presumably attributed to the difference in the treatment regimen and/or background of patients.

As mentioned above, interferon-ribavirin combination therapy has become the standard treatment for chronic hepatitis C. Results from recent studies have suggested that the prevalence of retinopathy associated with combination therapy may be higher than that associated with interferon monotherapy, which should be further investigated[8-10].

In spite of the high prevalence, risk factors for interferon-associated retinopathy are still unclear. Diabetes mellitus and the patients' age were reported to be possible risk factors for retinopathy associated with interferon monotherapy[4]. In interferon-ribavirin combination therapy, diabetes, hypertension[8], and response to treatment[10] were considered possible risk factors. However, the results are not conclusive because of the small number of patients examined.

The aim of the present study is to elucidate the prevalence and risk factors for retinopathy associated with interferon-ribavirin combination therapy.

MATERIALS AND METHODS

Patients

Seventy-three consecutive patients with histologically confirmed chronic hepatit is C (47 males and 26 females; median age, 53.4 years; ranges 26-73 years) were enrolled in this study from 2002 to 2004. The clinical backgrounds of the enrolled patients are shown in Table 1. All patients were treated with recombinant interferon α-2b (Intron A, Schering-Plough, Kenilworth, NJ, USA) and ribavirin (Rebetol; Schering-Plough, Kenilworth, NJ, USA) combination therapy. All the patients were treated daily with interferon α-2b at 6 MU for 2 wk followed by three times a wk treatment with interferon α-2b at 6 MU for 22 wk in combination with ribavirin. Ribavirin was given orally twice a day at a total daily dose of 600 mg for patients who weighed 60 kg or less and 800 mg for the remaining patients who weighed more than 60 kg for 24 wk.

All patients were assessed to determine the safety, tolerance, and efficacy of the treatment at the end of wk 1, 2, 4, and every 4 wk during the treatment. After the treatment was completed, patients were followed up on wk 4, 8, 12, and 24. The primary end point was indicated by a sustained loss of detectable HCV-RNA at 24 wk after the treatment.

Methods

Optic fundi were examined before, and 2, 4, 12 and 24 wk after the start of combination therapy. Ophthalmological examinations were carried out before the start of treatment and 2, 4, 12 and 24 wk after the start of treatment until the completion of treatment or until the retinopathy disappeared. Fundus photographs were taken for documentation and comparison when retinal abnormalities were detected.

Informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committees of our institutions.

Table 1 Profiles and initial laboratory data of patients with and without retinopathy during IFN-ribavirin combination therapy[1]

	Total	Retinopathy (+)	Retinopathy (-)
Patients			
Number	73	14	59
Age (yr)	53.4 ± 10.9	56.3 ± 10.5	52.8±38.6
Gender (M/F)	47/26	10/4	37/22
Hypertension (Yes/No)*	15/58	5/8	10/49
Diabetes mellitus (Yes/No)	2/71	1/13	1/58
Peripheral blood count			
Platelet count ($\times 10^4$/mm^3)	15.3 ± 6.0	12.5 ± 10.5	15.9 ± 38.6
White blood cell ($\times 10^3$/mm^3)	46.9 ± 12.6	46.5 ± 13.0	48.6 ± 10.9
Hemoglobin (×g/dL)	14.0 ± 1.3	14.0 ± 1.0	14.0 ± 1.4
Prothrombin time (%)	90.2 ± 13.3	87.1 ± 13.3	90.8 ± 13.3
ALT (IU/L)	109.4 ± 78.2	104.1 ± 41.0	110.4 ± 83.6
Viral factors			
Genotype (type 1/type 2)[2]	45/26	33/24	12/2
Viral load (kcopies/mL)	592.3 ± 271.2	505.6 ± 309.1	607.5 ± 271.2
Pretreatment/ Arteriosclerotic	12/61	7/7	5/54
changes in optic fundi (Yes/No)			
Response to therapy (SVR/non-SVR)	38/35	5/9	33/26

[1] Data are expressed as mean ± SD.
[2] Genotype could not be determined in 2 patients.
*$P = 0.004$

RESULTS

Before the start of the combinaiton therapy, one patient had scars from laser coagulation of a previous interferon-associated retinopathy and another patient had retinal central vein occlusion. Arteriosclerotic changes of the optic fundi were observed in 12 patients.

After the start of interferon-ribavirin combination therapy, 14 out of 73 patients (19%) developed retinopathy. The clinical profiles and laboratory data of the patients with and without retinopathy are shown in Table 1.

We compared the characteristics of patients who developed retinopathy and those who did not. The two groups showed no statistical differences in age, gender, subtype of virus, RNA level, white blood cell count, platelet count, prothrombin time before treatment or prevalence of pretreatment fundic arteriosclerotic changes. The patients with retinopathy were more frequently complicated by hypertension ($P = 0.004$) (Table 1).

Logistic regression analysis of factors affecting retinopathy was also carried out. Hypertension was found to be a factor for predicting retinopathy (Table 2).

Table 3 shows the optic fundi findings of the 14 patients with retinopathy. Retinopathy was initially diagnosed by the appearance of a cotton wool spot in 12 patients. In three of the 12 patients, retinal hemorrhage was also observed simultaneously or sequentially. Two of the 14 patients who developed retinopathy were diagnosed by retinal hemorrhage without a cotton wool spot. No patient complained of the visual disturbance.

Table 2 Logistic regression analysis of factors associated with retinopathy

Factor	P	Odds ratio	95% confidence interval
Sex	0.68	1.699	0.1-21.0
Age	0.203	1.099	1.0-1.3
Genotype	0.776	1.621	0.1-45.5
Levels of HCV RNA	0.114	1.006	0.99-1.0
Hypertension	0.004	246.32	5.5-10977.8
Diabetes mellitus	0.211	0.122	0.1-3.3
Abnormal findings in pretreatment optic fundi	0.904	1.192	0.1-20.3
Platelet	0.059	1.391	1.0-1.9
Prothrombin time	0.747	0.982	0.9-1.1
ALT	0.992	1	0.98-1.0
WBC	0.964	1.027	0.4-2.9
Response to therapy (SVR or non-SVR)	0.123	0.016	0.0-3.1

Table 3 Optic fundi findings of patients with retinopathy

No	Age	Sex	Underlying disease		Optic fundi before treatment		Optic fundi after treatment	
			Hypertension	Diabetes mellitus	H	S	Cotton wool spot	Retinal hemorrhage
1	38	M	+	+	0	0	4 wk-	4 wk-
2	52	M	+	-	1	0	4-12 wk	-
3	40	M	-	-	0	0	6-36 wk	-
4	62	F	-	-	0	0	4-36 wk	-
5	61	M	+	-	0	0	12 wk-	-
6	58	M	-	-	1	1	12 wk-	-
7	73	M	-	-	2	2	4-28 wk	-
8	65	F	+	-	0	0	24-36 wk	-
9	59	F	+	-	2	2	2 wk-	4-24 wk
10	40	M	-	-	0	0	4-20 wk	-
11	62	F	-	-	1	2	2 wk-	4 wk-
12	65	M	-	-	1	1	2-24 wk	-
13	40	M	-	-	0	0	-	8-16 wk
14	40	M	-	-	0	0	-	2-4 wk

Retinopathy disappeared in 9 of the 14 patients despite the continuation of combination therapy. However, it continued in three patients with retinal hemorrhage and two without retinal hemorrhage.

Ocular manifestations other than retinopathy (e.g., ocular pain, a mild watery eye and conjunctivitis) were not observed in any patients.

DISCUSSION

Interferon associated retinopathy was first recognized in 1990 when Ikebe and associates reported a 39-year-old patient who developed retinal hemorrhages and cotton wool spots following intravenous administration of interferon[11].

The exact mechanism of interferon-induced-retinopathy is not known but is presumably related to the disturbance in retinal microcirculation[12]. Therefore, preexisting arteriosclerosis that affects microcirculation may promote interferon-induced retinopathy.

Our study shows that hypertension is a more frequent complication in patients with interferon-induced-retinopathy. Chronic hypertension is associated with the thickening of the walls of the arteries and small arterioles[13]. Therefore, systemic hypertension predisposes patients to interferon-induced-retinopathy. The fact that hypertensive retinopathy induces the formation of flame-shaped hemorrhages and white cotton wool spots, which are also seen in interferon-induced-retinopathy, implies that systemic hypertension and interferon-induced-retinopathy may be related each other.

Statistical analysis did not indicate pretreatment optic fundic changes or diabetes as predictive factors of retinopathy. This may be attributed to the following reasons: (1) pretreatment changes in the optic fundi as a predictive factor are included in hypertension; and (2) the number of patients with diabetes is too small. Regardless of these reasons, systemic hypertension is an important risk factor for interferon-related retinopathy.

The frequencies of interferon-induced retinopathy associated with interferon monotherapy and interferon-ribavirin combination therapy are reported to be 24%-58%[4-7,14,15] and 16%-64%[8-10,16], respectively. The frequency in the present study (20%) was lower than that in previous reports. Furthermore, the ocular side effects of ribavirin, which include a mild watery eye and conjunctivitis, were not seen in this study. Therefore, the frequency of induced retinopathy associated with combination therapy may be considered as high as that associated with interferon monotherapy.

