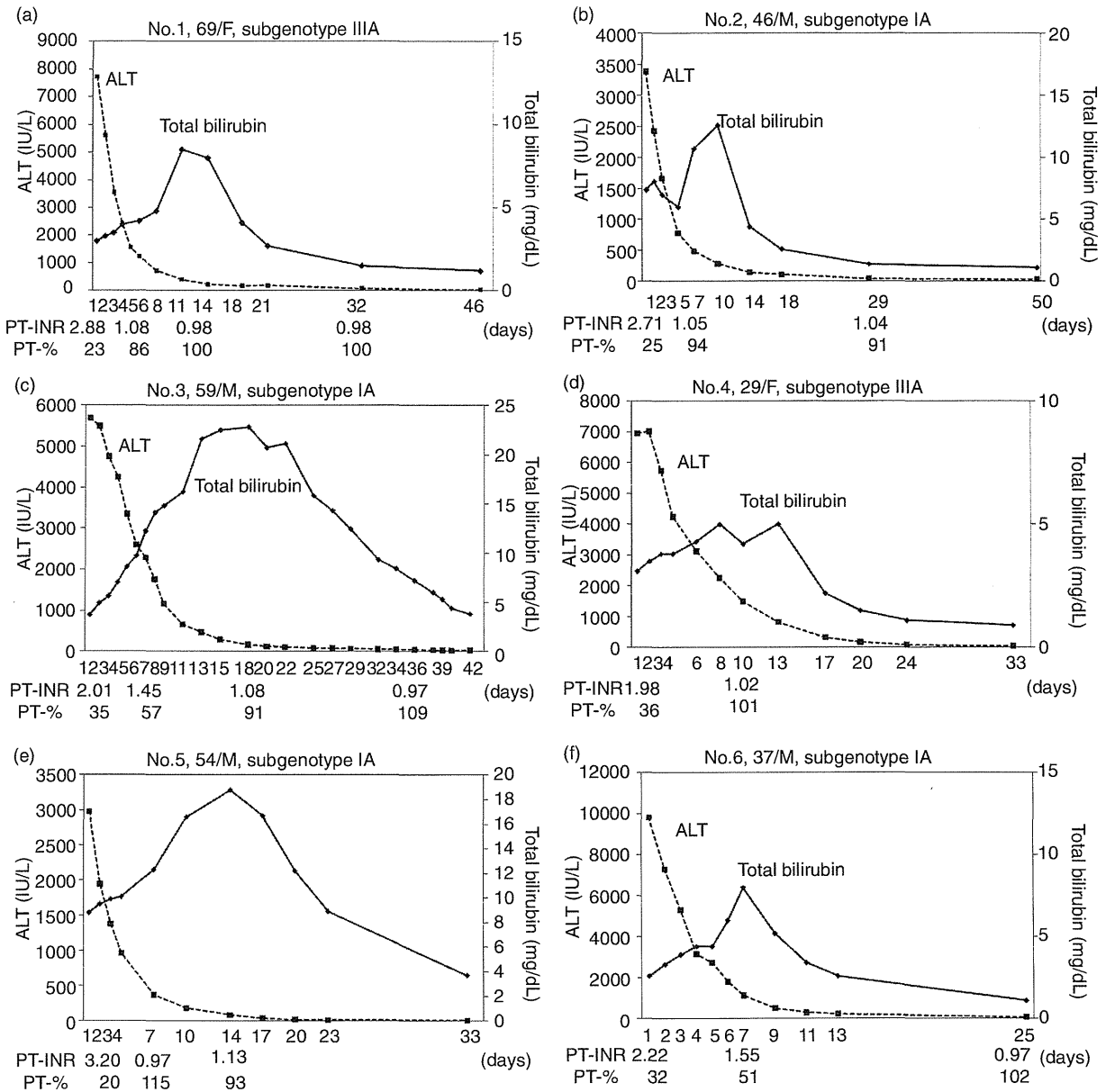
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Figure 1 Clinical course of six acute liver failure patients infected with hepatitis A virus (HAV) in Japan. (a), (b), (c), (d), (e) and (f) indicates patient no. 1, no. 2, no. 3, no. 4, no. 5 and no. 6 in Table 1, respectively. All patients presented with acute liver failure with coagulopathy but without encephalopathy (non-fulminant cases). PT, prothrombin time.

without liver transplantation, although patient no. 3 had interstitial pneumonia and was complicated by prolonged cholestasis while hospitalized and bone marrow suppression during the follow-up period, and patient no. 5 was complicated by mild acute kidney injury but recovered.

The nucleotide sequences of the six human HAV isolates in this study were compared with those of 24 published HAV sequences, and the genetic relatedness of the HAV isolates from different genotypes was investigated. Phylogenetic analysis of the nucleotide sequences from the VP1/2A region showed that four isolates (Ch23,

Ch26, Ch27 and Ch29) and two isolates (Ch24 and Ch25) belonged to subgenotype IA and IIIA, respectively (Fig. 2).

The sequences of the four isolates of subgenotype IA closely matched that of one well-characterized subgenotype IA virus: FH1 (GenBank accession no. AB020567) (96–97% nucleotide identity). Similarity of the nucleotide sequences of the VP1/2A region between the four isolates of subgenotype IA in this study ranged from 95% to 99%.

The sequences of the two isolates of subgenotype IIIA closely matched that of two well-characterized subgenotype IIIA viruses: A408 (GenBank accession no. AB046904) (99–100% nucleotide identity) and NOR-21 (GenBank accession no. AJ299464) (98% nucleotide identity). Similarity of the nucleotide sequences of the VP1/2A region between the two isolates of subgenotype IIIA in this study was 98%. Our two strains were clustered with A408 (Japan), NOR-21 (Norway), HA-JNG04-90F (Japan), HMH (Germany) and subgenotype IIIA strains reported from Japan in early 2010. Another subgenotype IIIA cluster was formed by two strains, HAJ95-8F (Philippines) and HA-JNG08-92F (Madagascar).

DISCUSSION

IN THE PRESENT study, of six recent patients with HAV-associated acute liver failure, two were caused by subgenotype IIIA. It was reported that almost all acute hepatitis A cases (93.6%) were caused by subgenotype IA and only 2.1% by subgenotype IIIA,[4] and that all acute liver failures were caused by subgenotype IA. Thus, the possibility of a changing pattern in circulating HAV genotypes such as that reported in Korea[7] might need to be entertained in Japan as well.

