Table 5
Comparison of distribution of background factors between patients who developed HCC and those who did not in the lamivudine group (matched case-controlled study)

Parameter	Patients with HCC ($n=4$)	Patients without HCC ($n=373$)	p-Value
Gender[a]			
Male	3 (75.0%)	273 (73.2%)	1.000[c]
Female	1 (25.0%)	100 (26.8%)	
Age (years)[b]	55.0 ± 19.5 ($n=4$)	41.3 ± 11.9 ($n=373$)	0.024
Follow-up period (years)[b]	1.5 ± 0.6 ($n=4$)	2.7 ± 2.1 ($n=373$)	0.236
Family clustering of hepatitis B[a]			
Yes	2 (50.0%)	236 (63.3%)	0.628[c]
No	2 (50.0%)	137 (36.7%)	
Drinking during the course of the study (>ethanol 80 g/day)[a]			
Yes	1 (25.0%)	37 (9.9%)	0.393[c]
No	3 (75.0%)	330 (88.5%)	
Unknown	0 (0.0%)	6 (1.6%)	
IFN therapy[a]			
Yes	0 (0.0%)	129 (34.6%)	0.387[c]
No	4 (100.0%)	232 (62.2%)	
Unknown	0 (0.0%)	12 (3.2%)	
Liver histology			
Grade of inflammation[a]			
A0	0 (0.0%)	6 (1.6%)	0.458[c]
A1	0 (0.0%)	110 (29.5%)	
A2	3 (75.0%)	154 (41.3%)	
A3	1 (25.0%)	97 (26.0%)	
Unknown	0 (0.0%)	6 (1.6%)	
Stage of fibrosis[a]			
F0	0 (0.0%)	7 (1.9%)	0.918[c]
F1	1 (25.0%)	102 (27.3%)	
F2	1 (25.0%)	94 (25.2%)	
F3	2 (50.0%)	105 (28.2%)	
F4	0 (0.0%)	65 (17.4%)	
HBeAg[a]			
+	3 (75.0%)	190 (50.9%)	0.648[c]
–	1 (25.0%)	177 (47.5%)	
Unknown	0 (0.0%)	6 (1.6%)	
HBeAb[a]			
+	2 (50.0%)	124 (33.2%)	0.632[c]
–	2 (50.0%)	243 (65.1%)	
Unknown	0 (0.0%)	6 (1.6%)	
Albumin (g/dL)[b]	4.23 ± 0.45 ($n=4$)	4.00 ± 0.51 ($n=373$)	0.384
AST (IU/L)[b]	47.0 ± 22.8 ($n=4$)	119.4 ± 156.2 ($n=326$)	0.356
ALT (IU/L)[b]	46.3 ± 24.2 ($n=4$)	193.2 ± 235.5 ($n=372$)	0.213
Platelet count ($\times 1000/mm^3$)[b]	141.0 ± 27.0 ($n=4$)	161.9 ± 52.9 ($n=373$)	0.431

[a] Data are expressed as positive numbers (%).
[b] Data are expressed as means ± S.D.
[c] Fisher's exact test.

and play an important role in hepatic carcinogenesis [28,29]. It is known that the repeat of necrosis and regeneration of liver might accelerate the mutation of oncogenes. In addition, de novo carcinogenesis is thought to be promoted in hepatitis B patients as a result of the increased genetic instability caused by the integration of the HBV genome into the host's chromosomes. When administered to patients with hepatitis B, lamivudine decreases the blood HBV-DNA concentration and markedly improves ALT levels, with consequent improvement of liver histological findings [7,11,13,14]. An early in vitro study showed that lamivudine decreases the amount of free HBV-DNA in hepatocytes but does not affect integrated HBV genes [30]. Therefore, lamivudine is thought to inhibit HCC by abating hepatitis and not by inhibiting viral gene integration. In fact, as shown in the matched case control study, all four patients who developed HCC in the lamivudine group had non-cirrhotic liver disease, whereas 23 (46%) of 50 patients who developed HCC had liver cirrhosis. Due to the small number of patients included, however, further studies are necessary to confirm this finding.

Table 6
Comparison of distribution of background factors between patients who developed HCC and those who did not in the control group (matched case-controlled study)

Parameter	Patients with HCC ($n=50$)	Patients without HCC ($n=327$)	p-Value
Gender[a]			
Male	40 (80.0%)	233 (71.3%)	0.236[c]
Female	10 (20.0%)	94 (28.7%)	
Age (years)[b]	50.6 ± 10.1	40.0 ± 11.9	<0.001
Follow-up period (years)[b]	5.3 ± 4.3	5.2 ± 4.8	0.951
Family clustering of hepatitis B[a]			
Yes	29 (58.0%)	213 (65.1%)	0.345[c]
No	21 (42.0%)	114 (34.9%)	
Drinking during the course of the study (>ethanol 80 g/day)[a]			
Yes	14 (28.0%)	48 (14.7%)	0.050[c]
No	36 (72.0%)	278 (85.0%)	
Unknown	0 (0.0%)	1 (0.3%)	
IFN therapy[a]			
Yes	16 (32.0%)	127 (38.8%)	0.578[c]
No	34 (68.0%)	197 (60.2%)	
Unknown	0 (0.0%)	3 (0.9%)	
Liver histology			
Grade of inflammation[a]			
A0	2 (4.0%)	16 (4.9%)	0.026[c]
A1	6 (12.0%)	95 (29.1%)	
A2	27 (54.0%)	159 (48.6%)	
A3	15 (30.0%)	57 (17.4%)	
Stage of fibrosis[a]			
F0	0 (0.0%)	6 (1.8%)	<0.001[c]
F1	7 (14.0%)	110 (33.6%)	
F2	8 (16.0%)	89 (27.2%)	
F3	12 (24.0%)	78 (23.9%)	
F4	23 (46.0%)	44 (13.5%)	
HBeAg[a]			
+	26 (52.0%)	194 (59.3%)	0.564[c]
–	22 (44.0%)	119 (36.4%)	
Unknown	2 (4.0%)	14 (4.3%)	
HBeAb[a]			
+	20 (40.0%)	101 (30.9%)	0.319[c]
–	27 (54.0%)	210 (64.2%)	
Unknown	3 (6.0%)	16 (4.9%)	
Albumin (g/dL)[b]	3.63 ± 0.59	4.06 ± 0.49	<0.001
AST (IU/L)[b]	96.9 ± 100.8	95.3 ± 130.0	0.934
ALT (IU/L)[b]	132.8 ± 165.5	154.4 ± 182.7	0.431
Platelet count ($\times 1000/mm^3$)[b]	126.8 ± 50.7	170.0 ± 58.7	<0.001

[a] Data are expressed as positive numbers (%).
[b] Data are expressed as means ± S.D.
[c] Fisher's exact test.

Seven HBV genotypes (A–G) have been identified to date, and their distribution shows regional variations [31–36]. In Japan, genotypes C, B, and the other five account for 85, 12, and 3% of hepatitis B patients [36]. The virological differences between HBV genotype B and genotype C might influence not only on the natural course of hepatitis B but also the efficacy by lamivudine. The patients with HBV genotype B are frequently negative for HBeAg, have lower ALT levels and a better prognosis. In contrast, the patients with HBV genotype C tend to remain HBeAg-positive for a longer duration and tend to have elevated ALT levels and more advanced liver disease, such as liver cirrhosis and HCC. This indicates that the analysis of HBV genotypes will be needed in this study.

In conclusion, our multicenter, retrospective, matched case study indicated that lamivudine treatment might suppress the risk of HCC in patients with chronic hepatitis B. However, the study has several limitations, such as the relatively short duration of treatment and the lack of virological analyses (HBV genotype, YMDD mutation, and HBV-DNA volume). To relief these limitations, further long-term observation should be continued to clarify the conclusion.

