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Identification of Hepatitis C Virus (HCV) Subtype 1b Strains That Are Highly, or Only Weakly, Associated with Hepatocellular Carcinoma on the Basis of the Secondary Structure of an Amino-Terminal Portion of the HCV NS3 Protein

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The NS3 protein of hepatitis C virus subtype 1b (HCV-1b) isolates obtained from 89 patients with hepatocellular carcinoma (HCC) and 78 patients without HCC were analyzed. On the basis of the secondary structure of the amino-terminal 120 residues of NS3, HCV-1b isolates were classified into group A, group B, and an indeterminate group, each of which was further divided into a number of subgroups, such as A1-1, A1-2, A2-1, A2-2, B1-1, B1-2, B2-1, B2-2, C-1, C-2, and C-3. HCV-1b isolates of subgroup B1-1 were found in 53 (59.6%) of 89 patients with HCC and 19 (24.4%) of 78 patients without HCC, with the difference between the two patient groups being statistically significant ($P < 0.00001$). Although the number of isolates was small, subgroup B2-1 was also highly associated with HCC, with all five isolates in that subgroup being found in patients with HCC ($P < 0.05$). On the other hand, HCV-1b isolates of subgroup A1-1 were associated only weakly with HCC; they were found in 6 (6.7%) of 89 patients with HCC and in 25 (32.1%) of 78 patients without HCC, with the difference between the two patient groups being statistically significant ($P < 0.0001$). The other subgroups, such as A1-2, A2-1, B1-2, C-1, C-2, and C-3, were moderately associated with HCC; their distribution patterns among patients with HCC did not differ significantly from those among patients without HCC. Taken together, our results suggest that HCV-1b isolates of subgroups B1-1 and B2-1 are highly associated with HCC and that this secondary structure analysis may be useful for predicting the relative risk of developing HCC.

Hepatitis C virus (HCV) easily establishes chronic persistent infection, initially causing chronic hepatitis, followed by liver cirrhosis, from which hepatocellular carcinoma (HCC) arises at an estimated rate of 1 to 4% per year (17). However, it is still a matter of debate whether all HCV strains are associated with HCC to the same extent. The HCV genome exhibits a considerable degree of sequence variation, and HCV is presently classified into at least six genotypes and more than 60 subtypes (5, 22, 27). HCV subtype 1b (HCV-1b) is the most prevalent subtype in most parts of Asia, including Japan (4, 12, 13). HCV-1b has been considered to be associated with a poorer response to interferon (IFN) treatment, a more rapid disease progression, and a greater rate of development of HCC than the other HCV subtypes (3, 24, 30). In addition to the differences at the genotype or subtype level, other important sequence diversities have been observed even among isolates of a given subtype. For example, amino acid mutations in a limited portion of NS5A have been reported to be associated with IFN sensitivity, and therefore, this region is referred to as the IFN sensitivity-determining region (6, 31).

NS3 encodes serine protease in its amino-terminal one-third. This serine protease is essential for cleavage of the HCV precursor polyprotein at the NS3-NS4A, NS4A-NS4B, NS4B-NS5A, and NS5A-NS5B junctions (1, 11, 18, 26, 32). A minimum portion of the serine protease activity of NS3 has been mapped to a region between amino acids (aa) 1059 and 1204 (33). The serine protease activity of NS3 is enhanced by NS4A, which forms a stable complex with NS3 at its amino terminus spanning from aa 1027 to 1054 (2, 7, 19, 29). Besides the proteolytic cleavage activity, NS3 appears to be involved in hepatocarcinogenesis. It was reported (28, 34) that an amino-terminal portion of NS3 flanked by a short stretch of the carboxy terminus of NS2 (aa 1020 to 1295 and aa 1008 to 1246, respectively) has the capacity to transform NIH 3T3 and rat fibroblast cells. Also, we observed that an amino-terminal portion of NS3 (aa 1027 to 1459) rendered NIH 3T3 cells more resistant to DNA damage-induced apoptosis (8), which is thought to be a prerequisite for malignant transformation of the cell. Moreover, it was observed that NS3 interacted differentially with the p53 tumor suppressor in a sequence-dependent manner in the presence of NS4A (23), with the p53-binding region of NS3 being mapped to a sequence between aa 1055 and 1200, which is in the close vicinity of the NS4A-binding region (aa 1027 to 1054) (14). Therefore, we have been interested to know whether there is any correlation between the sequence diversity of an amino-terminal portion of NS3 and the development of HCC. It was previously reported (25) that most HCV-1b strains can be classified into two groups, groups A and B, on the basis of the secondary structure of the amino-terminal 180 residues of NS3 and that the isolates of

