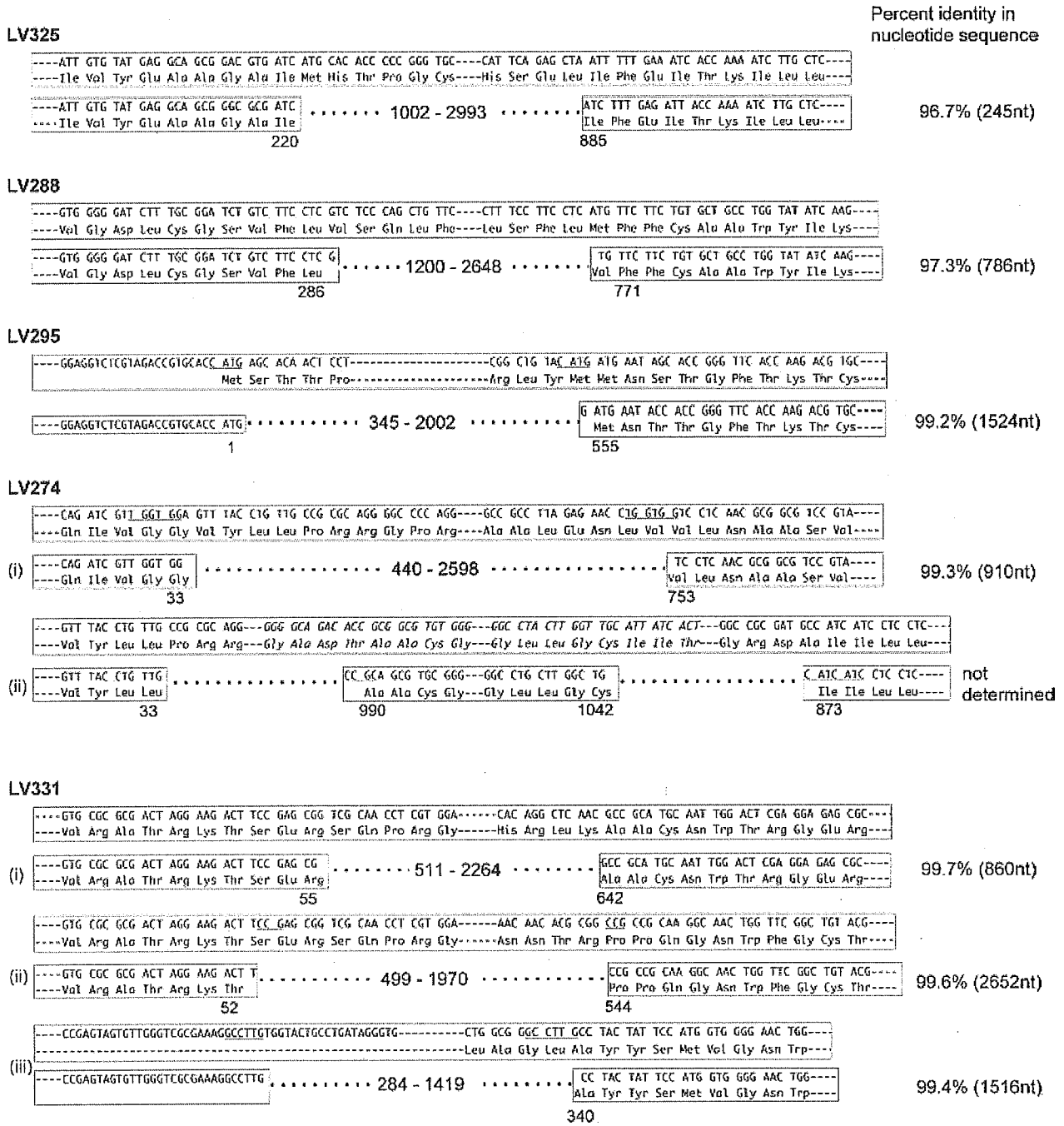
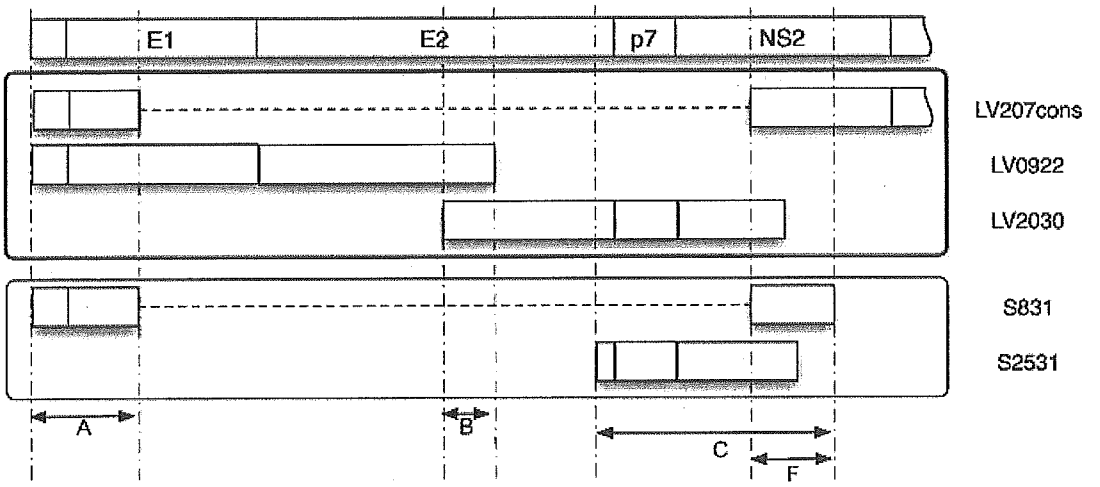


Fig. 4. Sequence comparisons between HCV genomes with and without deletions isolated from the same patient. Partial sequences of RT-PCR fragments isolated from liver biopsies are shown. cDNA fragments are labeled as described for previous figures. Nucleotide and amino acid positions are represented as the corresponding positions in HCV-J1. In these cases, both non-truncated and truncated HCV RNA were amplified from each biopsy specimen (Table I). The non-truncated and truncated RT-PCR fragments from Patient 325, 288, 295, 274, and 331 were obtained with primer sets d and d, d and d, b and b, b and e, and a and b (Table II), respectively. Numbers between tagged graphs represent deleted regions as they correspond to HCV-J1. Bottom lines show the corresponding amino acid positions of the boundaries. The right side of the sequences shows the identities of overlapping nucleotide sequences in the truncated and non-truncated genome, and the length of the overlap is given in parentheses. The LV274-(ii) had a translocation of the NS3 sequence inserted between the core and NS2 sequences. Because we did not clone the NS3 sequence from this case, the corresponding region of HCV-J1 is italicized in the figure and the sequence identity was not determined. Sequences that coincided between the boundaries of the deletions are underlined.

A



B

			Serum cDNA S831	Serum cDNA S2531
		Region	A	F	F
	LV207cons	Nucleotide	99.7% (315)	98.9% (180)	91.8% (98)
		Amino acid	100% (105)	98.3% (60)	81.8% (33)
		Region	A		
Liver cDNA	LV0922cons	Nucleotide	95.0% (303)		
		Amino acid	92.1% (101)		
		Region		F	C
	LV2030cons	Nucleotide		90.2% (82)	99.8% (513)
		Amino acid		81.4% (27)	99.4% (171)

			Liver cDNA LV0922cons	Liver cDNA LV2030cons
		Region	A	F
	LV207cons	Nucleotide	95.0% (303)	91.6% (98)
		Amino acid	92.1% (101)	82.1% (28)
Liver cDNA		Region	B	
	LV2030cons	Nucleotide	100% (76)	—
		Amino acid	100% (25)	

Number in parentheses expresses
The length of the overlapped sequences

C

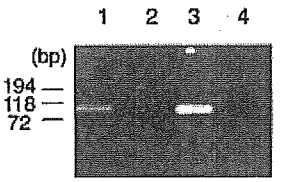


Fig. 5. Cloning of the non-truncated HCV RNA from patient 207. A: Schematic views of RT-PCR fragments for non-truncated HCV RNA in liver (LV0922 and LV2030) and in serum (S2531) from Patient 207, and for the truncated sequence in serum (S831). LV0922 fragment was obtained by RT-PCR with HC1b9405R for cDNA synthesis, HC841S and HC2199AS for 1st PCR, and HC948S and HC2199AS for 2nd PCR. LV2030 fragment was obtained by RT-PCR with XR58R for cDNA synthesis, HC2048SLV and LVC1392AS for 1st PCR, and HC2069S and LVC1280AS for 2nd PCR. S2531 fragment was obtained by RT-PCR with HC3174AS for cDNA synthesis, HC2430S and HC3174AS for 1st PCR, and HC2546S and HC3111AS for 2nd PCR. Arrows indicate regions being compared in the Tables (**B**), which show sequence identities between the non-truncated and truncated sequences in serum (**upper**), and those between sequences isolated from liver and liver (**lower**). **C**: Images of agarose gel electrophoresis of RT-PCR products amplified using junction site primer, from extracted RNA from Patient 207 serum (**lane 1**) and Donor G14 plasma (**lane 2**), truncated in vitro RNA transcripts of Patient 207 (**lane 3**) and non-truncated in vitro RNA transcripts of Donor G14 (**lane 4**).

protein at the 70-kDa position, which was the predicted mass of NS3, was detected by anti-NS3 polyclonal antibody. These data suggested that processing of the truncated HCV polyprotein is same as that of the full-length HCV polyprotein at the core–E1 and NS2–NS3 junctions.

DISCUSSION

Characteristics of HCV Subgenome With In-Frame Deletion

Novel truncated HCV genomes with in-frame deletions from E1 to NS2 were identified in the livers of two

