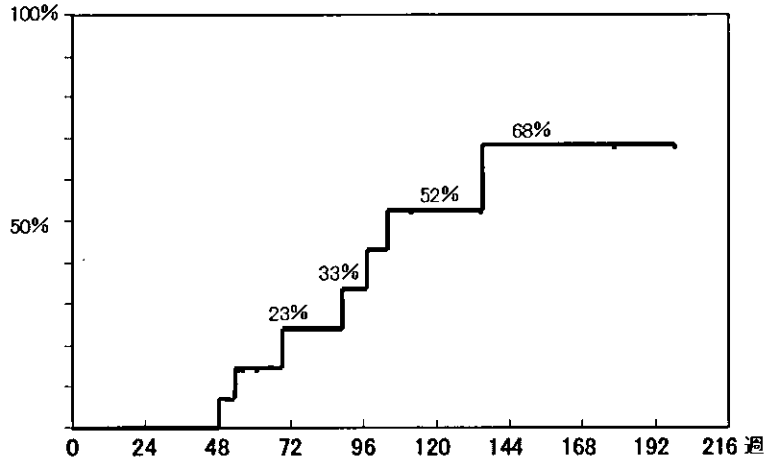


図9 ラミブジン継続投与時のviral breakthrough発現率

D．考察

投与中止例の検討において，症例1，2ではラミブジン中止後もpost-treatment flareがみられなかった．しかし症例3ではpost-treatment flareをきたし，ラミブジンを再投与したがHBe抗原が陰性化せず，HBV-DNAおよびALT値もその後変動を繰り返した．この経過から，ラミブジン中止後のpost-treatment flareによりラミブジン耐性ウイルスが誘導された可能性が示唆された．一般にpost-treatment flareはラミブジン再投与により速やかに改善する場合が多いが，このような症例が存在することから，ラミブジン投与の中止は慎重に行なわねばならないと考えられた．現時点ではラミブジン中止後にpost-treatment flareを起こすか否かを事前に予測することは困難であり，今後症例の蓄積によりこの点を明らかにする必要がある．

2年以上の長期継続投与例では，144週投与後に68％の症例にviral breakthroughが見られた．症例4，5はviral breakthroughから約70週経過後にALT上昇をきたしており，ラミブジン投与中にHBV-DNAが上昇した場合には，長期間経過した後肝炎が再燃する場合がある点に注意を要すると考えられた．症例6はviral breakthrough後の肝炎がコントロール困難であったが，今後このような症例では速やかにアデホビルの投与を開始すべきであると思われる．症例7，8は肝硬変例であり，viral breakthrough出現後に明らかなALTの上昇がみられなかったにもかかわらず，肝不全が進行して死亡または肝移植に至った．このことから肝硬変症例においては，明らかな肝炎再燃の徴候がみられない場合でも，viral breakthrough出現時には積極的にアデホビルの投与を検討する必要があると考えられた．

E．結論

1）ラミブジン投与中止後に肝炎が再燃した場合，ラミブジンを再投与しても十分な効果が得られない例があり注意を要する．また今後症例の蓄積により，post-treatment flareを起こす可能性を事前に予測する手段を見出す必要がある．

2）ラミブジン長期継続投与時には，viral breakthrough出現から1年以上経過した後肝炎が再燃する例があり注意を要する．

3）肝硬変例でviral breakthroughが出現した場合，肝炎再燃が明らかでなくとも肝不全が進行する可能性があり，早期にアデホビルの併用を検討する必要がある．

F．研究発表

1．論文発表

なし

2．学会発表

1) 佐藤丈顕，増本陽秀ほか：ラミブジン投与量の妥当性に関する検討．第8回日本肝臓学会大会(H16年10月21日，福岡)

2) 佐藤丈顕，岡田　誠ほか：当院におけるラミブジン治療の経験から．第11回北九州ウイルス性肝疾患研究会(H16年11月25日，北九州)

G．知的財産権の出願・登録状況

なし

厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業（肝炎分野））
分担研究報告書

肝炎ウイルス等の標準的治療困難例に対する治療法の確立に関する研究

Calcineurin inhibitorによるinterferonの肝細胞刺激伝達系への影響

分担研究者　中尾　一彦　長崎大学保健管理センター　助教授

研究要旨　現在肝移植の原因疾患の主流はHCV関連肝硬変，肝癌である．そしてHCVは肝移植後に全例再感染する．この時肝炎を発症すればInterferon (IFN)治療が必要となる．しかしその有効性は低い．高率の再感染，IFN治療の無効性の原因として免疫抑制剤の影響が考えられている．今回は肝移植後に通常使用されるCalcineurin inhibitorに注目し，特に同種の薬剤の中での差，タクロリムス(FK506)，サイクロスポリン(CyA)とラパマイシン(rapa)において抗ウイルス作用に与える影響が違うのではないかと考え実験を行った．1. FK506，rapaはIFN-αの刺激伝達系を抑制し，IFN作動性遺伝子の転写活性を減弱した．2. FK506はIFN-α誘導性抗ウイルス蛋白であるPKRの発現を抑制した．3. FK506はSTAT-1のチロシンリン酸化，活性型STAT-2とp48の核移行を抑制した．今回の検討でCalcineurin inhibitorはIFN-α刺激伝達系に対する影響があることが判明した．

研究協力者
濱崎 圭輔　長崎大学医学部歯学部附属病院第一内科
市川 辰樹　長崎大学医学部歯学部附属病院第一内科

A．研究目的

Calcineurin inhibitorの肝細胞におけるインターフェロン(IFN-α)刺激伝達系への影響を*in vitro*実験系を用いて検討する．

B．研究方法

(1) Calcineurin inhibitor添加によるIFN作動性遺伝子の転写活性，IFN誘導性抗ウイルス蛋白発現に対する影響

培養ヒト肝癌細胞HuH-7にIFN刺激対応配列(ISRE)を持つレポータープラスミドを遺伝子導入し，FK506, CyA, rapaを添加16時間培養後，IFN-αで刺激し，ルシフェラーゼアッセイにより転写活性に与える影響を検討した．またFK506前処置にてIFN-α誘導性抗ウイルス蛋白PKRの蛋白発現の変化をwestern blottingで検討した．

(2) FK506，CyAのIFN-α刺激伝達系に与える影響

培養ヒト肝癌細胞HuH-7をFK506で前処置後，IFN-αによるSTAT-1のリン酸化をwhole cell lysateを用いwestern blottingにより検討した．また，同様の治療を施したHuH-7を核，細胞質で分別しlysateを抽出し活性型STAT-1，STAT-2とp48の細胞内局在をwestern blottingで検討した．

C．研究結果

(1) Calcineurin inhibitor添加によるIFN作動性遺伝子の転写活性，IFN誘導性抗ウイルス蛋白発現に対する影響

IFN作動性遺伝子の転写活性はIFN-αに

より亢進するが，FK506 16時間前処置にて濃度依存性にその活性は減弱した．Rapaは低濃度でもIFN作働遺伝子の活性をshut downする事が解った．しかしCyAは今回検討した濃度において影響を与えなかった(図1)．また，抗ウイルス蛋白PKRの発現はIFN-α単独投与と比べFK506前処置では蛋白発現が減弱していた．

(2) FK506，CyAのIFN-α刺激伝達系に与える影響

HuH-7をFK506で前処置後し，IFN-αによるSTAT-1リン酸化の変化をwhole cell lysateを用いたwestern blottingで検討した結果，IFN-αによるSTAT-1のチロシンリン酸化がFK506前処置で抑制されていた．またIFN-α刺激による刺激伝達分子であるSTAT-1，STAT-2，p48の細胞質から核への以降に及ぼす検討では，FK506は活性型STAT-1の発現自体を抑制する事に加え，活性型STAT-2，p48の核移行を抑制した．CyAは活性型STAT-1の核移行はやや減弱するものの，p48の発現が増強した(図2)．

図1.

