LIVER DISEASE

Different hepatitis B virus core gene mutations in children with chronic infection and hepatocellular carcinoma

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Background: The significance of mutations of hepatitis B virus (HBV) precore/core antigen in causing persistent infection and subsequent liver diseases is debatable.
Aim: To investigate HBV core gene sequence changes in children with chronic HBV infection and their implications in hepatocellular carcinoma (HCC).
Methods: Thirty one chronic HBV infected children with documented hepatitis B e antigen seroconversion selected from 415 long term carrier children and 12 HBV related HCC children were studied. Four serial serum samples before and after hepatitis B e antigen seroconversion from each of the 31 children, and one serum sample taken from the 12 HCC children were subjected to HBV core gene sequence analysis.
Results: Mutations accumulated as chronic infection persisted and most frequently occurred at core gene codon 21 (29%), codon 147 (29%), codon 65 (16%), and precore stop codon 28 (74%) in the 31 chronic HBV infected children. Core gene mutation sites in HCC children were identified at core codons 74, 87, and 159. HCC children had more mutations in the core gene than those with chronic HBV infection (p=0.013).
Conclusion: Accumulation of mutations of HBV core region in HCC children differ from those in chronic HBV infected children. This may be a clue to the pathogenesis of paediatric HCC.

Several hot spots of the hepatitis B virus (HBV) nucleotide sequences are susceptible to mutation, perhaps due to various extrinsic pressures.[1] For example, precore G to A stop codon mutant at nucleotide position 1896 is detected in fulminant hepatitis, acute hepatitis, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC).[2–8] We have addressed the temporal changes in this mutation in chronic HBV infection in children.[9] Because hepatitis B core antigen (HBcAg) is the target for the cytotoxic T lymphocyte (CTL) mediated lysis of HBV infected hepatocytes,[10–12] it is also susceptible to mutation. Mutations of the core gene may change the conformation of the core protein and allow HBV to escape immune surveillance via loss or change of immunodominant epitopes. Alternatively, they could be virulent strains that induce persistent host immune attacks and lead to severe liver disease.

Previous studies of HBV core gene mutations showed that the core gene was highly conserved during the immune tolerance phase of chronic HBV infection.[13–15] As patients passed through the immune tolerance phase, an increasing number of mutations were noted in the core gene, where was rich in B and T cell epitopes.

All of these studies on HBV core gene variations were conducted in adult patients. The majority of chronic HBV infections in hyperendemic areas such as Taiwan begin in the perinatal period or early childhood.[16] In this study, a longitudinal follow up was conducted in 415 children to understand the temporal profile of core gene changes in HBV infection before and after hepatitis B e antigen (HBeAg) seroconversion. In the meantime, we compared the nucleotide sequences of HBV core gene of 12 children who had HBV related HCC with chronic HBV infected children. HCC is the gravest consequence of chronic HBV infection for both adults and children.[17] A previous study in Taiwanese adult HCC showed that there were 3–6 different missense mutations accumulated in the core gene which may relate to severe liver damage.[18] We thought it would be interesting to identify core gene mutations in childhood HCC. Moreover, if we could find any differences between childhood HCC and chronic HBV infection, these mutations might provide clues to the development of childhood HCC.

SUBJECTS AND METHODS

Children with chronic HBV infection

A total of 415 chronic HBV carrier children were followed up at our outpatient clinic. Among these 415 children, 169 had already undergone HBeAg seroconversion. We randomly selected 31 children (male:female ratio 17:14; mean (SD) age at enrolment 7.9 (3.5) years, range 2–15) from the aforementioned 169 subjects who fulfilled the criteria of being followed up for ⩾2 years before HBeAg seroconversion and ⩾2 years after HBeAg seroconversion. Thus we could observe the core gene variations for at least four years. Follow up duration was mean 7.1 (SD 2.7) years (range 4–13.1) for these 31 children. Their mean (SD) age at enrolment was 7.5 (3.9) years (range birth to 16 years). Mean age at HBeAg seroconversion was 10.9 (4.3) years. Four serum samples from each of the 31 children were studied for sequential nucleotide changes in the HBV core gene. The four serum samples were: (1) the earliest one available at their enrolment into the follow up study, 3.0 (2.0) years before HBeAg seroconversion; (2) immediately before (<6 months) HBeAg seroconversion; (3) immediately after (<6 months) seroconversion; and (4) the latest serum sample available which was 4.0 (2.0) years after HBeAg seroconversion. All 31 children were asymptomatic clinically.

