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## Declining Incidence of Hepatocellular Carcinoma in Osaka, Japan, from 1990 to 2003

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**Background:** Japan has the highest incidence rate of primary liver cancer attributed to chronic hepatitis C virus (HCV) infection among developed countries. Molecular clock analysis of HCV sequences revealed that the spread of HCV took place earlier in Japan than in other countries. This might influence recent temporal trends in hepatocellular carcinoma (HCC) incidence.

**Objective:** To characterize the contribution of HCV-related hepatocellular carcinoma (HCC) to recent changes in HCC incidence in Osaka, Japan.

**Design:** Population-based survey.

**Setting:** Osaka Cancer Registry and 10 hospitals in Osaka.

**Participants:** 63 862 patients with HCC that was diagnosed between 1981 and 2003 in Osaka Prefecture, including 5253 HCV-seropositive patients with HCC that was diagnosed between 1990 and 2003 at 10 hospitals.

**Measurements:** Incidence of HCC and estimated incidence rate of HCV-related HCC, measured by multiplying the prevalence of anti-HCV by the corresponding HCC incidence rate.

**Results:** Between 1981 and 2003, peak incidence of HCC among men age 50 to 59 years, 60 to 69 years, and 70 to 79 years occurred in 1986, 1995, and 2000, respectively, with marked downward trends thereafter (average annual change,  $-7.9$ ,  $-22.3$ , and  $-12.4$  per 100 000 persons, respectively). Similar trends were observed in women. Estimated sex- and age-specific incidence of HCV-related HCC (per 100 000 persons) decreased from 255 to 92 cases at the maximum in men age 60 to 69 years and from 61 to 34 cases in women age 60 to 69 years, whereas estimated incidence of non-HCV-related HCC did not change between 1990 and 2003.

**Limitation:** Infection was determined only by HCV seropositivity.

**Conclusion:** The incidence of HCC in Osaka started to decrease by 2000, mainly because of decreased HCV-related HCC.

*Ann Intern Med.* 2008;148:820-826.

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Primary liver cancer was the fifth most common cancer worldwide by 2000, with approximately 551 000 new cases recorded (1). In most countries, hepatocellular carcinoma (HCC) comprises 85% to 90% of primary liver cancer cases. With some exceptions, developed countries, including the United States, have been experiencing an increase in the incidence of primary liver cancer, considered to be due at least in part to increased prevalence of chronic hepatitis C virus (HCV) infection (2).

Japan has had one of the highest incidence rates of primary liver cancer among developed countries (age-standardized incidence rate in 1995, 25.5 per 100 000 men and 7.7 per 100 000 women) (3). Approximately 90% of liver cancer cases are HCC, which, in Japan, is mainly caused by chronic HCV infection rather than chronic hepatitis B virus infection (4). A recent report on the age-standardized incidence of primary liver cancer among Japanese men, which was calculated from 6 population-based

cancer registries, showed a sharp increase that started in the mid-1970s but leveled off in the mid-1990s (5). These distinctive trends were thought to be due to the spread of HCV infection, which began in the 1920s and increased after World War II (6-8). Thus, HCV penetrated Japan earlier than Spain, Egypt, the United States, the former Soviet Union, South Africa, and Hong Kong, as evidenced by molecular clock analysis of the sequences of HCV isolates (8). However, recent temporal trends regarding incidence rates of HCC and the contribution of HCV infection have not been clearly documented in the Japanese population.

We analyzed temporal trends for HCC incidence rates between 1981 and 2003 in Osaka Prefecture (population in 2005, 8.8 million) and interpreted these in the context of HCV infection rates.

### METHODS

#### Data Collection on Incident HCC Cases

We obtained data on incident HCC cases from the Osaka Cancer Registry, which was established by the Osaka Prefectural Government in 1962. The registry collects reports on patients with newly diagnosed cancer, including demographic and cancer-related information, from all medical institutions in Osaka Prefecture (9). These have been routinely supplemented by death certificates gathered

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by the Osaka Prefectural Government (9). For patients with cancer who were enrolled in the registry on the basis of their death certificate, we contacted the issuing hospital to obtain information on diagnosis and treatment and to establish the date of HCC incidence, which we determined to be the time of diagnosis at that hospital. We site-coded the data according to the International Classification of Diseases for Oncology, Third Edition (10). We included patients with HCC (codes 8170 through 8180). The protocol was approved by the ethics committee of the Osaka Medical Center for Cancer and Cardiovascular Diseases.

From 1981 to 2003, 48 166 men and 15 696 women with HCC were documented in the Osaka Cancer Registry. We calculated the annual age-standardized incidence rates of HCC (world population as a standard population) by sex between 1981 and 2003. To characterize temporal trends for HCC, we assessed 10-year, age-specific incidence rates of HCC between 1981 and 2003 in individuals age 50 to 79 years. We studied these particular age-specific rates because most HCV-related HCC cases in the Japanese population occur between the ages of 50 and 79 years (4). We used the annual population estimates from 1981 to 2003, which were based on the average population in each sex and age category for the Osaka Prefecture during the particular period, as denominators for calculating incidence rates. The annual population estimates were based on data from the 1980, 1985, 1990, 1995, 2000, and 2005 Japanese population censuses, with linear interpolation for the years in between.

### Statistical Analysis

To identify years when a statistically significant change in the slope of the temporal trend in the incidence occurred, we applied the joinpoint regression model by using the Joinpoint Regression Program, version 3.0 (U.S. National Cancer Institute, Bethesda, Maryland). We assumed constant variance and uncorrelated errors (11) because we could not detect heteroskedasticity by the White test or autocorrelation by the Durbin-Watson test in men or women in any age group.

We computed the estimated slopes describing the average annual change of incidence rate per 100 000 persons and the corresponding 95% CIs for each trend by fitting a piecewise regression line to the rates, using calendar year as a regression variable. We used the permutation test method to identify years when a statistically significant change had occurred ( $P < 0.05$ ) and set the number of randomly permuted data sets at 4499. We set the number of joinpoints to a minimum of 0 and a maximum of 3 in the Joinpoint Regression Program.

### Data Collection on Prevalence of HCV Infection among Patients with HCC

The Osaka Cancer Registry does not collect serologic data on HCV infection in the registered patients. Therefore, we used data on HCV seropositivity from patients with HCC that was diagnosed at 10 hospitals in Osaka

### Context

Hepatitis C virus (HCV) infection in Japan began to spread during the 1920s, increased after World War II with an explosion in parenteral amphetamine use and paid blood donation, and decreased in the 1950s to 1960s with voluntary blood donation and penalties against amphetamine use. Evidence linking the trends in HCV infection to hepatocellular carcinoma rates in Japan is limited.

### Contribution

Data from the Osaka Cancer Registry and 10 Osaka hospitals suggest that hepatocellular carcinoma rates began to decrease in 2000, mainly because of a decrease in HCV-associated cancer.

### Implication

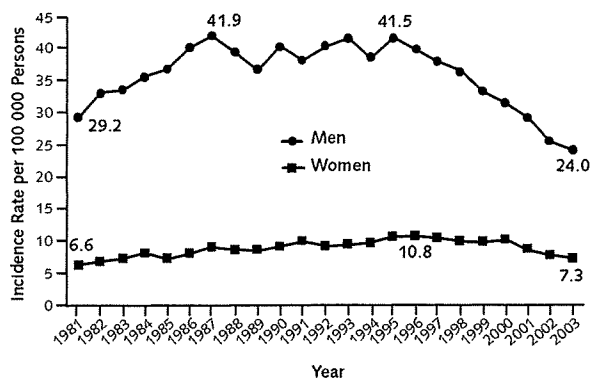
Control of HCV transmission within a population seems to be followed by a decrease in hepatocellular carcinoma.

—The Editors

Prefecture (1 university hospital, 2 cancer centers, and 7 general hospitals) to estimate the prevalence of HCV infection in patients with HCC. We considered the HCC diagnosis confirmed when the patient had positive histologic or positive radiologic results by enhanced computed tomography or hepatic angiography. We collected data on the patient's sex, date of birth, date of diagnosis between 1990 and 2003, first Chinese letter of the family name, and presence of hepatitis B surface antigen and antibody to hepatitis C (anti-HCV) as assessed by any commercially available kit. We did not collect the full first and family name for reasons of confidentiality. Because anti-HCV testing first became available in Japan in 1990, we collected data on patients whose HCC diagnosis was between 1990 and 2003. One investigator checked for duplication of the data set, because some patients might have been registered multiple times among the participating hospitals as a result of referrals and recurrence of HCC. We defined HCV-related HCC as occurring in patients who were HCV-seropositive at the time of diagnosis.

We calculated the sex-specific, age-specific (50 to 59, 60 to 69, or 70 to 79 years), and period-specific (1990 to 1992, 1993 to 1995, 1996 to 1998, 1999 to 2001, or 2002 to 2003) prevalences of HCV seropositivity for patients with HCC. We then multiplied prevalence rates by the corresponding strata of the HCC incidence rate obtained from the Osaka Cancer Registry data. Thus, we derived the denominators from the general population in Osaka through the denominators of the HCC incidence rate and obtained the numerators by multiplying the prevalence rates by the HCC incidence rate. We calculated the incidence rate of non-HCV-related HCC by subtracting HCV-related HCC from total HCC. Thus, we describe trends for the estimated incidence rates of HCV-related

**Figure 1.** Trends in age-standardized (world population) incidence of hepatocellular carcinoma in Osaka, Japan, 1981–2003.



and non-HCV-related HCC between 1990 and 2003 in Osaka Prefecture. We calculated the CI of the estimated rates by multiplying the lower and upper limits of the CI of the prevalence based on SE by the corresponding HCC incidence rate.

**Role of the Funding Source**

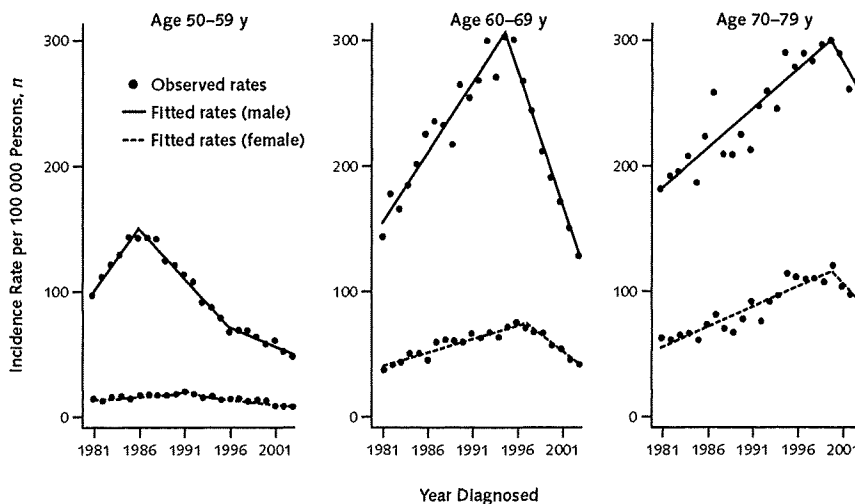
This study was supported by the Osaka Prefectural Government between 1990 and 2000 and Grants-in-Aid for Hepatitis Research of the Japanese Ministry of Health, Labor, and Welfare. There is no conflict of interest in the study. The funding sources had no role in the collection, management, or analysis of data.