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Appendix A

The Inuyama Hepatitis Study Group consists of the following 30 institutions and members: Dr. Sumio Watanabe (Akita University School of Medicine, Akita, Yamagata), Dr. Sumio Kawada (Yamagata University School of Medicine, Yamagata), Dr. Osamu Yokosuka (Chiba University, Graduate School of Medicine, Chiba), Dr. Kunihiko Hino (Delta Clinic, Tokorozawa), Dr. Hiromasa Ishii (Keio University, School of Medicine, Tokyo), Dr. Hiromitu Kumada (Toranomon Hospital, Tokyo), Dr. Gotaro Toda (Jikei University School of Medicine, Tokyo), Dr. Yasuyuki Arakawa (Nihon University School of Medicine, Tokyo), Dr. Nobuyuki Enomoto (Yamanashi University, School of Medicine, Kofu), Dr. Kendo Kiyosawa (Shinshu University School of Medicine, Matsumoto), Dr. Takafumi Ichida (Niigata University, Graduate School of Medical and Dental Science, Niigata), Dr. Tomoteru Kamimura (Niigata Saiseikai Hospital Dai-2, Niigata), Dr. Masashi Mizogami (Nagoya City University Graduate School of Medical Science, Nagoya), Dr. Shinichi Kakumu (Aichi Medical University, Nagoya), Dr. Hisataka Moriwaki (Gifu University School of Medicine, Gifu), Dr. Shuichi Kaneko (Kanazawa University, Graduate School of Medical Science, Kanazawa), Dr. Takeshi Okanoue (Kyoto Prefectural University, Graduate School of Medical Science, Kyoto), Dr. Norio Hayashi (Osaka University Graduate School of Medicine, Osaka), Dr. Masatoshi Kudo (Kinki University School of Medicine, Sayama), Dr. Yasushi Shiratori (Okayama University, Graduate School of Medicine and Dentist[r]y, Okayama), Dr. Gotaro Yamada (Kawasaki Hospital, Kawasaki Medical School, Okayama), Dr. Kazuaki Chayama (Hiroshima University, Graduate School of Biomedical Science, Hiroshima), Dr. Kiwamu Okita (Yamaguchi University, School of Medicine, Ube), Dr. Shigeki Kuriyama (Kagawa Medical University, Takamatsu), Dr. Morikazu Onji (Ehime University School of Medicine, Juushin-cho), Dr. Saburo Ohnishi (Kochi University School of Medicine, Nangoku), Dr. Michio Sata (Kurume University School of Medicine, Kurume), Dr. Shigetoshi Fujiyama, and Dr. Hiroshi Sasaki (Kumamoto University, Faculty of Medical and Pharmaceutical Science, Kumamoto), Dr. Hirohito Tsubouchi (Miyazaki University School of Medicine, Miyazaki), and Dr. Hiromi Ishibashi and Dr. Hiroshi Yatsuhashi (Nagasaki Medical Center, Omura).

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Age-specific antibody to hepatitis E virus has remained constant during the past 20 years in Japan

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SUMMARY. We investigated the presence of antibodies to hepatitis E virus (anti-HEV) and hepatitis A virus (anti-HAV) by enzyme immunoassays in sera from 1015 individuals collected in 1974, 1984 and 1994. Age-specific profiles of anti-HEV remained unchanged with a peak at 40–49 years, while those of anti-HAV started to increase in individuals aged 20–29 years in 1974, 30–39 years in 1984 and 40–49 years in 1994. These results suggest that a silent HEV infection has been taking place in the last 20 years or so in Japan, while HAV infection has been terminated at least since 1974.

Keywords: hepatitis A virus, hepatitis A virus antibody, hepatitis E virus, hepatitis E virus antibody, seroepidemiology.

INTRODUCTION

Hepatitis E virus (HEV) is transmitted mainly by the faecal–oral route, and causes waterborne outbreaks and sporadic cases of acute hepatitis in developing countries with poor sanitary conditions [1]. Outbreaks of HEV have been primarily noted in developing countries, whereas cases in developed countries were considered to have been exposed in foreign countries. However, cases of acute hepatitis because of indigenous HEV strains were reported in patients in the United States, Europe and Japan who had never travelled abroad [2–4]. Recently, HEV strains have been isolated from pigs in developed countries, which are closely related to local human HEV strains, suggesting zoonotic infection [5–7]. Because of these lines of evidence, HEV has attracted increasing attention even in developed countries where HEV is not endemic. For example, in Japan, more than 20% of acute, sporadic hepatitis cases are non-A, non-B, non-C [8].

The impact of HEV infection in developed countries, however, has not been fully explored. Past exposure to HEV can be examined by enzyme-linked immunosorbent assay (ELISA) by detecting antibody to HEV (anti-HEV). In the present study, we surveyed the extent and changes of HEV infection in Japan by testing sex- and age-specific prevalence of anti-HEV in serum samples collected in 1974, 1984 and 1994. Using the same samples, we also tested for antibodies to hepatitis A virus (anti-HAV) for comparison.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HAV, hepatitis A virus; HEV, hepatitis E virus; VLP, virus-like particles.

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MATERIALS AND METHODS

Serum samples

A total of 1015 samples were selected at random from the Serum Reference Bank of the National Institute of Infectious Diseases, Tokyo. They were obtained from healthy volunteers aged from 0 to 89 years (median 35.6 years) living in seven prefectures in the central part of Japan. Of them, 349 were collected in 1974, 324 in 1984 and 342 in 1994. The present study, was reviewed by the ethical committee of the National Institute of Infectious Diseases.

Hepatitis viral markers

Anti-HAV (total antibody) was determined by radioimmunoassay using a commercial kit (HAV-AB RIA kit; Dainabot Co., Ltd, Tokyo, Japan). Positive and negative results were judged according to the manufacturer's instructions with intermediate results recorded as negative. Anti-HEV was determined by ELISA using the method of Li *et al.* [9]. Briefly, wells of microtitre plates were coated with purified virus-like particles (VLP) of HEV expressed by a recombinant baculovirus. One hundred microlitres of test serum was then added to each well and the plate was incubated at 37 °C for 1 h. The plate was washed six times with 10 mM phosphate

buffered saline containing 0.05% Tween 20, and then 100 μL of buffer containing goat anti-human IgG conjugated with horseradish peroxidase was delivered to each well. The plate was incubated for a further 1-h period at 37 °C, washed six times, and thereafter, each well received 100 μL of buffer containing orthophenylenediamine. The plate was incubated at room temperature for 30 min, and then 50 μL of 4 N H_2SO_4 are added to each well. The absorbance at 492 nm was recorded and positive and negative results were scored as described by Li *et al.* [9].

Statistical analyses

Statistical analyses were performed using the chi-square test, and $P < 0.05$ was considered significant.

RESULTS

Age-specific prevalence of anti-HEV and anti-HAV

Basic patterns of age-specific prevalence of anti-HEV were similar in the three examination years (Fig. 1). The prevalence of anti-HEV increased gradually until 40–49 years old, and then decreased slowly with age. In contrast, the prevalence of anti-HAV was almost nil in people younger than 20 years, increased steeply at a certain age range, and then reached 80–90% in older people in all of the three years of examination. The age range at which the prevalence of anti-HAV started to increase sharply was 20–29 years in 1974, 30–39 years in 1984 and 40–49 years in 1994. Thus, it shifted by 10 years at each examination year. Anti-HAV was significantly more prevalent than anti-HEV in all age ranges over 30 years in 1974 ($P < 0.001$ in all). Similarly, anti-HAV was significantly more prevalent in all age ranges over 40 years in 1984 ($P < 0.001$ in all), and in all age ranges over 50 years in 1994 ($P < 0.001$ in all).

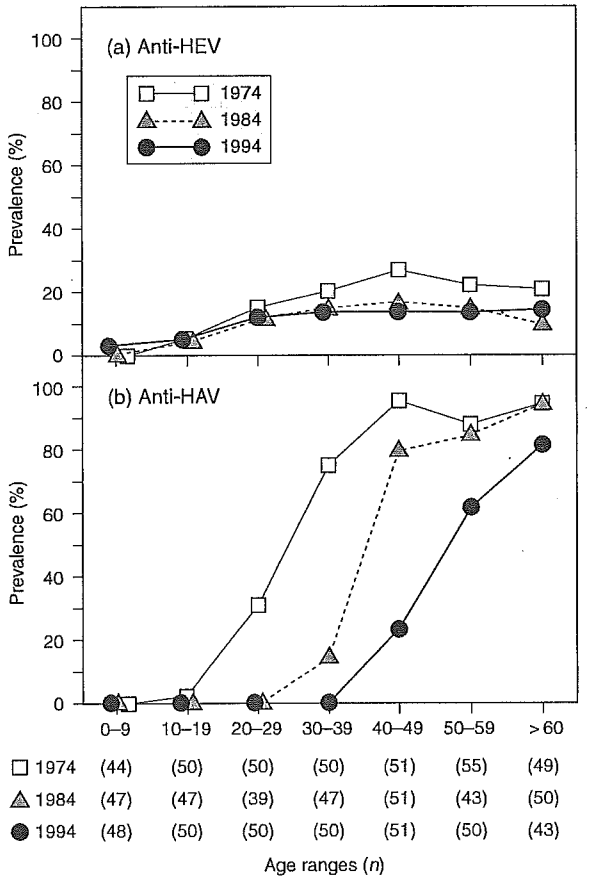

Fig. 1 Age-specific prevalence of anti-HEV and anti-HAV in Japan at three different times. Number of individuals tested in each age group and year is indicated below in parentheses.