What about the transmission route? Many high-risk groups such as travelers visiting highly endemic areas, the military, healthcare workers, sewage workers,
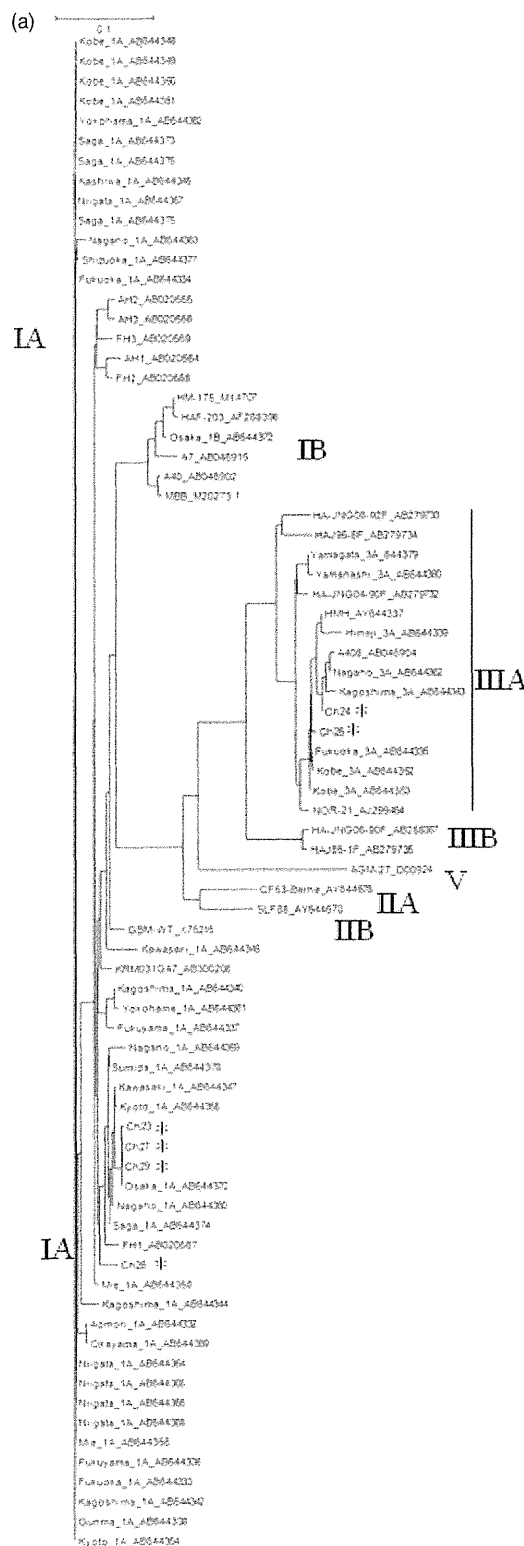
Figure 2 Phylogenetic analysis of hepatitis A virus (HAV) isolates from patients with acute liver failure from Japan. (a), (b) The neighbor joining tree was constructed based on a partial sequence of 451 nt in the VP1/2A region of HAV. Selected reference strains were also included in the phylogenetic analysis to represent the following subtypes: HAV-IA, IB, IIA, IIB, IIIA, IIIB, and V. *Strains sequenced in this study are indicated (Ch23, Ch24, Ch25, Ch26, Ch27 and Ch29), aligned with all the available reference sequences retrieved from data bases (DDBJ/EMBL/Gene Bank).
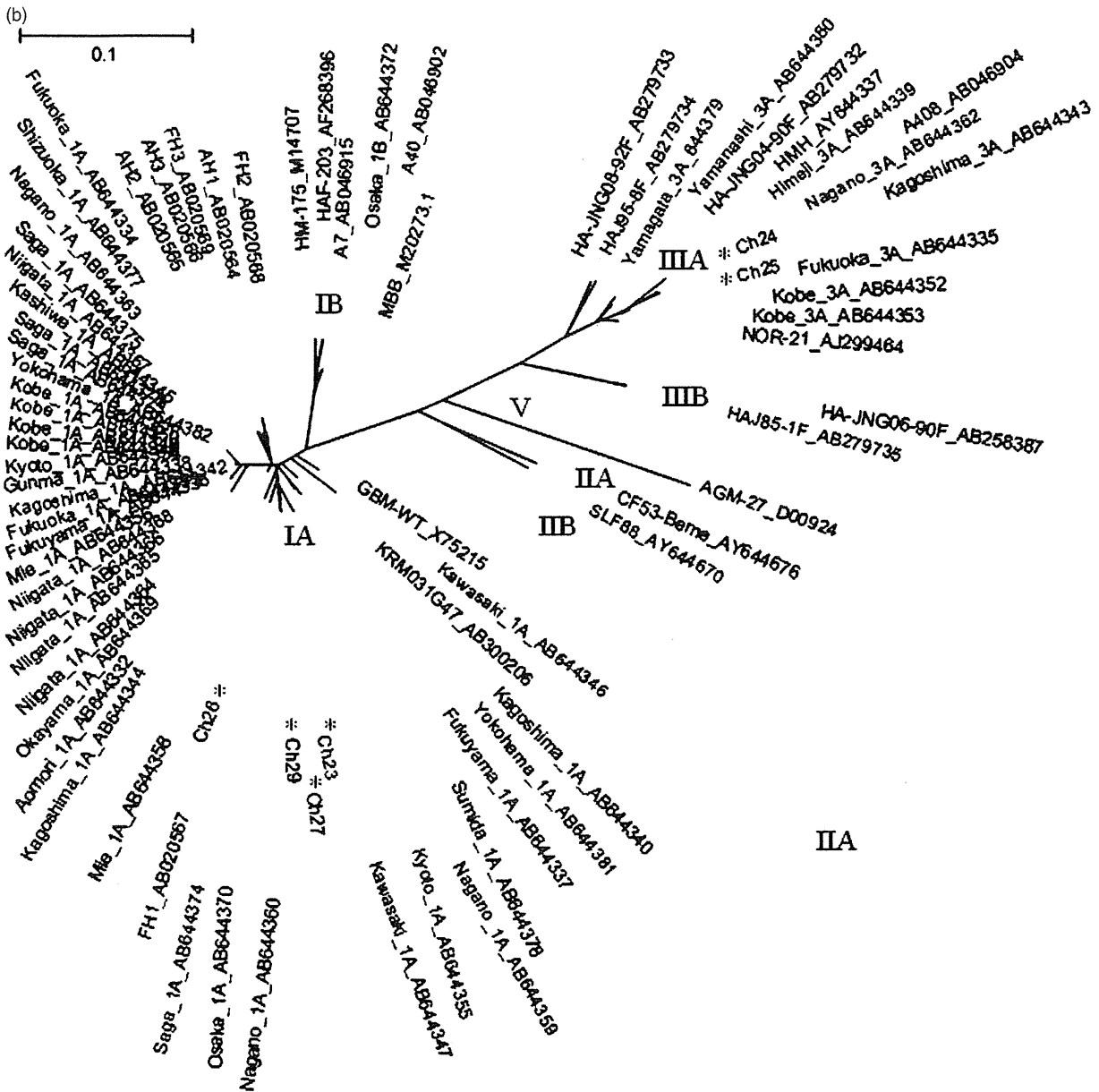
(b)

Figure 2 *Continued.*

day-care assistants, drug addicts, and homosexual people have been identified for potential HAV infection.[10] In the present study, four patients with subgenotype IA were male and two with subgenotype IIIA were female. We do not know why there were sex differences between the two subgenotypes. None in the present study was homosexual or HIV-positive. Patients no. 5

and no. 6 were associated with a recent HAV outbreak at a sushi shop in the Chiba area (Table 1).[11] None of the patients had traveled abroad, including to South Korea, during more than one year before admission. That is, all patients were infected with HAV in our country, suggesting that HAV subgenotype IIIA might be widespread in our country. Of interest is that these two patients (no. 1

and no. 4) had eaten raw scallops and raw oysters, respectively (Table 1).

The clinical spectrum of HAV infection ranges from asymptomatic infection to fulminant hepatitis.[12] Clinical presentation of hepatitis A depends on the age of the patient, being more severe in adults than in children.[13] In the present study, the mean age of subgenotype IA and IIIA patients was 49 ± 9.6 and 49.5 ± 27.5 years, respectively. A recent study from Korea reported that HAV genotype influences the severity of liver disease and that a higher ALT level (>1000 IU/L) and longer hospitalization were significantly associated with subgenotype IIIA.[7] All HAV-associated acute liver failure patients in the study of Fujiwara *et al.*[4] belonged to subgenotype IA. In this regard, we also examined whether HAV genotype is directly related to the disease severity of hepatitis A. Two of the six acute liver failure patients in the present study were subgenotype IIIA. It is well-known that viral genotypes occasionally affect disease progression, severity and treatment response in hepatitis B and C.[14,15] Mean ALT levels of subgenotype IA and IIIA patients were 5470 ± 3130 and 7340 ± 546 IU/L, respectively. Further studies will be needed to examine whether there are associations between HAV genotypes and disease severities, as the number of patients was limited and most of the patients in Chiba University Hospital were cases with acute liver failure.