**RESULTS**

The age-standardized incidence rate of HCC in men increased between 1981 and 1987 from 29.2 to 41.9 cases per 100 000 persons, then fluctuated until 1995. After that, it steadily decreased to 24.0 cases per 100 000 persons in 2003 (Figure 1). Among women, the age-standardized incidence rate of HCC increased between 1981 and 1996 from 6.6 to 10.8 cases per 100 000 persons, then gradually decreased to 7.3 cases per 100 000 persons in 2003 (Figure 1).

Figure 2 shows the trends in the incidence of HCC among men and women age 50 to 59 years, 60 to 69 years, and 70 to 79 years in Osaka between 1981 and 2003. The HCC incidence rate increased from 1981 to 1986 among men age 50 to 59 years, from 1981 to 1995 among men age 60 to 69 years, and from 1981 to 2000 among men age 70 to 79 years (average annual change of the incidence rate [per 100 000 persons], 10.0, 10.7, and 6.2, respectively) (Table 1). A striking downward trend occurred after the year of peak incidence in the 3 age groups (−7.9 until 1996, −22.3 until 2003, and −12.4 until 2003, respectively). Among men age 50 to 59 years, there was a second jointpoint (a change from rapid to moderate decrease) in 1996, resulting in a slope of −3.1 until 2003. Among women age 50 to 59 years, 60 to 69 years, and 70 to 79 years, the incidence rates of HCC peaked in 1991, 1997, and 2000, respectively (Table 1). The rates in women seemed to increase slightly from 1981 until the year of the jointpoint, with slopes of 0.43, 2.07, and 3.10, respectively. Thereafter, HCC incidence rates in women decreased through 2003 at a statistically significant average annual rate of −0.9, −5.7, and −7.9, respectively (Table 1).

**Figure 2.** Joinpoint analysis of the incidence rate of hepatocellular carcinoma among individuals age 50 to 79 years in Osaka, Japan, 1981–2003.



**Table 1. Joinpoint Analysis of the Hepatocellular Carcinoma Incidence Rate per 100 000 Persons in Osaka, Japan, 1981–2003**

Age Range	Peak Year	Incidence Rate per 100 000 Persons	Trend 1		Trend 2		Trend 3	
			Years	Slope (95% CI)	Years	Slope (95% CI)	Years	Slope (95% CI)
<b>Men</b>								
50–59 y	1986	142.0	1981–1986	10.0 (8.2 to 11.8)*	1986–1996	–7.9 (–8.6 to –7.1)*	1996–2003	–3.1 (–4.2 to –2.1)*
60–69 y	1995	299.6	1981–1995	10.7 (9.1 to 12.3)*	1995–2003	–22.3 (–26.0 to –18.6)*	–	–
70–79 y	2000	296.4	1981–2000	6.2 (4.8 to 7.5)*	2000–2003	–12.4 (–35.7 to 10.9)	–	–
<b>Women</b>								
50–59 y	1991	19.7	1981–1991	0.4 (0.2 to 0.7)*	1991–2003	–0.9 (–1.1 to –0.7)*	–	–
60–69 y	1997	68.5	1981–1997	2.1 (1.7 to 2.4)*	1997–2003	–5.7 (–7.3 to –4.1)*	–	–
70–79 y	2000	118.1	1981–2000	3.1 (2.5 to 3.7)*	2000–2003	–7.9 (–18.1 to 2.4)	–	–

\*  $P < 0.001$ .

Table 2 shows the prevalence of anti-HCV antibodies among 5253 patients age 50 to 79 years with HCC that was diagnosed at 10 hospitals in Osaka between 1990 and 2003. The prevalence was highest in men with HCC that was diagnosed in 1993 to 1995 (82.4%). The proportion of HCV-seronegative patients ranged from 18% to 29% through the observation period. The prevalence of anti-HCV was almost constant (81% to 83%) among women with HCC that was diagnosed between 1993 and 2003 (Table 2).

Figure 3 shows changes in the estimated incidence rate of HCV-related and non-HCV-related HCC from 1990 to 2003. Among men, the estimated incidence rate of HCV-related HCC steadily decreased among Osaka residents age 50 to 59 years from 83 (95% CI, 77 to 89) cases per 100 000 persons in 1990 to 1992 to 26 (CI, 21 to 30) cases per 100 000 persons in 2002 to 2003. Among men

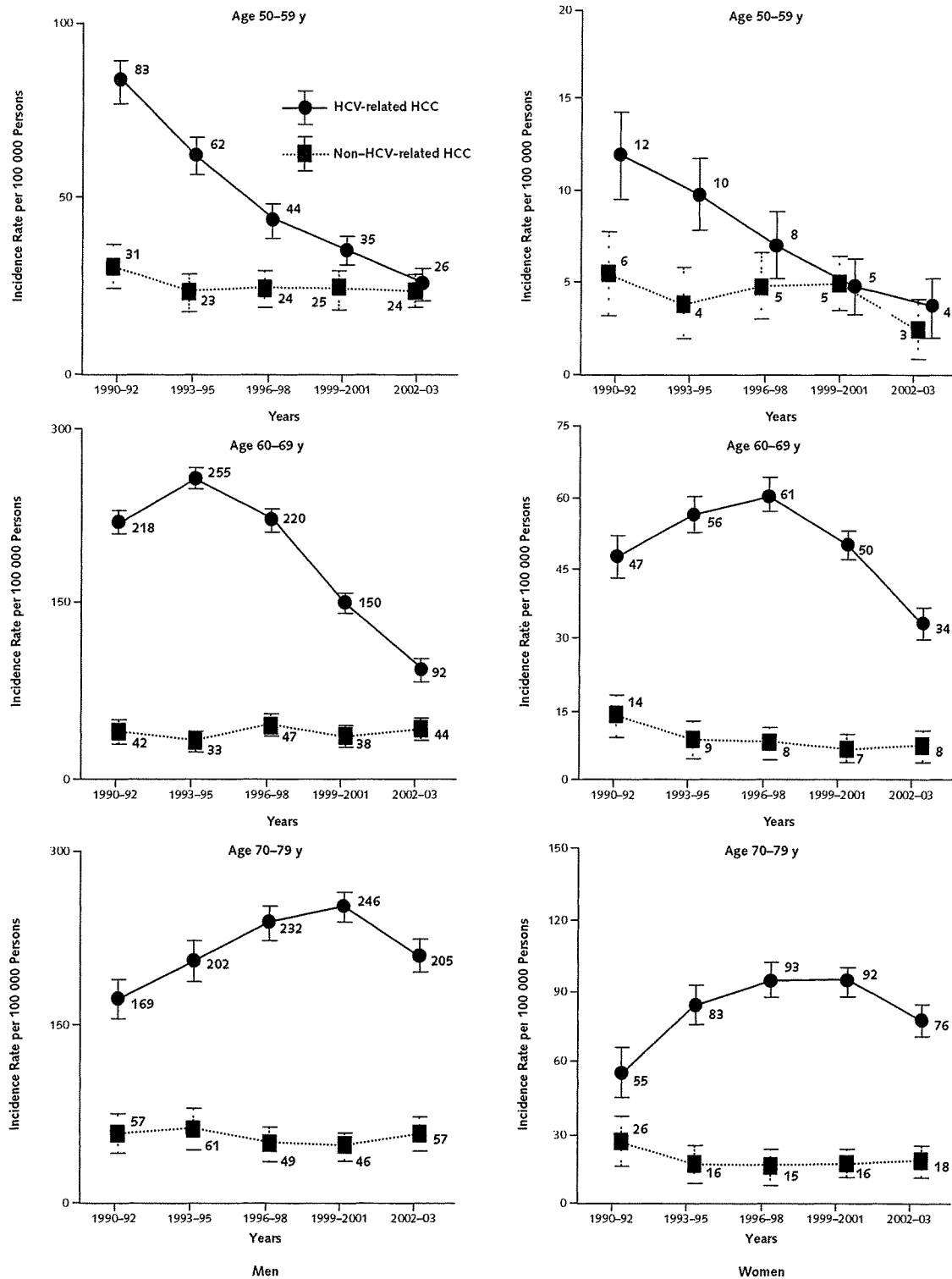
age 60 to 69 years, incidence seemed to peak (255 [CI, 247 to 264] cases per 100 000 persons) from 1993 to 1995. Among men age 70 to 79 years, the incidence rate increased from 1990 to 1992 (169 [CI, 153 to 186] cases per 100 000 persons) to 1999 to 2001 (246 [CI, 234 to 258] cases per 100 000 persons) and leveled off afterward. The estimated incidence rate of HCV-related HCC among women age 50 to 59 years decreased from 12.4 (CI, 10.1 to 14.7) cases per 100 000 persons during 1990 to 1992 to 4.2 (CI, 2.5 to 5.8) cases per 100 000 persons during 2002 to 2003, whereas among women age 60 to 69 years, the incidence peaked (61 [CI, 57 to 64] cases per 100 000 persons) during 1996 to 1998. The trend in women age 70 to 79 years seemed to be similar to that in men of the same age: increasing during the 1990s and leveling off in the early 2000s (Figure 3). The estimated incidence rate of non-HCV-related HCC was lower than that of HCV-

**Table 2. Prevalence of Anti-HCV among 5253 Patients Age 50 to 79 Years with Hepatocellular Carcinoma at 10 Hospitals in Osaka, Japan, 1990–2003\***

Variable	1990–1992		1993–1995		1996–1998		1999–2001		2002–2003	
	Patients, n	Prevalence (±SE), %	Patients, n	Prevalence (±SE), %	Patients, n	Prevalence (±SE), %	Patients, n	Prevalence (±SE), %	Patients, n	Prevalence (±SE), %
<b>Men</b>										
Anti-HCV(+)	602	78.3 ± 1.5	677	82.4 ± 1.3	651	78.7 ± 1.4	709	76.6 ± 1.4	385	70.9 ± 1.9
Anti-HCV(+) and HBsAg(+)	18	2.3 ± 0.5	17	2.1 ± 0.5	11	1.3 ± 0.4	16	1.7 ± 0.4	8	1.5 ± 0.5
Anti-HCV(+) and HBsAg(–)	584	75.9 ± 1.5	660	80.3 ± 1.4	640	77.4 ± 1.5	693	74.8 ± 1.4	377	69.4 ± 2.0
Anti-HCV(–)	167	21.7 ± 1.5	145	17.6 ± 1.3	176	21.3 ± 1.4	217	23.4 ± 1.4	158	29.1 ± 1.9
Anti-HCV(–) and HBsAg(+)	60	7.8 ± 1.0	57	6.9 ± 0.9	71	8.6 ± 1.0	106	11.4 ± 1.0	68	12.5 ± 1.4
Anti-HCV(–) and HBsAg(–)	107	13.9 ± 1.2	88	10.7 ± 1.1	105	12.7 ± 1.2	111	12.0 ± 1.1	90	16.6 ± 1.6
Total	769	100.0	822	100.0	827	100.0	926	100.0	543	100.0
<b>Women</b>										
Anti-HCV(+)	165	73.0 ± 3.0	211	82.7 ± 2.4	248	82.9 ± 2.2	274	80.8 ± 2.1	200	81.0 ± 2.5
Anti-HCV(+) and HBsAg(+)	8	3.5 ± 1.2	2	0.8 ± 0.6	5	1.7 ± 0.7	2	0.6 ± 0.4	2	0.8 ± 0.6
Anti-HCV(+) and HBsAg(–)	157	69.5 ± 3.1	209	82.0 ± 2.4	243	81.3 ± 2.3	272	80.2 ± 2.2	198	80.2 ± 2.5
Anti-HCV(–)	61	27.0 ± 3.0	44	17.3 ± 2.4	51	17.1 ± 2.2	65	19.2 ± 2.1	47	19.0 ± 2.5
Anti-HCV(–) and HBsAg(+)	21	9.3 ± 1.9	17	6.7 ± 1.6	29	9.7 ± 1.7	29	8.6 ± 1.5	18	7.3 ± 1.7
Anti-HCV(–) and HBsAg(–)	40	17.7 ± 2.5	27	10.6 ± 1.9	22	7.4 ± 1.5	36	10.6 ± 1.7	29	11.7 ± 2.0
Total	226	100.0	255	100.0	299	100.0	339	100.0	247	100.0

\* HBsAg = hepatitis B surface antigen; HCV = hepatitis C virus.

