types (Table I): no amplified fragments (five cases); fragments corresponding to the non-truncated HCV genome (eight cases); fragments corresponding to both the truncated and non-truncated genomes (six cases); and fragments corresponding only to the truncated genome (four cases, including Patient 207 and 373).

RT-PCR of HCV RNA in Patient 1, 368, and 203 yielded only fragments corresponding to the truncated HCV genome. The isolated molecular clones contained in-frame deletions of amino acids extending from E1 to NS2, similarly to those isolated from Patient 207 and 373. Although cDNA from Patient 2 contained two in-frame deletions in E1 and E1–NS2, it shared the characteristics of the truncated genome; entire core, partial E1, and NS2 (Fig. 3B).

In cases with both truncated and non-truncated HCV cDNA, the predominant PCR product was non-truncated (Table I, Fig. 3A). Figure 4 shows the structures of the cDNA isolates. Deletions similar to those described above were found only in Patient 325. Patient 295, 288, 274, and 331 had out-of-frame deletions, and except for a clone from Patient 331 [LV331-(i)], sequence comparison between truncated and non-truncated HCV cDNA indicated sequence identity (3–8 nucleotides in length) in these out-of-frame deletions between the deletion donor and acceptor regions.

In Patient 207, Both Truncated and Non-Truncated HCV RNA Co-Existed for Years

E2 primers for PCR were designed to avoid PCR bias under competitive PCR conditions in order to confirm the presence of the non-truncated HCV genome in Patient 207 [Alvarez et al., 2000]. Two overlapping HCV cDNA sequences (LV0922 and LV2030) were isolated from a liver biopsy specimen from Patient 207 (Fig. 5A). The overlapping regions of the consensus sequences (LV0922cons and LV2030cons in Fig. 5B) were identical; however, the amino acid sequence identity to the truncated HCV genome (LV207cons: consensus sequence determined with isolates) was 92.1% in the E1 region, and 82.1% in the NS2 region (Fig. 5B).

Both the truncated and non-truncated genomes were detected by RT-PCR in serum from Patient 207 (Fig. 5A). The sequence of an isolate (S831) showed 99.4% nucleotide and amino acid sequence identity with LV207cons and the same in-frame deletions (Fig. 5A). The cDNA for the non-truncated genome (S2531) was also isolated from the serum by PCR with primers for E2 sequences. This cDNA was nearly identical (99.8% in nucleotide sequence) to that from the liver (LV2030cons), but differed from the truncated HCV genome identified in the liver (LV207cons) and serum (S831) (Fig. 5B).

From the RNA extracted from Patient 207 serum, a PCR fragment of the expected length was amplified using anti-sense primer for junction site at the deleted position between E1 and NS2 of the truncated genome of Patient 207. However, the PCR fragment was not amplified from the RNA of Donor G14 plasma, in which only the full-length HCV genome was detected. Similarly, the PCR fragment was amplified from truncated RNA transcripts, but not amplified from non-truncated RNA transcripts (Fig. 5C).

In order to examine the persistence of both HCV genomes, a series of serum specimens consisting of samples obtained from Patient 207 were examined. Only the truncated RNA was detected by RT-PCR with primers for the core and NS2 in all samples. The sequences of PCR fragment clones were conserved at both the nucleotide and amino acid level (92–99% identity, Fig. 6A). All clones had the same deletions as observed in biopsy samples (Fig. 6A) at the E1–NS2 junction. Novel quasispecies with amino acid deletions at 13 amino acids upstream of the junction were found in March 1998 (designated as 9803). Another quasispecies was identified in March 1999. HCV E2 cDNAs of the non-truncated genome were isolated by RT-PCR from all serum samples, and were found to have conserved sequences (Fig. 6B).

Significant Difference in the Ratio of Truncated to Non-Truncated Genome Between Serum and Liver

HCV RNA from Patient 207 was measured by real-time RT-PCR for the 5′-UTR and E2 sequences. The quantity of 5′-UTR is indicative of the entire HCV genome, while that of E2 is only indicative of the non-truncated genome. The ratio of HCV E2 RNA to 5′-UTR RNA in serum was nearly constant throughout the 15-months monitoring period (Table III). Significant differences in the ratio of truncated to non-truncated genome were observed between liver and serum RNA samples; the ratio of HCV 5′-UTR to E2 RNA in liver specimens was about a hundred times of those in serum.

Expression of Truncated HCV cDNA In Vitro

HCV proteins processed from subgenomic HCV cDNA were investigated in a transient cDNA expression experiment. Chimeric cDNA for the truncated genome, LV207cont, consisted of cDNA fragments selected from 4 cDNA isolates from Patient 207 and the 3′-end of NS5B to the 3′-UTR sequence isolated from another patient (Fig. 7A). For expression of full-length HCV cDNA, chimeric cDNAs having the core–NS2 sequence of LV207cont instead of the corresponding region of J1 HCV cDNA (J1NLV) were used.

Figure 7B shows the Western blots of lysates from cells transfected with cDNA expression vectors. Anti-core monoclonal antibody detected a 19-kDa protein, thus indicating that the truncated polyprotein was correctly processed at the core-E1 junction. Each 35-kDa protein expressed from HCV cDNA, which was reactive to anti-E1 monoclonal antibody, was susceptible to endoglycosidase (Endo H), and converted to different molecular masses by enzyme treatment. The migration of deglycosylated E1 reactive peptide from the truncated HCV cDNA corresponded to the predicted molecular mass of the E1–NS2 fusion protein, 24 kDa, which differed from that of the non truncated E1 (19 kDa). The

