Analysis of full-length hepatitis B virus genome from chronic hepatitis B-patients with higher alanine aminotransferase elevation

日本大学大学院医学研究科博士課程

内科系消化器内科学専攻

氏名　高橋　央

修了年　2021 年

指導教員　森山　光彦

Research Article

For reprint orders, please contact: reprints@futuremedicine.com

Analysis of full-length hepatitis B virus genome from chronic hepatitis B-patients with higher alanine aminotransferase elevation

Hiroshi Takahashi[1], Tatsuo Kanda*[,1], Naoki Matsumoto[1], Toshikatsu Shibata[1], Kazushige Nirei[1], Akinori Tamura[1], Shunichi Matsuoka[1], Kazumichi Kuroda[1] & Mitsuhiko Moriyama[1]

[1]Division of Gastroenterology & Hepatology, Department of Medicine, Nihon University School of Medicine, 30–1 Oyaguchi-kamicho, Itabashi-ku, Tokyo 173-8610, Japan

*Author for correspondence: Tel.: +81 3 3972 8111 (Ext. 2424); Fax: +81 3 3956 8496; kanda.tatsuo@nihon-u.ac.jp

Background & aim: Higher elevation of alanine aminotransferase (ALT) occasionally leads to severe outcomes in hepatitis B virus (HBV)-infected patients. Our aim is to investigate the HBV sequence mutations associated with higher ALT elevation. **Materials & methods:** We analyzed full-length HBV sequences from patients with or without higher ALT elevation. **Results:** Nucleotide mutations in precore and core regions, which are associated with severe hepatitis B, were found in two HBV-infected patients with higher ALT elevation. Amino acid mutations within the pre-S1, pre-S2 and S regions were also found in a patient with HBV virologic breakthrough during the use of nucleoside analogs. **Conclusion:** It may be useful for HBV-infected patients with higher ALT elevation to analyze full-length HBV genome.

First draft submitted: 22 April 2020; Accepted for publication: 15 June 2020; Published online: 2 July 2020

Keywords: acute exacerbation • ALT • full-length HBV sequence • HBV • HCC • reactivation • virologic breakthrough

Hepatitis B virus (HBV) infection causes acute hepatitis, chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) [1,2]. Among chronic hepatitis B patients treated with or without nucleos(t)ide analogs (NUCs), elevation of both serum alanine aminotransferase (ALT) and HBV DNA levels occasionally occurs with or without triggers such as the use of immunosuppressants or anti-cancer drugs [2–5]. These conditions were called acute exacerbation of HBV, HBV reactivation and virologic breakthrough.

In the Asian Pacific Association for the Study of the Liver (APASL) HBV guidelines [2], they define acute exacerbation as the condition of patients with intermittent elevations of aminotransferase to more than five-times the upper limit of normal and more than twice the baseline value. They also define the reactivation of HBV as the reappearance of active necroinflammatory diseases of the liver in a patient who has an inactive chronic HBV infection state or resolved HBV infection [2].

In the American Association for the Study of Liver Diseases (AASLD) HBV guidelines [3], HBV reactivation is defined as loss of HBV immune control in HBV surface antigen (HBsAg)-positive, anti-HBV core antibody (anti-HBc)-positive or HBsAg-negative, anti-HBc-positive patients receiving immunosuppressive therapy for a concomitant medical condition; a rise in HBV DNA compared with baseline (or an absolute HBV DNA level when a baseline is unavailable); and reverse seroconversion (seroreversion) from HBsAg-negative to HBsAg-positive for HBsAg-negative, anti-HBc–positive patients. They also define the virologic breakthrough as a >1 log10 (tenfold) increase in serum HBV DNA levels from nadir during treatment in a patient who had an initial virologic response and who is adherent [3].

HBV reactivation is one of multiple life-threatening conditions during chronic HBV infection [6], and NUCs are effective for these conditions [7]. Immunosuppressants such as corticosteroids and anticancer drugs occasionally induce HBV reactivation [4,5], but HBV reactivation is often observed in patients without inducing factors [8]. Chronic hepatitis B patients receiving NUCs and achieving virologic suppression occasionally experienced virologic

Table 1. Characteristics of patients at the collection of sera in this study.

Patients	Points of serum collection	Age (years)	Sex	Cirrhosis	HCC	ALT (IU/l)	HBV DNA (LIU/ml)	HBeAg	NUCs
C1	Point 1	35	F	–	–	22	7.9	+	–
C2	Point 1	54	F	–	–	340	7.1	+	ETV
C3	Point 1	48	F	–	–	81	4.2	–	ETV
Case 1	Point 1	43	M	–	–	350	7.1	–	–
Case 2	Point 1	52	M	Unknown	–	31	4.3	+	–
Case 2	Point 2	58	M	+	+	35	5.2	–	LAM
Case 2	Point 3	69	M	+	+	1232	6.7	–	ETV

+/–: With/without; ALT: Alanine aminotransaminase; C: Control; ETV: Entecavir (0.5 mg daily); F: Female; HBeAg: HBV e antigen; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; LAM: Lamivudine (100 mg daily); LIU/ml: Log International Unit (IU)/ml; M: Male; NUC: Nucleos(t)ide analog.

breakthrough and higher ALT elevation, which are usually associated with NUC-resistance mutations of the HBV polymerase region [9].

It is possible that HBV reactivation may be associated with other host and virologic factors. In the present study, we analyzed full-length HBV sequences from HBV-infected patients with higher ALT elevation, who were treated with or without NUCs. Our aim is to examine the nucleotide and amino acid mutations that have responsibility for higher ALT elevation in HBV-infected patients. The present study will provide invaluable information on the mechanism of HBV reactivation.

