Author's personal copy

Table 1
Prevalence of hepatitis B virus (HBV) markers among 487 residents in Eastern Bolivia

Features	Total ($n = 487$)	Japanese immigrants ($n = 287$)	Native Bolivians ($n = 200$)	P^a
Sex (M/F)	193/294	120/167	72/128	NS
Median age[b]	51 (8–95)	63 (8–95)	37 (8–67)	<0.0001
Anti-HBc[c]	217 (44.5)	162 (56.4)	55 (27.5)	<0.0001
Anti-HBs[c]	162 (33.3)	108 (37.6)	53 (26.5)	0.011
HBsAg[c]	22 (4.5)	10 (3.4)	12 (6)	NS
HBV DNA[c]	17 (3.5)	9 (3.1)	8 (4)	NS

[a] p-value, Japanese immigrants vs. native Bolivians.
[b] Years (range).
[c] n (%).

al., 2004) The genetic diversity of HBV and the geographical distribution of its subgenotypes provide a tool to reconstruct the evolutionary history of HBV (Norder et al., 2004). The eight genotypes of HBV have also been divided into subgenotypes with distinct ethnic or geographical origin (Campos et al., 2005; Sakamoto et al., 2006). The most prevalent genotype of Central and South America, genotype F is subdivided into two subgenotypes, F1 and F2 (Norder et al., 2003) and a total of five clusters. Subgenotype F1 includes two clusters, 1a and 1b, with strains isolated mainly in Central America and Argentina, respectively. Subgenotype F2 including three clusters with strains found in Nicaragua, Venezuela and Brazil (cluster II), in Panama, Venezuela, and Columbia (cluster III) and in Argentina and Bolivia (Cluster IV) (Huy et al., 2006; Mbayed et al., 2001).

Although HBV/F is the most prevalent genotype of Central and South America, other HBV genotypes found in different Latin American countries are considered a reflection of migration of human population from other geographical areas into the region (Campos et al., 2005). It is thought that the first Japanese who arrived at the Bolivian territory came by the way of Peru and passing through Andean mountains settled in Amazonas. In 1950s the Japanese community in Bolivia began to grow composed of Colonia Okinawa and Colonia San Juan, both in Santa Cruz (Eastern Bolivia). Keeping in view the inter-marital and cultural contacts with native Bolivians, the inter-communities transmission of HBV genotypes seems a natural phenomenon.

The present study was conducted to examine Japanese immigrants and natives in Bolivia for HBV infection and distribution of HBV genotypes in both communities.

2. Methods

2.1. Subjects

Sera were collected from 487 residents in Eastern Bolivia (randomly selected healthy carriers), these included Japanese immigrants ($n = 287$) and native population ($n = 200$). Japanese immigrants and native Bolivian were defined according to their birth place; Japan and Bolivia, respectively. After isolation of the serum fraction from whole blood, the samples were stored at −40 °C until use. The number of subjects studied, their ages and sexes are summarized in Table 1.

2.2. Serological analysis

HBV serological markers (HBsAg, anti-HBc, and anti-HBs) were examined by chemiluminescence with commercial assay kits (Fujirebio Inc., Tokyo, Japan) (Table 1). Serum samples

Table 2
HBV DNA oligonucleotide primers for complete genomes of genotype F

Primer	Nucleotide sequence (5′–3′)	Position	Polarity
(1) Fragment A			
HBVH55F	TCCTGCTGGTGGCTCC	55–70	Sense
HBVH1801R	GTTGCATGGTGCTGGTGAAC	1820–1801	Antisense
HB6R	AACAGACCAATTTATGCCTA	1803–1784	Antisense
(2) Fragment B			
HBVH1611F	GAGACCACCGTGAACGCCC	1611–1629	Sense
HBVH285R	GCCAGGACACCCGGGTGGTA	304–285	Antisense
HBVH229R	CGAGTCTAGACTCTGTGGTATTGTGAGG	256–229	Antisense
(3) Sequencing primers			
HB2F	TGCTGCTATGCCTCATCTTC	414–433	Sense
HBVH760F	GCCAAATCTGTGCAGCATCTTGAG	760–783	Sense
HB5F	CTCTGCCGATCCATACTGCGGAA	1256–1278	Sense
HBVH1859F	ACTGTTCAAGCCTCCAAGCTGT	1859–1880	Sense
HBVH2415F	GTCGCAGAAGATCTCAATCTC	2415–2435	Sense
HBVH2814F	GGGTCACCATATTCCTGGGAA	2814–2834	Sense

Author's personal copy

Table 3
Clinical data of HBsAg-positive subjects in Eastern Bolivia

S. no.	Nationality	Origin	Age	Sex	ALT	HBV DNA	Genotype EIA and/or Sequencing (Subgenotype)
1	JPN	Nagasaki	60	F	8	+	B (B2)
3	JPN	Nagasaki	54	F	14	+	F (F4)
7	JPN	Nagasaki	68	M	5	−	C
71	JPN	Nagasaki	42	F	4	+	F (F4)
182	JPN	Nagasaki	59	F	7	+	C (C2)
414	JPN	Okinawa	53	F	8	+	C (C2)
577	JPN	Nagasaki	74	F	5	+	C (C2)
583	JPN	Nagasaki	73	F	3	+	C (C2)
584	JPN	Nagasaki	73	M	9	+	F (F4)
585	JPN	Nagasaki	72	M	4	+	F (F4)
51	BOL	San Juan	38	M	7	+	F (F4)
67	BOL	San Juan	39	M	10	+	F (F4)
75	BOL	San Juan	39	F	14	−	F
93	BOL	San Juan	43	F	5	+	F (F4)
95	BOL	San Juan	42	M	5	−	C
102	BOL	San Juan	40	M	4	+	F (F4)
113	BOL	San Juan	44	F	4	+	F (F4)
123	BOL	San Juan	41	M	17	+	C (C2)
168	BOL	San Juan	40	M	7	−	F
192	BOL	San Juan	38	M	16	−	F
196	BOL	San Juan	46	M	9	+	C (C2)
431	BOL	Santa Cruz	52	M	11	+	B (B2)

S. no. = sample number; JPN = native Japanese immigrated to Bolivia; BOL = native Bolivian; ALT = alanine amino transferase.

positive for HBsAg were also examined for their HBeAg status (Fujirebio Inc., Tokyo, Japan) and ALT (Table 3).

2.3. Genotyping of HBV

HBsAg-positive samples were subjected to HBV genotyping using commercially available enzyme-linked immunoassay kits (Institute of Immunology Co. Ltd., Tokyo, Japan). The method allows discrimination among the seven major HBV genotypes (A–G) by monoclonal antibodies targeted to the preS2 epitopes (Usuda et al., 1999). HBV DNA was extracted by QIAamp DNA Blood Mini Kit (Qiagen, Inc., Hilden, Germany) from 100 ml of all HBsAg-positive sera. The complete and/or partial genomes of HBV/F strains were amplified as two partially overlapping fragments in nested PCR using two sets of primers and consecutively were directly sequenced using sequencing primers (Table 2). The sequences for genotype B and C strains were obtained by using the previously reported primers (Sugauchi et al., 2001).

Table 4
Comparison of HBV genotypes, determined by EIA and sequencing

	Genotypes by sequencing				*n*
	F	C	B	ND[a]	
Genotypes by EIA					
F	7			2	9
C		6			6
B			2		2
ND[a]	3	2			5
n	10	8	2	2	22

[a] Not determinable.

2.4. Phylogenetic analysis

PCR products were directly sequenced with prism big dye (Applied Biosystems, Foster City, CA) in an ABI 3100 DNA automated sequencer. All sequences were analyzed in both forward and reverse directions. Complete and partial HBV genomes were assembled using GENETYX Version 11.0 (Software Development Co.). The sequences for phylogenetic analysis were retrieved from DDBJ/EMBL/GeneBank corresponding to the accession numbers mentioned in the trees. The phylogeny of all sequences was carried by Neighbor-Joining method using the Mega software (Version 3).

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number(s) "AB365437–AB365453".

Table 5
Prevalence of BCP, PC mutations among different HBV genotypes

Type of mutation (s)	Genotype B ($n = 2$)	Genotype C ($n = 4$)	Genotype F ($n = 7$)
Age[a]	56 ± 5.6	55 ± 14.7	47 ± 12.6
Sex (M/F)	1/1	2/2	4/3
HBeAg-positive	0	0	0
T1653[b]	0	3 (75)	2 (28.6)
C1753[b]	0	0	6 (85.7)
T1762/A1764[b]	0	2 (50)	2 (28.6)
A1896[b]	0	3 (75)	5 (71.4)
A1899[b]	0	1 (25)	2 (28.6)

[a] Years (mean ± S.D.).
[b] *n* (%).

Author's personal copy

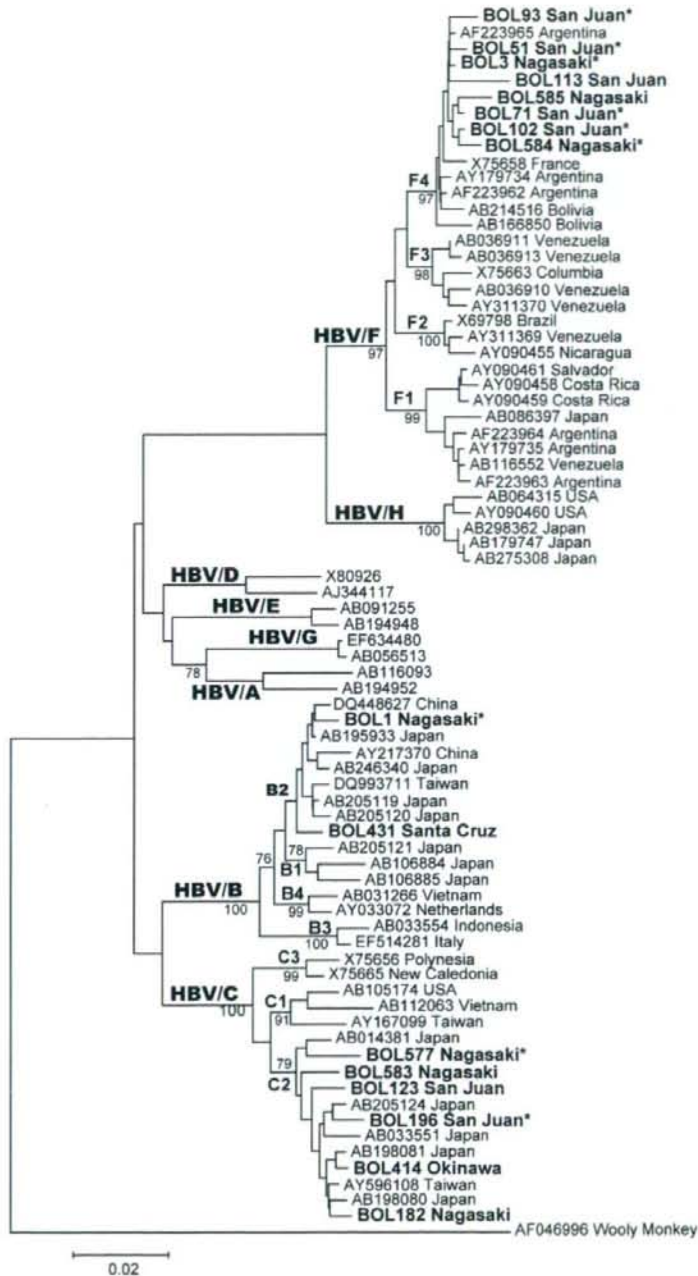

Fig. 1. A phylogenetic analysis of the hepatitis B virus (HBV) in S region of genome, sequences from Bolivia and other countries. The sequences of Japanese immigrants are labeled according to their origin in Japan, i.e., Nagasaki and Okinawa. Similarly, the sequences obtained from native Bolivians are labeled according to their origin in Bolivia, i.e., San Juan and Santa Cruz. The study isolates, e.g. BOL196, BOL577, etc. were subjected to bootstrap resampling with all available complete genome sequences retrieved from the EMBL/DDBJ/GeneBank database. Sequences used for the phylogenetic tree are indicated under the corresponding accession numbers from DDBJ/EMBl/GeneBank and country of origin. (*) Strains were subjected to complete genome analysis (Fig. 2).