Sex- and age-specific prevalence of anti-HEV

Figure 2 illustrates the prevalence of anti-HEV in serum samples from different age groups of healthy Japanese volunteers collected in 1974, 1984 and 1994 stratified by

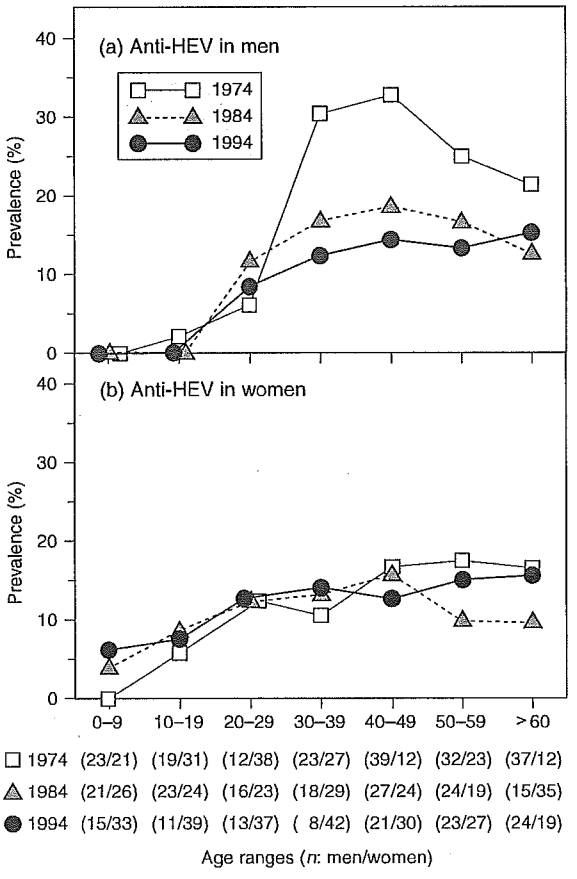

Fig. 2 Sex and age-specific prevalence of anti-HEV in Japan at three different times. Numbers of men/women tested in each age group and year are indicated below in parentheses.

sex and age. Although basic profiles of the prevalence of anti-HEV did not differ between men and women, anti-HEV in men was significantly more frequent in 1974 (21.6%) than in 1984 (11.1%, $P = 0.012$) and 1994 (10.4%, $P = 0.013$); the difference is attributed to a high frequency of anti-HEV in age groups older than 30 years in 1974. In sharp contrast, the age-specific prevalence of anti-HEV in women stayed unchanged in the three different years examined (11.0% in 1974, 10.6% in 1984 and 11.9% in 1994). Differences in the prevalence of anti-HEV between men and women were significant only in the year 1974 ($P = 0.008$). Age-specific prevalence of anti-HAV was quite similar between males and females in each year of examination (data not shown).

DISCUSSION

Many immunological methods have been developed for the determination of anti-HEV utilizing natural and recombinant viral proteins as antigens. They are, however, disappointingly nonspecific and have been shown to yield discrepant results using the same panel of sera with or without anti-HEV [10]. Lack of reliable serological assays for the detection of anti-HEV has hampered the accurate examination of exposure to HEV in various epidemiological and clinical settings.

Recently, Li *et al.* [11] succeeded in developing VLP using a recombinant baculovirus containing the coding sequences for capsid protein of the virus. The VLP appear to have similar antigenicity to the authentic HEV particles [12]. Using these VLP, a novel ELISA for anti-HEV has been developed that is sensitive and specific in seroepidemiological surveys for HEV infection. A considerable proportion of Japanese adults (around 10–20%) appear to have had previous exposure to HEV, although Japan is not endemic for hepatitis E [9,13].

The clinical features of HAV infection are similar to those of HEV infection, in that they both are transmitted by the faecal–oral route and cause acute hepatitis without chronic sequelae. In the present study, serological markers of HAV and HEV infection were determined and compared among healthy Japanese volunteers at three different time points (1974, 1984 and 1994). Age-specific prevalence of anti-HAV increased steeply and reached 90% at a certain age range dependent on the year of examination. The age range at which anti-HAV increased shifted by 10 years in subsequent time points, indicating that HAV infection was endemic several decades ago in Japan and has been contained thereafter. This would be mainly because of an improvement of sanitary conditions in Japan since the 1950s.

Age-specific profiles distinct between anti-HAV and anti-HEV during the last 20 years in Japan would be not only of epidemiological but also of clinical relevance. The prevalence of anti-HAV increasing with age involving by far the most aged individuals signifies a life-long immunoprotection against HAV. By sharp contrast, the prevalence of anti-HEV did not increase linearly with age, and peaked in individuals aged 40–49 years. Furthermore, unlike anti-HAV that has become increasingly absent in younger age groups, anti-HEV was detected in younger individuals aged 20–29 years in both men and women, and among women aged <20 years, in the three examination years. Similar age-specific profiles of anti-HEV have been reported in India [14]. Prevalence of anti-HEV in Japanese individuals younger than 30 years old was somewhat higher in the present study, than the almost zero prevalence reported by Li *et al.* [9]. Although there was some difference in percentages, the basic pattern of age-specific prevalence of anti-HEV was almost similar between the two studies.

Based on the age-specific distribution of anti-HEV, the exposure to HEV has not decreased during the past 20 years in Japan, unlike that to HAV. This implies that the principal transmission route of HEV may be different from that of HAV and would not be prevented only by improved sanitary conditions, despite the lower infectivity and transmissibility of HEV than HAV [14]. Zoonotic transmission of HEV through domestic and wild animals may account for the perpetuation of HEV infection, and deserves to be examined in future studies. Recently, transmission of HEV from pigs and deer to human beings has been reported in Japan [15,16].

In conclusion, exposure to HEV has stayed unchanged during the last 20 years in Japan, contrasting with HAV, which has diminished over the same period. These results warrant closer attention to infection with HEV, especially because it can induce fulminant hepatitis not only in pregnant women in developing countries [14], but also in sporadic cases in developed countries [15].

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Genetic analysis of the HLA region of Japanese patients with type 1 autoimmune hepatitis

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***Background/Aims*: Genetic predisposition to type 1 autoimmune hepatitis (AIH) is linked mainly to HLA-class II genes. The aim of this study is to scan the HLA region for additional genes which may contribute to type 1 AIH susceptibility.**

***Methods*: We performed association analysis using HLA class I and II alleles and 18 polymorphic microsatellite markers distributed throughout the HLA region. We specifically assessed tumor necrosis factor (TNF)-α gene polymorphisms.**

***Results*: The frequencies of HLA-DRB1*0405, DRB4 and DQB1*0401 alleles were significantly higher in AIH patients. The association study revealed the presence of three segments in the HLA region showing significantly low *P* (Pc) values. The first segment was located around the HLA-DR/-DQ subregion, the second was around the HLA-B54 allele, and the third was around two microsatellites near the TNF gene cluster. However, stratification analysis for the effect of DRB1*0405 eliminated association of the latter two segments. Haplotype D of the TNF-α promoter gene polymorphisms was weakly associated with susceptibility, but was found to be not significant after stratification analysis.**

***Conclusions*: The most influential gene on type 1 AIH pathogenesis in Japanese is the HLA-DRB1. Other genes in the HLA region, including TNF-α, have little or no association with type 1 AIH susceptibility.**

***Keywords*: Autoimmune hepatitis; Gene polymorphisms; Genetic susceptibility; HLA class II antigens; Microsatellite markers; Single nucleotide polymorphism; Tumor necrosis factor α**

1. Introduction

Autoimmune Hepatitis (AIH) is an organ-specific autoimmune disease characterized by chronic inflammation of the liver, hypergammaglobulinemia and autoantibodies [1–3]. Several studies from ethnically different countries have clarified strong genetic bases for both disease susceptibility and behavior [4–14]. Recently, molecular analysis by the use of PCR-based DNA typing techniques have shown that the susceptibility to develop type 1 AIH is associated specifically with the DRB1*0301 and DRB1*0401 alleles in Caucasians [6–10], the DRB1*0405 allele in Japanese [11] and Argentine adults [12], DRB1*0404 in Mexicans [13], and DRB1*1301 in Argentine children [12] and Brazilians [14] at the HLA class II DRB1 locus, which encodes a polymorphic β chain of the HLA-DR antigen.