Materials & methods

Definition of HBV reactivation & virologic breakthrough

In patients chronically infected with HBV, acute exacerbation is defined as intermittent elevations of aminotransferase to more than five-times the upper limit of normal and more than twice the baseline value [2]. HBV reactivation is defined as the reappearance of active necroinflammatory disease of the liver in a patient known to have an inactive chronic HBV infection state or resolved HBV infection [2]. Virologic breakthrough is defined as an increase in serum HBV DNA >1 log IU/ml from the nadir of the initial response during therapy, as confirmed 1 month later in HBV-infected patients who are treated with NUCs [2].

Patients

A total of five patients infected with HBV genotype C (GT-C) were analyzed. Three patients (C1, C2 and C3) chronically infected with HBV were used as controls (Table 1). Case 1 experienced acute exacerbation in the course of his chronic active hepatitis B before the use of NUCs. Case 2 had experienced virologic breakthrough after the use of NUCs. This patient was serially analyzed (Table 1). All five patients were positive for HBV DNA at the points of examination and negative for anti-HCV or anti-HIV.

This study was approved by the ethics committee of Nihon University School of Medicine Itabashi Hospital (No. RK-180911-13). For participation in the study, written informed consent was obtained from all patients. This study protocol conformed to the ethical guidelines of the Declaration of Helsinki (1964).

Serological markers

All biochemical tests were performed in the clinical laboratories of Nihon University School of Medicine Itabashi Hospital with routine automated techniques. HBsAg, HBV e antigen (HBeAg) and anti-HBV e antigen antibody (anti-HBe) were determined by chemiluminescent enzyme immunoassay (CLEIA) (Lumipulse Presto, Fujirebio, Tokyo, Japan), electrochemiluminescence immunoassay (ECLIA) (Roche Diagnostics, Tokyo, Japan) and ECLIA (Roche Diagnostics), respectively. Serum HBV DNA levels were determined with TaqMan PCR (Roche Diagnostics). HBV GTs were determined by enzyme-linked immunosorbent assay (ELISA)-based assay [10].

Extraction of serum DNA & amplification of HBV DNA by polymerase chain reaction

Sera collected from all patients were stored at –80°C until analysis. DNA was extracted from 200 µl sera with the Qiagen DNA Blood Mini Kit (Qiagen, Hilden, Germany). These DNA templates were amplified by long range single step PCR using Taq polymerase (KOD FX NEO, Toyobo Life Science, Osaka, Japan) under the following

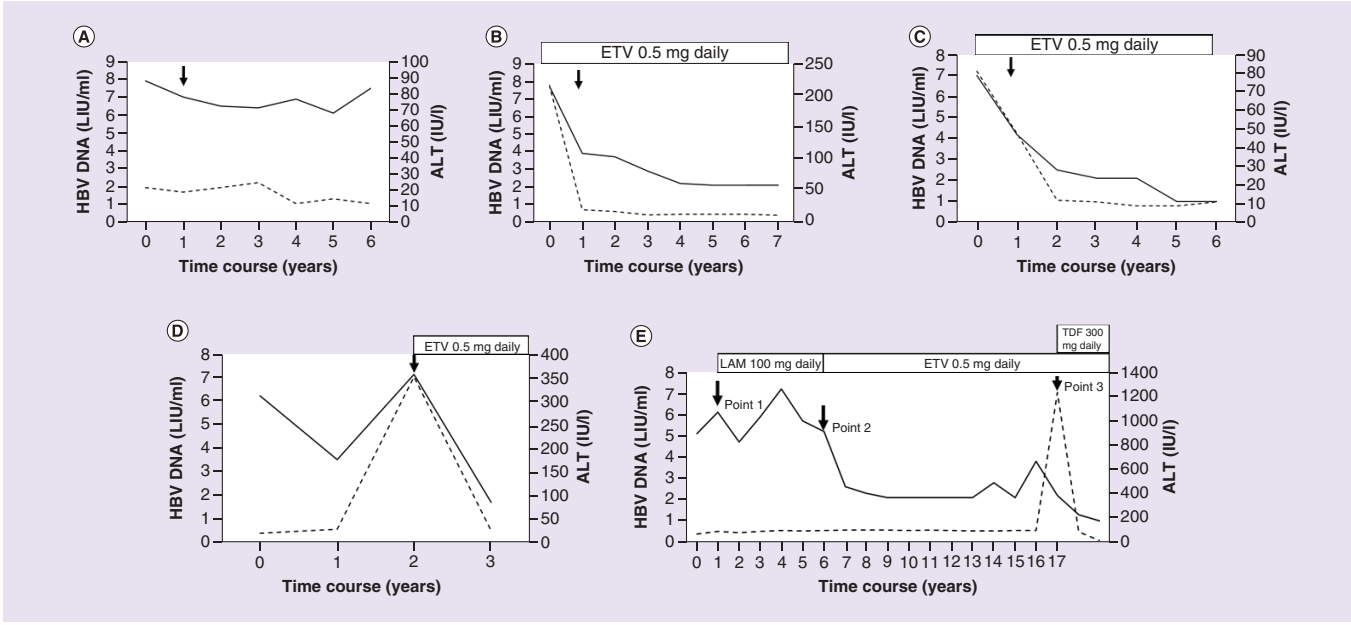

Figure 1. Clinical course of three control patients and two patients with hepatitis B virus reactivation or virologic breakthrough. (A), C1; **(B)**, C2; **(C)**, C3; **(D)**, case 1, patient with HBV reactivation; and **(E)**, case 2, patient with virologic breakthrough during the use of LAM or ETV. Dotted line: ALT; solid line: HBV DNA; LIU/ml; IU/ml; TDF. The arrow indicates the points of collection of sera.
ALT: Alanine aminotransaminase; C1: Control 1; C2: Control 2; C3: Control 3; ETV: Entecavir; HBV: Hepatitis B virus; IU/ml: International unit/ml; LAM: Lamivudinel; LIU/ml: Log international unit/ml; TDF: Tenofovir disoproxil fumarate.

