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.

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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

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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.

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

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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.

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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

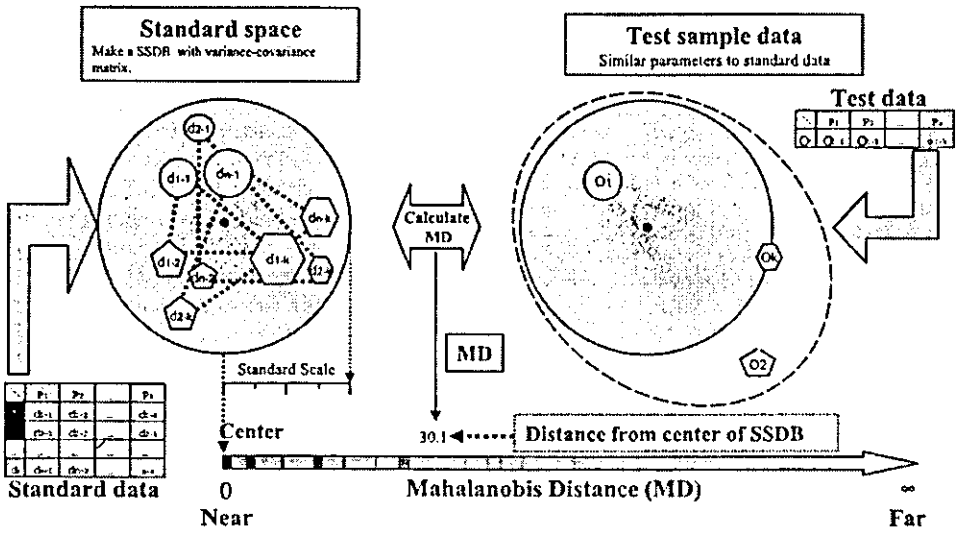


Fig. 1. Pattern recognition for establishment of SSDB and MD calculations. The standard space database (SSDB) was established based on a training data set. The parameter (1 to k) represents each data point (signal level for each gene) in the patient sample. A second parameter (1 to n) represents each 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.

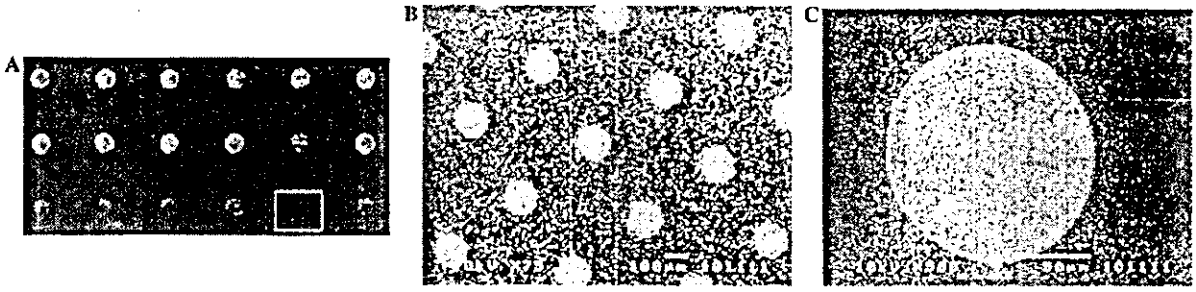


Fig. 2. Spot geometry on the cDNA microarray. (A) A typical fluorescent image was presented. The negative control (firefly luciferase) gene was spotted inside of the white square. Scanning electron microscopic images at (B) low power (75×) and (C) higher power (500×) are shown.