Retinopathy developed by 12 wk in most (13/14, 93%) of the patients after the start of combination therapy and disappeared in majority (10/14, 71%) of the patients during the 4-8 wk period, in which the patients were receiving the treatment. This suggests that treatment can be continued despite the development of retinopathy in many patients. However, two patients who developed cotton wool spots early in the therapy (2 wk) therafter suffered from retinal hemorrhage in a prolonged manner. Therefore, patients who develop cotton wool spots early in the therapy should be carefully monitored. However, as reported in previous studies[4,8,17], most of the patients with retinopathy in this study were asymptomatic. Therefore, combination therapy may be continued in most patients.

The fact that retinopathy occurred more frequently in patients with hypertension, suggests that these patients should be carefully monitored. With periodic examination of the optic fundi, major bleeding that causes visual symptoms may be prevented or detected at an early stage. Therefore, patients who undergo interferon-ribavirin combination therapy, particularly those with hypertension, should undergo periodic examination of the optic fundi.

To conclude, retinopathy associated with combination therapy of interferon α-2b and ribavirin tends to develop in patients with hypertension.

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REVIEW ARTICLE

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Antiviral treatment of hepatitis C: present status and future prospects

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Abstract Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis. A substantial proportion of patients with chronic hepatitis C eventually develop hepatocellular carcinoma (HCC), which is one of the leading causes of death worldwide. Therefore, efficient antiviral treatments for HCV have long been needed. A recently developed combination therapy of pegylated interferon and ribavirin has dramatically improved the outcome of antiviral therapy for HCV infection. In genotype 1b HCV infection, 48 weeks of the combination therapy achieved eradication of the virus in 50% of patients, and in genotype 2 HCV infection, 24 weeks of the therapy resulted in viral eradication in 80%–90% of patients. By this eradication, an improvement in the hepatic fibrosis, an inhibition of HCC development, and an improvement in life expectancy were attained. Patients who did not respond to the combination therapy may be treated with long-term interferon monotherapy, which is not intended to eradicate HCV, but will lower the serum alanine aminotransferase (ALT) level. Thus, the treatment for HCV infection has progressed significantly, but therapies with new modalities, such as inhibitors of viral protease or RNA polymerase, are still being awaited.

Key words Hepatitis C · Interferon · Treatment

Introduction

Hepatitis viruses mainly infect the liver, causing hepatic diseases in humans. To date, five types of hepatitis virus, B, A, D, E, and C, have been found, in this order, and subjected to medical treatment. Hepatitis C virus (HCV) and hepatitis B virus (HBV) infections can develop into persistence, while hepatitis A virus and hepatitis E virus cause only transient infection. In Japan, chronic hepatitis caused by HCV infection currently poses the greatest problem because of the large number of patients affected and the high rate of patient mortality from complications, particularly hepatocellular carcinoma (HCC).[1]

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Chronic hepatitis C

It is estimated that there are approximately 170 million HCV carriers or patients with persistent HCV worldwide, and approximately 1.8 million patients in Japan. HCV infection occurs when blood contaminated with HCV enters the body directly. The infection routes include blood transfusion with HCV-contaminated blood products obtained a long time ago, sharing of needles among drug abusers, and the use of inappropriately disinfected acupuncture needles and tattoo needles, among others.[2] People undergoing folk remedies and hair-removal treatments should also be regarded as susceptible to HCV infection if these are invasive practices and nondisposable devices are used.

The problem with HCV infection resides in the very high rate of general HCV infections which are becoming chronic (approximately 70%). However, in the case of HCV infection via blood transfusion, the rate of reaching chronicity has been reported to reach 80%, probably because of a high virus load.

Virus markers of HCV infection required for the treatment of hepatitis

Some virus markers of HCV infection are available, as described below. Figure 1 shows a progress observation flowchart for anti-HCV antibody-positive patients obtained using these virus markers.

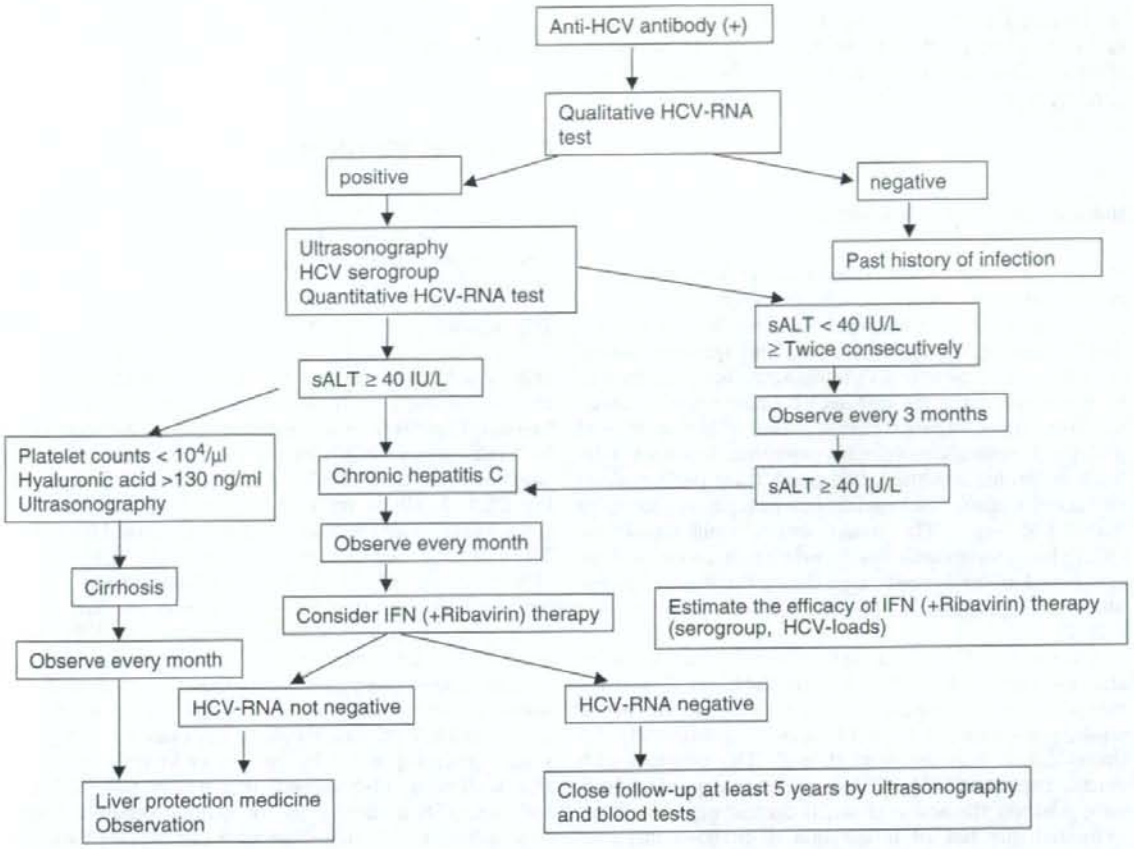

Fig. 1. Progress observation flow chart for anti-HCV antibody-positive patients. *HCV*, hepatitis C virus; *ALT*, alanine aminotransferase; *IFN*, interferon

Anti-HCV antibody

Anti-HCV antibody of low titer is frequently detected using sensitive HCV kits currently available in Japan. Patients with low anti-HCV antibody titers mostly have a history of remote HCV infection, while those with high titers generally have an ongoing infection. Hence, patients who test positive for anti-HCV antibodies are not necessarily infected with HCV at present. When the antibody titer is found to be low, a history of infection (i.e., currently cured) should be suspected. To verify this, a sensitive qualitative HCV–RNA measurement is required (reverse transcriptase–polymerase chain reaction (RT–PCR) method).

Meanwhile, it should be noted that during the early stage of HCV infection (2–3 months from the initial HCV exposure), patients do not test positive for anti-HCV antibody (window period).

HCV–RNA

To confirm the presence of HCV, we use an HCV–RNA assay by RT–PCR. There are two types of RT–PCR assay, a qualitative one and a quantitative one. However, the latter has a relatively low sensitivity. Therefore, the qualitative RT–PCR assay PCR is used to monitor the presence or absence of HCV, and hence the efficiency of an antiviral drug. For an estimation of the efficacy of antiviral treatment with interferon (IFN), a quantitative RT–PCR assay must be used.

Genotypes and serogroups of HCV

Many genotypes of HCV have been identified (i.e., there are HCV groups whose gene or genomic sequences differ to some extent). HCV genotypes are clinically important because the efficacy of IFN therapy varies depending on the HCV genotype. In Japan, the majority of HCV patients have HCV genotypes 1 or 2. Because the HCV genotype is determined on the basis of restriction fragment length polymorphism (RFLP) by PCR assay, the determination procedure is somewhat complicated. In order to determine the responsiveness of patients with chronic hepatitis C to IFN therapy easily (rapidly and accurately), serogroup (SG) identification by enzyme immunoassay is useful.[3] Patients

are classified as SG-1 (corresponding to HCV genotype 1) or SG-2 (corresponding to HCV genotype 2). Many patients classified as SG-1 are resistant to IFN, whereas many patients classified as SG-2 are generally responsive to IFN therapy.