In conclusion, the current study suggested that HAV subgenotype IIIA is also associated with acute liver failure in Japan. We need to make a cautious interpretation of the relation between HAV genotypes and their disease severities.

ACKNOWLEDGMENTS

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ウイルス肝炎のすべて

Ⅱ 経口感染するウイルス肝炎

1. A型肝炎

（1）A型肝炎のウイルス学

藤原 慶一[*1]　神田 達郎[*2]　横須賀 收[*3]

　わが国では，かつてのような粗悪な衛生環境にともなう糞口感染によるA型肝炎の大流行はみられなくなったが，輸入食品・性行為など感染経路の多様化によりA型肝炎は今なおウイルス性急性肝炎中10%以上を占め，決して過去の疾患ではない。一般にその予後はきわめて良好であるが，肝炎の重症化・劇症化例も散見される。従来から指摘されている宿主側の因子（高齢化，他の肝疾患の合併など）のみではなく，我々の検討では，肝炎の重症化とHAV遺伝子の複数の領域の変異が関連している可能性が示唆されたが，特異的変異は同定されなかった。また，A型劇症肝炎の集団発生の報告はまれであることから，A型肝炎の重症化については，ウイルス因子・宿主因子，そして免疫応答等のさまざまな要素が関与しているものと思われる。

Key Words：A型肝炎ウイルス，ウイルス変異，重症化

Ⅰ はじめに

　A型肝炎はA型肝炎ウイルス（hepatitis A virus：HAV）の糞口感染によって起こる急性肝炎である。わが国においては，衛生環境の悪さにともなうかつてのような大流行はみられなくなったが，散発性急性肝炎中，A型肝炎の占める割合は20%前後あり，決して過去の疾患ではない。

　現在のHAV感染は感染源・感染経路の多様化とも関連していると考えられる。教科書的な生牡蠣などの二枚貝の摂取による季節的発生から，輸入食品（魚介類，野菜，果物等）の関与が考えられる通年的発生，さらには，同性愛者を含む性行為の関与が考えられる感染などが増加している。

　一般にA型肝炎の予後はきわめて良好であるが，ときに急性腎不全などの重篤な合併症を生じたり，肝炎の重症化・劇症化例や死亡例も認められる。

Hepatitis A virus

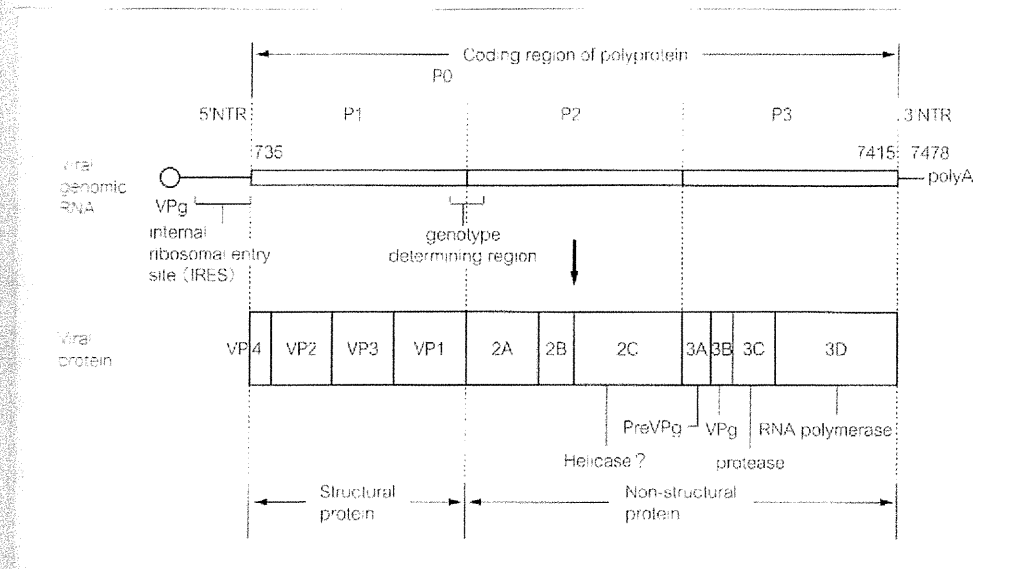[*] 千葉大学大学院腫瘍内科学　[1] 講師　*Keiichi Fujiwara*　[2] 特任講師　*Tatsuo Kanda*
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図1　HAV の遺伝子構造

HAV 遺伝子は約 7500 塩基の（＋）鎖1本鎖 RNA で，VPg タンパクが共有結合する 734 塩基の 5' 非翻訳領域（5'NTR），6681 塩基のひとつの ORF，63 塩基の 3' 非翻訳領域（3'NTR），polyA tail より成る．
HAV：A型肝炎ウイルス，VPg：genome-linked viral protein，ORF：open reading frame

（文献2より改変）

I ウイルス粒子の性状

HAV はピコルナウイルス科ヘパトウイルス属に分類される RNA ウイルスであり，直径 27 nm のエンベロープをもたない正二十面体粒子である[1]．血清型は1種類であるが遺伝子型（genotype）は6種類に区別されている．HAV は熱・エーテル・クロロホルム・界面活性剤・タンパク分解酵素・乾燥などに対して抵抗性であるが，高圧滅菌・UV 照射・ホルマリン・塩素剤・ヨウ素などで失活する．肝炎ウイルスの中で唯一，細胞培養可能であり，ヒトかサル由来の培養細胞に感染するが，他のピコルナウイルスと比較して増殖速度が遅い．このため，細胞培養によるウイルス分離は長時間を要し診断には適し

ていない．また，一般に宿主感染細胞の高分子合成系を阻害せず細胞変性効果（cytopathic effect：CPE）を示さない．

II ウイルスゲノムの構造と機能

HAV 遺伝子は約 7500 塩基の（＋）鎖1本鎖 RNA で，VPg（genome-linked viral protein）タンパクが共有結合する 734 塩基の 5' 非翻訳領域（5' non-translated region：5'NTR），6681 塩基のひとつの open reading frame（ORF），63 塩基の 3' 非翻訳領域（3'NTR），polyA tail より成る（図1）[2]．

5' および 3'NTR は高度に保存されており，ウイルスの翻訳・複製に重要な stem loop の高次構造が存在する．5'NTR には 151 〜 734 塩基領域に internal ribosomal

HAV（hepatitis A virus：A型肝炎ウイルス）　　CPE（cytopathic effect：細胞変性効果）
VPg（genome-linked viral protein）　　5'NTR（5' non-translated region：5' 非翻訳領域）
ORF（open reading frame）　　3'NTR（3' 非翻訳領域）　　IRES（internal ribosomal entry site）

entry site(IRES)が存在し、リボソームが直接相互作用して翻訳を開始させる。また、細胞培養馴化株では特定部位の塩基の置換や欠失がみられ、これらの変異により増殖速度が上昇する。IRES の翻訳効率や RNA の複製効率が上昇する変異も報告されている。3′NTR はウイルスゲノムの(−)鎖 RNA の合成に関与する。