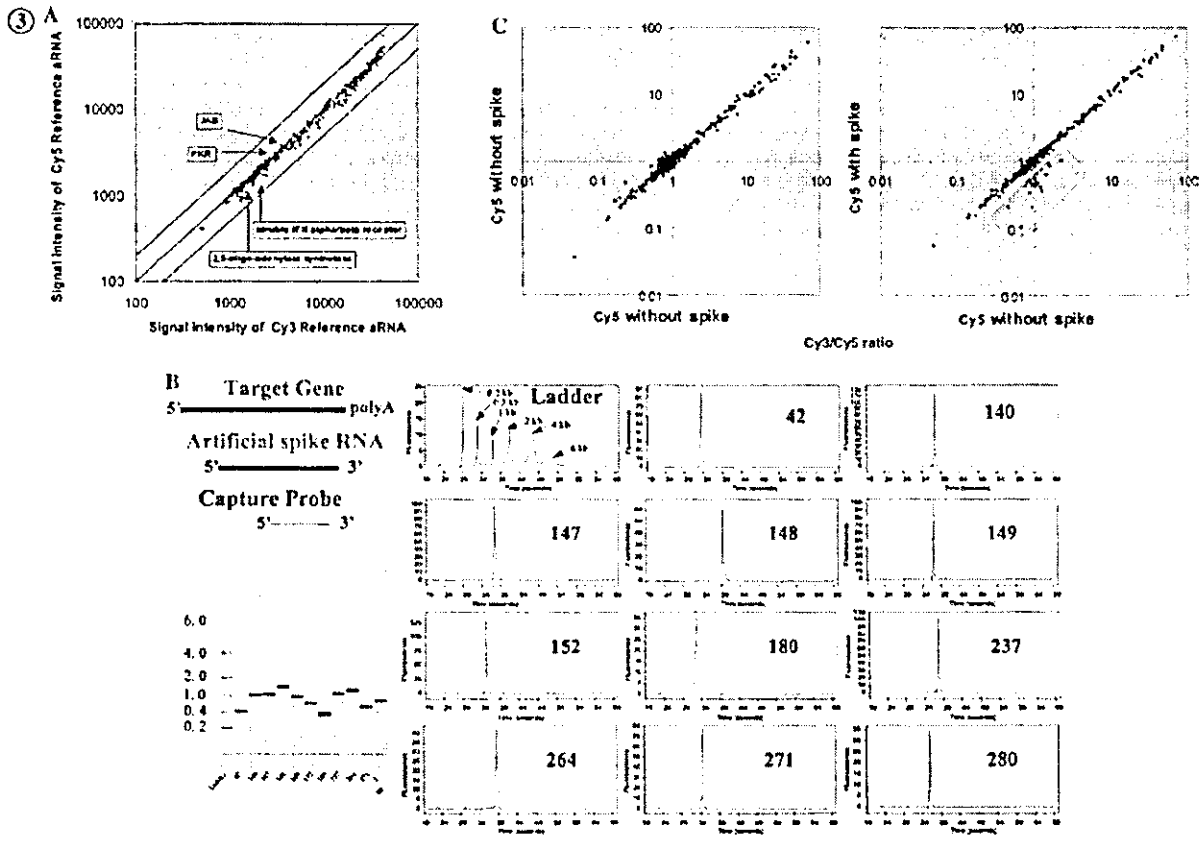
RNAs is shown in Fig. 3B. The level of the synthetic reference RNAs is shown as a scatter plot in Fig. 3C. These results show that the level of the synthetic RNA corresponds to the range of pseudo-inflammatory conditions. Thus, we spiked these 48 synthetic RNAs to the average signal level of patients 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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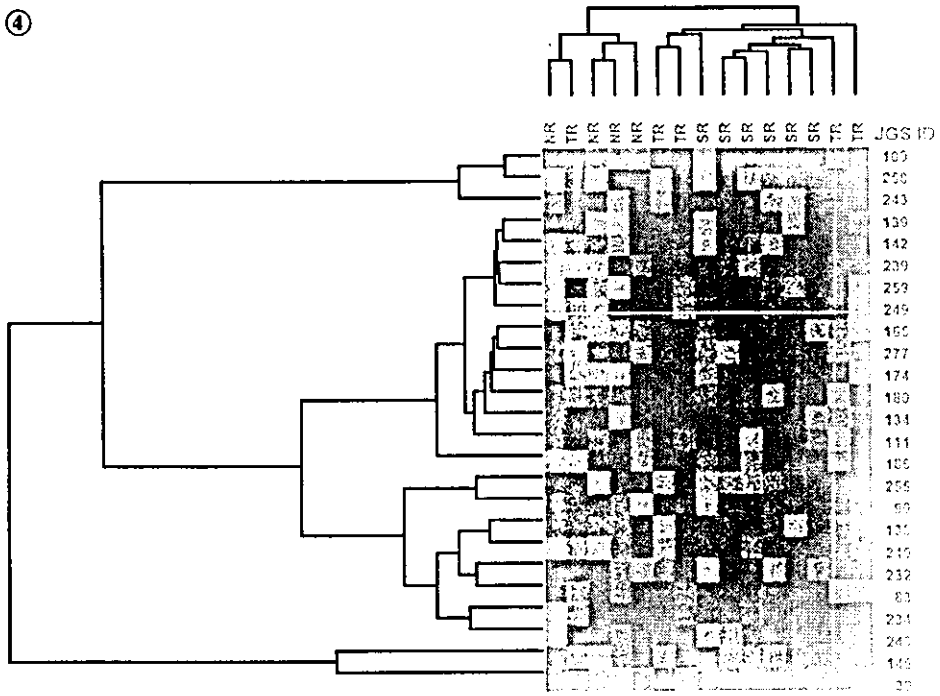
A
Signal Intensity of Cy5 Reference aRNA
Signal Intensity of Cy3 Reference aRNA
C
Cy5 without spike
Cy5 with spike
Cy3/Cy5 ratio
B
Target Gene
polyA
Artificial spike RNA
Capture Probe
Ladder
42
140
147
148
149
152
180
237
264
271
280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JGS ID

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).