**Figure 3.** Trends in estimated incidence rates of hepatitis C virus (HCV)-related and non-HCV-related hepatocellular carcinoma (HCC) in Osaka, Japan, 1990–2003.



Information on anti-HCV status only became available after 1989. Error bars indicate 95% CIs.

related HCC in most strata. We observed no distinctive changes in the temporal trends for non-HCV-related HCC during the study period.

## DISCUSSION

Our analysis of HCC incidence in the Japanese population between 1981 and 2003 identified calendar years in which significant changes in temporal trends occurred. The HCC incidence rates in men and women age 50 to 59 years peaked during 1986 and 1991, respectively; in men and women age 60 to 69 years during 1995 and 1997, respectively; and in men and women age 70 to 79 years in 2000. We also found that temporal trends for HCC incidence between 1990 and 2003 by age group were mainly determined by trends in the incidence rates of HCV-related HCC.

The most likely explanation for these observations is the particular mode of HCV transmission in Japanese society. According to a study on molecular tracing of endemic HCV (8), the exponential spread of HCV-1b infection, a dominant genotype of HCV in Japan, started in the 1920s. This was associated with treatment of *Schistosoma japonicum* beginning in 1921 (12). Later, HCV infection coincided with an increase in parenteral amphetamine use in the devastated country during and after World War II (6, 7). Subsequently, viral spread was considered to be amplified through blood transfusions and parenteral medical procedures in the 1950s and 1960s (6, 7). Data on first-time blood donor candidates in Osaka indicate that the prevalence of anti-HCV antibodies among those born in 1925 to 1935 was much higher (7% to 10%) than that in the younger generation born in 1936 to 1955 (13). It is plausible that Japanese people born between 1925 and 1935, who were adolescents in the early 1950s, were most susceptible to HCV transmission under these circumstances. Age groups with peak incidence of HCC in men and women in the current study (1986 and 1991, respectively, for 50 to 59 years; 1995 and 1997, respectively, for 60 to 69 years; and 2000 for 70 to 79 years) included the generation for which prevalence of anti-HCV was high in Osaka (born in 1925 and 1935) (13). Stiffening of legal penalties against amphetamine use starting in 1954 and conversion from paid to voluntary blood donation in the late 1960s may have reduced HCV transmission, thereby resulting in the lower prevalence of HCV infection in generations born after 1935. Indeed, the spread of HCV in Japan essentially ended by the early 1990s at the latest, as evidenced by the current very low incidence of HCV infection among repeat blood donors (14, 15). Better detection methods introduced in the early 1980s for HCC in patients with cirrhosis through ultrasonography and measurement of  $\alpha$ -fetoprotein may have contributed to the apparent increase in the incidence of HCC found in this study. However, the distinctive changes we observed in the age-specific incidence of HCC during the 1990s through

the early 2000s cannot be explained by the increased ability to detect HCC, because the different joinpoints in age-specific incidence rates would not be derived from a single period effect of detection of HCC.

Increases in the incidence of and deaths from liver cancer in the 1970s to 1990s have been reported in Japan (5, 16), Australia (2), the United Kingdom (17), France (2, 18), Italy (2, 18), and the United States (2, 19). The increases in Japan and the United States are attributable to increased seroprevalence of HCV (6, 13, 20, 21), whereas this relationship has not been clearly established in the other countries.

Certain limitations of this study should be considered. First, because cancer reporting in Osaka is not mandated by law, HCC could have been underreported. However, because it is fatal, most of the unreported cases should have been detected by examination of the death certificate. In addition, because the proportion of persons with HCC included only on the basis of their death certificate was almost constant (22% to 25%) during the observation period (22–24), such underreporting would not be expected to affect the temporal trends for HCC incidence rates shown in our study. Second, the proportion of HCV-seropositive patients among the 5253 cases diagnosed at 10 hospitals might differ somewhat from the entire cohort of patients with HCC in Osaka. However, all Japanese patients, including those with HCC, have easy access to hospitals because of the national medical insurance system, and the 10 participating hospitals did not select patients with HCC on the basis of their etiologic background. Therefore, it is realistic to suppose that selection bias on prevalence of anti-HCV among these 5253 patients would have been limited. Finally, the temporal trends seen in the present study might differ from those among the entire Japanese population. We previously reported age-specific incidence rates of liver cancer by birth year in Japanese men between 1962 and 1997 (5) by using 6 population-based cancer registries from Cancer Incidence in Five Continents (9) (registries for Miyagi, Yamagata, Osaka, Hiroshima, Saga, and Nagasaki). Our previous study found the peak incidence of HCC among those born between 1931 and 1935 (5). In addition, the age-dependent prevalence of anti-HCV among first-time blood donors in Osaka (13) was similar to those in other areas of Japan (25). These findings may indicate that the timing of the outbreak of HCV infection and its reduction were similar in the different geographic areas of the country.

In conclusion, our calculation of HCC incidence rates demonstrated that they are already decreasing in both sexes in Osaka, Japan. That the outbreak of HCV infection in Japan after World War II and its termination occurred earlier in Japan than in the rest of the world is the most likely explanation for these observations. These findings confirm that HCV-related HCC is a preventable disease that can be decreased by controlling parenteral HCV transmission. In the early 1990s, interferon therapy for patients

with chronic HCV infection was started in Japan to reduce the risk for HCC (26, 27). A nationwide, community-based anti-HCV screening system targeting individuals age 40 to 70 years was introduced by municipal governments in Japan in 2002. Further observation of the temporal trends of HCC incidence is needed to assess the efficacy of these interventions in Japan.

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**Acknowledgment:** The authors thank the Osaka Cancer Registry for allowing use of their data and Ms. Yasue Kotani for assistance with statistical analysis.

**Grant Support:** By the Osaka Prefectural Government (1999–2000) and Grants-in-Aid for Hepatitis Research of the Japanese Ministry of Health, Labor, and Welfare.

**Potential Financial Conflicts of Interest:** None disclosed.

**Reproducible Research Statement:** *Study protocol:* Available by contacting Dr. Tanaka (e-mail, hitanaka@aichi-cc.jp). The protocol is only available in Japanese. *Statistical code and data set:* Not available.

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# Serum levels of soluble major histocompatibility complex (MHC) class I-related chain A in patients with chronic liver diseases and changes during transcatheter arterial embolization for hepatocellular carcinoma

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(Received January 16, 2008/Revised April 8, 2008/Accepted April 11, 2008/Online publication July 29, 2008)

Soluble forms of major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) are increased in the sera of patients with malignancy and impair the antitumor immune response by downregulating expression of their cognate immunoreceptor natural killer group 2, member D (NKG2D). Recently, soluble MICA/B were reported to appear even in some premalignant diseases, raising questions about the impact of soluble MICA/B produced from tumors on the expression of NKG2D. The present study examined soluble MICA/B in chronic liver disease and hepatocellular carcinoma (HCC) and their involvement in the immune-cell expression of NKG2D during transcatheter arterial embolization for HCC. The levels of soluble MICA/B were significantly higher in chronic liver disease and HCC patients than in healthy volunteers. The progression of liver disease and that of the tumor were independent determinants for soluble MICA/B levels. Immunohistochemistry revealed that MICA/B were expressed not only in HCC tissue but also on hepatocytes in cirrhotic livers. The transcatheter arterial embolization therapy significantly decreased serum levels of soluble MICA, but not soluble MICB, and increased the NKG2D expression on natural killer cells and CD8-positive T cells; there was an inverse correlation between changes in soluble MICA levels and in NKG2D expression. In conclusion, although soluble MICA/B are produced from both HCC and premalignant cirrhotic livers, therapeutic intervention for HCC can reduce the levels of soluble MICA and thereby upregulate the expression of NKG2D. Cancer therapy may have a beneficial effect on NKG2D-mediated antitumor immunity. (*Cancer Sci* 2008; 99: 1643–1649)

**M**HC class I-related chain A and B, glycoproteins expressed on the cellular membrane, are ligands for NKG2D expressed on a variety of immune cells.<sup>(1)</sup> In contrast to classical MHC class I molecules, MICA/B are expressed rarely on normal cells but frequently on tumor cells, including colon cancer, prostate cancer, HCC, and brain tumors.<sup>(2–5)</sup> The engagement of MICA/B and NKG2D strongly activates NK cells and costimulates T cells, enhancing their cytolytic ability and cytokine production.<sup>(6)</sup> Thus, the MICA/B–NKG2D pathway is an important mechanism by which the host immune system recognizes and kills transformed cells.<sup>(7)</sup> In addition to those membrane-bound forms, MICA/B are also cleaved proteolytically from tumor cells and appear as soluble forms in sera of patients with malignancy.<sup>(8–10)</sup> The levels of NKG2D expression tend to be decreased in patients with high levels of soluble MICA/B.<sup>(4)</sup> In addition, sera from those patients can downregulate NKG2D expression *in vitro*.<sup>(5,11)</sup> These data

suggest that soluble MICA/B in the circulation downregulate NKG2D expression and disturb NKG2D-mediated antitumor immunity, raising the possibility that cancer therapy might reduce the serum levels of soluble MICA/B and thereby improve the NKG2D-related immune environment. However, this possibility has not been addressed directly by examining soluble MICA/B and NKG2D expression in a cohort of patients before and after cancer therapy. Furthermore, recent reports by Holdenrieder *et al.* demonstrating that soluble MICA/B are increased not only in malignant disease but also in some benign diseases, such as of the gastrointestinal tract, gynecologic organs, and lungs, raise questions about the impact of cancer therapy on modulating soluble MICA/B levels.<sup>(12,13)</sup>

Hepatocellular carcinoma is one of the leading causes of cancer death worldwide. Chronic liver disease caused by hepatitis virus infection and non-alcoholic steatohepatitis leads to a pre-disposition for HCC; liver cirrhosis, in particular, is considered to be a premalignant condition.<sup>(14,15)</sup> With regard to treatment, surgical resection or percutaneous techniques such as ethanol injection and radiofrequency ablation are considered to be choices for curable treatment of localized HCC, whereas TAE is a well-established technique for unresectable HCC.<sup>(16)</sup> We reported previously that soluble MICA could be detected in sera of HCC patients.<sup>(17)</sup> However, the clinical significance of the soluble forms of NKG2D ligands in liver disease has not yet been established in a comprehensive manner, because the previous study was conducted on a small number of patients, did not include patients with premalignant conditions such as liver cirrhosis, and did not analyze its closely related molecule MICB. Furthermore, influences of therapeutic intervention on soluble NKG2D ligands in patients have been unclear. In the present study, we examined soluble MICA and soluble MICB in sera from a large number of patients with chronic liver diseases and HCC and their impact on NKG2D expression on immune cells during TAE therapy for HCC.