ORF は 2227 アミノ酸残基の前駆体ポリタンパク質 P0 をコードする。P0 は P1、P2、P3 領域からなり、後述のウイルスプロテアーゼ 3C によって各ウイルスタンパク質へ切断される。P1 は構造タンパク質を構成し VP4・VP2・VP3・VP1 に切り出され、ウイルス粒子を形成する。P2、P3 は非構造タンパク質でウイルスの複製に関与する。P2 からは 2A・2B・2C が切り出され、細胞馴化株では 2B・2C 領域に多くの変異がみられ、ウイルスの複製に関与している。また、2C には NTP 結合モチーフが存在し、RNA ヘリカーゼ活性があると考えられている。P3 からは 3A・3B・3C・3D が切り出され、3C はプロテアーゼである。3B は VPg でゲノム RNA の 5′末端に共有結合する。3D は RNA 依存 RNA ポリメラーゼで、(+)、(−)鎖ウイルスゲノム RNA の合成を行うと考えられている。3AB は 3CD、ゲノム RNA と相互作用することにより膜に固定され複製複合体を形成すると考えられている。

HAV の VP1/2A 接合領域には hypervariable region が存在する。この領域の 168 塩基の相同性 85% を基準として 6 種類の genotype が決定される。さらに、I 型、II 型、III 型は 92.5% を基準として 2 種類の亜型(sub-genotype)に分けられる。従来、VII 型とされていたものは II B 型となった。ヒトからは、I(I A、I B)、II(II A、II B)、III(III A、III B)の 3 つの genotype が検出さ

れた。IV、V、VI の 3 つの genotype はサルのみから分離された。Genotype を決定することにより、A 型肝炎の感染・伝播に関して分子疫学的な検討を行うことができるようになった。

IV HAV と臨床病態

A 型肝炎の重症化については宿主側の因子(高齢化、慢性肝疾患の存在など)に依存すると考えられてきたが、我々の検討では、宿主側には有意差をもって重症化の因子と考えられるものは認めなかった。HAV 側の因子については、これまで培養細胞やサルでの検討はなされてきたが、ヒトの臨床病態との関連についての検討は少ない。ここでは、血清中 HAV 遺伝子変異と A 型肝炎の重症化との関連について筆者らの知見をもとに述べる。

1. 血清中 HAV の検出

A 型肝炎のウイルス血症の期間は潜伏期までに限られ、かつては発症後はみられないと考えられてきたが、Yotsuyanagi らは、トランスアミナーゼがピークに達する前の発症早期であれば RT-PCR(逆転写ポリメラーゼ連鎖反応)法により血中に HAV RNA を検出しうることを示した。

我々は nested RT-PCR 法を用いて HAV RNA の検出を検討した結果、初期血清では約 90% の症例で HAV が検出され、ほとんどの症例で HAV を解析することが可能となった。血清中 HAV はほとんどの症例では発症後約 3 週まで検出されたが、一部の症例では約 6 週まで認められた(3〜114 日間、平均 18±14 日間)(**図2**)。ほとんどの遷延例、重症化例においても特にウイルス血症期間の延長は認めなかった。

HAV の検出期間について Bower らは、米国の B 型肝炎ワクチン試験中の患者血清を用いて ALT(アラニンアミノトランスフェラー

RT-PCR(逆転写ポリメラーゼ連鎖反応)　　ALT(アラニンアミノトランスフェラーゼ)

図2　血清中の HAV 検出期間

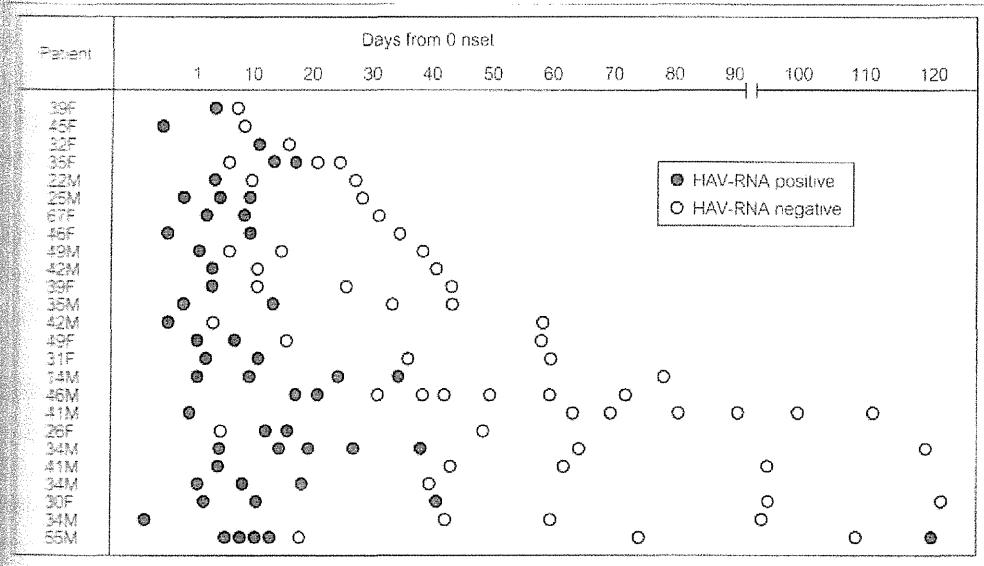　血清中 HAV はほとんどの症例では発症後約3週まで検出されたが，一部の症例では約6週まで認められた（3～114 日間，平均 18±14 日間）。

　HAV：A型肝炎ウイルス

（文献7より）

　　ピークの 17 日前から 79 日後まで HAV が検出されたことを報告している[5]。その他国々から 20 ～ 42 日間の検出期間の報告があるが，これら検出期間の違いは，使用された primer の設定部位・個数，血清の保存状態等によるところが大きいと考えられる[6]。

　このように，ウイルス血症は従来考えられていたよりも長期間にわたりみられることが明らかになった。糞便中にはさらに長期に（3～4カ月後まで）検出されることが報告されている[3,4]。

2．全ゲノム配列

　劇症肝炎（fulminant hepatitis：FH）および通常の急性肝炎（acute hepatitis：AH），各3例由来の HAV の全塩基配列を解析すると，5'NTR の中央部において FH では AH に

比べてヌクレオチド変異が少なく，また，非構造タンパク領域の 2B において FH で変異がやや多い傾向が認められた。FH と AH のそれぞれに特異的な変異はみられなかった（図3，4）[12]。前述のように，5'NTR には IRES が存在し RNA の翻訳・ウイルスの複製に関与し，2B・2C タンパクはウイルス RNA の複製に関与する。培養細胞にて細胞障害効果を示すウイルス株においては非構造タンパク領域 2B，2C の変異が必要であり，5'NTR の変異と相互作用しているとの報告がある[3]。ヒト臨床検体においてもそれに合致する所見を認めた。

3．遺伝子型（genotype）

　1990 年代の全国のさまざまな病型の症例血清で genotype を検討すると，FH と急性肝炎重症型（acute hepatitis severe type：

FH (fulminant hepatitis：劇症肝炎)　　AH (acute hepatitis：急性肝炎)

AHs (acute hepatitis severe type：急性肝炎重症型)