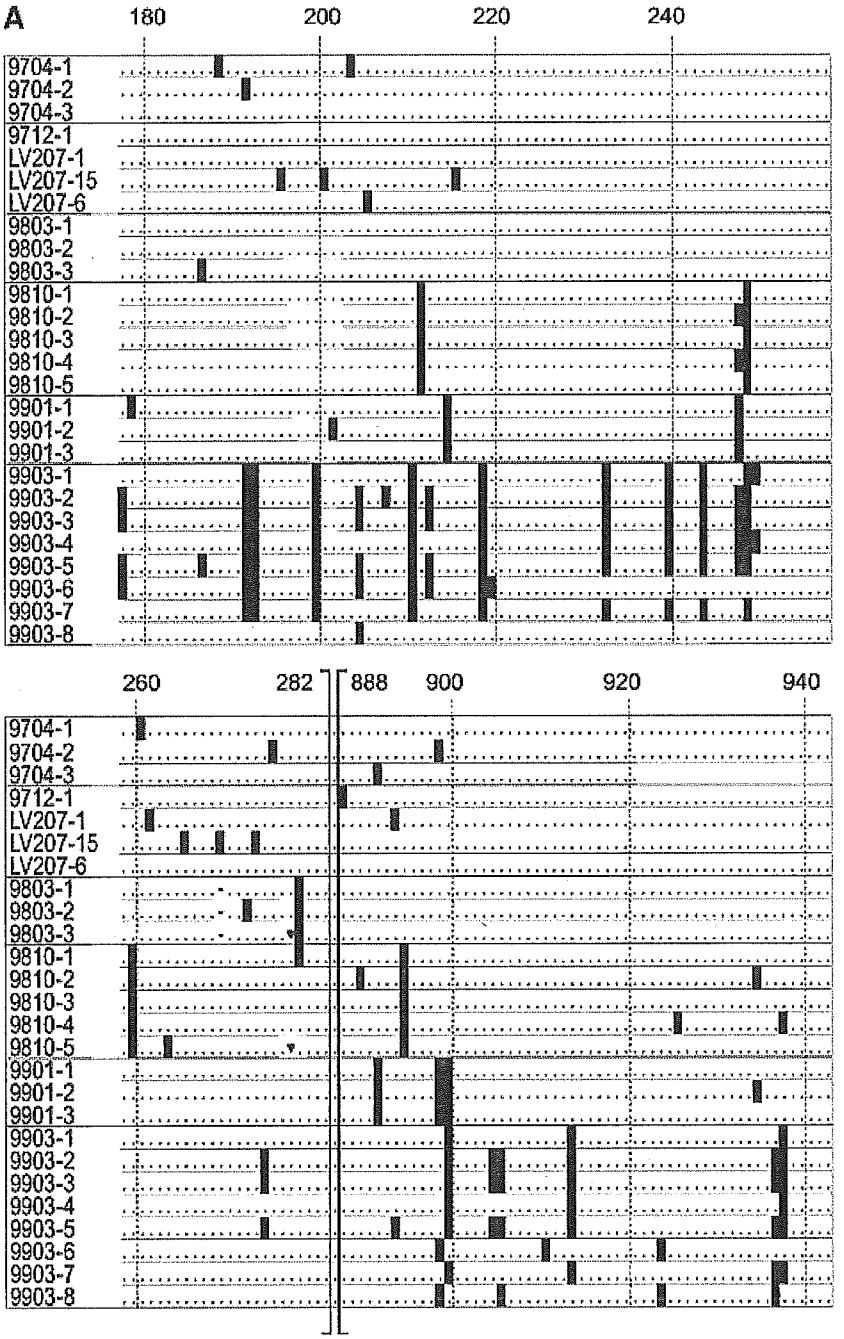


Fig. 6. Alignment of amino acid sequences of cDNA isolates from a series of serum samples from Patient 207. Comparison of nucleotide sequences of truncated genome (A) and non-truncated genome (B) was carried out using Clustal W algorithm. Numbers on top of the alignment show the corresponding amino acid positions of HCV J1. Names of the sequences indicate the date the serum was taken; 9712, for example, refers to December 1997. Bold bar represents amino acid position that differs from consensus sequence. Coinciding amino acid positions are represented by dots. All cDNA isolates from March 1998 (9803) had an amino acid deletion at the position marked by the horizontal bar. Bold vertical bars in A show the boundaries of the deletion. Inverted triangles indicate positions of nucleotide deletions in cDNA isolates. Deduced amino acid sequences with nucleotide deletions were obtained by inserting a nucleotide at the position. Fragments of the truncated genome (A) were obtained by RT-PCR with HC3297R for cDNA synthesis, HC813S and HC3297R for 1st PCR, and HC841S and HC3174AS for 2nd PCR. Fragments of the non-truncated genome (B) were obtained by RT-PCR with HC2378R for cDNA synthesis, HC1979S and HC2378R for 1st PCR, and HC1979S and HC2300R for 2nd PCR.

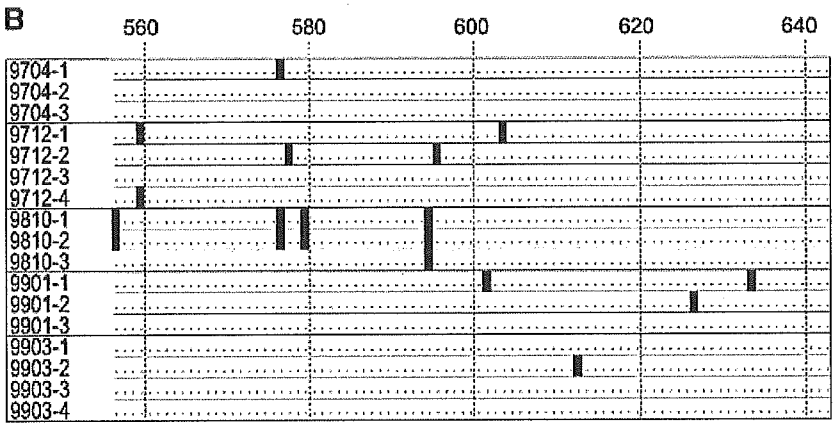


Fig. 6. (*Continued*)

chronic active hepatitis C patients. These HCV subgenomes encoded a single polyprotein for the entire core, five NS proteins (from NS3 to NS5B), and E1–NS2 fusion protein. This type of HCV subgenome was found in 4 of 23 chronic active hepatitis patients (16 cases with positive results by RT-PCR with primer sets tested), and in 2 hepatocellular carcinoma patients. These data suggested that HCV subgenomic RNA is generated in a certain number of chronic active hepatitis C patients.

HCV polypeptide expressed in cells with the subgenomic cDNA was processed in the same way as the authentic HCV polyprotein. The E1–NS2 fusion protein from subgenomic HCV cDNA was glycosylated and was susceptible to Endo H treatment, thus suggesting that it was located in the ER. NS2 is a membrane protein located in the ER region [Santolini et al., 1995; Kim et al., 1999]. On the other hand, localization and modification of E1 protein was governed by E2 [Cocquerel et al., 1998; Duvet et al., 1998]. These data indicate that ER retention in the E1–NS2 fusion protein is determined by NS2.

TABLE III. Quantitation of HCV RNA in a Series of Serum Samples Taken From Patient 207

Date	5′-UTR (copies/ml)	Percentage of E2/UTR
December 8, 1997	7.75×10^4	19.5%
	(5.75×10^3	0.2%)
	Numbers in parenthesis represents HCV quantity in liver biopsy specimen	
March 6, 1998	2.93×10^3	36.2%
March 9, 1998	1.19×10^4	22.1%
	IFN treatment From March 9, 1998 to September 2, 1998	
September 29, 1998	4.17×10^4	23.8%
October 28, 1998	5.25×10^4	21.8%
December 8, 1998	2.24×10^4	26.1%
January 12, 1999	5.81×10^4	28.5%
February 10, 1999	2.57×10^4	20.3%
March 31, 1999	5.30×10^4	23.1%

Do HCV Subgenomes Replicate Autonomously in Patients?

Viral subgenomes have been isolated from viruses closely related to HCV, such as flavivirus (Murray Valley encephalitis virus) [Lancaster et al., 1998], pestivirus (classical swine fever virus; SFV [Aoki et al., 2001], and bovine viral diarrhea virus; BVDV [Tautz et al., 1994; Kupfermann et al., 1996]. The HCV subgenomes shared common structural features with these subgenomic RNAs.

The HCV subgenomes fulfilled the minimal requirements for autonomous RNA replication; the 5′-UTR, nonstructural proteins (NS3–NS5B), and the 3′-UTR, as demonstrated using artificial HCV subgenomic replicons [Lohmann et al., 1999; Blight et al., 2000]. In addition, defective genomes of DI autonomously replicate their RNA [Behrens et al., 1998]. Furthermore, sequence comparisons of the truncated and non-truncated HCV genome sequences, which were isolated from a serum series obtained from a single patient, suggested that both genomes have been replicating independently for years. These data suggest that HCV subgenomes with in-frame deletions in structural proteins replicate themselves. However, it is possible that the full-length genome is required for the replication of the subgenome, as both genomes were present for years despite the dominance of the subgenome.

The dominance of the subgenome over the full-length genome (approximately 500-fold in the liver) was indicated by real-time RT-PCR analysis for the HCV 5′-UTR and E2. The dominance of HCV subgenome suggests an advantage in RNA replication. The length of the genome is probably a key factor in viral replication. If processing velocities in translation and transcription are equal over the HCV genome, the HCV subgenome would be replicated about 20% faster than the non-truncated genome. However, other mechanisms affecting efficiency are likely present.

Heterogeneous molecular clones with out-of-frame deletions, which shared sequences with the full-length

genome, indicated that the HCV subgenome frequently arises from its full-length genome by such mechanisms found in other RNA viruses [Nagy and Simon, 1997]. However, the fact only one type of subgenome with an in-frame deletion persisted suggested that a competent subgenome for replication is selected. The NS2 in all HCV subgenomes, which preserved their NS2–NS3 protease domains [Grakoui et al., 1993a; Hijikata et al., 1993], indicated protease activity is involved in the persistence of the HCV subgenome. Furthermore, we believe that core protein is required for virus replication in vivo, because the core sequence in the HCV subgenomes was preserved among the dominant HCV subgenomes.

Comparison With HCV Subgenomes or Recombinants Described Previously

Quadri and Negro [2001] identified recently a positive-strand subgenomic RNA starting from the 5′-UTR without the 3′-UTR, and a negative-strand subgenomic RNA with the 3′-UTR lacking the 5′-UTR. Although we did not clone the 3′-UTR from the Patient 207 sample, an HCV subgenome with same deletion was isolated from cDNA with a primer corresponding to the X-region in the 3′-UTR(data not shown), and we isolated the 3′-UTR from Patient 373 liver RNA. Based on these observations, it is considered that the subgenomic HCV RNA contains the entire 3′-UTR, rather than their proposed RNA populations.

Intergenotypic recombination has been described between genotype 2k and genotype 1b HCV at between nucleotides 3175 and 3176, about 200 nucleotides from the recombination region of the HCV subgenomes [Kalinina et al., 2002]. We did not examine the possibility of this type of recombination because the number of HCV cDNAs covering this region was too few in the present study.

HCV Subgenome and Pathogenesis

The question whether the HCV subgenome involved in the mechanism of viral persistence and pathogenesis in a similar manner as the DI particles of other viruses