Author's personal copy

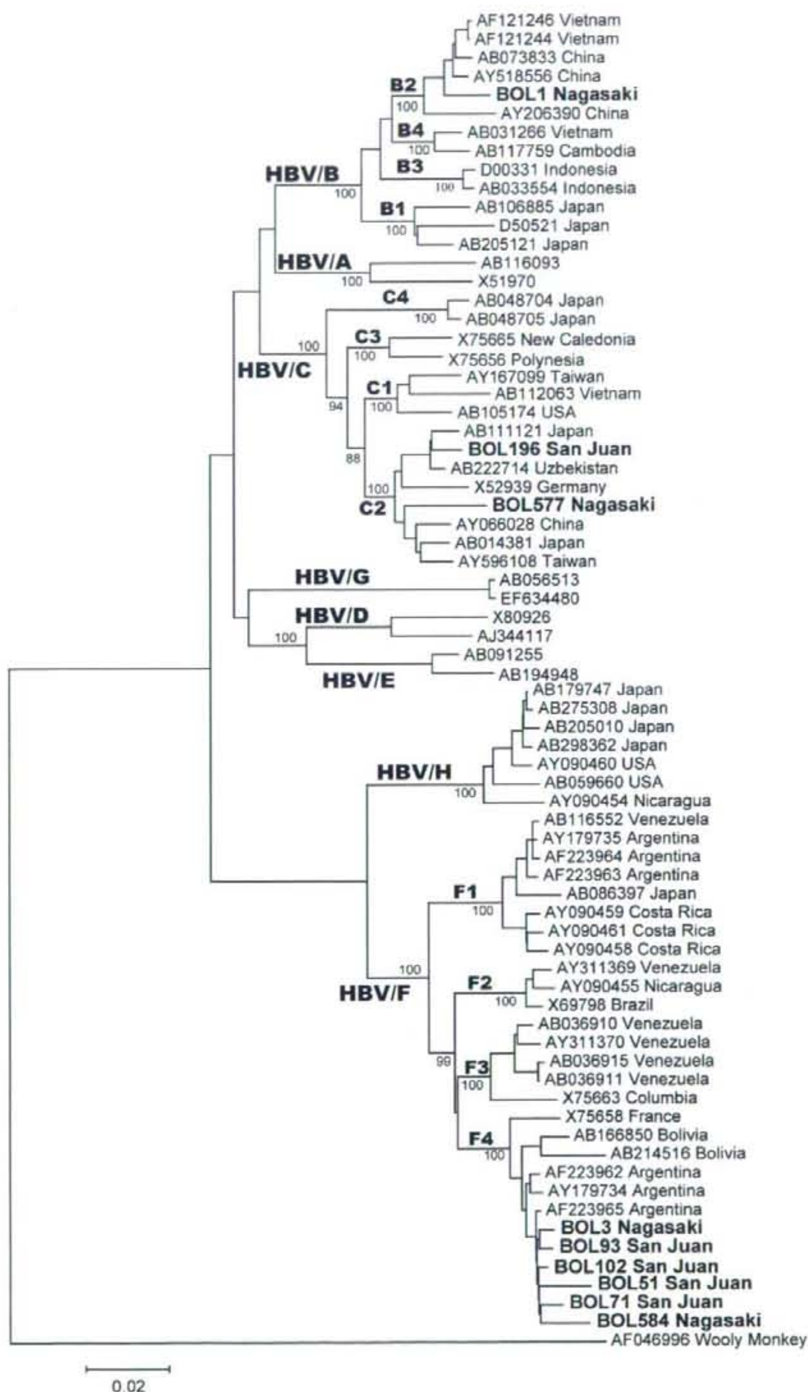

Fig. 2. A pylogenic analysis of the HBV in complete genome. Sequences from Bolivia and other countries. The sequences of Japanese immigrants are labeled according to their origin in Japan, i.e., Nagasaki. Similarly, the sequences obtained from native Bolivians are labeled according to their origin in Bolivia, i.e., San Juan. The study isolates, e.g. BOL196, BOL577, etc. were subjected to bootstrap resampling with all available complete genome sequences retrieved from the EMBL/DDBJ/GeneBank database. Sequences used for the phylogenetic tree are indicated under the corresponding accession numbers from DDBJ/EMBl/GeneBank and country of origin.

Author's personal copy

3. Results

3.1. Characteristics of subjects and serological prevalence of HBV markers

Among 487 subjects screened, 193 (39.6%) were male and 294 (60.3%) were female. The median age of the subjects was 51 years (mean: 52.5 years; range: 8–95 years). Japanese immigrants (group 1) was significantly older than native Bolivians (group 2) ($p < 0.0001$). Overall the seroprevalence of anti-HBc was 44.5% and significantly higher in group 1 than group 2 (Table 1, $p < 0.0001$). Similarly, the overall seroprevalence of anti-HBs was 33.3%, significantly higher in group 1 (37.6%) than group 2 (26.5%) ($p = 0.011$). Overall, 22 (4.5%) of 487 subjects were positive for HBsAg, which were subsequently found positive for anti-HBc but negative for HBeAg. The background and clinical features of the HBsAg-positive subjects is summarized in Table 3.

3.2. HBV genotypes

HBV genotypes were successfully determined in 20 (91%) of the 22 HBsAg-positive cases by EIA genotyping method, while 17 (77.3%) of the subjects were positive for HBV DNA (Table 4). Three genotypes of HBV were found: genotypes B, C and F. Overall, HBV/F was found in 12 (54.5%) of the 22 subjects, HBV/C in 8 (36.4%), and HBV/B in 2 (9.1%).

3.3. Phylogenetic analysis

Complete genome sequences were obtained from 6 genotype F strains, 1 genotype B and 2 genotype C strains; while rest of DNA positive subjects were sequenced for S gene and pre-C/C gene except 1 genotype F strain whose only pre-C/C sequence was available for genotypic analysis. Complete genome analysis (Fig. 1) and partial S sequence analysis (Fig. 2) showed that genotype F strains obtained in this study belonged to cluster IV of genotype F along with previously published Bolivian, French and Argentinean isolates. The genotype B strains belonged to subgenotype B2 (Ba) while genotype C strains were of subgenotype C2 (Ce), which were originated from Asia.

3.4. Variability of HBV in enhance II, basal core promoter (BCP), pre-C/C region

The prevalence of the nucleotide substitutions along with the 13 sequences bearing the enhancer II/BCP, pre-C/C regions of the HBV genome corresponding to each genotype is summarized in Table 5. The frequency of C1753 and A1896 mutations was relatively high in genotype F strains that is 85.7% and 71.4%, respectively. T1653 mutation was found in 75% of genotype C and 28.6% of genotype F strains. T1762/A1764 double mutation was present in 50% of genotype C strains compared to 28.6% of genotype F strains. Statistical analysis was not performed because of the reduced number of subjects in comparison.

4. Discussion

Viral hepatitis is one of the main infectious health problems existing in the world. An important aspect of the epidemiological behavior of this virus causing chronic liver disease is the tendency to establish closed epidemiological cycles in isolated populations due to geographical, social or cultural factors (Echevarria and Leon, 2003). Significant differences in the prevalence of viral agents can be observed in relation to the population specific behaviors that may increase the transmission (Echevarria and Leon, 2003).

HBV genotype distribution may reflect the different patterns of migration to the Americas (Arauz-Ruiz et al., 1997; Kidd-Ljunggren et al., 2002). HBV/F is the indigenous genotype of South and Central America while other HBV genotypes mainly reflect human migration from other geographical areas into the region (Campos et al., 2005). The present study was carried out on Japanese immigrants and "native Bolivians" residing in the same region of the country. HBV/F was found in 54.5%, HBV/C in 36.4%, and HBV/B in 9.1% individuals of the studied population. Based on the partial and complete genome analyses, HBV/B sequences belonged to subgenotype B2 and HBV/C sequences belonged to subgenotype C2, both of Asian origin. The close alignment of these sequences on phylogenetic tree suggested that Japanese immigrants might be a source of introduction of these genotypes in their close contacts "native Bolivians". The subgenotype B2 of HBV/B is not very representative of Japan; 7% were found in acute hepatitis patients (Ozasa et al., 2006) and 12% in chronic hepatitis patients with genotype B (Sugauchi et al., 2004). Therefore, possibility of carry over of this subgenotye by Japanese immigrants may not be excluded, as also evidenced by phylogenetic trees. Moreover, due to small number (only 2 HBV/B patients), further studies are needed to confirm this.

The phylogenetic analysis of HBV/F sequences also showed similar kind of results; the HBV/F sequences aligned closely together and belonged to cluster IV of the HBV/F which had already identified in this area (Huy et al., 2006), providing convincing evidence of transmission of HBV/F from Bolivian natives into Japanese immigrants.

The seroprevalence of anti-HBc was found to be high (44.5%), followed by anti-HBs (34%) and low detection of HBsAg carriers (4.5%). Japanese immigrants were significantly older in age than native Bolivians and had significantly higher seroprevalence of anti-HBc. The high detection of anti-HBc indicates that most individuals had past infection of HBV, whereas the low HBsAg prevalence may be explained by seroclearance of HBsAg due to natural seroconversion (Lee, 1997). The relative low detection of anti-HBc in native Bolivians may be due to younger age in this population.

Increasing evidence add that HBV genotypes affect the clinical course of infection and antiviral therapy. Mutations in the basal core promoter region (T1762/A1764) and a stop-codon generating mutation in the precore region (A1896) have been found to be associated with viral replication as well as HBeAg seroconversion. Similarly, the T1653 and V1753 (not T) mutations were previously found to be associated with the progression

Author's personal copy

of liver disease from chronic hepatitis to liver cirrhosis or HCC (Ito et al., 2006; Takahashi et al., 1999; Tanaka et al., 2006). These mutations were found in the genotype C and F strains in the present study. The subjects of this study being asymptomatic carriers (normal ALT level and HBeAg-negative), presence of these mutations may indicate disease prognosis. However, the host genetic factors and environmental factors possibly interact and determine the natural history of hepatitis B as a function of genotype (Tanaka and Mizokami, 2007).

Control of HBV infection is an immediate target that can only be achieved by knowing the epidemiological behavior of the virus and by designing specific immunization strategies accordingly. We conclude that Japanese immigrants might be responsible for introduction of HBV genotypes B and C in native Bolivians conversely, exposed to the indigenous genotype F. This is a report providing evidence of inter-communities transmission of HBV genotypes, which would contribute to the investigation of the worldwide spread of HBV. Further study is required to investigate peculiarities of the genotypes in different ethnic groups in Bolivia.

Acknowledgements

This study was supported in part by Ministry of Health, Labor and Welfare of Japan. A.K. was supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

References

Arauz-Ruiz, P., Norder, H., Visona, K.A., Magnius, L.O., 1997. Molecular epidemiology of hepatitis B virus in Central America reflected in the genetic variability of the small S gene. J. Infect. Dis. 176 (4), 851–858.

Campos, R.H., Mbayed, V.A., Pineiro, Y.L.F.G., 2005. Molecular epidemiology of hepatitis B virus in Latin America. J. Clin. Virol. 34 (Suppl. 2), S8–S13.

Chen, C.H., Chen, Y.Y., Chen, G.H., Yang, S.S., Tang, H.S., Lin, H.H., Lin, D.Y., Lo, S.K., Du, J.M., Chang, T.T., Chen, S.C., Liao, L.Y., Kuo, C.H., Lin, K.C., Tai, D.I., Changchien, C.S., Chang, W.Y., Sheu, J.C., Chen, D.S., Liaw, Y.F., Sung, J.L., 2004. Hepatitis B virus transmission and hepatocarcinogenesis: a 9 year retrospective cohort of 13,676 relatives with hepatocellular carcinoma. J. Hepatol. 40 (4), 653–659.

Dumpis, U., Holmes, E.C., Mendy, M., Hill, A., Thursz, M., Hall, A., Whittle, H., Karayiannis, P., 2001. Transmission of hepatitis B virus infection in Gambian families revealed by phylogenetic analysis. J. Hepatol. 35 (1), 99–104.

Echevarria, J.M., Leon, P., 2003. Epidemiology of viruses causing chronic hepatitis among populations from the Amazon Basin and related ecosystems. Cad. Saude Publica 19 (6), 1583–1591.