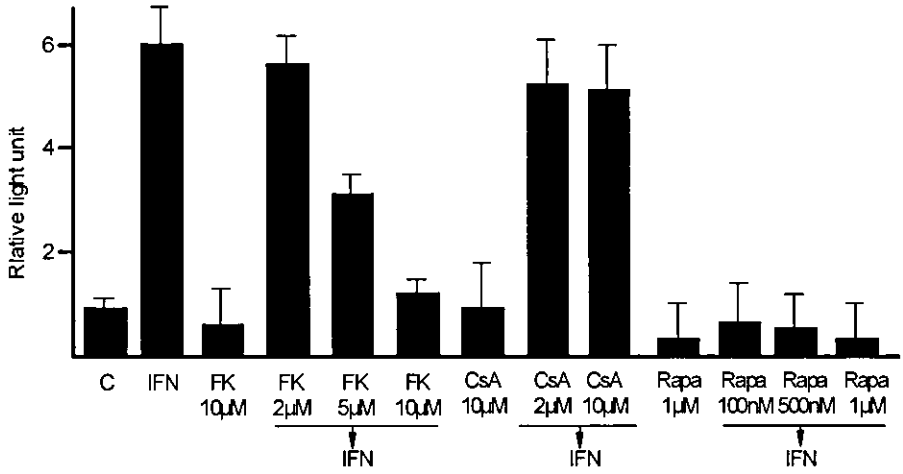
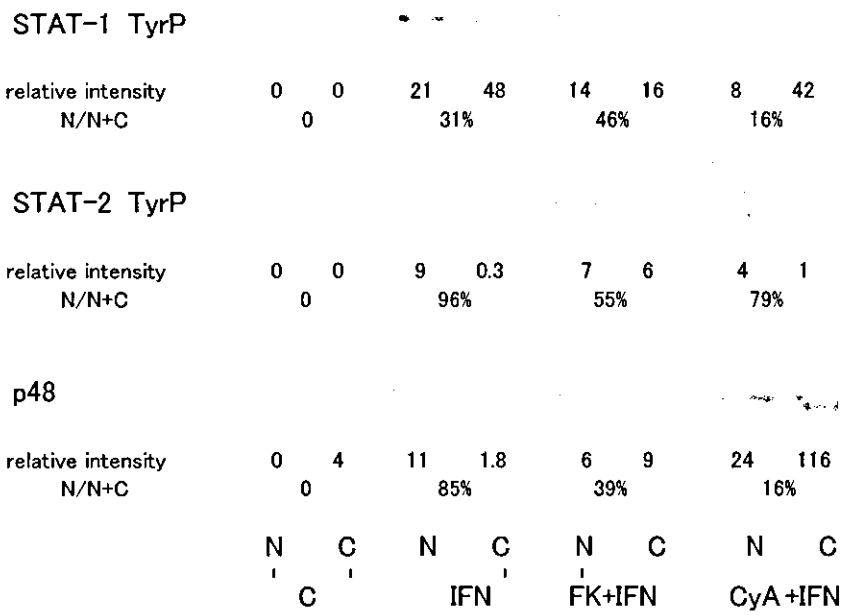


図2.



D．考察

今回の検討では，Calcineurin inhibitorはIFN-αによる抗ウイルス蛋白発現を抑制する事が判明した．この機序はこれらの蛋白のpromoter活性が抑制 されることに起因し，その原因は刺激伝達物質のリン酸化や核移行の阻害によると考えられる．

Calcineurin inhibitorは細胞内の細胞内シグナルの中でMAP kinaseを変化させることによりセリン，スレオニンのリン酸化を抑制 することが知られている．IFN-αの刺激伝達系において通常の刺激はレセプターにリガンドが結合した後，Jak-1，Tyk-2のチロシンリン酸化がおきてその後STAT-1，STAT-2のチロシンのリン酸化が誘導され活性型になることが知られている．最近の検討ではSTAT-1の転写活性にはセリンのリン酸化も必要とされており，このためにはMAP系の刺激が重要とされている．RapaやFK506はその作用であるセリン，スレオニンリン酸化抑制効果によりSTATの活性化に影響を与えておいる可能性が考えられる．

現在までCalcineurin inhibitorの肝細胞に及ぼす影響，サイトカイン刺激に対する影響は知られていない．今回の検討ではRapa，FK506，CyAの順でIFN-αのpromoter活性を抑制 する可能性が示された．種々の免疫抑制剤間でその作用には違いがあり，IFN-α刺激伝達系に対しても異る作用を示す．今後この差異を明確にし特徴を把握する事がよりよい治療効果につながると思われる．

E．結論

Calcineurin inhibitorのなかでFK506は肝細胞におけるIFN-αの抗ウイルス蛋白発現を抑制する事が示された．その機序は細胞内刺激伝達系に対する抑制効果である．

F．研究発表

1．論文発表

1) Matsumoto K, Ishikawa H, Nishimura D, Hamasaki K, Nakao K, Eguchi K. Antiangiogenic property of pigment epithelium-derived factor in hepatocellular carcinoma. Hepatology. 2004 Jul;40(1):252-9.

2) Fujikawa K, Ohata K, Honda T, Miyazoe S, Ichikawa T, Ishikawa H, Hamasaki K, Nakao K, Toriyama K, Eguchi K. Nonalcoholic steatohepatitis with improved hepatic fibrosis after weight reduction. Intern Med. 2004 Apr;43(4):289-94.

3) Ohata K, Hamasaki K, Toriyama K, Ishikawa H, Nakao K, Eguchi K. High viral load is a risk factor for hepatocellular carcinoma in patients with chronic hepatitis B virus infection. J Gastroenterol Hepatol. 2004 Jun;19(6):670-5.

4) Ishikawa H, Nakao K, Matsumoto K, Nishimura D, Ichikawa T, Hamasaki K, Eguchi K. Bone marrow engraftment in a rodent model of chemical carcinogenesis but no role in the histogenesis of hepatocellular carcinoma. Gut. 2004 Jun;53(6):884-9.

5) Saeki A, Nakao K, Nagayama Y, Yanagi K, Matsumoto K, Hayashi T, Ishikawa H, Hamasaki K, Ishii N, Eguchi K. Abstract Diverse efficacy of vaccination therapy using the alpha-fetoprotein gene against mouse hepatocellular carcinoma. Int J Mol Med. 2004 Jan;13(1):111-6.

２．学会発表

1) 田浦直太，濱崎圭輔，松本幸二郎，福田麻里子，後藤貴史，西村大介，大畑一幸，石川博基，中尾一彦，江口勝美：肝細胞癌（HCC）発見のための画像検査の有無と予後への影響．第101回日本内科学会，2004.04.08-04.10，東京

2) 後藤貴史，石川博基，佐伯　哲，川口康久，松本幸二郎，喜多由子，福田麻里子，田浦直太，西村大介，大畑一幸，濱崎圭輔，中尾一彦，江口勝美：肝炎後再生不良性貧血(Hepatitis-associated aplastic anemia）の2例．第90回日本消化器病学会総会2004.04.21-04.23，仙台

3) 中尾一彦，濱崎圭輔，石川博基，福田麻里子，大畑一幸，西村大介，喜多由子，江口勝美，石井伸子：ヒストン脱アセチル化酵素阻害剤（HDACI）による肝癌細胞 TRAIL 誘導アポトーシスの増強．第40回日本肝臓学会総会，2004.06.03-06.04，千葉

4) 西村大介，石川博基，松本幸二郎，後藤貴史，田浦直太，大畑一幸，濱崎圭輔，中尾一彦，江口勝美：新規の Nuculear FactorkB 阻害剤 dehy-droxymethyl epoxyquinomicin（DHMEQ）の肝癌細胞に対する増殖抑制効果の検討．第40回日本肝臓学会総会，2004.06.03-06.04，福岡

5) 喜多由子，松本幸二郎，福田麻里子，後藤貴史，田浦直太，西村大介，大畑一幸，石川博基，濱崎圭輔，中尾一彦，江口勝美：当科で経験した劇症肝炎14例の検討．第8回日本肝臓学会大会，2004.10.21-10.22，福岡

6) 田浦直太，濱崎圭輔，中尾一彦，石川博基，大畑一幸，西村大介，後藤貴史，福田麻里子，松本幸二郎，喜多由子，江口勝美：HCV 関連肝細胞癌における発症年齢の検討．第8回日本肝臓学会大会，2004.10.21-10.22，福岡

7) 石川博基，松本幸二郎，喜多由子，福田麻里子，後藤貴史，田浦直太，西村大介，大畑一幸，濱崎圭輔，中尾一彦，江口勝美：肝細胞癌における Pigment Epithelium Derived Factor (PEDF) の血管新生に対する影響と遺伝子治療への応用．第8回日本肝臓学会大会，2004.10.21-10.22，福岡

G．知的財産権の出願，登録状況

なし

厚生労働科学研究費補助金

肝炎等克服緊急対策研究事業(肝炎分野)

肝炎ウイルス等の標準的治療困難例に対する治療法の確立に関する研究

平成16年度

総括研究報告書
分担研究報告書
(2／2)

論　　文　　集

主任研究者
八　橋　　弘

平成17(2005)年3月

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tamada Y, Yano K, Yatsuhashi H, Inoue O, Mawatari F, Ishibashi H	Consumption of wild boar linked to cases of hepatitis E	J Hepatol	40 (5)	869-870	2004
Yatsuhashi H	Epidemiological and clinical features of hepatitis E in Japan	J Gastroenterol	39 (7)	702-703	2004
Daiba A, Inaba N, Ando S, Kajiyama N, Yatsuhashi H, Terasaki H, Ito A, Ogasawara M, Abe A, Yoshioka J, Hayashida K, Kaneko S, Kohara M, Ito S	A low-density cDNA microarray with a unique reference RNA: pattern recognition analysis for IFN efficacy prediction to HCV as a model	Biochem Biophys Res Commun	315 (4)	1088-1096	2004
Ohata K, Yano K, Yatsuhashi H, Daikoku M, Koga M, Eguchi K, Yano M	Natural interferon α treatment and interferon α receptor 2 levels in acute hepatitis C	Dig Dis Sci	49 (2)	289-294	2004
八橋 弘	わが国のC型肝炎の現状 日本の肝癌は，なぜ多いのか	日本消化器病学会雑誌	101 (6)	578-584	2004
八橋 弘	B型肝炎ウイルス（HBV） B型肝炎ウイルスのゲノム疫学	日本臨牀 ウイルス性肝炎（下） －基礎・臨床研究の進歩	62（増8)	186-189	2004
八橋 弘	特集／B型肝炎診療の進歩 IFN治療の適応と問題点	肝胆膵	49 (4)	471-477	2004
八橋 弘，矢野公士，玉田陽子	日本におけるE型肝炎	現代医療	36 (11)	131-136	2004
六波羅明紀，松本晶博，武藤英知，沖山 洋，田中直樹，丸山敦史，山浦高裕，折井幸司，一條哲也，吉澤 要，小林正和，袖山 健，田中榮司，清澤研道	B型慢性肝炎に対するラミブジン治療のウイルス学的効果予測式の作成	肝臓	44 (4)	182-183	2003
Hayashi S	Practice of interferon therapy－Chronic hepatitis C (Therapy with consensus interferon) －	Jap Med Assoc J	47(2)	69-72	2004
Huy TTT, Ushijima H, Quang VX, Ngoc TT, Hayashi S, Sata T, Abe K	Characteristics of core promoter and precore stop codon mutants of hepatitis B virus in Vietnam	J Med Virol	74	228-236	2004
橋本直明，桜林 真，平野正憲，滝川 一，佐藤芳之，池田有成，正木尚彦，林 茂樹	C型慢性肝炎における遺伝子発現－cDNAマイクロアレイを用いて－	肝臓	45(2)	129	2004
Kato M, Yuki N, Kaneko A, Yamamoto K, Masuzawa M, Hayashi N	Changes in virus loads and precore mutations in chronic hepatitis B patients treated with 4 weeks of daily interferon alfa-2a therapy	Hepatol Res	28	73-78	2004