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Abbreviations: APC, allophycocyanin; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HCC, hepatocellular carcinoma; MFI, mean fluorescence intensity; MICA/B, major histocompatibility complex (MHC) class I-related chain A and B; NK, natural killer; NKG2D, natural killer group 2, member D; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; TAE, transcatheter arterial embolization; TNM, tumor node metastasis.

**Table 1. Control and patient characteristics**

Characteristic	Healthy control	Chronic hepatitis	Liver cirrhosis	HCC
Number	104	141	104	232
Sex (male/female)	49/55	78/63	60/44	177/55*
Age (years)	62 ± 15	55 ± 13**	61 ± 12	68 ± 9***
Etiology				
HBV/HCV	–	27/107	12/78	37/187
Alcohol/NASH	–	0/5/	2/1/	4/0/
AIH/PBC/others	–	2/0/0	1/6/4	0/0/3
Child–Pugh (A/B/C)	–	–	34/27/26	131/84/17****
TNM stage (I/II/III/IV)	–	–	–	59/68/64/39

AIH, autoimmune hepatitis; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NASH, non-alcoholic steatohepatitis; PBC, primary biliary cirrhosis; TNM, tumor node metastasis.  
 \* $P < 0.05$  vs control, hepatitis, and cirrhosis by  $\chi^2$ -test; \*\* $P < 0.05$  vs control, cirrhosis, and HCC by ANOVA and post hoc Bonferroni test; \*\*\* $P < 0.05$  vs control, hepatitis, and cirrhosis by ANOVA and post hoc Bonferroni test; \*\*\*\* $P < 0.05$  vs cirrhosis by  $\chi^2$ -test.

**Materials and Methods**

**Stock sera from patients with chronic liver disease and HCC.** We used frozen stock sera obtained from consecutive patients with chronic liver disease who had been registered at our institute from February 2002 to April 2006. They included 141 patients with chronic hepatitis, 104 patients with liver cirrhosis, and 232 patients with HCC. The differential diagnosis between chronic hepatitis and liver cirrhosis was basically from liver biopsy ( $n = 98$ ), but for those who had not undergone biopsy the diagnosis was based on clinical findings from the aspartate aminotransferase/platelet ratio index (APRI) score.<sup>(18)</sup> Diagnosis of HCC was based on unequivocal clinical and imaging data. The control group consisted of 104 healthy volunteers of an age range similar to the liver cirrhosis group. Table 1 summarizes the control and patient characteristics of age, sex, etiology of liver disease, Child–Pugh classification, and TNM staging of HCC. Child–Pugh classification is a well-established index for progression of liver disease in cirrhotic patients where A, B, and C indicate compensated cirrhosis, mildly decompensated cirrhosis, and severely decompensated cirrhosis, respectively. The TNM staging adopted in the present study was that modified by the Liver Cancer Study Group of Japan.<sup>(16)</sup>

**Detection of soluble MICA/B by ELISA.** Serum levels of soluble MICA and soluble MICB were determined differentially by commercially available ELISA kits (R & D Systems, Minneapolis, MN, USA). In preliminary experiments, we determined the median intra-assay variation ( $n = 5$ ) to be between 3.5 and 5.6% for soluble MICA and between 2.4 and 7.8% for soluble MICB, and the median interassay variation ( $n = 5$ ) to be between 12.8 and 18.9% for soluble MICA and between 15.2 and 18.7% for soluble MICB.

**Detection of MICA/B on liver tissues by immunohistochemistry.** The human liver tissues examined were one normal liver, three from those at fibrosis stages 1 and 2 of chronic hepatitis, five from liver cirrhosis (fibrosis stage 4) patients, and five from HCC patients. Paraffin-embedded liver sections were deparaffinized, heat-inactivated by a microwave oven and then subjected to immunohistochemical staining using the ABC procedure (Vector Laboratories, Burlingame, CA, USA). The primary antibody used was 6D4 monoclonal antibody, which recognizes the  $\alpha 1$  and  $\alpha 2$  domains of MIC molecules shared by both MICA and MICB.<sup>(2)</sup> To confirm the specificity of the staining, the 6D4 antibody was incubated with recombinant MICA (R & D Systems) for 2 h and then applied to liver sections in parallel with staining of the primary antibody as the absorption test.

**Table 2. Characteristics of hepatocellular carcinoma patients**

Characteristic	TAE-treated group	Non-treated group
Number	38	21
Sex (male/female)	28/10	17/4
Age (years)	75 ± 11	74 ± 8
Etiology (HBV/HCV)	2/36	1/21
Child–Pugh (A/B/C)	29/9/0	16/5/0
TNM stage (I/II/III/IV)	4/20/14/0	2/11/8/0

HBV, hepatitis B virus; HCV, hepatitis C virus; TAE, transcatheter arterial embolization; TNM, tumor node metastasis.

**Detection of membrane-bound and soluble forms of MICA/B on cultured cells.** HepG2 hepatoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Human non-transformed hepatocytes were purchased from Cambrex Bio Science (Charles City, IA, USA) and cultured according to the manufacturer's instructions. For detection of membrane-bound MICA/B, a single-cell suspension was stained with PE-labeled 6D4 monoclonal (R & D Systems) antibody, fixed with 2% paraformaldehyde, and then subjected to flow cytometric analysis. The culture supernatants were subjected to analysis of soluble forms of MICA and MICB using the above-mentioned ELISA assay.

**Patients with HCC and TAE therapy.** Thirty-eight patients with HCC admitted to our institution for TAE therapy were enrolled prospectively in the present study. TAE was carried out by the standard procedure using an emulsion of farnorubicin and lipiodol followed by gelatin sponge particles. Blood samples were collected before and 2 weeks after TAE therapy. Twenty-one patients with HCC, matching the TAE group with respect to TNM stage and Child–Pugh score, were also enrolled as controls (Table 2). Blood samples were collected twice at a 2-week interval. Written informed consent was received from all patients and the study protocol was approved by the Ethical Committee of Clinical Research at Osaka University Hospital.

**Natural killer cell analysis.** PBMC were isolated from heparinized venous blood by a standard procedure. PBMC were stained with FITC-labeled anti-CD3 antibody, APC-labeled anti-CD56 antibody, and PE-labeled anti-NKG2D antibody. They were also stained with FITC-labeled anti-CD3 antibody, APC-labeled anti-CD8 antibody, and PE-labeled anti-NKG2D antibody. All antibodies were purchased from Becton Dickinson (San Jose, CA, USA). NKG2D expression on NK cells (defined as CD56-positive and CD3-negative cells) and CD8-positive T cells (defined as CD3-positive and CD8-positive cells) were analyzed by flow cytometry. As a control, corresponding fluorescence-labeled irrelevant antibodies were used. As most NK and CD8-positive T cells express NKG2D, the levels of expression were evaluated by the mean fluorescence intensity of the stained cells.

**Statistics.** Values were expressed as the median and interquartile range as a box plot, and the 10th and 90th percentiles as a horizontal bar. For comparison of more than two groups, the Kruskal–Wallis rank sum test was used. If the Kruskal–Wallis test was significant, post hoc multiple comparisons were carried out using the Steel–Dwass procedure. Differences between pretreatment and post-treatment values were tested by paired  $t$ -test.  $P < 0.05$  was considered statistically significant.

**Results**

**Soluble MICA and soluble MICB in chronic liver disease and HCC.** Soluble MICA and soluble MICB were assessed in sera from patients with chronic hepatitis, liver cirrhosis, and HCC as well as healthy volunteers. There was a stepwise increase in the levels of both soluble MICA and soluble MICB from hepatitis

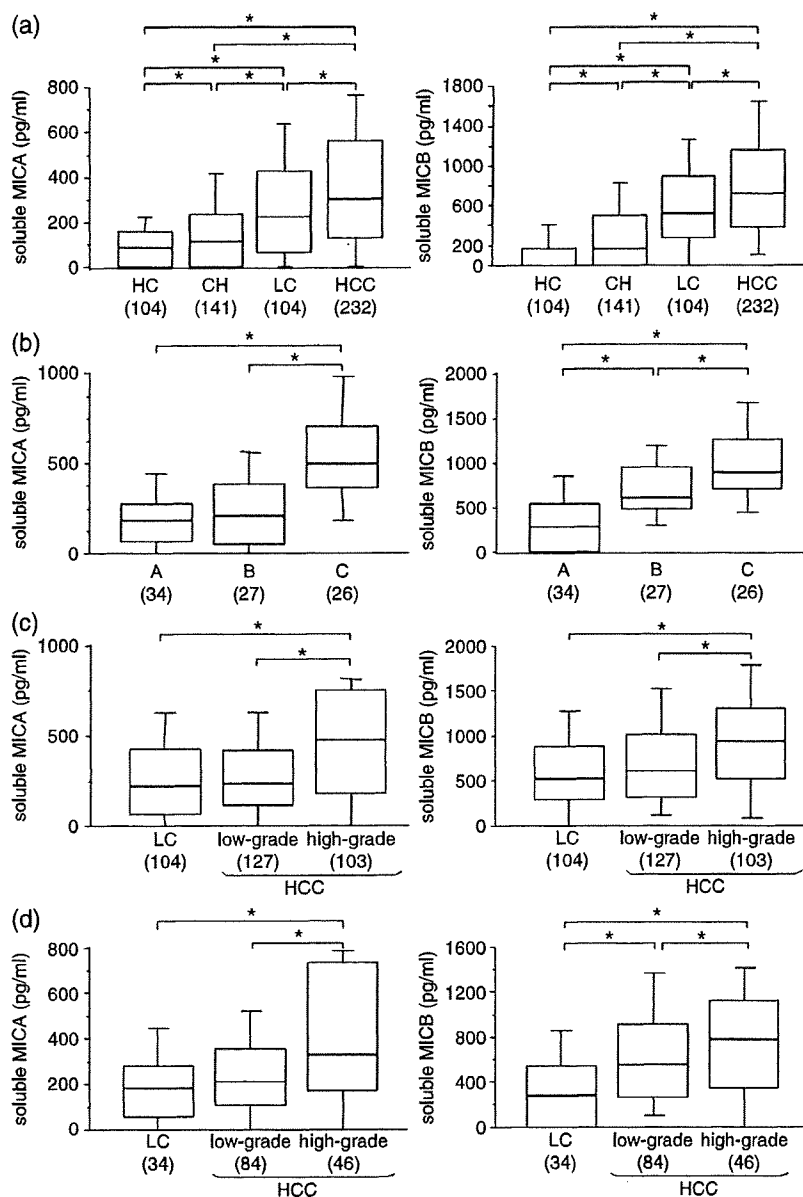


Fig. 1. Serum levels of soluble major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) in chronic liver disease and hepatocellular carcinoma (HCC). (a) Soluble MICA and soluble MICB levels in serum samples of healthy controls (HC), chronic hepatitis (CH), liver cirrhosis (LC), and HCC. (b) Soluble MICA and soluble MICB are associated with the progression of liver disease. Data on cirrhotic patients were stratified based on Child-Pugh classification. (c,d) Soluble MICA and soluble MICB are associated with the progression of tumors. (c) Data on cirrhosis and HCC patients were classified into three groups: patients with absence of HCC (cirrhosis), patients with low-grade HCC (tumor node metastasis [TNM] stage I and II), and patients with high-grade HCC (TNM stage III and IV). (d) To exclude the possibility of progression of liver disease being involved in increase in soluble MICA/B, soluble MICA/B levels were compared among the three groups of Child-Pugh classification A. Data are represented as box plots (median values, 10th, 25th, 75th, and 90th percentiles). The number in parentheses indicates the number of patients in each group. \* $P < 0.05$  by Kruskal-Wallis test and post hoc Steel-Dwass test.

