JOURNAL OF CLINICAL MICROBIOLOGY, July 1996, p. 1815–1818 Vol. 34, No. 7
0095-1137/96/$04.00+0

Precore Mutant of Hepatitis B Virus Prevails in Acute and Chronic Infections in an Area in Which Hepatitis B Is Endemic

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Received 29 December 1995/Returned for modification 26 February 1996/Accepted 2 April 1996

By using an amplification-created restriction site method, the precore TAG mutant of hepatitis B virus was detected in 6 (75%) of 8 acute fulminant hepatitis B patients, 7 (58%) of 12 acute self-limiting hepatitis B patients, 35 (81%) of 43 hepatitis B virus surface antigen carriers with fulminant hepatitis, and 42 (70%) of 60 hepatitis B virus surface antigen carriers with chronic hepatitis. The precore TAG mutant prevails in acute and chronic hepatitis B of various severity in this area where hepatitis B is endemic.

The development of fulminant hepatitis B virus (HBV) infection has long been suggested to be closely related to the host-virus interaction (23, 24). Only recently has the role of virological factors in the pathogenesis of fulminant hepatitis B been explored. In 1989, a precore defective HBV mutant with a G-to-A substitution at nucleotide 1896 resulting in a TAG stop codon, and failure to produce hepatitis B e antigen (HBeAg) was found in hepatitis B surface antigen (HBsAg) carriers with antibody against HBeAg (anti-HBe) and severe chronic liver disease (6). Since then, the association of the precore mutant with fulminant hepatitis B has been investigated in several studies, but the results remain controversial (1, 12, 16–18, 21).

The role of the precore mutant in fulminant hepatitis B in Taiwan has been studied only in one series of pediatric patients (14). The precore sequence in adult fulminant hepatitis B patients in this area where hepatitis B is endemic has never been investigated before, possibly because these cases are extremely rare in this area (8). On the other hand, the majority of HBsAg-positive adults with acute hepatitis in Taiwan are negative for immunoglobulin M (IgM) class antibody against hepatitis B core antigen (IgM anti-HBc) (9, 10). Such patients actually were previously unrecognized HBsAg carriers with reactivation of HBV or unrelated forms of acute hepatitis. The precore sequence in these patients with fulminant hepatitis has been examined in only a few studies (12, 21).

The precore TAG mutant has been shown to account for more than 95% of the precore mutants described so far (2). The study described here was conducted to determine the prevalence of the precore TAG mutant in adult fulminant hepatitis B patients and HBsAg carriers with superimposed fulminant hepatic failure by an amplification-created restriction site (ACRS) method (11). The results were compared with those for acute self-limiting and chronic hepatitis B patients.

Patients. The study population consisted of 8 patients with acute fulminant hepatitis B and 46 HBsAg carriers with superimposed fulminant hepatic failure. Of the latter group of patients, fulminant hepatitis could be attributed to hepatitis C virus (HCV) and/or hepatitis D virus (HDV) superinfection in 17 patients, reactivation of HBV in 16 patients, and no identifiable cause in 13 patients. The serodiagnosis of acute viral hepatitis in these patients was based on testing of the following virological markers: hepatitis A virus (HAV)–IgM class antibody against HAV; HBV–HBsAg, IgM anti-HBc, HBV DNA, HBeAg, and anti-HBe; HCV–antibody against HCV and HCV RNA; HDV–IgM and IgG class antibodies against HDV and HDV antigen; hepatitis E virus (HEV)–IgG class antibody against HEV and HEV RNA; cytomegalovirus–IgM class antibody against cytomegalovirus; and Epstein-Barr virus–IgM class antibody against Epstein-Barr virus capsid antigen, as previously reported in detail (10). The controls consisted of 12 acute self-limiting and 62 chronic hepatitis B patients. Of the latter, 29 patients had chronic persistent hepatitis and 33 had chronic active hepatitis, according to the standard criteria (15).