Hiramatsu N, Kasahara A, Nakanishi F, Toyama T, Tsujii M, Tsuji S, Kanto T, Takehara T, Kato M, Yoshihara H, Naito M, Katayama K, Hijioka T, Hagiwara H, Kubota S, Oshita M, Meren H, Masuzawa M, Hruna Y, Mita E, Suzuki K, Hayashi N	The significance of interferon and ribavirin combination therapy followed by interferon monotherapy for patients with chronic hepatitis C in Japan	Hepatol Res	29	142-147	2004
加藤道夫，伊与田賢也，結城暢一，山本佳司，分島一，里見絵理子，道田知樹，林紀夫	HBVマーカーと発癌リスクよりみたHBVキャリアのステージ分類 －適切な抗ウィルス治療の選択に向けて－	肝臓	45 (11)	581-588	2004
西田真佐夫，島田志美，斎藤誠，加藤道夫，長谷川健次	C型慢性肝炎に対するインターフェロンα-2bとリバビリン併用療法におけるヘモグロビン減少に関する検討	医療薬学	30 (1)	53-58	2004
加藤道夫	くり返しTwo-step Interferon Rebound Therapyが奏功した難治性C型慢性肝炎の1例	治療学	38 (9)	73-75	2004
加藤道夫，結城暢一，伊与田賢也，山本佳司，林 紀夫	C型肝炎ウイルス(HCV) C型慢性肝炎の治療 Two-step interferon rebound therapyとその適応	日本臨床 ウイルス性肝炎（上）－基礎・臨床研究の進歩－	62 （増7)	497-501	2004
伊与田賢也，加藤道夫	C型肝炎ウイルス(HCV) C型慢性肝炎の治療 C型慢性肝炎に対するIFN再治療の成績とその適応	日本臨床 ウイルス性肝炎（上）－基礎・臨床研究の進歩－	62 （増7)	502-505	2004
結城暢一，加藤道夫	HBV replication のマーカーとしてのウイルス関連蛋 HBV-related proteins as a marker of viral replication	日本臨床 ウイルス性肝炎（下）－基礎・臨床研究の進歩－		33-35	2004
Kakiuchi Y, Yuki N, Iyoda K, Sugiyasu Y, Kaneko A, Kato M	Circulating soluble Fas levels in patients with hepatitis C virus infection and interferon therapy	J Gastroenterol	39	1189-1195	2004
原田英治，矢倉道泰，上司裕史，時田 元，田中晃久，福井秀雄，多賀谷健，亀井宏治	B型肝炎ウイルスマーカーの意味とその選択法，これを用いた臨床診断	診断と治療	92 (10)	1849-1856	2004
矢倉道泰，原田英治	急性肝炎の最近の動向と対処法	治療	86 (9)	2479-2486	2004
矢倉道泰，田中晃久，時田元，上司裕史，原田英治	結婚50年後に感染したHCV夫婦感染の1例	肝臓	46 (1)	19-25	2005
矢倉道泰，福井秀雄，時田元，上司裕史，原田英治	C型肝炎に対するIFN治療後10年経過観察例についての検討	肝臓	45 (4)	192-201	2004
竹崎英一，津田敏孝，水野重樹，三好栄司，福原達磨，横山聡恵，岡本泰治	慢性HBV感染患者のHBVプレコア／コアプロモーター変異の検討	広島医学	57 (5)	461-464	2004

Matsumoto K, Ishikawa H, Nishimura D, Hamasaki K, Nakao K, Eguchi K	Antiangiogenic property of pigment epithelium- derived factor in hepatocellular carcinoma	Hepatology	40 (1)	252-259	2004
Fujikawa K, Ohata K, Honda T, Miyazoe S, Ichikawa T, Ishikawa H, Hamasaki K, Nakao K, Toriyama K, Eguchi K	Nonalcoholic steato- hepatitis with improved hepatic fibrosis after weight reduction	Intern Med	43 (4)	289-294	2004
Ohata K, Hamasaki K, Toriyama K, Ishikawa H, Nakao K, Eguchi K	High viral load is a risk factor for hepatocellular carcinoma in patients with chronic hepatitis B virus infection	J Gastroenterol Hepatol	19 (6)	670-675	2004
Ishikawa H, Nakao K, Matsumoto K, Nishimura D, Ichikawa T, Hamasaki K, Eguchi K	Bone marrow engraft- ment in a rodent model of chemical carcinogenesis but no role in the histogenesis of hepato- cellular carcinoma	Gut	53 (6)	884-889	2004
Saeki A, Nakao K, Nagayama Y, Yanagi K, Matsumoto K, Hayashi T, Ishikawa H, Hamasaki K, Ishii N, Eguchi K	Diverse efficacy of vaccination therapy using the α-fetoprotein gene against mouse hepato- cellular carcinoma	Int J Mol Med	13 (1)	111-116	2004

ELSEVIER

Journal of Hepatology 40 (2004) 869–873

Journal of Hepatology

www.elsevier.com/locate/jhep

Letters to the Editor

Consumption of wild boar linked to cases of hepatitis E

To the Editor:

A zoonotic transmission of hepatitis E virus (HEV) has been suggested, based on indirect [1,2] and direct [3] evidence. However, the extent and impact of zoonotic transmission of HEV infection have not been fully elucidated and should be further clarified.

We encountered two acute hepatitis patients who visited one of our hospitals in April 2003 (Table 1; cases 1 and 2). Serum samples were all negative for Hepatitis A-Immunoglobulin (Ig) M antibody, hepatitis B surface antigen and hepatitis C virus RNA. However, they were positive for HEV-IgM and -IgG antibodies as well as HEV-RNA, indicating a diagnosis of acute hepatitis E.

Careful history taking revealed that the two patients had a single occasion of eating at a barbecue party where they exclusively ate charcoal-grilled, but partially undercooked, wild boar (*Sus scrofa leucomystax*) meat on March 13 (39 days prior to the admission of case 1).

A survey was done on August 26 (166 days after the meeting) for a total of 12 members of a local senior association who participated in the party. All participants were males with a median age of 79 years (Table 1). None of the members had traveled to HEV endemic areas. Surprisingly, eight persons (67%) were positive for HEV-IgM and 11 persons (92%) were positive for HEV-IgG antibodies. These results indicate that at least eight persons were recently infected by HEV, suggesting that the barbecue party was the occasion of the infection. It is even possible that all but one (case 12) became infected at the party in March but HEV-IgM subsequently disappeared, as of case 1. Two individuals (cases 3 and 4) had been admitted to other hospitals because of acute hepatitis. One (case 5) visited a clinic on May 15 where an elevation of liver enzymes was noted. Although no acute phase serum samples of such patients were available, these three patients positive for HEV-IgM, are considered to be infected with acute hepatitis E. All five patients recovered within 3–4 weeks.

A phylogenetic analysis of 317 nucleotide within the open reading frame 1 [4] performed on the HEV isolates obtained from the first two patients sera revealed that the strains belong to genotype III, with 99.4% homology to each other. These isolates were neighbored by, yet with a notable distance, some Japanese isolates, such as JRA1 (AB003430; 92.1% homology) and JJT-Kan (AB091394; 92.1% homology),

Table 1
Data of members who ingested boar meat on March 13

Data obtained in April and May						Case No	Age (year)	Sex	Sx	Survey on August 26 (166 days after boar meat ingestion)			
HEV-IgG	HEV-IgM	HEV-RNA	ALT (IU/mL)	T.Bil (mg/dL)	Time* (Days)					ALT (IU/mL)	HEV-IgG	HEV-IgM	HEV-RNA
+	+	+	1,172	1.9	39	1	69	M	Yes	10	+	−	−
+	+	+	751	0.9	43	2	69	M	Yes	15	+	+	−
			290	2.6	40	3	78	M	Yes	15	+	+	−
			531	3.9	36	4	80	M	Yes	6	+	+	−
			312	1.2	55	5	81	M	Yes	12	+	+	−
						6	80	M	No	13	+	+	−
						7	64	M	No	11	+	+	−
						8	81	M	No	12	+	+	−
						9	78	M	No	36	+	−	−
						10	75	M	No	9	+	−	−
						11	82	M	No	23	+	−	−
						12	80	M	No	18	−	−	−

Sx., Symptoms; ALT, alanine aminotransferase; ALP, alkaline phosphatase; T.Bil, total bilirubin; M, male; +, positive; −, negative.
GenBank accession numbers obtained are: AY427956 for Case 1 (ENK-NGS03) and AY427957 for Case 2 (EMN-NGS03).
* ; Time of blood examination (days after boar meat ingestion) when peak ALT (for cases 1–4) levels were observed. "Yes" indicates existence of fatigue or jaundice. Blanks indicate data not available.

strongly suggesting that the two patients were infected by HEV of a common source.