Natural course of HCV infection

HCV patients commonly develop "acute hepatitis" 2 or 3 months after the initial exposure. However, many patients are unaware of this development because they have minor subjective symptoms and hardly exhibit jaundice. About 20% to 30% of patients exhibiting acute hepatitis recover spontaneously from the disease, but acute hepatitis develops into chronic hepatitis in the remaining 70% to 80% of patients (hepatitis persisting for more than 6 months is defined as chronic hepatitis). In general, these patients enter an "inactive phase" of hepatitis C, which persists for more than 10–15 years. The serum alanine aminotransferase (ALT) level, which indicates the extent of hepatocytic damage, is within the normal range during the inactive phase, but viral replication continues even during this period (Fig. 2).

Chronic hepatitis C frequently enters the "active phase" after an inactive phase of 10–15 years; however, this period varies greatly depending on the individual. In the active phase, the serum ALT level becomes approximately 2–3 times higher than the normal level. The problem with chronic hepatitis C is that it does not resolve spontaneously once it enters the active phase. If chronic hepatitis is left untreated, the risk of progression to cirrhosis increases without the patient realizing it. Thus, hepatitis C is characterized by its gradual but steady progression.[4]

With the progression to cirrhosis, there is an increasing risk of developing HCC. This risk has been reported to have an annual rate of 5% to 7%.[5] Ideally, HCV-infected patients should have the disease diagnosed during the inactive phase of chronic hepatitis so that, upon transition to the active phase, the patients can start receiving antiviral therapy for HCV.

Treatment of HCV infection

HCV infection is treated using mainly IFN preparations. These IFN preparations are outlined below in their order of development.

IFN monotherapy

IFN monotherapy was first introduced for the treatment of chronic hepatitis C. In Japan, the treatment of chronic hepatitis C generally starts with the daily administration of 6–10 million units of IFN for 2–4 weeks, followed by administration three times weekly for 6 months. In Europe and the USA, 3 million units of IFN are administered three times weekly from the start, and this is continued for a year. The efficacy of the therapy is evaluated after 6 months of IFN treatment. If an HCV–RNA test is negative by a qualitative RT–PCR assay at this time, it indicates that the patient obtained a sustained virological response (SVR) and is considered to be practically free of HCV.

IFN monotherapy had conventionally been used for non-A/non-B hepatitis from around 1985, prior to the discovery of HCV. A nationwide survey carried out by a research group supported by the former Ministry of Health and Welfare in 1995 showed that the overall SVR rate following IFN monotherapy for chronic hepatitis C (the administration of 6–10 million units per day) for 6 months was approximately 30%. SVR rates at facilities across Japan were nearly equal to this value. However, among patients with HCV genotype 1, who accounted for approximately 70% of all Japanese patients infected with HCV, and particularly those with a high viral load (defined as HCV–RNA >100 KIU/ml in Japan), a SVR was obtained in only 2% to 7% of cases; i.e., the efficacy of treatment by IFN

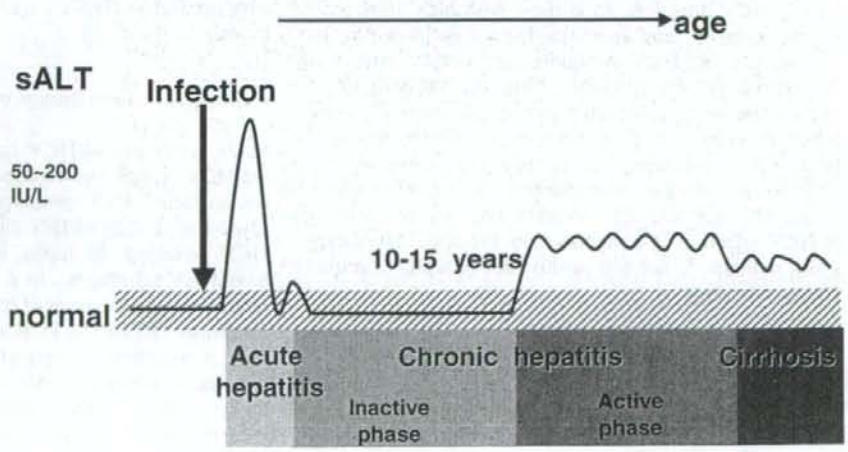

Fig. 2. Natural course of HCV-infected patients. Approximately 70% of acutely HCV-infected people develop persistent infection. After 10–15 years of the inactive phase, most chronic hepatitis C patients move into the active phase. One-third of chronic hepatitis C patients are assumed to develop cirrhosis. *sALT*, serum alanine aminotransferase

monotherapy was low. These patients with HCV genotype 1 at a high viral load have what is called "intractable hepatitis C."

IFN therapy in combination with ribavirin

IFN is also administered in combination with ribavirin, an antiviral drug. In Japan, the use of ribavirin was approved in December 2001. Ribavirin (600–800 mg daily, divided into two doses) is taken orally throughout the period of IFN injections. Ribavirin is a synthesized nucleic acid derivative and, when administered in combination with IFN, shows an increased antiviral activity.

In clinical studies of IFN therapy in combination with ribavirin conducted in Japan, a SVR rate of approximately 20% was obtained even in patients with HCV genotype 1 at a high viral load, i.e., "intractable hepatitis C," and who were less responsive to IFN monotherapy. Because patients on IFN monotherapy used as the control showed a SVR rate of only 2.3%, the concomitant use of ribavirin contributed to an approximately 10-fold increase in antiviral activity.[6]

The efficacy of IFN therapy in combination with ribavirin after its inclusion in the health insurance program is very similar to that found in a clinical study in Japan. However, the adverse effects of this combinational therapy have generally been more severe than those observed during the clinical study period. The drop-out rates of patients who could not complete the combinational therapy were as high as 15%–20%, and this led to a decrease in SVR rate calculated on intention-to-treat (ITT). In other words, the number of patients who dropped out of the treatment is added to the denominator. Adverse drug reactions that reduce the quality of life (QOL), such as hemolytic anemia, severe malaise, anorexia, and taste disorders, are frequently observed, particularly in many elderly patients. Indications for IFN therapy in combination with ribavirin should be considered carefully for patients aged 65 years or older.

Long-term interferon therapy

In cases of long-term IFN therapy, IFN is administered two or three times a week for a period of 2 years or more. The purpose of this therapy is not the eradication of HCV, but the normalization of serum ALT levels and eventually the suppression of HCC development. This is a promising therapy for patients who cannot be treated with ribavirin because of its adverse effects, or for those who were not able to continue with the combination therapy of IFN and ribavirin.

PEG–IFN therapy in combination with ribavirin

PEG–IFN is an interferon molecule covalently bonded to polyethylene glycol (PEG), which shows a sustained release. PEG–IFN characteristically requires subcutaneous administration only once weekly, as compared with the conventional type of IFN which requires administration three times a week. PEG–IFN therapy alone has a higher efficacy than the conventional IFN monotherapy, but it has been demonstrated that PEG–IFN therapy used in combination with ribavirin shows an even higher efficacy[7,8] (Fig. 3).

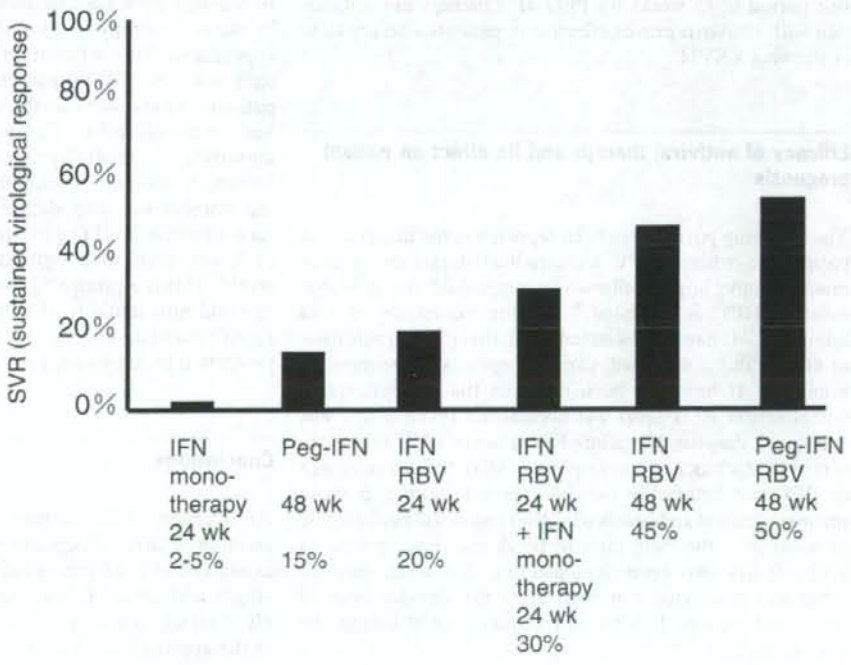

Fig. 3. Changes in anti-HCV therapy, including interferon for intractable (genotype 1b, high viral loads) chronic hepatitis C patients. After the introduction of IFN monotherapy for chronic hepatitis C, the efficacy of IFN therapy has gradually increased with the addition of ribavirin, the introduction of pegylated IFN, and an extension of the duration of therapy. *IFN*, interferon; *RBV*, ribavirin

PEG–IFN therapy in combination with ribavirin is expected to increase the SVR rate to approximately 50% even in patients infected with HCV genotype 1 at a high viral load, and to approximately 60% in all patients infected with HCV. The efficacy in those infected with genotype 2 HCV reaches 80%–90%. In Japan, treatment with PEG–IFN α-2a (Pegasys) alone was approved in December 2003. The combined use of PEG–IFN α-2b (PegIntron) and ribavirin (Rebetol) was also approved in December 2004. These treatments with PEG–IFN are generally administered for 48 consecutive weeks. Continuation of the treatments for 48 consecutive weeks is important, although it may be necessary to decrease the dose owing to adverse drug effects.