ACRS 1896 G→A mutation test. Serum (100 μl) was processed as described previously (25). The following oligonucleotide primers were used in the ACRS 1896 G→A mutation test: P1 (5′-GCCTCCAAGCTGTGCCTTCCATGGCTTT-3′; nucleotides [nt] 1868 to 1895; sense), with the underlined mismatch introducing a *Bst*XI cleavage site ($CCAN_6TGG$) in the absence of a TAG stop codon mutation, and P2 (5′-GTATGG TGAGGTGAACAATG-3′; nt 2058 to 2039; antisense). Samples that were negative after 40 cycles of amplification and examination by ethidium bromide staining of gels were subjected to nested PCR. The outer primers used were P3 (5′-TAGGAGGCTGTAGGCACAAATTGG-3′; nt 1776 to 1799; sense) and P4 (5′-AAGGAAGGAGTTTGCCATTCAGG-3′; nt 2542 to 2520; antisense). Two serum samples containing only mutant or wild-type HBV precore sequences were used as positive controls. Contamination controls consisted of water and a negative serum sample from a subject with no HBV markers.

Ten microliters of the PCR product was mixed with 1.6 μl of 10× buffer H (Boehringer Mannheim), 3.4 μl of H_2O, and 1 μl (10 U) of *Bst*XI (Boehringer Mannheim), and the mixture was incubated at 45°C for 2 h. The mixture was electrophoresed onto 8% polyacrylamide gels. Ten microliters of the PCR product with 1.6 μl of 10× buffer H and 4.4 μl of H_2O but without *Bst*XI was electrophoresed in the parallel lane. The presence of the TAG mutant was detected by comparing the positions of the DNA bands in these two parallel lanes. The *Bst*XI digestion revealed three electrophoresis patterns: (i) one, representing the precore TAG mutant, was a single band that was the same size (191 bp) as the band obtained when *Bst*XI was not added; (ii) the second pattern was a single smaller band (167 bp) resulting from cleavage of the wild-type HBV DNA; and (iii) the third pattern was a double band (191 and 167 bp) interpreted as mixed wild-type HBV and the precore TAG mutant.

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TABLE 1. Clinical details for the study patients and controls

Group	No. of patients	Age (median yr [range])	Sex[a]	No. of patients with HBeAg/no. of patients with anti-HBe	No. of patients who survived
Fulminant hepatitis patients					
Acute hepatitis B	8	30 (20–66)	6:2	0/7[b]	2
Chronic hepatitis B with HCV and/or HDV superinfection	15	34 (19–59)	13:2	4/11	3
Chronic hepatitis B with reactivation of HBV	16	46 (18–66)	10:6	5/8[b]	2
Chronic hepatitis B without identified causes	12	38 (23–61)	10:2	4/6[b]	2
Controls					
Acute self-limiting hepatitis B	12	24 (20–35)	7:5	6/4[b]	
Chronic persistent hepatitis B	27	39 (18–64)	21:6	13/13[b]	
Chronic active hepatitis B	33	36 (20–62)	25:8	20/12[b]	

[a] Number of males:number of females.
[b] The remaining patients were negative for both HBeAg and anti-HBe.

Sequencing. The sequence of 191 bp (nt 1868 to 2058), including part of precore region (nt 1814 to 1900), was determined in selective cases by the dideoxy chain-termination method (22) with Sequi Therm Cycle Sequencing kits (Epicentre Technologies, Madison, Wis.). The detailed procedure of direct sequencing was outlined in the manufacturer's instructions accompanying the kit.

Results and discussion. HBV DNA could be amplified from the sera of 123 of 128 study patients; for sera from 6 patients nested PCR was needed. The essential clinical details for these 123 patients are listed in Table 1. Results of the ACRS test revealed wild-type HBV in 33 patients, mixed wild-type HBV and the precore TAG mutant in 55 patients, and the precore TAG mutant in 35 patients. Direct sequencing of codon 28 in five serum samples with wild-type HBV, five serum samples with mixed wild-type HBV and precore TAG mutant, and five serum samples with the precore TAG mutant agreed completely with the results of the ACRS test.