Animals including boars, pigs and deer have been suggested as candidate reservoirs for HEV [1–3]. In fact, severe hepatitis E after the ingestion of uncooked boar liver has been recently reported [5]. Although we could not prove that the boar was the source of infection because there were no left-overs, it seems reasonable to speculate that this small outbreak of acute hepatitis E was caused by the simultaneous ingestion of wild boar meat.

Our observations provide further evidence of HEV transmission as zoonosis. We suggest that the consumption of wild boar is a risk of HEV transmission and that the ingestion of such animals plays at least a partial role in prevailing HEV in non-endemic countries.

Yoko Tamada[1], Koji Yano[1], Hiroshi Yatsuhashi[1], Osami Inoue[2], Fumihiro Mawatari[2], Hiromi Ishibashi[1]
[1]*Clinical Research Center, WHO Collaboration Centre for Reference and Research on Viral Hepatitis, National Nagasaki Medical Center, 2-1001-1 Kubara, Omura, Nagasaki 856-8562, Japan*
[2]*Labour Welfare Corporation, Nagasaki Rosai Hospital, Nagasaki, Japan*
E-mail address: kyano-kkr@umin.ac.jp

References

[1] Emerson SU, Purcell RH. Hepatitis E virus. Rev Med Virol 2003;13: 145–154.
[2] Nishizawa T, Takahashi M, Mizuo H, Miyajima H, Gotanda Y, Okamoto H. Characterization of Japanese swine and human hepatitis E virus isolates of genotype IV with 99% identity over the entire genome. J Gen Virol 2003;84:1245–1251.
[3] Tei S, Kitajima N, Takahashi K, Mishiro S. Zoonotic transmission of hepatitis E virus from deer to human beings. Lancet 2003;362: 371–373.
[4] Takahashi K, Iwata K, Watanabe N, Hatahara T, Ohta Y, Baba K, et al. Full-genome nucleotide sequence of a hepatitis E virus strain that may be indigenous to Japan. Virology 2001;287:9–12.
[5] Matsuda H, Okada K, Takahashi K, Mishiro S. Severe hepatitis E virus infection after ingestion of uncooked liver from a wild boar. J Infect Dis 2003;188:944.

doi:10.1016/j.jhep.2003.12.026

J Gastroenterol 2004; 39:702–703
DOI 10.1007/s00535-004-1392-z

Journal of Gastroenterology

Editorial

Epidemiological and clinical features of hepatitis E in Japan

Article on page 640

Epidemiological and clinical study of sporadic acute hepatitis E caused by indigenous strains of hepatitis E virus in Japan compared with acute hepatitis A

Sainokami S, Abe K, Kumagai I, et al.

Hepatitis E virus (HEV) is the causative agent of acute hepatitis E, which is now recognized as the major cause of enterically transmitted non-A, non-B hepatitis in many developing countries in Asia and Africa (as well as in Mexico), where the disease usually occurs as epidemics.[1] HEV is transmitted primarily via a fecal-oral route. In these areas, epidemics and the spread of HEV, the etiological agent of hepatitis E, are predominantly caused by contaminated water, due to inadequate sanitation practices; the highest incidence of HEV infections occurs in young adults, and the mortality rate of HEV-infected pregnant women is approximately 20%. Sporadic cases of HEV infection have also been reported in industrialized countries, where the occurrence is usually associated with travel to countries endemic for HEV. However, recent studies have documented that HEV-associated hepatitis also occurs among individuals in industrialized countries with no history of travel to areas endemic for HEV.

HEV is a non-enveloped virus, approximately 27–30 nm in diameter, with a positive-sense, single-stranded RNA genome. The genome of HEV is approximately 7·2 kb long and contains a short 5′ untranslated region (UTR), three open reading frames (ORFs 1–3), and a short 3′ UTR terminated by a poly(A) tract.[2] HEV was previously classified in the family Caliciviridae. However, it is now reclassified in the hepatitis E-like viruses with an unassigned genus.[3] ORF-1 likely encodes nonstructural viral proteins (putative RNA helicase, protease, and RNA-dependent RNA polymerase). ORF-2 encodes the putative capsid protein, and ORF-3 encodes a cytoskeleton-associated phosphoprotein.

HEV isolates are generally classified into four genotypes, based on the phylogenetic analysis of the full-length genome. These include genotypes I (HEV isolates from several countries in Asia and Africa), II (HEV isolates from Mexico and Nigeria), III (HEV isolates from the United States, European countries, and Argentina), and IV (HEV isolates from China and Taiwan).[4] In Japan, multiple HEV strains of genotype III or IV have been isolated from patients with acute hepatitis of non-ABC etiology who had never been abroad, and swine HEV strains of genotype III have been isolated from farm pigs in Japan.[5,6]

Zoonotic spread of HEV has been suggested, as human and swine HEV strains are closely related, and experimental cross-species infection of swine HEV to non-human primate, (such as chimpanzees, rhesus monkeys, and cynomolgus macaques) and that of human HEV to swine, have been demonstrated.[7,8] Recently, it was reported that veterinarians working with swine in the United States,[9] and pig handlers in China, Taiwan, and Thailand were at increased risk for HEV infection. In addition, high levels of anti-HEV antibodies have been detected in several animal species (including pigs, cattle, dogs, rodents, and monkeys) that lived both in countries of HEV endemicity and in countries of nonendemicity. These facts suggest that animals are an important reservoir of HEV infections in humans.

In Japan, hepatitis E is rarely reported and most, if not all, cases of hepatitis E observed thus far have been regarded as "imported" hepatitis. Recently, however, the seroprevalence of antibodies against HEV (anti-HEV) in healthy individuals was reported to range from 1.9% to 14.1%,[10] depending on the geographic area in Japan. In addition, an HEV strain of genotype III (strain JRA1) has been isolated from a Japanese patient with acute hepatitis who had never been abroad,[11] and a swine HEV strain with the highest degree of similarity to isolate JRA1 among the known HEV isolates has been isolated from a farm pig in Japan. These results indicate that HEV infection may be circulating in Japan.

In most hepatitis E outbreaks, the highest rates of clinically evident disease have been reported in young

Reprint requests to: H. Yatsuhashi

to middle-aged adults; the lower disease rates reported in younger age groups may be the result of anicteric (i.e., without the elevation of serum bilirubin that is used as a marker of clinical jaundice) and/or subclinical HEV infection. Hepatitis E is more severe than hepatitis A, with mortality rates in the range of 1%–2%, compared with 0.2% for hepatitis A. One distinctive clinical feature of hepatitis E, compared with other forms of viral hepatitis, is its increased incidence and severity in pregnant women, which results in up to 20% mortality. By contrast, none of the other recognized hepatitis viruses causes such severe hepatitis in pregnancy. The mechanism(s) to explain the pathogenesis of fulminant hepatitis E in pregnancy is not known. Although the symptoms of hepatitis E are typical of acute viral hepatitis and the infection follows a natural history that is similar to that of hepatitis A, an exact comparison between acute hepatitis E and A has never been investigated.

In this issue of the *Journal of Gastroenterology*, Sainokami et al.[12] report an epidemiological and clinical comparison between acute hepatitis E and acute hepatitis A in Japan, and they provide some interesting findings. Clinical features obtained from patients admitted to six hospitals indicated that acute hepatitis E was more common in male patients and older patients than acute hepatitis A. Laboratory data indicated a weak immunological reaction and early appearance of jaundice in acute hepatitis E. Although the number of patients in their study was limited, such a clinical study should be regarded as valuable in a nonendemic, industrialized country.

A review by Okamoto et al.[13] regarding hepatitis E in Japan reported 46 Japanese patients infected with hepatitis E. These patients had no history of travel to endemic areas and they had had no contact with travelers who had been abroad or with people from other countries. The clinical and epidemiological characteristics of hepatitis E in these 46 patients were summarized as follows: (1) the age of patients ranged from 38 to 86 years, with a mean age of 59.6 years, (2) 40 patients (87%) were male, (3) 6 (13%) of the 46 patients developed severe prolonged jaundice, and 5 other patients (11%) contracted fulminant hepatitis, (4) the months of onset of hepatitis E were distributed almost equally over the year, and there was no particular season when hepatitis E predominantly occurred; and (5), there was wide variation in the geographical distribution of hepatitis E, with a higher prevalence in the northern part of Japan. Therefore, in Japan, clinical HEV infection should be taken into consideration when a clinician is confronted with patients with sporadic acute or fulminant hepatitis of non-ABC etiology, and special attention should be paid to their age, sex, and location of residence.