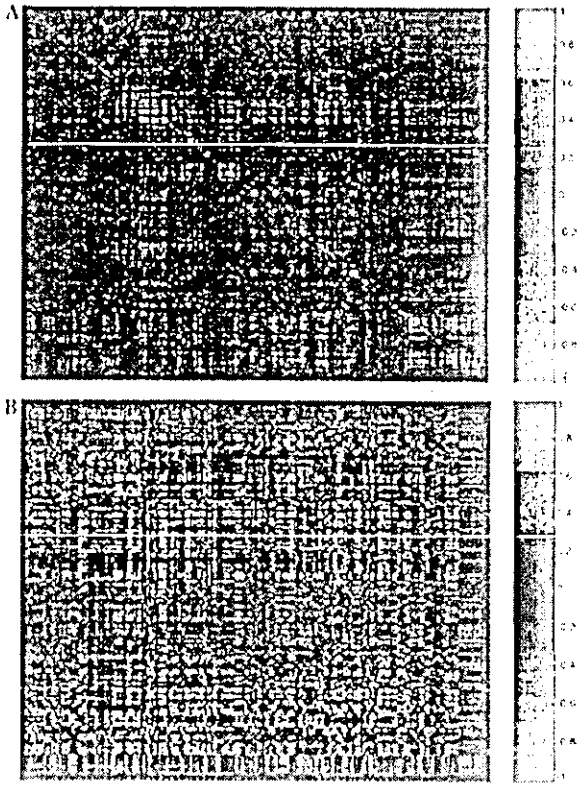

Fig. 5. Typical expression pattern: correlation matrix of 295 gene signals. (A) This expression pattern represented the established standard data as SSDB. Each axis represents genes in consideration. (B) The expression pattern shows an example of NR data as new test sample data. Color and brightness 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	0.24	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.

Genomic Profiler software. Because of our interest in predicting IFN efficacy in hepatitis C, we compared the NR group against SR/TR group [33–35]. We chose genes with a 5% discriminated expression pattern (T test) between two groups ([SR vs. NR]∩[(SR + TR) vs. NR]). The hierarchical tree for these genes is shown in Fig. 4. Appropriate groups were assigned to all but one case, a TR case that was classified into the NR group.

Development of a new algorithm for hierarchical clustering

Although the classical method dealing with multi-parameters allowed satisfactory classification of the patients into two groups, this method is not useful for practical purposes. Thus, the entire data set is necessary for interpretation of the results from the classical analysis even for the analysis of a single test sample. In general, clinical diagnosis requires the common scale to compare the analytical data from samples. But a classical classification presents only a relative scale among samples for comparison.

For these reasons, we have developed a new algorithm. This method is based on the calculation of MD. The concept of the MD calculation is outlined in Fig. 1. This is one of the pattern recognition analysis and determines how close or how far from the standard group of interest. Thus, it deals with multi-parameters leading to single parameter, Mahalanobis distance (MD) as a scale, from the center of gravity of SSDB established by a training set shown in Fig. 1. Prior to the MD calculation, it was necessary to establish a SSDB with a training data set randomly selected but with clear and known clinical outcome. The standard expression pattern obtained from the SR/TR group, which was the established data source for SSDB, is shown in Fig. 5A and the new test sample pattern of the NR group is shown in Fig. 5B. The red color represents the higher expression profile and green depicts the lower expression profile with an interrelating style at the same time. Then, the pattern recognition algorithm Eqs. (1)–(3) was applied to compare the two groups. Among these expression patterns, we selected the stably and differentially expressed gene set data. Then, every selected gene expression pattern was correlated to each other like connecting a network. Thus, based on Eqs. (1)–(3), the elements that distinguish the groups shown were selected to create the SSDB. The SSDB was created based on variance and covariance. Once we established the SSDB and the center of gravity of the SSDB, we calculated a MD value for each new test sample (Eq. (4)). We utilized sMD value (divided the MD value by the number of parameters) to reduce MD value (Eq. (5)) and simplify understanding of the results[25]. The classification of IFN efficacy prediction to hepatitis C patients is shown in Fig. 6 and clearly shows that this analysis generates the NR and SR/TR groups and they are predicted with 100% accuracy ($p < 0.005$). The sample size (15 cases) was too small for statistical validation. Further detailed analyses and validation are ongoing in our laboratory and will be reported elsewhere.

Although the MD method is popular in even biological system publications [36,37], an application of MD method to microarray data is not so popular yet. The current studies are not the first published report where MD for analysis of gene expression data [38]. However, that report focused on the differential expression of a causative gene in conjunction with a standard hierarchical clustering algorithm. In our studies, we have attempted to scale the test sample position as a simple understandable parameter with a new pattern recognition algorithm. Once the SSDB scale has been established for a project, the MD can be easily used to classify new data according to the NR and TR/SR groups on an absolute scale (Fig. 6). This system will be acceptable for clinicians as a simple system for understanding the microarray data.

Conclusions

Besides the technical issues, there are many factors that control the variability within a microarray system, including individual differences between patients, the duration of the disease of each patient, different therapeutic protocols, and complications with other diseases. Some of these factors interact with each other, while others are independent. Therefore, an algorithm that allows some variability in the measurements is needed for prediction of therapeutic outcomes. The algorithm presented here appears to satisfy this requirement and it simplifies handling of large data sets. Finally, this algorithm should be generally applicable to the prediction of therapeutic outcome of diseases.