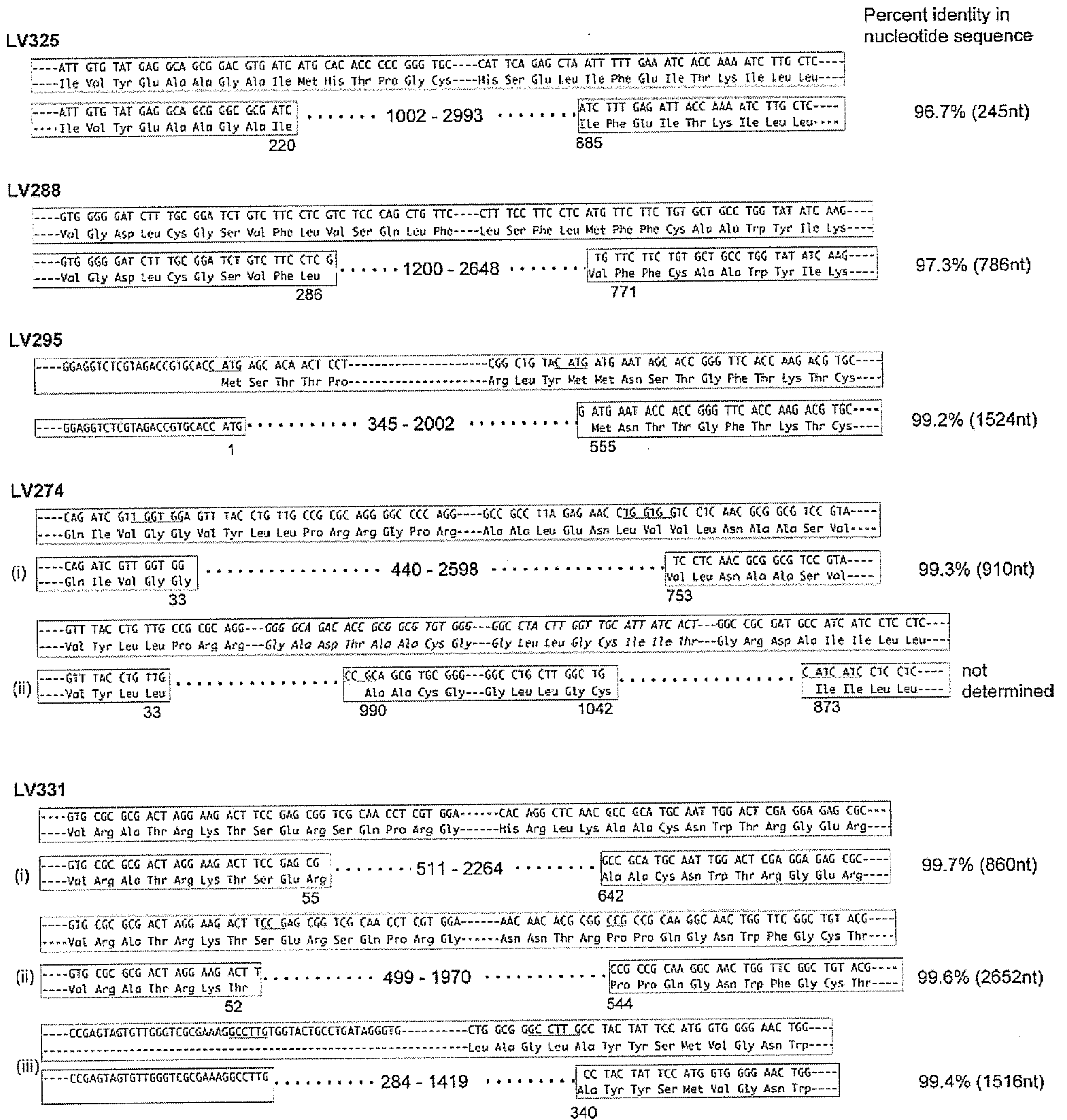
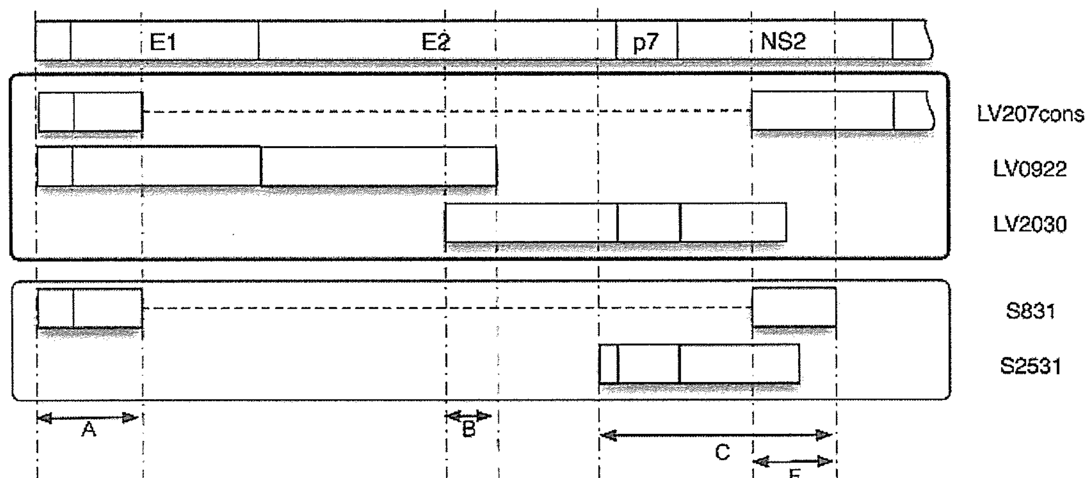


Fig. 4. Sequence comparisons between HCV genomes with and without deletions isolated from the same patient. Partial sequences of RT-PCR fragments isolated from liver biopsies are shown. cDNA fragments are labeled as described for previous figures. Nucleotide and amino acid positions are represented as 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.



B

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

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

Number in parentheses expresses
The length of the overlapped sequences

C

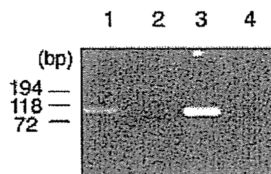


Fig. 5. Cloning of the non-truncated HCV RNA from patient 207. A: Schematic views of RT-PCR fragments for non-truncated HCV RNA in liver (LV0922 and LV2030) and in serum (S2531) from Patient 207, and for the truncated sequence in serum (S831). LV0922 fragment was obtained by RT-PCR with HC1b9405R for 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