Hiroshi Yatsuhashi, M.D.
Clinical Research Center, WHO Collaborating Center for Reference and Research on Viral Hepatitis, National Nagasaki Medical Center, 2-1001-1 Kubara, Omura 856-8562, Japan

References

1. Purcell RH, Emerson SU. Hepatitis E virus. In: Knipe DM, Howley PM, Griffin DE, Martin MA, Lamb RA, Roizman B, Straus SE, editors. Fields virology 4th ed. Philadelphia: Lippincott Williams & Wilkins; 2001. p. 3051–60.
2. Tam AW, Smith MM, Guerra ME, Huang CC, Bradley DW, Fry KE, et al. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. Virology 1991;185: 120–31.
3. Pringle C. Minutes of the 27th International Committee on Taxonomy of Viruses Meeting. Arch Virol 1998;143:1449–59.
4. Schlauder GG, Mushahwar IK. Genetic heterogeneity of hepatitis E virus. J Med Virol 2001;65:282–92.
5. Mizuo HK, Suzuki Y, Takikawa Y, Sugai H, Tokita Y, Akahane K, et al. Polyphyletic strains of hepatitis E virus are responsible for sporadic cases of acute hepatitis in Japan. J Clin Microbiol 2002;40:3209–321.
6. Takahashi M, Nishizawa T, Miyajima H, Gotanda Y, Iita T, Tsuda F, et al. Swine hepatitis E virus strains in Japan form four phylogenetic clusters comparable with those of Japanese isolates of human hepatitis E virus. J Gen Virol 2003;84:851–62.
7. Meng XJ. Zoonotic and xenozoonotic risks of hepatitis E virus. Infect Dis Rev 2000;2:35–41.
8. Meng XJ. Novel strains of hepatitis E virus identified from humans and other animal species: is hepatitis E a zoonosis? J Hepatol 2000;33:842–5.
9. Meng XJ, Wiseman B, Elvinger F, Guenette DK, Toth TE, Engle RE, et al. Prevalence of antibodies to hepatitis E virus in veterinarians working with swine and in normal blood donors in the united states and other countries. J Clin Microbiol 2002;40:117–22.
10. Tanaka E, Takeda N, Li T-C, Orii K, Ichijo T, Matsumoto A, et al. Seroepidemiological study of hepatitis E virus infection in Japan using a newly developed antibody assay. J Gastroenterol 2001;36:317–21.
11. Takahashi K, Iwata K, Watanabe N, Hatahara T, Ohta Y, Baba K, et al. Full-genome nucleotide sequence of a hepatitis E virus strain that may be indigenous to Japan. Virology 2000;287:9–12.
12. Sainokami S, Abe K, Kumagai I, Miyasaka A, Endo R, Takikawa Y, et al. Epidemiological and clinical study of sporadic acute hepatitis E caused by indigenous strains of hepatitis E virus in Japan compared with acute hepatitis A. J Gastroenterol 2004;39:640–8.
13. Okamoto H, Takahashi M, Nishizawa T. Features of hepatitis E virus infection in Japan. Intern Med 2003;42:1065–71.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 315 (2004) 1088–1096
BBRC
www.elsevier.com/locate/ybbrc

A low-density cDNA microarray with a unique reference RNA: pattern recognition analysis for IFN efficacy prediction to HCV as a model☆

Akito Daiba,[a] Niro Inaba,[a] Satoshi Ando,[a] Naoki Kajiyama,[a] Hiroshi Yatsuhashi,[b] Hiroshi Terasaki,[a] Atsushi Ito,[a] Masanori Ogasawara,[a] Aki Abe,[a] Junichi Yoshioka,[a] Kazuhiro Hayashida,[c,1] Shuichi Kaneko,[d] Michinori Kohara,[e] and Satoru Ito[a,*,2]

[a] *JGS Japan Genome Solutions, Inc. 51 Komiya-cho, Hachioji, Tokyo 192-0031, Japan*
[b] *National Nagasaki Medical Center, Kubara 2-1001-1 Omura City, Nagasaki 856-8562, Japan*
[c] *Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*
[d] *Kanazawa University Graduate School of Medical Sciences, 13-1 Muro-machi, Kanazawa 920-8641, Japan*
[e] *Tokyo Metropolitan Institute of Medical Science, 3-18-22 Hon-komagome, Bunkyo-ku, Tokyo 113-8613, Japan*

Received 9 January 2004

Abstract

We have designed and established a low-density (295 genes) cDNA microarray for the prediction of IFN efficacy in hepatitis C patients. To obtain a precise and consistent microarray data, we collected a data set from three spots for each gene (mRNA) and using three different scanning conditions. We also established an artificial reference RNA representing pseudo-inflammatory conditions from established hepatocyte cell lines supplemented with synthetic RNAs to 48 inflammatory genes. We also developed a novel algorithm that replaces the standard hierarchical-clustering method and allows handling of the large data set with ease. This algorithm utilizes a standard space database (SSDB) as a key scale to calculate the Mahalanobis distance (MD) from the center of gravity in the SSDB. We further utilized sMD (divided by parameter k: MD/k) to reduce MD number as a predictive value. The efficacy prediction of conventional IFN mono-therapy was 100% for non-responder (NR) vs. transient responder (TR)/sustained responder (SR) ($P < 0.0005$). Finally, we show that this method is acceptable for clinical application.

Keywords: Low-density microarray; Artificial reference RNA; Efficacy prediction; Mahalanobis distance

Large data sets can be collected from cDNA microarrays for the study of expression profiles in biological systems. The large amount of data generated can be especially useful to help cluster genes into interest groups. Such genome-wide information can be used for clinical applications (see reviews, [1–5]), for example, for the identification of the cDNA expression patterns associated with different stages of tumor development. However, translating complex microarray data into practical clinical applications has been difficult. New algorithms are being developed to solve this problem, for example for the prognosis of cancer treatment [6,7]. Also, low-density microarrays with selected genes of interest can simplify the analysis of microarray data.

Another critical issue in understanding microarray data is the level of precision in the data set. Solid phase DNA hybridization is not as quantitative as hybridization in solution, and scanners have limited dynamic

☆ *Abbreviations:* SAGE, serial analysis of gene expression; SSDB, standard space database; MD, Mahalanobis distance; sMD, scaled MD; FL, firefly luciferase gene; RL, *Renilla* luciferase gene; GP, baculovirus glycoprotein gene; LD, lambda DNA; MEP, microcapillary electrophoretic; aRNA, amplified RNA; NR, non-responder; SR, sustained responder; TR, transient responder.

* Corresponding author. Fax: +81-426-45-0461.
E-mail address: sr-itou@jgs-inc.co.jp (S. Ito).
[1] Present address: Sasebo Kyosai Hospital, 10-17 Shimaji, Sasebo, Nagasaki 857-8575, Japan.
[2] Visiting position: Medical Research Institute, Tokyo Medical and Dental University.

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.01.160

ranges. In addition, sample variability can result from variations in sampling conditions, RNA amplification, RNA degradation, and cDNA labeling conditions. These factors are not well understood, and, currently, precision is primarily controlled at the level of data collection [8–10].

One means of enhancing the precision of data collection is to use reference controls for each individual. This can be accomplished by laser-captured microdissection of tissues into diseased and counter part areas [11,12]. In case of hepatitis C, this is difficult because inflammatory damage occurs throughout the liver. Another possible approach is to utilize artificial reference RNAs as a reference [13–15] in conjunction with RNA from established cell lines, such as hepatocellular carcinoma cell lines. However, the stages and characteristics of the disease in vivo and in vitro can differ, and expression of some RNAs of interest, especially some inflammatory genes, may be too low in the cultured cells to produce a satisfactory signal-to-noise ratio.

In the current studies, we identified inflammatory genes that can be used for a low-density microarray to predict the efficacy of INF treatment in hepatitis C patients. We found sufficient levels of expression for these genes in patient samples, but very low levels of expression in established cell lines. We overcame this problem by using a low-density cDNA microarray system in conjunction with a unique artificial reference RNA. In addition, we describe an algorithm for analysis of the microarray data.

Methods

cDNA Microarray. We selected 295 genes for the cDNA microarray based on publicly available data, including SAGE analysis and other DNA microarray data from HCV patients and a normal subject [16,17]. From 2000 candidate genes, we omitted low frequency-tag genes based on the SAGE data. Genes previously identified as predictive host factors for IFN efficacy [18–20] were given a high priority. For most of the genes, each cDNA was designed approximately 500–600 bp and within approximately 1 kb region from the 3′-poly(A) tail and all cDNAs for microarray probe were cloned into the pGEM vector (Promega, Madison, WI). We also selected and cloned external control genes (approximately 0.5–1 kb) into the pGEM vector to establish the dynamic range of the microarray. These genes were firefly luciferase (FL; negative control), *Renilla* luciferase (RL) [21], baculovirus envelope gp64 (GP), and lambda phage DNA (LD). All clones for capture probe on microarray were sequenced and validated by comparison with the GenBank sequence. The aminosilane surfaces of SuperAmine glass slides (TeleChem International, Sunnyvale, CA) were stamped with triplicate spots of cDNA probe corresponding to each of the remaining 295 genes. The average spot size was 150 μm and separated each other with a distance (500 μm) as shown in Fig. 2B.