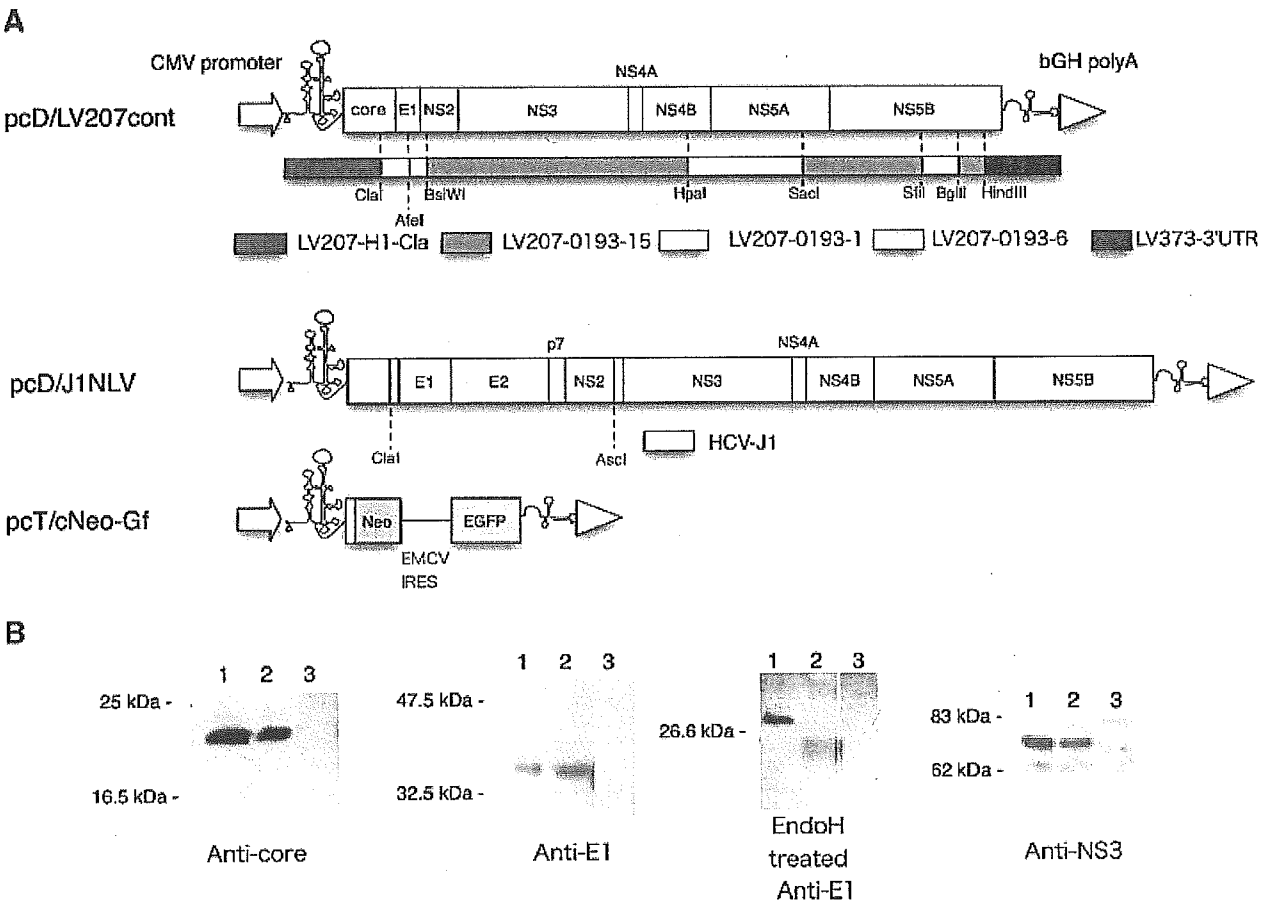


Fig. 7. Expression of HCV proteins from truncated and non-truncated HCV cDNA in mammalian cells. A: Chimeric HCV subgenomic cDNA, LV207cont, was composed of fragments from four cDNAs from patient liver (LV207-H1-Cla, 0193-15, 0193-1, and 0193-6) and a fragment from Patient 373 (LV373-3′-UTR) by using restriction sites depicted. Other chimeric cDNAs consisted of LV207cont and a fragment of HCV-J1 (J1NLV), as indicated. Dicistronic DNA constructs of HCV core-Neo resistant fusion and EGFP genes, cNeo-EGFP, was used as a negative control for HCV protein expression. All DNA constructs were transiently expressed in HEK293 cells under the control of the CMV promoter in pcDNA3.1. B: Western blotting analysis results for core, E1 and NS3 in transfected cells are shown. The positions of pre-stained molecular weight markers are indicated on the left side of the images.

[Tautz et al., 1994; Kupfermann et al., 1996] is remained. The amounts of core protein in patients with the HCV subgenome were larger than in patients without the subgenome (not statically significant). Transgenic mice expressing core protein in liver developed steatosis and later cancer, indicating that the core protein is a potent carcinogen in mice [Moriya et al., 1997, 1998]. It was found that two HCC patients had this subgenome. These data suggested that the involvement of the truncated genome in pathogenesis; however, we must examine more cases in order to elucidate any correlations between HCV subgenome and disease, particularly for progression of the disease to HCC.

The heterogeneous nature of the HCV genomes in patients may contribute to the persistence of HCV in escaping the host defense system. Particularly, the deletion of E1/E2 proteins may have a great impact on host immune response to the virus; E1/E2 is believed to be a target molecule for neutralizing antibodies, which block the binding of virions to virus receptor [Beyene et al., 2002]. The function of the truncated HCV genome in the life cycle of HCV is uncertain, but we believe the presence of this subgenomic RNA in both the liver and serum is important for illustrating that much about the nature of HCV remains unknown.

Added in Proof

Wakita et al. recently reported that a man-made HCV subgenomic RNA lacking E1/E2 replicated in vitro. [Wakita et al., 2005, NatureMedicine, published online 12 June]

ACKNOWLEDGMENTS

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REFERENCES

Aizaki H, Aoki Y, Harada T, Ishii K, Suzuki T, Nagamori S, Toda G, Matsuura Y, Miyamura T. 1998. Full-length complementary DNA of hepatitis C virus genome from an infectious blood sample. Hepatology 27:621–627.

Alter HJ, Seeff LB. 2000. Recovery, persistence, and sequelae in hepatitis C virus infection: A perspective on long-term outcome. Semin Liver Dis 20:17–35.

Alter HJ, Purcell RH, Shih JW, Melpolder JC, Houghton M, Choo QL, Kuo G. 1989. Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. N Engl J Med 321:1494–1500.

Alvarez MJ, Depino AM, Podhajcer OL, Pitossi FJ. 2000. Bias in estimations of DNA content by competitive polymerase chain reaction. Anal Biochem 287:87–94.

Aoki H, Ishikawa K, Sakoda Y, Sekiguchi H, Kodama M, Suzuki S, Fukusho A. 2001. Characterization of classical swine fever virus associated with defective interfering particles containing a cytopathogenic subgenomic RNA isolated from wild boar. J Vet Med Sci 63:751–758.

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.

Behrens SE, Grassmann CW, Thiel HJ, Meyers G, Tautz N. 1998. Characterization of an autonomous subgenomic pestivirus RNA replicon. J Virol 72:2364–2372.

Beyene A, Basu A, Meyer K, Ray R. 2002. Hepatitis C virus envelope glycoproteins and potential for vaccine development. Vox Sang 83:27–32.

Blight KJ, Kolykhalov AA, Rice CM. 2000. Efficient initiation of HCV RNA replication in cell culture. Science 290:1972–1974.

Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244:359–362.

Cocquerel L, Meunier JC, Pillez A, Wychowski C, Dubuisson J. 1998. A retention signal necessary and sufficient for endoplasmic reticulum localization maps to the transmembrane domain of hepatitis C virus glycoprotein E2. J Virol 72:2183–2191.

Dries V, von Both I, Muller M, Gerken G, Schirmacher P, Odenthal M, Bartenschlager R, Drebber U, Meyer zum Buschenfeld KH, Dienes HP. 1999. Detection of hepatitis C virus in paraffin-embedded liver biopsies of patients negative for viral RNA in serum. Hepatology 29:223–229.

Duvet S, Cocquerel L, Pillez A, Cacan R, Verbert A, Moradpour D, Wychowski C, Dubuisson J. 1998. Hepatitis C virus glycoprotein complex localization in the endoplasmic reticulum involves a determinant for retention and not retrieval. J Biol Chem 273:32088–32095.

Fagan EA, Ellis DS, Tovey GM, Lloyd G, Smith HM, Portmann B, Tan KC, Zuckerman AJ, Williams R. 1992. Toga virus-like particles in acute liver failure attributed to sporadic non-A, non-B hepatitis and recurrence after liver transplantation. J Med Virol 38:71–77.

Grakoui A, McCourt DW, Wychowski C, Feinstone SM, Rice CM. 1993a. A second hepatitis C virus-encoded proteinase. Proc Natl Acad Sci USA 90:10583–10587.

Hijikata M, Kato N, Ootsuyama Y, Nakagawa M, Shimotohno K. 1991. Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. Proc Natl Acad Sci USA 88:5547–5551.

Hijikata M, Mizushima H, Akagi T, Mori S, Kakiuchi N, Kato N, Tanaka T, Kimura K, Shimotohno K. 1993. Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. J Virol 67:4665–4675.

Hiramatsu N, Hayashi N, Haruna Y, Kasahara A, Fusamoto H, Mori C, Fuke I, Okayama H, Kamada T. 1992. Immunohistochemical detection of hepatitis C virus-infected hepatocytes in chronic liver disease with monoclonal antibodies to core, envelope and NS3 regions of the hepatitis C virus genome. Hepatology 16:306–311.

Houghton M, Selby M, Weiner A, Choo QL. 1994. Hepatitis C virus: Structure, protein products and processing of the polyprotein precursor. Curr Stud Hematol Blood Transfus 1–11.

Iino S, Hino K, Yasuda K. 1994. Current state of interferon therapy for chronic hepatitis C. Intervirology 37:87–100.

Infantolino D, Bonino F, Zanetti AR, Lesniewski RR, Barbazza R, Chiaramonte M. 1990. Localization of hepatitis C virus (HCV) antigen by immunohistochemistry on fixed-embedded liver tissue. Ital J Gastroenterol 22:198–199.

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.

Kasahara A, Tanaka H, Okanoue T, Imai Y, Tsubouchi H, Yoshioka K, Kawata S, Tanaka E, Hino K, Hayashi K, Tamura S, Itoh Y, Kiyosawa K, Kakumu S, Okita K, Hayashi N. 2004. Interferon treatment improves survival in chronic hepatitis C patients showing biochemical as well as virological responses by preventing liver-related death. J Viral Hepat 11:148–156.

Kashiwakuma T, Hasegawa A, Kajita T, Takata A, Mori H, Ohta Y, Tanaka E, Kiyosawa K, Tanaka T, Tanaka S, Hattori N, Kohara M. 1996. Detection of hepatitis C virus specific core protein in serum of patients by a sensitive fluorescence enzyme immunoassay (FEIA). J Immunol Methods 190:79–89.

Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, Shimotohno K. 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci U S A 87:9524–9528.

Kato T, Miyamoto M, Date T, Yasui K, Taya C, Yonekawa H, Ohue C, Yagi S, Seki E, Hirano T, Fujimoto J, Shirai T, Wakita T. 2003. Repeated hepatocyte injury promotes hepatic tumorigenesis in hepatitis C virus transgenic mice. Cancer Sci 94:679–685.

Kim JE, Song WK, Chung KM, Back SH, Jang SK. 1999. Subcellular localization of hepatitis C viral proteins in mammalian cells. Arch Virol 144:329–343.

Kiyosawa K, Sodeyama T, Tanaka E, Gibo Y, Yoshizawa K, Nakano Y, Furuta S, Akahane Y, Nishioka K, Purcell RH, et al. 1990. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: Analysis by detection of antibody to hepatitis C virus. Hepatology 12:671–675.

Kiyosawa K, Tanaka E, Sodeyama T, Furuta S. 1994. Natural history of hepatitis C. Intervirology 37:101–107.

Kiyosawa K, Umemura T, Ichijo T, Matsumoto A, Yoshizawa K, Gad A, Tanaka E. 2004. Hepatocellular carcinoma: Recent trends in Japan. Gastroenterology 127:S17–S26.

Kuo G, Choo QL, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, Miyamura T, Dienstag JL, Alter MJ, Stevens CE, et al. 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 244:362–364.

Kupfermann H, Thiel HJ, Dubovi EJ, Meyers G. 1996. Bovine viral diarrhea virus: Characterization of a cytopathogenic defective interfering particle with two internal deletions. J Virol 70:8175–8181.

Lancaster MU, Hodgetts SI, Mackenzie JS, Urosevic N. 1998. Characterization of defective viral RNA produced during persistent infection of Vero cells with Murray Valley encephalitis virus. J Virol 72:2474–2482.

Lau GK, Davis GL, Wu SP, Gish RG, Balart LA, Lau JY. 1996. Hepatic expression of hepatitis C virus RNA in chronic hepatitis C: A study by in situ reverse-transcription polymerase chain reaction. Hepatology 23:1318–1323.

Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285:110–113.

McHutchison JG, Fried MW. 2003. Current therapy for hepatitis C: Pegylated interferon and ribavirin. Clin Liver Dis 7:149–161.

Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, Miyamura T, Koike K. 1997. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J Gen Virol 78:1527–1531.

Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, Matsuura Y, Kimura S, Miyamura T, Koike K. 1998. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med 4:1065–1067.

Nagy PD, Simon AE. 1997. New insights into the mechanisms of RNA recombination. Virology 235:1–9.

Nielsen SU, Bassendine MF, Burt AD, Bevitt DJ, Toms GL. 2004. Characterization of the genome and structural proteins of hepatitis C virus resolved from infected human liver. J Gen Virol 85:1497–1507.