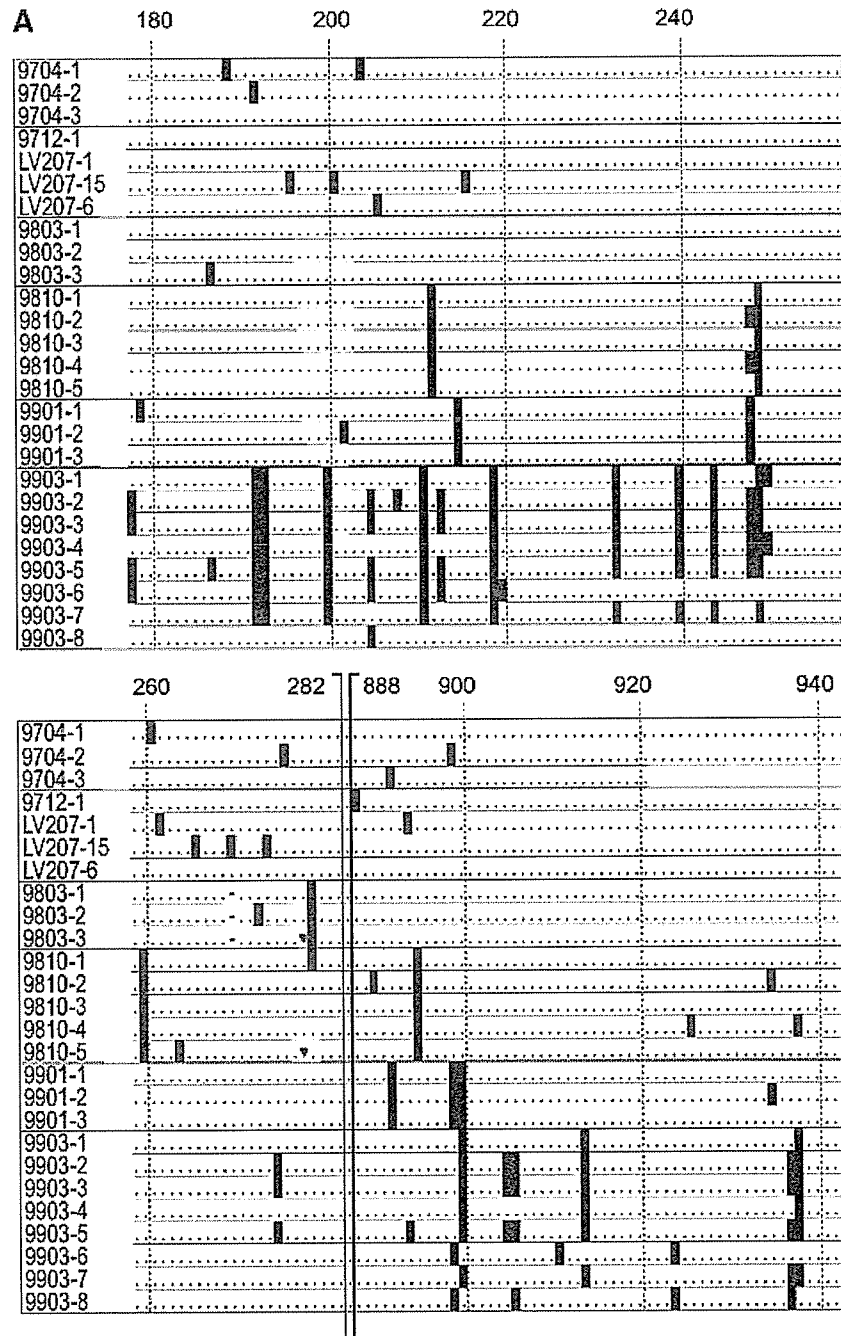


Fig. 6. Alignment of amino acid sequences of cDNA isolates from a series of serum samples from Patient 207. Comparison of nucleotide sequences of truncated genome (**A**) and non-truncated genome (**B**) was carried out using Clustal W algorithm. Numbers on top of the alignment show the corresponding 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.

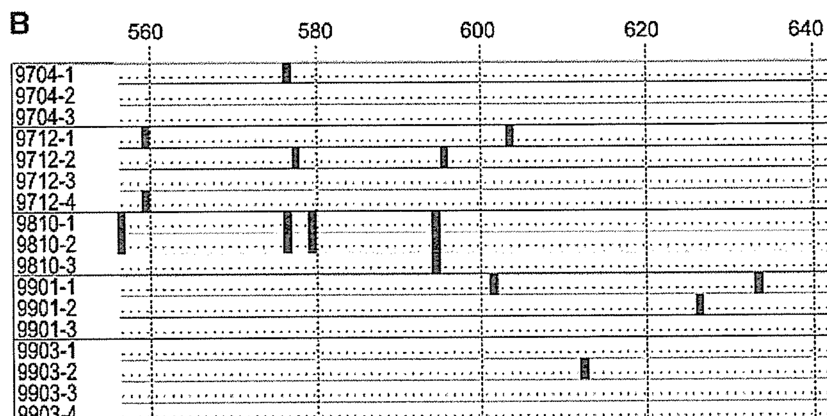


Fig. 6. *(Continued)*

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

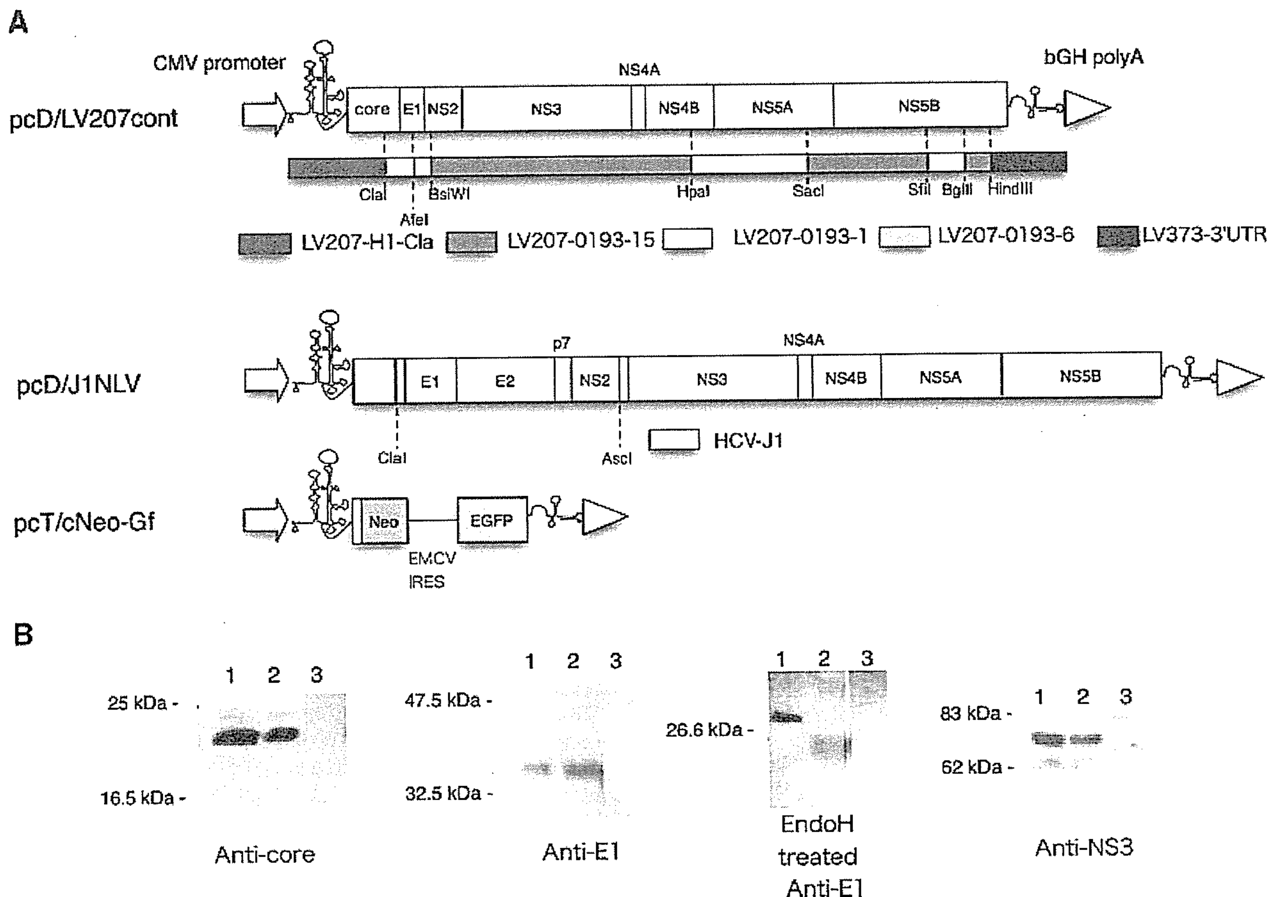


Fig. 7. Expression of HCV proteins from truncated and non-truncated HCV cDNA in mammalian cells. **A:** Chimeric HCV subgenomic cDNA, LV207cont, was composed of fragments from four cDNAs from patient liver (LV207-H1-Cla, 0193-15, 0193-1, and 0193-6) and a fragment from Patient 373 (LV373-3′-UTR) by using 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

We thank Dr. Y. Matsuura (Research Institute for Microbial Diseases, Osaka University) for providing the HCV-J1 cDNA clone, the members of Advanced Life Science Institute, Inc. for HCV Core Antigen Assay, Dr. N. Maki and Dr. Y. Komatsu for their insightful comments.