conditions: activation at 94°C for 2 min, 45 cycles with denaturation at 98°C for 10 s, annealing at 50°C for 10 s and extension at 68°C for 120 s in a DNA thermal cycler (GeneAtlas 322/324, Astec, Fukuoka, Japan).

The primers used for PCR were 5′-GGTTTTTCACCTCTGCCTARTCATCTCWTGTWCATGT-3′ (HBV-full P1C: sense, nt 1821-1855) and 5′-GGAAAAAGTTGCATGGTGCTGGTGMRCAGACCAATTT-3′ (HBV-full-P2C: antisense, nt 1826-1791) [11]. These two sets of amplification primers were made at the position of the HBV X region based on the sequences of HBV GT-C (AB014376) [12]. HBV DNA amplified by PCR resulted in an ~3,200 bp fragment, which contained the full-length HBV genome [13]. The PCR products were separated on 1.0% agarose gels and visualized with ethidium bromide and ultraviolet light.

Direct sequencing of PCR products by Sanger methods

After the PCR products were purified with the QIA-quick Spin Kit (Qiagen), PCR products (60–150 ng for each reaction) were directly sequenced to determine their nucleotide sequences using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Tokyo, Japan) and the ABI 3730xl DNA Genetic Analyzer (Thermo Fisher Scientific), according to the manufacturer's instructions. Primers for sequencing have been previously described [14]. All nucleotide sequences from this study have been deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers LC516604 – LC516610. Nucleotide and amino acid sequences were analyzed with GENETYX 10 (GENETYX Corp., Tokyo, Japan).

Statistical analysis

Statistical analysis was conducted using the $\chi 2$ test with or without Yates correction and Student's t-test where appropriate.

Results

Patient characteristics

Table 1 demonstrates the characteristics of the patients in the present study. Among control patients, C1 did not take any NUCs until analysis, and C2 and C3 had entecavir when they were analyzed (Figure 1A-1C). We diagnosed C1 as a HBeAg-positive asymptomatic carrier and NUC treatment-naive patient, and her sera were collected at 1 year after starting follow-up. We also diagnosed C2 and C3 as HBeAg-positive and negative chronic hepatitis,

10.2217/fvl-2020-0104

respectively, and their sera were collected at 1.5 years after starting entecavir. At the collection of sera, ALT levels were 22, 340 and 81 IU/l and HBV DNA were 7.1, 4.2 and 7.1 LIU/ml, in patients C1, C2 and C3, respectively (Table 1). C1 was NUC treatment-naive although C2 and C3 were treated by entecavir at the collection of their sera. These three control patients have not experienced higher ALT elevation after sera was collected.

Case 1 is an NUC treatment-naive patient who experienced naturally occurring acute exacerbation (Figure 1D). Case 2 experienced virologic breakthrough at points 2 or 3 during the course of disease after the use of lamivudine or entecavir, respectively, following lamivudine treatment. Case 2 had HCC at points 2 and 3 (Figure 1E).

Nucleotide sequence & deduced amino acid residue of full-length HBV

We amplified the full-length HBV genome in all samples in the present study. By using full-length PCR-based direct sequencing, we determined the sequence of the HBV genomes in seven serum samples (Supplementary Table 1). We also deduced full-length HBV amino acid sequences (Supplementary Table 2).

Although basal core promoter (BCP) mutations also lead to mutations at HBx protein codons 130/131 [15,16], double mutations (130M/131I) of HBx were found in cases 1 and 2 (Table 2A). Although 131I was found in four patients, 130M was found in cases 1 and 2. Of note, 130M was observed at points 1, 2 and 3 in case 2. Among HBx region, 123S and 22E were found in cases 1 and 2, respectively.

Precore stop codon were observed in all HBeAg-negative patients in the present study (Table 2B). Of interest, precore stop codons were observed at the acute exacerbation in patients with or without NUCs (Table 2B). In the core region, amino acid mutations (35A, 49T, 77D, 97V, 131P and 176T) were observed in patient C3. In case 1, amino acid mutations of the core region (74G, 84A, 97L, 100I, 113D, 130T and 149I) were distinct from those of others (Table 2C). In case 2 (at points 2 and 3), 87G and 180G were observed. In both cases 1 and 2 (at points 2 and 3), 97L was found in the core region. Thus, HBV sequence mutations associated with severe hepatitis B [16–18] were observed in patients with higher ALT elevation in the present study.

In patient C3, amino acid mutations of the pre-S1 (4P, 17A, 32L, 35R, 51P and 90V) and S (126T, 198I and 199L) were observed (Table 3). In case 2 (at point 2), amino acid mutations of the pre-S1 (10K), pre-S2 (1T, 22L, 23Del, and 45T) and S (19Y, 47A, 122R, 190A and 203R) were distinct from those of the other subjects (Table 3).