Reference RNA preparation. Extracted total RNAs from four hepatocellular carcinoma cell lines (HepG2, Hep3B, Huh7, and IMY-4 [22]) purified through RNeasy column (Qiagen, Hilden, Germany) were mixed as a reference source. In order to find a mixing ratio of four cell derived RNAs and provide reliable reference source, we measured the copy number of certain genes in each cell line by real-time PCR method. Using real-time PCR with the PRISM 7900HT system (Applied Biosystems, Foster City, CA), we measured the copy number of several genes, including the IFN-α/β receptor, double-strand RNA-activated protein kinase (PKR), 2′,5′-oligoadenylate synthetase (2,5-AS), interferon regulatory factor-1 (IRF-1), interferon regulatory factor-3 (IRF-3), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sets were as follows: IFN-α/β receptor (forward: GTGACCTCACAGATGAGTGG; reverse: CCCTCTGA CTGTTCTTCAATGA; and probe: CACCGTCCTAGAAGGA TTCAGCGG), PKR (forward: CCTGTCCTCTGGTTCTTTTG; reverse: TGTCAGGAAGGTCAAATCTG; and probe: CTACGTGT GAGTCCCAAAGCAAC), 2,5-AS (forward: CTCAGAAATAC CCCAGCCAAATC; reverse: GTGGTGAGAGGACTGAGGAA; and probe: CCAGGTCAGCGTCAGATCGGCCTC), IRF-1 (forward: GCAAGGCCAAGAGGAAGTCA; reverse: TCATCAGG CAGAGTGGAGCT; and probe: TTCCAGCCCTGATACCTTC TCTGATGG), IRF-3 (forward: AAGGAAGGAGGCGTGTTTGA; reverse: ATTTCTACCAAGGCCCTGAGG; and probe: CGTCCGC TTCCTTCCGTGAAGGTAAT), GAPDH (forward: GAAGGTGA AGGTCGGAGT; reverse: GAAGATGGTGATGGGATTTC; and probe: CAAGCTTCCCGTTCTCAGCC). The RNA mixture was amplified using MessageAmp aRNA kit (Ambion, Austin, TX). The resulting aRNA was used as the reference aRNA. Moreover, we cloned genes (RL, GP, and LD; ~1000 bp) into pCRII TOPO vector (Invitrogen, Carlsbad, CA) as scanning range markers as well as 48 genes (in the same TOPO vector) of inflammatory genes to spike into reference aRNA. Each cloning region was designed to be larger than the size of capture probes on the microarray. Then three external control RNAs and additional spike RNAs of 48 genes were synthesized by Megascript T7 kit (Ambion, Austin, TX). Three external control RNAs were mixed as spike control mixture in both target sample and reference aRNAs. Other 48 spike RNAs were added to the reference aRNA.

Sample RNA preparation, labeling, hybridization, and scanning. Total RNA of liver biopsy samples was isolated by Isogen (Nippon Gene, Tokyo, Japan) extraction according to manufacturer's instruction. The total RNA quality was confirmed with a Bioanalyser 2100 microcapillary-electrophoretic (MEP) analyzer (Agilent Technologies, Palo Alto, CA). The 28S/18S ratio of the total RNA was >1.0. Then total RNA (1–2 μg) was transcribed and amplified to produce amplified sample RNA (aRNA) using the MessageAmp aRNA kit (Ambion, Austin, TX) according to manufacturer's instructions. Next, an external control RNA mixture (LD, GP, and RL) was added to both the sample and reference aRNAs. These mixed sample and reference aRNAs were labeled using SuperScript II kit with random hexamer (TaKaRa, Kyoto, Japan) with Cy3-dUTP and Cy5-dUTP (Perkin–Elmer, Boston, MA), respectively. Competitive hybridization of Cy3-labeled sample and Cy5-labeled reference cDNAs on the microarray was carried out according to Brown and coworkers [23]. Slides were scanned three times with ScanArray 5000 (Perkin–Elmer, Boston, MA). Each scan was carried out using the external spike level around 30,000. The data were converted from tif image data to signal using ImaGene software (BioDiscovery, El Segundo, CA) for further statistical analysis. Three file data of each three spot data of each gene were merged to establish the single representative data for each gene (Patent pending: PCT/JP03/06677). The Cy3 (patient sample)/Cy5 (reference sample) ratio of each mRNA signal was calculated for further analysis.

Patients. Liver biopsy samples from five patients receiving IFN-α monotherapy and 10 patients receiving a combination therapy (a mixture of IFN-α, IFN-β, and IFN-α/β) were obtained during 1992–2000 from Kyushu University Hospital and Nagasaki National Medical Center, respectively. Biopsy samples were stored at −80 °C. Informed consent was obtained from all patients in accordance with the Helsinki protocol. The samples were classified into three groups: sustained responders (SR) had an absence of serum HCV RNA both during the therapy and 6 months after the completion of therapy, non-responders (NR) were positive for serum HCV RNA during the

therapy, and transient responders (TR) had an absence of serum HCV RNA during the therapy or at the end of IFN treatment, but has serum HCV RNA after cessation of the therapy. Because RNA degradation may have occurred during storage, and because this can be a major source of variation in microarray data [24], we verified the quality of the extracted RNA by assessing the ribosomal RNA 28S/18S ratio.

Statistical data analysis. The merged data were subjected for hierarchical clustering to noise reduction and normalization (patent pending, PCT/JP03/06677) using the reference control and then analyzed with Genomic Profiler software (Mitsui Knowledge Industry, Tokyo, Japan). In addition, we developed a novel algorithm to calculate the Mahalanobis distance (MD) for the data from 15 patients using a standard space database (SSDB) (Eqs. (1)–(5) and Fig. 1). To establish the SSDB, we searched a gene set representing the differences between the SR/TR and NR groups. The necessary genes for the SSDB and MD calculations were selected using MATLAB (MathWorks, Natick, MA). We have calculated a graded scale utilizing variance–covariance to valuate dispersion and correlation of the standard group as a training set to establish the center of the gravity of SSDB. Once the SSDB was established, new test sample data were applied to the equations to calculate the MD. We utilize sMD as a predictive value. The sMD was presented from the center of gravity of SSDB (0:zero) along its scale to theoretically ∞. This method is one of the pattern recognition analysis dealing with correlation of multi-parameters [25].

$$d_{xi} = \frac{D_{xi} - \overline{D_x}}{\sigma_x} \quad \text{(auto scale)}, \quad (1)$$

$$S_{xx'} = \frac{\sum_{i=1}^{n} (d_{xi} - \overline{d_x})(d_{x'i} - \overline{d_{x'}})}{n-1} \quad \text{(variance–covariance)}, \quad (2)$$

$$S = \begin{bmatrix} S_{11} & S_{12} & \cdots & S_{1(k-1)} & S_{1k} \\ S_{21} & S_{22} & \cdots & S_{2(k-1)} & S_{2k} \\ \vdots & \vdots & \vdots & \vdots & \vdots \\ S_{(k-1)1} & S_{(k-1)2} & \cdots & S_{(k-1)(k-1)} & S_{(k-1)k} \\ S_{k1} & S_{k2} & \cdots & S_{k(k-1)} & S_{kk} \end{bmatrix}$$

(variance–covariance matrix), (3)

$$\mathrm{MD}^2 = [d_1 \ \cdots \ d_k] S^{-1} \begin{bmatrix} d_1 \\ \vdots \\ d_k \end{bmatrix} \quad \text{(Mahalanobis distance)}, \quad (4)$$

$$\mathrm{sMD} = \frac{\mathrm{MD}^2}{k} \quad \text{(scaled Mahalanobis distance)}. \quad (5)$$

Results and discussion

The low-density cDNA microarray

High-density microarray data were so hard to handle its huge data for analysis and difficult to understand their meaning. One approach is to minimize gene set for collection of mRNA profiling data to each category of research field. Chang et al. [26] have described the selection of data from high-density microarrays for prediction of "docetaxel" therapy efficacy of breast cancer. Specifically, they omitted low level signals of genes from the high-density microarray data at first. We followed a similar approach to select genes on our microarray, also omitting low frequency tag genes from the SAGE data. This ensured a steady state signal amongst the target samples. Based on this selection, we chose 295 genes for a low-density microarray system. The DNA sequence of each cloned gene fragment (500–600 bp) was validated by comparison with the published sequences in the GenBank database. To provide replicate data, the cDNAs were spotted in triplicate on the aminosilane-coated slide glass [8]. Scanning electron microscopy confirmed that the spots were round, smooth, and homogeneous without any doughnut features (Fig. 2). To obtain stable signals, we used three independent internal RNA references, including RL for the high expression range, GP for the middle range, and LD for the low range. The signals from the microarray were adjusted so that the ScanArray would give reliable signal of 30,000, which should be within the linear and stable range of the scanner (maximum signal = 65,535). We also carried out noise reduction and normalization of data using the artificial external spike genes as well as some housekeeping genes. Validation of the low-density cDNA microarray system was carried out using the RNA from HepG2 (data not shown).