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.

PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

ELSEVIER

World J Gastroenterol 2005;11(42):6607-6612
World Journal of Gastroenterology ISSN 1007-9327
© 2005 The WJG Press and Elsevier Inc. All rights reserved.

Assessment of KL-6 as a tumor marker in patients with hepatocellular carcinoma

Amal Gad, Eiji Tanaka, Akihiro Matsumoto, Moushira Abd-el Wahab, Abd el-Hamid Serwah, Fawzy Attia, Khalil Ali, Howayda Hassouba, Abd el-Raoof el-Deeb, Tetsuya Ichijyo, Takeji Umemura, Hidetomo Muto, Kaname Yoshizawa, Kendo Kiyosawa

Amal Gad, Eiji Tanaka, Akihiro Matsumoto, Moushira Abd-el Wahab, Tetsuya Ichijyo, Takeji Umemura, Hidetomo Muto, Kaname Yoshizawa, Kendo Kiyosawa, Second Department of Internal Medicine, Shinshu University School of Medicine, Matsumoto, Japan
Amal Gad, Abd el-Hamid Serwah, Fawzy Attia, Khalil Ali, Howayda Hassouba, Abd el-Raoof el-Deeb, Suez Canal University School of Medicine, Ismailia, Egypt
Kendo Kiyosawa, Shinshu University Graduate School of Medicine, Institutes of Organ Transplants, Reconstructive Medicine and Tissue Engineering, Matsumoto, Japan
Supported by the Takeda Foundation, Osaka, Japan
Correspondence to: Eiji Tanaka, MD, Second Department of Internal Medicine, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621,
Japan. etanaka@hsp.md.shinshu-u.ac.jp
Telephone: +81-263-37-2634 Fax: +81-263-32-9412
Received: 2005-03-13 Accepted: 2005-04-30

Abstract

AIM: To investigate the clinical significance of KL-6 as a tumor marker of HCC in two different ethnic groups with chronic liver disease consecutively encountered at outpatient clinics.

METHODS: Serum KL-6 was measured by the sandwich enzyme immunoassay method using the KL-6 antibody (Ab) as both the capture and tracer Ab according to the manufacturer's instructions (Eisai, Tokyo, Japan). Assessment of alpha fetoprotein (AFP) and protein induced vitamin K deficiency or absence (PIVKA-II) was performed in both groups using commercially available kits.

RESULTS: A significantly higher mean serum KL-6 (556±467 U/L) was found in HCC in comparison with non-HCC groups either with (391±176 U/L; P<0.001) or without (361±161 U/L; P<0.001) liver cirrhosis (LC). Serum KL-6 level did not correlate with either AFP or PIVKA-II serU/Levels. Using receiver operating curve analysis for KL-6 as a predictor for HCC showed that the area under the curve was 0.574 (95%CI = 0.50-0.64) and the KL-6 level that gave the best sensitivity (61%) was found to be 334 U/L but according to the manufacturer's instructions; a cut-off point of 500 U/L was used that showed the highest specificity (80%) in comparison with AFP and PIVKA-II (78% *vs* 72% respectively). Combining the values of the three markers improved specificity of AFP for HCC diagnosis from 78% for AFP alone; 93% for AFP plus PIVKA-II to 99% for both plus KL-6 value (P<0.001). Mean serum alkaline phosphatase level was significantly higher in KL-6 positive (564±475) in comparison with KL-6 negative (505±469) HCC patients (P = 0.021), but such a difference was not found among non-HCC corresponding groups.

CONCLUSION: KL-6 is suggested as a tumor for HCC. Its positivity may reflect HCC-associated cholestasis and/ or local tumor invasion.

© 2005 The WJG Press and Elsevier Inc. All rights reserved.

Key words: Tumor markers; Liver disease; Hepatocellular carcinoma

Gad A, Tanaka E, Matsumoto A, Wahab MA, Serwah AeH, Attia F, Ali K, Hassouba H, el-Deeb AeR, Ichijyo T, Umemura T, Muto H, Yoshizawa K, Kiyosawa K. Assessment of KL-6 as a tumor marker in patients with hepatocellular carcinoma. *World J Gastroenterol* 2005; 11(42): 6607-6612
http://www.wjgnet.com/1007-9327/11/6607.asp

INTRODUCTION

Hepatocellular carcinoma (HCC) is the 4th most common cancer worldwide, and it is a well-known complication of chronic hepatitis[1,2]. Asymptomatic patients diagnosed as HCC through screening programs are more likely to be candidates for curative treatment and have improved short- and medium-term survival[3,4]. Although serum alpha-fetoprotein (AFP) had been shown to be associated with HCC since 1963[5], unfortunately it is also elevated in a wide variety of non-hepatic malignancies[6,7] and benign hepatic conditions[8,9]. Moreover, it is uncertain whether serum AFP is a useful marker for HCV-related HCC in some ethnic groups e.g., North American patients of African origin[10]. Thus, searching another tumor marker, that together with AFP could improve the diagnostic utility of the later, seemed to be justified. KL-6 was originally found using a murine monoclonal antibody that recognized an undefined sialylated carbohydrate chain on a mucin-like glycoprotein[11] which was also defined as MUC1[12]. The cell membrane MUC1 was found to regulate cell adhesion properties[13]. KL-6 has been first shown to be

Table 1 Background data of the study groups

	Egyptian			Japanese		
	HCC (+)	HCC (-)	P	HCC (+)	HCC (-)	P
	n = 65	n = 106		n = 45	n = 128	
Mean age (SD, yr)	57±11[b]	47±9	<0.001	66±10[b]	63±10	NS
Age <50 yr	16 (25)[d]	65 (61)	<0.001	3 (7)[d]	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)[1]	4 (4)	NS	8 (18)[1]	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)[f]	17 (16)	0.001	4 (9)[f]	1 (1)	0.017
Mean±(SD)						
ALT (IU/L)	73±95	66±45	0.08	55±35	50±39	NS
Serum Albumin (g/L)	3.0±0.7	3.0±0.5	NS	3.6 ±0.5	4.2±0.4	<0.001
Platelet count×1 000/mL^3	186±107[h]	89±53	0.001	130±51[h]	170±71	<0.001
AFP >10 ng/mL (+)	64 (99)	28 (26)	<0.001	30 (67)	23 (18)	<0.001
PIVIKA>40 mAU/L (+)	51 (79)	38 (36)	<0.001	16 (36)	27 (21)	0.047