As shown in Table 2, the precore TAG mutant alone or as a mixture with the wild-type HBV was found in 58% of the patients with self-limiting hepatitis and in 75% of the patients with fulminant hepatitis, without a significant difference. Previous studies reported from Japan and Israel have shown that the precore TAG mutant was found in 80 to 100% of the patients with fulminant hepatitis B, but in none of the patients with acute self-limiting hepatitis B (1, 16, 18, 21). The precore TAG mutant was also frequently detected in Greek patients (80%; four of five patients) and British patients (50%; four of eight patients) with fulminant hepatitis B (4). Finally, the direct role of the precore TAG mutant in the pathogenesis of fulminant hepatitis B was supported by the finding of the same mutation in the contaminant and in the recipient (16, 18, 21, 26). However, the prevalence of the precore TAG mutant in fulminant hepatitis B patients from the United States and France was less than 10% (12, 17). A recent study from Taiwan revealed that only 36% (5 of 14) of childhood fulminant hepatitis B patients were found to have the precore TAG mutant. Furthermore, this mutation was also detected in 30% (3 of 10) of children with acute self-limiting hepatitis B (14). The present results have revealed a high prevalence of the precore TAG mutant in adult fulminant or self-limiting hepatitis B patients in Taiwan. This finding suggests that the precore TAG mutant is not exclusively associated with fulminant hepatitis. Whether mutations elsewhere in the HBV genome may contribute to a more fulminant course of hepatitis B needs further study.

Although the emergence of the precore TAG mutant has been demonstrated in patients with chronic HBV infection (3, 13, 20), whether this process occurs in patients with acute HBV infection remains controversial (5, 16, 21). Notably, the emergence of the precore TAG mutant in patients with acute HBV infection occurred around the time of anti-HBe seroconversion (5), when the hepatitis activity is diminishing. Serum samples from acute hepatitis B patients in the present series were collected on the day of admission when the hepatitis activities were usually at their peak. It seems likely that the acute hepatitis B patients harboring the precore TAG mutant in the present series were infected with the precore TAG mutant, ab initio, although the possibility of the early emergence of the precore TAG mutant because of an unusually potent host selective pressure cannot be excluded.

The high prevalence of the precore TAG mutant in adult

TABLE 2. Prevalence of the wild-type and precore TAG mutant of HBV in patients with acute self-limiting versus fulminant hepatitis B

Group	No. of patients	No. (%) of patients		
		Wild-type HBV	Mixed wild-type HBV and precore TAG mutant	Precore TAG mutant
Acute self-limiting hepatitis B				
HBeAg positive	6	4 (67)	2 (33)	0 (0)
HBeAg negative	6	1 (17)[a]	3 (50)[a]	2 (33)[a]
Subtotal	12	5 (42)[a]	5 (42)[a]	2 (17)[b]
Acute fulminant hepatitis B, HBeAg negative	8	2 (25)	0 (0)	6 (75)

[a] $P > 0.1$ (by chi-square test with Yates' correction).
[b] $P < 0.05$ versus acute fulminant hepatitis B patients.

TABLE 3. Prevalence of wild-type and precore TAG mutant of HBV in HBsAg carriers with chronic hepatitis versus HBsAg carriers with fulminant hepatitis