Huy, T.T., Ushijima, H., Sata, T., Abe, K., 2006. Genomic characterization of HBV genotype F in Bolivia: genotype F subgenotypes correlate with geographic distribution and T(1858) variant. Arch. Virol. 151 (3), 589–597.

Ito, K., Tanaka, Y., Orito, E., Sugiyama, M., Fujiwara, K., Sugauchi, F., Kato, T., Tokita, H., Izumi, N., Kato, M., Yuen, M.F., Lai, C.L., Gish, R.G., Ueda, R., Mizokami, M., 2006. T1653 mutation in the box alpha increases the risk of hepatocellular carcinoma in patients with chronic hepatitis B virus genotype C infection. Clin. Infect. Dis. 42 (1), 1–7.

Kidd-Ljunggren, K., Miyakawa, Y., Kidd, A.H., 2002. Genetic variability in hepatitis B viruses. J. Gen. Virol. 83 (Pt 6), 1267–1280.

Lee, W.M., 1997. Hepatitis B virus infection. N. Engl. J. Med. 337 (24), 1733–1745.

Mbayed, V.A., Barbini, L., Lopez, J.L., Campos, R.H., 2001. Phylogenetic analysis of the hepatitis B virus (HBV) genotype F including Argentine isolates. Arch. Virol. 146 (9), 1803–1810.

Miyakawa, Y., Mizokami, M., 2003. Classifying hepatitis B virus genotypes. Intervirology 46 (6), 329–338.

Norder, H., Arauz-Ruiz, P., Blitz, L., Pujol, F.H., Echevarria, J.M., Magnius, L.O., 2003. The T(1858) variant predisposing to the precore stop mutation correlates with one of two major genotype F hepatitis B virus clades. J. Gen. Virol. 84 (Pt 8), 2083–2087.

Norder, H., Courouce, A.M., Coursaget, P., Echevarria, J.M., Lee, S.D., Mushahwar, I.K., Robertson, B.H., Locarnini, S., Magnius, L.O., 2004. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. Intervirology 47 (6), 289–309.

Ozasa, A., Tanaka, Y., Orito, E., Sugiyama, M., Kang, J.H., Hige, S., Kuramitsu, T., Suzuki, K., Tanaka, E., Okada, S., Tokita, H., Asahina, Y., Inoue, K., Kakumu, S., Okanoue, T., Murawaki, Y., Hino, K., Onji, M., Yatsuhashi, H., Sakugawa, H., Miyakawa, Y., Ueda, R., Mizokami, M., 2006. Influence of genotypes and precore mutations on fulminant or chronic outcome of acute hepatitis B virus infection. Hepatology 44 (2), 326–334.

Sakamoto, T., Tanaka, Y., Orito, E., Co, J., Clavio, J., Sugauchi, F., Ito, K., Ozasa, A., Quino, A., Ueda, R., Sollano, J., Mizokami, M., 2006. Novel subtypes (subgenotypes) of hepatitis B virus genotypes B and C among chronic liver disease patients in the Philippines. J. Gen. Virol. 87 (Pt 7), 1873–1882.

Sugauchi, F., Kumada, H., Sakugawa, H., Komatsu, M., Niitsuma, H., Watanabe, H., Akahane, Y., Tokita, H., Kato, T., Tanaka, Y., Orito, E., Ueda, R., Miyakawa, Y., Mizokami, M., 2004. Two subtypes of genotype B (Ba and Bj) of hepatitis B virus in Japan. Clin. Infect. Dis. 38 (9), 1222–1228.

Sugauchi, F., Mizokami, M., Orito, E., Ohno, T., Kato, H., Suzuki, S., Kimura, Y., Ueda, R., Butterworth, L.A., Cooksley, W.G., 2001. A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. J. Gen. Virol. 82 (Pt 4), 883–892.

Takahashi, K., Ohta, Y., Kanai, K., Akahane, Y., Iwasa, Y., Hino, K., Ohno, N., Yoshizawa, H., Mishiro, S., 1999. Clinical implications of mutations C-to-T1653 and T-to-C/A/G1753 of hepatitis B virus genotype C genome in chronic liver disease. Arch. Virol. 144 (7), 1299–1308.

Tanaka, Y., Mizokami, M., 2007. Genetic diversity of hepatitis B virus as an important factor associated with differences in clinical outcomes. J. Infect. Dis. 195 (1), 1–4.

Tanaka, Y., Mukaide, M., Orito, E., Yuen, M.F., Ito, K., Kurbanov, F., Sugauchi, F., Asahina, Y., Izumi, N., Kato, M., Lai, C.L., Ueda, R., Mizokami, M., 2006. Specific mutations in enhancer II/core promoter of hepatitis B virus subgenotypes C1/C2 increase the risk of hepatocellular carcinoma. J. Hepatol. 45 (5), 646–653.

Usuda, S., Okamoto, H., Iwanari, H., Baba, K., Tsuda, F., Miyakawa, Y., Mayumi, M., 1999. Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. J. Virol. Methods 80 (1), 97–112.

Journal of Medical Virology 80:268–276 (2008)

Epidemiological and Clinical Evaluation of Hepatitis B, Hepatitis C, and Delta Hepatitis Viruses in Tajikistan

Anis Khan,[1] Fuat Kurbanov,[1] Yasuhito Tanaka,[1] Abeer Elkady,[1] Masaya Sugiyama,[1] Abdusamad Dustov,[2] and Masashi Mizokami[1]*

[1]Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya, Japan
[2]Institute of Gastroenterology of the Academy of Sciences of the Republic of Tajikistan, Dushanbe, Republic of Tajikistan

The implication of genotypes is recognized increasingly in the clinical course of hepatitis B virus (HBV) and in response to anti-viral drugs of hepatitis C virus (HCV). Genotypic prevalence of both etiological agents varies geographically and no data are available for Tajikistan. To investigate the epidemiology and clinical significance of HBV and HCV genotypes in chronic hepatitis (group 1) and liver cirrhosis/hepatocellular carcinoma (HCC) (group 2) patients in Tajikistan, 124 patients with chronic liver disease (group 1 = 84 and group 2 = 40) were enrolled. Genotypes of HBV, HCV, and delta hepatitis virus (HDV) were determined by sequencing. The overall prevalence of anti-HCV, HCV core antigen (HCVcAg) and HBsAg was 46% (57/124) and 41.1% (51/124), respectively. Coinfection of HCV/HBV, HBV/HDV, and HCV/HBV/HDV was found in 4.8% (6/124), 11.2% (12/124), and 0.8% (1/124) of cases, respectively. HDV genotype 1 was found in 19.6% (10/51) of HBsAg-positive patients. The HBV/HDV coinfection was relatively high in group 2 compared to group 1 (15% vs. 7.1%). HCV/1b detected in 84.6% (44/52) of HCV RNA-positive patients, followed by 3a (7.6%), 2a (5.7%), and 2c (1.9%). HBV/D was detected in 94.1% (48/51) of HBsAg-positive patients, followed by HBV/A [5.8% (3/51)]. T1762/A1764 double mutation was associated with liver cirrhosis/HCC in HBV-infected patients ($P = 0.0004$). This is the first study on the molecular epidemiology of hepatitis viruses among chronic liver diseases patients in Tajikistan. Among HBV-infected patients, the T1762/A1764 mutation was associated with liver cirrhosis/HCC. ***J. Med. Virol. 80:268–276, 2008.***

© 2007 Wiley-Liss, Inc.

KEY WORDS: Tajikistan; HBV; HCV; HDV; genotype; molecular epidemiology; mutations BCP

INTRODUCTION

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are common etiological agents of viral hepatitis. Approximately 350 million people are infected with HBV worldwide and the World Health Organization (WHO) estimates that approximately 170 million people are infected with HCV. Chronic HBV and/or HCV infection can progress to liver cirrhosis and hepatocellular carcinoma (HCC).

HCV displays a high degree of genetic variability and is classified into six major genotypes that show sequence similarities of only 66–69% [Pawlotsky, 2003a]. Each of the genotypes contains multiple subtypes with >75% nucleotide sequence similarity [Simmonds et al., 2005]. The HCV genotypes have different geographical distributions, that is, some strains are distributed worldwide, whereas others are found only in specific geographical regions [Pawlotsky, 2003a; Verbeeck et al., 2005]. Investigation of circulating HCV genotypes is useful as an epidemiological tool, and it is helpful for improvement of diagnostic tests and treatment efficiency [Zein, 2000; Hui et al., 2003; Pawlotsky, 2003b]. Similarly, HBV has been classified into eight major genotypes (A–H) by sequence divergence in the entire genome in excess of 8% and are distributed geographically [Miyakawa and Mizokami, 2003; Norder et al., 2004]. A number of studies have demonstrated that HBV genotypes influence the course of disease, therapeutic responsiveness and clinical outcomes [Chu et al., 2002; Miyakawa and Mizokami, 2003; Norder et al., 2004; Schaefer, 2005]. Previous reports have indicated that

*Correspondence to: Masashi Mizokami, MD, PhD, Prof., Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Kawasumi 1, Mizuho, Nagoya 467-8601, Japan.
E-mail: mizokami@med.nagoya-cu.ac.jp

Accepted 13 September 2007
DOI 10.1002/jmv.21057
Published online in Wiley InterScience
(www.interscience.wiley.com)

© 2007 WILEY-LISS, INC.

WILEY InterScience DISCOVER SOMETHING GREAT

TABLE I. Clinical Features and Hepatitis Markers Among 124 Subjects With Chronic Liver Diseases in Tajikistan

Features	Total (n = 124)	Chronic hepatitis (n = 84)	Liver cirrhosis/HCC (n = 40)	P
Sex (M/F)	72/52	52/32	20/20	NS
Mean age (mean ± SD)	38.4 ± 14.9	36.67 ± 14.4	42.2 ± 15.5	NS
ALT median (range)	330.5 (171.9–2001.6)	297.5 (171.9–1946)	383.6 (177.9–2001.6)	NS
AST median (range)	251.35 (14–1189.6)	217.6 (14–1189.6)	282.2 (150.1–1000.8)	NS
ALP median (range)	173.55 (35.8–428.2)	166.8 (35.8–428.2)	180.7 (147.3–428.1)	<0.01
T. Bil. median (range)	21.9 (7.6–239.3)	21 (7.6–239.3)	24.6 (11.4–140.2)	NS
Chol. median (range)	3.6 (2.3–7.8)	3.8 (2.3–7.2)	3.25 (2.4–7.8)	<0.05
Anti-HCV/HCVcAg	57/124 (46)	40/84 (47.6)	17/40 (42.5)	NS
HCV RNA	52/57 (76.4)	40/40 (100)	12/17 (70.5)	NS
Anti-HBc	121/124 (97.5)	79/84 (94)	35/40 (87.5)	NS
Anti-HBs	15/124 (12.1)	14/84 (16.6)	1/40(2.5)	<0.05
HBsAg	51/124 (41.1)	34/84 (40.4)	17/40 (42.5)	NS
HBeAg	2/51 (3.9)	1/34 (2.9)	1/17 (5.8)	NS
HBV DNA	37/51 (72.5)	24/34 (70.5)	13/17 (76.4)	NS
Anti-HDV	12/51 (23.5)	6/34 (17.6)	6/17 (35.2)	NS
HDV RNA	10/12 (83.3)	6/6 (100)	4/6 (66.6)	NS
Patterns of mono or co-infection				
HCV only	50/124 (40.3)	33/84 (39.2)	17/40 (42.5)	NS
HBV only	27/124 (21.7)	20/84 (23.8)	7/40 (17.5)	NS
HCV + HBV	6/124 (4.8)	4/84 (4.7)	2/40 (5)	NS
HBV + HDV	12/124 (9.7)	6/84 (7.1)	6/40 (15)	NS
HCV + HBV + HDV	1/124 (0.8)	1/84 (1.2)	0/40 (0)	NS

Normal ranges: Alanine aminotransferase (ALT): 33.3–200.1 nM/L, asparate aminotrasferase (AST): 27.8–150.1 nM/L, alkaline phosphatase (ALP): 44.5–219.6 nM/L, total bilurubin (T. Bilu): 8.5–20.5 mM/L, cholestrol (Chol.): 3–6.2 nM/L.

nucleotide substitutions in the basal core promoter (BCP) of HBV are associated with HCC in infected patients, these include the 1762T/1764A double mutation [Kao et al., 2003], 1653T and 1753V [Takahashi et al., 1999; Ito et al., 2006]. However most of the studies were carried out on genotype C, and little is known about the other genotypes.