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TABLE 1. Ages and sexes of the patients analyzed in this study

Patient location and clinical status	No. of patients (no. of males/no. of females)	Mean ± SD (range) age (yr)
Hyogo Prefecture		
With HCC	36 (32/4)	65.0 ± 5.3 (52–76)
Without HCC	35 (18/17)	62.6 ± 8.5 (49–80)
Yamagata Prefecture		
With HCC	53 (22/31)	70.3 ± 10.0 (36–79)
Without HCC	43 (24/19)	60.7 ± 8.7 (34–78)

group B are highly associated with HCC. It was noticed in the same study that groups A and B could each be further classified into a number of subgroups, depending on the number and positions of the turn structures. It was also noticed that secondary structure analysis of a shorter sequence (~120 residues) was sufficient for the subgroup classification of most isolates. In the present study, we focused on the secondary structure of the 120-aa sequence and analyzed the possible correlation of its secondary structure with the development of HCC in HCV-1b-infected patients.

MATERIALS AND METHODS

Serum samples. Sera were collected from patients with and without HCC in Hyogo and Yamagata Prefectures, Japan. The diagnosis of HCC was made on the basis of clinical and histopathological criteria. The sera were tested for anti-HCV antibodies and HBsAg by using commercial kits (Ortho HCV Ab ELISA Test III [Ortho Diagnostics, Tokyo, Japan] and AUSAB EIA [Abbott Diagnostics], respectively). Anti-HCV antibody-positive sera were tested for HCV RNA by reverse transcription (RT)-PCR, and the HCV subtypes were determined as reported previously (5, 21, 30). A total of 167 serum samples positive for HCV-1b RNA and negative for HBsAg were further analyzed, as described below. The age and sexes of the patients are summarized in Table 1.

NS3 analysis. RNA was extracted from 50 μl of serum with the RNeasy Mini kit (Qiagen). To amplify a portion of the HCV genome encoding an amino-terminal region of NS3, a one-step RT-PCR was performed in a tube by the Superscript One-Step RT-PCR with Platinum *Taq* (GIBCO BRL) and an outer set of primers, primer NS3-F1 (sense primer; 5′-ACACCGCGGCGTGTGGGGACAT-3′; nucleotides 3295 to 3316) and primer NS3-AS2 (antisense primer; 5′-GCTCTTGCCGCTGCCAGTGGGA-3′; nucleotides 4040 to 4019). PCR was initially performed at 45°C for 30 min for RT and then at 94°C for 2 min, followed by the first-round PCR over 40 cycles, with each cycle consisting of 1 min each at 94, 55, and 72°C. The second-round PCR was performed with *Pfu* DNA polymerase (Promega) and an inner set of primers, primer NS3-F3 (sense primer; 5′-CAGGGGTGGCGGCTCCTT-3′; nucleotides 3390 to 3407) and primer NS3-AS1 (antisense primer; 5′-GCCACTTGGAATGTTTGCGGTA-3′; nucleotides 4006 to 3985). The second-round PCR was performed for 35 cycles, with each cycle consisting of 1 min at 94°C, 1.5 min at 55°C, and 3 min at 72°C. This method allowed us to amplify the corresponding portion of the HCV genome from more than 90% of HCV-1b RNA-positive samples. The amplified fragments were purified with the QIAquick PCR Purification kit (Qiagen) and directly sequenced, without being subcloned, in both directions with the dRhodamine Terminator Cycle Sequencing Ready Reaction kit and an ABI 377 sequencer (PE Applied Biosystems). The secondary structure of the amino-terminal portion of NS3 was predicted by computer-assisted Robson analysis (9) with GENETYX-MAC software (version 10.1; Software Development Co., Ltd., Tokyo, Japan) with α-helix and extended decision constants of 0.

Statistical analysis. The data obtained were statistically analyzed by the χ^2 test for independence with a two-by-two contingency table and Student's *t* test. A *P* value of <0.05 was considered significant.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession numbers AB072043 through AB072113, AB089512 through AB089583, and AB100806 through AB100829.