to HCC (Fig. 1a). Although the difference between hepatitis patients and healthy volunteers was modest, both of the levels were clearly higher in patients with liver cirrhosis and HCC than in normal volunteers or hepatitis patients. To examine whether the progression of liver disease in cirrhotic patients affects the levels of soluble MICA/B, cirrhotic patients were stratified based on Child-Pugh classification. The levels of both soluble MICA and MICB were increased significantly with the progression of liver disease (Fig. 1b).

Hepatocellular carcinoma often develops from cirrhotic liver and most patients with HCC included in the present study had complications from cirrhosis. To examine whether the development and progression of HCC contributes to increasing soluble MICA/B, patients with liver cirrhosis and those with HCC were classified into three groups: those with an absence of HCC, low-grade HCC (TNM stage I/II) and high-grade HCC (TNM stage III/IV). There was no significant difference in soluble MICA or soluble MICB between patients without HCC and

low-grade HCC patients. However, the high-grade HCC patients showed significantly higher levels of soluble MICA or soluble MICB than patients without HCC or the low-grade HCC patients (Fig. 1c). To exclude the possibility of the progression of liver disease affecting the increases in soluble MICA/B in high-grade HCC, we selected and analyzed only the Child-Pugh A patients. In this subgroup of patients, the levels of soluble MICA/B were also significantly higher with high-grade HCC than with low-grade HCC or the absence of HCC (Fig. 1d). Thus, the progression of liver disease and that of the tumor independently affects the levels of soluble MICA or soluble MICB.

**MICA/B expression in liver tissues and production of soluble MICA/B.** The increase in soluble MICA/B in cirrhotic patients suggests that MICA/B may be expressed in cirrhotic livers. We therefore examined MICA/B expression by immunohistochemistry in various human tissues including normal liver, chronic hepatitis (F1 and F2 stage), liver cirrhosis, and HCC (Fig. 2a). MICA was detected clearly in four of five HCC tissues, agreeing with a

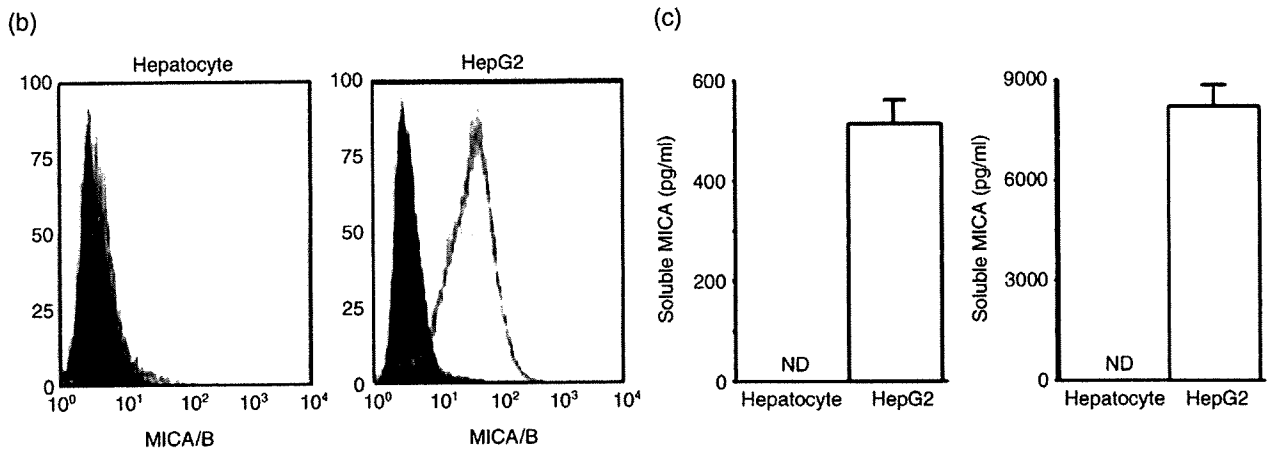
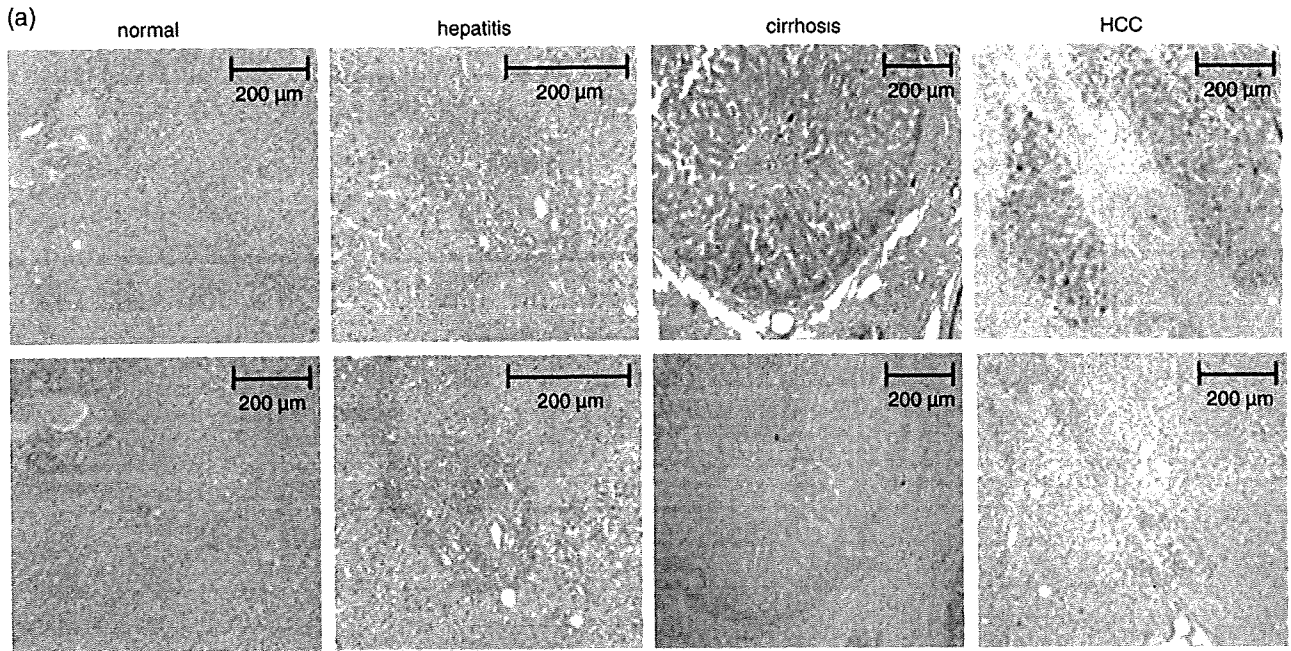


Fig. 2. Expression of major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) and production of their soluble forms. (a) Immunohistochemical detection of MICA/B in liver tissues. Representative staining with anti-MICA/B monoclonal antibody (6D4) is shown for normal liver, chronic hepatitis (F1 stage), liver cirrhosis (F4 stage), and hepatocellular carcinoma (HCC) (upper panel). As a control, 6D4 monoclonal antibody was preabsorbed with recombinant MICA and applied to the neighboring corresponding sections (lower panel). (b) Flow cytometric analysis of surface expression of MICA/B on HepG2 hepatoma cells and non-transformed hepatocytes. Open and closed histograms represent the staining of anti-MICA/B antibody (6D4) and control antibody, respectively. (c) Soluble MICA and soluble MICB released from HepG2 hepatoma cells and non-transformed hepatocytes. Cells were seeded in a subconfluent condition and cultured for 48 h. The culture supernatants were applied for analysis of soluble MICA and soluble MICB by enzyme-linked immunosorbent assay. ND, not detected.

previous report.<sup>(3)</sup> Importantly, hepatocytes in four of five cirrhotic livers were positive for MICA/B, whereas MICA/B were not detected in hepatocytes from normal liver or liver at the early stage of chronic hepatitis.

We also examined the expression of MICA/B on normal hepatocytes and HepG2 hepatoma cells. Flow cytometric analysis revealed that HepG2 cells expressed MICA/B on the cell surface (Fig. 2b). Both soluble forms of MICA and MICB were detected in the supernatant of HepG2 cells cultured for 48 h (Fig. 2c). In contrast, non-transformed hepatocytes expressed MICA/B faintly and soluble MICA/B could not be detected in their culture supernatant. This observation supported the idea that both soluble MICA and soluble MICB are produced from MICA/B-expressing hepatic cells.

**Downregulation of soluble MICA levels by TAE.** The above findings suggest that soluble MICA/B are produced from cirrhotic livers as well as HCC. In addition, the progression of the tumor is an important determinant of soluble MICA/B independent of the progression of liver disease. We then asked the question of whether therapeutic intervention of HCC would reduce the levels of soluble MICA or soluble MICB and affect the levels of NKG2D expression on immune cells. We prospectively analyzed the levels of soluble MICA/B and NKG2D expression in 38 HCC patients before and 2 weeks after TAE therapy. As a control, 21 HCC patients who did not receive TAE therapy but were matched to the TAE group with respect to clinical characteristics were analyzed over a 2-week interval.