Hepatitis delta virus (HDV), a defective RNA virus that requires the "help" of HBV for packaging and transmission, is known to aggravate the chronic liver damage in patients infected chronically with HBV [Lusida et al., 2003]. HDV has been classified into three genotypes distributed geographically. Several studies suggested that HDV genotypes also influence the course of the disease [Casey et al., 1996; Wu et al., 1998; Hsu et al., 2002].

There has been no report on the prevalence of the hepatitis B and C viruses in Tajikistan. Therefore, the aim of the present study was to investigate the genotypic prevalence and clinical significance of HCV, HBV, and/or HDV among chronic hepatitis patients with and without liver cirrhosis and/or HCC in Tajikistan. Furthermore, the association between coinfection patterns or HBV specific mutations and disease progression were also analyzed in this study.

METHODS

Patients

Sera were obtained from 124 consecutive chronic liver diseases patients over a period of 6 months between February to August 2006. Patients in this study were classified into two clinical groups (i) Chronic hepatitis: patients with constantly high serum alanine aminotrasferase level (ALT), and (ii) Liver cirrhosis and HCC: patients, as diagnosed clinically based on ultrasonography, detection of serological tumor markers (AFP) and in a few cases histologically.

Serological Testing

Anti-HCV, HBsAg, HBeAg, anti-HBc and anti-HBs were determined by chemiluminescence with commercial assay kits (Fujirebio, Inc., Tokyo, Japan). HCV core antigen (HCVcAg), was measured [Aoyagi et al., 1999] using enzyme immunoassay (Fujirebio, Inc.).

Delta antibody (anti-HDV) was assessed only in HBsAg positive specimens using the recombinant HDVAg [Semiletov Iu et al., 2002] diagnostic kit ELISA

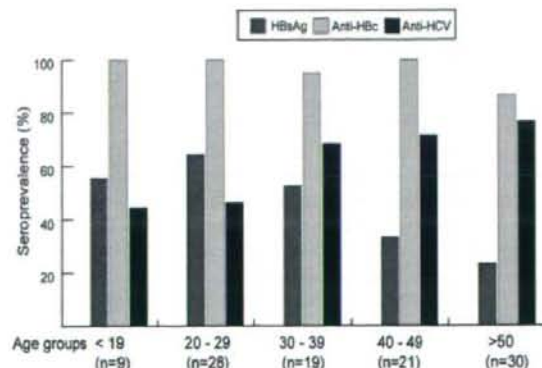

Fig. 1. Comparative prevalence of viral hepatitis markers in different age groups of studied chronic liver disease patients.

Anti-HDV (RPC Diagnostics Systems, Nizhnyi Novgorod, Russia).

Biochemical markers, that is, ALT, AST, Alkaline Phosphatase, cholestrol, bilurubin were measured in all samples at the local hospital.

Genotyping of HCV

Total RNA was extracted from serum, reverse transcribed into cDNA using random hexamer primer as described previously [Ohno et al., 1997]. Confirmation of HCV RNA in the samples was carried by amplifying highly conserved 5′UTR region and HCV genotypes were determined for both; structural (E1/Core) and nonstructural (NS5B) viral genes using either or both genotyping PCR [Hashimoto et al., 1996; Ohno et al., 1997] and/or direct sequencing with genotype-universal primers [Tanaka et al., 2002].

Screening for HCV Type RF1_2k/1b

Screening for natural recombinant type RF1_2k/1b strain was performed by the newly developed sensitive and specific method allowing detection up to 10 copies/

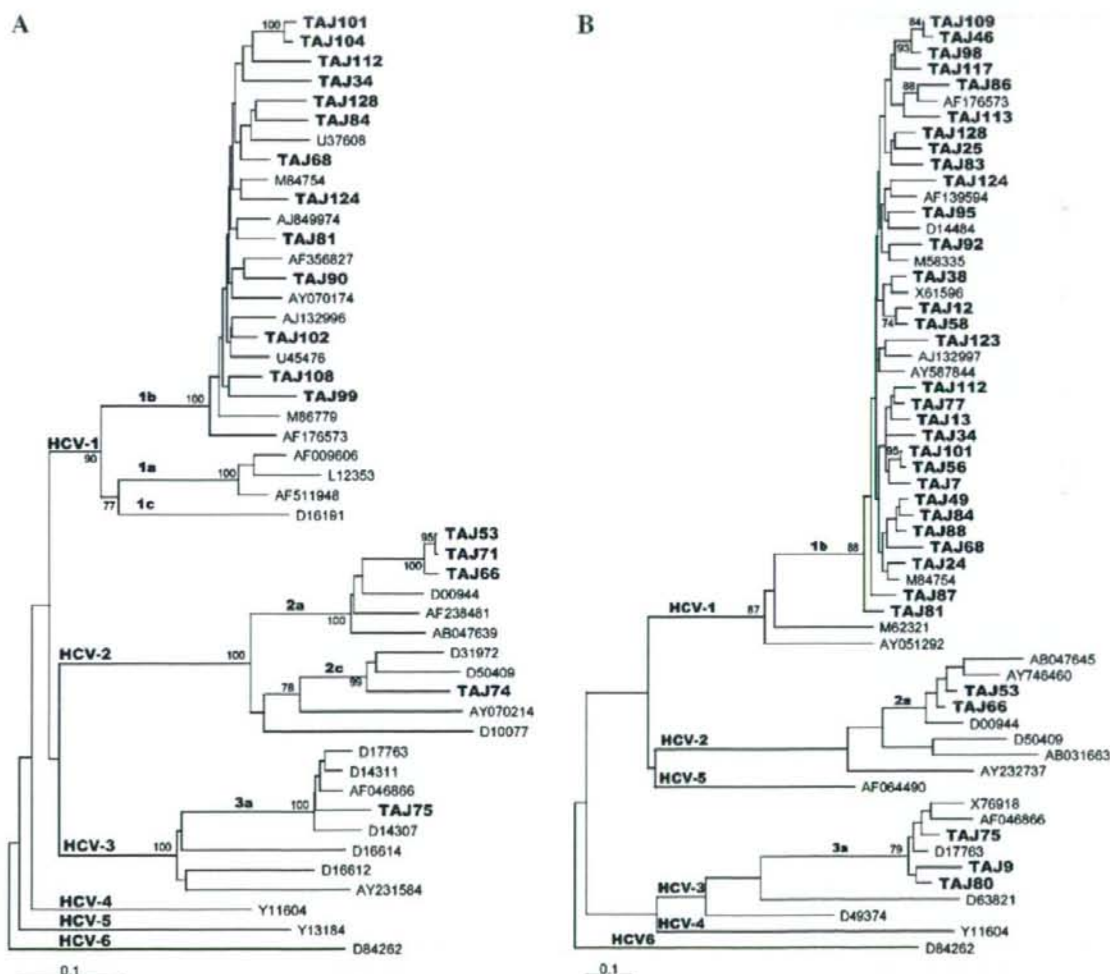

Fig. 2. **A**: Phylogenetic analysis of the hepatitis C virus (HCV) partial E1 region, sequences from Tajikistan and other countries. Tajikistan isolates (e.g., TAJ74, TAJ75, etc.) were subject to bootstrap resampling with all available sequences in E1 region retrieved from the EMBL/DDBJ/Gene Bank database. Closest neighbors used for phylogenetic tree are indicated under the corresponding accession numbers from DDBJ/EMBl/Gene Bank. **B**: Phylogenetic analysis of the HCV partial NS5B region, sequences from Tajikistan and other countries. Tajikistan isolates (e.g., TAJ108, TAJ75, etc.) were subject to bootstrap resampling with all available sequences in NS5B region retrieved from the EMBL/DDBJ/Gene Bank database. Closest neighbors used for phylogenetic tree are indicated under the corresponding accession numbers from DDBJ/EMBl/Gene Bank.

TABLE II. Comparison of Genotyping Results as Determined by Core, E1, and NS5B

Classification based on core and/or E1	Classification based on NS5B				
	1b	2a	3a	ND	n
1b	41			1	42
2a		3			3
3a			4		4
2c				1	1
ND	2				2
n	43	3	4	2	52

assay (100 copies/ml) of the target sequence even if it is present in minority in mixed heterogeneous template (Kurbanov et al., in submission). Briefly, the primers were designed to amplify a part of NS2 coding region between nt. 2986 and 3270 spanning the intergenotypic breakpoint described for RF1_2k/1b [Kalinina et al., 2002]. Target product's size was 347 base pair (bp) in the first round of PCR and 300 bp in the second round of the "hemi-nested" PCR.

Genotyping of HBV and HDV

All of the HBsAg-positive samples were subjected to genotyping by commercial EIA kit (Institute of Immunology Co., Ltd, Tokyo, Japan). The method allows discrimination among the seven major HBV genotypes (A–G) by monoclonal antibodies targeted to the preS2 epitopes [Usuda et al., 1999]. HBV DNA was extracted by QIAamp DNA Blood Mini Kit (Qiagen, Inc., Hilden, Germany) from 100 ml of all HBsAg-positive sera. Complete genome was successfully sequenced for 7 strains and a part of the HBV core and short S region was sequenced for other 30 strains using previously reported primers [Sugauchi et al., 2001].

HDV RNA was extracted from anti-HDV positive samples and transcribed reversely into a cDNA using random primer as previously described for HCV [Ohno et al., 1997]. A part of HDVAg coding region of the HDV was amplified using specific primers described previously [Nakano et al., 2001] and sequenced directly.

Phylogenetic Analysis

PCR products were sequenced directly with the Prism Big Dye (Applied Biosystems, Foster City, CA) in an ABI 3100 DNA automated sequencer. All sequences were analyzed in both forward and reverse directions. Complete and partial HBV genomes were assembled using GENETYX version 11.0 (GENETYX, Tokyo, Japan). The sequences for phylogenetic analysis were retrieved from DDBJ/EMBL/GeneBank corresponding to the accession numbers mentioned in the trees. Alignment was accomplished using CLUSTAL W and NJ tree were constructed with Tamura-Nei distance correction model using online tools of the HCV database [Kuiken et al., 2006].

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number(s) AB330313 to AB330383.

Stastical Analysis

Statistical differences were evaluated by Fisher's exact probability test and Chi-square test with Yates' correction, where appropriate, using the STATA software version 8.0 (Stata Corp. LP, College Station, TX). Differences were considered significant for P values less than 0.05.

RESULTS

The HCV, HBV, and HDV Prevalence and the Co-Infection Patterns

Among 124 hepatitis patients, 84 (67.7%) were assigned into chronic hepatitis (group 1) and 40 (32.2%) into liver cirrhosis/HCC (group 2). The overall male to female ratio was 1.4, and the mean age (standard deviation) was 38.4 (± 14.9). These estimations are summarized in Table I. There was no significant difference in age between both groups. The ALT, AST, and total bilirubin, were high without significant difference between the both groups. Overall alkaline phosphatases (ALP) levels were within normal range in both groups. HCV-infection was defined upon detection of HCV RNA

TABLE III. Hepatitis C Genotypes, Stratified With Clinical Groups

Genotypes	HCV genotypes determination		
	Chronic hepatitis (n = 40)	Liver cirrhosis/HCC (n = 12)	n = 52
1b	32 (80.0)	12 (100)	44 (84.6)
2a	3 (7.5)	0 (0)	3 (5.7)
3a	4 (10.0)	0 (0)	4 (7.6)
2c	1 (2.5)	0 (0)	1 (1.9)

Numbers in brackets represent percentage (%).