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Natural Interferon α Treatment and Interferon α Receptor 2 Levels in Acute Hepatitis C

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Efficacy of interferon (IFN) therapy during the acute phase of hepatitis C infection is promising, although the optimal regimen has yet to be determined. It is not known whether the known prognostic factors for chronic hepatitis C (CHC) influence the effect of IFN in acute hepatitis C (AHC). Seventeen patients with AHC were analyzed for hepatic IFN α receptor 2 (IFNAR2) prior to IFN treatment. All patients were subsequently treated with either 168 million units (MU) or 336 MU of natural IFN α. Seventeen age-matched samples of CHC were provided as controls. The overall sustained response rate was 64.7% (11/17). In patients who received a total dose of 168 MU IFN, the sustained response rate was 28.6% (2/7), and in those who received 336 MU of IFN, the sustained response rate was 90.0% (9/10). The peaks of ALT and HCV-RNA quantity were not associated with the response to IFN. The hepatic IFNAR2 levels were 1.52 ± 0.34 densitometry units and 0.92 ± 0.16 in AHC and CHC, respectively ($P = 0.042$). There was no difference in hepatic IFNAR2 levels between sustained virological responders (SVR) and nonsustained virological responders (NR). The hepatic receptor levels were higher in AHC than in CHC patients. The levels of hepatic IFNAR2 did not differ in SVR and NR, indicating that high-dose natural IFN α treatment is effective for AHC, irrespective of the levels of hepatic IFNAR2.

KEY WORDS: acute hepatitis C; natural interferon α; hepatic IFNAR2.

Hepatitis C infection poses a serious problem all over the world. About 30% of patients with chronic hepatitis C (CHC) will develop cirrhosis within 3 to 20 years, and a large number of these patients will develop complications such as liver failure, portal hypertension, and hepatocellular carcinoma (1). Interferon (IFN) is the most common treatment for chronic hepatitis C virus (HCV) infection but a substantial portion of patients does not respond, and many responders relapse after stopping treatment.

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It is well known that the virological efficacy of IFN monotherapy on chronic hepatitis C (CHC) is only about 30% (1, 2). IFN and ribavirin combination therapy improved response rate up to about 50% (3, 4). Patient selection, depending on the prognostic factors, however, allows virus eradication in a higher percentage of patients. Such prognostic factors include HCV viral load, HCV genotype, NS-5A mutation, and histological progression of the disease (1, 2). Moreover, others (5) and we (6) have reported a correlation between the effect of IFN and hepatic IFN α receptor 2 (IFNAR2), which play a pivotal role in IFN therapy against HCV infection.

Acute hepatitis C (AHC) infection has a tendency to progress to chronic hepatitis in 55–88% (7–10). Because of the high rate of chronic infection resulting from acute hepatitis C, several studies have been conducted to seek an effective therapy during the acute phase which may prevent progression to chronicity (11–14). Several uncontrolled (15, 16) and controlled (12, 14, 17, 18) studies of

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IFN therapy in patients with AHC have been performed aiming to assess the effects of treatment on serum alanine aminotransferase (ALT) level, viral markers, and histological necroinflammation. To increase the statistical power to resolve uncertainty and to improve estimates of effect size, a few meta-analysis based on the results of controlled trials have been performed (19–21). All but one (22) support IFN therapy in AHC, showing effectiveness ranging from 35 to 90%. However, the reason these high virological efficacies of IFN are obtained against AHC infection has not been elucidated to date.

Results of IFN therapy during the acute phase of HCV infection are promising, although the optimal regimen is yet to be determined. Moreover, it is not known whether the known prognostic factors for CHC influence the effect of IFN in AHC. In the current study, we retrospectively analyzed 17 cases of AHC and assessed the efficacy of IFN with respect to pretreatment values including HCV viral load, HCV genotype, and the levels of hepatic IFNAR2.

METHODS

Patient Selection and Diagnosis. Diagnosis of acute hepatitis was based on the finding of elevated values of serum ALT at least five times the normal values, associated with either HCV antibody seroconversion of second-generation ELISA (ELISA III; Ortho Diagnostic System), or positivity of HCV-RNA by reverse-transcription polymerase chain reaction (RT-PCR). Patients for whom other causes of liver disease were suspected were excluded. Seventeen patients (6 males and 11 females) treated at National Nagasaki Medical Center between 1987 and 1999 fulfilled the inclusion criteria.

Liver biopsy specimens obtained from CHC patients between 2000 and 2001 were provided as controls for immunohistochemistry. CHC patients were routinely subjected to liver biopsy at National Nagasaki Medical Center. Seventeen samples were selected of consecutively obtained 74 CHC samples on a crudely age-matching basis.

Written informed consent was obtained from each patient upon enrollment for the study.

HCV Genotype and HCV-RNA Quantification. HCV genotyping was performed by PCR amplification on core region sequences with universal and five subtype-specific primers according to Okamoto *et al.* (23) and their modified version (24). According to this classification, HCV genotypes can be classified into five distinct categories. Genotypes I, II, III, IV, and V correspond to 1a, 1b, 2a, 2b, and 3a in Simmonds' classification (25), respectively.

HCV-RNA quantification was performed by the branched DNA signal amplification method (Quantiplex 2.0; Chiron Diagnostics), which is based on hybridization with specific probes located in the 5′ noncoding region of HCV, with a detection limit of 0.3 Meq/ml.

IFN Therapy and Follow-up. Natural IFN α (Sumiferon; Sumitomo Pharmaceutics, Tokyo) was administered subcutaneously at doses of 6 million units (MU) daily for either 4 weeks (total, 168 MU) or 8 weeks (total, 336 MU). All patients were hospitalized during the treatment. Patients were followed up as outpatients monthly for at least 24 weeks after the end of treatment.