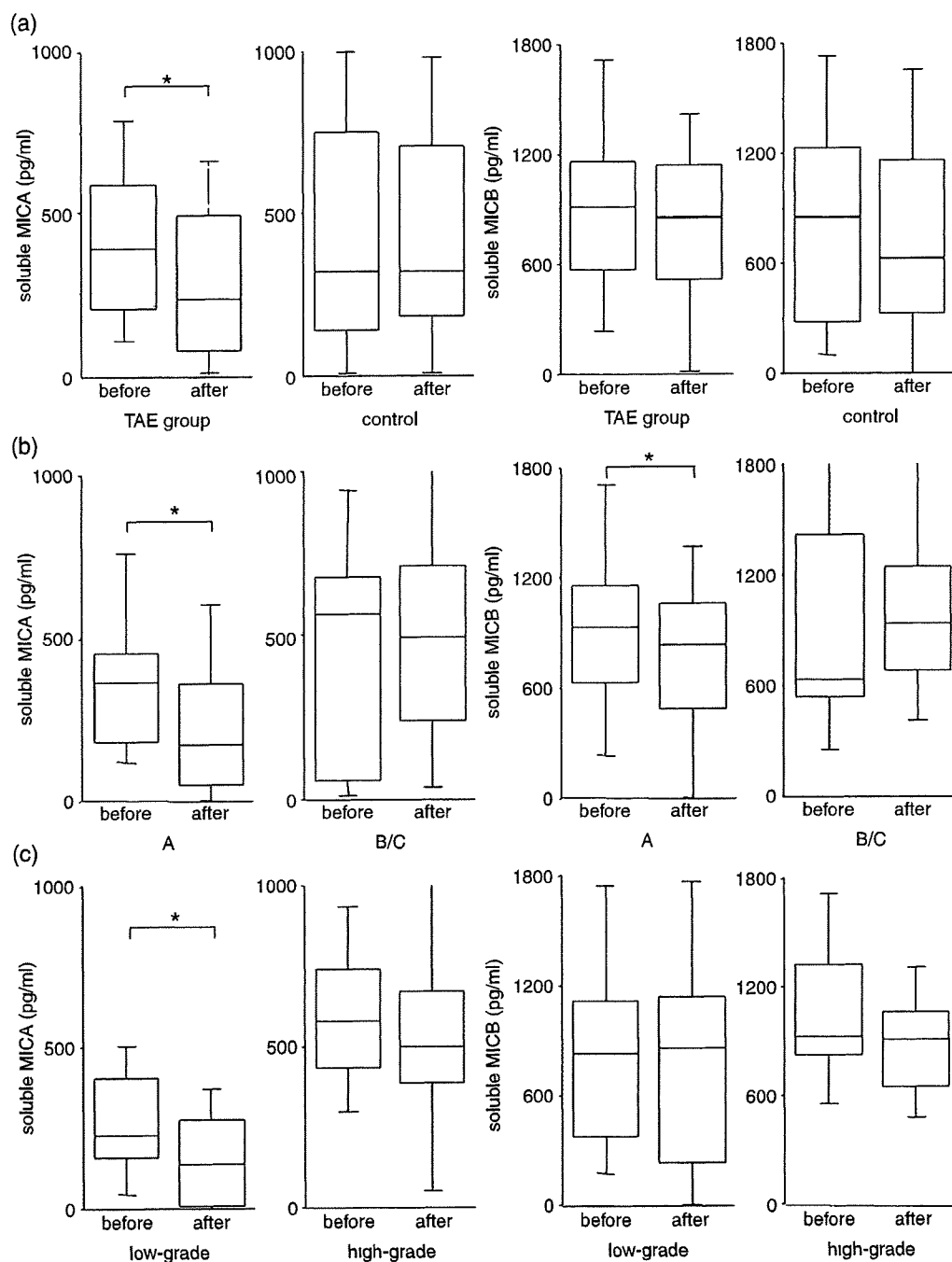


Fig. 3. Soluble major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) during transcatheter arterial embolization (TAE) therapy. (a) Soluble MICA and soluble MICB were measured for 38 patients before and 2 weeks after TAE therapy. Twenty-one patients who did not receive TAE therapy served as controls, with soluble MICA/B being measured twice with a 2-week interval. (b) TAE-treated patients were divided into two groups: Child-Pugh A ( $n = 29$ ) and Child-Pugh B and C ( $n = 9$ ). (c) TAE-treated patients were divided into two groups: low-grade hepatocellular carcinoma (HCC) ( $n = 24$ ) and high-grade HCC ( $n = 14$ ). \* $P < 0.05$  by paired  $t$ -test.

In the TAE-treated group, the levels of soluble MICA were decreased significantly 2 weeks after TAE therapy compared with those before TAE (Fig. 3a). In contrast, TAE did not affect the levels of soluble MICB. Neither the levels of soluble MICA nor those of soluble MICB changed during the 2-week interval in HCC patients not receiving TAE therapy. As the progression of liver disease and that of the tumor affects the levels of soluble

MICA/B, TAE-treated patients were divided according to their Child-Pugh stage or tumor stage. The levels of soluble MICA decreased significantly after TAE therapy in Child-Pugh A patients but not in Child-Pugh B and C patients (Fig. 3b). Interestingly, Child-Pugh A patients showed a significant decrease even in soluble MICB levels after TAE therapy but Child-Pugh B and C patients did not. As for tumor stage, a significant decrease in

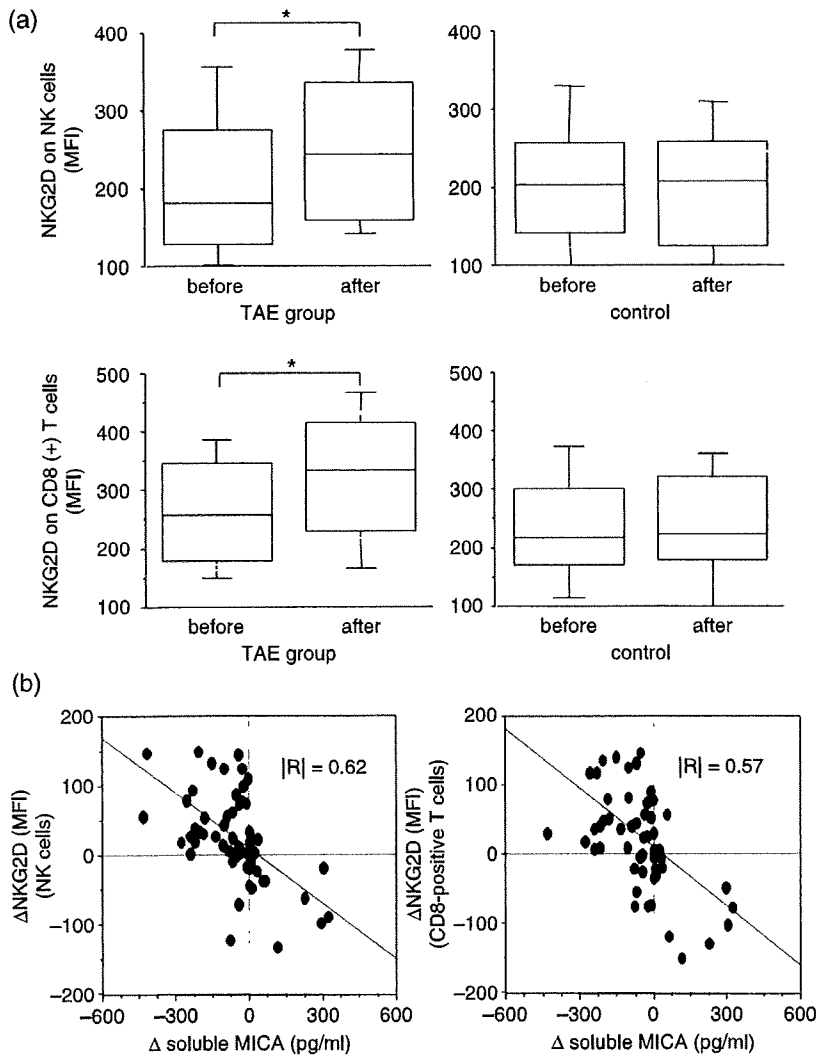


Fig. 4. Natural killer group 2, member D (NKG2D) expression during transcatheter arterial embolization (TAE) therapy. (a) NKG2D expression on natural killer (NK) cells and CD8-positive T cells. NKG2D expression on immune cells was analyzed in 38 patients before and 2 weeks after TAE therapy. Twenty-one patients who did not receive TAE therapy served as a control by measuring NKG2D expression for 2-week interval. NKG2D expression on each cell type was evaluated by mean fluorescence intensity (MFI). \* $P < 0.05$  by paired *t*-test. (b) Correlation between change of soluble MICA and that of NKG2D expression on NK cells or CD8-positive T cells.

soluble MICA levels after TAE therapy was found in low-grade HCC but not in high-grade HCC (Fig. 3c). The levels of MICB did not change in the low-grade or high-grade HCC groups.

**Upregulation of NKG2D expression by TAE.** The number of PBMC as well as NK and T-cell subsets did not change over the 2-week interval in both the control and TAE-treated patients (data not shown). However, the levels of NKG2D expression on NK and CD8-positive T cells increased significantly upon TAE therapy, but not in the control group (Fig. 4a). To examine the involvement of soluble MICA in NKG2D expression, we analyzed the relationship of changes between soluble MICA and NKG2D expression in HCC patients. Change in soluble MICA was correlated inversely with changes in NKG2D expression on NK and CD8-positive T cells (Fig. 4b). There was no significant correlation between changes in soluble MICB and NKG2D expression (data not shown).

## Discussion

In the present study, we demonstrated that soluble MICA/B increases with the progression of chronic liver disease as well as the progression of HCC. Increases in soluble MICA/B in advanced stages of tumors have been reported in some malignancies.<sup>(12)</sup> However, little is known about soluble MICA/B in the premalignant

condition. Recently, Holdenrieder *et al.* examined soluble MICA/B levels in benign as well as malignant diseases from heterogeneous organs.<sup>(12,13)</sup> They found that benign diseases, such as gastrointestinal tract adenoma, pulmonary infectious disease, and gynecologic benign tumors, showed intermediate levels of soluble MICA/B between healthy controls and malignant disease. Our present findings not only agree with theirs, but also provide evidence that soluble MICA/B increases in premalignant conditions such as liver cirrhosis.

Malignant disease is known to lead frequently to the expression of MICA/B.<sup>(2)</sup> In contrast, their expression in premalignant tissues has not been fully elucidated. In the present study, MICA/B were found to be expressed in liver cirrhosis as well as HCC tissues, but not in the early stages of chronic hepatitis or in normal liver. This finding is consistent with the tendencies observed for serum-soluble MICA/B levels in chronic liver disease and HCC. Analysis of cultured cells also revealed that MICA/B expressed on hepatoma cells is released spontaneously into the culture supernatant as soluble forms, supporting the idea that MICA/B expressed in the liver may be released into the circulation. In contrast, MICA/B were not expressed on nor released from cultured non-transformed hepatocytes, which is consistent with the *in vivo* immunohistochemical finding. An issue to be resolved is the underlying mechanism by which non-transformed



hepatocytes express and release MICA/B in pathological conditions such as liver cirrhosis. Recently, it was reported that non-transformed pulmonary epithelial cells can express MICA/B under oxidative stress-inducing conditions.<sup>(19)</sup> It was also reported that MICA/B are upregulated in non-tumor cell lines by genotoxic stress.<sup>(20)</sup> It has been speculated that oxidative and genotoxic stresses may accumulate in hepatocytes in chronic diseased liver. Thus, it is possible that those stresses may contribute to MICA/B expression in chronic diseased liver. Further study is needed to clarify this issue.