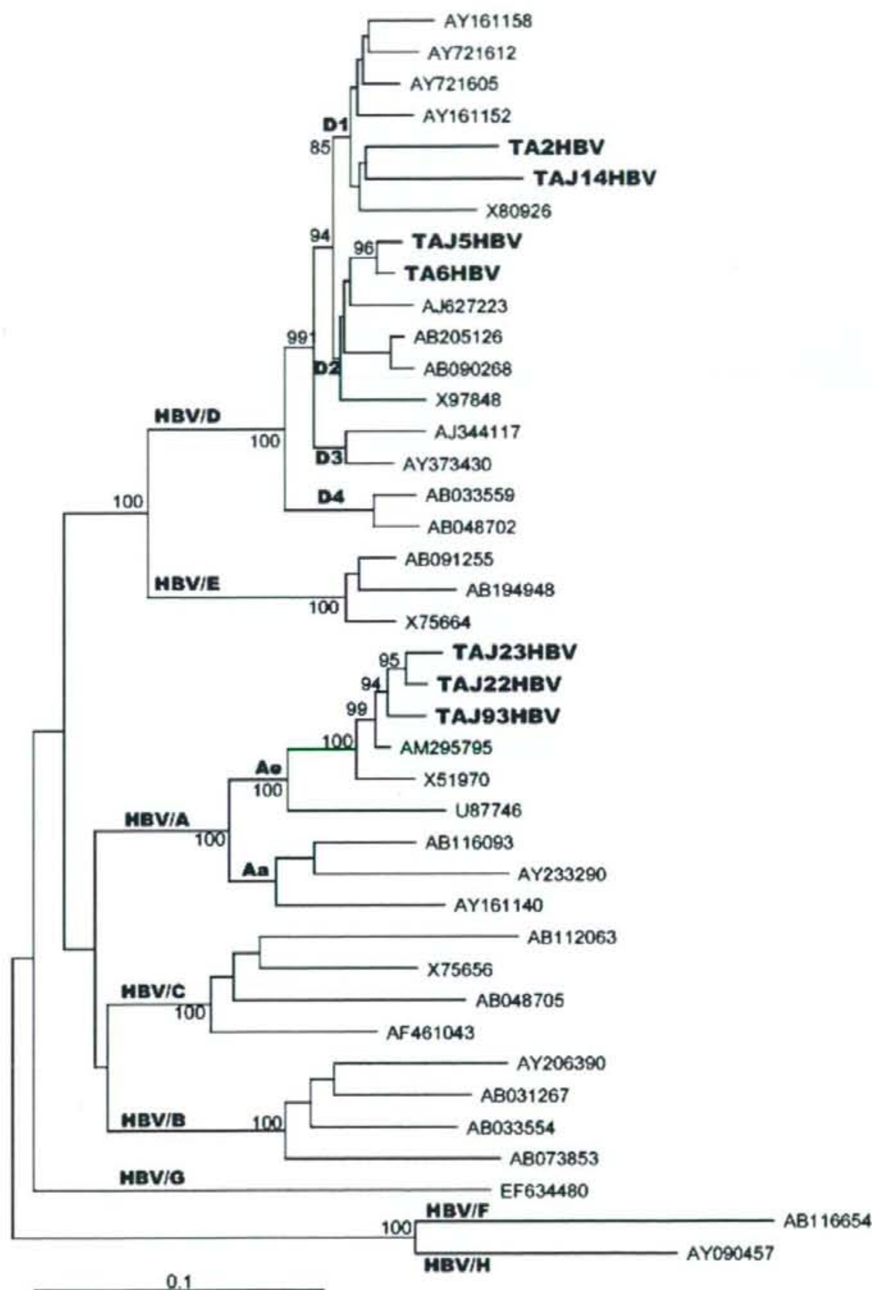

Fig. 3. Phylogenetic analysis of the hepatitis B virus (HBV) eight full genomes, sequences from Tajikistan and other countries. Tajikistan isolates (e.g., TAJ5, TAJ6, etc.) were subject to bootstrap resampling with all available full genome sequences retrieved from the EMBL/DDBJ/Gene Bank database. Closest neighbors used for phylogenetic tree are indicated under the corresponding accession numbers from DDBJ/EMBl/Gene Bank.

or HCVcAg positive and HCV recovered as anti-HCV positive but negative for HCVcAg or HCV RNA. HCV core antigen (HCVcAg) was measured in serum, where the cutoff value was set tentatively at 50 fmol/L. In this study 15 out of 68 anti-HCV positive samples were negative for HCV RNA. The HCVcAg was positive in only 4 of the 15 cases, suggesting that HCV RNA was degraded in these samples, while remaining 11 cases had resolved acute HCV infection. HCV infection was found to be high in both groups (group 1 = 47.6% and group 2 = 42.5%). HCV RNA was detected in 91% of cases (100% in group 1 and 70.5% in group 2). Anti-HBc was found to be high in both groups (94% and 87.5%, respectively). Although seroprevalence of anti-HBs was very low (12.1%) in the studied population but significantly high in group 1 (16.6%) than group 2 ($P = 0.0356$) while a few cases were positive for anti-HBeAg (2.9% and 5.8%, respectively). Positivity for HBsAg was regarded as indicating HBV infection. The seroprevalence of HBsAg was equally high in both groups, that is, group 1 = 40.4% and group 2 = 42.5%, whereas DNA positivity was 70.5% and 76.4%, respectively. Overall anti-HDV seroprevalence was 23.5%, 12 out of 51 HBsAg positive cases, relatively higher in group 2 (35.2%) compared to group 1 (17.6%) $P = 0.1990$. HDV viremia was detected in 83.3% of cases (100% in group 1 and 66.6% in group 2) (Table I). Based on the serological findings for hepatitis viruses, the patients were categorized into five groups, HCV only (40.3%), HBV only (21.7%), HCV/HBV (4.8%), HBV/HDV (9.7%), and HCV/HBV/HDV (0.8%) in Tajikistan (Table I).

When the seropositivity of hepatitis markers was analyzed in relation to age (Fig. 1), it was found that the prevalence of HBsAg was relatively high in younger group (<29 years old) and tended to decline with age. The opposite trend was observed in anti-HCV prevalence, which was increasing with the age of the patients. The seroprevalence of anti-HBc was high in all age groups, indicating that most patients had past HBV infection during childhood.

HBV, HCV, and HDV Genotypes

HCV genotyping was carried by two independent methods, that is, by type specific primers for core, NS5B region and phylogenetic analyses based on nucleotide sequences in E1 or NS5B region. No discrepancy was observed among the results between either method (Fig. 2A,B), however, one case (TAJ74) was determined as HCV genotype 2c (HCV/2c) only by the phylogenetic analysis in E1 region (Fig. 2A). A total of 7.6% of cases were untypeable by one of either method (Table II). Overall, HCV/1b was a predominant genotype (84.6%) in Tajikistan, followed by HCV/3a (7.6%), 2a (5.7%), and 2c (1.9%). None of the HCV-infected in this study carried recombinant 2k/1b strain. Other potential forms of recombination were also excluded by matching result of genotyping based on both structural and nonstructural genomic parts (Table II). Interestingly, all 12 LC/HCC patients had HCV/1b, not 3a or 2a (Table III).

HBV genotype D (HBV/D) was the predominant genotype (94.1%) in both groups, that is, group 1 = 97% and group 2 = 88.2%, followed by genotype A (2.9% and 11.7%, respectively). HBV genotypes were determined in 45 (88%) of 51 HBsAg-positive cases by EIA. The entire HBV genome was sequenced successfully for seven cases (Fig. 3), and the precore/core promoter region was sequenced in the other 25 cases. The remaining five cases were only positive by PCR for a short S region. The full genome analysis revealed that of the four HBV/D strains, two belonged to subgenotype D1, and the remaining two to subgenotype D2. All three HBV/A strains in this study were belonged to subgenotype Ae. The results of the phylogenetic genotyping were all concordant with EIA-genotyping results. For HDV strains, the phylogenetic analysis of the HDVAg region showed that all of the PCR-positive cases (10/12) belonged to the genotype 1 (Fig. 4).

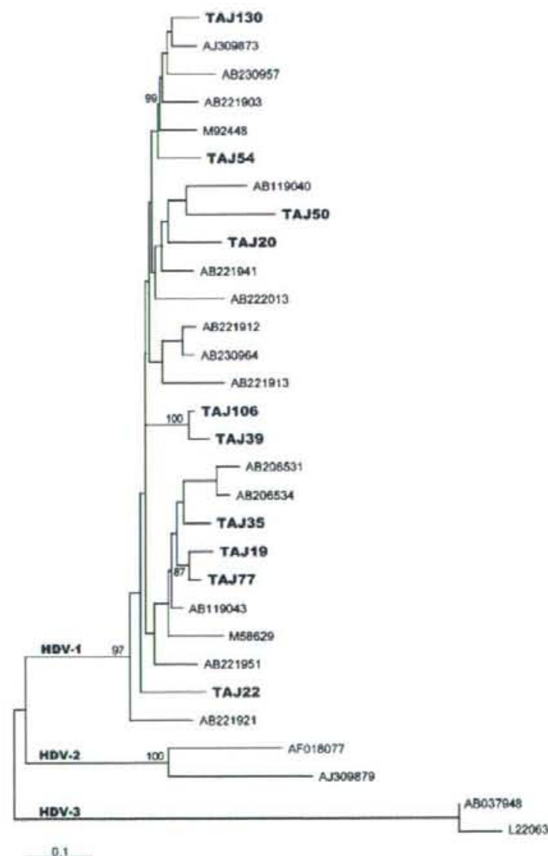

Fig. 4. Phylogenetic analysis of the hepatitis D virus (HDV) partial HDVAg coding region, sequences from Tajikistan and other countries. Tajikistan isolates (e.g., TAJ20-HDV, TAJ50-HDV, etc.) were subject to bootstrap resampling with all available sequences in partial HDVAg region retrieved from the EMBL/DDBJ/Gene Bank database. Closest neighbors used for phylogenetic tree are indicated under the corresponding accession numbers from DDBJ/EMBl/Gene Bank.

Variability of HBV in Enhance II, Basal Core Promoter (BCP), Pre-C/C Region

The prevalence of the nucleotide substitutions along the 33 sequence bearing the enhancer II/core promoter/precore/core regions of the HBV genome is summarized for both clinical groups (22 group 1 vs. 11 group 2) in the Table IV. Due to the small numbers of patients with liver cirrhosis (n = 8) and HCC (n = 3), these were combined in the "Group 2" to enable the comparative analysis with chronic hepatitis patients without the complications; "Group 1." The frequency of A1757 was high in both of the groups without the difference significant statistically. The T1762/A1764 double mutation was more frequent in the Group 2 and this was significant; $P = 0.0004$. The difference in prevalence of the A1896, A1899, and T1912 mutations did not reach statistical significance between the groups.

DISCUSSION

In the present study, the seroprevalence and molecular-genetic characteristics of the hepatitis viruses were investigated among chronic hepatitis (group 1) and liver cirrhosis/HCC (group 2) patients in Tajikistan. The HCV seroprevalence was equally high in both of the groups (47.6% and 42.5%, respectively) followed by HBsAg (40.4% and 42.5%, respectively). These results are slightly higher than the previous studies carried in neighboring Uzbekistan, where anti-HCV was detected in 26.8–41.9% and HBsAg in 25.6% of chronic hepatitis patients [Ruzibakiev et al., 2001; Kurbanov et al., 2003], such a difference may be explained by the different ages of studied patients different collecting time and also regional characteristics. A rather old study carried on the healthy population in Tajikistan and Azerbaijan showed that HBsAg detection was significantly higher in Tajikistan (7.2%) than in Azerbaijan (2.8%) suggesting that HBV is endemic in this country [Vorozhbieva et al., 1985]. Tajikistan introduced universal immunization against HBV for newborn children since 2002. Although, new anti-viral drugs have the potential to alter the natural progression of disease but their high cost prevents the mass use of therapy in Tajikistan (Dustov, personal communication).

One of the interesting observations in this study was the trend of seroprevalence of anti-HCV to increase and HBsAg do decrease with the age of the patients. Interestingly, the prevalence of anti-HBc was very high regardless the age of the patients, indicating that most of the patients had present or past infection of HBV. The age-related decline of the HBsAg prevalence observed in this study may be explained by either; seroclearance of HBsAg due to natural seroconversion [Lee, 1997] or by death of the HBV-infected patients at age younger than those with HCV, due to earlier development of the end stage liver disease [Oyunsuren et al., 2006; Kurbanov et al., 2007]. Alternatively, suppressive effect of the concurrent HCV infection might had caused the loss of the HBV antigenia [Sheen et al., 1994; Chu et al., 1998].