Histological Analysis. All liver tissue specimens were obtained by needle biopsy. Specimens were fixed in 10% formalin, embedded in paraffin, cut to a thickness of 4 μm, and stained with hematoxylin–eosin and Azan. In each patient, the liver biopsy sample confirmed the diagnosis of acute hepatitis.

Analysis for Hepatic IFNAR2 Levels. Liver biopsy samples were evaluated using an indirect immunoperuoxidase staining technique as described previously (6). Briefly, samples embedded in paraffin and cut into 4-μm slices were deparaffinized with xylene and 100, 90, 80, and 70% ethanol, microwaved for 5 min, then subjected to immunostaining using monoclonal anti-IFNAR2. Mouse monochronal antibody against human IFNAR2 (kindly provided by Otuka Pharmaceutical, Tokushima, Japan) at a 1:10,000 dilution was applied to the slide and incubated at 4°C overnight. Standard streptavidin–biotin (Histofine SAB-PO(M) kit; Nichirei, Tokyo) method was utilized for immunohistochemical reaction. As a negative control, normal, nonimmune mouse serum was used instead of the anti-IFNAR2. The staining intensity in each image was quantified by computer-image quantative analysis. The expression intensity was assessed in at least three lobular areas, and the average value for each specimen was determined. The expression intensity in one normal control sample was defined as 1.0 densitometry unit (DU).

Assessment of Efficacy. The treatment was considered to be effective when a sustained virological response (SVR), defined by the absence of detectable levels of HCV-RNA in serum 24 weeks after the end of treatment, was achieved with normal ALT levels. All patients who did not meet the criteria of SVR were defined as nonsustained virological responders (NR).

Statistical Analysis. Data are expressed as mean ± SD. Differences between groups were examined for statistical significance using nonparametric test (Mann–Whitney U test). Chi-square test was also utilized where appropriate. A P value less than 0.05 was considered a statistically significant difference.

RESULTS

General Characteristics of the Patients Studied. The source of infection included blood transfusion (before July 1990; 10 cases; 59%) and needle-stick injury (2 cases; 12%). In five cases (29%), the mode of infection was unclear. The average time from infection to the first signs or symptoms of disease was 43.5 days (median; range, 23 to 180 days). All patents received IFN therapy for 4 or 8 weeks. Genotypes 1b, 2a, and 2b were detected in seven, seven, and one, respectively. All patients completed therapy and follow-up. Patients' clinical backgrounds are listed in Table 1.

Response to IFN Therapy. Overall sustained response rate was 64.7% (11/17). In patients whose total dose of inteferon was 168 MU, sustained response rate was 28.6% (2/7). In patients whose total dose was 336 MU, sustained response rate was 90.0% (9/10). No apparent associations were seen between peak of ALT and HCV-RNA quantity or response to IFN. Although modes of infection were not clear in five patients the time from

TABLE 1. PATIENT CHARACTERISTICS

Patient No.	*Age (yr)*	*Gender*	*Mode of infection*	*Peak of ALT (IU/L)*	*Pre-IFN HCV RNA (Meq/ml)*	*HCV genotype*	*IFN, total dose*	*Effect of IFN*
1	40	F	BT	1154	<0.3	1b	6 MU, 4 weeks, daily (168 MU)	SVR
2	33	F	BT	470	<0.3	2a		SVR
3	35	F	BT	1240	21.8	1b		NR
4	48	F	BT	923	<0.3	1b		NR
5	43	M	BT	350	0.4	1b		NR
6	44	F	Unknown	1192	1.4	1b		NR
7	64	M	BT	440	1.6	Mixed (1b and 2a)		NR
8	32	M	BT	380	4.3	1b	6 MU, 8 weeks, daily (336 MU)	SVR
9	41	M	BT	395	12.3	Mixed (1b and 2a)		SVR
10	68	M	Unknown	2412	<0.3	2a		SVR
11	32	F	BT	550	<0.3	2b		SVR
12	28	F	Unknown	1238	0.3	2a		SVR
13	30	M	Needle stick	682	0.8	2a		SVR
14	59	F	Needle stick	300	<0.3	2a		SVR
15	24	F	Unknown	1352	10.0	2a		SVR
16	42	F	Unknown	2304	<0.3	2a		SVR
17	33	F	BT	1138	<0.3	1b		NR

Note. SVR, sustained virological responder; NR, nonsustained virological responder; BT, blood transfusion.

infection to initiation of IFN therapy of the SVR and NR groups were among patients whose transmission route was known.

Comparison of IFN Receptor Levels Between AHC and CHC. Because the mean age of consecutive 74 CHC patients admitted to our hospital in 2000 and 2001 was much higher than that of AHA patients, 17 samples were selected on an age-matching basis and were provided as controls for immunohistochemical study. Table 2 shows the levels of hepatic IFNAR2 levels as well as other parameters of these two groups. Male gender was predominant in the CHC group and female gender was more frequent in the AHC group, but the difference was not significant. Hepatic IFNAR2 levels were 1.52 ± 0.34 and 0.92 ± 0.16 DU in AHC and CHC, respectively ($P = 0.042$). The significance was even higher when all 74 CHC patients (0.95 ± 0.06 DU) were included in the analysis ($P = 0.005$: data not shown).