NUC resistance mutations of the HBV polymerase region

Amino acid mutations of HBV polymerase region were shown in Table 4. Lamivudine resistance mutations (L180M and M204V) were observed in patient C2 and at points 2 and 3 in case 2, although we did not confirm whether patient C2 had previously taken lamivudine (Table 4D). Entecavir resistance mutations (L180M, M204V, T184I and S202G) were also seen at point three in case 2 (Table 4D), suggesting that these mutations contributed to HBV virologic breakthrough. We successfully treated this case by the introduction of tenofovir disoproxil fumarate. Patient C2 did not take any NUCs or experience HBV virologic breakthrough. In general, NUC-associated resistance mutations resulted in HBV virologic breakthrough.

Discussion

In the present study, we analyzed full-length HBV sequences from chronic HBV infected patients (one was naturally occurring acute HBV exacerbation (Table 1, Figure 1D) and the other was HBV virologic breakthrough (Table 1, Figure 1E), and compared them with those of the controls and characterized them. The prognosis of acute exacerbation of HBV infection to acute liver failure (ALF) demonstrated a poor prognosis [19]. We found nucleotide mutations in precore and core regions, which are associated with severe hepatitis B, in two patients with chronic HBV infection and higher ALT elevation with or without NUCs. Amino acid mutations within the pre-S1, pre-S2 and S regions were also found in a patient with HBV virologic breakthrough.

Both the patient with acute exacerbation and the patient with HBV virologic breakthrough during the treatment of NUCs were negative for HBeAg at the higher elevation of ALT. In general, HBV reactivation is observed in both HBeAg-positive and HBeAg-negative patients. HBeAg-positive status is usually observed in patients with an immune-tolerant phase or immune-reactive phase during the natural history of chronic HBV infection [2]. In general, HBeAg-positivity indicates higher HBV replication status and higher HBV DNA levels in sera [2]. As ALF occasionally occurs in persons who are negative for HBeAg [17], careful attention should also be paid to the HBeAg-negative patients with ALT elevation.

For nucleotide mutation of the HBV genome, double point mutations in the BCP, from A to T at nt 1762 and from G to A at nt 1764, were found in cases 1 and 2, although a single mutation was observed in three controls.

Table 2. Amino acid changes of hepatitis B virus X, precore and core regions in the present study.

(A) HBV X region															
AA	5	22	36	38	52	78	80	95	117	123	127	130	131	144	149
AB014376	V	G	H	P	H	R	E	H	L	L	T	M	I	S	N
C1	–	–	T	–	–	–	A	–	V	–	I	K	V	A	–
C2	–	–	–	S	Y	–	–	–	–	–	I	K	–	–	–
C3	–	–	T	–	–	–	–	–	–	–	I	K	–	A	–
Case 1	M	–	–	–	–	–	–	–	–	S	–	–	–	–	–
Case 2/point 1	M	E	–	–	–	–	A	–	–	–	I	–	–	–	–
Case 2/point 2	–	E	–	S	–	H	A	Y	–	–	I	–	–	A	R
Case 2/point 3	–	E	–	S	–	H	A	Y	–	–	I	–	–	–	–

(B) Precore region				
AA	3	9	13	28
AB014376	L	I	S	W
C1	–	V	–	–
C2	–	–	T	–
C3	–	V	T	Stop
Case 1	–	V	–	Stop
Case 2/point 1	–	–	–	–
Case 2/point 2	V	V	–	Stop
Case 2/point 3	–	V	–	Stop

(C) Core region										
AA	5	26	35	38	49	69	74	77	84	87
AB014376	P	S	S	H	S	V	S	E	L	S
C1	–	–	–	Y	–	L	–	–	–	–
C2	H	A	–	Y	–	L	–	–	–	–
C3	–	–	A	Y	T	L	–	D	–	–
Case 1	–	–	–	Y	–	L	G	–	A	–
Case 2/point 1	–	–	–	Y	–	L	–	–	–	–
Case 2/point 2	T	–	–	–	–	L	–	–	–	G
Case 2/point 3	–	–	–	Y	–	L	–	–	–	G

AA	97	100	113	117	130	131	149	176	180
AB014376	I	L	E	E	P	A	V	S	E
C1	–	–	–	–	–	–	–	–	–
C2	–	–	–	–	–	–	–	–	–
C3	V	–	–	–	–	P	–	T	–
Case 1	L	I	D	–	T	–	I	–	–
Case 2/point 1	–	–	–	–	–	–	–	–	–
Case 2/point 2	L	–	–	–	–	–	–	–	G
Case 2/point 3	L	–	–	–	–	–	–	–	G

– refers to amino acid identical to AB014376.
AA: Amino acid number; C: Control; HBV: Hepatitis B virus; Point: Point of serum collection; Stop: Stop codon.

These mutations are occasionally observed in HBV strains from patients with fulminant hepatitis B in Japan [20], although BCP mutations at positions 1762 and 1764 are rarely observed in those in USA [21]. These mutations in BCP affect the transcription of the HBeAg coding region [16]. Takahashi *et al.* observed that the most common mutations observed in their 40 HBV isolates from HCC patients in Japan were double point mutations in the BCP (frequency was ~90%) [12]. HCC occurred during the course of case 2, but HCC did not occur in case 1.