[b]P<0.001, [d]P<0.001, [f]P<0.001, [h]P<0.001 *vs* Japannese, [1]P = 0.001.

elevated in patients with interstitial pneumonia[14]. It was also reported to have a high positive rate in different non-hepatic malignancies and its expression was also correlated with metastatic potential of the primary tumor in some of them[15-17]. It has also been studied as a fibrosis marker in patients with HCV-related chronic liver disease[18] and was found to correlate with the degree of irregular regeneration of hepatocytes[19]. A recent study addressed its clinical significance as a tumor marker in HCV-related HCC[20]. However, all these studies investigated KL-6 in HCV-related disease only so that its actual significance as a marker for screening HCC in patients with different chronic liver disease is not yet fully understood. In this study, we aimed to investigate KL-6 as a tumor marker in consecutive patients with chronic liver disease seen at outpatient settings in two different ethnic groups of possible different risk factors for HCC, so that we could get a wider spectrum of disease in order to assess KL-6 validity for HCC screening.

MATERIALS AND METHODS

Study population

We conducted a cross-sectional study between October 2001 and November 2002. Data were gathered from two Affiliations; Shinshu University (Japan) and Suez Canal University (Egypt) Hospitals. A total of 334 consecutive patients with chronic liver disease seen at outpatient liver clinics in the two settings (who met our inclusion/exclusion criteria) were included; of them: 110 patients were diagnosed as HCC with a mean age of 61±11 years and M:F (4:1). Sixty-five were Egyptians and 45 Japanese with viral-related liver disease accounting for 94% and 98% of them respectively. Non-HCC patients were 234 with a mean age of 56±13 years; M:F (7/3). One hundred and six were Egyptians and 128 Japanese with viral-related liver disease accounting for 91% and 84% of them respectively (Table 1).

Chronic liver disease and cirrhosis were identified and diagnosed according to liver biopsy findings, clinical and/or radiological evidence of portal hypertension. HCC was excluded by imaging studies (abdominal ultrasound (US), computed tomography (CT), magnetic resonance imaging (MRI) and/or hepatic angiography), one of which must have been performed at least 6 months following the measurement of AFP.

HCC was diagnosed when meeting our inclusion criteria of positive cytology and/or histology 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 vascularization).

We excluded patients with alcoholic and schistosomal liver diseases from our study populations. We had also excluded patients known from their medical history to have interstitial lung fibrosis or any other lung disease from our study population.

Tumor markers measurement

Serum KL-6 was measured by the sandwich enzyme immunoassay method using the KL-6 antibody (Ab) as both the capture and tracer Ab (14) according to the manufacturer's instructions (Eisai, Tokyo, Japan). KL-6 cut-off point was set at 500 U/L for this study. Assessment of alpha fetoprotein (AFP) and protein-induced vitamin K deficiency or absence (PIVKA-II) was performed using commercially available kits. Cut-off points were set at 10 ng/mL for AFP and 40 mAU/L for PIVKA-II.

Statistical analysis

Univariate statistical analysis was performed using Student's t-test for quantitative and χ^2 test with Yates' correction for qualitative data. Fisher's exact test was used

for comparison of small numbers; statistical significant level was set at $P<0.05$. Statistical analysis was performed using a computer software (SPSS, version 6.0).

RESULTS

Population background

A difference in mean age, prevalence of advanced Child class and HBV infection was observed between Egyptian and Japanese patients with HCC (Table 1). However, no difference in tumor characteristics was found between the two studied populations (Table 2).

KL-6 and other tumor markers in HCC

A significantly higher mean serum KL-6 (556±467) was found in HCC in comparison with non-HCC groups of patients with (391±176; $P<0.001$) and without (361±161; $P<0.001$) liver cirrhosis (LC). Serum KL-6 level did not correlate with either AFP (Figure 1) or PIVKA-II (Figure 1) serU/Levels. Using receiver operation characteristic (ROC) curve, the KL-6 level that gave the best sensitivity (61%) was found to be 334 U/L with a specificity of 50%, while PIVKA-II and AFP showed a sensitivity/specificity of (60/72)% and (80/78)% respectively. However, according to the manufacturer's instructions; a cut-off point of 500 U/L was used in this study that showed the highest specificity (80%) for KL-6 in comparison with the other two markers. Combining the values of KL-6; AFP and PIVKA-II resulted in improvement in the specificity of AFP for HCC diagnosis from 78% for AFP alone; 93% for AFP plus PIVKA-II to 99% for both plus KL-6 ($P<0.001$) (Table 3).

Table 2 Comparison of background tumor characteristics between Egyptian and Japanese HCC 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			
<3 cm	32 (49)	20 (44)	>0.2
3:< 5cm	15 (23)	14 (31)	>0.2
≥5 cm	18 (28)	11 (25)	0.13
Metastases	01 (2)	>0.2	
Tumor grade[1]			
Well differentiated	5 (16)	2 (8)	>0.2
Poorly differentiated	5 (16)	4 (16)	>0.2

[1]Tumor grade is analyzed in 32 of the Egyptian and 22 of the Japanese groups who passed HCC resection operation during the study period.

Factors associated with positive KL-6 in the study population

Univariate analysis (Table 4) of possible factors that could be associated with elevated serum KL-6 in our study group showed that elevated AFP ($P<0.001$), Child's class C (P = 0.002), Egyptian race (P = 0.003) and HCC (P = 0.008) were significantly associated with positive serum KL-6. Also mean serum alkaline phosphatase level was significantly higher in KL-6 positive (564±475) in comparison with KL-6 negative (505±469) HCC patients (P = 0.021), but such a difference was not found among non-HCC corresponding group (Table 5). Mean serum bilirubin was found to be higher in KL-6 positive subgroups in both HCC and non-HCC (P = 0.077, 0.023) respectively, while mean serum albumin was significantly lower in both groups (P = 0.029, 0.041), respectively (Table 5).