RESULTS

Classification of HCV-1b strains on the basis of the secondary structure of an amino-terminal portion of NS3 and their possible association with HCC. On the basis of the secondary structure of the amino-terminal 120 residues of NS3, HCV-1b isolates were classified into group A, group B, and an indeterminate group, each of which was further divided into a number of subgroups (Fig. 1). The criteria for the group classification, which should match the reported one (25), are as follows. In group A isolates, the carboxy-terminal portion of 20 residues (aa 1125 to 1146) goes leftward relative to a domain composed of the remaining amino-terminal portion. In group B isolates, on the other hand, the carboxy-terminal portion of 20 residues goes rightward relative to the amino-terminal domain. It should be noted that the turn structures at about positions 1048 and 1125 were conserved among all 167 isolates tested (data not shown). Isolates of group A and group B were further classified into subgroups, such as subgroups A1-1, A1-2, A2-1, A2-2, B1-1, B1-2, B2-1, and B2-2, on the basis of the positions of the additional turn structures. For example, subgroups A1-1 and B1-1 have a turn structure at about position 1083, but not at about position 1075. On the other hand, subgroups A1-2 and B1-2 have a turn structure at about position 1075 but not at about position 1083. Subgroups A2-1 and B2-1 have two turn structures at about positions 1075 and 1083, while subgroups A2-2 and B2-2 do not have any turn structure between positions 1048 and 1125. Eighteen (10.8%) of 167 isolates had an additional turn structure near the amino terminus at about positions 1033 to 1037, with the partial secondary structure thereafter resembling that of one of the subgroups mentioned above. Those isolates were classified into an indeterminate group and were further divided into a number of subgroups, such as subgroups C-1 (six isolates), C-2 (five isolates), and C-3 (four isolates). Three isolates of the indeterminate group differed from each other and were tentatively classified into a subgroup referred to as C-etc. (Table 2).

HCV-1b isolates of subgroup B1-1 were found in 53 (59.6%) of 89 patients with HCC and in 19 (24.4%) of 78 patients without HCC, with the difference in the prevalence between the two patient groups being statistically significant ($P < 0.00001$) (Table 2). Thus, subgroup B1-1 was highly associated with HCC. Although the number of isolates was small, subgroup B2-1 was also highly associated with HCC, with all five isolates being found in patients with HCC ($P < 0.05$). Isolates of subgroup A1-1, which was weakly associated with HCC, were found in 6 (6.7%) of 89 patients with HCC and in 25 (32.1%) of 78 patients without HCC, with the difference between the two patient groups being statistically significant ($P < 0.0001$). The other subgroups, such as A1-2, A2-1, and B1-2 and the indeterminate ones, C-1 to C-3, were moderately associated with HCC; their distribution patterns among patients with HCC did not significantly differ from those among patients without HCC.

Sequence alignment of the amino-terminal 120 residues of NS3 of HCV-1b strains. The amino acid sequences of the isolates were aligned with each other, and a consensus sequence was determined on the basis of the alignment result (Fig. 2). The consensus sequence differed from the sequence of a standard strain, strain HCV-J (GenBank accession number