Nuovo GJ, Holly A, Wakely P, Jr., Frankel W. 2002. Correlation of histology, viral load, and in situ viral detection in hepatic biopsies from patients with liver transplants secondary to hepatitis C infection. Hum Pathol 33:277–284.

Perillo RP. 1997. The role of liver biopsy in hepatitis C. Hepatology 26(3 supp 1):57S–61S.

Quadri R, Negro F. 2001. Are there any subgenomic forms of hepatitis C virus RNA in the liver? Dig Liver Dis 33:480–486.

Rice P, Longden I, Bleasby A. 2000. EMBOSS: The European molecular biology open software suite. Trends Genet 16:276–277.

Saito M, Hasegawa A, Kashiwakuma T, Kohara M, Sugi M, Miki K, Yamamoto T, Mori H, Ohta Y, Tanaka E, et al. 1992. Performance of an enzyme-linked immunosorbent assay system for antibodies to hepatitis C virus with two new antigens (c11/c7). Clin Chem 38: 2434–2439.

Sakamoto N, Enomoto N, Kurosaki M, Marumo F, Sato C. 1994. Detection and quantification of hepatitis C virus RNA replication in the liver. J Hepatol 20:593–597.

Santolini E, Pacini L, Fipaldini C, Migliaccio G, Monica N. 1995. The NS2 protein of hepatitis C virus is a transmembrane polypeptide. J Virol 69:7461–7471.

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.

Tautz N, Thiel HJ, Dubovi EJ, Meyers G. 1994. Pathogenesis of mucosal disease: A cytopathogenic pestivirus generated by an internal deletion. J Virol 68:3289–3297.

Tellier R, Bukh J, Emerson SU, Miller RH, Purcell RH. 1996. Long PCR and its application to hepatitis viruses: Amplification of hepatitis A, hepatitis B, and hepatitis C virus genomes. J Clin Microbiol 34:3085–3091.

Yanagi M, Purcell RH, Emerson SU, Bukh J. 1997. Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. Proc Natl Acad Sci USA 94:8738–8743.

Yanagi M, St Claire M, Shapiro M, Emerson SU, Purcell RH, Bukh J. 1998. Transcripts of a chimeric cDNA clone of hepatitis C virus genotype 1b are infectious in vivo. Virology 244:161–172.

Yoshida H, Shiratori Y, Moriyama M, Arakawa Y, Ide T, Sata M, Inoue O, Yano M, Tanaka M, Fujiyama S, Nishiguchi S, Kuroki T, Imazeki F, Yokosuka O, Kinoyama S, Yamada G, Omata M. 1999. Interferon therapy reduces the risk for hepatocellular carcinoma: National surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of hepatocarcinogenesis by interferon therapy. Ann Intern Med 131: 174–181.

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Nucleotide Mutations Associated With Hepatitis B e Antigen Negativity

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One hundrded and forty four patients with chronic hepatitis B were tested to identify new mutations associated with hepatitis B e antigen (HBeAg) negativity, using a full genome sequence analysis. All the patients were Chinese and had hepatitis B virus infection of genotype C. Patients with none of the pre-core or core promoter mutations were significantly ($P < 0.001$) less common in the group with anti-HBe (13%) than in the group with HBeAg (56%). The complete nucleotide sequence was determined in four anti-HBe-positive patients who had neither pre-core nor core promoter mutations and in five HBeAg-positive patients who also had neither of these mutations (the groups were matched for age and sex). Six mutations were found to be significantly more common in the former group than in the latter: G529A (3/4 vs. 0/5), C934A (4/4 vs. 1/5), A1053G (4/4 vs. 1/5), G1915T/A (4/4 vs. 0/5), T2005C/A (4/4 vs. 0/5), and C3026T (3/4 vs. 0/5). Three of the six mutations were significantly more common in the four anti-HBe-positive patients who had neither pre-core nor core promoter mutations, compared to 11 HBeAg-positive patients who had pre-core and core promoter mutations, and also compared to 15 anti-HBe-positive patients who had pre-core and core promoter mutations, suggesting further the specificity of these mutations. Of the six mutations, two resulted in amino acid substitution in the polymerase protein, and one is located near the enhancer I region. The results suggest that the six newly discovered mutations are associated with HBeAg negativity. ***J. Med. Virol. 76:170–175, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: hepatitis B e antigen (HBeAg); genotype; nucleotide mutation

INTRODUCTION

Approximately 350 million people are chronic carriers of hepatitis B virus (HBV) worldwide [Maynard, 1990; Maddrey, 2000]. Chronic HBV infection is the cause of up to 50% of cirrhosis and 70–90% of hepatocellular carcinomas (HCC) in China, South-East Asia, and Africa [Lok, 1992; Fattovich, 1998], and in Asian countries, almost all patients with chronic HBV infection have been infected perinatally from hepatitis B e antigen (HBeAg)-positive mothers [Okada et al., 1976]. HBeAg is considered to be a marker for viral replication, but some HBeAg-negative patients remain viremic and continue to have active liver disease [Hadziyannis et al., 1983; Lok et al., 1984; Bonino et al., 1986]. Many of these patients are found to have a G to A change at nucleotide 1896, which creates a stop codon (TAG) in the precore (Pre-C) open reading frame, which in turn prevents translation of the Pre-C protein and aborts HBeAg production [Carman et al., 1989]. Other patients have mutations in the core promoter (CP) region, including an A to T mutation at nucleotide 1762 and a G to A mutation at nucleotide 1764 [Okamoto et al., 1994]. In vitro studies of this double mutation show decreased transcription of Pre-C messenger RNA and hence a resultant decrease in HBeAg production by 70% [Buckwold et al., 1996; Chan et al., 1999]. A recent follow-up study on Pre-C and CP mutations has also

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shown that the presence of these mutations is useful for predicting seroconversion [Yamaura et al., 2003].

Besides the G1896A mutation and the A1762T/G1764A mutation, a number of point mutations, as well as deletions and insertions of nucleotides, have been detected in the Pre-C region and CP region that could correlate with seroconversion [Okamoto et al., 1990; De Castro et al., 2001]. In the present study, the complete HBV genome was examined for other nucleotide mutations associated with HBeAg negativity, in addition to mutations in the Pre-C and CP regions.

MATERIALS AND METHODS

Patients

A cohort of 193 Chinese patients with chronic HBV infection who visited the Liver Disease Clinic of the Second Hospital of HeBei Medical University in Shijiazhuang city, North China, between June and August 2001 were enrolled in the study. These patients comprised 124 men and 69 women and had a median age of 29.1 years old (range: 5–73 years old). Patients who were co-infected with hepatitis C or D virus or with the human immunodeficiency virus and patients with other concomitant causes of chronic liver disease were excluded. According to the consensus diagnostic criteria for HBV infection, 182 patients were diagnosed with chronic hepatitis B. The remaining 11 patients had persistently normal alanine aminotransferase (ALT: normal range 10–21 IU/L) levels, suggesting an inactive HBV carrier stage. None of the 193 patients were treated with antiviral agents such as interferon or lamivudine. Of the 193 patients, 169 (87.6%) were of genotype C, 21 (10.9%) of genotype B, and 3 (1.5%) of genotype A. For the mutation analysis, 144 patients who were positive for either HBeAg or anti-HBe were selected from the 169 genotype C patients. Informed consent was obtained from each patient.

Conventional HBV Markers and Genotyping of HBV

Hepatitis B surface antigen (HBsAg), HBeAg and anti-HBe were measured using commercially available enzyme immunoassay kits (Abbott Japan, Tokyo, Japan). Serum concentration of HBV DNA was measured using the AMPLICOR HBV Monitor test (Roche Diagnostics K.K., Tokyo, Japan), which has a quantitative range of 2.6–7.6 log copies/ml. When the concentration to be tested was beyond this range, the actual concentration was determined using a serum sample diluted 100-fold with normal human serum. The HBV genotype was determined using the restriction fragment length polymorphism (RFLP) method on an S-gene sequence amplified by polymerase chain reaction (PCR) with nested primers [Mizokami et al., 1999].

Determination of Pre-C and CP Mutations

The 1,896th nucleotide in the Pre-C region of G or A was detected with an enzyme-linked mini-sequence assay kit (Roche Diagnostics), and the results were expressed as the percentage mutation rate, as defined by Aritomi et al. [1998]. If the mutation rate was 0%, the strain was considered to be Pre-C mutation-negative, while a Pre-C mutation-positive strain was recorded when the mutation rate exceeded 0%. The double mutation in the CP region (A1762/T1764) was detected using an HBV CP mutation detection kit (Smitest: Genome Science Laboratories, Tokyo, Japan), and the results were classified into three categories: wild, mixed, and mutant types. A wild type strain was considered to be CP mutation-negative, while mixed and mutant types were recorded as CP mutation-positive strains. The detection limits of the pre-C and the CP mutation detection kits are both 1,000 copies/ml.

Determination of Nucleotide Sequence

The complete genome sequence was determined according to the method described by Rokuhara et al. [2000]. Briefly, nucleic acids were extracted from a serum sample of 100 μl with a DNA/RNA extraction kit (Smitest EX-R&D: Genome Science Laboratories Co., Ltd.). Two microliters of each DNA solution were used for amplification by PCR. The reaction was carried out in 25 μl of PCR-mixture containing 250 μmol/L of each dNTP, 1× PCR buffer [50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$, 0.001% gelatin], 0.25 U EX-Taq DNA polymerase (TaKaRa, Tokyo), and 0.25 μM of a primer pair. The PCR was initiated using the hot-start technique.

To determine the full-length nucleotide sequence of HBV, two fragments (fragments A and B) were amplified by PCR, using the primers shown in Table I. Fragment A (1,498 bases in length; nt 457–nt 1954) was amplified with nested pairs of outer (SB1 and CB2) and inner primers (SB3 and CB4), while fragment B (2162 bases in length; nt 1611–nt 557), was amplified with nested pairs of outer primers (es2 and PS4) and inner primers (is2 and PS3). The first round of PCR was performed with an outer primer set for 40 cycles (94°C for 1.5 min, 55°C for 1 min, and 72°C for 2 min), and was followed by an extension reaction at 72°C for 7 min. The second round was undertaken with an inner primer set for 30 cycles, and was also followed by an extension reaction. PCR products were subjected to electrophoresis on a 1.0% agarose gel with ethidium bromide staining and visualization with an UV transilluminator. The band containing the target sequence was removed and DNA was isolated using GFX™ PCR DNA and a Gel Band Purification kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The nucleotide sequence was directly determined by the dideoxy method, using the sequencing primers shown in Table I. The accuracy of the sequence was ensured by comparison of the sequence data for the complete genome obtained with sense-sequencing primers and that obtained with anti-sense-sequencing primers.