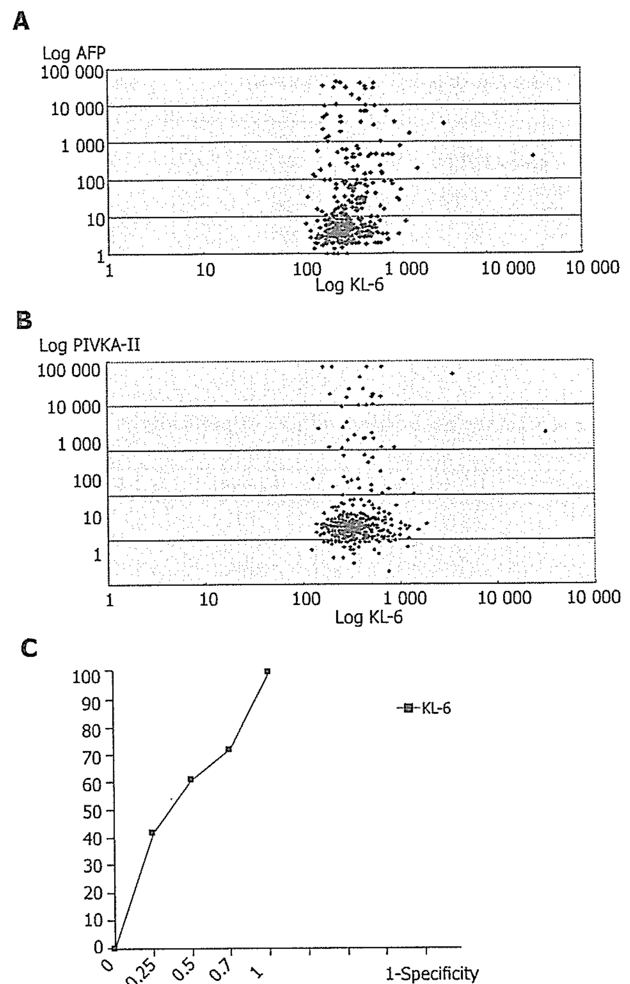


Figure 1 A: Correlation between KL-6 U/L and AFP ng/ml serU/Levels in the study population. C = 0.04, $P>0.1$. The Log values of both markers are shown; **B:** Correlation between KL-6 U/L and PIVKA-II mAU/L serU/Levels in the study population. C = 0.03, $P>0.5$. The Log values of both markers are shown; **C:** Receiver operating characteristic curves for KL-6 as predictors of HCC. The area under the ROC was found to be 0.574 (95%CI = 0.50–0.64). The best KL-6 sensitivity was obtained at a cut-off point = 334 U/L.

KL-6 in Egyptian vs Japanese

Mean KL-6 was significantly higher in Egyptians (576±522) in comparison with Japanese (510±300) HCC

Table 3 Comparison of the result of different tumor markers between HCC and non-HCC group

Tumor marker (cut-off point)	Sensitivity %	Specificity %
AFP (10 ng/mL)	86	78
PIVKA-II (35 mAU/L)	61	72
KL-6 (500 U/L)	34	80
AFP+PIVKA-II	86[1]	93
AFP+KL-6	87[2]	94
AFP+PIVKA-II+KL-6	87	99

[1]All PIVKA-II (+) HCC patients are AFP (+). [2]One KL-6 (+) HCC patient is AFP (-).

Table 4 Factors associated with KL-6 positivity in the study population

Factors	Total	KL-6 (+)	KL-6 (-)
Age (yr)			
>=50	244	60 (24)	184 (75)
<50	100	24(24)	76 (76)
P	NS		
Sex			
Male	257	61 (24)	196 (76)
Female	87	23 (26)	64 (73)
P	NS		
Ethnicity			
Egyptian	171	53 (31)	118 (69)
Japanese	173	31 (18)	142 (82)
P	0.003		
Underlying liver disease			
HCV-related	267	71 (27)	196 (73)
HBV-related	42	5 (12)	37 (88)
Non-viral	35	8 (23)	27 (77)
P	NS		
Cirrhosis			
(+)	169	46 (27)	123 (73)
(-)	175	38 (22)	137 (78)
P	NS		
Child's class			
C	47	20 (43)	27 (57)
A&B	297	64 (21)	233 (78)
P	0.002		
HCC:			
(+)	110	37 (34)	73 (66)
(-)	234	47 (20)	187 (80)
P	0.008		
AFP			
(+)	145	49 (34)	96 (66)
(-)	199	35 (17)	164 (82)
P	<0.001		

patients (P = 0.041) (Table 6). Although a significant difference in mean KL-6 level between HCC and non-HCC was observed in both Egyptian and Japanese patients with chronic liver disease (P<0.001 respectively), the difference was not statistically significant among Japanese patients with HCV-related disease (Table 6). No difference in mean KL-6 level was found between cirrhotic and non-cirrhotic in either HCC or non-HCC patients.

KL-6 and tumor characteristics

In the HCC groups of both Egyptian and Japanese patients; KL-6 showed no significant association with tumor site, echogenicity or multiplicity. However, a significantly lower mean KL-6 (Table 7) was noticed in larger size tumors of >5 cm (371±168 U/L) in comparison with tumors of less than or equal to 5 cm (537±323) (P<0.05 in the Japanese group).

DISCUSSION

KL-6 was studied as a tumor marker in different malignancies like breast, lung and pancreatic cancer and it was reported to be elevated in up to 50% of these malignancies[14]. Two previous studies by Moriyama *et al.*[19, 20] addressed KL-6 as a tumor marker for HCC in patients with HCV-related chronic liver disease, and his results showed that the estimated cumulative incidence of HCC development in HCV-related chronic liver disease patients was significantly greater in patients with positive KL-6[19] and suggested KL-6 to be used as a serological marker for HCC development in HCV-positive patients[20]. In our study, we included consecutive patients with chronic liver disease seen at outpatient settings in two different ethnic groups of possible different risk factors for HCC[22, 23] in order to have a wider spectrum of disease to judge KL-6 validity as a diagnostic test for HCC; however, one limitation was that most of the encountered patients in the two settings were actually with HCV-related disease with low proportion of HBV and non-viral-related disease. Our results showed a significantly higher mean KL-6 in HCC compared with non-HCC; either with or without LC; in addition no difference in mean KL-6 was found among HCC patients with and without LC; such findings together point to KL-6 association with HCC independent on the presence or absence of LC. A significantly higher mean KL-6 level was found in HBV-related in comparison with HCV-related HCC in both Egyptian and Japanese populations; a finding that deserves future study on a larger population of HBV-related disease. Our results also showed a significantly higher mean KL-6 level in HCC patients of Egyptian compared with Japanese race. The finding of a difference in the clinical background between both in terms of lower mean age and lower prevalence of HBV-related HCC could reflect a difference in the risk factors for HCC in both groups. Also, a higher prevalence of advanced Child class in the HCC Egyptian patients was observed that could stand behind the finding of higher mean KL-6 level in this group compared to their corresponding Japanese group. Although we excluded patients with overt schistosomal from this study, still some Egyptian patients had a past history of schistosomiasis with US evidence of hepatic periportal fibrosis (denoting a background of schistosomal liver disease) that could also explain the finding of higher mean KL-6 level in Egyptian HCC patients, if we consider the possibility that KL-6 could be a fibrosis marker too[21]. This topic is highly suggested for future study.