The adverse effects of PEG–IFN therapy in combination with ribavirin are almost the same as those of conventional IFN therapy. However, such adverse effects are generally minor (for example, fever), and the therapy requires administration only once per week, thereby improving the patient's QOL. Because there is the possibility of drug accumulation in the body and an associated exacerbation of adverse effects owing to the sustained-release formulation, very careful observation of patients is required. There have been reports of other problematic adverse effects of this combinational therapy compared with those of the conventional IFN preparation, e.g., decreased counts of leukocytes, and particularly of neutrophils. Some patients exhibit severe thrombocytopenia. It is mandatory to confirm neutrophil count immediately before every administration.

It is currently specified that PEG–IFN therapy used in combination with ribavirin is the best choice in the treatment of intractable hepatitis C of genotype 1 at a high viral load. This combinational therapy is thus administered first. It has recently been suggested that an extended administration period of 72 weeks for PEG–IFN therapy in combination with ribavirin proves effective in patients who are slow in showing a SVR.

Efficacy of antiviral therapy and its effect on patient prognosis

The following points have been reported in the literature: in patients in whom HCV was eradicated mainly by IFN monotherapy, hepatic fibrosis is improved,[9] the development of HCC is inhibited,[10] and life expectancy is also improved.[11] It has thus been indicated that if the eradication of HCV can be achieved, chronic hepatitis C prognosis is improved. It has also been reported that in patients in whom serum ALT level was normalized (even if this was transient), despite the failure to eradicate HCV (cases referred to as a biochemical response (BR)), the development of HCC was delayed in the short term. However, because no improvement in fibrosis was observed, it will probably be impossible in the long term to block the development of HCC. It has also been demonstrated that when curative treatment is carried out even after the development of HCC, subsequent IFN-based treatment could inhibit the recurrence of HCC.

Treatment of hepatitis C patients who do not respond sufficiently to IFN

Liver-protection therapy

Liver-protection therapy aims to delay the progression of chronic hepatitis by controlling inflammation in patients in whom HCV could not be eradicated. An ursodeoxycholic acid preparation (Urso) and a glycyrrhizin preparation (Stronger Neo Minophagen C) are used in combination as a liver-protection therapy. These drugs inhibit hepatic inflammation and decrease serum ALT level, but they do not decrease HCV load. It was reported that Stronger Neo Minophagen C delays the progression of chronic hepatitis and the onset of HCC.[12] The ursodeoxycholic acid preparation decreases serum ALT level, but its action of delaying the progression of chronic hepatitis has not yet been verified.

Hepatitis C generally progresses slowly and is less likely to aggravate rapidly, unlike hepatitis B, which may aggravate very rapidly, and progresses steadily. Liver-protection therapy, which retards the progression of the disease by controlling inflammation, can therefore be considered significant in hepatitis C. This therapy is applied mainly when it is impossible to use IFN due to its adverse effects, or when patients do not respond sufficiently to IFN therapy, including in combination with ribavirin. Liver-protection therapy is also administered as a temporary measure until a therapy in combination with IFN is started.

Phlebotomy

Iron deficiency leads to a decrease in serum ALT level, and its use as a therapy for chronic hepatitis C has begun to be appreciated. This is based on the observation that reactive oxygen species (ROS) production increases in hepatitis C patients, which leads to the development of liver disease and eventually HCC. Because intrahepatic iron plays an important role in ROS production (Fenton reaction), phlebotomy is designed to suppress ROS production by inducing intrahepatic iron deficiency. In fact, decreasing the serum ferritin level (an indicator of iron store) to 10ng/ml or lower leads to a significant decrease in serum ALT level.[13] This is a promising therapy for patients who do not respond sufficiently to IFN therapy, or who are unable to receive it and do not respond to the above-mentioned liver-protection therapy either.

Conclusions

An overview of the current status of research on the progression of chronic hepatitis C and the treatment methods available has been presented and discussed in terms of the effects and limits of these methods. The early detection of HCV infection makes it possible to apply antiviral therapy at the appropriate time. It is particularly worth noting that

it has become possible for antiviral therapies to eradicate viruses in a majority of HCV patients, and to suppress and control the progression of HCV infection (or acute hepatitis C) to chronic hepatitis and subsequently to HCC. However, the limits of the current IFN-based therapies have also become evident. Specific antiviral drugs targeting HCV enzymes (including viral proteases, helicase, and RNA polymerase) have recently been developed. The development of one antiviral drug has advanced to phase II clinical trials as of 2006.

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Molecular epidemiology of hepatitis A virus in metropolitan areas in Japan

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Background. Transmission routes of hepatitis A virus (HAV) in Japan have changed. The present study investigated changes of transmission routes in relation to genetic drift. ***Methods.*** All 60 patients who were admitted between 1993 and 2003 with a diagnosis of hepatitis A were retrospectively analyzed. Nucleotide sequences of the VP1/2A region of the HAV recovered from their sera were determined. ***Results.*** The suspected transmission routes were household contact, 19 (31%); food or waterborne, 16 (27%); homosexual activity, 11 (18%); international travel, 4 (7%); and unknown 10. (17%). The 11 patients presumably infected through homosexual activity were found exclusively in 1998 and 1999. The proportion of patients exposed through homosexual behavior and household contact was higher in those 2 years than in other years. Nucleotide sequences could be determined for 58 patients. Fifty-seven of the 58 sequences belonged to genotype IA HAV, with less than 10% nucleotide diversity. Of the 27 sequences isolated during 1998 and 1999, 25 had an identical nucleotide sequence regardless of the suspected transmission route. In contrast, sequences obtained in the other years differed from one another. A phylogenetic tree constructed from sequences recovered from patients without a history of travel abroad showed several clusters. ***Conclusions.*** Our results suggest that (1) HAV acquired through homosexual activity may be transmitted to nonhomosexual individuals; (2) hepatitis A in metropolitan areas in Japan is caused mainly by sporadic infection with genotype IA HAV; and (3) several subtypes of genotype IA HAV are endemic in Japan.

Key words: hepatitis A virus, epidemiology, transmission route, sexuality, Japan

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None of the authors has a potential conflict of interest.

Introduction

Hepatitis A virus (HAV) is one of the major causes of viral hepatitis and a worldwide problem. The annual incidence of hepatitis A is 1.5 million cases of clinical disease, and the true incidence, including subclinical disease, may be much higher.[1] Fulminant hepatic failure is a complication for some patients with clinical disease.[2] Therefore, preventing the spread of HAV is an important issue.

Good sanitation and a sterilized water supply are essential for the prevention of hepatitis A. In developing countries with a high incidence of hepatitis A, the main transmission route of hepatitis A is the fecal–oral route caused by poor sanitation, which increases the chance of ingesting contaminated food or water.[2,3] In contrast, in developed countries with good sanitation and a sterilized water supply, such as North America, Western Europe, Australia, and New Zealand, the incidence of hepatitis A is low and transmission is caused by personal contact with an infected person, homosexual activity, or transfusion of contaminated blood products, in addition to ingestion of contaminated food or water.[2–5]

The incidence of hepatitis A in Japan has markedly decreased recently. National surveillance of HAV in Japan has shown that more than 90% of people over 65 but fewer than 10% of people under 34 are positive for anti-HAV.[6] The difference can probably be attributed to changes in sanitation. If this hypothesis is true, then the transmission route of HAV in Japan may have changed with time. Studying changes in HAV transmission routes in Japan may therefore elucidate the influence of sanitation on transmission routes.

Molecular epidemiological approaches may also be useful for studying transmission routes. Studies from European countries have shown that several clusters of viral strains from various genotypes prevail in those countries.[7,8] The heterogeneity of isolated strains

suggests multiple transmission routes. Information on transmission routes is, however, not available in these reports. Sequential molecular epidemiological studies linked to transmission routes may elucidate native strains in Japan and provide new information for the control of this disease.

The aim of this study was to understand both clinical and molecular epidemiology of HAV infection in Japan.

Methods

Patients

Sixty patients admitted to our institutions between 1993 and 2003 who were diagnosed with hepatitis A were analyzed retrospectively. The patients comprised 39 men (65%) and 21 women (35%), and their median age was 34.0 years (range, 22–55 years). The diagnosis of hepatitis A was based on a high titer serum IgM anti-HA level with acute liver injury. Coinfection with hepatitis B virus, hepatitis C virus, or other hepatotropic viruses was excluded by serological testing. Serum samples were available from all patients on admission. Fifty-eight of the 60 samples were positive for HAV RNA by reverse transcription (RT)-nested polymerase chain reaction (PCR) with the protocol outlined below. None of the 58 patients had fulminant hepatic failure. Intrahepatic cholestasis was a complication in one patient. The other 57 patients underwent a noncomplicated and self-limited clinical course.

Informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committees of our institutions.

Detection of hepatitis A viral RNA in serum

RNA was extracted from sera using the acid guanidinium-phenol-chloroform method.[9] In brief, 100 µl of sample was mixed with 300 µl of solution D (guanidinium solution), 60 µl of chloroform, and 40 µl of NaOAc (pH 5.2) and precipitated with 1 ml of ethanol. The RNA pellet was washed twice with 70% ethanol and dissolved in 25 µl of RNase-free distilled water.

For reverse transcription, 1 µl of RNA solution, extracted from 100 µl of sera using the acid guanidinium-phenol-chloroform method and dissolved in 25 µl of RNase-free distilled water, was heat-denatured at 68°C for 10 min. It was chilled rapidly on ice and mixed with 4 µl of 1.5 mM $MgCl_2$ solution, 2 µl of 10× RNA PCR buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl), 8.5 µl of RNase-free distilled H_2O, 2 µl of dNTP mixture (10 mM dATP, dCTP, dGTP, dTTP), 1 µl of random 9-mers (5′-NNNNNNNNN-3′), 0.5 µl of RNase inhibitor (Takara-Shuzo, Kyoto, Japan), and 1 µl of reverse transcriptase (Takara-Shuzo). After incubation at 30°C for 10 min, reverse transcription reaction was carried out at 42°C for 30 min, followed by inactivation at 95°C for 5 min.

In the first PCR, 5 µl of the 20 µl cDNA solution was used. The first PCR was performed in 50 µl of reaction mixture containing 1.0 µM each of outer sense primer (5′-GGTTTCTATTCAGATTGCAAATTA-3′ nt. 2891–2914) and antisense primer (5′-AGTAAAAACT CCAGCATCCATTTC-3′ nt. 3398–3375), 200 µM of each dNTP, 5 µl of 10× PCR buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl; 15 mM $MgCl_2$; 0.01% (w/v) gelatin), and 2.5 U of *Ex Taq* polymerase (Takara) with proofreading activity. The amplification conditions were 94°C for 16 min followed by 40 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min.

One microliter of the first PCR product was used for the second PCR. The reaction mixture contained 1.0 µM each of inner sense primer (5′-TTGCAAATTACAAT CATTCTG-3′ nt. 2905–2925) and inner antisense primer (5′-TTCAAGAGTCCACACACTTCT-3′ nt. 3377–3367), 5 µl of 10× PCR buffer, 35 µl of RNase-free dH_2O, 5 µl of dNTP mixture (2 mM dATP, dCTP, dGTP, dTTP), and 0.5 µl of amplitaq gold (Roche Diagnostics, Branchburg, NJ, USA). The amplification conditions for the second PCR were the same as those of the first PCR. The second PCR products were analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV transilluminator. Standard precautions to avoid contamination were taken during PCR, with a negative control serum included in each run.

Sequencing of PCR products

Amplification products were purified on Wizard PCR Preps DNA purification resin (Promega, Madison, WI, USA), and sequenced bidirectionally with the Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA, USA) using the above PCR primers. Sequencing was performed on an automated DNA sequencer ABI 377 (PE Applied Biosystems).

The nucleotide sequences of HAV isolates from the patients were compared with those of seven reference HAV strains retrieved from the DDBJ/EMBL/GenBank databases, representing each of the seven major genotypes (I–VII). Phylogenetic trees were constructed with the Mega program version 2.1 using the Kimura two-parameter matrix and the neighbor-joining method.[10] To confirm the reliability of the phylogenetic tree analysis, bootstrap resampling and reconstruction were carried out 500 times.

Statistical analysis

Data were analyzed by a χ-squared test. P values less than 0.05 were regarded as statistically significant.

Results

Transmission routes

Table 1 shows the numbers of patients and transmission routes. An epidemic of hepatitis A among homosexuals was reported in metropolitan areas in Japan between 1998 and 1999.[11,12] Our results showed that more patients were admitted during that period. The increase was caused not only by patients involved in homosexual activity but also by individuals without that risk factor.

The suspected transmission routes for the patients were as follows: household contact, 19 (31%); food or water, 16 (27%); homosexual activity, 11 (18%); international travel, 4 (7%); and unknown, 10 (17%). In 1998 and 1999, the suspected transmission routes were as follows: household contact, 13 (45%); homosexual activity, 11 (38%); food or water, 1 (3%); international travel, 1 (3%); and unknown, 3 (11%). The proportion of cases associated with homosexuality and household contact was higher in those 2 years than in the other years (homosexuality-associated, $P = 0.0006$; household contact-associated, $P = 0.034$). Figure 1 shows the time of onset for all patients over the 2 years. The times of onset for those 2 years were from July 1998 to July 1999 for homosexual patients and from February 1998 to September 1999 for nonhomosexual patients. After excluding two patients with different sequences, the onset of nonhomosexual patients varied from August 1998 to September 1999. The periods of transmission were similar between homosexual and nonhomosexual groups.

Sequence analysis of HAV RNA

The sequence between nucleotides 3024 and 3191 of the VP1/2A region was determined for the 58 patients. Many nucleotide sequences were closely related to HAS-15, a representative HAV genotype IA strain. Twenty-five of the 27 viral sequences recovered during 1998–1999 were identical. This sequence is identical to IMSTU, which is prevalent among homosexuals in metropolitan areas.[12] As shown in Table 2, all sequences except one showed more than 90% identity with the reference sequences.

We then performed a phylogenetic analysis of the region between nucleotides 3024 and 3191 and classified the virus strains (Fig. 2). Bootstrap analysis to evaluate the statistical reliability of the phylogenetic tree revealed 500/500 (100%) reliability. All strains belonged to genotype IA, except for one that belonged to genotype IIIA. The patient harboring the genotype IIIA virus had a history of travel to Africa 1 month before admission.

Table 1. Numbers of patients and routes of transmission

	1993–1997	1998–1999	2000–2004	Total
Household contact	4 (29%)	13 (45%)*	2 (12%)	19 (31%)
Food or waterborne	4 (29%)	1 (3%)	11 (64%)	16 (27%)
Homosexual activity	0	11 (38%)**	0	11 (18%)
Foreign travel	1 (7%)	1 (3%)	2 (12%)	4 (7%)
Unknown	5 (35%)	3 (11%)	2 (12%)	10 (17%)
Total	14	29	17	60

* $P = 0.034$, ** $P = 0.0006$; χ-squared test

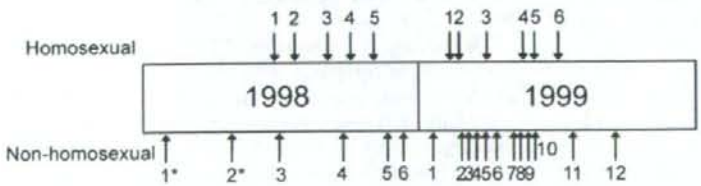

Fig. 1. Time of first visit of patients with hepatitis A in 1998 and 1999. *Arrows* in the upper part show the times of visit of homosexual patients. *Arrows* in the lower part show those of nonhomosexual patients. All except two (cases 1 and 2) had the same sequences. The period of transmission was similar for homosexual and nonhomosexual groups. * shows the sequences that were different

Table 2. Homology with recovered sequences and representative strains (HAS-15 and IMSTU)

Homology with IMSTU			Homology with HAS-15		
	Nucleic acid	Amino acid		Nucleic acid	Amino acid
HAS-15	95.7	98.2	IMSTU	95.7	98.2
1993	97.6	100	1993	94.4	98.2
1994	77.1	89.1	1994	73.8	87.3
1995	95.7–98.8	98.2–100	1995	93.8–94.4	96.4–98.2
1996	97.6	100	1996	94.4	98.0
1997	95.7–98.8	96.4–100	1997	92.4–94.4	94.5–98.2
1998	95.7–100	98.2–100	1998	93.7–95.7	96.4–98.2
1999	100	100	1999	95.7	98.2
2000	90.4–95.7	92.7–98.2	2000	86.8–92.4	90.9–96.4
2001	93.7–97.6	96.4	2001	92.4–94.4	94.5–98.2
2002	93.1–96.3	100	2002	92.4–96.3	94.5–98.2
2003	96.7	100	2003	95.0	98.2