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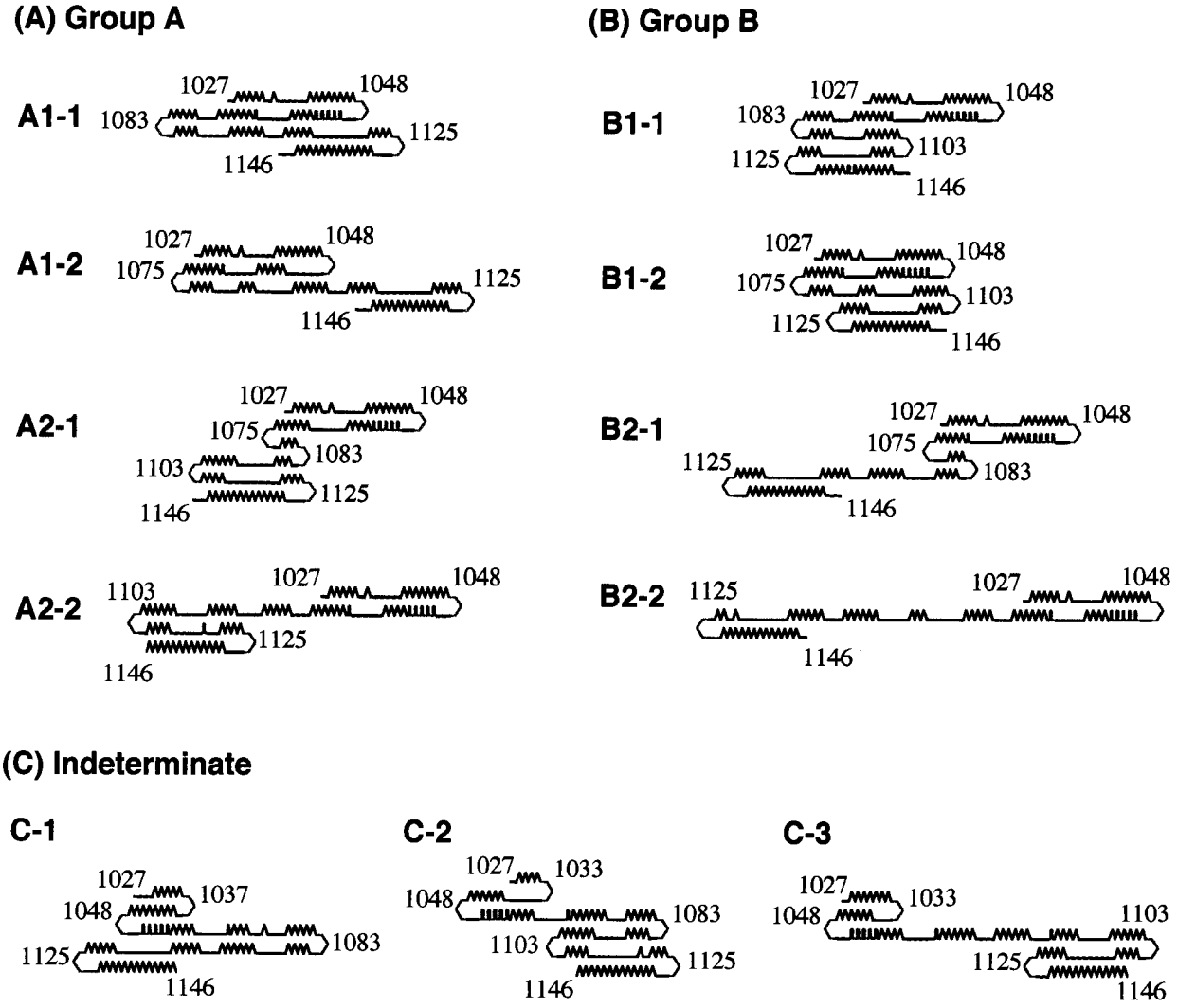

FIG. 1. Schematic representation of secondary structure of the amino-terminal 120 residues of NS3 of HCV-1b isolates obtained from patients with and without HCC. (A) Representative forms of group A, which can be further classified into subgroups A1-1, A1-2, A2-1, and A2-2, based on the number and positions of turn structures; (B) representative forms of group B, which can be further classified into subgroups B1-1, B1-2, B2-1, and B2-2; (C) representative forms of an indeterminate group, which can be further classified into subgroups C-1, C-2, C-3, C-etc. The looped, zigzag, straight, and bent lines represent α-helix, β-sheet, coil, and turn structures, respectively. The numbers along the secondary structure indicate amino acid positions.

D90208) (15), by five residues at positions 1056 (Asp to Glu), 1062 (Leu to Val), 1112 (Pro to Gln), 1120 (Met to Leu), and 1140 (Val to Ile). Subgroup B1-1 had the consensus sequence, whereas subgroup A1-1 had the HCV-J sequence. Sequences of representative isolates of each of the HCV subgroups were aligned with the consensus sequence (Fig. 2). Tyr-1082 was associated, in general, with the presence of a turn structure at about position 1083, as observed in subgroups A1-1, A2-1, B1-1, and B2-1, with some exceptions. On the other hand, Phe-1082 was associated with the absence of the turn structure at about position 1083 and generation of a turn structure at about position 1075, as observed in subgroups A1-2 and B1-2. Also, a mutation(s) upstream of position 1082 appeared to affect the turn structures at about positions 1075 and 1083. It was difficult to predict the presence or absence of a turn structure at about position 1103 simply by comparing amino acid sequences. Isolates of the indeterminate subgroups, C-1, C-2, C-3, and C-etc., had a unique mutation(s) near the amino terminus, such as Leu to Phe at position 1040 (13 isolates) and Ser to Cys or Thr at position 1033 (3 isolates), that created an additional turn structure.