Ⅱ　経口感染するウイルス肝炎 ─────────────
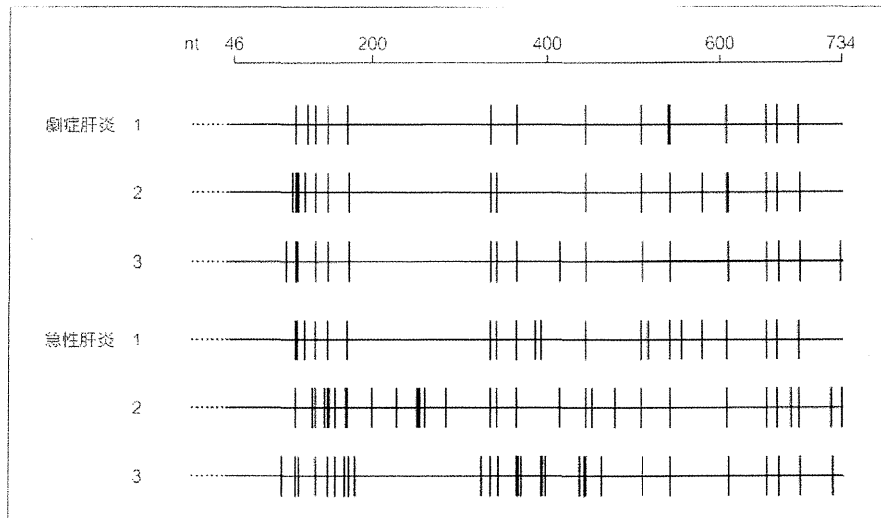
図3　全ゲノム解析（5'NTR領域の変異）
5'NTR の中央部において FH では AH に比べてヌクレオチド変異が少なかった。
5'NTR：5'非翻訳領域，FH：劇症肝炎，AH：急性肝炎　　　　　（文献12より）
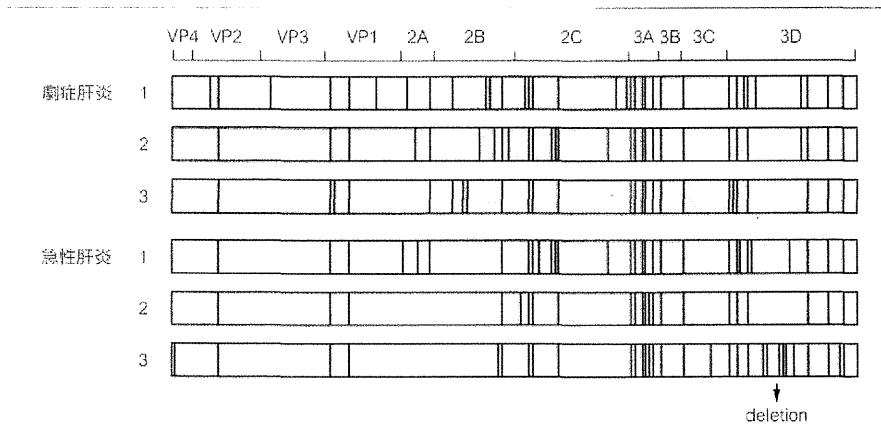
図4　全ゲノム解析（タンパク翻訳領域の変異）
非構造タンパク領域の 2B において FH で変異がやや多い傾向が認められた。FH と AH のそれぞれに特異的な変異はみられなかった。　　　（文献12より）

AHs）の全例を含む94％においてⅠAであり，ⅠBを4％，ⅢAを2％に認めた。Geno-type と重症化との関連はみられなかった。同時期，同地域でも多様なⅠAの感染がみられた（表）[11]。

2010年に全国的にA型肝炎が多発したが，フィリピン由来と考えられるⅠA株や韓国で流行したⅢA株に近似したものが認められ，ⅢAが30％を占めた。海外の株がわが国に侵淫してきたためと考えられた[15][16]。

4．5'NTR，2B，2C 領域の変異

5'NTR の中央部（nt 200-500）のヌクレオチド変異を調べた我々の成績では，AH では多様な strain がみられたが，FH・AHs では

表　A型肝炎の重症度とHAV遺伝子型

	strain	DDBJ accession number	genotype	location	year
FH (劇症肝炎)	A5	AB046906	IA	Kanto	1993
	A10	AB046878	IA	Kanto	1994
	A204	AB046889	IA	Tohoku	1990
	A205	AB046890	IA	Tohoku	1994
	A206	AB046891	IA	Kanto	1993
	A414	AB046905	IA	Shinetsu	1989
	A601	AB046911	IA	Shinetsu	1997
	A1	AB046877	IA	Kanto	1992
AHs (急性肝炎 重症型)	A6	AB046910	IA	Kanto	1992
	A16	AB046881	IA	Kanto	1994
	A160	AB046882	IA	Kanto	1998
	A197	AB046885	IA	Kanto	1993
	A201	AB046888	IA	Tohoku	1993
	A302	AB046895	IA	Chugoku	1997
	A702	AB046916	IA	Kanto	1999
	A811	AB046921	IA	Kanto	1996
	A159	AB046880	IA	Kanto	1998
	A196	AB046884	IA	Kanto	1993
	A200	AB046887	IA	Tohoku	1995
AH (急性肝炎)	A9	AB046923	IA	Kanto	1992
	A32	AB046899	IA	Kanto	1991
	A38	AB046900	IA	Kanto	1991
	A39	AB046901	IA	Kanto	1991
	A51	AB046907	IA	Kanto	1993
	A55	AB046908	IA	Kanto	1993
	A58	AB046909	IA	Kanto	1993
	A62	AB046912	IA	Kanto	1993
	A65	AB046913	IA	Kanto	1993
	A75	AB046918	IA	Kanto	1994
	A157	AB046879	IA	Kanto	1997
	A195	AB046883	IA	Kanto	1999
	A301	AB046894	IA	Chugoku	1994
	A303	AB046896	IA	Chugoku	1998
	A306	AB046897	IA	Chugoku	1996
	A307	AB046898	IA	Chugoku	1997
	A406	AB046903	IA	Shinetsu	1986
	A814	AB046922	IA	Kanto	1997
	A20	AB046886	IA	Kanto	1991
	A28	AB046893	IA	Kanto	1991
	A68	AB046914	IA	Kanto	1993
	A80	AB046920	IA	Kanto	1994
	A207	AB046892	IA	Kanto	1995
	A713	AB046917	IA	Kanto	1998
	A77	AB046919	IA	Kanto	1993
	A7	AB046915	IB	Kanto	1992
	A40	AB046902	IB	Kanto	1992
	A408	AB046904	IIIA	Shinetsu	1987

　1990年代の全国のさまざまな病型の症例血清でgenotypeを検討すると，FHと急性肝炎重症型（AHs）の全例を含む94％においてIAであり，IBを4％，IIIAを2％に認めた．Genotypeと重症化との関連はみられなかった．同時期，同地域でも多様なIAの感染がみられた．

HAV：A型肝炎ウイルス，FH：劇症肝炎　　　　　　　　　　　　　　（文献14より）
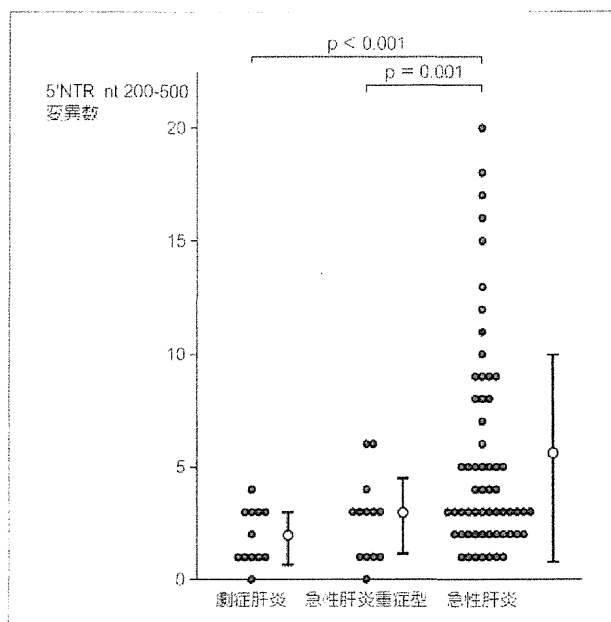
Ⅱ　経口感染するウイルス肝炎 ────────────

図5　A型肝炎の重症度と5' 非翻訳領域の変異数
　　Prototype に対する1症例当たりの平均変異数は重症例で有意に
変異が少なかった。　　　　　　　　　　　　　　　（文献5より）