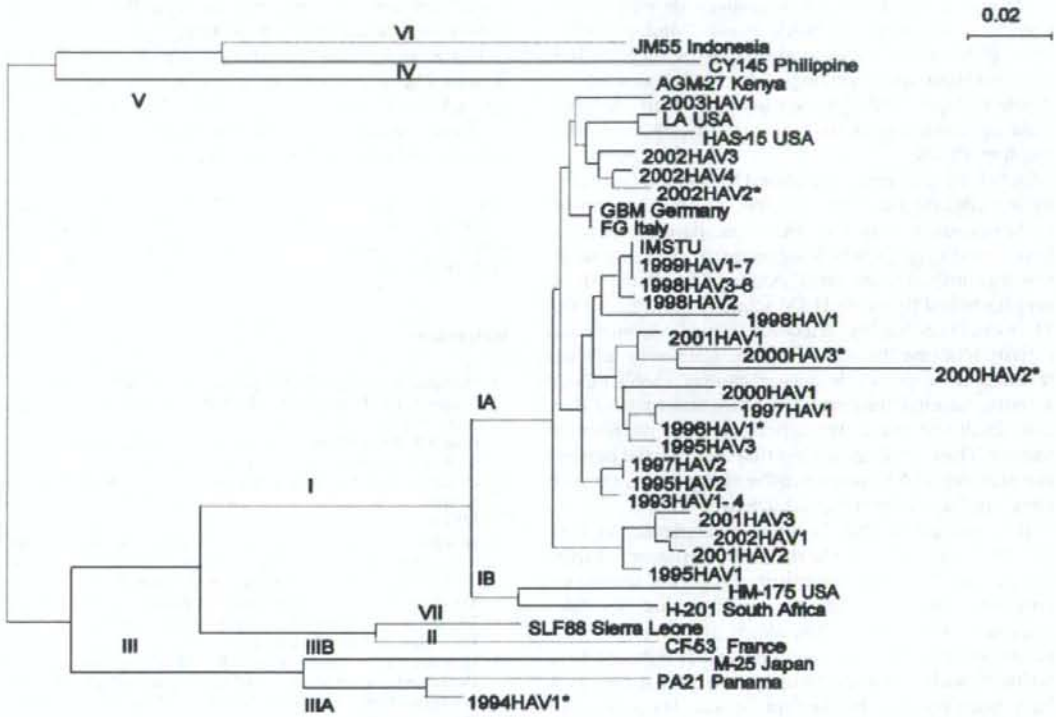

Fig. 2. A phylogenetic tree constructed for RNA sequences located in the VP1/2A region of hepatitis A virus (HAV) genomes reported previously. Accession numbers are shown for the isolates that have been deposited in the DDBJ/EMBL/GenBank databases. Many nucleotide sequences were close to that of HAS-15, a representative HAV genotype IA strain. Twenty-five of 27 viral sequences recovered during 1998–1999 were identical to IMSTU. *shows the sequences that were acquired abroad

Discussion

In this study, the most frequent transmission route was personal contact, which is also the case in the United States (www.cdc.gov/ncidod/diseases/hepatitis/resource/PDFs/hep_surveillance_60.pdf). The next most frequent cause was contaminated food, which is different from the United States. Additionally, the

proportion of patients whose hepatitis was caused by contaminated food was highest in recent years. As mentioned above, anti-HAV prevalence, which may reflect poor sanitation, has decreased in Japan. Therefore, the high incidence of hepatitis A with food/water as a transmission route may not result from poor sanitation. The reason for the high percentage is presumably related to diet. Japanese people often eat raw fish or shellfish, which increases the chances of transmission. Indeed an outbreak caused by eating raw oysters has been reported.[13] In other words, the transmission routes of HAV in Japan are different from those in other developed countries irrespective of improved sanitation.

The molecular epidemiological study showed interesting results. The sequences recovered in the years other than 1998 and 1999 were heterogeneous (Fig. 1). Furthermore, the phylogenetic analysis showed several clusters of genotype IA HAV strains, after excluding cases presumably acquired abroad. This suggests that several subgroups of genotype IA HAV strains are endemic in Japan and cause sporadic hepatitis. A large-scale epidemiological study may be useful for testing this hypothesis.

In contrast, an epidemic caused by homosexual activity was observed in 1998 and 1999.[11,12] The same nucleotide sequences were detected among patients for more than 1 year (Fig. 1), which suggests that the same strain was transmitted secondarily. Among 24 patients whose sera harbored the same HAV sequences in this period, 11 were presumably infected through homosexual activity. Among the other patients, four were infected through close person-to-person contact (heterosexual activity, familial transmission, or transmission in day-care facilities), while the other four had no relevant history. These findings suggest that homosexual persons can transmit HAV to nonhomosexual persons through close contact or heterosexual activity.

It is interesting that 24 separate sequences in 1998 and 1999 were identical to the IMSTU recovered from ten patients in another institution. A serum sample recovered 2 months before the epidemic had a similar sequence, with 99.4% homology. Because a homosexual patient may have sexual contact with multiple partners within that community, the 34 patients may have been infected by the same strain. Therefore, our results suggest that the rate of mutation in this region of 168 base pairs is probably very low. A previous study has shown that the mutation rate of HAV within a person is very low;[14] our results are consistent with that observation.

A previous report has shown that sequences recovered in Japan in the early 1990s are variable and belong to multiple genotypes.[14] Our results are contrary to those of that study. This suggests that improvements in sanitation decrease both the number of patients and viral heterogeneity. The endemicity in low-HAV-prevalence countries may be caused by highly related viral strains. A report from the United States showing that most patients infected in a community-wide outbreak were infected by the same strain supports this hypothesis.[15]

In developed countries, a substantial number of patients with hepatitis A acquired HAV through homosexual activity (www.cdc.gov/nciod/diseases/hepatitis/h96surve.htm). Recent studies using PCR analysis have shown that the fecal excretion of HAV continues even after recovery,[16] which suggests that hepatitis A patients may transmit the virus even after recovery. This is in accordance with the fact that positivity for anti-HAV among homosexual people is very high.[17–19] Therefore, people who engage in homosexual activity should be considered for HAV vaccination regardless of human immunodeficiency virus coinfection.

To conclude, recent hepatitis A in metropolitan areas is caused predominantly by sporadic infection by genotype IA HAV. Homosexual activity may cause an HAV epidemic among not only homosexuals but also heterosexuals, and involve homogeneous viral strains.

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Clinical Studies

Premature telomere shortening and impaired regenerative response in hepatocytes of individuals with NAFLD

Nakajima T, Moriguchi M, Katagishi T, Sekoguchi S, Nishikawa T, Takashima H, Kimura H, Minami M, Itoh Y, Kagawa K, Tani Y, Okanoue T. Premature telomere shortening and impaired regenerative response in hepatocytes of individuals with NAFLD.
Liver International 2006: 26: 23–31. © Blackwell Munksgaard 2005

Abstract: *Aims:* The risk factors associated with poor prognosis of nonalcoholic fatty liver disease (NAFLD) are not fully understood. Our aim was to assess the role of progressive hepatocellular telomere shortening in the clinical course of NAFLD. *Methods:* We measured average telomere lengths in liver tissue samples from 44 patients with NAFLD by quantitative fluorescence *in situ* hybridization using a telomere-specific probe. Patients in which telomeres measured at least 80% of the lengths of age-matched controls were categorized as group A. Those patients with telomeres measuring less than 80% of the control lengths formed group B. *Results:* Within group B, some samples showed a remarkable shortening of hepatocyte telomeres in younger patients, whereas some group A patients showed almost normal telomere lengths until their seventies. Among clinicopathological factors, body mass index (BMI), homeostasis model assessment insulin resistance (HOMA-IR), histological degree of steatosis and intensity of 8-hydroxy-2′-deoxyguanosine (8-OHdG) immunostaining were all significantly higher in group B than in group A. Ki-67 immunohistochemistry demonstrated that group B liver tissues were significantly less proliferative than those from group A, despite no significant difference in the necroinflammatory activities of group A and B samples. In group B patients, the ratios of Ki-67 positive index to alanine aminotransferase value were significantly lower than group A. *Conclusions:* Greater insulin resistance can result in more severe hepatic steatosis among group B patients, leading to an overproduction of reactive oxygen species, which may accelerate telomere erosion. Furthermore the regenerative response of hepatocytes with prominent telomere shortening may be impaired, making these cells vulnerable to the effect of a 'second-hit' insult.

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Key words: nonalcoholic fatty liver disease – quantitative fluorescence *in situ* hybridization – replicative senescence – telomere

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Nonalcoholic fatty liver disease (NAFLD) represents a spectrum of clinicopathologic conditions, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), with varying risks for progression to cirrhosis. A study by Matteoni et al. (1) revealed that 25% of those initially diagnosed with histologic evidence of hepatocellular necrosis (with or without fibrosis) progressed to cirrhosis in less than 20 years. Fassio and colleagues documented that progression of fibrosis was found by the second liver biopsy from seven out of 22 NASH patients, 4.3 years after the first biopsy (2). One prospective study showed that nine out of 23 patients with NASH-associated cirrhosis died of liver-related causes in less than 10 years (3). To date, limited information on the natural history of NAFLD has hampered discovery of the risk factors associated with poor prognosis of this disease.

Telomeric regions present at the ends of chromosomes consist of hexameric DNA repeat sequences $(TTAGGG)_n$ in association with telomere-binding

Abbreviations: NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; BMI, body mass index; HOMA-IR, homeostasis model assessment insulin resistance; ROS, reactive oxygen species; Q-FISH, quantitative fluorescence *in situ* hybridization; ALT, alanine aminotransferase.

proteins (4). Telomeric repeat sequences effectively 'cap' the ends of linear chromosomes, thus preventing fusion between chromosome ends. Telomere repeat sequences are subject to shortening with each cell division because they cannot be replicated completely during normal DNA synthesis (5). Normally, telomere shortening places an upper limit on the number of divisions undergone by a somatic cell, since critically short telomeres function as targets for cell-cycle checkpoint systems that induce apoptosis or an irreversible cell-cycle arrest, termed replicative senescence (6, 7).