Table 5 Comparison of the clinical profile of KL-6 positive and negative patients with and without HCC

	HCC (+)			HCC (-)		
	KL-6 (+) *n* = 37	KL-6 (-) *n* = 73	*P*	KL-6 (+) *n* = 47	KL-6 (-) *n* = 187	*P*
Mean age (yr)[1]	59±12	62±10	NS	57±12	56±13	NS
Cirrhosis	39 (81)	55 (76)	NS	16 (34)	65 (35)	NS
Child's C	13 (35)	15 (21)	NS	7 (15)	11 (6)	0.045
Mean ALT	74±101	62±61	NS	59±33	57±45	NS
Serum						
Albumin (g/L)[1]	2.9±0.7	3.3±0.7	0.029	3.5±0.9	3.8±0.8	0.041
Bilirubin (mmol/L)[1]	2.7±2.8	2.5±3.0	0.077	2.4±2.9	1.4±1.9	0.023
ALP (IU/L)[1]	564±475	505±469	0.021	316±139	299±152	NS
AFP (+)	36 (97)	58 (80)	0.013	13 (28)	37 (20)	NS
PIVIKA (+)	23 (62)	44 (60)	NS	14 (30)	50 (27)	NS

[1]Data is shown as mean±SD. Other data is shown as *n* (%).

Table 6 Comparison of mean serum KL-6 level among different study sub-groups

	Egyptian			Japanese		
	HCC (+)	HCC (-)	*P*	HCC (+)	HCC (-)	*P*
Chronic liver disease[1]:	576 (522)	398 (185)	0.001	510 (300)	350 (147)	<0.001
HCV-related	558 (524)	400 (172)	0.008	356 (290)	382 (209)	>0.2
HBV-related	778 (663)	246 (72)	>0.2	877 (292)	340 (163)	<0.001
Non-viral	729 (538)	446 (309)	>0.2	262[2] ()	357 (160)	-
Cirrhotics	599 (586)	406 (159)	0.035	510 (350)	374 (196)	0.035
Non-cirrhotics	518 (325)	398 (185)	0.045	225 (73)	349 (222)	<0.001

[1]The KL-6 values are shown as mean (SD) U/L. [2]Only one patient's data.

Table 7 Difference in mean KL-6 level according to HCC size

	Egyptian (*n* = 65)	*P*[1] value	Japanese (*n* = 45)	*P* value
Tumor size				
<3 cm	485±227		618±361	
3:<5 cm	643±685	>0.1	456±285	0.17
≥5 cm	581±420	>0.1	371±168	0.04

[1]*P* value is shown for the difference group (<3 cm) and the other two groups.

We used a cut-off point of 500 U/L for KL-6 positivity in this study; however, applying the ROC analysis showed that a cut-off point of 334 U/L would give the best sensitivity in our study population of 60% compared with only 32% for a cut-off (500 U/L); however, the best specificity was obtained using the later. Moriyama *et al.* used a cut-off point of 300 U/L in his analysis of KL-6 in HCV-related disease[19,20]. KL-6 serU/Level did not correlate with either serum AFP or PIVKA-II levels, which points to its behavior independently from either of them and this may justify its clinical significance as an independent tumor marker for HCC diagnosis when considered with both AFP and PIVKA-II. Our results also supported this finding as AFP specificity for HCC diagnosis improved from 78% for AFP alone and 93% of both AFP and PIVKA-II to 99% when combined with KL-6. Univariate analysis showed that low serum albumin, hyperbilirubinemia and elevated ALP were significantly associated with positive KL-6 in HCC patients, while KL-6 showed no association with LC in turn, and this denotes a possible association between positive KL-6 and deterioration of hepatic condition in HCC patients independent from their cirrhotic status; a finding that might point to KL-6 as a predictor of tumor aggression and/or local or systemic metastasizing potential. A follow-up study is needed to confirm its exact role in this regard.

ACKNOWLEDGMENTS

We would like to thank Takeda Foundation, Osaka, Japan for their financial support. We also thank Dr. Alla Sad, Dr. Essam Abd Alla, Dr. Khaled Gad for their help with various laboratory techniques.

REFERENCES

1 **Sherman M.** Hepatocellular carcinoma: epidemiology, risk factors, and screening. *Semin Liver Dis* 2005; **25**: 143-154
2 **Szilagyi A**, Alpert L. Clinical and histopathological variation in hepatocellular carcinoma. *Am J Gastroenterol* 1995; **90**: 15-23
3 **Yuen MF**, Cheng CC, Lauder IJ, Lam SK, Ooi CG, Lai CL. Early detection of hepatocellular carcinoma increases the chance of treatment: Hong Kong experience. *Hepatology* 2000; **31**: 330-335
4 **Wong LL**, Limm WM, Severino R, Wong LM. Improved survival with screening for hepatocellular carcinoma. *Liver Transpl* 2000; **6**: 320-325
5 **Johnson PJ.** The role of serum alpha-fetoprotein estimation in the diagnosis and management of hepatocellular carcinoma.

Clin Liver Dis 2001; **5:** 145-159

6 **Iwai M**, Kashiwadani M, Takino T, Ibata Y. Demonstration by light and ultrastructural immunoperoxidase study of alpha-fetoprotein-positive non-hepatoma cells and hepatoma cells during 3'-methyl-4-dimethylaminoazobenzene hepatocarcinogenesis. *Virchows Arch B Cell Pathol Incl Mol Pathol* 1988; **55:** 117-123

7 **McIntire KR**, Waldmann TA, Moertel CG, Go VL, Serum alpha-fetoprotein in patients with neoplasms of the gastrointestinal tract. *Cancer Res* 1975; **35:** 991-996

8 **Gallo V**, Cerutti E, Riberi A, Re M, Petrino R, Pecchio F. Alpha-fetoprotein and tissue polypeptide antigen in non neoplastic hepatic disorders. *J Nucl Med Allied Sci* 1989; **33:** 89-93

9 **Alpert E**, Feller ER. Alpha-fetoprotein (AFP) in benign liver disease. Evidence that normal liver regeneration does not induce AFP synthesis. *Gastroenterology* 1978; **74:** 856-858

10 **Nguyen MH**, Garcia RT, Simpson PW, Wright TL, Keeffe EB. Racial differences in effectiveness of alpha-fetoprotein for diagnosis of hepatocellular carcinoma in hepatitis C virus cirrhosis. *Hepatology* 2002; **36:** 410-417

11 **Kohno N**, Kyoizumi S, Awaya Y, Fukuhara H, Yamakido M, Akiyama M. New serum indicator of interstitial pneumonitis activity. Sialylated carbohydrate antigen KL-6. *Chest* 1989; **96:** 68-73

12 **Stahel RA**, Gilks WR, Lehmann HP, Schenker T. Third International Workshop on Lung Tumor and Differentiation Antigens: overview of the results of the central data analysis. *Int J Cancer Suppl* 1994; **8:** 6-26