HCV/1b has been found as the dominant genotype (84.6%) in Tajikistan, followed by 3a (7.6%), 2a (5.7%), and 2c (1.9%). The similar epidemiological situation was also described in the former Soviet Union Republics (Russia, Belarus, Moldova, and Uzbekistan) [Viazov et al., 1997; Kurbanov et al., 2003; Shustov et al., 2005] as well as in most of Asian countries, namely Mongolia [Lvov et al., 1996], Taiwan [Yu et al., 2001], China

TABLE IV. BCP and PC Mutations and Clinical Disease

	Clinical diagnosis		
	Chronic hepatitis (n = 22)	Liver cirrhosis/HCC (n = 11)	P
Age (mean ± SD)	39.0 ± 15.6	41.6 ± 17.3	NS
Gender (M/F)	15/7	8/3	NS
Genotype D	21 (95.4)	9 (81.8)	NS
Genotype A	1 (4.5)	2 (18.1)	NS
Nucleotide substitutions			
T1653	3 (13.6)	4 (36.3)	NS
T1678	15 (68.2)	3 (27.2)	0.0613
G1737	13 (59)	5 (45.4)	NS
C1752	5 (22.7)	2 (18.2)	NS
V1753 (not T)	6 (27.2)	4 (36.3)	NS
A1757	17 (77.2)	7 (63.6)	NS
T1762/A1764	2 (9)	8 (72.7)	0.0004
T1764/G1766	2 (9)	0	ND
A1768 alone	0	2 (18.2)	ND
Deletions 1766–1773	4 (18.2)	0	ND
C1773 alone	3 (13.6)	3 (27.2)	NS
C1772/C1773	2 (9)	1 (9)	NS
G1775	3 (13.6)	1 (9)	NS
A1896	7 (31.8)	6 (54.5)	NS
A1899	4 (18.2)	5 (45.4)	NS
T1912	8 (36.3)	5 (45.4)	NS
G1915	1 (4.5)	2 (18.2)	NS

Numbers in brackets represent percentage (%).

[Suzuki et al., 1997], and Japan [Noguchi et al., 1997] where genotype 1b was predominant.

The HCV core antigen (HCVcAg) has significant corelation with HCV RNA [Tanaka et al., 2000]. In this study 15 out of the 68 anti-HCV positive simples, had the HCV RNA undetectable by PCR method, however only 4 of the 15 cases had the HCVcAg detectable, suggesting that the viral RNA has degraded in these samples prior study [Tanaka et al., 2003], while the remaining 11 cases may have had an acute HCV infection resolved in the past.

HBV genotypes have a characterstic geographical distribution [Miyakawa and Mizokami, 2003; Norder et al., 2004]. Particularly, genotype D is widespread, predominating in the Mediterranean area and in the Middle East region. Genotype A is present in Europe, India, Africa, and North America. The present results have shown that 94% of HBV isolates in Tajikistan belonged to genotype D and 5.8% to genotype A. Similar epidemiological situation was observed in a previous study carried in Uzbekistan, where HBV/D found in 87% and HBV/A in 13% of HBV infected patients [Kato et al., 2002]. Complete genome analysis of the four HBV/D strains determined in present study allowed distinction into two subgenotypes; D1 and D2. Previous reports indicate that HBV/D subgenotypes have no specific geographical distribution; however strains from the Middle East mainly belong to subgenotype D1 and those from India, Russia and Europe to D2 [Norder et al., 2004]. No specific clustering was observed in Tajikistan strains, which is in agreement with previous observations regarding HBV/D molecular epidemiology. HBV genotype A has three major geographical clusters, the European-North American (Ae), the Afro-Asian (Aa) [Sugauchi et al., 2004], and the Central African (Ac) [Kurbanov et al., 2005]. The HBV genotype A strains isolated in this study were all belonged to the subgenotype Ae.

Increasing evidence suggests that specific HBV genomic mutations may increase the risk of HCC in the patients infected chronically. Particularly, a high incidence of the T1762/A1764 double mutation was found among HBV/B or C infected Asian population [Kao et al., 2003] and with HBV/E infected African patients [Baptista et al., 1999]. Similarly, in the present study, the frequency of the double mutation was significantly high in group 2 (6HBV/D and 2 HBV/A) as compared to group 1 (1HBV/D and 1HBV/A). In vitro studies [Buckwold et al., 1996], indicated that the T1762/A1764 mutants show enhanced replication and might be associated with high incidence of HCC.

The prevalence of coinfection with HBV and HDV found to be relatively higher in group 2 compared to group 1 (15% vs. 7.1%), however the difference was not statistically significant possibly due to the small size of cases ($P = 0.1990$). Previous studies have demonstrated that the coinfection with these viruses was also contributing in high HCC incidence in Mongolia [Oyunsuren et al., 2006].

It is concluded that HBV and HCV are the major cause of viral hepatitis in Tajikistan. Among HBV-infected patients, the T1762/A1764 mutations were associated with liver cirrhosis/HCC in this population.

REFERENCES

Aoyagi K, Ohue C, Iida K, Kimura T, Tanaka E, Kiyosawa K, Yagi S. 1999. Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. J Clin Microbiol 37:1802–1808.

Baptista M, Kramvis A, Kew MC. 1999. High prevalence of 1762(T) 1764(A) mutations in the basic core promoter of hepatitis B virus isolated from black Africans with hepatocellular carcinoma compared with asymptomatic carriers. Hepatology 29:946–953.

Buckwold VE, Xu Z, Chen M, Yen TS, Ou JH. 1996. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. J Virol 70:5845–5851.

Casey JL, Niro GA, Engle RE, Vega A, Gomez H, McCarthy M, Watts DM, Hyams KC, Gerin JL. 1996. Hepatitis B virus (HBV)/hepatitis D virus (HDV) coinfection in outbreaks of acute hepatitis in the Peruvian Amazon basin: The roles of HDV genotype III and HBV genotype F. J Infect Dis 174:920–926.

Chu CM, Yeh CT, Liaw YF. 1998. Low-level viremia and intracellular expression of hepatitis B surface antigen (HBsAg) in HBsAg carriers with concurrent hepatitis C virus infection. J Clin Microbiol 36:2084–2086.

Chu CJ, Hussain M, Lok AS. 2002. Quantitative serum HBV DNA levels during different stages of chronic hepatitis B infection. Hepatology 36:1408–1415.

Hashimoto M, Chayama K, Tubota A, Kobayashi M, Saitou S, Arase Y, Ikeda K, Kobayashi M, Nakano A, Takagi K, Koike H, Okamoto K, Handa H, Kumada H. 1996. Typing six major hepatitis C virus genotypes by polymerase chain reaction using primers derived from nucleotide sequences of the NS5 region. Int Hepatol Commun 4: 263–267.

Hsu SC, Syu WJ, Sheen IJ, Liu HT, Jeng KS, Wu JC. 2002. Varied assembly and RNA editing efficiencies between genotypes I and II hepatitis D virus and their implications. Hepatology 35:665–672.

Hui CK, Yuen MF, Sablon E, Chan AO, Wong BC, Lai CL. 2003. Interferon and ribavirin therapy for chronic hepatitis C virus genotype 6: A comparison with genotype 1. J Infect Dis 187:1071–1074.

Ito K, Tanaka Y, Orito E, Sugiyama M, Fujiwara K, Sugauchi F, Kato T, Tokita H, Izumi N, Kato M, Yuen MF, Lai CL, Gish RG, Ueda R, Mizokami M. 2006. T1653 mutation in the box alpha increases the risk of hepatocellular carcinoma in patients with chronic hepatitis B virus genotype C infection. Clin Infect Dis 42:1–7.

Kalinina O, Norder H, Mukomolov S, Magnius LO. 2002. A natural intergenotypic recombinant of hepatitis C virus identified in St. Petersburg. J Virol 76:4034–4043.

Kao JH, Chen PJ, Lai MY, Chen DS. 2003. Basal core promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers. Gastroenterology 124:327–334.

Kato H, Ruzibakiev R, Yuldasheva N, Hegay T, Kurbanov F, Achundjanov B, Tuichiev L, Usuda S, Ueda R, Mizokami M. 2002. Hepatitis B virus genotypes in Uzbekistan and validity of two different systems for genotyping. J Med Virol 67:477–483.

Kuiken C, Mizokami M, Deleage G, Yusim K, Penin F, Shin IT, Charavay C, Tao N, Crisan D, Grando D, Dalwani A, Geourjon C, Agrawal A, Combet C. 2006. Hepatitis C databases, principles and utility to researchers. Hepatology 43:1157–1165.

Kurbanov F, Tanaka Y, Sugauchi F, Kato H, Ruzibakiev R, Zalyalieva M, Yunusova Z, Mizokami M. 2003. Hepatitis C virus molecular epidemiology in Uzbekistan. J Med Virol 69:367–375.

Kurbanov F, Tanaka Y, Fujiwara K, Sugauchi F, Mbanya D, Zekeng L, Ndembi N, Ngansop C, Kaptue L, Miura T, Ido E, Hayami M, Ichimura H, Mizokami M. 2005. A new subtype (subgenotype) Ac (A3) of hepatitis B virus and recombination between genotypes A and E in Cameroon. J Gen Virol 86:2047–2056.

Kurbanov F, Tanaka Y, Elkady A, Oyunsuren T, Mizokami M. 2007. Tracing hepatitis C and Delta viruses to estimate their contribution in HCC rates in Mongolia. J Viral Hepat 14:667–674.

Lee WM. 1997. Hepatitis B virus infection. N Engl J Med 337:1733–1745.

Lusida MI, Surayah, Sakugawa H, Nagano-Fujii M, Soetjipto, Mulyanto, Handajani R, Boediwarsono, Setiawan PB, Nidom

CA, Ohgimoto S, Hotta H. 2003. Genotype and subtype analyses of hepatitis B virus (HBV) and possible co-infection of HBV and hepatitis C virus (HCV) or hepatitis D virus (HDV) in blood donors, patients with chronic liver disease and patients on hemodialysis in Surabaya, Indonesia. Microbiol Immunol 47:969–975.

Lvov DK, Samokhvalov EI, Tsuda F, Selivanov NA, Okamoto H, Stakhanova VM, Stakhgildyan IV, Doroshenko NV, Yashina TL, Kuzin SN, Suetina IA, Deryabin PG, Ruzaeva LA, Bezgodov VN, Firsova LA, Sorinson SN, Mishiro S. 1996. Prevalence of hepatitis C virus and distribution of its genotypes in Northern Eurasia. Arch Virol 141:1613–1622.

Miyakawa Y, Mizokami M. 2003. Classifying hepatitis B virus genotypes. Intervirology 46:329–338.

Nakano T, Shapiro CN, Hadler SC, Casey JL, Mizokami M, Orito E, Robertson BH. 2001. Characterization of hepatitis D virus genotype III among Yucpa Indians in Venezuela. J Gen Virol 82:2183–2189.

Noguchi S, Sata M, Suzuki H, Mizokami M, Tanikawa K. 1997. Routes of transmission of hepatitis C virus in an endemic rural area of Japan. Molecular epidemiologic study of hepatitis C virus infection. Scand J Infect Dis 29:23–28.

Norder H, Courouce AM, Coursaget P, Echevarria JM, Lee SD, Mushahwar IK, Robertson BH, Locarnini S, Magnius LO. 2004. Genetic diversity of hepatitis B virus strains derived worldwide: Genotypes, subgenotypes, and HBsAg subtypes. Intervirology 47: 289–309.

Ohno O, Mizokami M, Wu RR, Saleh MG, Ohba K, Orito E, Mukaide M, Williams R, Lau JY. 1997. New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. J Clin Microbiol 35:201–207.

Oyunsuren T, Kurbanov F, Tanaka Y, Elkady A, Sanduijav R, Khajidsuren O, Dagvadorj B, Mizokami M. 2006. High frequency of hepatocellular carcinoma in Mongolia; association with mono-, or co-infection with hepatitis C, B, and delta viruses. J Med Virol 78: 1688–1695.

Pawlotsky JM. 2003a. Hepatitis C virus genetic variability: Pathogenic and clinical implications. Clin Liver Dis 7:45–66.

Pawlotsky JM. 2003b. Mechanisms of antiviral treatment efficacy and failure in chronic hepatitis C. Antiviral Res 59:1–11.

Ruzibakiev R, Kato H, Ueda R, Yuldasheva N, Hegay T, Avazova D, Kurbanov F, Zalalieva M, Tuichiev L, Achundjanov B, Mizokami M. 2001. Risk factors and seroprevalence of hepatitis B virus, hepatitis C virus, and human immunodeficiency virus infection in uzbekistan. Intervirology 44:327–332.