Adverse Events. Therapy was tolerated in all patients. The spectrum of side effects was similar to that reported in previous reports on IFN monotherapy for CHC, including flu-like symptoms, arthralgia, neutropenia, and thrombocytopenia. There were no serious adverse effects during therapy.

TABLE 2. COMPARISON OF PATIENTS WITH CHRONIC HEPATITIS C (CHC) AND ACUTE HEPATITIS C (AHC)

	CHC (n = 17)	*AHC (n = 17)*	P
Gender (male:female)	11:6	6:11	0.086
Age (yr)	42.1 ± 2.1	40.9 ± 9.9	0.755
Hepatic IFNAR2 (DU)	0.92 ± 0.16	1.52 ± 0.34	0.042
Genotype (1b:other type)	9:7 (mixed in 1)	7:10	

Levels of Hepatic IFNAR2 in AHC. Table 3 shows the comparison between SVR and NR for IFN therapy in acutely HCV-infected patients ($n = 17$). There are no significant difference in gender, age, peak ALT levels, HCV-RNA levels, or hepatic IFNAR2. Genotype 1b was more common in NR according to univariate analysis. Total IFN dose (169 vs 336 MU) strongly affected the outcome of acute hepatitis C ($P = 0.009$).

Figure 1 indicates the levels of hepatic IFNAR2 in AHC and CHC. There was no difference in hepatic IFNAR2 levels between SVR (1.43 ± 0.35) and NR (1.70 ± 0.25) patients with AHC, whereas they were both significantly higher than the hepatic IFNAR2 levels of CHC patients ($P = 0.028$ and $P = 0.012$, respectively).

DISCUSSION

Unlike hepatitis A and hepatitis B virus infection, the rate of chronic evolution of HCV infection is very high. The rate of chronicity in HCV infection has been reported

TABLE 3. COMPARISON OF SUSTAINED VIROLOGICAL RESPONDERS (SVR) AND NONSUSTAINED VIROLOGICAL RESPONDERS (NR)

	SVR	*NR*	P
Gender (male:female)	4:7	2:4	>0.999
Age (yr)	39.0 ± 13.4	44.5 ± 11.1	0.410
Mode of infection (blood transfusion:others)	5:6	5:1	0.308
Peak of ALT (IU/L)	1021.5 ± 756.2	880.5 ± 392.4	0.679
HCV-RNA (Meq/ml)	5.5 ± 5.4	6.3 ± 10.3	0.890
Hepatic IFNAR2 (DU)	1.43 ± 0.35	1.70 ± 0.25	0.120
Genotype (1b:other type)	2:9	5:1	0.036
Total dose of IFN (336:168 MIU)	9:2	1:5	0.036

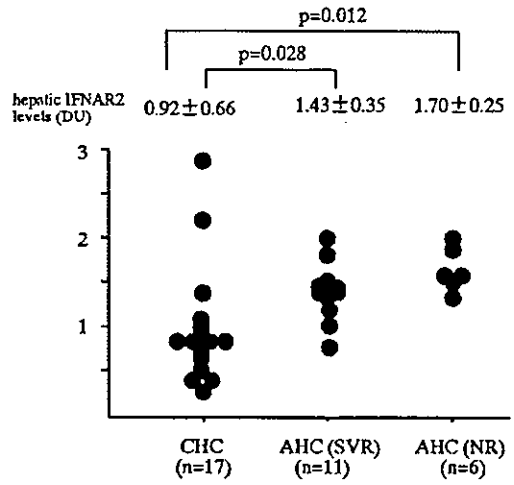

Fig 1. Hepatic IFNAR2 levels in patients with acute hepatitis C ($n = 17$: sustained virological responders, 11; nonsustained virological responders, 6) and chronic hepatitis C ($n = 17$). AHC, acute hepatitis C; CHC, chronic hepatitis C; SVR, sustained virological responders; NR, nonsustained virological responders.

to be 59–88% (7, 8) in transfusion-associated cases and 55–62% (9, 10) in sporadic cases. Once HCV infection progresses to chronic stages, the long-term response to IFN monotherapy is poor. Recently, combination therapy with ribavirin has been established, but it still has limits, including insufficient HCV clearance and significant side effects (26).

On the other hand, high rates of resolution of AHC after IFN therapy have been reported. Hence, it would be rational to challenge stopping infection during the acute phase. For example, recently, a German group (27) reported a nationwide, prospective study in which 44 patients were enrolled. In the study, patients received 5 MU of interferon α-2b daily for 4 weeks and then three times per week for another 20 weeks. Ninety-eight percent of patients had undetectable levels of HCV-RNA in serum at the end of follow-up (24 weeks after the therapy). Based on the results, they suggested that all patients with AHC should be treated, admitting that about 30% of their patients would have had self-limited disease, regardless of whether they received IFN.

Others claim, however, that treating the acutely infected could be harmful, in providing drug therapy for patients whose hepatitis C would have resolved spontaneously (13, 28). Moreover, there is no consensus on the optimal dose, daily versus three times a week injections, or duration of therapy in the setting of AHC.

We reported a comparative study of IFN treatment for AHC in which administration of 6 MU of natural IFN α daily for 4 weeks (total, 168 MU) and 8 weeks (total, 336 MU) was compared. Apparently, the 8-week protocol resulted in a favorable outcome regarding the disappearance of HCV-RNA (90% [9/10] vs. 28% [2/7]).