13 **Wesseling J**, van der Valk SW, Vos HL, Sonnenberg A, Hilkens J. Episialin (MUC1) overexpression inhibits integrin-mediated cell adhesion to extracellular matrix components. *J Cell Biol* 1995; **129:** 255-265

14 **Kohno N**. Serum marker KL-6/MUC1 for the diagnosis and management of interstitial pneumonitis. *J Med Invest* 1999; **46:** 151-158

15 **Sagara M**, Yonezawa S, Nagata K, Tezuka Y, Natsugoe S, Xing PX, McKenzie IF, Aikou T, Sato E. Expression of mucin 1 (MUC1) in esophageal squamous-cell carcinoma: its relationship with prognosis. *Int J Cancer* 1999; **84:** 251-257

16 **Utsunomiya T**, Yonezawa S, Sakamoto H, Kitamura H, Hokita S, Aiko T, Tanaka S, Irimura T, Kim YS, Sato E. Expression of MUC1 and MUC2 mucins in gastric carcinomas: its relationship with the prognosis of the patients. *Clin Cancer Res* 1998; **4:** 2605-2614

17 **Tanimoto T**, Tanaka S, Haruma K, Yoshihara M, Sumii K, Kajiyama G, Shimamoto F, Kohno N. MUC1 expression in intramucosal colorectal neoplasms. Possible involvement in histogenesis and progression. *Oncology* 1999; **56:** 223-231

18 **Suzuki K**, Takada H, Oka S, Kanouzawa S, Iimuro M, Kitazumi Y, Arima T, Ohyama R, Kuwayama H. Clinical significance of KL-6, a marker of interstitial pneumonia, in cases of HCV-associated chronic liver disease. *Intern Med* 2003; **42:** 650-654

19 **Moriyama M**, Matsumura H, Mikuni M, Arkawa Y, Ohshiro S, Aoki H, Yamagami H, Kaneko M, Shioda A, Saito H, Tanaka N, Arakawa Y. The clinical significance of serum KL-6 levels in patients with type C liver diseases. *Hepatol Res* 2003; **25:** 385-395

20 **Moriyama M**, Matsumura H, Watanabe A, Nakamura H, Arakawa Y, Oshiro S, Aoki H, Shimizu T, Yamagami H, Kaneko M, Shioda A, Tanaka N, Arakawa Y. Detection of serum and intrahepatic KL-6 in anti-HCV positive patients with hepatocellular carcinoma. *Hepatol Res* 2004; **30:** 24-33

21 **Hirasawa Y**, Kohno N, Yokoyama A, Inoue Y, Abe M, Hiwada K. KL-6, a human MUC1 mucin, is chemotactic for human fibroblasts. *Am J Respir Cell Mol Biol* 1997; **17:** 501-507

22 **Hassan MM**, Zaghloul AS, El-Serag HB, Soliman O, Patt YZ, Chappell CL, Beasley RP, Hwang LY. The role of hepatitis C in hepatocellular carcinoma: a case control study among Egyptian patients. *J Clin Gastroenterol* 2001; **33:** 123-126

23 **Tanaka K**, Sakai H, Hashizume M, Hirohata T. A long-term follow-up study on risk factors for hepatocellular carcinoma among Japanese patients with liver cirrhosis. *Jpn J Cancer Res* 1998; **89:** 1241-1250

Science Editor Guo SY **Language Editor** Elsevier HK

Asia–Pacific Journal of Clinical Oncology 2005; 1: 64–70

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

Amal GAD,[1,2] Eiji TANAKA,[1] Akihiro MATSUMOTO,[1] Abd EL-HAMID SERWAH,[2] Fawzy ATTIA,[2] Adel HASSAN,[2] Ahmed SANNY,[2] Khalil ALI,[2] Amro ABBAS,[2] Abd EL-RAOOF EL-DEEB,[2] Xiao Hong SUN,[1] Takeji UMEMURA,[1] Tetsuya ICHIJO,[1] Takashi EHARA,[3] Kaname YOSHIZAWA[1] and Kendo KIYOSAWA[1,4]

[1]*Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan,* [2]*Suez Canal University School of Medicine, Ismailia, Egypt,* [3]*Department of Pathology, Shinshu University School of Medicine and* [4]*Shinshu University Graduate School of Medicine, Institutes of Organ Transplants, Reconstructive Medicine and Tissue Engineering, Matsumoto, Japan*

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.

Correspondence: Dr Eiji Tanaka, Department of Medicine, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan.
Email: etanaka@hsp.md.shinshu-u.ac.jp

Accepted for publication 9 June 2005.

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 $P < 0.05$.

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)

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.

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.

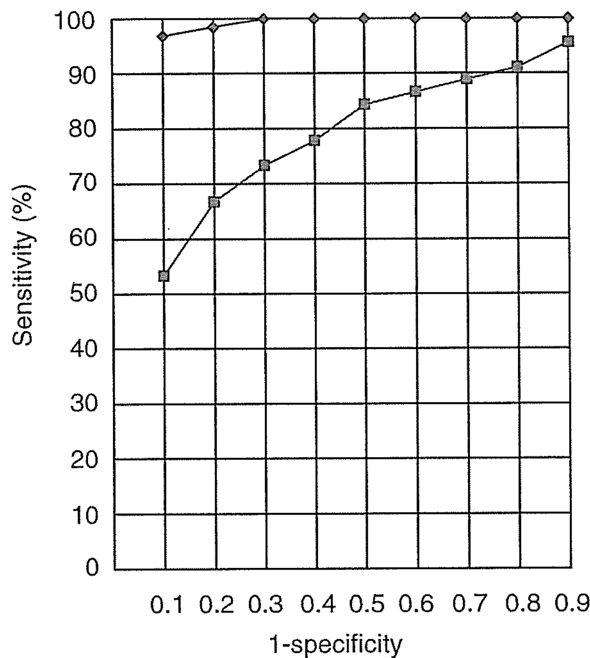


Figure 1 Receiver operating characteristic curves (ROC) for α-fetoprotein (AFP) as predictors of HCC for Egyptians (◆) and Japanese (■). The area under the ROC was found to be 0.98 (95%CI = 0.969–0.997) for Egyptians with the highest AFP sensitivity obtained at AFP = 20.5 ng/mL and 0.77 (95%CI = 0.686–0.864)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

				95% C.I.		
Variable	AFP </≥ cut-off point *(ng/dL)	No.	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.

ACKNOWLEDGMENTS

We would like to thank Dr Alla Sad, Dr Essam Abd Alla, Dr Khaled Gad and Dr Azza Gad for their help with various investigative techniques. We also thank the Pathology Department of Suez Canal School of Medicine for their technical help. Finally, we are grateful to the Takeda Foundation, Osaka, Japan for their financial support.

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, Rạdl J, Koutecky J, Kotal L. Occurrence of fetoprotein in patients with neoplasms and non-neoplastic diseases. *Inter J Cancer* 1968; 3: 364–73.