核酸配列が近似した変異の少ない strain が
検出された。Prototype に対する1症例当た
りの平均変異数は重症例で有意に変異が少な
かった[5]（図5）。多数例で2Bの変異を検討
すると、重症例で aa100-200 にてアミノ酸
変異が多い傾向がみられた[17]。2C において
は FH で AH より有意にアミノ酸変異が少な
かった[18]。いずれの3領域においても重症例
に特異的な変異は認めなかった。

　5'NTR、2B、2C の3領域の揃った症例で
の系統樹解析（図6）では FH・AHs が近傍に
集積する部位があり、臨床的な重症化におい
ても細胞系と同様に、HAV の複数の領域の
変異が関与している可能性が考えられた。ま
た、そうした FH・AHs 症例では AH 症例よ
りウイルス量が多いことが観察された[19]。

5. ウイルス量

　多数例で real-time RT-PCR を用いてウイ
ルス定量を行うと、FH・AHs では AH に比
べて有意にウイルス量が多いことが確認され

た（図7）[20]。発症時の高度のウイルス増殖が
過度の免疫応答を引き起こし、多くの感染肝
細胞が破壊される結果、臨床的な重症化が生
じ、また、血中ウイルスは急速に減少するこ
とが推測された。

Ⅴ　おわりに

　A型肝炎の臨床的な重症化と、HAV 遺伝
子の複数の領域の変異が関連している可能性
が示唆されたが、B型肝炎にみられるような
特異的変異は同定されなかった。また、透析
施設等における B型劇症肝炎の集団発生は多
数報告されているが、A型劇症肝炎の集団発
生の報告はまれであることより、臨床的な立
場から、A型肝炎の重症化についてはウイル
ス因子・宿主因子、そして免疫応答等のさま
ざまな要素が関与しているものと思われる。

　しかし、多少なりとも HAV が重症化にか
かわっているならば、なおさら感染予防が重
要である。A型肝炎に対しては優れた不活化

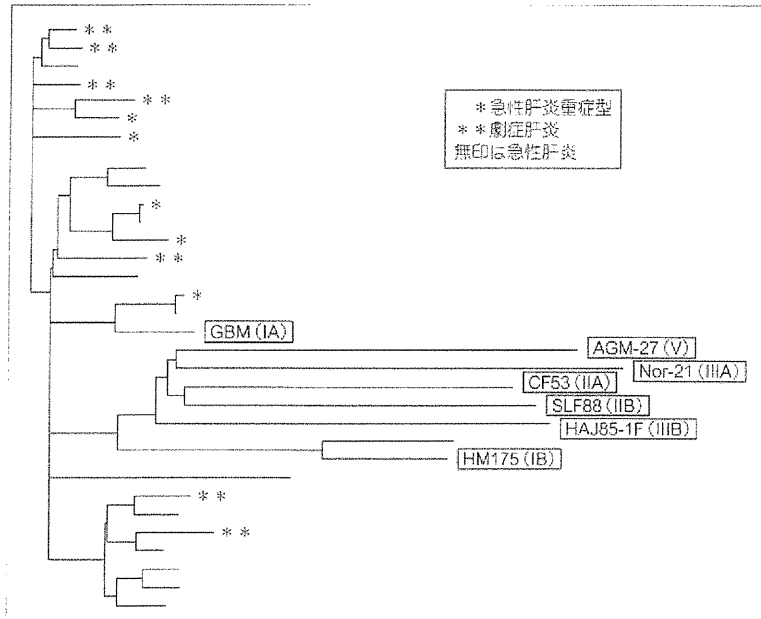**図6　2B＋2C領域の系統樹解析**

　5'NTR，2B，2Cの3領域の揃った症例での系統樹解析ではFH・AHsが近傍に
集積する部位があり，臨床的な重症化においても細胞系と同様に，HAVの複数の領
域の変異が関与している可能性が考えられた．

　5'NTR：5'非翻訳領域，FH：劇症肝炎，AHs：急性肝炎重症型

　HAV：A型肝炎ウイルス　　　　　　　　　　　　　　　　　　（文献19より）
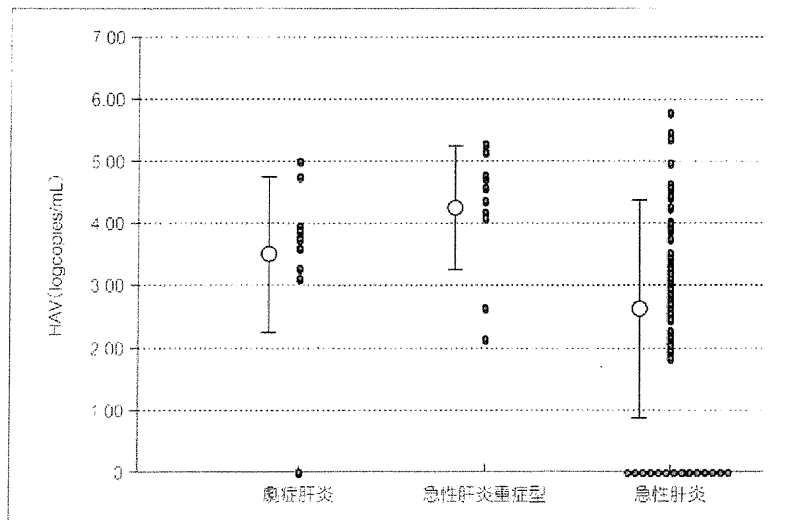

図7　A型肝炎の重症度とHAV定量

　FH・AHsではAHに比べて有意にウイルス量が多いことが確認された．

　HAV：A型肝炎ウイルス，FH：劇症肝炎，AHs：急性肝炎重症型，AH：急性肝炎

　　　　　　　　　　　　　　　　　　　　　　　　　　　　　（文献20より）

Ⅱ 経口感染するウイルス肝炎 ————

ワクチンが国内生産され利用可能であり，
universal vaccination を含めた検討も必要
と思われる。

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Demonstration of intrahepatic accumulated microbubble on ultrasound represents the grade of hepatic fibrosis

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Hidehiro Kamesaki · Keiichi Fujiwara · Fumio Imazeki · Osamu Yokosuka

Abstract

Objectives To examine the feasibility of perflubutane-based ultrasound for grading hepatic fibrosis.

Methods This prospective study included 202 subjects; main study (controls:33, F0–1:35, F2:26, F3:23, cirrhosis:29) and subsequent study (controls:16, F0–1:7, F2:20, F3:7, cirrhosis:6). Diagnostic abilities for assessing fibrosis grade were compared between contrast findings and FIB4 (age × AST/[platelet count × ALT$^{0.5}$]).

Results High-power emission produced an intrahepatic band-like structure, and the three-layer appearance was less frequent and monolayer appearance was more frequent in cirrhosis than controls/chronic hepatitis ($P<0.0001$). Intensity difference at 15-min phase showed most significant correlation with fibrosis grade ($\rho=0.79$, $P<0.0001$), and the best areas under the receiver operating characteristic curves are 0.88 for marked fibrosis, 0.95 for advanced fibrosis and 0.97 for cirrhosis, which were significantly higher than those of FIB4, 0.85 for marked fibrosis, 0.89 for advanced fibrosis and 0.90 for cirrhosis. Sensitivity, specificity and efficiency of the intensity difference were 88%, 72% and 81% for marked fibrosis, 85%, 91% and 89% for advanced fibrosis and 97%, 90% and 91% for cirrhosis, respectively. The subsequent study validated the main study results; significant correlation between the intensity difference and the fibrosis grade ($\rho=0.73$–0.77, $P<0.0001$).

Conclusions Perflubutane-based ultrasound accurately predicts the grade of hepatic fibrosis.