Schaefer S. 2005. Hepatitis B virus: Significance of genotypes. J Viral Hepat 12:111–124.

Semiletov Iu A, Pimenov VK, Ianina MV, Zubov SV, Shibnev VA. 2002. Test for specific antibodies to virus hepatitis delta based on synthetic peptides. Vopr Virusol 47:21–24.

Sheen IS, Liaw YF, Lin DY, Chu CM. 1994. Role of hepatitis C and delta viruses in the termination of chronic hepatitis B surface antigen carrier state: A multivariate analysis in a longitudinal follow-up study. J Infect Dis 170:358–361.

Shustov AV, Kochneva GV, Sivolobova GF, Grazhdantseva AA, Gavrilova IV, Akinfeeva LA, Rakova IG, Aleshina MV, Bukin VN, Orlovsky VG, Bespalov VS, Robertson BH, Netesov SV. 2005. Molecular epidemiology of the hepatitis C virus in Western Siberia. J Med Virol 77:382–389.

Simmonds P, Bukh J, Combet C, Deleage G, Enomoto N, Feinstone S, Halfon P, Inchauspe G, Kuiken C, Maertens G, Mizokami M, Murphy DG, Okamoto H, Pawlotsky JM, Penin F, Sablon E, Shin IT, Stuyver LJ, Thiel HJ, Viazov S, Weiner AJ, Widell A. 2005. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology 42:962–973.

Sugauchi F, Mizokami M, Orito E, Ohno T, Kato H, Suzuki S, Kimura Y, Ueda R, Butterworth LA, Cooksley WG. 2001. A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: Complete genome sequence and phylogenetic relatedness. J Gen Virol 82:883–892.

Sugauchi F, Kumada H, Acharya SA, Shrestha SM, Gamutan MT, Khan M, Gish RG, Tanaka Y, Kato T, Orito E, Ueda R, Miyakawa Y, Mizokami M. 2004. Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. J Gen Virol 85:811–820.

Suzuki K, Mizokami M, Cao K, Wu XS, Wu RR, Hata A, Sasaki M, Fang ZX, Zhang LY, Lau JY, Iino S. 1997. Prevalence of hepatitis C virus infection in Nanjing, southern China. Eur J Epidemiol 13:511–515.

Takahashi K, Ohta Y, Kanai K, Akahane Y, Iwasa Y, Hino K, Ohno N, Yoshizawa H, Mishiro S. 1999. Clinical implications of mutations C-to-T1653 and T-to-C/A/G1753 of hepatitis B virus genotype C genome in chronic liver disease. Arch Virol 144:1299–1308.

Tanaka E, Ohue C, Aoyagi K, Yamaguchi K, Yagi S, Kiyosawa K, Alter HJ. 2000. Evaluation of a new enzyme immunoassay for hepatitis C virus (HCV) core antigen with clinical sensitivity approximating that of genomic amplification of HCV RNA. Hepatology 32:388–393.

Tanaka Y, Hanada K, Mizokami M, Yeo AE, Shih JW, Gojobori T, Alter HJ. 2002. Inaugural article: A comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. Proc Natl Acad Sci USA 99:15584–15589.

Tanaka Y, Takagi K, Fujihara T, Kitsugi K, Fujiwara K, Hiramatsu K, Ito Y, Takasaka Y, Sakai M, Mizokami M. 2003. High stability of enzyme immunoassay for hepatitis C virus core antigen-evaluation before and after incubation at room temperature. Hepatol Res 26:261–267.

Usuda S, Okamoto H, Iwanari H, Baba K, Tsuda F, Miyakawa Y, Mayumi M. 1999. Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. J Virol Methods 80:97–112.

Verbeeck J, Maes P, Wollants E, Van der Merwe S, Song E, Nevens F, Van Ranst M. 2005. Use of a commercially available line probe assay for genotyping of hepatitis C virus 5a strains. J Clin Microbiol 43: 6117–6119.

Viazov S, Kuzin S, Paladi N, Tchernovetsky M, Isaeva E, Mazhul L, Vasychova F, Widell A, Roggendorf M. 1997. Hepatitis C virus genotypes in different regions of the former Soviet Union (Russia, Belarus, Moldova, and Uzbekistan). J Med Virol 53:36–40.

Vorozhbieva TE, Iasinskii AV, Alieva G, Mikhailov MI, Iavorkovskaia EK. 1985. Characteristics of the distribution of the markers of hepatitis B vir us infection among the healthy population of the Tadzhik SSR and Azerbaijan SSR. Zh Mikrobiol Epidemiol Immunobiol 10:35–39.

Wu JC, Chiang TY, Sheen IJ. 1998. Characterization and phylogenetic analysis of a novel hepatitis D virus strain discovered by restriction fragment length polymorphism analysis. J Gen Virol 79:1105–1113.

Yu ML, Chuang WL, Chen SC, Dai CY, Hou C, Wang JH, Lu SN, Huang JF, Lin ZY, Hsieh MY, Tsai JF, Wang LY, Chang WY. 2001. Changing prevalence of hepatitis C virus genotypes: Molecular epidemiology and clinical implications in the hepatitis C virus hyperendemic areas and a tertiary referral center in Taiwan. J Med Virol 65:58–65.

Zein NN. 2000. Clinical significance of hepatitis C virus genotypes. Clin Microbiol Rev 13:223–235.

Differential MicroRNA Expression Between Hepatitis B and Hepatitis C Leading Disease Progression to Hepatocellular Carcinoma

Shunsuke Ura,[1] Masao Honda,[1,2] Taro Yamashita,[1] Teruyuki Ueda,[1] Hajime Takatori,[1] Ryuhei Nishino,[1] Hajime Sunakozaka,[1] Yoshio Sakai,[1] Katsuhisa Horimoto,[3] and Shuichi Kaneko[1]

MicroRNA (miRNA) plays an important role in the pathology of various diseases, including infection and cancer. Using real-time polymerase chain reaction, we measured the expression of 188 miRNAs in liver tissues obtained from 12 patients with hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) and 14 patients with hepatitis C virus (HCV)-related HCC, including background liver tissues and normal liver tissues obtained from nine patients. Global gene expression in the same tissues was analyzed via complementary DNA microarray to examine whether the differentially expressed miRNAs could regulate their target genes. Detailed analysis of the differentially expressed miRNA revealed two types of miRNA, one associated with HBV and HCV infections (n = 19), the other with the stage of liver disease (n = 31). Pathway analysis of targeted genes using infection-associated miRNAs revealed that the pathways related to cell death, DNA damage, recombination, and signal transduction were activated in HBV-infected liver, and those related to immune response, antigen presentation, cell cycle, proteasome, and lipid metabolism were activated in HCV-infected liver. The differences in the expression of infection-associated miRNAs in the liver correlated significantly with those observed in Huh7.5 cells in which infectious HBV or HCV clones replicated. Out of the 31 miRNAs associated with disease state, 17 were down-regulated in HCC, which up-regulated cancer-associated pathways such as cell cycle, adhesion, proteolysis, transcription, and translation; 6 miRNAs were up-regulated in HCC, which down-regulated anti-tumor immune response. *Conclusion:* miRNAs are important mediators of HBV and HCV infection as well as liver disease progression, and therefore could be potential therapeutic target molecules. (HEPATOLOGY 2009;49:000-000.)

MicroRNA (miRNA) is an endogenous, small, single-strand, noncording RNA consisting of 20 to 25 bases and regulates gene expression of various cell types. It plays an important role in various biological processes, including organ development and differentiation as well as cellular death and proliferation, and is also involved in various diseases such as infection and cancer.[1-3]

miRNAs are produced as follows. A primary miRNA with a hairpin loop structure is cleaved into a precursor miRNA and transported out of the nuclei with a carrier protein (Exportin-5). The precursor miRNA is then processed by Dicer and converted into an active single-strand RNA in the cytoplasm. The miRNA binds to a target messenger RNA in a sequence-dependent manner and induces degradation of the target messenger RNA and translational inhibition. One miRNA regulates the expression of multiple target genes; bioinformatics analyses have suggested that the expression of more than 30% of human genes is regulated by miRNAs.[4-7]

Abbreviations: cDNA, complementary DNA; CH, chronic hepatitis; CH-B, chronic hepatitis B; CH-C, chronic hepatitis C; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCC-B, hepatitis B-related hepatocellular carcinoma; HCC-C, hepatitis C-related hepatocellular carcinoma; HCV, hepatitis C virus; miRNA, microRNA; RTD-PCR, real-time detection polymerase chain reaction; SVM, support vector machine.

From the Departments of [1]Gastroenterology and [2]Advanced Medical Technology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan; and the [3]Biological Network Team, Computational Biology Research Center, National Institute of Advanced Industrial Science and Technology, 2-42 Aomi, koto-ku, Tokyo 135-0064, Japan.

Received July 3, 2008; accepted November 15, 2008.

Address reprint requests to: Masao Honda, M.D., Ph.D., Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, Takara-Machi 13-1, Kanazawa 920-8641, Japan. E-mail: mhonda@m-kanazawa.jp; fax: (81)-76-234-4250.

Copyright © 2009 by the American Association for the Study of Liver Diseases.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.22749

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

Infection of the human liver with hepatitis B virus (HBV) and hepatitis C virus (HCV) induces the development of chronic hepatitis (CH), cirrhosis, and in some instances hepatocellular carcinoma (HCC).[8] The virological features of these two distinct viruses are completely different; however, the viruses infect the liver and cause CH, which is not distinguished by histological examination or clinical manifestations. We previously reported that gene expression profiles in chronic hepatitis B (CH-B) and chronic hepatitis C (CH-C) are different. Proapoptotic and DNA repair responses were predominant in CH-B, and inflammatory and antiapoptotic phenotypes were predominant in CH-C. However, factors inducing these differences in gene expression remain to be elucidated.[9,10]

We examined miRNA expression in liver tissue with HBV-related liver disease (CH-B and HCC-B) and HCV-related liver disease (CH-C and HCC-C) and in normal liver tissue via real-time detection polymerase chain reaction (RTD-PCR). We also performed global analysis of messenger RNA expression in these tissues using complementary DNA (cDNA) microarray. These analyses allowed us to find characteristic miRNAs associated with HBV or HCV infection as well as the progression of liver disease.

Materials and Methods

Patients. The study subjects included 12 patients with CH-B complicated by HCC and 14 patients with CH-C complicated by HCC. Gene expression analysis was approved by the ethics committee of the Graduate School of Medicine, Kanazawa University Hospital, Japan, between 1999 and 2004. In addition, nine normal liver tissue samples obtained during surgery for metastatic liver cancer were used as control samples. Surgically removed liver tissues were stored in liquid nitrogen until analysis. Histological classification of HCC and histological evaluation of hepatitis in noncancerous regions for each patient are shown in Table 1. HCV viremia in two patients with CH-C was persistently cleared by interferon therapy before HCC development. There were no significant differences in the histological findings of HCC and noncancerous regions, as well as in sex, age, and hepatic function between the HBV and HCV infection groups.

Quantitative RTD-PCR. Approximately 1 mg of each liver tissue sample stored in liquid nitrogen was ground with a homogenizer while still frozen, and total RNA containing miRNA was isolated according to the protocol of the mirVana miRNA Isolation kit (Ambion, Austin, TX) and stored at −80°C until analysis. miRNA expression levels were quantitated using the TaqMan MicroRNA Assays Human Panel Early Access kit (Applied Biosystems, Foster City, CA). cDNA was prepared via reverse transcription using 10 ng each of the isolated total RNA and 3 μL each of the reverse transcription primers with specific loop structures. Reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's protocol. Then, a mixture of 6.67 μL of nuclease-free water, 10 μL of TaqMan 2 × Universal PCR Master Mix (No AmpErase UNG; Applied Biosystems), and 2 μL of TaqMan MicroRNA Assay Mix, which was included in the kit, was prepared for each sample on a 384-well plate; 1.33 μL of the reverse transcription product was added to the mixture, and amplification reaction was performed on an ABI PRISM 7900HT (Applied Biosystems). Expression levels of 188 miRNAs in each sample were quantitated.