A total dose of 336 MU of natural IFN α in total is considered to be the "medium" dose in therapy for CHC, suggesting that the amount is safe even for treatment of AHC. Takano *et al.* (12) previously showed a good response (83%) with the same dose of IFN β (336 MU), compared to lower doses, in a randomized, controlled-dose study. Increasing the dose of IFN significantly increased the rate of virological response (as shown in Refs. 12, 14, 18, and 29). Indeed, the German study utilized 440 MU of IFN α2b in total. They admitted that shorter periods of treatment might have been sufficient in patients in whom serum levels of HCV-RNA quickly became undetectable. Thus, the optimal regimen of IFN for AHC should be further elaborated with care.

The reason these high virological efficacies of IFN are obtained against acute HCV infection has not been elucidated. Additionally, factors that determine the virological response rate after IFN therapy are poorly understood. Gursoy *et al.* (30) studied the effects of IFN α2b treatment in hemodialysis patients with acute HCV infection to identify factors that predict the response to this therapy. They found that pretreatment HCV load and genotype were not significantly associated with virological sustained response and that quasispecies heterogeneity was the only parameter that predicted virological response in their 53 patients.

On the other hand, Toyoda *et al.* (31) reported a patient with AHC whose HCV was transmitted by a needle-stick accident from a patient with CHC who had failed to eradicate HCV with IFN therapy. The transmitted HCV was successfully eradicated from the patient with AHC, suggesting some host immune responses in patients with acute viral infections distinct from those in patients with chronic infection.

Human IFN α and β have been considered to elicit their effect via their receptor, IFNAR2 (32). We have reported a strong association between the effect of IFN and the levels of hepatic IFNAR2 mRNA (33) and protein (6) in chronic HCV-infected patients. In the current study, we evaluated if the levels of IFN receptor influence the effect of IFN for those acutely infected by HCV.

As stated above, many studies have shown high resolution rates of AHC after IFN therapy but the reason is yet to be clarified. We conducted a retrospective, immunohistochemical study to clarify whether the IFN receptor levels affect the response of acute hepatitis to IFN therapy. The results indicate that acutely HCV-infected liver expresses higher levels of IFN receptor than does chronically infected liver.

Univariate analysis revealed that there was no difference in the levels of IFN receptor between SVR and NR, suggesting a distinct factor that regulates IFN efficacy. Although the limited number of samples did not allow us to perform multivariate analysis to reveal independent factors associated with the effect of IFN therapy, previous data suggest that genotype is not a strong determinant of IFN efficacy in AHC (27, 30, 31). To reveal the impact of IFN receptors on IFN therapy for AHC, further controlled study is warranted.

In conclusion, we showed that the higher-total dose regimen was superior to the lower total dose in the treatment of AHC. The hepatic receptor level was higher in AHC patients compared to CHC patients, suggesting that the IFN receptor is one of the causes of the high resolution rate of IFN α. The levels of IFNAR2 did not differ in SVR and NR, indicating that high-dose natural IFN α treatment is effective for AHC, irrespective of the levels of hepatic IFNAR2.

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今月のテーマ●わが国のC型肝炎の現状

日本の肝癌は，なぜ多いのか

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要旨：日本は，HCV感染率では高罹患国でないにもかかわらず，世界中の中で日本ほどHCV感染による肝発癌のリスクが高い国はない．HCV感染者における肝発ガンのリスク要因としては，アルコールや性差などの多くの因子が考えられるが，その中でも年齢の因子が大きく関与していると思われる．すなわち，日本のHCV感染者に肝癌が多い理由としては，1）HCV感染による肝癌好発年齢が60歳代である，2）現在の日本のHCV感染者が60歳以後の高齢者に大きな集団を形成している，という二つの要因が現在重なっているからと考えられる．

索引用語：C型肝炎，肝癌，遺伝子多型

はじめに

わが国には約150万人から200万人のHCV（C型肝炎ウイルス）キャリアーが存在すると推定されている．一方，わが国の年間肝癌死亡者数は3万2000人を超し，肝癌は主要悪性新生物死亡順位の男性では第3位，女性では第4位に位置している[1]．また，その肝癌死亡者の約8割がHCV感染に由来し，日本ではHCV感染者は非感染者に比較して1000倍近くの肝癌のリスクを有することも明らかとなっている．一方，世界のHCV感染者は1億7000万人存在すると推定されている[2]．今までに得られた知見によると，HCV感染率では日本は高罹患国でないにもかかわらず日本ほどHCV感染による肝発癌のリスクが高い国はないことが明らかとなってきた．各種国際会議においても，日本でのHCV感染と肝発癌との関係，日本の特殊性が話題となっているも，なぜ日本人はHCV感染による高発癌リスクを有するのか，その理由は明確にはされていない．わが国のHCV感染と肝発癌の状況を諸外国と比較することにより，その特殊性をクローズアップしながら，わが国のHCV感染の状況を現在までに得られている知見をもとに考察したい．