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Key Points

- *The behaviour of intrahepatic microbubbles depends on the severity of hepatic fibrosis.*
- *Layer enhancement pattern simply represents the degree of chronic liver disease.*
- *Parenchymal intensity change due to high-power emission predicts the hepatic fibrosis grade.*

Introduction

Liver biopsy remains the gold standard for grading hepatic fibrosis, although many studies have been carried out to find alternative methods [1]. Liver biopsy, however, has some shortcomings; invasiveness in patients with impaired coagulation and the possibility of sampling error owing to the heterogeneous distribution of fibrosis [1, 2]. Furthermore, because repeated assessment of the grade of hepatic fibrosis may be required during the management of a prolonged clinical course, a non-invasive technique would be preferred to replace this invasive procedure.

Ultrasound has the advantages of being simple, non-invasive, and enables real-time observation. Recent studies with sulfur hexafluoride (Sonovue; Bracco, Milan, Italy) have shown the effectiveness of haemodynamic assessment of microbubbles based on time-intensity analysis to diagnose advanced stages of fibrosis, but failed to find a significant difference in the parameters between the different fibrosis grades [3–5].

Sonazoid™ (GE Healthcare, Oslo, Norway) is a second generation microbubble agent, with the feature of being captured in reticuloendothelial tissue, such as

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the liver and spleen [6]. As the behaviour of micro-bubbles depends on the acoustic power, instantaneous high-power emission (IHPE) after the injection of Sona-zoid™ allows ultrasound to depict the difference in the parenchymal intensity change between cirrhosis and idiopathic portal hypertension, because an ultrasound beam with greater power than the threshold level destroys microbubbles immediately [7, 8]. Basically, as this novel technique estimates the amount of intrahepatic micro-bubbles by subtraction of the image before and after the microbubble breakdown, it may have the potential to assess the grade of hepatic fibrosis. The aim of this study was to determine the efficacy of contrast-enhanced ultrasound with Sonazoid™ as a non-invasive tool for the evaluation of the grade of hepatic fibrosis.

Materials and methods

Enrolment of the subjects

This prospective study was performed in Chiba University Hospital after approval by the ethics committee. The study was composed of two sub-studies; the main study (January 2008 to August 2009) aimed to classify the contrast-enhanced patterns of liver parenchyma, and to determine the relationship between contrast-enhanced findings and the grade of hepatic fibrosis, and the subsequent study (August 2009 to October 2010) investigated the agreement and variation of the data in the main study. The study enrolled the following subjects: chronic liver disease (CLD) patients who were scheduled for contrast-enhanced ultrasound before providing a liver sample (liver biopsy or liver transplantation) and healthy volunteers without signs of hepatic disease as controls (Fig. 1). However, we excluded patients with hepatic tumours diagnosed by ultrasound or egg allergy, which is a contraindication for Sonazoid™. Written informed consent was obtained from all partici-pants. Blood samples were collected from all CLD patients within the 3-day period before the ultrasound examinations, and FIB4 (age $\times$ AST/[platelet count $\times$ ALT$^{0.5}$]) was calculated as an indirect marker of fibrosis [9].

Ultrasound examinations

Ultrasound examinations (AplioXG, Toshiba, Tokyo, Japan; 3.75 MHz convex probe) were performed under the supine position after more than four hours of fasting. We screened the abnormalities such as intrahepatic arterio-portal/portal-venous communications and portal vein thrombosis, because these might affect the contrast-enhanced findings. The settings were changed for the contrast-enhanced study; harmonic mode with a low mechanical index

(MI, 0.25), a depth to cover the whole right lobe of the liver, a focus point 8 cm below the skin surface and a dynamic range of 55 dB. The perfluorobutane microbubble agent (Sonazoid™, 0.0075 mL/kg) was injected manually into the antecubital vein, followed by a 3 ml flush of normal saline. All the cine images were stored digitally on the hard disk of the ultrasound system. Clinical symptoms, including blood pressure and oxygen saturation before and after the contrast-enhanced ultrasound examinations were monitored to screen adverse events.

Main study

The liver parenchyma was examined via a right inter-costal approach at three different phases, 5, 10 and 15 min after injection of the agent, using IHPE at maximum acoustic power level (MI, 1.4–1.6; 1.5 s at 20 Hz), according to the previous study [8]. A different imaging plane was selected carefully for the observation of the next phase, because microbubble breakdown caused by the previous IHPE might affect the subsequent contrast enhancement. All the ultrasound examinations were performed by H.I., a hep-atologist with seven years' experience with ultrasound at the time of the initial case. Second contrast-enhanced ultrasound examination was performed to evaluate inter-operator agreement in the subjects who agreed to it and whose examination could be scheduled within 1 week after the initial examination. This was carried out by M.T., a hepatologist with more than eight years' experience with ultrasound.
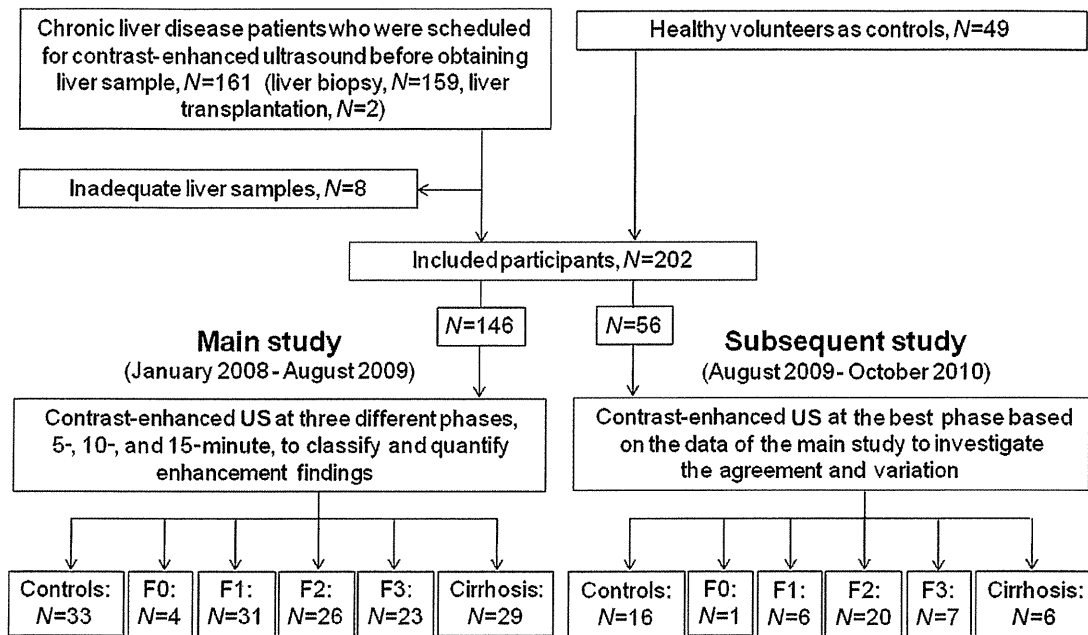
Subsequent study

It was the final step of our study to investigate the agreement and variation of IHPE data. The ultrasound observations were carried out for three different imaging planes in one of the three phases, which provided the enhancement findings closest to the fibrosis grade based on the data from the main study. All the ultrasound examinations were also performed by H.I.

Analysis of contrast-enhanced ultrasound data

Parenchymal enhancement

The initial review was performed by H.I. for the post-IHPE sonograms. Layer appearance was defined when the parenchymal enhancement showed band-like structure which appeared horizontally on the sonogram. Then the findings were reviewed by two reviewers (T.S. and H.K., hepatologists with 6 years' experience with ultrasound) who examined the inter-reviewer agreement with no prior knowledge of the pathological data or any other informa-

Fig. 1 The main study (January 2008 to August 2009) classified and quantitated the enhancement findings and the subsequent study (August 2009 to October 2010) investigated agreements and variations in the findings

tion of the patients, and provided a final result for layer appearances in this study by consensual decision-making.