Children with HCC

Twelve children (male:female ratio 9:3; mean age 9.6 (2.8) years, range 4.5–13.2) with histologically proved HCC were studied. They were age matched to the chronic HBV infection

Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBcAg, hepatitis B core antigen; CTL, cytotoxic T lymphocytes; PCR, polymerase chain reaction; ALT, alanine aminotransferase.

Table 1 Hot spots of mutation in the precore/core gene of the 31 long term hepatitis B virus infected children with hepatitis B e antigen (HBeAg) seroconversion

	Before seroconversion		After seroconversion	
	1st (n=31)	2nd (n=31)	3rd (n=28)	4th (n=23)†
Age (y) (mean (SD))	7.5 (3.9)	9.6 (4.8)	10.9 (4.3)	14.3 (4.9)
Precore/28 (%)	W→X, 9.7*	W→X, 45.2	W→X, 57.1	W→X, 52.2*
Core/21 (%)	S→P/A,12.9	S→P/A,12.9	S→P/A,14.3	S→P/A,21.7
Core/65 (%)	L→W, 6.5	L→W, 3.2	L→W/V,10.7	L→W/V,17.3
Core/147 (%)	T→S/C, 9.7	T→S/C, 6.4	T→S/C, 10.7	T→S/C, 21.6

X, stop codon; W, tryptophan; S, serine; P, proline; A, alanine; L, leucine; V, valine; T, threonine; C, cysteine; P/A, mutant codon is either proline or alanine.
The four serum samples were obtained at: 1st, the earliest available at enrolment; 2nd, immediately before HBeAg seroconversion; 3rd, immediately after seroconversion; and 4th, latest after HBeAg seroconversion.
†HBV DNA was detected by PCR in 28/31 of the third serum samples, and in 23/31 of the fourth serum samples.
*p=0.006.

group. All were seropositive for antibodies to HBeAg and hepatitis B surface antigen (HBsAg). All had a HBsAg (+) mother. Serum samples were collected when they were diagnosed. Three survived long term after surgical resection of the tumour. The other nine cases died. Cirrhosis of the liver was excluded by surgical findings and histological examinations in three. The other nine cases had tumours with cirrhosis, as evidenced by either imaging studies or histological findings. Duration after HBeAg seroconversion was not known for all of these cases as they presented with a liver tumour and were not recruited into the long term follow up study.

Analysis of nucleotide sequences of the HBV core gene

HBV DNA was extracted from 20 µl of serum, as described previously.[2] Polymerase chain reaction (PCR) was performed to amplify the precore and core genes of HBV from extracted serum DNA. The PCR reaction mixture consisted of outer primers (sense: 5′-ATAAG, AGGAC, TCTTG, GACTC-3′ nucleotide position 1654–1673, and antisense: 5′-AAAGA, CAGGT, ACAGT, AGAAG-3′ nucleotide position 2503–2522, position number according to EcoRI restriction site), dNTP 2.5 mM, Taq polymerase 2.5 U, and PCR buffer to a total volume of 20 µl. One tenth of the first PCR product was subjected to nested PCR using inner primers (sense: 5′-TCCAA, GCTGT, GCCTT, GGGTG-3′ nucleotide position 1871–1890, and antisense: 5′-GAAGA, ATAAA, GCCCA, GTAAA-3′ nucleotide position 2487–2506). The expected nested PCR product size is 639 bp and PCR conditions and procedures have been described previously.[19] The sensitivity of our nested PCR was 10^{-5} pg of HBV DNA. Nested PCR products were then subjected to direct sequencing by the automatic cyclic sequencing analyser (ABI prism, dye labelled terminators and cycle sequencing ready reaction kit; Perkin Elmer, Foster city, California, USA). To prevent the artefacts of point mutations created by PCR, the sequencing reactions were done in both directions. All tests were duplicated to confirm the results.

Comparisons of the nucleotide sequences

To calculate the mutation rate of HBV core gene in children with chronic infection, we took the nucleotide differences between the earliest serum sample and the latest serum sample available for each case, divided by the number of follow up years and the sequenced precore/core gene length. For example, if the nucleotide difference was 6 bp, followed for six years, and the sequenced PCR product size was 639 bp, the mutation rate would be 6/6/639 bp = 7.8×10^{-4} nucleotide/site/year. To compare codon mutations between children with chronic HBV infection and HCC, Fisher's exact test with Yates' correction was used. A p value <0.05 was considered significant.