At the primary structure level, we did not find any particular residue that is unique to and yet common among the majority of isolates from patients with HCC (data not shown). It should be noted, however, that isolates of subgroup B2-1, which are highly associated with HCC but small in number (Table 2), have more mutations or a unique mutation from a hydrophobic residue (Val) to a hydrophilic residue (Thr) at about positions 1055 to 1080 (Fig. 2).

The average number of mutations in the 120-residue sequence differed by subgroup. Subgroup A1-1 had significantly more mutations than B1-1 but significantly fewer mutations than A1-2, B1-2, and B2-1 (Table 3). When isolates from patients with HCC and those without HCC were compared,

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TABLE 2. Distributions of different HCV subgroups in patients with and without HCC

HCV subgroup[a]	Hyogo Prefecture			Yamagata Prefecture			Total		
	No. of isolates from:		*P* value	No. of isolates from:		*P* value	No. of isolates from:		*P* value
	HCC patients	Non-HCC patients		HCC patients	Non-HCC patients		HCC patients	Non-HCC patients	
A1-1	2	11	<0.01[b]	4	14	<0.01[b]	6	25	<0.0001[b]
A1-2	3	3	NS[c]	1	4	NS	4	7	NS
A2-1	0	0	NS	2	2	NS	2	2	NS
A2-2	0	0	NS	1	0	NS	1	0	NS
B1-1	21	5	<0.001[b]	32	14	<0.01[b]	53	19	<0.00001[b]
B1-2	5	8	NS	3	6	NS	8	14	NS
B2-1	1	0	NS	4	0	NS	5	0	<0.05[d]
B2-2	1	0	NS	2	0	NS	3	0	NS
C-1	1	4	NS	1	0	NS	2	4	NS
C-2	1	3	NS	1	0	NS	2	3	NS
C-3	0	1	NS	2	1	NS	2	2	NS
C-etc.	1	0	NS	0	2	NS	1	2	NS
Total	36	35		53	43		89	78	

[a] HCV subgroups were determined on the basis of the secondary structure of the amino-terminal 120 residues of NS3.
[b] χ^2 test.
[c] NS, not significant.
[d] Fisher's exact test.

there was no significant difference in the average number of mutations between them (2.9 ± 1.8 and 3.2 ± 1.5, respectively).

DISCUSSION

Little is known about the relationship between the NS3 sequence diversity of HCV and the development of HCC. Giménez-Barcons et al. (10) reported that there was no difference in the mutation rate of a limited portion of NS3 between NS3 from samples from patients with HCC and NS3 from samples from patients without HCC. Their result is consistent with the observations presented in a previous report (25) and in this study. On the other hand, it was reported that HCV isolates from cancerous tissues, but not those from noncancerous liver tissue or sera, of patients with HCC had unique mutations in the vicinity of the catalytic sites of the HCV serine protease (35). It was observed in the previous study (25) that the sequence of the amino-terminal 180 residues was identical between the HCV isolates from cancerous tissues and those from surrounding noncancerous tissues of patients with HCC for 9 of 12 pairs of isolates compared. As for the remaining three pairs of isolates, one or two mutations were observed between the isolates from cancerous tissues and those from noncancerous tissues at positions 1033, 1062, 1115, and 1140 (data not shown), which are in the vicinity of the catalytic sites of the serine protease (His-1083, Asp-1107, and Ser-1165). However, those mutations were found in only 3 of 12 pairs of isolates tested and were even found in the sera of patients without HCC. Therefore, it was concluded that those mutations are not unique to HCC. In this connection, the present data have revealed that isolates of B2-1, which are highly associated with HCC but small in number (Table 2), have more mutations upstream of His-1083, although the positions and the residues of the mutations varied from one isolate to another (Fig. 2). Overall, at the primary structure level, we did not find any HCC-specific consensus sequence that is unique to yet common among the majority of HCV-1b isolates obtained from patients with HCC, with the result being in agreement with previous observations (25). Thus, it is rather difficult to identify HCV-1b isolates that are highly associated with HCC merely on the basis of their primary structure.