MICA/B expression in the premalignant condition raises the question of which contributes more to the production of soluble MICA/B, malignant tissues or non-malignant tissues. To address this question we analyzed the levels of soluble MICA/B in HCC patients before and after therapeutic intervention. Among treatments for HCC, TAE is a well-established technique for unresectable, advanced HCC.<sup>(16)</sup> To include HCC patients who show relatively high levels of soluble MICA/B, we chose a cohort of patients who received the TAE therapy in the present study. The data indicated that the levels of soluble MICA, but not those of soluble MICB, decreased after TAE therapy. It is not clear why soluble MICB did not change during TAE therapy. One possibility is that soluble MICB production from non-tumor livers may be relatively high compared with that of soluble MICA. In our subpopulation analysis, Child-Pugh A patients showed a significant decrease in soluble MICB levels after TAE therapy. In general, TAE therapy is more effective for Child-Pugh A patients than Child-Pugh B or C patients because the former is better able to tolerate the large dose of lipiodol emulsion and gelatin sponge that is necessary for efficient antitumor effect. Indeed, Child-Pugh A patients in our cohort showed a larger decrease in  $\alpha$ -fetoprotein levels after TAE therapy than Child-Pugh B and C patients, although the difference did not reach a significant level (our unpublished data). Thus, TAE therapy might reduce the levels of soluble MICB when it achieves substantial antitumor effect. Most importantly, the data also indicated that NKG2D expression on immune cells was clearly ameliorated with TAE therapy. Furthermore, there was an inverse correlation between a reduction in soluble MICA and upregulation of NKG2D, suggesting the link between soluble MICA and NKG2D expression in cancer patients.

It is generally speculated that soluble MICA/B produced from tumors may deactivate NKG2D-mediated immune responses.<sup>(8,9)</sup> *In vitro* experiment indicates that soluble MICA could down-regulate NKG2D expression and effector cell function. However, the regulation by soluble forms of NKG2D ligands would be more complicated *in vivo*. First, soluble forms of NKG2D ligands could be produced not only from malignant tissues but also from non-malignant tissues, as shown in the present study. Second, MHC-encoded MICA/B may not be the sole family of proteins serving as NKG2D ligands. Non-MHC-encoded UL16-binding proteins also act as NKG2D ligands and were very recently found to be cleaved proteolytically from tumor cells.<sup>(21)</sup> The present study provides evidence that soluble MICA is derived, at least in part, from HCC and regulates NKG2D expression on NK and CD8-positive T cells. Although several species of soluble NKG2D ligands may exist in the circulation, the present study suggests that soluble MICA regulates NKG2D expression directly in cancer patients.

In conclusion, soluble MICA and MICB are significantly increased in the sera of patients not only with HCC but also with chronic liver disease. Soluble MICA/B increases together with the progression of liver disease as well as the tumor. Therapeutic intervention for HCC leads to reduction of soluble MICA levels in association with upregulation of NKG2D on immune cells, offering *in vivo* evidence of soluble MICA regulating NKG2D expression. Thus, cancer therapy may have a beneficial effect on the NKG2D-mediated immune response even if some of the soluble NKG2D ligands are produced from non-cancerous premalignant tissues.

#### Acknowledgments

We sincerely thank Dr Veronika Groh and Dr Thomas Spies (Fred Hutchinson Cancer Research Center, Seattle, WA, USA) for providing 6D4 antibody and Dr Alexander Steinle (University Tübingen, Tübingen, Baden-Württemberg, Germany) for providing the RSV-MICA\*04 and control plasmids. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan and a Grant-in-Aid for Research on hepatitis and bovine spongiform encephalopathy (BSE) from the Ministry of Health, Labor and Welfare, Japan.

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## Supportive Role Played by Precore and PreS2 Genomic Changes in the Establishment of Lamivudine-Resistant Hepatitis B Virus

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**Background.** Hepatitis B virus (HBV) establishes lamivudine resistance via the resistance-causative rtM204V/I mutation and the replication-compensatory rtL180M mutation. However, both lamivudine-resistant viruses with and those without rtL180M can exist in clinical settings. To elucidate the differences between viruses with and those without rtL180M, we conducted full-length sequencing analysis of HBV derived from patients with type B chronic hepatitis showing lamivudine resistance.

**Methods.** The full-length HBV DNA sequences derived from 44 patients showing lamivudine resistance were determined by polymerase chain reaction direct sequencing. Viral replicative competence was examined by in vitro transfection analysis using various HBV-expressing plasmids.

**Results.** Throughout the HBV genome, a precore-defective A1896 mutation and a short deletion in the preS2 gene were detected more frequently in viruses without rtL180M than in those with it (64% vs. 17% [ $P < .005$ ] and 50% vs. 10% [ $P < .01$ ], respectively). In vitro transfection analysis revealed that the level of reduction in intracellular viral replication caused by the introduction of lamivudine resistance-associated mutations was lower in precore-defective and preS2-deleted viruses than in wild-type virus.

**Conclusions.** Both the precore-defective mutation and the preS2 deletion may play a supportive role in the replication of lamivudine-resistant HBV, which may be a reason for there being no need for the compensatory rtL180M mutation in lamivudine-resistant HBV possessing the precore and preS2 genomic changes.

Therapeutic concepts for hepatitis B virus (HBV) infection have been strikingly modified by the introduction of nucleos(t)ide analogues. Nucleos(t)ide analogues, such as lamivudine, adefovir dipivoxil, entecavir, tenofovir disoproxil fumarate, emtricitabine, telbivudine, and clevudine, have been shown to lead to suppression of viral replication and improvement of liver diseases in chronic HBV infection [1–10]. However, the effective-

ness of nucleos(t)ide analogues is debilitated by the emergence of drug-resistant mutant virus. Treatment with lamivudine has been shown to lead to a higher rate of emergence of drug-resistant virus than with other newly developed nucleos(t)ide analogues, such as adefovir dipivoxil and entecavir [11–14]. The incidence of lamivudine resistance has been reported to be 24% at 1 year and 70% at 4 years of therapy [11].

Lamivudine resistance is known to be caused by a point mutation within the reverse transcriptase (rt) domain of the HBV polymerase gene, either rtM204V or rtM204I [15–17]. In addition, an rtL180M mutation has been shown to be frequently found together with the rtM204V/I mutation associated with lamivudine resistance [15–17]. Previous studies using in vitro transfection with the HBV-expressing plasmid have demonstrated that the rtM204V/I mutation principally confers lamivudine resistance but results in a decrease in viral replicative activity [18, 19]. It has also been shown that

Received 7 January 2008; accepted 22 April 2008; electronically published 19 August 2008.

Potential conflicts of interest: none reported.

Financial support: Ministry of Education, Culture, Sports, Science, and Technology of Japan (Grant-in-Aid for Scientific Research); Ministry of Health, Labor, and Welfare of Japan (Grant-in-Aid for Research on Hepatitis and BSE).

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The Journal of Infectious Diseases 2008; 198:1150–8

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0022-1899/2008/19808-0009\$15.00

DOI: 10.1086/591941

the rtL180M mutation has no relevance to lamivudine resistance in itself but restores the reduced replicative activity caused by the lamivudine resistance-associated rtM204V/I mutation [20]. In light of these findings, the rtL180M mutation has been recognized as being compensatory for the support of replication in lamivudine-resistant HBV. Among patients with type B chronic hepatitis (CH-B) showing lamivudine resistance, almost all rtM204V mutations have been detected in conjunction with the rtL180M mutation, and rtM204I mutations have been found either in isolation or together with the rtL180M mutation [15–17]. Thus, the compensatory rtL180M mutation is not always necessary for generating replicative-competent lamivudine-resistant HBV in the clinical setting. Virus without the rtL180M mutation is speculated to possess specific features in the genome that support viral replicative activity, compared with virus with the mutation. However, differences between lamivudine-resistant viruses with and those without the rtL180M mutation have not been elucidated.

To clarify this, we determined the nucleotide sequences of full-length HBV DNA in 44 patients with CH-B who showed lamivudine resistance, by means of the direct sequencing method. Differences in the whole HBV genome were comprehensively investigated in relation to the presence or absence of the rtL180M mutation.

## METHODS

**Patients.** The subjects were 44 consecutive patients with CH-B (37 males and 7 females) who received lamivudine therapy and became refractory to it at Osaka University Hospital and National Hospital Organization Osaka National Hospital. At the beginning of therapy, all patients tested positive for hepatitis B surface antigen (HBsAg) and were positive for HBV DNA by a branched DNA assay (Quantiplex HBV DNA; Chiron) or a polymerase chain reaction (PCR)-based assay (Amplicor HB Monitor; Roche Diagnostics). All patients were negative for antibodies to hepatitis C virus and HIV; none showed evidence of alcoholic liver disorder, autoimmune hepatitis, or drug-induced liver injury. Eight patients (18%) had previously received interferon (IFN) therapy.

All patients were treated with 100 mg of lamivudine daily, and liver function tests and monitoring of HBV markers were conducted during follow-up. In 16 patients (36%), natural IFN- $\alpha$  therapy (Sumiferon; Sumitomo Pharmaceuticals) was administered in combination with lamivudine for the initial 24 weeks (total dose, 432 million units). For all 44 patients, the lamivudine-resistant rtM204V/I mutation was detected by a PCR enzyme-linked minisequence assay (Sumitomo Metal Industries) [21] after an initial reduction and subsequent increase in HBV DNA during therapy. All serum samples for sequencing analysis of full-length HBV DNA were collected after the emergence of the lamivudine-resistant mutant virus and were stored

at  $-80^{\circ}\text{C}$  until use. The serum sampling points ranged from 0.8 to 5.5 years (median, 2.7 years) after the commencement of lamivudine therapy. In addition, pairwise serum samples obtained before therapy were used to determine the nucleotide sequences in portions of HBV DNA as baseline controls for 23 patients (52%).