Statistical Analysis

Mann–Whitney's *U* test was utilized for quantitative data, and Fisher's exact test and a Chi-square test were

TABLE I. Primers Used for PCR and Sequencing of HBV DNA

Primer		Sequence	nt position
Primers for PCR of fragment A			
SB1	Sense	5-TGCTGCTATGCCTCATCTTC	(414–433)
CB2	Anti-sense	5-GGAAAGAAGTCAGAAGGCAA	(1974–1955)
SB3	Sense	5-AGGTATGTTGCCCGTTTCTC	(457–476)
CB4	Anti-sense	5-AAAAGAGAGTAACTCCACAG	(1954–1935)
Primers for PCR of fragment B			
es2	Sense	5-ACGTCGCATGGAGACCACCG	(1601–1620)
PS4	Anti-sense	5-CAGTTTCCGTCCGAAGGTTTTG	(594–573)
is2	Sense	5-GAGACCACCGTGAACGCCCA	(1611–1630)
PS3	Anti-sense	5-GAAACATAGAGGTGCCTTGAGCAG	(557–534)
Primers for sequencing			
SB3	Sense	5-AGGTATGTTGCCCGTTTCTC	(457–476)
as1	Anti-sense	5-TGCGAAAGCCCAGGATGATG	(631–612)
s2	Sense	5-TGCGAAAGCCCAGGATGATG	(760–783)
as2	Anti-sense	5-AGTTGGCGAGAAAGTGAAAGCCTG	(1107–1084)
s3	Sense	5-CTCTGCCGATCCATACTGCGGAA	(1256–1278)
as3	Anti-sense	5-CGGGACGTAGACAAAGGACGT	(1434–1414)
is2	Sense	5-GAGACCACCGTGAACGCCCA	(1611–1630)
ea1	Anti-sense	5-TGAAAAAGTTGCATGGTGCTGGTG	(1827–1804)
s4	Sense	5-TATCGGGAGGCCTTAGAGTCTCCG	(2012–2035)
as4	Anti-sense	5-ATAGGGGCATTGGTCT	(2314–2298)
s5	Sense	5-CGCAGAAGATCTCAATCTCGG	(2417–2437)
as5	Anti-sense	5-GGATAGAACCTAGCAGGCAT	(2654–2635)
s6	Sense	5-GGGTCACCATATTCTTGGGAA	(2814–2834)
as6	Anti-sense	5-GGGTTGAAGTCCCAATCTGGATT	(2987–2965)
is1	Sense	5-AAGCTCTGCTAGATCCCAGAGT	(18–39)
ea2	Anti-sense	5-TAGAAAATTGAGAGAAGTCCACCA	(280–257)
s1	Sense	5-CATCCTGCTGCTATGCCTCATC	(409–430)
as1	Anti-sense	5-TGCGAAAGCCCAGGATGATG	(631–612)

Nucleotides are numbered from the unique *Eco*RI site of HBV.

used for qualitative data. P values less than 0.05 were considered significant. Analyses were carried out using SPSS version 10.0J (SPSS Inc., Chicago, IL).

RESULTS

Of the 144 patients selected for mutation analysis, 90 (62.5%) were HBeAg-positive and the remaining 54 (37.5%) were anti-HBe-positive. The clinical and virological backgrounds of the two groups of patients are compared in Table II. The 90 HBeAg-positive patients tended to be younger and have a higher concentration of HBV DNA than the 54 anti-HBe patients. Patients with none of the Pre-C and CP mutations were significantly ($P < 0.001$) more common in the HBeAg-positive patients (56%) than in the anti-HBe-positive patients (13%).

A comparison of the clinical background of seven anti-HBe-positive patients who had neither Pre-C nor CP mutations and 47 anti-HBe-positive patients who had at least one of the mutations is shown in Table III. Distributions of age, gender, ALT level, and HBV DNA concentration did not differ between the two groups.

Nucleotide sequences of the complete genome were determined in four out of seven anti-HBe-positive patients who had neither Pre-C nor CP mutations and in 5 out of 50 HBeAg-positive patients who also had neither mutation. All nine of the genome sequences

TABLE II. Comparison of Clinical and Virological Backgrounds of Patients With HBeAg and Those With Anti-HBe

	HBeAg-positive n = 90	Anti-HBe-positive n = 54	P
Age[a]	25 (5–53)	36 (11–73)	<0.001[b]
Gender (M:F)	58:32	30:24	>0.2[c]
ALT[a]	89 (11–2100)	62 (13–458)	>0.2[b]
HBV DNA (log copies/mL)[a]	8.3 (4.4–7.9)	5.0 (3.2–8.8)	<0.001[b]
Pre-C/CP mutations			
Both negative	50 (56%)	7 (13%)	<0.001[c]
Pre-C mutation only	13 (14%)	20 (37%)	
CP mutation only	12 (13%)	5 (9%)	
Both positive	15 (17%)	22 (41%)	

[a]Data are expressed as median values (range).
[b]Mann–Whitney test.
[c]Chi-square test.

TABLE III. Comparison of Clinical and Virological Backgrounds of Anti-HBe-Positive Patients With Neither Pre-C nor CP Mutations and Anti-HBe Patients With at Least one of These Mutations

	Pre-C and CP mutation-negative n = 7	Pre-C and/or CP mutation-positive n = 47	P
Age[a]	37 (18–60)	36 (11–73)	>0.2[b]
Gender (M:F)	4:3	26:21	>0.2[c]
ALT[a]	44 (18–86)	65 (13-458)	0.17[b]
HBV DNA (log copies/ml)*	4.7 (3.3–5.5)	5.0 (3.2–8.8)	>0.2[b]

[a]Data are expressed as median values (range).
[b]Mann–Whitney test.
[c]Chi-square test.

determined had nucleotide lengths of 3,215 bases, and thus there were no insertions or deletions. When the full genome sequences were compared, the six mutations shown in Table IV were significantly more common in the four anti-HBe-positive patients than in the five HBeAg-positive patients. The positions of the six mutations in the HBV genome are shown in Figure 1. Of the four mutations located in the polymerase gene, the G529A and C934A mutations cause amino acid substitutions in the polymerase protein. The C3026T mutation does not cause an amino acid substitution in the polymerase, but rather in the pre-S1 protein, while the A1053G mutation does not lead to an amino acid substitution, but the mutation is located near the enhancer I region. The G1915T/A and T2005C/A mutations are located in the core gene, but do not result in an amino acid substitution. Patients with at least one of the three mutations (G529A, C934A, and A1053G) which might affect HBV replication had a significantly ($P = 0.029$) lower level of HBV DNA (n = 22, median 5.3 copies/ml, range 3.8–8.9) than those patients who had no mutations (n = 13, median 8.5 copies/ml, range 3.8–8.9).

To examine further the specificity of the six mutations, these mutations were also determined in 11 HBeAg-positive patients who were positive for Pre-C and CP mutations and in 15 anti-HBe-positive patients who were also positive for Pre-C and CP mutations. The frequencies of the six mutations were compared between groups of patients classified according to their HBeAg/anti-HBe and Pre-C/CP mutation status. Three (G1915T/A, T2005C/A, and C3026T) of the six mutations were found to be significantly more common in anti-HBe-positive patients who had neither a Pre-C nor a CP mutation than in the two groups of patients with Pre-C and CP mutations, as shown in Table V.

The nucleotide sequence data reported in this paper have been registered in the DDBJ/EMBL/GenBank nucleotide sequence databases, with the accession numbers AB198076-84.

DISCUSSION

Studies to date have shown that the stop codon mutation in the Pre-C region (G1896A) and the double mutation in the CP region (A1762T/G1764A) are independently associated with the seroconversion of HBeAg, and that the Pre-C mutation is more directly associated with seroconversion than the core promoter mutation [Okamoto et al., 1994; Yamaura et al., 2003]. Only a small number of anti-HBe-positive patients (13%) were both negative for the Pre-C and CP mutations, and in the present study this rate was significantly lower than that (56%) in HBeAg-positive patients. These results are consistent with previous reports, suggesting that the two mutations are the main causes of seroconversion. However, there are also patients in whom HBeAg secretion discontinues without

TABLE IV. Comparison of Full Nucleotide Sequences of HBV With Neither Pre-C nor CP Mutations for HBeAg-Positive and Anti-HBe-Positive Patients

Nucleotide mutation	Amino acid substitution (viral protein)	HBeAg Pre-C and CP mutation-negative n = 5	Anti-HBe Pre-C and CP mutation-negative n = 4	P
G529A	D480N (P) None (S)	0	3	0.048
C934A	L615I (P)	1	4	0.040
A1053G	None (P)	1	4	0.040
G1915T/A	None (C)	0	4	0.008
T2005C/A	None (C)	0	4	0.008
C3026T	A60V (Pre-S1) None (P)	0	3	0.048

Six mutation sites with significant differences are shown. Data are expressed as the number of positives. Statistical analysis was performed with a chi-square test. P, polymerase protein; S, surface protein; C, core protein; Pre-S1, pre-surface 1 protein.

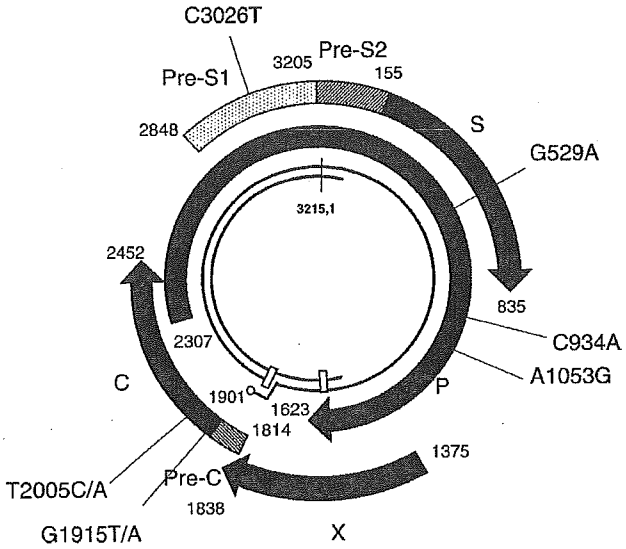

Fig. 1. Organization of the hepatitis B virus genome (genotype C) and the approximate positions of the six nucleotide mutations in the HBV genome. The inner circles represent the minus and plus DNA strands of the viral genome. The different open reading frames encoded by the genome, designated as S, C, P, and X, are indicated by the arrows. Abbreviations: S, surface antigen; C, core; P, polymerase; Pre-C, precore.

appearance of Pre-C and/or CP mutations. Thus, we speculated that some other mutations might be associated with HBeAg seroconversion. A variety of other mutations in the CP and Pre-C regions have been detected in previous studies [Carman et al., 1989; Tillmann et al., 1995; Baumert et al., 1996; Laras et al., 1998; Chan et al., 2000; De Castro et al., 2001; Yoo et al., 2003], but other regions of the HBV genome have not been analyzed sufficiently for mutations associated with HBeAg seroconversion.

When the full nucleotide sequences of HBV genomes of HBeAg-positive and anti-HBe-positive patients with neither Pre-C nor CP mutations were compared, six mutations (G529A, C934A, A1053G, G1915T/A, T2005C/A, C3026T) were found to be significantly more common in the anti-HBe-positive patients. The six mutations were also more common in anti-HBe-positive patients who had neither Pre-C nor CP mutations than in HBeAg-positive patients or in anti-HBe-positive patients who had Pre-C and CP mutations, with the results being statistically significant for three (G1915T/A, T2005C/A, C3026T) of the six mutations. These results suggest that the six mutations are associated with HBeAg negativity.

The mechanisms through which the six mutations facilitate HBeAg negativity were not investigated in the present study. However, some possible mechanisms can be considered, based on the locations of these mutations in the HBV genome. The G529A and C934A mutations cause amino acid substitutions in the polymerase protein. Thus, these two mutations may attenuate HBV replication through changes in the enzymatic activity of the polymerase. The A1053G mutation is located near the enhancer I region, which may affect the replication of HBV [Bock et al., 2000]. Patients who had at least one of the three mutations associated with HBV replication tended to have a lower level of HBV DNA than those who had none of these mutations, providing further support for a replication-associated mechanism. It has been reported that amino acid substitutions in immunogenic epitopes in the core protein are found most frequently during or after seroconversion from HBeAg to anti-HBe [Akarca and Lok, 1995; Carman et al., 1995]. We found two mutations in the core gene, but these mutations did not cause amino acid substitutions. Thus, the mechanisms through which the G1915T/A and T2005C/A mutations exert their effects remains unclear.