In the present study we observed that, on the basis of the secondary structure of the amino-terminal 120-residue sequence of NS3, HCV-1b isolates could be classified into group A, group B, and an indeterminate group, each of which was further divided into a number of subgroups, such as A1-1, A1-2, A2-1, A2-2, B1-1, B1-2, B2-1, B2-2, C-1, C-2, and C-3, according to the positions of the turn structures (Fig. 1). Interestingly, subgroups B1-1 and B2-1 were found significantly more frequently in patients with HCC than in those without HCC (Table 2). On the other hand, subgroup A1-1 was only weakly associated with HCC. The other subgroups, such as A1-2, A2-1, B1-2, C-1, C-2, and C-3, were moderately associated with HCC. This analysis of the secondary structure of NS3 may be useful for prediction of the relative risk of developing HCC among individuals infected with different HCV-1b isolates. In this connection, it should be noted that when the secondary structure was predicted by using the Chou-Fasman method (GENETYX-MAC, version 10.1) instead of the Robson method, such a significant correlation was not observed (data not shown).

Crystal structure analysis of the ~190 amino-terminal residues of NS3 has demonstrated that the amino-terminal portion of ca. 20 residues (aa 1027 to 1046) of strain BK was shown to extend away from the molecule, while the corresponding portion of strain H was shown to stay closer to the molecule (16, 20). On the basis of our secondary structure analysis of the amino-terminal portion of NS3, strain BK is classified into subgroup A1-1, while strain H is classified into subgroup A2-1 (data not shown). Meanwhile, it was previously demonstrated that NS3 is physically associated with p53 through a region between aa 1055 and 1200 (14) and that their physical interaction is influenced by a difference(s) in the NS3 sequence

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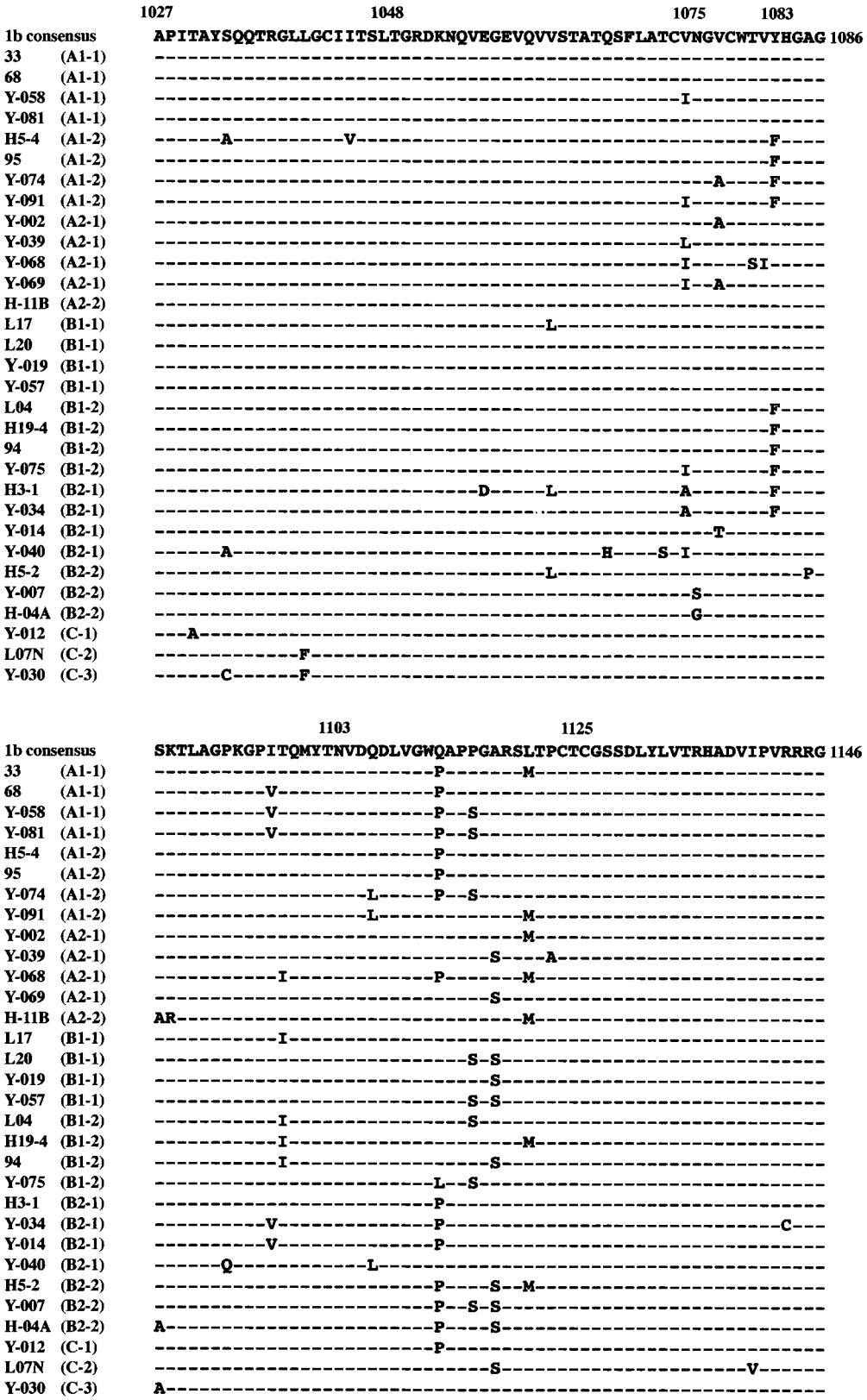