A point mutation from G to A at nucleotide 1896 in the precore region, which introduces a precore stop codon and prevents HBV from producing HBeAg, was observed in both cases 1 and 2 (at points 2 and 3). Of note, the patient with acute exacerbation and the patient with HBV virologic breakthrough during the treatment of NUCs have severe hepatitis-associated nucleotide mutations in their HBV genomes. Takahashi *et al.* observed this mutation from G to A at nucleotide 1896 in the precore region in 45% of their 40 HBV isolates from HCC

Table 3. Amino acid changes in the pre-S1, pre-S2 and S regions in the present study.

(A) Pre-S1 region									
AA	**4**	**6**	**10**	**17**	**32**	**35**	**51**	**84**	**90**
AB014376	**W**	**F**	**R**	**S**	**P**	**G**	**H**	**I**	**A**
C1	–	S	Q	–	–	–	–	–	–
C2	–	S	–	–	–	–	–	–	–
C3	P	S	–	A	L	R	P	T	V
Case 1	–	S	–	–	–	–	–	–	–
Case 2/point 1	–	S	Q	–	–	–	–	–	–
Case 2/point 2	–	S	K	–	–	–	–	T	–
Case 2/point 3	–	S	Q	–	–	–	–	–	–

(B) Pre-S2 region							
AA	**1**	**18**	**22**	**23**	**45**	**46**	**55**
AB014376	**M**	**K**	**F**	**P**	**I**	**F**	**N**
C1	–	R	–	–	–	–	T
C2	V	R	–	–	–	S	–
C3	–	R	–	–	–	–	–
Case 1	–	R	–	–	–	–	–
Case 2/point 1	–	R	–	–	T	–	–
Case 2/point 2	T	R	L	Del	T	–	–
Case 2/point 3	–	R	–	–	T	–	–

(C) S region																	
AA	**3**	**19**	**47**	**68**	**79**	**98**	**122**	**126**	**184**	**190**	**195**	**198**	**199**	**203**	**204**	**207**	**213**
AB014376	**S**	**F**	**T**	**I**	**R**	**L**	**K**	**I**	**A**	**V**	**I**	**M**	**W**	**P**	**R**	**N**	**L**
C1	N	–	–	–	–	–	–	–	V	–	–	–	–	–	S	–	–
C2	N	–	–	–	–	V	–	–	–	–	M	–	–	–	S	–	–
C3	–	–	–	–	–	–	–	T	V	–	–	I	L	–	S	–	–
Case 1	–	–	–	T	H	–	–	–	–	–	–	–	–	–	–	T	I
Case 2/point 1	N	–	–	–	–	–	R	–	–	–	–	–	–	Q	S	–	–
Case 2/point 2	N	Y	A	–	–	–	R	–	–	A	M	–	–	R	S	–	–
Case 2/point 3	N	–	–	–	–	–	R	–	–	–	M	–	–	R	S	–	–

– refers to amino acid identical to AB014376.
AA: Amino acid number; C: Control; Del: Deletion; Point: Point of serum collection.

patients in Japan [12]. This mutation, as well as the BCP and HBx mutations, may play an important role in the disease progression of severe hepatitis B [22].

Ehata *et al.* found that clustering substitutions (codon 48–60 from the start of the core gene) in 7 of 8 HBV subtype adw (mainly GT-C)-infected patients with fulminant and severe exacerbation in Japan [18]. We found the several substitutions in different parts of core region in cases 1 and 2 (Table 2C). HBV core antigen could be one of the immunological targets of cytotoxic T cells [23–25].

HBV S gene overlaps polymerase gene. Therefore, S gene mutations could be introduced by the administration of NUCs [26]. NUCs could also help the introduction of HBV vaccine escape mutations [27]. We also observed amino acid mutations of the pre-S1, pre-S2 and S regions of patients with virologic breakthrough during the treatment of NUCs. Of interest, in case 2 (at point 2), amino acid mutations within the pre-S2 and S regions as well as the spacer domain of the polymerase region were found (Table 3). Sueki *et al.* also reported the presence of amino acid substitutions in HBV pre-S1 and pre-S2, may be related to the emergence of lamivudine resistance during chronic HBV infection [28]. In HBeAg-negative and HBV GT-D carriers, pre-S/S heterogeneity increases with severity of liver disease [29]. Next generation sequencing-based platform may provide an improvement of the clinical application of pre-S mutants in serving as predictive and prognostic markers for HBV-related HCC [30]. HBV pre-S/S variants may be associated with the development of progressive liver damage, hepatocarcinogenesis and the NUC-resistance.

Recently, NUC-resistance mutations of HBV polymerase region may exist in NUC-naive patients [31–34]. In the case of ALF patients who have consciousness disturbance, it is too difficult for us to take information of their

Table 4. Amino acid changes in the hepatitis B virus polymerase region in the present study.