During the process of chronic liver cell death and regeneration, hepatocyte-specific telomere shortening and replicative senescence are linked to progressive fibrosis and the development of cirrhosis (8–10), and it is argued that process can account for the development of cirrhosis due to hepatitis viruses, autoimmune diseases and alcohol in an age-independent manner (10). For NAFLD, neither the degree of telomere shortening nor the association between telomere shortening and hepatic fibrosis has been studied.

In addition to the shortening of telomeres during cell division, recent reports demonstrate that telomeres also suffer damage by reactive oxygen species (ROS) (11–13), since oxidative damage is not repaired as efficiently in telomeric DNA as elsewhere in the chromosome. Since severe hepatic steatosis can lead to increases in intracellular ROS, it may accelerate telomere loss. The relationship between these processes in NAFLD remains to be determined.

In this study, we assessed the progression of hepatocellular telomere shortening in NAFLD by quantitative fluorescence *in situ* hybridization (Q-FISH) using a telomere-specific probe (14–16). This method differs from conventional Southern blot analysis in that the lengths of telomeres can be measured specifically in hepatocytes from the biopsy specimens used for analysis. We evaluated telomere shortening with respect to hepatic regenerative indices, as well as to oxidative stress measures in patients with NAFLD vs age-matched controls. Our findings contribute to an understanding of the biological significance of telomere shortening in NAFLD, especially its effect on replicative response to cell injury.

Materials and methods

Tissue preparation

The patients diagnosed as NAFLD in 2003–2004 were selected from the files of Department of Surgical Pathology of Kyoto Prefectural University of Medicine. Each liver biopsy specimen was read and diagnosed blindly by two hepatologists (T.N. and T.O.), according to the histopathologic criteria summarized by attendees of the AASLD Single Topic Conference 2002 (17). Patients with a history of alcoholism (consumed more than 20 g/day), showing evidence of hepatitis B or C infection, taking known hepatotoxic drugs, or demonstrating symptoms of another specific liver disease were excluded from the study.

A total of 44 paraffin-embedded liver tissue biopsies were selected. Eleven histologically normal liver tissues obtained by partial hepatectomy for metastatic liver tumors from the patients without NAFLD, and negative for hepatitis B surface antigen and anti-hepatitis C antibody, were selected as controls. Informed consent to our using these tissues for this study was obtained from all patients in a written form.

Of four sections (5 μm each) cut serially from each paraffin block, one each was used for hematoxylin and eosin (HE) staining, Masson trichrome staining, immunohistochemical staining for Ki-67 antigen or Q-FISH for the telomeric region. The degree of steatosis was assessed by percent fat in each tissue section. The degree of fibrosis was graded as stage 1, zone 3 perisinusoidal fibrosis; stage 2, as above with portal fibrosis, stage 3, as above with bridging fibrosis, or stage 4, cirrhosis, based on the standards proposed by Brunt et al. (18). The necroinflammatory activity was graded as none or mild (grade 1), moderate (grade 2), or severe (grade 3), based on the modified standards also proposed by Brunt et al. (18). Activity was graded 1 in 18 cases, graded 2 in 21 cases, and graded 3 in 5 cases; fibrosis stage was F0 in 14 cases, F1 in 15 cases, F2 in 7 cases, F3 in 6 cases and F4 in 2 cases.

The clinicopathological variables analyzed were body mass index (BMI), homeostasis model assessment insulin resistance (HOMA-IR), serum values of alanine aminotransferase (ALT) and ferritin at the time of liver biopsy. (HOMA-IR = fasting immunoreactive insulin (μU/ml) × fasting blood glucose (mg/dl)/405). High HOMA-IR values indicate the presence of insulin resistance, and the upper limit of HOMA-IR is defined to be 2.5 (19, 20). Values for serum ferritin in 8 cases, and HOMA-IR in 14 cases, were not available in archived clinical charts and thus were excluded from analyses of these samples.

Ki-67 immunohistochemistry

Proliferative activity was scored by the frequency of immunohistochemical detection of the Ki-67 antigen. A paraffin section was dewaxed, dehy-

drated, and then immersed in tap water. Endogenous peroxidase was inactivated by incubating the sections in 0.3% H_2O_2. Antigen retrieval was performed by autoclaving the sections at 121 °C for 10 min in Target Retrieval Solution (catalog no. S1700; DakoCytomation, Kyoto, Japan). Nonspecific reactions were blocked by incubating the sections in Tris-buffered saline (TBS) containing 2% fetal bovine serum. The section was incubated overnight at 4 °C following application of one drop of mouse anti-Ki-67 antigen monoclonal primary antibody (MIB-1; DakoCytomation, Carpinteria, CA). After three 5-min washes in TBS, they were incubated with biotin-conjugated anti-mouse immunoglobulins (DakoCytomation, Kyoto, Japan) for 60 min at room temperature. After three 5-min washes in TBS, they were incubated with horseradish peroxidase (HRP)-conjugated streptavidin (DakoCytomation, Kyoto, Japan) for 60 min at room temperature. After three 5-min washes in TBS, immunospecific reactivity was visualized by peroxidase oxidation of diaminobenzidine substrate (DAB; Wako, Osaka, Japan). The section was counterstained with hematoxylin. Negative control slides without the primary antibody were included for each staining. The Ki-67-positive index (Ki-67-PI, the percent cells immunoreactive to anti-Ki-67 antibody) was calculated from a minimum of 1000 scored hepatocytes.

8-Hydroxy-2′-deoxyguanosine (8-OHdG) immunohistochemistry

Hepatic expression of 8-OHdG, a reliable marker of oxidative DNA damage (21), was immunohistochemically investigated in NAFLD following the procedure of Kato et al. (22). After visualizing sites of immunoreactivity with DAB, each section was counterstained using Meyer's hematoxylin. Three grades of staining intensity were defined as follows: Grade 1; most of the hepatocytes were 8-OHdG-negative and only stained by hematoxylin (Fig. 1a), grade 2; most of the hepatocytes were 8-OHdG-positive but faintly stained (Fig. 1b), and grade 3; most of the hepatocytes were 8-OHdG-positive and strongly stained (Fig. 1c).

Q-FISH for telomere length

The combined length of telomeres within single cells was determined by the intensity of fluorescence detected in telomere FISH of each paraffin section, measured according to the procedure of Meeker et al. (14). Paraffin sections were deparaffinized with xylene, hydrated through a graded ethanol series, and then placed in deionized water (DW). Slides were then autoclaved at 121 °C for 20 min in Target Retrieval Solution (catalog no. S1700; DakoCytomation, Kyoto, Japan). After cooling to room temperature for 20 min, the slides were washed in DW for 1 min three times, and immersed in 0.3% H_2O_2/absolute methanol at room temperature for 20 min to block endogenous peroxidase. Slides were rinsed in DW twice for 3 min, in 70% ethanol for 1 min, in 95% ethanol for 1 min, in 100% ethanol for 1 min, and then air dried. Ten microliters of a fluorescent isothiocyanate (FITC)-labelled telomere-specific peptide nucleic acid (PNA) probe (vial 2 in K 5325, DakoCytomation, Copenhagen, Denmark) was applied to each sample, which was then covered with an 18 × 18 mm coverslip, and denatured on a heat block at 90 °C for 5 min. Slides were then incubated in a dark, moist chamber at 45 °C overnight. Coverslips were then carefully removed in Tris buffered saline with 0.1% Tween 20 (TBST) (item no. 003178 in K0618, DakoCytomation, Kyoto, Japan) and the slides were washed in Wash Solution (vial 4 in K 5325) at a dilution of 1:50 at 52 °C for 20 min, followed by five 3-min washes in TBST. The slides were incubated in anti-FITC-HRP (item no. 004404 in K0618) diluted 1:100 in anti-FITC-HRP Diluent (item no. 004407 in K0618) at room temperature for 30 min, followed by five 3-min washes in TBST. Seventy microliters of Fluorescyl Tyramide (item no. 004409 in K0618) were applied to each slide at room temperature for 15 min. The slides were then

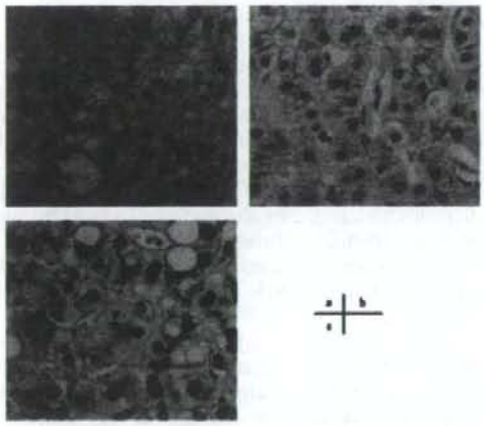

Fig. 1. Representative photographs of 8-hydroxy-2′-deoxyguanosine (8-OHdG) immunostaining. After immunoreactivity was visualized by diaminobenzidine, each section was counterstained by Meyer's hematoxylin. The staining intensity was classified into three grades (a) grade 1; most of the hepatocytes were 8-OHdG-negative and only stained by hematoxylin, (b) grade 2; most of the hepatocytes were 8-OHdG-positive but faintly stained, and (c) grade 3; most of the hepatocytes were 8-OHdG-positive and strongly stained.

immersed in TBST for five 3-min incubations, counterstained with 4′-6-diamidino-2-phenylindole (DAPI) (1000 ng/ml, VYS-32-804830, Vysis, Downers Grove, IL) and finally coverslipped for image analysis.