FIG. 2. Sequence alignment of the amino-terminal 120 residues of NS3 of HCV-1b isolates obtained from patients with and without HCC. Representative sequences of each of the subgroups are aligned with a consensus sequence, shown on the top. Dashes indicate residues identical to the residue in the consensus sequence. The numbers along the consensus sequence indicate amino acid positions.

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TABLE 3. Average number of mutations in the amino-terminal 120 residues of NS3 of various HCV subgroups[a]

HCV subgroup[b]	No. of mutations (mean ± SD)
A1-1 (n = 31)	3.1 ± 1.3[c,d,e,f]
A1-2 (n = 11)	4.1 ± 1.4[c,g]
A2-1 (n = 4)	3.5 ± 1.7
B1-1 (n = 72)	2.3 ± 1.4[d,g,h,i,j,k]
B1-2 (n = 22)	4.1 ± 1.6[e,h]
B2-1 (n = 5)	5.2 ± 1.9[f,i]
B2-2 (n = 3)	4.3 ± 1.2[j]
C[l] (n = 18)	3.4 ± 1.5[k]

[a] P values were determined by Student's t test.
[b] Data for subgroup A2-2 (one isolate with three mutations) are not shown.
[c] $P < 0.05$ (A1-1 versus A1-2).
[d] $P < 0.01$ (A1-1 versus B1-1).
[e] $P < 0.05$ (A1-1 versus B1-2).
[f] $P < 0.01$ (A1-1 versus B2-1).
[g] $P < 0.001$ (A1-2 versus B1-1).
[h] $P < 0.001$ (B1-1 versus B1-2).
[i] $P < 0.001$ (B1-1 versus B2-1).
[j] $P < 0.05$ (B1-1 versus B2-2).
[k] $P < 0.01$ (B1-1 versus C).
[l] An indeterminate group that includes C-1 to C-3 and C-etc. (Fig. 1 and 2 and Table 2).

between the two HCV-1b isolates tested, strains BK and M094AJ (23). Strain M094AJ was classified into subgroup B1-2 on the basis of the secondary structure of the amino-terminal portion of its NS3 protein (data not shown). It is possible that the difference in the secondary structure of NS3 might reflect a conformational difference, which would affect the interaction with p53. Another interesting issue to be studied is whether the other biological functions of NS3, such as the serine protease activity and antiapoptotic capacity, vary with different subgroups. The results of the present study have revealed that the catalytic triad of the serine protease (His-1083, Asp-1107, and Ser-1165) and the ligands to the zinc ion (Cys-1123, Cys-1125, Cys-1171, and His-1175) were completely conserved in all 167 isolates tested (data not shown). However, the conformation of the catalytic sites and the surrounding portion could be altered by certain mutations. Even at the primary structure level, some mutations clustered near the catalytic sites of the serine protease, as was most evidently seen with subgroup B2-1 (Fig. 2). Those mutations as well as the possible conformational alteration may affect the serine protease activity. Studies are now in progress in our laboratory to elucidate the issues outlined above.

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