Group	No. of patients	No. (%) of patients		
		Wild-type HBV	Mixed wild-type HBV and precore TAG mutant	Precore TAG mutant
HBsAg carriers with chronic hepatitis				
HBeAg positive CPH[a]	13	10 (77)	3 (23)	0 (0)
HBeAg positive CAH[b]	20	6 (30)[c]	12 (60)	2 (10)
HBeAg negative CAH	13	1 (8)[d]	7 (54)	5 (38)[e]
HBeAg negative CPH	14	1 (7)[f]	6 (43)	7 (50)[g]
Subtotal	60	18 (30)	28 (47)	14 (23)
HBsAg carriers with fulminant hepatitis				
Reactivation of HBV				
HBeAg positive	5	0 (0)	5 (100)	0 (0)
HBeAg negative	11	0 (0)	7 (67)	4 (33)
HCV and/or HDV superinfection				
HBeAg positive	4	3 (75)[h]	1 (25)[h]	0 (0)
HBeAg negative	11	1 (9)	5 (46)	5 (46)
Undetermined cause				
HBeAg positive	4	3 (75)[h]	1 (25)[h]	0 (0)
HBeAg negative	8	1 (13)	3 (38)	4 (50)
Subtotal	43	8 (19)	22 (51)	13 (30)

[a] CPH, chronic persistent hepatitis.
[b] CAH, chronic active hepatitis.
[c] $P < 0.05$ (by chi-square test with Yates' correction).
[d] $P < 0.005$.
[e] $P < 0.05$.
[f] $P < 0.001$ versus HBeAg-positive chronic persistent hepatitis patients.
[g] $P < 0.05$ versus HBeAg-positive chronic persistent hepatitis and chronic active hepatitis patients, respectively.
[h] $0.05 < P < 0.1$ versus HBeAg-positive patients with reactivation of HBV.

acute hepatitis B patients in the present series might be closely related to the common occurrence of the precore mutant in chronically infected HBsAg carriers in this area (Table 3). The prevalence of the precore TAG mutant in chronic hepatitis B patients varied with the status of HBeAg in serum and the inflammatory activity in liver. It has been suggested that the natural history of chronic HBV infection could be divided into three sequential stages: (i) the immune tolerance phase, characterized by HBeAg reactivity in serum and only minor histological activity; (ii) the immune clearance phase, during which serum is positive for HBeAg or anti-HBe and histologic signs of chronic active hepatitis are prominent; and (iii) the residual integration phase, when the patient is anti-HBe positive and there is no evidence of inflammatory liver disease (7). The data presented here thus suggest that wild-type HBV is predominant during the early immune tolerance phase and that the precore TAG mutant emerges in the ensuing immune clearance phase and then prevails over wild-type HBV after seroconversion from HBeAg to anti-HBe.

The precore sequence in HBsAg carriers with superimposed fulminant hepatic failure appears to be closely related to the status of HBeAg in serum as well as the etiological factors of acute hepatitis (Table 3). All patients with reactivation of HBV in this series had the precore TAG mutant, which usually occurred as a mixture with the wild-type HBV. This finding seems to support the previous suggestion of other investigators that reactivation of HBV in patients with chronic infection is associated with the emergence of the precore TAG mutant in wild-type HBV carriers (3). Patients with HCV and/or HDV superinfection tended to be infected with virus with the precore sequence, similar to the respective HBeAg-positive or -negative carriers with minor hepatitis activity. About 30% of HBsAg carriers with fulminant hepatitis in the present series did not have identified causes of infection; i.e., they were negative for HBV DNA by spot hybridization techniques and did not have serological evidence of infection with other known viruses. However, the possibility of reactivation of HBV with an early clearance of viremia in these subjects still cannot be completely excluded (19). An interesting finding is that these subjects usually were infected with virus with the precore sequence, similar to those subjects with HCV and/or HDV superinfection rather than those subjects with reactivation of HBV. These findings probably suggest that fulminant hepatic failure in these subjects is more likely due to other unidentified viral or nonviral liver disease rather than reactivation of HBV.

In conclusion, the precore TAG mutant prevails in adult patients with acute and chronic HBV infection in Taiwan, and its occurrence cannot be correlated with fulminant viral liver disease.

Grant support was received from the National Institute of Health, Republic of China (DOH85-HR-522) and a Chang Gung Medical Research Grant (CMRP 431).

We thank M. H. Tsai for secretarial assistance.

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