Fluorescent microscopy and image analysis of telomeres

Following the protocol of Meeker et al. (14), image-processed telomeric signals were quantified from digitized fluorescence microscopic images using the image analysis software package IP Labs (version 3.54, Scanalytics, Fairfax, VA). Lymphocytes, which maintain relatively stable telomere length, especially in adults 40 years and older, were used as measures for the normal-length telomere fluorescence signal intensity within each tissue sample. Telomere erosion of lymphocytes is reported to be only 1.68 kb from age 40 to 80 years and 2.00 kb from age 20 to 40 years (23). Therefore, we compared the intensities of telomere-specific fluorescence signals from hepatocytes and lymphocytes from groups A and B with those measured from biopsies from two age-matched populations without liver disease, age 20–40 years and 40–80 years.

As reported previously (10), three different cell populations were distinguishable by morphological features following DAPI staining of each Q-FISH section and HE staining of each serial section. Hepatocytes showed round nuclei and a large area of cytoplasmic space. Stellate cells were recognized as elongated cells with elongated nuclei. Lymphocytes were characterized by round nuclei and very little cytoplasm. Telomeric pixel intensities of individual hepatocyte and lymphocyte nuclei were recorded. To control for different amounts of DNA in the sectioned nuclei, telomeric signal intensity was adjusted by dividing each telomere fluorescence sum for a given nucleus by the sum of the pixels of the DAPI signal within that nucleus, as reported previously (14). For each field of view, the adjusted telomeric signal intensities of each hepatocyte nucleus and lymphocyte nucleus were designated as Tel-H and Tel-L, respectively. The ratio of mean Tel-H/mean Tel-L was calculated for each field of view after assessing 15–20 hepatocytes and more than 10 lymphocytes. Relative telomere intensity is defined as the mean of the Tel-H/Tel-L ratios measured from least five different view fields per sample under various histological conditions.

Classification of NAFLD patients into group A or B required comparisons of telomere shortening in these liver biopsies with normal controls. Furthermore, both NAFLD and normal control values needed to reflect reductions in telomere length that occur normally during aging. Towards this end, the ratios of age to relative telomere intensity among control samples were used to calculate a best-fit linear regression, which indicated the decrease in relative telomere intensity relative to age among control samples. Among NAFLD study patients, those with liver biopsies at least 80% of the normal ratio of relative telomere intensity to age were classified into group A, and those showing less than 80% of the normal ratio were designated group B.

Results

Twenty-seven male patients and 17 female patients were studied, with an average age of 50 ± 15 years. Photographs of representative samples of Q-FISH and Ki-67 immunostained tissues are shown in Fig. 2. The slope of the regression line for age v–s relative telomere intensity of hepatocytes in normal individuals was calculated to be $y = -0.0157x + 1.9576$ ($R^2 = 0.9077$, $P < 0.001$, $n = 11$), where x is patient age and y is relative telomere intensity (Fig. 3a). Twenty-two of the 44 liver tissues analyzed met the criterion for classification in group B. The distributions of relative telomere intensities within groups A and B are shown in Fig. 3b and c, respectively. In both groups, relative telomere intensity and age were negatively correlated. The slopes of the regression lines for age vs relative telomere intensity in group A patients and group B patients were calculated to be $y = -0.0127x + 1.872$ ($R^2 = 0.2314$, $P < 0.05$, $n = 22$) and $y = -0.0069x + 1.0694$ ($R^2 = 0.4588$, $P < 0.01$, $n = 22$), respectively.

The average relative telomere intensity of normal control patients from 20 to 40 years old was 1.51 (Fig. 3a). In group B patients in this age range, the average relative telomere intensity was 0.90, which was 60.0% of that of normal controls (Fig. 3c). Thus, the telomere lengths of some NAFLD patients in group B were already shortened remarkably in early age. Without a longitudinal study, the relative telomere intensity of these group B patients after age 40 cannot be predicted. However, the regression slopes of group B and that of control group were significantly different ($df = 27$, $t = 3.551$, $P = 0.001$). Therefore, the rate of age-related telomere shortening within group B was significantly slower than the control group. It appears that a minimum telomere length is achieved at an early age in some group B patients. Alternatively, several group A NAFLD patients of all ages showed normal telomere length (Fig. 3b). At age 70, the average relative telomere intensities in the normal control

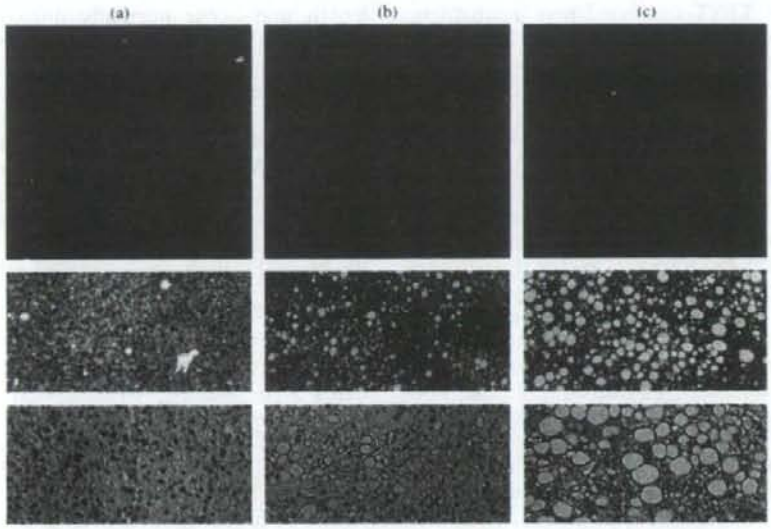

Fig. 2. Representative photographs of quantitative fluorescence *in situ* hybridization (upper), hematoxylin and eosin (HE) staining (middle) and Ki-67 immunostaining (bottom). The Tel(H)/Tel(L) calculated for one field of view from samples (a) control group, (b) group A and (c) group B were 1.633 (Tel(H) = 0.119 ± 0.027, Tel(L) = 0.073 ± 0.044), 1.850 (Tel(H) = 0.107 ± 0.029, Tel(L) = 0.058 ± 0.024) and 0.678 (Tel(H) = 0.043 ± 0.006, Tel(L) = 0.063 ± 0.024), respectively.

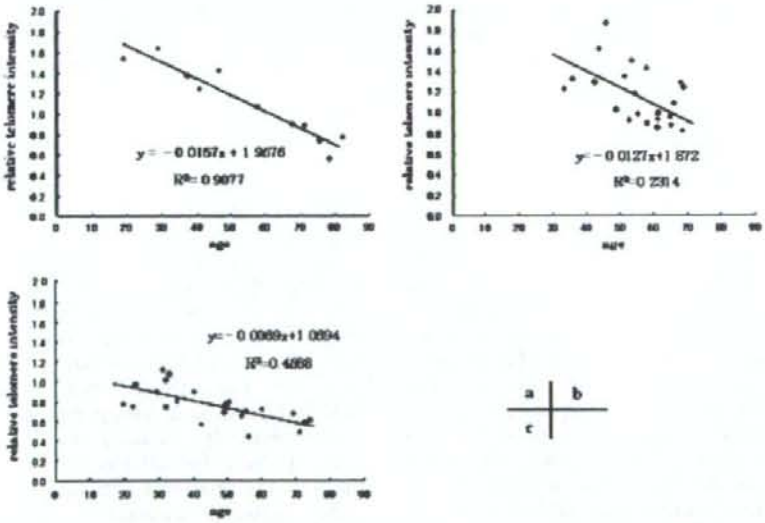

Fig. 3. Telomeric pixel intensities of individual hepatocyte and lymphocyte nuclei were recorded in samples from normal individuals, group A and group B patients. For each field, the value derived from the adjusted telomeric signal intensities of each hepatocyte nucleus or each lymphocyte nucleus was designated Tel-H or Tel-L, respectively. The ratio of mean Tel-H/mean Tel-L was calculated for each field after assessing 15–20 hepatocytes and more than 10 lymphocytes. Relative telomere intensity was determined by the average value of mean Tel-H/mean Tel-L following analysis of at least 5 different fields per sample. (a) The slope of the linear regression for age vs relative telomere intensity in normal individuals was calculated to be $y = -0.0157x+1.9576$, where x was patient's age and y was the relative telomere intensity. Those with liver biopsies at least 80% of the normal ratio of relative telomere intensity to age were classified into group A, and those showing less than 80% of the normal ratio were designated group B. (b) Group A. Several group A patients of all ages showed normal telomere length. The slope of the linear regression for age vs relative telomere intensity was calculated to be $y = -0.0127x+1.872$. (c) Group B. The telomere lengths of some patients in group B were already shortened remarkably in early age. The slope of the linear regression for age vs relative telomere intensity was calculated to be $y = -0.0069x+1.0694$.