In anti-HBe-positive patients, the clinical background, including the mean age, gender distribution, ALT level and HBV DNA level, were similar in patients with and without Pre-C and/or CP mutations. Although these comparisons were cross-sectional, the results suggest that mutations other than those in the Pre-C and CP regions have a similar impact in patients in whom seroconversion occurs, compared to Pre-C and CP mutations.

The six mutations identified in the present study have not been described previously. These mutations

TABLE V. Comparison of Six Mutations Among Three Groups Classified According to Their HBeAg/anti-HBe and Pre-C/CP Mutation Status

Mutation site	Anti-HBe Pre-C and CP mutation-negative n = 4	HBeAg Pre-C and/or CP mutation-positive n = 11	Anti-HBe Pre-C and/or CP mutation-positive n = 15
G529A	3	3	3
C934A	4	6	10
A1053G	4	4	9
G1915T/A	4[a]	3	1
T2005C/A	4[b]	3	4
C3026T	3[c]	0	1

Data are expressed as the number of positives. Statistical analysis was performed with Fisher's exact test. Other comparisons were not statistically significant.
[a] $P = 0.026$ versus 11 patients with HBeAg, and $P = 0.001$ versus 15 patients with anti-HBe.
[b] $P = 0.026$ versus 11 patients with HBeAg, and $P = 0.018$ versus 15 patients with anti-HBe.
[c] $P = 0.009$ versus 11 patients with HBeAg, and $P = 0.016$ versus 15 patients with anti-HBe.

are thought to be associated with HBeAg negativity because they were found specifically in anti-HBe-positive patients with neither a Pre-C nor a CP mutation. However, several issues remain to be resolved to clarify the real significance of the six mutations, including the mechanisms through which they facilitate HBeAg negativity, their universality in genotypes other than genotype C, and their clinical relevance. Furthermore, it is possible that immune-based selection pressures that cause loss of HBeAg are responsible for the selection of the mutations identified in the present study [Locarnini, 2004]. Therefore, it is not possible to conclude that the new mutations are definitely associated with seroconversion, but they do provide new clues regarding the nature of seroconversion.

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REFERENCES

Akarca US, Lok AS. 1995. Naturally occurring core-gene-defective hepatitis B viruses. J Gen Virol 76:1821–1826.

Aritomi T, Yatsuhashi H, Fujino T, Yamasaki K, Inoue O, Koga M, Kato Y, Yano M, 1998. Association of mutations in the core promoter and precore region of hepatitis virus with fulminant and severe acute hepatitis in Japan. J Gastroenterol Hepatol 13:1125–1132.

Baumert TF, Rogers SA, Hasegawa K, Liang TJ. 1996. Two core promoter mutations in a hepatitis B virus strain associated with fulminant hepatitis result in enhanced viral replication. J Clin Invest 98:2268–2276.

Bock CT, Malek NP, Tillmann HL, Manns MP, Trautwein C. 2000. The enhancer I core region contributes to the replication level of hepatitis B virus in vivo and in vitro. J Virol 74:2193–2202.

Bonino F, Rosina F, Rizzetto M, Rizzi R, Chiaberge E, Tardanico R, Callea F, Verme G. 1986. Chronic hepatitis in HBsAg carriers with serum HBV-DNA and anti-HBe. Gastroenterology 90:1268–1273.

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

Carman WF, Jacyna MR, Hadziyannis S, Karayiannis P, McGarvey MJ, Makris A, Thomas HC. 1989. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. Lancet ii:588–590.

Carman WF, Thursz M, Hadziyannis S, McIntyre G, Colman K, Gioustoz A, Fattovich G, Alberti A. 1995. Hepatitis B e antigen negative chronic active hepatitis: Hepatitis B virus core mutations occur predominantly in known antigenic determinants. J Viral Hepat 2:77–84.

Chan HLY, Hussain M, Lok ASF. 1999. Different hepatitis B virus genotypes are associated with different mutations in the core promoter and precore regions during hepatitis B e antigen seroconversion. Hepatology 29:976–984.

Chan HLY, Leung NW, Hussain M, Wong ML, Lok AS. 2000. Hepatitis B e antigen-negative chronic hepatitis B in Hong Kong. Hepatology 31:763–768.

De Castro L, Niel C, Gomes SA. 2001. Low frequency of mutations in the core promoter and precore regions of hepatitis B virus in anti-HBe positive Brazilian carriers. BMC Microbiol 1:10.

Fattovich G. 1998. Progression of hepatitis B and C to hepatocellular carcinoma in Western countries. Hepatogastroenterology 45:1206–1213.

Hadziyannis SJ, Lieberman HM, Karvountzis GG, Shafritz DA. 1983. Analysis of liver disease, nuclear HBcAg, DNA viral replication, and hepatitis B virus DNA in liver and serum of HBeAg vs. anti-HBe positive carriers of hepatitis B virus. Hepatology 3:656–662.

Laras A, Koskinas J, Avgidis K, Hadziyannis SJ. 1998. Incidence and clinical significance of hepatitis B virus precore gene translation initiation mutations in e antigen-negative patients. J Viral Hepatitis 5:241–248.

Locarnini S. 2004. Molecular virology of hepatitis B virus. Semin Liver Dis 24:3–10.

Lok ASF. 1992. Natural history and control of perinatally acquired hepatitis B virus infection. Dif Sis 10:46–52.

Lok ASF, Hadziyannis S, Weller IVD, Karvountzis MV, Monjardino J, Karayiannis P, Montano L, Thomas HC. 1984. Contribution of low level HBV replication to continuing inflammatory activity in patients with anti-HBe positive chronic hepatitis B virus infection. Gut 25:1283–1287.

Maddrey WC. 2000. Hepatitis B: An important public health issue. J Med Virol 61:362–366.

Maynard JE. 1990. Hepatitis B: Global importance and need for control. Vaccine 8:S18–20.

Mizokami M, Nakano T, Orito E, Tanaka Y, Sakugawa H, Mukaide M, Robertson BH. 1999. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. FEBS lett 450:66–71.

Okada K, Kamiyama I, Inomata M, Imai M, Miyakawa Y. 1976. E antigen and anti-e in the serum of asymptomatic carrier mothers as indicators of positive and negative transmission of hepatitis B virus to their infants. N Engl J Med 294:746–749.

Okamoto H, Yotsumoto S, Akahane Y, Yamanaka T, Miyazaki Y, Sugai Y, Tsuda F, Tanaka T, Miyakawa Y, Mayumi M. 1990. Hepatitis B viruses hosts along with seroconversion to the antibody against e antigen. J Virol 64:1298–1303.

Okamoto H, Tsuda F, Akahane Y, Sugai Y, Yoshiba M, Moriyama K, Tanaka T, Miyakawa Y, Mayumi M. 1994. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. J Virol 68:8102–8110.

Rokuhara A, Tanaka E, Yagi S, Mizokami M, Hashikura Y, Kawasaki S, Kiyosawa K. 2000. De novo infection of hepatitis B virus in patients with orthotopic liver transplantation: Analysis by determining complete sequence of the genome. J Med Virol 62:471–478.

Tillmann H, Trautwein C, Walker D, Michitaka K, Kubicka S, Boker K, Manns M. 1995. Clinical relevance of mutations in the precore genome of the hepatitis B virus. Gut 37:568–573.

Yamaura T, Tanaka E, Matsumoto A, Rokuhara A, Orii K, Yoshizawa K, Miyakawa Y, Kiyosawa K. 2003. A case-control study for early prediction of hepatitis B e antigen seroconversion by hepatitis B virus DNA levels and mutations in the precore region and core promoter. J Med Virol 70:545–552.

Yoo BC, Park JW, Kim HJ, Lee DH, Cha YJ, Park SM. 2003. Precore and core promoter mutations of hepatitis B virus and hepatitis B e antigen-negative chronic hepatitis B in Korea. J Hepatol 38:98–103.

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Ethnicity affects the diagnostic validity of alpha-fetoprotein in hepatocellular carcinoma

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Abstract

Introduction: Hepatocellular carcinoma (HCC) is the fourth most common cancer worldwide with a high morbidity and mortality. Alpha-fetoprotein (AFP) is considered the main tumor marker for HCC diagnosis, but the variation in its diagnostic validity among studies justifies further investigation of the underlying contributing factors. Ethnic difference could be one of the factors that has not been well studied. We aimed at investigating the ethnic difference in AFP validity between Egyptian (representing Arabic North African) and Japanese (representing Asian) for HCC diagnosis.

Methods: Four cohorts with chronic liver diseases (CLD) were studied: 171 Egyptian (65 HCC/106 non-HCC), and 173 Japanese (45 HCC/128 non-HCC). Laboratory tests including serum AFP, protein-induced vitamin K deficiency or absence (PIVKA-II), alanine aminotransferase (ALT), total bilirubin, platelet count, HBsAg, anti-HCV, and HCV core antigen were conducted using standard commercially available assays.

Results: A significantly higher sensitivity of AFP in Egyptian in comparison with Japanese for HCC diagnosis (99 *vs* 67%, $P < 0.001$) was observed using an AFP cut-off point of 10 ng/mL, with a comparable specificity (75 *vs* 82%) While a sensitivity of 98 versus 56%, $P < 0.001$ and a specificity of 83 versus 89% was found for AFP cut-off point of 20 ng/mL, respectively. The area under the receiver operating characteristic curve (ROC) was found to be 0.98 (95%CI = 0.969–0.997) for Egyptian and 0.77 (95%CI = 0.686–0.864) for Japanese. The highest sensitivity for the former group occurred at AFP = 20.5 ng/mL and at AFP = 10.2 ng/mL for the latter. Univariate analysis showed no effect for age, sex, underlying liver disease, cirrhosis, Child's class or tumor characteristics (size, pathological grade) on AFP sensitivity, while race significantly contributed to the higher sensitivity among Egyptians in comparison with the Japanese. Using ROC analysis, the AFP cut-off point for HCC detection in each subgroup of patients with and without each of the risk factors of interest was determined and the subgroups were again subclassified according to AFP positivity (< or ≥ the decided cut-off point for each group). Logistic regression analysis of those factors combined showed that Egyptian ethnicity with an AFP level >20.5 ng/mL ($P = 0.007$), older age (>50 years) with an AFP level >26 ng/mL ($P = 0.010$), and cirrhosis with an AFP level >10.5 ng/mL ($P = 0.014$) were the independent risk factors for HCC.

Conclusion: There is an ethnic variation in AFP validity between Egyptian and Japanese patients with a significantly lower sensitivity in the latter. Alpha-fetoprotein should not be the only marker used for screening HCC among Asian Japanese and younger age groups (<50 years) with CLD. In addition, an AFP cut-off point of 20 ng/mL is recommended when screening patients of Asian origin for HCC.