(A) Terminal protein domain													
AA	40	45	46	81	90								
AB014376	D	N	L	N	N								
C1	–	–	–	H	–								
C2	–	–	–	–	–								
C3	E	–	–	H	–								
Case 1	–	–	P	–	D								
Case 2/point 1	–	–	–	–	D								
Case 2/point 2	–	D	–	–	–								
Case 2/point 3	–	D	–	–	–								
(B) RNase H domain													
AA	2	23	89	93	107	113	117	136	138	149	151		
AB014376	S	R	Y	A	I	H	Q	L	D	A	K		
C1	A	Q	–	–	L	–	R	–	–	–	R		
C2	–	–	–	–	L	R	R	–	–	–	R		
C3	–	–	–	–	L	R	R	–	–	–	R		
Case 1	–	–	–	–	L	–	–	–	G	–	–		
Case 2/point 1	–	Q	–	K	L	–	R	P	–	–	R		
Case 2/point 2	–	Q	–	K	L	–	R	P	–	T	R		
Case 2/point 3	–	Q	S	K	L	–	R	P	–	T	R		
(C) Spacer domain													
AA	1	7	12	13	14	15	16	30	31	32	49	66	71
AB014376	L	R	E	S	F	C	S	C	V	R	L	P	S
C1	–	–	–	–	–	–	–	–	–	–	–	–	–
C2	–	–	–	–	–	–	–	–	F	–	–	–	P
C3	S	–	K	–	C	–	–	–	–	Q	M	–	–
Case 1	–	–	–	–	–	G	–	G	–	–	–	–	–
Case 2/point 1	–	–	–	–	–	–	–	–	–	–	–	–	–
Case 2/point 2	–	K	–	P	–	–	A	G	–	–	–	–	–
Case 2/point 3	–	–	–	–	–	–	–	G	–	–	–	T	–
AA	78	83	87	89	100	110	117	123	131	138	157	160	
AB014376	G	D	S	A	K	K	H	N	S	F	T	V	
C1	–	–	–	T	–	–	–	–	–	–	–	–	
C2	–	N	–	T	–	–	R	–	–	–	–	I	
C3	–	–	–	T	–	–	–	–	P	L	–	–	
Case 1	S	–	G	S	–	–	–	–	–	–	–	–	
Case 2/point 1	–	–	–	–	E	–	–	H	–	–	A	–	
Case 2/point 2	–	–	C	–	E	–	–	H	–	Del	A	–	
Case 2/point 3	–	–	–	–	E	E	–	H	–	–	A	–	
(D) Reverse transcriptase domain													
AA	8	55	75	106	109	122	123	124	180	184			
AB014376	E	H	S	S	P	I	N	Y	L	T			
C1	D	–	–	–	–	L	H	–	–	–			
C2	–	–	–	C	–	–	–	–	M	–			
C3	–	–	T	–	–	–	–	N	–	–			
Case 1	–	–	–	–	S	–	–	–	–	–			
Case 2/point 1	–	–	–	–	–	–	–	H	–	–			
Case 2/point 2	–	R	–	–	–	–	–	H	M	–			
Case 2/point 3	–	–	–	–	–	–	–	H	M	I			

– refers to amino acid identical to AB014376.
AA: Amino acid number; C: Control; Del: Deletion; Point: Point of serum collection.

Table 4. Amino acid changes in the hepatitis B virus polymerase region in the present study (cont.).

AA	202	204	207	215	221	238	266	267	271
AB014376	S	M	V	Q	F	N	V	H	Q
C1	–	–	–	–	–	–	–	–	–
C2	G	V	–	–	–	–	I	–	–
C3	–	–	I	–	–	–	–	–	–
Case 1	–	–	–	H	Y	–	–	S	–
Case 2/point 1	–	–	–	–	–	–	–	–	R
Case 2/point 2	–	V	–	–	–	–	–	–	–
Case 2/point 3	G	V	–	–	–	H	–	–	–

– refers to amino acid identical to AB014376.
AA: Amino acid number; C: Control; Del: Deletion; Point: Point of serum collection.

previous use of NUCs. In these situations, attention should be paid to the existence of NUC-resistance mutations of the HBV polymerase region, providing useful information for their treatment. As virologic breakthroughs are also dependent on adherence to NUCs, attention should be paid to the adherence to medication [35].

Sequencing of reverse transcriptase (RT) domain may be enough to know the details of drug-resistance mutations. However, the cost of RT domain and that of full-length HBV genome by Sanger's direct sequencing methods are 800 JPY (~7.00 USD) and 2000 JPY (~18.5 USD), respectively. Therefore, full-length HBV genome analysis may be better if HBV-infected patient with higher ALT elevation, wants to have these tests.

The rate of HBV evolution in HBeAg-positive subjects has been estimated to be $1.4 \times 10^{-5} \sim 3.2 \times 10^{-5}$ nucleotide substitutions/site/year [36]. In HBeAg-negative subjects including asymptomatic carriers, the calculated mean number of nucleotide substitutions/site/year was $\sim 7.9 \times 10^{-5}$, and nucleotide hypervariability was observed in the polymerase and pre-S/S overlap region and core region [37]. We did not examine the sequence changes from the baseline where there was no ALT elevation in patient C1 with no use of NUCs, according to patient's will. However, Fujiwara *et al.* demonstrated that 10 HBeAg-positive asymptomatic carriers did not show any amino acid substitutions in the precure and core regions [38]. Zhang *et al.* also demonstrated that HBeAg-positive asymptomatic carriers had less amino acid substitutions in full genome than those of HBV-infected patients with chronic liver diseases [39].

Conclusion & future perspective

We compared full-length HBV sequences from patients with acute exacerbation and virologic breakthrough. Of interest, at the ALT elevation, NUC-resistance mutations of the HBV polymerase region were observed only in the patient with virologic breakthrough during the use of NUCs. We found nucleotide mutations in precore and core regions, which are associated with severe hepatitis B, in two HBV-infected patients with higher ALT elevation. Amino acid mutations within the pre-S1, pre-S2 and S regions were also found in a patient with HBV virologic breakthrough. Number of subjects was too small to generalize the result. But it is the beginning of our project. These limitations could be addressed in future prospective study. Our work could shed light again on full-length HBV genome analysis and treatment strategy of HBV infection. Next-generation sequencing approach could also provide a new information in this area.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/fvl-2020-0104

Author contributions

H Takahashi, T Kanda, K Kuroda and M Moriyama contributed to the study conception and design, data acquisition, data analysis and interpretation. H Takahashi, T Kanda, T Shibata and A Tamura performed the experiments. H Takahashi, T Kanda, N Matsumoto, K Nirei, S Matsuoka and M Moriyama saw the patients. H Takahashi and T Kanda drafted the manuscript. All authors contributed to making critical revisions and contributed to the final approval of the version of the article to be published.