However, the association with these DRB1 antigens and susceptibility to type 1 AIH is not complete because not all AIH patients possess these antigens. This suggests that additional susceptibility genes (either HLA or non-HLA) and/or environmental factors may contribute to the development of type 1 AIH, such that finding candidate genes has been compared to searching for a needle in a haystack [15]. Historically, candidate genes were searched for on

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an individual level. The current study searched for them comprehensively throughout the HLA region on the short arm of human chromosome 6.

HLA, which encompasses 3.6 Mb, is divided into three regions, class II (1.1 Mb), class III (0.7 Mb) and class I (1.8 Mb), from centromere to telomere. More than 200 genes are localized in the HLA region and many genes located at the telomeric end of the class III region are involved in immune and inflammatory responses, and possess a marked degree of polymorphism [16]. Association analysis using microsatellite markers is a powerful method for mapping candidate susceptibility genes of multifactorial genetic diseases [17,18]. If the frequencies of some microsatellite markers show significant differences between patients and controls, susceptibility genes might exist near them and be analyzed by sequencing. To search for additional genes influencing the development of type 1 AIH in the HLA region, we performed association analysis using HLA class I and class II alleles and 18 microsatellites densely distributed within or just outside the HLA region.

In addition to HLA-DR, the tumor necrosis factor α (TNF-α) gene, which lies in the HLA class III region, is another candidate susceptibility gene. Czaja et al. described that polymorphisms in the promoter region of the TNF-α gene at position −308 was associated with severity of AIH-1 in European and North-American patients in synergy with HLA-DR3 [8,9,19,20]. To our knowledge, this phenomenon in Japanese type 1 AIH patients has not yet been demonstrated. Therefore, we investigated polymorphisms in the promoter region of the TNF-α gene as well.

2. Patients and methods

2.1. Subjects

Seventy seven patients with type 1 AIH and 248 healthy Japanese controls were enrolled for investigation of genetic association with polymorphic genetic markers around the HLA region. Forty-six of these 77 patients were included in our earlier study [11]. They were all residents of Nagano Prefecture, Japan, and their racial backgrounds were all Japanese. All of the patients were diagnosed as probable or definite cases according to the scoring system from the International Autoimmune Hepatitis Group (Table 1) [21]. All patients were classified as having type 1 AIH based on antibody profiles. The average age of the AIH onset in this study was 55.8. The youngest and the oldest patients were 29 and 85 y.o., respectively (Table 1). No viral markers, such as Hepatitis B surface antigen, anti-hepatitis B core antibody, anti-hepatitis C virus antibody (second generation) or hepatitis C virus RNA were detected in the serum. This study was approved by the Ethics Committee of Shinshu University School of Medicine. Informed consent, in writing, was obtained from each subject.

Table 1
Clinical data of Japanese type 1 AIH patients

Features	AIH (n=77)
Age (year)	55.8±12.7 (29–85)
Women:men	64:13
ALT (nl: 7–45 U/L)	701.5±589.9 (40–2644)
Bilirubin (nl: 0.3–1.2 mg/dl)	5.4±7.3 (0.5–30.2)
Alkaline phosphatase (nl: 124–367 U/L)	468.5±288.8 (144–1405)
IgG (nl: 800–2000 mg/dl)	3189.9±1122.3 (1312–7248)
IgM (nl: 40–350 mg/dl)	348.2±323.5 (57–1498)

ALT, alanine aminotransferase; AIH, autoimmune hepatitis; nl, normal range.

2.2. DNA preparation

Genomic DNA from patients and controls were isolated by phenolic extraction of sodium dodecyl sulfate-lysed and proteinase K-treated cells as described previously [18,22].

2.3. HLA typing

HLA class I and II alleles were determined using Micro SSP™ DNA Typing Kit (One Lambda, Canoga Park, CA). DNA typing of DRB1 and DQB1 alleles was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis as previously described [18,22,23].

2.4. Microsatellite

To determine the number of repeat units of 18 microsatellite loci around the HLA region (Fig. 1), forward primers were synthesized by labeling at the 5′ end with the fluorescent reagents 6-FAM, HEX, or TET (PE Biosystems, Foster City, CA). PCR primers and conditions for amplifying C3-2-11, C2-4-4, C1-3-1, C1-2-5, C1-4-1, MICA-TM(GCT)n (MHC class I chain-related gene A transmembrane region) and C1-2-A were the same as described previously [24–27]. PCR primers and conditions were comparable to those of the previous papers, for D6S276 [28,29], MIB [30], TNFa [31,32], TNFd [32], D6S273 [33], DQ-CARII [34], T16CAR [34], D6S2443 [35], D6S2444 [35], TAP1 [36], and D6S439 [27]. PCR-amplified products were denatured for 5 min at 100 °C, mixed with formamide-containing stop buffer, then electrophoresed on a 4% polyacrylamide denaturing gel containing 8 M urea in a Model 377 automated DNA sequencer (PE Biosystems). Fragment sizes were determined automatically by means of GeneScan software (PE Biosystems) as described previously [18].

2.5. Genotyping of polymorphisms in the 5′-flanking region of the TNF-α gene

Five single-nucleotide polymorphisms at nucleotide positions −1031, −863, −857, −308, and −238 in the promoter region of the TNF-α gene were determined for these samples by direct sequencing as previously reported [18].

2.6. Statistical analysis

Gene and phenotype frequencies at polymorphic loci or sites including 18 microsatellites, HLA-DRB1, -DQB1 genes, and promoter haplotypes of the TNF-α gene were estimated by direct counting. The significance of the distribution of alleles between patients with AIH and normal controls was tested by the χ^2 method with continuity correction. The P value was corrected by multiplication by the number of alleles observed in each locus tested (corrected P value: Pc value). A Pc value of less than 0.05 was evaluated as statistically significant. To control for the effect of linkage disequilibrium between loci, Mantel-Haenszel weighted odds ratio (OR) was calculated [37], and again a P value of less than 0.05 was accepted as statistically significant.

3. Results

Tables 2 and 3 show the HLA-DRB1, 3, 4, 5 and DQB1 alleles in type 1 AIH patients and controls.

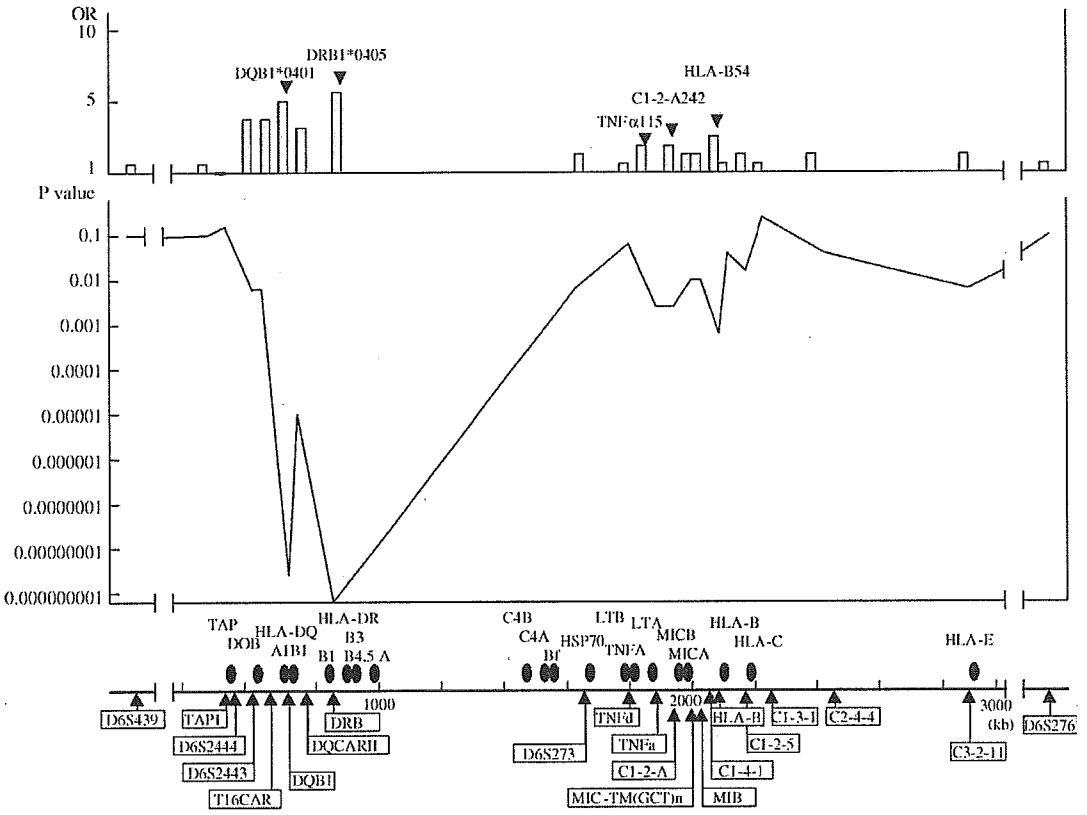

Fig. 1. AIH susceptibility gene mapping by association analysis using genetic markers in the HLA region. OR and *P* values obtained by association tests between control and patient groups are displayed with the location of genetic markers used for mapping. The gene map at the bottom indicates the location of these genetic markers in boxes, and representative genes in the HLA region are indicated by black ovals.