I わが国のHCV感染者数の推定

日赤血液センターでは年間700万検体の献血血液が採取されており，この検査成績より，わが国のHCVキャリアー数の推測が可能である．日赤血液センターでは，献血時の検査でHCV抗体陽性が判明した者には結果を通知し，安全な血液の確保の観点から以後の献血の辞退の勧告をおこなっており，また献血辞退勧告を受けない一般献血者の中には1年間に複数回献血する者も少なくない．1995年4月から1996年3月までの1年間に献血した全献血者の中から，このようなバイアスを除く目的で，初回献血者272343人を抽出し，その集団内におけるHCV抗体陽性率が日赤より報告されている[3]．それによると各年代でのHCV抗体陽性率は，20歳代で0.38%，30歳代で1.06%，40歳代で1.29%，50歳～64歳では2.54%と，年齢が高くなるにつれてHCV抗体陽性率も明らかに上昇している．一方，日赤調査では65歳以上の対象群でのHCV抗体陽性率は不明であるが，各都道府県，市町村単位で実施された各住民検診の結果によると，65歳以上の人口集団でのHCV抗体

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Hepatitis C infection in Japan

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Table 1. 日本におけるHCV抗体陽性者数の推定

年齢階級	HCV抗体陽性率(%)	年齢階級別人口(万人)	HCV抗体陽性者数(万人)(%)
20～	0.38	1800	6.8 (3)
30～	1.06	1600	17.0 (8)
40～	1.29	1900	24.5 (11)
50～64	2.54	2400	61.0 (28)
65～	(5.0)	2200	110.0 (50)
			219.3 (100)

陽性率は5～10%であると報告しているものが多い[4]．65歳以上の高齢者は，わが国では最もHCV感染率の高い集団である．2000年の人口動態統計調査では65歳以上の人口は約2200万人存在することから，この年齢層でのHCV抗体陽性率を仮に5.0%と仮定した場合，この年齢集団には110万人のHCV抗体陽性者が存在すると算出される．以上のように，各年代別の推定HCV抗体陽性者数をそれぞれ加算し，わが国のHCV抗体陽性者推定数を算出したものがTable 1である．この概算によると219万人のHCV抗体陽性者数が存在し，その約50%は65歳以上，約80%は50歳以上の集団で，わが国のHCV抗体陽性者は高齢者に多いことが容易に理解できる．このHCV抗体陽性者の概数は，献血集団における各年代のHCV抗体陽性率から算出され，既にC型肝炎と診断されて通院，入院加療されているものは対象外で，有病者は含まれないことを考慮しなければならない．また一方で，献血者集団でのHCV抗体陽性者中約70%がHCV-RNA陽性のHCVキャリアー，約30%が抗体陽性HCV-RNA陰性の既往の感染であることが過去の調査より明らかとなっている．以上のような状況を総合的に判断すると，わが国には200～250万人のHCV抗体陽性者，150～200万人のHCVキャリアー，ウイルス保有者が存在すると推定される．

II 諸外国のHCV感染状況

今までに文献上，各国の供血者，一般人口を対象とした場合のHCV抗体陽性率の報告を列記する[5]．HCV抗体陽性率で最も低い国は英国，スカンジナビアなどで0.04～0.09%の範囲内であった．次に0.15～0.5%の範囲内の陽性率を示したのは，米国，西ヨーロッパ，イスラエル，0.6～0.9%と中等度の陽性率を示した国は，南ヨーロッパ，ケニア，タイ，台湾などであった．1.0～1.5%の範囲内の陽性率を示したのは，インド，中国，キューバ，エチオピア，韓国，フランスなどであった．1.6～3.5%とやや高い陽性率を示した国は，日本，インドネシア，トルコ，ロシア，ブラジル，中近東，シンガポールであった．3.6%以上の陽性率を報告したのは，カメルーン，エジプトで，特にエジプトでは，血液供血者の14%がHCV抗体陽性と報告している．欧米や開発途上国では，HCV抗体陽性率が低いのに対して，アジアやアフリカの中でも比較的医療の発達している国では抗体陽性率が高い傾向がみられ，これらの国では，手術，輸血などの医療行為でHCV感染が広まったと考えられる．

これらのHCV抗体陽性率は，各国によって，調査対象者，調査方法，HCV抗体測定時期により測定感度と特異性が異なるため，一律に評価することは困難ではあるが，そのような問題点を考慮しても世界のHCV抗体陽性率の平均は1.0ないし2.0%の範囲内であり，日本のHCV抗体陽性率が著しく高くないことが理解できる．一般にアジアやアフリカに比較して欧米ではHCV抗体陽性率は低く，世界の中で一部に高罹患国は存在するもののHBV感染とは異なり国単位で一般人口の5%を超える高罹患国は極めてまれである．

III 日本と米国との間でのHCV感染，肝癌に関する疫学の比較

日本と米国との間でのHCV感染の状況，肝癌の発生状況に大きな差が存在する．お互いのデータを対比することにより，グローバルな観点から日本の特異なHCV感染と発癌との関連をクローズアップすることが可能である(Table 2)．日本の人口は2000年の人口統計で1億2669万人，米国は1995年の調査で2億6275万人である．HCV抗体陽性者数に関しては，日本は約200万人（全人口の1.6%)，米国は約400万人(全人口の2.0%)と推定されている．米国と日本のHCV抗体陽性