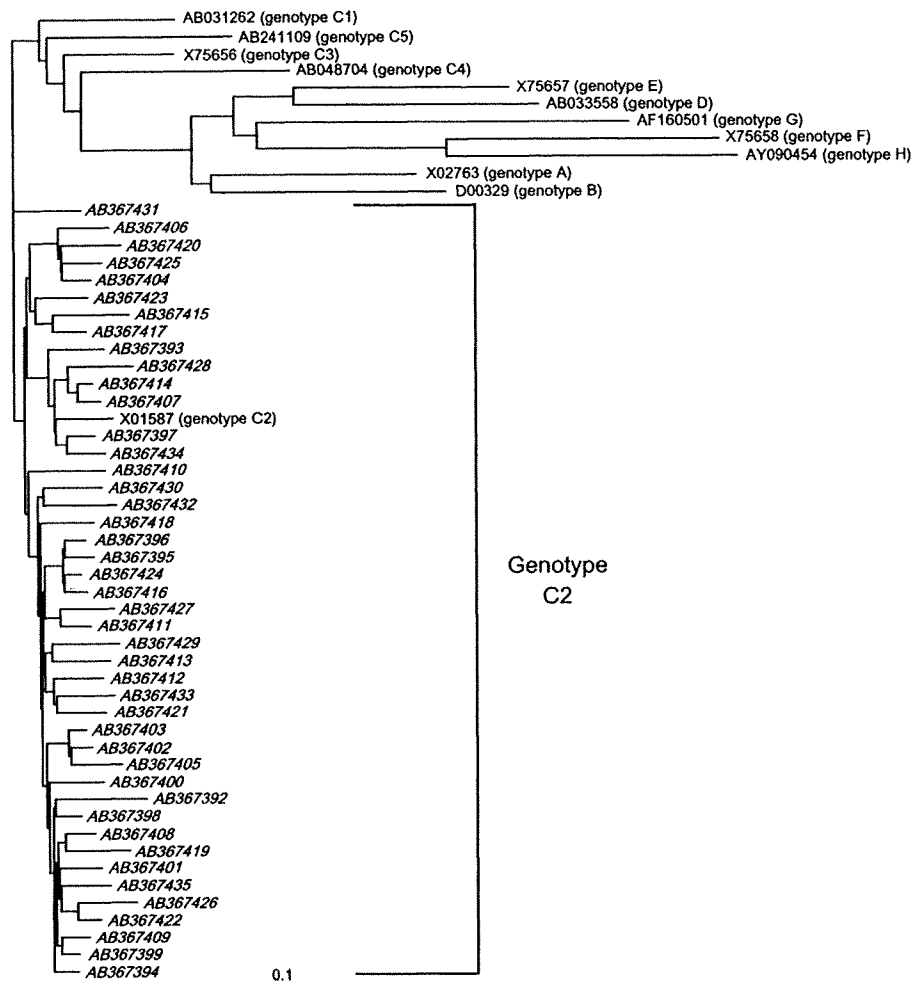
Patient characteristics at the point of analysis were as follows. Age ranged from 25 to 74 years (median, 51 years). Hepatitis B e antigen (HBeAg) was found in 31 patients (70%), and antibody to HBeAg developed in all 13 HBeAg-negative patients (30%). Serum HBV DNA levels ranged from 3.5 to  $>7.6 \log_{10}$  copies/mL (median, 7.2  $\log_{10}$  copies/mL). Serum alanine aminotransferase (ALT) levels ranged from 11 to 393 IU/L (median, 66 IU/L). Chronic hepatitis was diagnosed in 34 patients (77%), cirrhosis in 6 (14%), and hepatocellular carcinoma in 4 (9%), on the basis of liver biopsy and/or abdominal imaging procedures. Informed consent was obtained from all patients.

**Genomic analysis of full-length HBV nucleotide sequences.** From the serum sample, full-length HBV DNA was amplified by PCR and directly sequenced as described elsewhere [22]. The full-length HBV DNA sequences derived from the 44 patients with lamivudine-resistant CH-B (GenBank accession numbers AB367392–AB367435) were aligned together with the 12 representative HBV strains of various genotypes by means of CLUSTALW software. Phylogenetic tree analysis was then conducted [23, 24].

**Plasmid and transfection.** The HBV-expressing plasmid pHBC was derived from the genotype C2 HBV strain adr4 (GenBank accession number X01587) [25]. pHBC was constructed by inserting the 1.2-fold HBV genome into pBluescriptII SK<sup>+</sup>. pHBC-PC and pHBC- $\Delta$ PS2, which were generated by site-directed mutagenesis, possessed the precore-defective A1986 mutation and the short deletion of 45 bp (nt 11–55) within the preS2 gene. Further site-directed mutagenesis was done to introduce rtM204V and rtL180M, rtM204I and rtL180M, or rtM204I alone into pHBC, pHBC-PC, and pHBC- $\Delta$ PS2. pCMV-SEAP was a secreted alkaline phosphatase-expressing plasmid.

For transfection,  $3 \times 10^5$  Huh7 cells were seeded on a 35-mm-diameter culture dish and transfected with 1  $\mu\text{g}$  of various HBV-expressing plasmids and 0.06  $\mu\text{g}$  of pCMV-SEAP, using FuGENE6 reagent (Roche Diagnostics). On day 5, the culture supernatant and cell lysate were collected. Transfection efficiency was evaluated by measuring the secreted alkaline phosphatase activity.

**Detection of viral progeny DNA and antigen in HBV-expressing cells.** For detection of intracellular HBV DNA, cells were lysed in buffer containing 50 mmol/L Tris-Cl (pH 7.5), 1 mmol/L EDTA, and 1% Nonidet-P40. After a 15-min incubation on ice, nuclei were removed by brief centrifugation. Then, the sample was incubated at  $37^{\circ}\text{C}$  for 30 min in the presence of 0.1 mg/mL DNaseI and 10 mmol/L  $\text{MgCl}_2$ . After the reaction was stopped by adding EDTA, the sample was subjected to over-



**Figure 1.** Phylogenetic tree analysis including 44 lamivudine-resistant hepatitis B virus (HBV) strains obtained in the present study and 12 representative HBV strains of various genotypes. All HBV strains are represented as GenBank accession nos., and the 44 lamivudine-resistant HBV strains are indicated by italics.

night incubation at 37°C in buffer containing 1% sodium dodecyl sulfate and 0.5 mg/mL proteinase K, followed by phenol/chloroform extraction and ethanol precipitation. The DNA sample was subjected to Southern blot analysis to detect HBV DNA, using a nonradioactive detection system (Alkphos Direct; GE Healthcare Life Sciences). Finally, the signals were analyzed quantitatively using image analyzing software (ImageJ; version 1.38). To detect extracellular HBV DNA, the transfection was scaled up to the 60-mm-diameter culture dish. After clarification by centrifugation at 8300 g for 30 min, the culture medium (3 mL) was centrifuged through a 20% sucrose cushion at 192,000 g for 4 h, using the Beckmann SW55Ti rotor. Then, DNA was extracted from the pellet and subjected to Southern blot analysis as described above. HBsAg and HBeAg in the culture medium were measured by chemiluminescent immunoassay.

**Statistical analysis.** Statistical analysis was performed by the  $\chi^2$  test, Fisher's exact test, and the Mann-Whitney *U* test. The

results for the in vitro transfection study were examined by 1-way analysis of variance, and pairwise comparison was done by Fisher's protected least significant difference test.  $P < .05$  was considered to indicate statistical significance.

## RESULTS

**Patient clinical characteristics and lamivudine resistance-associated mutations.** All 44 HBV strains obtained from the patients with lamivudine-resistant CH-B comprised 3161–3230 nt in length and belonged to genotype C2, the most prevalent type in Japan (figure 1). As for lamivudine resistance-associated mutations in these strains, the rM204V mutation was observed in 16 strains (36%), whereas the remaining 28 strains (64%) had the rM204I mutation. The compensatory rL180M mutation was found in 30 strains (68%). All 16 strains with rM204V and 14 (50%) of the 28 strains with rM204I possessed the rL180M

**Table 1. Clinical features of patients with lamivudine-resistant type B chronic hepatitis, according to the mutational status of rt180 and rt204.**

Clinical feature	rt180 status		P	rt204 status		P
	rtL180M positive (n = 30)	rtL180M negative (n = 14)		rtM204V (n = 16)	rtM204I (n = 28)	
Age, years	48 (25–74)	55 (27–71)	NS	48 (25–74)	51 (27–71)	NS
Sex, M/F, no.	25/5	12/2	NS	13/3	24/4	NS
Liver disease, chronic hepatitis/cirrhosis/hepatocellular carcinoma, no.	26/3/1	8/4/2	NS	13/2/1	21/5/2	NS
ALT level, IU/L	66 (11–331)	67 (25–393)	NS	85 (17–261)	54 (11–393)	NS
HBeAg, positive/negative, no.	23/7	8/6	NS	13/3	18/10	NS
HBV DNA level, log <sub>10</sub> copies/mL	7.5 (3.5 to >7.6)	7.1 (3.6 to >7.6)	NS	7.5 (3.8 to >7.6)	7.1 (3.5 to >7.6)	NS
Previous IFN therapy, no. (%)	6 (20)	2 (14)	NS	3 (19)	5 (18)	NS
Combination therapy with IFN, no. (%)	10 (33)	6 (43)	NS	4 (25)	12 (43)	NS
Duration of lamivudine administration until point of analysis, years	2.9 (1.5–5.5)	2.2 (0.8–4.8)	NS	2.9 (1.5–5.5)	2.2 (0.8–4.8)	NS

**NOTE.** Data are median (range) values, unless otherwise indicated. HBeAg, hepatitis B e antigen; IFN, interferon; NS, not significant.

mutation, in agreement with previous reports with respect to the emergence pattern of the rtM204V/I and rtL180M mutations [15–17].

Various patient clinical characteristics were first correlated with the presence or absence of the rtL180M mutation or with the alternative of the rtM204V or rtM204I mutation in our 44 patients with CH-B (table 1). No differences were observed between patients with and those without the rtL180M mutation with respect to age, sex ratio, disease severity, ALT level, HBeAg positivity, serum HBV DNA level, frequency of previous IFN therapy, frequency of combination therapy with IFN, and total duration of lamivudine administration until the point of analy-

sis. Also, there were no significant differences concerning these 9 characteristics between patients with virus having the rtM204V mutation and those with virus having the rtM204I mutation.

**Genomic changes throughout the HBV genome associated with lamivudine resistance-associated mutations.** Next, the genomic changes, which were significantly correlated with the occurrence of rtL180M or the preference for rtM204V or rtM204I, were investigated for the 44 HBV strains derived from the patients. As shown in table 2, 8 mutations and 1 deletion were identified as viral genomic changes significantly associated with the presence or absence of rtL180M. Among them, the A1896 mutation, which forms the in-frame stop codon in the

**Table 2. Differences in the viral genome between lamivudine-resistant hepatitis B virus (HBV) strains with and those without the rtL180M mutation.**

Viral genomic changes	Consensus nucleotide <sup>a</sup>	Amino acid substitution	rtL180M, no. (%)		P
			Positive (n = 30)	Negative (n = 14)	
<b>Mutation</b>					
A373	C	Pol-L428M (rtL82M)	0 (0)	3 (21)	<.05
T619	C	None	0 (0)	3 (21)	<.05
G739	T	Pol-M550V (rtM204V), surface-I95R	16 (53)	0 (0)	<.001
T/C/A741	G	Pol-M550I (rtM204I), surface-W96L/S/stop	14 (47)	14 (100)	<.001
A1896	G	Precore-W28stop	5 (17)	9 (64)	<.005
T2102	C	None	0 (0)	3 (21)	<.05
A/G2660	C	Pol-N118K	0 (0)	3 (21)	<.05
A2860	T	PreS1-S5T, pol-V184D <sup>b</sup>	0 (0)	4 (29)	<.01
<b>Deletion</b>					
6–54-bp deletion within nt 1–55		Truncation of 2–18 amino acids in preS2 <sup>c</sup>	3 (10)	7 (50)	<.01

<sup>a</sup> Consensus nucleotides are derived from the genotype C2 HBV strain adr4 (GenBank accession no. X01587) [25].

<sup>b</sup> One patient had the pol-V184Q amino acid substitution due to a mutation in the adjacent nucleotide position.

<sup>c</sup> Detailed patterns of the preS2 deletion are shown in figure 2.