DRB1*0405 ($Pc < 2.9 \times 10^{-8}$; OR 4.97), DRB4 ($Pc < 8.1 \times 10^{-7}$; OR 4.92) and DQB1*0401 ($Pc < 5.9 \times 10^{-8}$; OR 4.70) alleles were markedly significant in the patients.

The association study investigating disease susceptibility to type 1 AIH using polymorphic markers revealed the presence of three segments in the HLA region showing significantly low *P* (Pc) values (Table 4 and Fig. 1). All of the alleles in each microsatellite marker were named on the basis of the amplified fragment size length. The first segment was located around the HLA-DR and -DQ subregion, with the most significant associations observed in the DRB1 gene (DRB1*0405), the DQB1 gene, (DQB1*0401) and allele 193 of the DQCARII microsatellite marker, which is located between the DRB1 and DQB1 loci ($Pc < 0.00015$, OR 3.25).

The second segment was found around the HLA-B locus. Since strong linkage disequilibrium between HLA-B54 and DRB1*0405 had earlier been noted in a Japanese population [5], the significant association of HLA-B54 with AIH was tested for the possible confounding effect of DRB1*0405 and disappeared after stratification (Table 5; $0.88 < OR < 3.36$).

Lastly, there were also significant associations of two microsatellites (TNFa and C1-2-A) giving rise to the third

Table 2
HLA-DRB1 alleles in type 1 AIH patients and controls

DR-B1*	AIH $n=77$ (%)	Control $n=248$ (%)	OR	χ^2	*P*	Pc
0101	5 (6.5)	28 (11.3)	0.55	1.48	0.22	
1501	5 (6.5)	34 (13.7)	0.44	2.90	0.089	
1502	20 (26.0)	63 (25.4)	1.03	0.01	0.92	
1602	2 (2.6)	2 (0.8)	3.28	1.55	0.21	
0401	2 (2.6)	3 (1.2)	2.18	0.75	0.39	
0403	5 (6.5)	7 (2.8)	2.39	2.23	0.14	
0405	48 (62.3)	62 (25.0)	4.97	36.6	1.5×10^{-9}	2.9×10^{-8}
0406	4 (5.2)	13 (5.2)	0.99	0.00	0.99	
0410	1 (1.3)	8 (3.2)	0.39	0.81	0.37	
1001	1 (1.3)	2 (0.8)	1.62	0.16	0.69	
1101	3 (3.9)	15 (6.0)	0.63	0.52	0.47	
1201	11 (14.3)	16 (6.5)	2.42	4.73	0.030	
1202	2 (2.6)	5 (2.0)	1.30	0.09	0.76	
1301	1 (1.3)	2 (0.8)	1.62	0.16	0.69	
1302	7 (9.1)	33 (13.3)	0.65	0.97	0.33	
1403	1 (1.3)	6 (2.4)	0.53	0.35	0.55	
1406	2 (2.6)	13 (5.2)	0.48	0.93	0.33	
0802	4 (5.2)	18 (7.3)	0.70	0.40	0.53	
0803	12 (15.6)	39 (15.7)	0.99	0.00	0.98	
0901	15 (19.5)	76 (30.6)	0.55	3.63	0.057	

OR, odds ratio; Pc, corrected *P*.

Table 3
HLA-DRB3, 4, 5 and HLA-DQB1 alleles in type 1 AIH patients and controls

DRB3, 4, 5 DQB1*	AIH $n=77$ (%)	Control $n=248$ (%)	OR	χ^2	P	Pc
DRB3	24 (31.2)	89 (35.9)	0.81	0.58	0.45	
DRB4	64 (83.1)	124 (50.0)	4.92	26.4	2.7×10^{-7}	8.1×10^{-7}
DRB5	24 (31.2)	82 (33.1)	0.92	0.10	0.76	
DQ-B1*0301	14 (18.2)	44 (17.7)	1.03	0.01	0.93	
0302	8 (10.4)	26 (10.5)	0.99	0.00	0.98	
0303	15 (19.5)	69 (27.8)	0.63	2.13	0.14	
0401	47 (61.0)	62 (25.0)	4.70	34.2	4.9×10^{-9}	5.9×10^{-8}
0402	5 (6.5)	15 (6.0)	1.08	0.02	0.89	
0501	5 (6.5)	29 (11.7)	0.52	1.70	0.19	
0502	2 (2.6)	7 (2.8)	0.92	0.01	0.92	
0503	0 (0)	11 (4.4)	0.13	3.53	0.06	
0601	17 (22.1)	77 (31.0)	0.63	2.30	0.13	
0602	1 (1.3)	28 (11.3)	0.10	7.22	0.0072	0.084
0603	1 (1.3)	1 (0.4)	3.25	0.77	0.38	
0604	4 (5.2)	29 (11.7)	0.41	2.72	0.010	

OR, odds ratio; Pc, corrected P.

lowest P (Pc) value peak near the TNF gene cluster in the class III region. Within this segment, allele 115 of the TNFa microsatellite marker gave the lowest Pc value ($\chi^2=9.09$, $Pc<0.033$). However, stratification for the effect of DRB1*0405 showed no significant association for TNFa115 and C1-2-A242 microsatellites with AIH (Table 5).

TNF-α promoter gene polymorphisms were also evaluated by the direct sequence method. Previously, five different haplotypes were assigned in terms of allelic combination at five polymorphic sites in the 5′ flanking region in a Japanese population and were named haplotypes A-E [38]. Haplotype D of the TNF-α gene polymorphisms was weakly associated with susceptibility to AIH (OR 2.12, $P=0.026$, $Pc=0.13$, Table 6). However, stratification for the effect of DRB1*0405 eliminated this possibility ($P=0.075$, Table 7).

4. Discussion

This is the first comprehensive study to search for candidate genes responsible for type 1 AIH susceptibility in the HLA region, and confirms our previous findings that the HLA-DRB1 and/or -DQB1 loci are strongly associated with susceptibility to AIH.

We showed that DRB1*0405 ($Pc<2.9\times10^{-8}$; OR 4.97), DRB4 ($Pc<8.1\times10^{-7}$; OR 4.92) and DQB1*0401 ($Pc<5.9\times10^{-8}$; OR 4.70) alleles were markedly significant in the patients. We already reported that a predisposition to type 1 AIH in Japanese was associated with the HLA-B54/ DRB1*0405/ DQB1*0401 haplotype [11]. In Japanese, DQB1*0401 is in very strong linkage disequilibrium with DRB1*0405 and only one patient with the DRB1*0405 allele did not have the DQB1*0401 allele. Therefore, it is not possible to evaluate the association of DQB1*0401 with AIH susceptibility from a statistical point of view. DRB4 is in linkage disequilibrium with DRB1*04, DRB1*0701 and DRB1*0901 and all patients with the DRB1*0405 allele have the DRB4 allele. We previously reported that the DQA1 allele was also associated with AIH, but was not as strongly associated as the serological DR4 antigen. We suggested that its association may be explained by linkage disequilibrium with DR4 because DR4 is tightly linked to DQA1*0301 [11].

In our earlier study, we proposed that the basic amino acid at position 13, which is present only on the DR2 and DR4 B1 molecules (arginine on DR2 and histidine on DR4), contributes to the susceptibility to type 1 AIH among Japanese, since all of the 6 DR4-negative patients with AIH ($n=53$) had DR2 [11]. However, our current study revealed that 10 out of the 77 AIH patients were negative for both DR4 and DR15 (DR2), and 32.5% of AIH patients and 39.1% of controls had DR15 (DRB1*1501; OR 0.44, DRB1*1502; OR 1.03). Therefore, we can no longer conclude that type 1 AIH in Japan is associated with DRB1 alleles encoding arginine or histidine at position 13.