RESULTS

Core gene variations and precore stop codon

Throughout the follow up period, the mean (SD) mutation rate of the core gene in the 31 children was (1.5 (1.0)) $\times10^{-3}$/site/year. Precore stop codon 28 was the commonest hot spot mutation. Nine children had wild-type precore codon 28 throughout the HBeAg seroconversion process. Another nine children had the wild-type before HBeAg seroconversion and the mutant emerged after seroconversion. In the remaining 13 children this stop codon emerged even before HBeAg seroconversion. Core gene codons 21, 65, and 147 were the hot spots for mutations (table 1). Comparing the frequency of mutations, we found that only for the precore codon 28 was there a statistically significant difference between the first and fourth serum samples (p=0.0006) and not for the other three codons (Fisher's exact test with Yates' correction). The following were sporadic mutations detected on one occasion: codons 5, 13, 14, 19, 20, 35, 54, 55, 79, 80, 97, 101, 103, 135, 143, 163, 180, and 182.

Among the 22 children who had a precore stop codon 28 mutation, 12 had coexisting core gene mutations: six had codon 21 mutations, two had codon 65 mutations, and six had codon 147 mutations (two cases had double mutations of codons 21 and 65). The existence of a precore stop codon 28 was not associated with the emergence of the aforementioned three common core gene mutations. For nine children without a precore stop codon mutation, six had core gene mutations: three had codon 21 mutations, four had codon 65 mutations, and one had a codon 147 mutation (double mutations of

Table 2 Peak alanine aminotransferase (ALT) levels preceding hepatitis B e antigen (HBeAg) seroconversion in chronic hepatitis B virus infected children with precore codon 28 and core gene codon 21, 65, and 147 mutations

Mutation	Peak ALT (IU/l)	p Value*
Precore 28		
Yes (n=22)	176.8 (259.0)	0.24
No (n=9)	246.0 (196.7)	
Core 21		
Yes (n=9)	164.7 (136.5)	0.035
No (n=22)	347.7 (296.9)	
Core 65		
Yes (n=6)	204.8 (325.6)	0.43
No (n=25)	230.9 (187.8)	
Core 147		
Yes (n=7)	247.8 (234.7)	0.06
No (n=24)	150.7 (102.2)	

*Kruskal-Wallis test.

Table 3 Percentage of children with hepatocellular carcinoma (HCC) and chronic hepatitis B virus (HBV)* who had HBV core gene mutations

Codon	HCC (n=12) (% (n))	Chronic HBV infection (n=23) (% (n))	Mutation	p Value†
Precore 28	58% (7)	52.2% (12)	W→X	0.73
Core 21	8% (1)	21.7% (5)	S→PorA	0.32
Core 65	33% (4)	17.3% (4)	L→WorV	0.29
Core 74	33% (4)	0	S→G	0.0032
Core 87	33% (4)	0	S→G	0.0032
Core 131	8% (1)	0	A→D	0.16
Core 143	33% (4)	4.3% (1)	L→P	0.015
Core 147	8% (1)	21.6% (5)	T→Cor S	0.32
Core 159	42% (5)	0	R→S	0.0006
Core 182	42% (5)	4.3% (1)	Q→X	0.0035

W, tryptophan; X, stop codon; S, serine; P, proline; L, leucine; V, valine; G, glycine; A, alanine; D, aspartic acid; T, threonine; C, cysteine; R, arginine; Q, glutamine.
*From the 23 latest serum sample (after HBeAg seroconversion).

codons 21+65 or 65+147 in one case each). There was no difference between the emergence of mutations at codon 21 and codon 147, irrespective of the existence of a precore stop codon (p=0.74 and 0.33, Fisher's exact test).

Core gene variations and ALT changes

We took mutations in the precore codon 28 and codons 21, 65, and 147 as an index, and divided children into two groups based on the presence or absence of these mutations. Peak alanine aminotransferase (ALT) levels before HBeAg seroconversion were compared between these two groups. Those without a core gene codon 21 mutant had higher peak ALT levels than those with a mutation (p=0.035) (table 2). After HBeAg seroconversion, 28 children had normal ALT concentrations while three had elevated ALT levels. Levels of ALT were 59–68 IU/l (normal <35 IU/l) and occurred one, five, and seven years after HBeAg seroconversion. No specific core gene mutants were clustered in these three children.