Key words: alpha-fetoprotein, diagnostic validity, ethnicity, hepatocellular carcinoma.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers with an incidence of one million affected per year.[1] It accounts for more than 90% of all hepatic primary tumors in Asia[2] and it is considered the fourth most common cancer worldwide.[3]

Alpha-fetoprotein (AFP), which was discovered in 1956,[4] is a glycoprotein produced by the fetal yolk sac and liver. Its level falls rapidly to less than 10 ng/mL immediately after birth, but in certain pathological conditions it rises again.[5] Elevated serum AFP has been reported to be associated with hepatocellular carcinoma;[6,7] however, positive cases are also seen in non-hepatic tumors[8–10] and benign liver disease such as chronic hepatitis and cirrhosis.[11–13] Protein-induced vitamin K deficiency or absence (PIVKA-II) is another marker for HCC that was shown to be sensitive in early diagnosis of small sized HCC, and becomes more sensitive when combined with AFP.[14]

Previous studies have found variations in AFP validity in the context of HCC diagnosis;[15–20] however the factors contributing to these variations have not well been studied. Some studies addressed a variation in AFP utility in HCC patients according to the underlying liver disease,[15,16] while others reported no difference.[17] Alpha-fetoprotein sensitivity varied significantly between studies from Asia,[15,16] the USA[18] and Europe,[19,20] which might point to an ethnic difference in its validity. Two studies have addressed the effect of ethnic difference on AFP.[18,21] A lower sensitivity of AFP for HCC diagnosis was reported in Americans of African origin compared with those of non-African origin.[18] However, in that study, the African population with HCC was too small for the author to draw a solid conclusion, and the study was limited to HCV-associated cirrhosis and HCC only. Another population-based study addressed an age-dependant difference in the AFP level between six different ethnic groups, and showed a significant difference in distribution between a black African and Asian normal population.[21] In our study we investigated the hypothesis that AFP would be less sensitive in Egyptian patients (representing Arabs of North Africa) than in Japanese patients (representing Asian) among different categories of chronic liver disease patients seen in outpatient settings for HCC diagnosis. We also investigated other contributing factors that could affect AFP validity using a multivariate analysis.

MATERIALS AND METHODS

Study population

This is a cross-sectional study conducted from April to November 2003. Data were gathered from two hospitals; Shinshu Medical School University Hospital in Japan and Suez Canal University Hospital in Egypt. A total of 110 consecutive HCC patients were included, with a mean age of 61 ± 11 years and a male to female ratio of 4:1; 65 were Egyptian and 45 were Japanese. The HCC-negative control group included 234 patients with chronic liver disease with a mean age of 56 ± 13 years and a male to female ratio of 7:3; 106 were Egyptian and 128 were Japanese.

All patients were screened for HCC by abdominal ultrasound (US) and computed tomography (CT). Chronic liver disease and cirrhosis were identified and diagnosed according to liver biopsy findings and/or clinical or radiological evidence of portal hypertension. Hepatocellular carcinoma was excluded by imaging studies including US, CT, magnetic resonance imaging (MRI) and/or hepatic angiography, one of which must have been performed at least 6 months after the measurement of AFP. Diagnosis of HCC was made when our inclusion criteria of positive cytology and/or histology were met or by the presence of characteristic hepatic masses on liver CT, MRI and/or hepatic angiography (i.e. enlarging tumors and/or tumors with typical arterial vascularaization). We excluded purely schistosomal liver disease (4 HCC, 9 non-HCC) and alcoholic liver disease (4 HCC and 20 non-HCC) patients from our study populations.

Laboratory tests including serum AFP, PIVKA-II, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, platelet count, HBsAg, anti-HCV, and HCV core antigen were performed using standard commercially available assays. The AFP assay was performed using ELISA 2-AFP; an immunoradiometric assay for direct quantitation. The AFP cut-off point was set to 10 ng/mL. The PIVKA-II assay was performed using ELISA according to the manufacturer instructions (Esai Laboratory, Tokyo, Japan); its cut-off point was set to 35 mAU/mL.

Pathological assessment

Tumor differentiation was determined for 32 of the Egyptian and 22 of the Japanese patients who had a HCC resection operation during the study period. Paraffin-impeded tumor sections were stained using haematoxlin–eosin and HCC was graded as either well, moderately or poorly differentiated.[22]

Table 1 Background data of the study group

	Egyptian			Japanese		
	HCC (+) $n = 65$	HCC (–) $n = 106$	P	HCC (+) $n = 45$	HCC (–) $n = 128$	P
Mean age (years) (SD)	57 ± 11*	47 ± 9	<0.001	66 ± 10*	63 ± 10	NS
Age < 50	16 (25)†	65 (61)	<0.001	3 (7)†	17 (13)	NS
Male	50 (77)	82 (77)	NS	38 (84)	87 (68)	0.024
Liver disease						
Viral:	61 (94)	96 (91)	44 (98)	107 (84)		
HCV related	59 (91)	92 (87)	NS	36 (80)	81 (63)	0.031
HBV related	2 (3)#	4 (4)	NS	8 (18)#	28 (22)	NS
Non viral:	4 (6)	10 (9)	NS	1 (2)	20 (16)	0.010
Cirrhosis	46 (71)	45 (42)	<0.001	40 (89)	40 (31)	<0.001
Child's C	25 (38)‡	17 (16)	=0.001	4 (9)‡	1 (1)	0.017
Mean ± SD:						
ALT (IU/dL)	73 ± 95	66 ± 45	=0.08	55 ± 35	50 ± 39	NS
Serum Albumin(gm/dL)	3.0 ± 0.7	3.0 ± 0.5	NS	3.6 ± 0.5	4.2 ± 0.4	<0.001
Platelet count (per 1000/mL)	186 ± 107§	89 ± 53	=0.001	130 ± 51§	170 ± 71	<0.001
α-fetoprotein >10 ng/mL	64 (99)	28 (26)	<0.001	30 (67)	23 (18)	<0.001
PIVIKA >35 mAU/mL	51 (79)	38 (36)	<0.001	16 (36)	27 (21)	0.047

The P-value for the difference between Egyptian and Japanese hepatocellular carcinoma (HCC) patients was <0.001 for *,†,‡,§, and = 0.001 for #. All the categorical data were shown as n (%).

Statistical analysis

Univariate statistical analysis was performed using Student's t-test for continuous and chi-square test for categorical data (Fisher exact test was used for small numbers comparison). Different risk factors for HCC (age, sex, ethnicity, underlying liver disease, cirrhosis, Child's class, PIVKA-II positivity) and tumor characteristics (tumor size, differentiation) were studied for their effect on AFP sensitivity using univariate analysis. The AFP cut-off point for each risk factor of interest was determined separately using the ROC analysis (the point that gave the highest sensitivity of AFP for HCC diagnosis). Each factor was then considered using multivariate analysis under four subgroups (+/<, +/≥, –/<, –/≥) that included each factor (+) versus (–) patients with AFP < or = the cut-off point. Multivariate analysis was performed using a logistic regression model with a stepwise method and a statistical computer program (SPSS, version 6.0). The level of significance was set at $P < 0.05$.

Table 2 Comparison of background tumor characteristics between Egyptian and Japanese hepatocellular carcinoma patients

Tumor characteristic	Egyptian ($n = 65$)	Japanese ($n = 45$)	P
Tumor multiplicity:			
Solitary	25 (38)	22 (49)	>0.2
Multiple	40 (62)	23 (51)	0.06
Tumor size:			
<3cm	32 (49)	20 (44)	>0.2
3:<5cm	15 (23)	14 (31)	>0.2
≥5cm	18 (28)	11 (25)	0.13
Metastases	0	1 (2)	>0.2
Tumor grade#:			
Well differentiated	5 (16)	2 (8)	>0.2
Poorly differentiated	5 (16)	4 (16)	>0.2

Tumor grade was analyzed in 32 of the Egyptian and 22 of the Japanese groups who had a hepatocellular carcinoma resection operation during the study period.

RESULTS

Background data

Our study (which included consecutively seen Egyptian and Japanese patients at outpatient liver clinics) found some background difference between the two groups. The Egyptian patients (Table 1) had a younger mean age ($P < 0.001$), lower prevalence of HBV-related disease ($P = 0.001$) and a higher prevalence of Child's class C ($P < 0.001$). No difference in tumor background characteristics was found between the two groups (Table 2)

Alpha-fetoprotein validity

Results showed an overall AFP positivity of 86% in the HCC group in comparison with 21% in the control group ($P < 0.001$) using an AFP cut-off point of 10 ng/mL. A significantly higher sensitivity of AFP was observed in Egyptian patients in comparison with Japanese patients for HCC diagnosis (99 *vs* 67%, $P < 0.001$) for an AFP greter than 10 ng/mL, with a comparable specificity (75 *vs* 82%, NS), while a sensitivity of 98 versus 56% and a specificity of 83 versus 89% were found for an AFP greater than 20 ng/mL.

Receiver operating characteristic curve

The area under the receiver operating characteristic curve (Fig. 1) was found to be 0.98 (95% CI = 0.969–0.997) for Egyptian and 0.77(95% CI = 0.686–0.864) for Japanese patients, which gave the highest sensitivity of AFP for HCC diagnosis at 20.5 ng/mL in the former and 10.2 ng/mL in the latter.

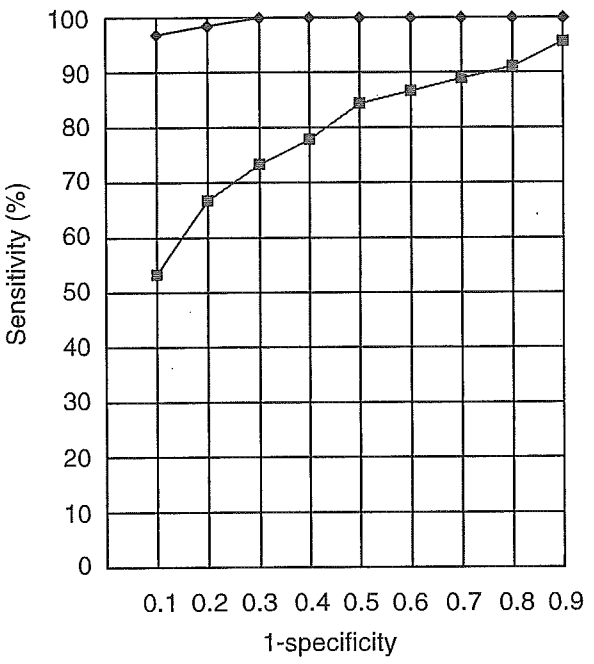

Figure 1 Receiver operating characteristic curves (ROC) for α-fetoprotein (AFP) as predictors of HCC for Egyptians (◆) and Japanese (■). The area under the ROC was found to be 0.98 (95%CI = 0.969–0.997) for Egyptians with the highest AFP sensitivity obtained at AFP = 20.5 ng/mL and 0.77 (95%CI = 0.686–0.864) for Japanese with the highest AFP sensitivity obtained at AFP = 10.2 ng/mL.

Factors affecting α-fetoprotein validity

Univariate analysis of different risk factors of HCC (Table 3) in relation to AFP cut-off point for each factor (calculated using the ROC curve for each factor as a risk for HCC) showed that there was no effect for age, sex, underlying liver disease, cirrhosis, Child's class or PIVKA-II positivity on AFP sensitivity in the studied population, while only race significantly contributed to a higher sensitivity among Egyptians in comparison with the Japanese. However, age less than 50 years ($P = 0.02$), male gender ($P < 0.001$), non-viral liver disease ($P = 0.03$), non-cirrhotic ($P = 0.007$), and Child's C group ($P = 0.008$) showed a significantly higher specificity for AFP in the context of HCC diagnosis. In addi-

Table 3 Factors affecting the cut-off point for α-fetoprotein (AFP) for diagnosing hepatocellular carcinoma

	AFP		
Variable	*Cut-off point	Sensitivity	Specificity
Age (years):			
≥50	26	77	90
<50	123	94	98
P		0.067	0.02
Sex:			
Male	8.45	91	94
Female	23.8	86	72
P		NS	<0.001
Ethnicity:			
Egyptian	20.5	98	83
Japanese	10.15	66	81
P		<0.001	NS
Liver disease:			
Viral	25	80	86
Non-viral	100	80	100
P		NS	0.03
Cirrhosis:			
(+)	24	80	53
(–)	10.5	92	87
P		NS	0.007
Child's class:			
C	217	83	100
A and B	8.3	86	75
P		NS	0.008
HCV core Ag:			
(+)	27.5	84	76
(–)	31.3	72	74
P		NS	NS

*AFP cut-off point was decided for each variable using the Reciever Operating Characteristic (ROC) analysis (the point that showed the highest AFP sensitivity for hepatocellular carcinoma diagnosis).

tion, data showed no effect of tumor size or grade on AFP validity in the studied population in general or specifically in either the Egyptian or Japanese group (Table 4). Multivariate analysis of those factors divided according to AFP positivity using a different cut-off point (see statistical analysis) for each factor (Table 5) showed that Egyptian ethnicity with an AFP level greater than 20.5 ng/mL ($P = 0.007$), an older age (>50 years) with an AFP level greater than 26 ng/mL ($P = 0.010$), and cirrhosis with an AFP level greater than 10.5 ng/mL ($P = 0.014$) were the independent risk factors for HCC.