Table 4
Statistically significant alleles associated with type 1 AIH patients

Marker	No. of alleles	Significant allele	AIH ($n=77$)%	Control ($n=248$)%	OR	χ^2	P	Pc
D6S2443	8	185	9.1	2.4	4.03	6.81	0.0091	
T16CAR	12	211	9.1	2.4	4.03	6.81	0.0091	
DQB1	12	0401	61.0	25.0	4.70	34.24	4.9×10^{-9}	5.9×10^{-8}
DQCARII	10	193	70.1	41.9	3.25	18.70	1.6×10^{-5}	1.6×10^{-4}
DRB1	20	0405	62.3	25.0	4.97	36.58	1.5×10^{-9}	2.9×10^{-8}
D6S273	7	134	63.6	46.4	2.02	7.01	0.0081	
TNFa	13	115	48.1	29.4	2.22	9.09	0.0026	0.033
C1-2-A	14	242	62.3	42.7	2.22	9.05	0.0026	0.037
MICA-TM(GCT)n	5	179	44.2	29.8	1.85	5.32	0.021	
HLA-B	21	54	33.8	14.5	3.09	14.89	0.00011	0.0023
C1-2-5	21	202	19.5	10.5	2.07	4.31	0.038	
C2-4-4	13	251	19.5	10.1	2.16	4.81	0.028	
C3-2-11	18	213	28.6	14.5	2.36	7.92	0.0049	

OR, odds ratio; Pc, corrected P.

Table 5
Association of HLA-B54 and microsatellites TNFa115 and C1-2-A242 in type 1 AIH patients after stratification for the effect of DRB1*0405

DRB1*0405	HLA-B54	Control	AIH	Weighted OR	χ^2	*P*	95% CI
Present	Negative	42	24	1.72	1.91	0.17	0.88<OR<3.36
	Positive	20	24				
Absent	Negative	171	27				
	Positive	15	2				
DRB1*0405	TNFa115	Control	AIH	Weighted OR	χ^2	*P*	95% CI
Present	Negative	32	20	1.50	1.53	0.22	0.85<OR<2.64
	Positive	30	28				
Absent	Negative	143	20				
	Positive	43	9				
DRB1*0405	C1-2-A242	Control	AIH	Weighted OR	χ^2	*P*	95% CI
Present	Negative	25	12	1.55	1.85	0.17	0.88<OR<2.73
	Positive	37	36				
Absent	Negative	117	17				
	Positive	69	12				

OR, odds ratio; 95% CI, 95% confidence interval.

Table 6
TNF-α promoter gene polymorphisms

Haplotype	Polymorphism in the 5′-flanking region					AIH (n=56)%	Control (n=210)%	OR	*P*
	−1031	−863	−857	−308	−238				
A	T	C	C	G	G	78.6	90.0	0.41	0.042
B	C	A	C	G	G	25.0	28.6	0.83	1.192
C	C	C	C	G	A	5.4	3.3	1.64	0.959
D	T	C	T	G	G	48.2	30.5	2.12	0.026*
E	T	C	C	A	G	1.8	1.4	1.25	1.691

OR, odds ratio. *Corrected P=0.13.

Genetic susceptibility to type 1 AIH in Caucasians is related to HLA alleles encoding the six amino acid sequence LLEQKR at position 67–72 of the DRB1 polypeptide [6,8–10]. In Japan and Argentina, AIH susceptibility is linked to DRB1*0405. In Mexico, it is linked to DRB1*0404. DRB1*0405 and DRB1*0404 encode arginine (R) at position 71, which is at the lip of the antigen-binding groove of the HLA DR molecule and influences the interaction between antigen presenting cells and helper T cells. DRB1*0405 and DRB1*0404 share the LLEQ-R motif with DRB1*0301 and DRB1*0401. It is likely that this motif presents the same or similar auto-antigen(s) since lysine at position 71 of DRB1*0301 and 0401 and arginine at the same position of DRB1*0405 and 0404 are basic and highly charged polar amino acids. Therefore, we can conclude that type 1 AIH susceptibility in Japanese maps to the DRB1 locus, and that arginine at position 71 of DRB1 is primarily associated with type 1 AIH, as proposed by Doherty DG et al. [6].

In Caucasians, patients with DRB1*0301 present with disease at a significantly younger age than patients with DRB1*0401 [7,9,10]. DRB1*0301 is in strong linkage disequilibrium with DRB3*0101, which encodes the LLEQKR motif at DRβ position 67–72. DRB1*0401 is in strong linkage disequilibrium with DRB4*0103, which encode the LLERRR at DRβ position 67–72. It has therefore proposed that number of LLEQKR motifs on the antigen presenting cells determines the clinical expression of AIH, called dose effects [7,9,10]. Japanese patients having the DRB1*0405-DRB4 haplotype express similar clinical

Table 7
Association of TNF-α promoter gene haplotype D with type 1 AIH patients after stratification for the effect of DRB1*0405

DRB1*0405	Allele D	Control	AIH	Weighted OR	χ^2	*P*	95% CI
Present	Negative	30	10	1.91	3.18	0.075	1.01<OR<3.60
	Positive	32	22				
Absent	Negative	116	19				
	Positive	32	5				

OR, odds ratio; 95% CI, 95% confidence interval.

features as Caucasoid patients with DRB1*0401; Disease onset is late and response to corticosteroid therapy is good. Only 6 out of the 77 patients were homozygous for DRB1 alleles (4 for DRB1*0405, and 1 each for 1302 and 1502). As a result, it was difficult to analyze the dose effects and clinical feature differences between patients with homozygous alleles and heterozygous ones.

Type 1 AIH in Brazilian patients and Argentine children is associated with DRB1*1301, which runs contrary to the shared motif model. Position 71 of DR13 is glutamine, which belongs to a different group of amino acids from arginine or lysine. The only amino acid difference between DRB1*1301 and 1302 is in position 86, so researchers in the above studies proposed an alternative hypothesis of susceptibility based on a valine/glycine dimorphism at position 86 of DRβ [14]. Of the 16 Japanese patients without DR4 (20.8%), 5 had DR13 (4 had 1302 and 1 had 1301). One of them was homozygous for the 1302 allele. Position 86 of DRβ of DRB1*0405 is also glycine. Therefore, valine at position 86 is not considered to be related to AIH in Japanese patients.

We also showed that a large number of oligoclonal αβT cells infiltrated into the liver of type 1 AIH patients [39]. T cells recognize antigens presented by MHC class I or class II molecules via a specific T cell receptor and clonally expand. Therefore, specific T cells of AIH patients might be stimulated by unknown auto-antigen(s) in the groove of the HLA class II molecule. Taken together, our data strongly suggest that HLA-DRB1 and/or -DQB1 genes alone are the susceptibility genes in the HLA region.

TNF-α is a key cytokine in the inflammatory response. In Caucasian patients with type 1 AIH, the frequency of the −308A allele (TNF*2) was significantly increased in cases with AIH [19,20]. The TNF*2 allele is carried on the extended 8.1 ancestral haplotype (A1-B8-DRB1*0301) of northern Europeans [40], and previous researchers described that TNF*2 allele may work in synergy with HLA DR3 to affect clinical manifestations and disease severity (autoimmune promoter hypothesis) [9,10]. However, AIH in Brazilian patients was found not to be linked to TNF-α gene polymorphisms at position −308 [41]. Type 1 AIH in Brazil is also not associated with HLA DR3. It is therefore suggested that region-specific etiologic factors may affect disease susceptibility [42]. In our study, the frequency of the −308A allele (TNF*2) was very rare in both Japanese patients and controls (1.8% in AIH patients and 1.4% in controls, Table 4). TNF*2 is found in strong linkage disequilibrium with HLA DRB1*0301, which is also very rare in Japanese [11].

Lastly, we analyzed the TNF-α polymorphisms in the 5′ flanking region to investigate whether other polymorphisms influenced the development of type 1 AIH in Japanese. Haplotype D of the TNF-α gene polymorphisms was weakly associated with susceptibility to AIH, but was found to be not significant after stratification analysis.

In conclusion, microsatellite analysis of the HLA region on the short arm of human chromosome 6 clearly shows that there are no further genes associated with the development of type 1 AIH besides HLA-DRB1 in Japanese. We are currently searching for other candidate susceptibility genes outside of the HLA region using microsatellite and single nucleotide polymorphism analysis. Future studies are needed to confirm the shared motif model and the autoimmune promoter hypothesis in Japanese patients, and to search for the auto-antigen(s) that trigger the disease. Taken together, these results may provide the specific tools for therapeutic intervention.