Core gene variations in HCC

Peak ALT concentration for the 12 children with HCC was 114 (125) IU/l (mean (SD)) (range 21–392). Mutations were found mostly at precore codon 28 and core gene codons 65, 74, 87, 143, 159, and 182 (table 3). Core gene codon 21 and 147 mutations were seen in only one HCC case. These findings were significantly different from those found in children with chronic HBV infection except for precore codon 28 and core gene codon 65 mutations which were common in both groups.

We took the core gene sequence of genotype B HBV, which is the most common in Taiwan,[20] as a standard with which to compare core genes of chronic HBV infected and HCC children. Mean synonymous mutated points in these 12 HCC children were (4.9 (3.6)) × 10^{-3}/nucleotide position while mean synonymous mutated points in the last serum sample of the 31 chronic HBV infected children were (2.0 (0.8)) × 10^{-3}/nucleotide position (p=0.013).

DISCUSSION

This long term follow up paediatric study provides additional data on the significance of precore/core gene mutations, particularly in relation to HCC children. Few related data have been reported in the literature.

Precore codon 28 was the commonest mutation overall. It has been regarded as a virulent viral factor causing liver damage.[21] However, this mutation alone may not account for the increased virulence, and additional mutations in the core region were suggested to be necessary.[2 9 22] Our study showed that peak ALT levels, which may reflect the degree of liver damage, were not different between the groups with and without the precore codon 28 mutation in chronic HBV infected children (table 2).

Core gene codons 21, 65, and 147 were the three common mutation sites in children with chronic HBV infection. All were located in the HBcAg epitopes of CTL,[23] namely codons 18–27,[24] 50–69,[25] 74–83,[26] and 141–151.[27] It is thus conceivable these three sites were the most preferred targets of CTL.

Contrary to previous reports,[14 15] the number of core gene mutations did not increase significantly after HBeAg seroconversion in our patients. The reason may be related to the age of the different study populations. All of the subjects in the previous reports were adults and perhaps had already undergone HBeAg seroconversion for a much longer time than our patients. The latest serum samples were withdrawn about four years after HBeAg seroconversion in this study. Such a duration may not be long enough to accumulate mutations to the same extent as in adults. On the other hand, this finding is consistent with one study in chronic HBV infected children which showed that core gene mutations did not increase significantly after HBeAg seroconversion.[28]

HCC is regarded as the gravest finale of chronic HBV infection. This study revealed that children with HCC had more point mutations in the core gene. There are two possible explanations for this phenomenon. (1) With the passage of time, there are more chances for the host immune system to attempt to clear the virus and hence more mutated virus species appear to escape the host immune system. In these HCC children, more core gene mutations reflect the fact that they have undergone more episodes of host-viral immune interactions. (2) Some core gene mutations are virulent and may relate to the development of HCC. Codon 74, 87, and 159 mutations were found in HCC children but not in their chronically infected counterparts in this study. In this study, children in the chronic carrier group and the HCC group were relatively age matched (mean (SD) 7.9 (3.5) years *v* 9.6 (2.8) years) and the former group was followed up for 7.1 (2.7) years. One of the four serum samples during the follow up period was obtained at a comparable age to the HCC children. Both groups had an equal chance of experiencing host-viral immune interactions but had different outcomes. A previous report described clusters of mutations in the sequences involved in viral encapsidation, replication, and immune epitopes (codons 55–63, 96–108, and 151–155) in adult HCC tissues.[29] Hosono *et al* used HCC samples from Taiwanese adults and found that core antigen mutations of adult HCC located in domains coincided with HLA class II restricted T cell epitopes.[18] These mutations could modify the antigenicity of the core protein as well as viral replication and could participate in the establishment of chronic viral infection. The core gene codon 74 and 87 mutations in childhood HCC are in a major antigenic region of the core gene[30] while the codon 159 mutation is in the region initiating viral encapsidation. We speculate that the surviving mutated virus may take

advantage of these mutations to escape the host immune system, facilitating viral integration, expanding viral proteins, and finally causing cancer development.[31]

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