Financial & competing interests disclosure

T Kanda and M Moriyama perform joint research with Towa Pharmaceutical Co., Ltd., Kyoto, Japan. The funders had no role in the designing the study in the collecting, analyzing or interpreting the data; in writing of the manuscript, or in deciding to publish the results. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

This study was approved by the ethics committee of Nihon University School of Medicine Itabashi Hospital (No. RK-180911-13). For participation in the study, written informed consent was obtained from all patients. This study protocol conformed to the ethical guidelines of the Declaration of Helsinki (1964).

Summary points

- Higher alanine aminotransferase (ALT) elevation is associated with critical condition in hepatitis B virus (HBV)-infected patients.
- These conditions include acute exacerbation, reactivation and virologic breakthrough.
- Nucleotide mutations in precore and core regions, which are associated with severe hepatitis B, were observed in two patients with chronic HBV infection and higher ALT elevation.
- Amino acid mutations within the pre-S1, pre-S2 and S regions were also found in a patient with HBV virologic breakthrough.
- Clinicians should pay a careful attention to HBV mutations in daily clinical practice.
- It should be useful for HBV-infected patients with higher ALT elevation to analysis full-length HBV genome.

References

Papers of special note have been highlighted as: • of interest

1. Yokosuka O, Kurosaki M, Imazeki F *et al.* Management of hepatitis B: Consensus of the Japan Society of Hepatology 2009. *Hepatol. Res.* 41(1), 1–21 (2011).
2. Sarin SK, Kumar M, Lau GK *et al.* Asian-Pacific clinical practice guidelines on the management of hepatitis B: a 2015 update. *Hepatol. Int.* 10(1), 1–98 (2016).

• **Demonstrates the Asian Pacific Association for the Study of the Liver (APASL) Guidelines for hepatitis B virus (HBV) infection.**

3. Terrault NA, Lok ASF, McMahon BJ *et al.* Update on prevention, diagnosis, and treatment of chronic hepatitis B: AASLD 2018 hepatitis B guidance. *Hepatology* 67(4), 1560–1599 (2018).

• **Demonstrates the AASLD Guidelines for the treatment of chronic hepatitis B.**

4. Takahashi H, Ikeda M, Kumada T *et al.* Multicenter cooperative case survey of hepatitis B virus reactivation by chemotherapeutic agents. *Hepatol. Res.* 45(12), 1220–1227 (2015).
5. Fujita M, Sugiyama M, Sato Y *et al.* Hepatitis B virus reactivation in patients with rheumatoid arthritis: analysis of the National Database of Japan. *J. Viral Hepat.* 25(11), 1312–1320 (2018).
6. Oketani M, Ido A, Nakayama N *et al.* Etiology and prognosis of fulminant hepatitis and late-onset hepatic failure in Japan: summary of the annual nationwide survey between 2004 and 2009. *Hepatol. Res.* 43(2), 97–105 (2013).
7. Harigai M, Mochida S, Mimura T, Koike T, Miyasaka N. A proposal for management of rheumatic disease patients with hepatitis B virus infection receiving immunosuppressive therapy. *Mod. Rheumatol.* 24(1), 1–7 (2014).
8. Mori N, Suzuki F, Kawamura Y *et al.* Determinants of the clinical outcome of patients with severe acute exacerbation of chronic hepatitis B virus infection. *J. Gastroenterol.* 47(9), 1022–1029 (2012).
9. Sheppard-Law S, Zablotska-Manos I, Kermeen M *et al.* Factors associated with HBV virological breakthrough. *Antivir. Ther.* 22(1), 53–60 (2017).
10. Usuda S, Okamoto H, Iwanari H *et al.* Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. *J. Virol. Methods* 80(1), 97–112 (1999).
11. Ogawa M, Kamimura S, Kanda T *et al.* Long-term follow-up of a Japanese patient with hepatitis B virus genotype H and human immunodeficiency virus coinfection. *Future Virol.* 14(10), 633–639 (2019).

12. Takahashi K, Akahane Y, Hino K, Ohta Y, Mishiro S. Hepatitis B virus genomic sequence in the circulation of hepatocellular carcinoma patients: comparative analysis of 40 full-length isolates. *Arch. Virol.* 143(12), 2313–2326 (1998).
13. Günther S, Li BC, Miska S, Krüger DH, Meisel H, Will H. A novel method for efficient amplification of whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutants in immunosuppressed patients. *J. Virol.* 69(9), 5437–5444 (1995).
14. Zhang KY, Imazeki F, Fukai K *et al.* Analysis of the complete hepatitis B virus genome in patients with genotype C chronic hepatitis and hepatocellular carcinoma. *Cancer Sci.* 98(12), 1921–1929 (2007).
15. Kanda T, Yokosuka O, Imazeki F *et al.* Hepatitis B virus X protein (HBx)-induced apoptosis in HuH-7 cells: influence of HBV genotype and basal core promoter mutations. *Scand. J. Gastroenterol.* 39(5), 478–485 (2004).
16. Okamoto H, Tsuda F, Akahane Y *et al.* Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. *J. Virol.* 68(12), 8102–8110 (1994).
17. Omata M, Ehata T, Yokosuka O, Hosoda K, Ohto M. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. *N. Engl. J. Med.* 324(24), 1699–1704 (1991).