Analysis of RTD-PCR Data. The measured 188 miRNAs included RNU6B, which is commonly used as a control for miRNA. β-Actin and glyceraldehyde 3-phosphate dehydrogenase were also measured simultaneously for correcting RNA amount. The mean Ct values and standard deviations of each miRNA were calculated from expression data of all patients obtained by RTD-PCR. miRNA with the lowest expression variation was used as the internal control. Ct values of each miRNA were then corrected by the Ct value of the internal control to yield $-\Delta Ct$ values defined as relative miRNA expression levels and used for analyses. Statistical analyses and hierarchical cluster analyses of expression data were performed using BRB ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html). Relative miRNA expression levels were further normalized using the median over the all patients so that the normalized expression levels of each patient have a median log ratio of 0. A class prediction method was used for classifying two patient groups based on the supervised learning method, and a binary tree classification method was used for classifying three or more patient groups with a statistical algorithm of the support vector machine (SVM). Class prediction was performed using SVM incorporating genes differentially expressed at a univariate parametric significance level of $P = 0.01$. The prediction rate was estimated via cross-validation and the bootstrap method for small sample data.[11] (It is worth noting that the prediction rate may be likely an overestimate of the true rate, given the weaknesses of cross-validation and bootstrapping methods in a strict sense.)

Microarray Analysis. cDNA microarray slides (Liver chip 10k) were used as described.[10] RNA isolation, amplification of antisense RNA, labeling, and hybridization were performed according to the protocols described.[9,10] Quantitative assessment of the signals on the slides was

Table 1. Characteristics of Patients Used for Analysis of miRNA and Microarray Samples

Patient No.	Virus	Age	Sex	ALT	Histology of Activity	Background Liver Fibrosis	Histological Grade of HCC	Tumor Size (mm)	TNM Staging	HCV-RNA (KIU/mL)	HBV-DNA (LEG/mL)
1	HBV	57	M	16	2	4	Moderate	20	II	–	3.4
2	HBV	51	M	57	1	2	Moderate	48	II	–	< 2.6
3	HBV	61	M	17	1	4	Well	16	II	–	< 3.7
4	HBV	47	M	19	1	4	Moderate	15	I	–	< 3.7
5	HBV	72	M	19	1	1	Well	25	II	–	NA
6	HBV	73	M	62	1	3	Moderate	45	III	–	5.7
7	HBV	42	M	36	1	4	Moderate	18	I	–	< 3.7
8	HBV	63	M	13	1	2	Moderate	15	I	–	2.8
9	HBV	68	F	54	1	2	Well	56	II	–	4.1
10	HBV	70	M	13	0	2	Well	40	II	–	< 3.7
11	HBV	58	M	29	1	4	Moderate	35	IVA*	–	3.3
12	HBV	72	M	22	1	4	Moderate	18	I	–	6
13	HCV	66	F	33	2	4	Well	25	II	423	–
14	HCV	67	M	89	1	4	Well	30	II	> 850	–
15	HCV	64	M	31	1	4	Moderate	75	III	< 5 (+)	–
16	HCV	68	M	30	0	4	Well	23	II	> 850	–
17	HCV	46	M	98	2	3	Moderate	20	I	> 850	–
18	HCV	68	F	32	2	4	Moderate	25	III	< 5 (+)	–
19	HCV	66	F	46	2	4	Well	25	II	> 850	–
20	HCV	47	M	246	1	3	Moderate	20	I	262	–
21	HCV	75	M	27	1	3	Moderate	19	II	85.1	–
22	HCV	77	M	21	0	1	Moderate	20	II	< 5 (−)	–
23	HCV	66	M	46	2	2	Well	60	II	50.3	–
24	HCV	65	M	89	1	1	Poorly	25	III	850	–
25	HCV	53	M	54	0	1	Moderate	28	II	< 5 (−)	–
26	HCV	75	F	212	1	4	Well	19	I	580	–
27	–	51	F	18	0	0	–	–	–	–	–
28	–	78	F	13	0	0	–	–	–	–	–
29	–	75	M	20	0	0	–	–	–	–	–
30	–	34	M	12	0	0	–	–	–	–	–
31	–	64	M	30	0	0	–	–	–	–	–
32	–	78	M	9	0	0	–	–	–	–	–
33	–	53	M	19	0	0	–	–	–	–	–
34	–	64	F	12	0	0	–	–	–	–	–
35	–	60	F	20	0	0	–	–	–	–	–

HCV RNA was assayed via Amplicor Monitor Test (KIU/mL); HBV DNA was assayed via transcription-mediated amplification (LEG/mL).

Abbreviations: ALT, alanine aminotransferase; F, female; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; M, male; TNM, tumor-node–metastasis.

*Vascular invasion (+).

performed by scanning on the ScanArray 5000 (General Scanning, Watertown, MA) followed by image analysis using GenePix Pro 4.1 (Axon Instruments, Union City, CA) as described.[10]

Preliminary Survey of Independency of Paired Samples from the Same Patient. CH and HCC expression data were derived from the same patient. Before further analysis, we examined whether the miRNA expression of paired samples was similar or independent. We compared differences in the expressions of paired and nonpaired CH and HCC samples using the Dunnett test[12] (Supplementary Data). All possible tests performed for data pairs represented no dependency due to the paired data from the same patients. For data analysis, we used the standard pairwise class comparison and prediction tool in BRB ArrayTools.

Identification of Candidate miRNA Target Genes. Candidate target genes predicted to be regulated by miRNAs based on sequence comparison were selected using MIRANDA Pro3.0 (Sanger Institute). Of the selected genes, those represented on a microarray chip were then examined for expression (Fig. 4). The number of genes showing a significant ($P < 0.05$) expression difference among the candidate target genes represented on the chip was statistically analyzed to evaluate the significance of expression regulation by miRNAs. Analysis of significance was performed using Hotelling T2 test (BRB ArrayTools).

Pathway Analysis. Of the candidate miRNA target genes, those showing a significant ($P < 0.01$) expression difference between N, CH-B, HCC-B, CH-C, and HCC-C samples were analyzed for pathways involving

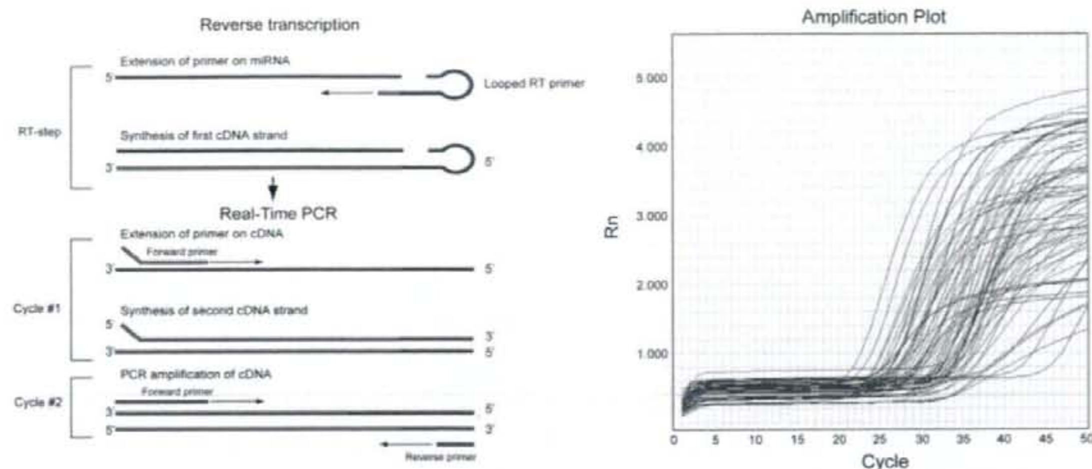

Fig. 1. (A) miRNA-specific RTD-PCR using sheet hairpin primers. (B) miRNA amplification curves by RTD-PCR.

these genes using MetaCore software suite (GeneGo, St. Joseph, MI). Significance probability was calculated using the hypergeometrical distribution based on gene ontology terms. Because one gene is frequently involved in multiple pathways, all pathways corresponding to the genes with significance probability were listed.

Verification of Regulation of Candidate Target Genes by miRNAs. Small interfering RNAs (Ambion) specific to 13 miRNAs (has-miR-17*, has-miR-20a, has-miR-23a, has-miR-26a, has-miR-27a, has-miR-29c, has-miR-30a, has-miR-92, has-miR-126, has-miR-139, has-miR-187, has-miR-200a, and has-miR-223) showing significant differences in expression were transfected into Huh7 cells using TransMessenger transfection reagent (QIAGEN, Valencia, CA), and loss of function of each miRNA was evaluated. Similarly, precursor miRNAs of five miRNAs (has-miR-23a, has-miR-26a, has-miR-27a, has-miR-92, and has-miR-200a) were also transfected into Huh7 cells, and gain of function of each miRNA was evaluated. The loss- and gain-of-function of miRNAs were evaluated via RTD-PCR. In addition, different gene expressions regulated by miRNAs were also evaluated via RTD-PCR.

HBV/HCV Infection Model Using Cultured Cells. The plasmid pHBV 1.2 coding the 1.2-fold length of the HBV genome was transfected into Huh7.5 cells using Fugene6 transfection reagent (Roche Applied Science, Indianapolis, IN). HBeAg production in culture medium was measured using Immunis HBeAg/Ab EIA (Institute of Immunology Co., Ltd., Tokyo, Japan).[13] The amount of HBV-DNA was measured via RTD-PCR (Supplementary Fig. 1A,B). JFH1-RNA was transfected into Huh7.5 cells using TransMessenger transfection reagent (QIAGEN) and the expression of the core protein was examined via immunofluorescence staining using anti-HCV core antibody (Affinity BioReagent, CO).[14,15] HCV-RNA amount was also measured via RTD-PCR (Supplementary Fig. 1A,B). JFH1/GND was used as a negative control. miRNA expression was quantitated by RTD-PCR 48 hours after transfection.

Results

Expression of miRNA in Liver Tissue. A panel of miRNA was successfully amplified from liver tissues via RTD-PCR. The representative amplification profile of miRNA as determined with RTD-PCR is shown in Fig. 1. To assess the reliability and reproducibility of this assay system, we first measured RNU6B in duplicate from all samples in different plates. The mean difference in Ct values of RNU6B expression within the same samples was 0.08 ± 0.05 (mean $\pm$ standard deviation), indicating the high reproducibility of this assay. All Ct values from each reaction were collected, and Ct variation obtained by each probe from all patients was calculated. Although RNU6B was frequently used as the internal control, the standard Ct variation was relatively high (Ct, 27 ± 1.94), suggesting that the variances in its value depend on the state of liver disease (N, CH and HCC). Therefore, we selected has-miR-328 as the internal control with the smallest standard deviation (Ct, 30 ± 0.60). The relative expression ratio of individual miRNA to has-miR-328 was calculated and applied to the following analysis using a BRB-array tool.

Hierarchical cluster analysis revealed that the expression profiles of the 188 miRNAs from each patient were

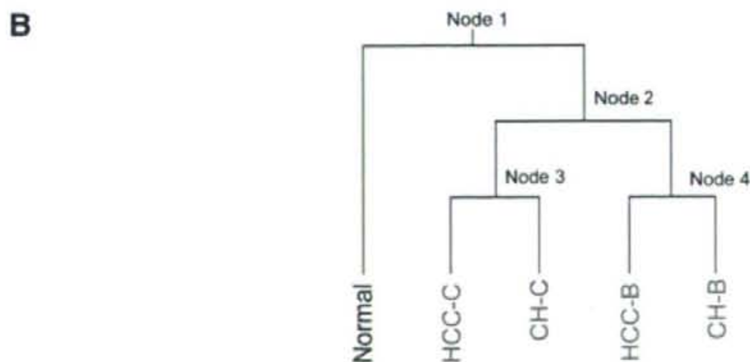

Fig. 2. (A) Hierarchical cluster analysis using total miRNA. Chronic hepatitis is indicated by histological stage and grade (F, fibrosis; A, activity) and type of infecting virus (B or C). HCC is indicated by histological grade (w, well differentiated; m, moderately differentiated; p, poorly differentiated) and type of infecting virus (B or C), with the patient number added at the end. (B) Relationship between five classes divided by binary tree classification. Expression profiles were first classified into normal liver and non-normal liver groups (node 1), then into HBV and HCV groups (node 2). The HBV group was further divided into HCC-B and CH-B (node 3), and the HCV group into HCC-C and CH-C (node 4).