Intensity analysis

The analysis was carried out using image analysing software (ImageLab-avi; Toshiba, Tokyo, Japan) with reference to the methodology described in the literature [8]. Two images, before and after IHPE, were prepared for each subject and three round-shaped regions of interests (ROIs, 10 mm in diameter) were placed manually and longitudinally at the centre of each image, from 10 to 30 mm below the liver surface. The difference of signal intensity (dB) before and after IHPE was calculated (Fig. 2). Considering the variance caused by the measurement processes, the average difference in signal intensities obtained from three measurements was used as intensity difference data.
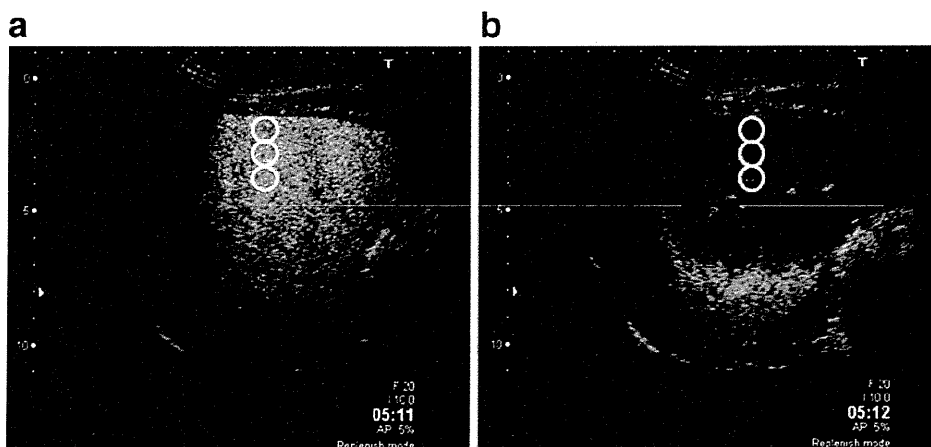
Fig. 2 Contrast-enhanced images of a 56-year-old woman with chronic hepatitis, in the 5-min phase. **a** Before instantaneous high-power emission (IHPE): the liver parenchyma showed homogeneous enhancement. Three regions of interests (ROIs, *white circles*) were placed longitudinally in the centre of the image from 10 to 30 mm below the liver surface. **b** After IHPE: the liver parenchyma around the liver surface appeared as a hypo-enhancement area because of the breakdown of microbubbles by IHPE. The difference in signal intensity (dB) between the two images was calculated and the average difference in the signal intensities was defined as "intensity difference"

Histological assessment

Liver samples were obtained within a week of the ultrasound examination. Paraffin-embedded specimens were stained with haematoxylin–eosin for assessment of cell morphology and Azan stain for assessment of fibrosis. Fibrosis grade and activity grade were assessed according to the METAVIR scoring system by the consensus reading of two expert hepatologists (F.I. and K.F., each with pathological examination experience of more than 20 years). Fibrosis was graded on a scale of 0–4 (F0, F1, F2, F3, cirrhosis) and activity grade was scored on a scale of 0–3 (A0–1, A2, A3). In this study, fibrosis grade was also evaluated quantitatively as a "fibrosis-ratio"; a digital image (40×) of an Azan-stained specimen was loaded into the image analysis software (Photoshop; Adobe systems, San Jose, CA, USA), using an off-line personal computer, and the collagen-fibre area, stained by aniline blue, and the entire tissue area were measured as pixel numbers using image binarisation techniques. The average ratio between them obtained by three-time measurements was defined as the fibrosis ratio (%) and the fibrosis ratio of controls was defined as 0% for data analysis [10].

Statistical analysis

The Spearman rank correlation was used for the correlation between discrete and continuous variables and Pearson's correlation coefficient was used for the correlation among continuous variables. The Chi-squared test was used to compare the layer appearances among controls and patients with chronic hepatitis and cirrhosis. The correlation between the fibrosis ratio and the intensity difference data at each phase in the main study and in the subsequent study were compared using Fisher's z-transformation. For the comparison of other parameters in more than two groups, analysis of variance with Scheffe post hoc test was used. Receiver operating characteristic curves were applied to determine the best cut-off values of the intensity difference with the best sensitivity and specificity in discriminating fibrosis stages. Diagnostic accuracy of the intensity difference was assessed by areas under the receiver operating characteristic curves (Az), 95% confidence interval, sensitivity, specificity, positive and negative predictive values, and efficiency for the prediction of significant fibrosis. Intra- or inter-observer variability and variations in intensity difference in the subsequent study were calculated by the coefficient of variation obtained by standard deviation/mean×100. Inter-operator and inter-reviewer agreement was assessed by Kappa value calculation. Agreement grade was defined as <0.2 for poor, 0.2–0.4 for moderate, 0.4–0.6 for fair, 0.6–0.8 for good and 0.8–1.0 for excellent. Probability values below 0.05 were considered to be significant. All statistical analyses were performed using the SPSS package (version 17.0 J; SPSS, Chicago, IL, USA). Az values were obtained using ROCKIT1.1B2.

Table 1 Clinical and biochemical data of all subjects

	Main study		Subsequent study	
	Controls, $n=33$	CLD, $n=113$	Controls, $n=16$	CLD, $n=40$
Age (years)	46±16 (26–82)	55±12 (23–78)	62±18 (29–86)	52±15 (24–74)
Gender (male/female)	21/12	37/76	9/7	18/22
BMI (kg/m²)	21.7±2.4 (16–26)	22.7±3.9 (16–37)	21.8±2.4 (17–25)	24.0±3.7 (16–35)
Presence of ascites (%)	0 (0)	12 (11)	0 (0)	2 (5)
AST (IU/L)	18.0±3.5 (13–24)	54.8±51.4 (16–447)	19.9±5.5 (13–32)	71.4±53.5 (19–236)
ALT (IU/L)	13.4±5.3 (9–30)	61±89 (10–867)	14.6±3.3 (10–19)	91.7±101 (16–526)
Total bilirubin (mg/dL)	0.87±0.33 (0.4–1.3)	1.0±1.4 (0.4–15)	0.6±0.14 (0.5–0.8)	1.2±2.2 (0.7–1.8)
Albumin (g/dL)	4.5±0.36 (3.9–5.0)	4.0±0.54 (1.7–5.4)	4.2±0.3 (3.9–4.8)	4.3±0.4 (3.2–5.2)
Platelets (10⁹/L)	244±44 (163–340)	177±70 (44–428)	227±43 (162–336)	182±59 (48–320)
FIB4	1.0±0.61 (0.4–2.6)	3.0±2.5 (0.40–15)	1.5±0.7 (0.5–3.0)	2.8±2.7 (0.7–16)
Aetiology, HCV/HBV/AIH/PBC /NASH/ Alcohol/ Cryptogenic	–	62/13/10/13/3/5/7	–	15/5/5/5/6/2/2
Activity grade, A0-1/A2/A3	–	53/50/10	–	21/9/10
Grade of fibrosis, F0/F1/F2/F3/ Cirrhosis	–	4/31/26/23/29	–	1/6/20/7/6
Child-Pugh class, A/B/C	–	19/9/1	–	5/0/1

CLD, chronic liver disease; BMI, body mass index; AST, aspartate transaminase; ALT, alanine transaminase; FIB4, age × AST/(Platelet Count/ ALT^0.5); HCV, hepatitis C virus; HBV, hepatitis B virus; AIH, autoimmune hepatitis; PBC, primary biliary cirrhosis; NASH, non-alcoholic steatohepatitis