Table 4 Comparison of the sensitivity of alpha-fetoprotein in Egyptian versus Japanese patients according to tumor size

	HCC			
	Egyptian		Japanese	
Tumor size	Total	AFP (+) *n* (%)	Total	AFP (+) *n* (%)
<3 cm	32	32 (100)	20	13 (65)
3:<5 cm	15	15 (100)	14	12 (84)
= 5 cm	18	17 (94)	11	7 (60)

P value is calculated in comparison with the <3 cm group. The difference was statistically insignificant.

DISCUSSION

The AFP positive rate varies markedly between studies; a fact that could reflect a difference in underlying liver disease, ethnic background or some other factors. To our knowledge, only two studies had addressed the question of racial difference as a factor that could influence AFP validity.[18,21] Although one study found a lower AFP sensitivity in African Americans compared with other ethnic origins, the study was limited to HCV-related liver disease, and included too small number of Africans to give a solid conclusion.[18] The other one was a population-based study which addressed an age dependant difference in AFP level between six different ethnic groups. However, the studied group was a normal population and the Egyptians included were all black Egyptian.[21] Our study could be the first to address ethnic difference in AFP validity in consecutively examined outpatient liver clinic patients of two different ethnicities.

We found a lower AFP sensitivity in Japanese compared with Egyptian, a difference which may be related to a genetically determined difference in the cut-off point for AFP. This possibility was supported by our finding of a higher cut-off point for AFP in Egyptian compared with Japanese patients using ROC curve analysis. Those results also agree with a previous study that

Table 5 Multivariate analysis of the factors affecting α-fetoprotein (AFP) validity for hepatocellular carcinoma diagnosis at different cut-off points

Variable	AFP </≥ cut-off point *(ng/dL)	No.	95% C.I.			
			OR	0.05	0.95	*P*
Age (years):						
<50	<123	80	1.00			
<50	≥123	20	1.9	0.138	27.3	0.600
≥50	<26	158	5.4	0.546	53.28	0.100
≥50	≥26	86	29.8	1.85	481.8	0.010
Ethnicity:						
Japanese	<10.15	118	1.00			
Japanese	≥10.15	55	0.17	0.020	1.971	0.100
Egyptian	<20.5	82	0.28	0.021	3.77	0.300
Egyptian	≥20.5	89	0.02	0.001	0.366	0.007
Cirrhosis:						
(–)	<10.5	134	1.00			
(–)	≥10.5	41	27.9	1.956	389.1	0.014
(+)	<24	78	26.3	5.031	138.1	0.000
(+)	≥24	91	81.5	4.46	1488.7	0.003

AFP cut-off point for each risk factor of interest was determined separately using the Reciever Operating Characteristic (ROC) analysis (the point that gave the highest sensitivity of AFP for hepatocellular carcinoma diagnosis). Each factor was then considered using multivariate analysis under four subgroups (+/<, +/≥, –/<, –/≥) that included each factor (+) versus (–) patients with AFP < or ≥ the cut-off point.

found an ethnic difference in maternal/fetal level for AFP cut-off point.[23]

It is also possible that there is an ethnic difference in tumor behavior and aggression and hence in AFP expression in HCC patients, as AFP is known to be less secreted in both early and severely advanced HCC.[16] However, our data showed no significant difference between Egyptian and Japanese with regards to tumor size, multiplicity or differentiation grade. In addition, data analysis showed no effect of either tumor size or grade on AFP sensitivity in either group. There is a possibility that our finding of a difference in the prevalence of some underlying chronic liver diseases of non-viral origin between Egyptian (e.g. schistosomal liver disease) and Japanese (e.g. alcoholic liver disease) might have influenced the difference in results. Although we excluded purely schistosomal liver disease and alcoholic liver disease patients from our study, some of the patients had a past history or a background of risk for such diseases.

Our Egyptian and Japanese study groups were not matched in terms of their background data because non-matching made it easier to study patients' background characteristics for possible predictors of AFP elevation and including consecutive patients encountered at out-patient clinics in both ethnic groups gave a better representation of the populations hepatologists are most likely to encounter in those settings.

Although our study found lower mean age, lower prevalence of HBV-related disease and higher prevalence of Child's class C in the Egyptian group these variations in fact indicated a lower rather than a higher AFP sensitivity in Egyptian HCC patients compared with Japanese.[24,25] This was also supported by our findings using multivariate analysis that the only independent factors that increased AFP validity in this study were Egyptian ethnicity, older age and cirrhosis. This limits the possibility that the existing background difference between the two groups had biased our finding of higher AFP sensitivity in the Egyptians and strongly suggests that Egyptian ethnicity was independently associated with higher AFP sensitivity compared with Japanese ethnicity. However, we recommend further study of the racial difference between Egyptians and Japanese with regard to risk factors, morbidity and survival rate of HCC, a difference that could also have affected the validity of the variation in tumor markers in our study.

We also reported a significantly higher PIVKA-II sensitivity in the Egyptian patients compared with the Japanese patients which also suggests the need for further study, as PIVKA-II has been proposed as a sensitive marker for early diagnosis of HCC in Asian populations,[14] although its significance in other races and specially Africans has still to be investigated. In addition, we found a higher PIVKA-II sensitivity in larger sized tumors in Egyptian and Japanese patients while no difference was found for AFP, a finding that might denote a difference in tumor behavior in the AFP and PIVKA-II mechanisms of secretion.

Finally we can conclude that there is an ethnic variation in AFP validity and this justifies our recommendation that the AFP cut-off point be reset in screening programs for HCC according to differences in ethnicity among different groups. Alpha-fetoprotein should not be the only marker to be used for screening HCC among Japanese and younger age groups (<50 years) with chronic liver disease.

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REFERENCES

1 World Health Organization. *Prevention Liver Cancer. WHO Tech Rep Series* 1983; 691: 7–12.
2 Liver Cancer Study Group of Japan. Primary liver cancer in Japan. Clinicopathological features and results of surgical treatment. *Ann Surg* 1990; 21:1 277–87
3 National Cancer Institute. 2001, Screening for hepatocellular cancer-screening detection- health professionals See Cancer net nci nih-gov, last updated May.
4 Bergstrand CG, Czar B. Demonstration of a new protein fraction in serum from the human fetus. *Scand J Clin Laboratory Invest* 1956; 8: 174.
5 Masseyeft R, Bonet C, Drouet J, SudakaP, Balanne C. Radioimmunoassayof alpha-fetoprotein: Technique and serum levels in the normal adults. *Digestion* 1974; 10: 17–28.
6 Johnson PJ. The role of serum Alpha-fetoprotein estimation in the diagnosis and management of hepatocellular carcinoma. *Clin Liver Dis* 2001; 5: 145–59.
7 Tatarinov YS. Presence of embryo specific Alpha-globulin in the serum of patients with primary hepatocellular carcinoma. *Vopr Med Khim* 1964; 10: 90–1.
8 Masopust J, Kithier K, Radl J, Koutecky J, Kotal L. Occurrence of fetoprotein in patients with neoplasms and non-neoplastic diseases. *Inter J Cancer* 1968; 3: 364–73.

9 Javadpour N. The role of biologic tumour markers in testicular cancer. *Cancer* 1980; **45**: 1755–61.
10 McIntire KR, Waldmann TA, Morrel CG, Go VL. Serum alpha-fetoprotein in patients with neoplasms of the gastrointestinal tract. *Cancer Res* 1975; **35**: 991–6.
11 Liaw YF, Tai DI, Chen TJ, Chu CM, Huang MJ. Alpha-fetoprotein changes in the course of chronic hepatitis: relation to bridging hepatic necrosis and hepatocellular carcinoma. *Liver* 1986; **6**: 133–7.
12 Delmont J, Kermarec J, Lafon J, Bonet C, Cassuto JP, Masseyeff R. Radioimmunoassay of Alpha-fetoprotein studies from patients suffering from hepatic disease. *Digestion* 1974; **10**: 29–39.
13 Silver HKB, Deneault J, Gold P, Thompson WG, Shuster J, Freedman SO. The detection of Alpha-fetoprotein in patients with viral hepatitis. *Cancer Res* 1974; **34**: 244–7.
14 Cui R, Wang B, Ding H, Shen H, Li Y, Chen X. Usefulness of determining a protein induced by vitamin K absence in detection of hepatocellular carcinoma. *Chin Med J (Engl)* 2002; **115**: 42–5.
15 Furui J, Furukawa M, Kanematsu T. The low positive rate of serum Alpha-fetoprotein levels in hepatitis C virus antibody-positive patients with hepatocellular carcinoma. *Hepatogasteroenterology* 1995; **42**: 445–9.
16 Peng YC, Chan CS, Chen GH. The effectiveness of serum Alpha-fetoprotein level in anti-HCV positive patients for screening hepatocellular carcinoma. *Hepatogastroenterology* 1999; **46**: 3208–11.
17 Cedrone A, Covino M, Caturelli E *et al.* Utility of Alpha-fetoprotein (AFP) in the screening of patients with virus related chronic liver disease: Does different viral etiology influence AFP levels in HCC? A study in 350 Western patients. *Hepatogastrenterology* 2000; **47**: 1654–8.
18 Nguyen MH, Garcia RT, Simpson PW, Wright TL, Keeffe EB. Ethnic difference in effectiveness of Alpha-fetoprotein for diagnosis of hepatocellular carcinoma in hepatitis C virus cirrhosis. *Hepatology* 2002; **36**: 410–7.
19 Raedle JJ, Roth WK, Oremek G, Caspary WF, Zeuzem S. Alpha-fetoprotein and p53 antoantibodies in patients with chronic hepatitis C. *Dig Dis Sci* 1995, 40–2587–, 2594.
20 Trevisani F, D'Intino PE, Morselli-Labate AM *et al.* Serum Alpha-fetoprotein for diagnosis of hepatocellular carcinoma in patients with chronic liver disease: influence of HbsAg and anti-HCV status. *J Hepatol* 2001; **34**: 570–5.
21 Sizaret P, Tuyns A, Martel N *et al.* Alpha-fetoprotein levels in normal males from seven ethnic groups with different hepatocellular carcinoma risks. *Ann NY Acad Sci* 1975 August 22; **259**: 136–55.
22 Edmonson HA. Tumors of the liver and intrahepatic bile ducts. In: *Atlas of Tumor Pathology*, Sect VII. AFIP, 1985; 37–80.
23 O'Brien JE, Drugan A, Chervank FA *et al.* Maternal serum alpha-fetoprotein screening: the need to use race/ethnic specific medians in Asians. *Ftal Diagn Ther* 1993; **8**: 367–70.
24 Johnson PJ. The role of serum alpha-fetoprotein in the diagnosis and management of hepatocellular carcinoma. *Clin Liver Dis* 2001; **5**: 145–59.
25 Tsai JF, Chang WY, Jeng JE, Ho MS, Lin ZY, Tsai ZH. Frequency of raised alpha-fetoprotein level among Chinese patients with hepatocellular carcinoma related to hepatitis B and C. *Br J Cancer* 1994; **69**: 1157–9.