Adjustment of reference RNA

Selection of an appropriate control reference is another important factor for accuracy in microarray analysis. One method has been to use laser microdissection to select normal tissue from the same patient as a reference. Although this is useful for single patients, it cannot be applied to comparison of multiple patients' samples because the baseline expression of specific genes can vary from patient to patient. Therefore, conditions including the duration of disease, the medications used, sampling conditions, storage conditions, and life style differences can cause variability in the microarray data. To eliminate this problem, we have used an artificial reference RNA isolated from cell lines as a reference. When we screened the signal levels of both reference and patient samples, we found 48 genes out of 295 genes in the microarray that were expressed in the patients but not or background level in the RNA mixture from the four cell lines. Typical data from the four cell reference mixture are shown in Fig. 3A. The IFN-receptor and some other well-known IFN-inducible factors are indicated. The graph shows that the levels of these mRNAs are in the low signal range. This includes the IFN-α/β receptor, even though it has been proposed as a possible marker for the prediction of IFN efficacy [18,19]. The problem in this case appears to be high variability in IFN-α/β receptor gene expression between different reference RNA preparations. To avoid this problem, we produced a large single preparation of reference RNA for future analyses. In addition, we have produced 48 synthetic RNAs, which we added to the reference RNA mixture. These synthetic RNAs were designed to be larger than the size of the capture probes on the microarray. The design and purity of some these synthetic

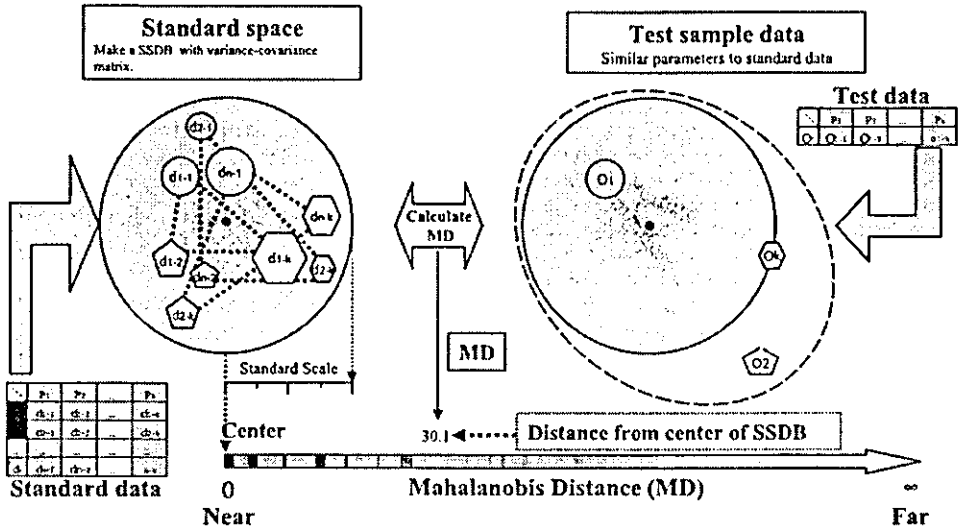


Fig. 1. Pattern recognition for establishment of SSDB and MD calculations. The standard space database (SSDB) was established based on a training data set. The parameter (1 to k) represents each data point (signal level for each gene) in the patient sample. A second parameter (1 to n) represents each patient. Both parameters were utilized along with the distinguishing genes for each group that were used in a variance–covariance matrix calculation to create the SSDB. Next, the test sample data were calculated to obtain the Mahalanobis distance (MD), where the MD represents the distance of new test sample data from the center of gravity in the SSDB. In theory, MD can be from 0 to ∞. A high MD value means that its distance is far away from the SSDB center of gravity.

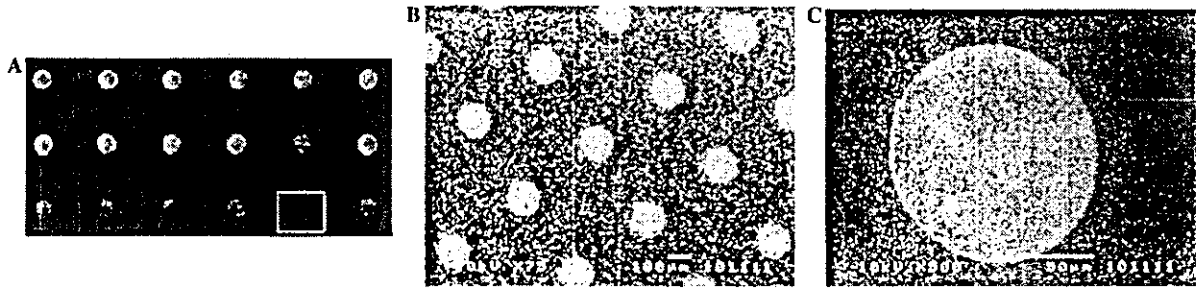


Fig. 2. Spot geometry on the cDNA microarray. (A) A typical fluorescent image was presented. The negative control (firefly luciferase) gene was spotted inside of the white square. Scanning electron microscopic images at (B) low power (75×) and (C) higher power (500×) are shown.

RNAs is shown in Fig. 3B. The level of the synthetic reference RNAs is shown as a scatter plot in Fig. 3C. These results show that the level of the synthetic RNA corresponds to the range of pseudo-inflammatory conditions. Thus, we spiked these 48 synthetic RNAs to the average signal level of patients which was surveyed at first. Without this synthetic reference RNA, it would be difficult to analyze and categorize the microarray data and use it to predict the efficacy of IFN treatment. Thus, the signal below the negative cut-off level will be treated as zero for further ratio calculation leads to nonsense.

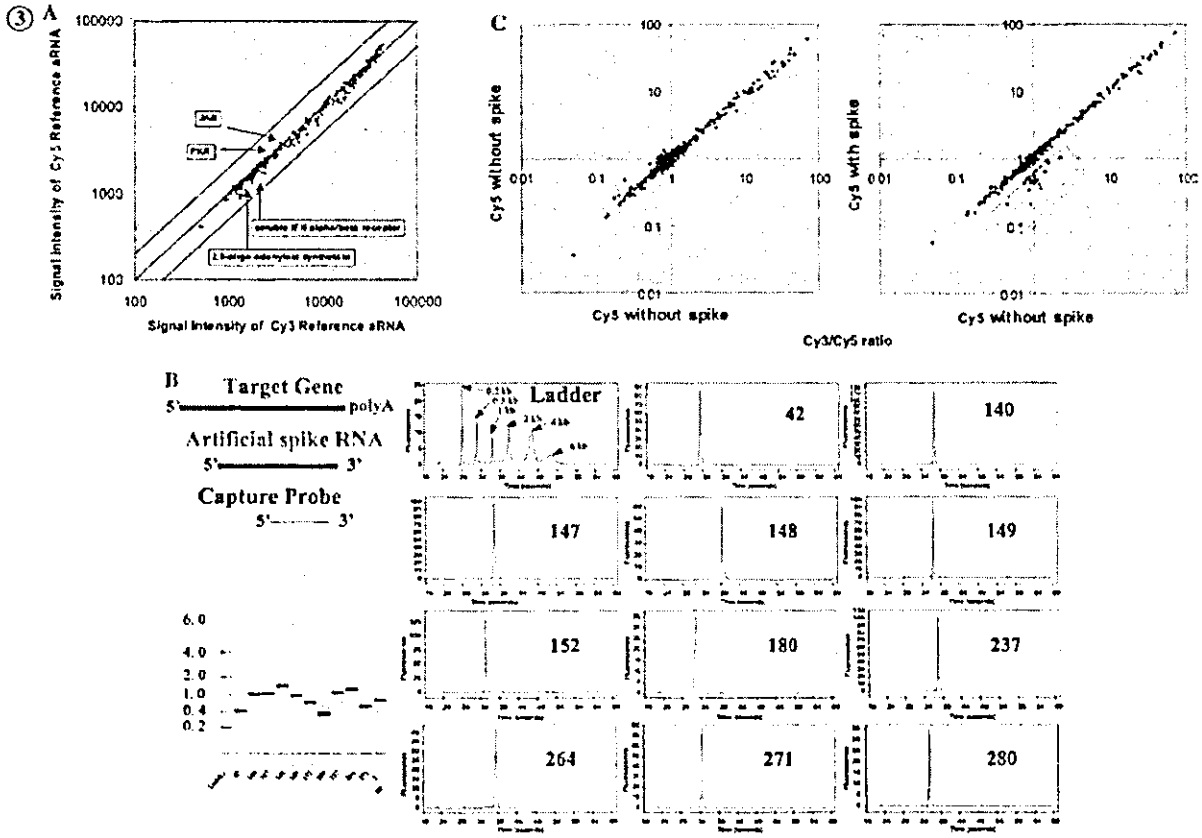
Statistical analysis

A variety of normalization techniques have been used for the analysis of DNA microarray data [9,27–30]. Many of these techniques rely on the expression of housekeeping genes. However, it is difficult to find suitable candidates, and it would be difficult to integrate a large set of housekeeping genes onto the low-density cDNA microarray [30–32]. For these reasons, we have added synthetic non-human genes as external controls. We have also utilized some type of housekeeping genes for normalization of the microarray data (patent pending, PCT/JP03/06677). Furthermore, to minimize variability in the calculations due to variability in the fluorescence measurements, we used six data files (three scans of each Cy3/Cy5 wavelength) to merge into a single representative data for each gene expression analysis.