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Case Report

A Case of Metachronous Cholangiocellular and Hepatocellular Carcinoma with Good Prognosis

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KEY WORDS: Hepatocellular carcinoma; Cholangiocellular carcinoma; Interferon; Chronic hepatitis C; Metachronous

ABBREVIATIONS: Cholangiocellular Carcinoma (CCC); Interferon (IFN); Percutaneous Ethanol Embolization (PEI); Radiofrequency Ablation (RFA); Transcatheter Arterial Embolization (TAE)

SUMMARY

A 63-year-old man was treated for 6 months with interferon (IFN) for chronic hepatitis C but the treatment failed to eradicate hepatitis C virus. Six months after completion of IFN therapy, cholangiocellular carcinoma (CCC) was detected in the posterior inferior segment and was resected surgically. He had been in good condition except for diabetic nephropathy progressing to renal failure at 3 years after the resection of CCC. Seven years after the resection of CCC, hepatocellular carcinoma (HCC) was detected in the posterior superior segment of the liver. The tumor was pathologically confirmed by fine needle aspiration biopsy. The patient was successfully treated with two courses of percutaneous ethanol injection and has been well 1 year after the treatment. HCV status did not change as genotype 1b with moderate viral load (300 to 500 kilo copies/mL by amplicore monitoring) during the follow-up. Thus, even though the patient was treated with IFN, hepatitis C could progress to not only HCC but also CCC in the same patient. Our patient is still alive, 9.5 years after detection of the first tumor.

INTRODUCTION

Chronic hepatitis C viral infection could lead to not only hepatocellular carcinoma (HCC) but also cholangiocellular carcinoma (CCC) (1). Interferon treatment for hepatitis C viral infection is effective in the eradication of hepatitis virus C (HCV) (2,3) but HCC is still one of the leading cancers worldwide. There are several reports of hepatocarcinogenesis even after treatment of IFN irrespective of whether HCV was eradicated or not (4-7). However, no cases of CCC in combination with HCC after treatment with IFN have been reported previously. We report the successful treatment of metachronous double cancers of HCC and CCC in a patient with hepatitis C who biochemically responded to IFN but without viral eradication. Based on the findings in this case, we recommend a close follow-up of patients with chronic hepatitis C to prevent the development of both HCC and CCC irrespective of whether they are treated with IFN or not.

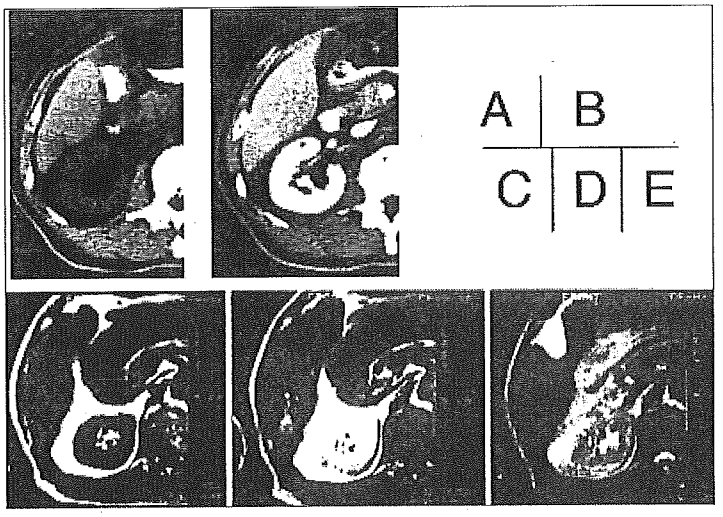

FIGURE 1 Abdominal CT; plain **(A)**, enhanced **(B)** Dynamic CT of the liver shows a small tumor, which is lightly enhanced at early phase in the posterior lobe.
Abdominal MRI; T1-weighted MR image **(C)** shows a isointense mass, T1-weighted MR enhanced by Gd **(D)** shows lightly enhanced mass, T2-weighted MR **(E)** shows a hyperintense mass in the posterior lobe.

CASE REPORT

A 63-year-old man was found positive for HCV and liver biopsy proved chronic active hepatitis in October 1992. He was followed as a case of chronic hepatitis for 10 years during which he also developed diabetes mellitus. He was found to have liver dysfunction at age 46 years and was followed as non-A non-B hepatitis. The patient was treated with 6 million units of natural IFN- from November 1992 to April 1993 for 78 days but the treatment failed to eradicate HCV. In May 1993, he was screened for HCC and was found to have a liver tumor in the posterior inferior segment (S6) on computed tomography **(Figure 1)**. Biopsy of the liver tumor proved it was CCC, which was subsequently treated by posterior inferior segmentectomy on September 16, 1993. The tumor was white in color and histopathological examination showed a moderately differentiated CCC **(Figure 2)**. The patient was free

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from recurrence but developed diabetic nephropathy, which progressed to renal failure 7 years after the operation. He was maintained on hemodialysis at 3 times/week and followed as chronic hepatitis. In January 2000, another liver tumor was detected on ultrasonography and computed tomography in the posterior and anterior segments of the remnant liver **(Figure 3)**. Angiography demonstrated a hypervascular tumor in the liver and tumor biopsy revealed moderately differentiated HCC **(Figure 4)**. The patient was informed of the diagnosis and chose the combination treatment of transcatheter arterial embolization (TAE) and percutaneous ethanol injection (PEI). HCC changed into non-enhanced tumor on computed tomography after 1 course of TAE with lipiodol and gelfoam spongel and 3 courses of PEI. One year later, recurrence of HCC in the right lobe and a new lesion in left lobe were detected. These tumors were treated with TAE and radiofrequency ablation (RFA). HCV status did not change as genotype 1b with moderate viral load (300 to 500 kilo copies/mL by amplicore monitoring) during the follow-up.

At the last follow-up in April 2003, one year after the last TAE and RFA, no recurrence was noted on abdominal CT **(Figure 5)**. The patient is currently being followed as part of a group at risk of HCC and CCC.

DISCUSSION

Patients with chronic HCV infection often develop cirrhosis and a significant proportion of these patients progress to HCC. Several groups have proposed various molecular mechanisms for HCV and HCC (9,10) but the precise mechanism(s) for the malignant transformation of hepatocytes infected by HCV has not been elucidated (11). Furthermore, only a few studies demonstrated a relationship between HCV and CCC but Tomimatsu et al. (1) showed a clear link between HCV and CCC (1). Furthermore, Horiguchi et al. (12) reported that 27% of patients with CCC were positive for HCV and a larger study reported by the Japan Liver Cancer Study Group showed that 24.7% of Japanese patients with CCC were positive for HCV (13). Multicentric hepatocarcinogenesis has been proposed based on histopathological findings (14) but the double cancer of HCC and CCC can most clearly tell the story of multicentric hepatocarcinogenesis (15-19). Metachronous and synchronous double cancer with HCC and CCC have been reported but none of these cases were treated by IFN for the associated chronic hepatitis C (15-29). Several cases of HCC occurring after treatment with IFN have been reported (4-8). According to these studies, HCC could develop with or without eradication of HCV because the carcinogenic process is often triggered by chronic liver damage such as fibrosis and inflammation, over a substantial period by HCV before viral eradication (30). Whether the cumulative rate of the development of HCC in chronic hepatitis C decreases or not after treatment with IFN is still controversial (31,32). The case presented here clearly showed that not only HCC but also HCC combined with CCC can occur after treatment with IFN and thus, hepatitis C can be a risk factor for both of these liver cancers.

A search of the Medline database for Japanese cases with double cancers of HCC and CCC reported between 1985 and 2003, identified 17 case reports, including our case **(Table 1)**. Eight cases (47.1%)

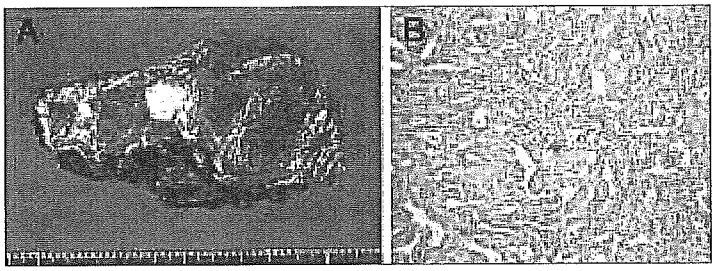

FIGURE 2 (A) Surgical resection of the posterior tumor was performed on September 16, 1993. The resected tumor was yellowish-white in color with a clear border, and measured approximately 1.4x1.4cm. **(B)** Microscopic examination of the surgical specimen showed ductal formation consisting of atypical bile duct epithelium, which is compatible with cholangiocellular carcinoma (hematoxylin and eosin, x200).