Hierarchical clustering by the classical method

Hierarchical clustering of the merged data was carried out using Euclid distance and Ward method with

③


A
Signal Intensity of Cy5 Reference aRNA
Signal Intensity of Cy3 Reference aRNA
C
Cy5 without spike
Cy5 with spike
Cy3/Cy5 ratio
B
Target Gene
5'——— polyA
Artificial spike RNA
5'——— 3'
Capture Probe
5'—— 3'
Ladder
42
140
147
148
149
152
180
237
264
271
280

④

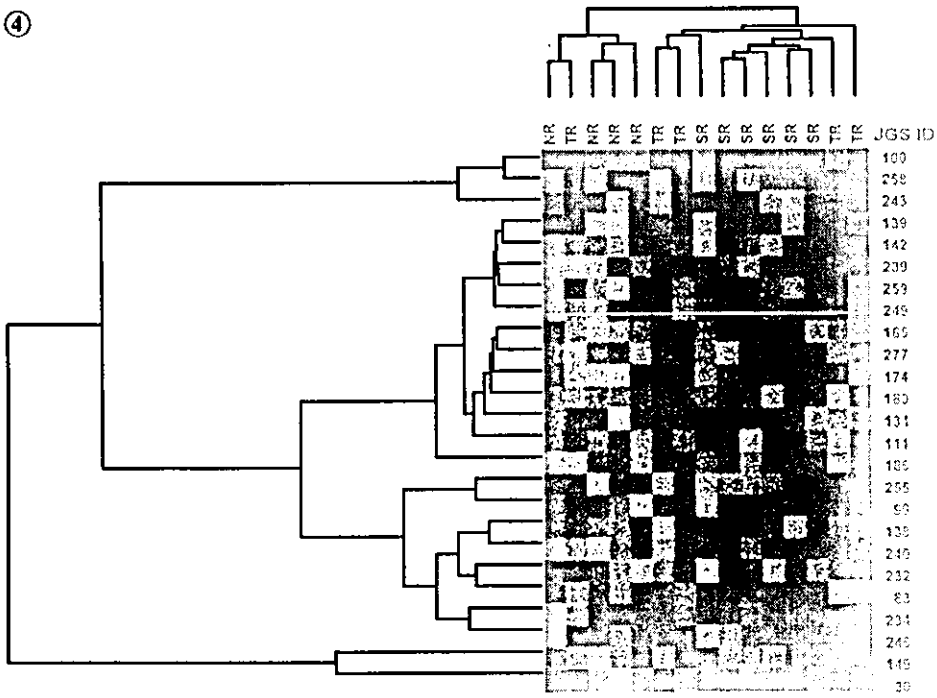

NR
TR
SR
JGS ID

Fig. 3. Establishment of the artificial reference RNA. (A) Scatter plot of the mRNA signal level in the four cell RNA mixture. The RNA mixture from four cell lines was covalently modified with Cy3 and Cy5 dyes. The signal levels for typical inflammatory genes, including 2,5-oligoadenylate synthetase (2,5-AS), IFN-α/β receptor, IFN-α receptor, and PKR, are indicated with arrows. (B) Design and purity of the synthetic RNA. The general design of the synthetic RNA (positional relation), including size and position of the probe and the artificial RNA, is shown on the left. Thus, each reference RNA was designed to be longer than the captured probe on the microarray, but not to exceed the size of the target RNA. The purity of the 11 of the synthetic RNA samples is shown. The corresponding gene numbers on our cDNA microarray are shown in each panel and are as follows (GenBank accession number in parentheses): 42, gamma-G globin (X55656); 140, T cell activation antigen (CD27) (M63928); 147, (2′,5′)-oligoadenylate synthetase (D00068); 148, p68 kinase (M35663); 152, CIS3 (AB006967); 180, calcium-binding protein in macrophages (MRP-14), also known as macrophage migration inhibitory factor-related protein (X06233); 237, interleukin 2 receptor β chain (p70-75) (M26062); 264, interferon-induced protein 44 (IFI44) (NM_006417); 271, interleukin 4 (M13982); and 280, hepatocyte growth factor (X16323). (C) Synthetic spiked RNA signal level. The panel on the left represents the scatter plot without any synthetic RNA added to the reference RNA, while the panel on the right shows the reference with added synthetic RNAs. The plot shows the ratio of sample Cy3/Cy5 rather than real signal level. The spots in the white rectangle represent the level of the added synthetic RNAs.

Fig. 4. Hierarchical clustering. cDNA microarray data of 15 patients' samples were analyzed with Genomic Profiler software (MKI, Japan). For clustering, normalization, filtering, and *T* test were essential. Because of interest in predicting clinical outcomes of IFN treatment, we tried to classify the data into two groups, including non-responders (NR) and transient responders (TR)/sustained responders (SR). The accuracy of this prediction was >93%. The corresponding microarray number and according GenBank accession number of the genes responsible for clustering are shown on the right and include: 100, cytoplasmic dynein light chain 1 (U32944); 258, thymosin β-10 (M92383); 243, stathmin (X53305); 139, homeobox 1.4 protein (M74297); 142, cAMP-dependent protein kinase regulatory subunit RI-bet (M65066); 239, alternatively spliced interferon receptor (IFNAR2) (L42243); 259, eukaryotic translation initiation factor 2, subunit 1 α, 35 kDa (BC002513); 249, brain-derived neurotrophic factor precursor (BDNF) (M61176); 165, interleukin 2 (X01586); 277, natural killer cell stimulatory factor (NKSF) (M65290); 174, IFN-responsive transcription factor subunit (M87503); 180, calcium-binding protein in macrophages (MRP-14) also known as macrophage migration inhibitory factor-related protein (X06233); 134, lunatic fringe U94354); 111, protein tyrosine kinase (Syk) (L28824); 186, leukocyte-associated molecule-1 α subunit (LFA-1 α subunit) (Y00796); 255, FLICE-like inhibitory protein short form (U97075); 99, CDK4-inhibitor (p16-INK4) (L27211); 138, α 7B integrin (X74295); 240, interferon-stimulated T-cell α chemoattractant precursor(AF030514); 232, Charcot–Leyden crystal protein (L01664); 83, NADH:ubiquinone oxidoreductase MLRQ subunit (U94586); 234, apoptotic cysteine protease Mch4 (Mch4) (U60519); 246, metallothionein-III (M93311); 149, interferon regulatory factor 1 (X14454); and 30, heat shock 70 kDa protein 1A (BC002453).

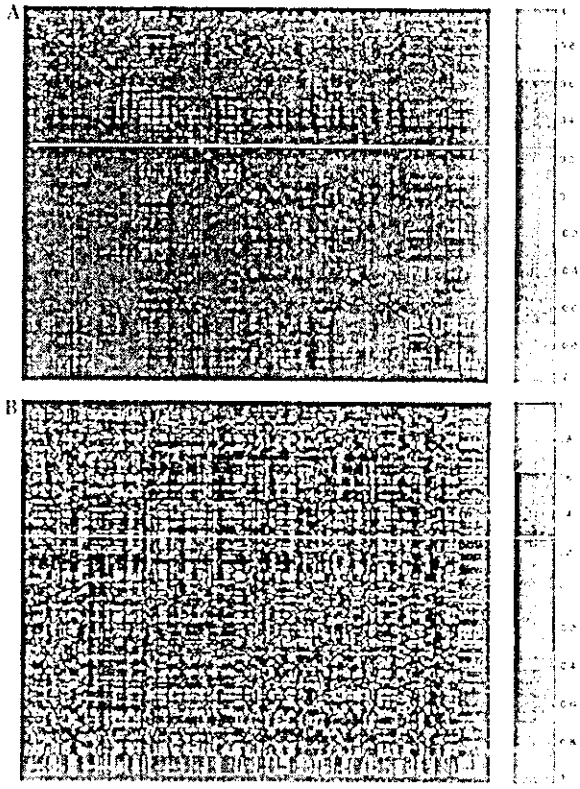


Fig. 5. Typical expression pattern: correlation matrix of 295 gene signals. (A) This expression pattern represented the established standard data as SSDB. Each axis represents genes in consideration. (B) The expression pattern shows an example of NR data as new test sample data. Color and brightness are adjusted according to Eqs. (1)–(3).

Patient No	Prediction	Clinical outcome	sMD (MD/k)*1	
10	NR	NR	6.31	so far from
9	NR	NR	5.97	SSDB center of gravity
8	NR	NR	4.38	
15	NR	NR	3.53	
4	SR or TR	SR	1.96	threshold =2.0
12	SR or TR	TR	1.90	
13	SR or TR	SR	1.44	
1	SR or TR	SR	1.36	
14	SR or TR	TR	1.09	
6	SR or TR	TR	0.98	
3	SR or TR	SR	0.73	
7	SR or TR	SR	0.67	
11	SR or TR	TR	0.38	
5	SR or TR	TR	0.24	
2	SR or TR	SR	0.21	SSDB center of gravity

		Clinical Outcome			
		SR or TR	NR	Total	%
Prediction	SR or TR	11	0	11	100%
	NR	0	4	4	100%
	Total	11	4	15	
	%	100%	100%		

$P < 0.0005$

Fig. 6. Mahalanobis distance (MD) and classification for efficacy prediction. The resulting MD data were arranged along the scale based on numbers of sMD value. The first column shows the patient number, the second column shows the prediction from our microarray analysis, and the third column shows the actual clinical outcome. Blue represents the NR group, purple represents TR, and red represents the SR group. We have set a threshold at the sMD level of 2.0 to set two groups from the sMD calculation. The hit ratio of prediction to clinical outcome was also shown.