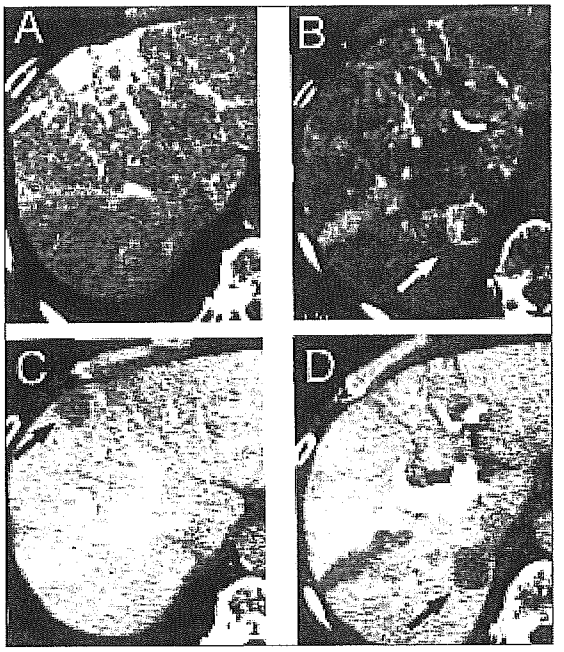

FIGURE 3 Computed tomography during hepatic arteriography (CTHA) **(A and B)** demonstrating a well-enhanced mass in segment 8 and ring-like enhancement mass in segment 7 (arrow). Computed tomography during arterial portography (CTAP) **(C and D)** demonstrating well-detected mass in segments 7 and 8 (arrow).

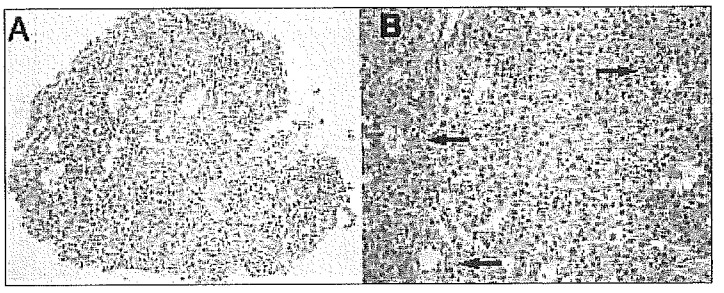

FIGURE 4 Echo-guided-percutaneous liver tumor biopsy performed January 14, 2000 showed a moderately differentiated hepatocellular carcinoma with ductular arrangement (**B**, arrow) (**A**; H&E x40, B; H&E x200).

TABLE 1. Reported Cases of Hepatocellular carCinoma combined with Cholangiocellular Carcinoma

Ref.	Year	Sex	Age	Etiology	Non-carcinoma liver tissue	S or M	Interval	Tumor Marker				HCC			CCC			Prognosis from first tumor detection		
								AFP	PIVKA-II	CEA	CA19-9	Size	S6	Therapy	Size	Site	Therapy		alive or dead	cause of death
15	1986	M	53	NANB	CH	S		UK	UK	1.8	UK	53x35, 21x20	S6, S6, S7	OPE	15x15, 6	S8	OPE	4M	alive	
16	1987	M	57	NANB	LC	S		864.1	UK	2.5	UK	35x30	ant	OPE	60x55	post	OPE	4M	dead	recurrence
17	1989	M	50	NANB	CH	M (C/H)	6Y	(-)/143	UK	(-)/N	UK	40	S4	OPE	15	S8	OPE	2Y	dead	multiple metastases
17	1989	M	53	NANB	CH	S		(-)	UK	N	UK	21x20	S6	OPE	53x35	S6	OPE	7Y	dead	traffic accident
18	1990	M	46	B	LC	M (C/H)	2Y	UK	<0.06	UK	UK	26x21	S4	PEI	11x10	S4	OPE	2Y	alive	
19	1993	M	64	B	LC	M (C/H)	12Y	(+)/2	UK/<0.06	UK	UK	25	S6	OPE	16x15	ant	OPE	13Y	alive	
20	1994	M	50	Alcohol	LC	M (H/C)	8Y	1.3	0.06	0.8	UK	20	S7	TAE	35x30	S4-S5	OPE	8Y	alive	
21	1994	M	59	Alcohol	CH	S		629	UK	1.3	15	68x50	lat	OPE	10x7	S4	OPE	UK		
22	1995	M	65	C	LC	M (H/C)	1Y	10.7/135.7	<0.07/<0.01	2.7/2.1	44.4/8.4	13 30x18, 10x10,	S7 S8, S7,	PEI	30	S6	PEI	1.5Y	alive	
23	1995	M	69	C	LC	S		20.4	<0.06	2.8	1300	10x10	S6	OPE	UK	lat	OPE	UK		
24	1995	M	73	C	LC	S		83.9	UK	3.8	52	80x90	S6	OPE	40x50	lat	OPE	1.5Y	alive	
25	1996	F	62	AIH	LC	M (C/H)	8M	2.5/2.2	UK	<0.9/UK	2251/>24000	50	S6	TAE	17	S6	OPE	13M	dead	multiple metastases
26	1996	M	68	C	CH	S		UK	UK	2.4/3.2	UK	17	lat	OPE	12	S7	OPE	2.5M	dead	cancer death
27	2000	M	65	C	LC	S		13.8	23	12.7	151.7	17x15	S8 S8, S7,	OPE	23x15	S8	OPE	11M	alive	
28	2001	M	70	C	UK	M (H/C)	6M	101/low	0.084/low	12.3/32.3	40/177	UK	S6	TAE	UK	S4	OPE	13M	dead	multiple metastases
29	2003	M	66	C	LC	S		12.7	(-)	(-)	UK	20x10	S8	OPE	15x7	S4	OPE	2Y	alive	
Present	2003	M	64	C	CH/LC	M (C/H)	6Y	12.9/15.3	<0.06/20.3	1.2/1.2	14/27	20,20	S7, S8	TAE, etc	14x14	S6	OPE	9.5Y	alive	

CH: chronic hepatitis; LC: liver cirrhosis; S: synchronous; M: metachronous; C/H: first tumor is CCC, second tumor is HCC; H/C: first tumor is HCC, second tumor is CCC; UK: unknown; OPE: operation; Size: scale in mm; (-): negative; (+): positive; N: normal. Tumor markers of metachronous cases are described as first tumor/second tumor.

were positive for HCV and 2 cases (11.8%) were positive for HBV. The other 2 cases had alcoholic liver injury and only one case had autoimmune hepatitis (25). Four cases had non-A non-B hepatitis but these cases could have been HCV-positive because they were diagnosed before the discovery of HCV. None of these patients had subjective symptoms, e.g. abdominal pain, at diagnosis of the tumor. Generally, it is difficult to diagnose CCC in the early stage but CCC was diagnosed in these cases during follow-up of chronic liver injury. Of these 17 cases, 16 (94.1%) with CCC and 11 (64.7%) with HCC were treated surgically. This rate of surgical treatment is higher than that for CCC and HCC alone, in which the rate of surgery is 29.2% and 54.6%, respectively (13). The 10-year survival rates of CCC treated by surgical resection is 17.3% (13), and thus the prognosis tends to be satisfactory **(Table 1)**. The characteristics of the double cancers with regard to the location in the liver and size of the tumor were not specific.

In conclusion, a careful follow-up, including diagnostic imaging, allowed detection of not only HCC but also CCC in a patient with chronic hepatitis C following treatment with IFN. Even though double cancers of CCC and HCC can develop in chronic hepatitis C patients, good prognosis can be provided by radical multidisciplinary treatment including hepatic resection, ethanol injection and radiofrequency ablation of each tumor.

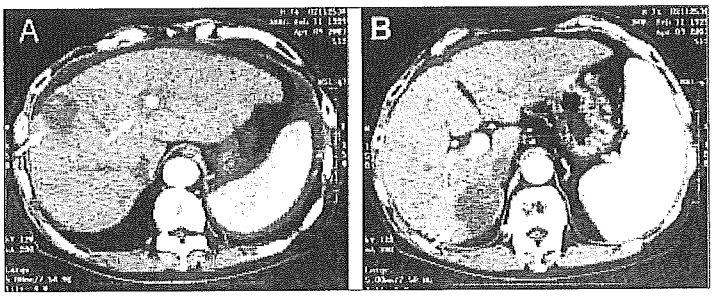

FIGURE 5 Abdominal computed tomography taken at last follow-up examination (9.5 years after detection of CCC) demonstrating three small masses in segments 3, 8 (**A**, arrow) and 7 (**B**, arrow). These tumors were subsequently treated by TAE, PEI or RFA.

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