- **Describes the association between HBV precure mutation and fulminant hepatitis B.**

18. Ehata T, Omata M, Chuang WL *et al.* Mutations in core nucleotide sequence of hepatitis B virus correlate with fulminant and severe hepatitis. *J. Clin. Invest.* 91(3), 1206–1213 (1993).
19. Kakisaka K, Suzuki Y, Jinnouchi Y *et al.* Unfavorable prognosis of patients with acute liver injury due to drug-induced liver injury and acute exacerbation of hepatitis B virus infection. *Hepatol. Res.* 49(11), 1286–1293 (2019).
20. Sato S, Suzuki K, Akahane Y *et al.* Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. *Ann. Intern. Med.* 122(4), 241–248 (1995).
21. Laskus T, Rakela J, Nowicki MJ, Persing DH. Hepatitis B virus core promoter sequence analysis in fulminant and chronic hepatitis B. *Gastroenterology* 109(5), 1618–1623 (1995).
22. Kaneko M, Uchida T, Moriyama M *et al.* Probable implication of mutations of the X open reading frame in the onset of fulminant hepatitis B. *J. Med. Virol.* 47(3), 204–208 (1995).
23. Mina T, Amini Bavil Olyaee S, Tacke F *et al.* Genomic diversity of hepatitis B virus infection associated with fulminant hepatitis B development. *Hepat. Mon.* 15(6), e29477 (2015).
24. Zhang Y, Wu Y, Deng M *et al.* CD8(+) T-cell response-associated evolution of hepatitis B virus core protein and disease progress. *J. Virol.* 92(17), e02120–17 (2018).
25. Khakpoor A, Ni Y, Chen A *et al.* Spatiotemporal Differences in Presentation of CD8 T Cell Epitopes During Hepatitis B Virus Infection. *J. Virol.* 93(4), e01457–18 (2019).
26. Shan M, Shen Z, Sun H, Zheng J, Zhang M. The enrichment of HBV immune-escape mutations during nucleoside/nucleotide analogue therapy. *Antivir. Ther.* 22(8), 717–720 (2017).
27. Pal A, Sarker N, Saha D *et al.* High incidence of lamivudine-resistance-associated vaccine-escape HBV mutants among HIV-coinfected patients on prolonged antiretroviral therapy. *Antivir. Ther.* 20(5), 545–554 (2015).
28. Sueki R, Maekawa S, Miura M *et al.* Correlation between pretreatment viral sequences and the emergence of lamivudine resistance in hepatitis B virus infection. *J. Med. Virol.* 84(9), 1360–1368 (2012).
29. Cavallone D, Ricco G, Oliveri F *et al.* Do the circulating pre-S/S quasispecies influence hepatitis B virus surface antigen levels in the HBeAg negative phase of HBV infection? *Aliment. Pharmacol. Ther.* 51(12), 1406–1416 (2020).
30. Teng CF, Huang HY, Li TC *et al.* A next-generation sequencing-based platform for quantitative detection of hepatitis B virus pre-S mutants in plasma of hepatocellular carcinoma patients. *Sci. Rep.* 8(1), 14816 (2018).
31. Kobayashi S, Ide T, Sata M. Detection of YMDD motif mutations in some lamivudine-untreated asymptomatic hepatitis B virus carriers. *J. Hepatol.* 34(4), 584–586 (2001).
32. Hayashi K, Katano Y, Ishigami M *et al.* Prevalence and clinical characterization of patients with acute hepatitis B induced by lamivudine-resistant strains. *J. Gastroenterol. Hepatol.* 25(4), 745–749 (2010).
33. Baxa DM, Thekdi AD, Golembieski A *et al.* Evaluation of anti-HBV drug resistant mutations among patients with acute symptomatic hepatitis B in the United States. *J. Hepatol.* 58(2), 212–216 (2013).
34. Fan J, Zhang Y, Xiong H *et al.* Nucleotide analogue-resistant mutations in hepatitis B viral genomes found in hepatitis B patients. *J. Gen. Virol.* 96(Pt 3), 663–670 (2015).
35. Kamezaki H, Kanda T, Wu S *et al.* Emergence of entecavir-resistant mutations in nucleos(t)ide-naive Japanese patients infected with hepatitis B virus: virological breakthrough is also dependent on adherence to medication. *Scand. J. Gastroenterol.* 46(9), 1111–1117 (2011).
36. Okamoto H, Imai M, Kametani M, Nakamura T, Mayumi M. Genomic heterogeneity of hepatitis B virus in a 54-year-old woman who contracted the infection through materno-fetal transmission. *Jpn J. Exp. Med.* 57(4), 231–236 (1987).
37. Osiowy C, Giles E, Tanaka Y, Mizokami M, Minuk GY. Molecular evolution of hepatitis B virus over 25 years. *J. Virol.* 80(21), 10307–10314 (2006).

38. Fujiwara K, Yokosuka O, Ehata T *et al.* The two different states of hepatitis B virus DNA in asymptomatic carriers: HBe-antigen-positive versus anti-HBe-positive asymptomatic carriers. *Dig. Dis. Sci.* 43(2), 368–376 (1998).

39. Zhang K, Imazeki F, Fukai K *et al.* Analysis of the complete hepatitis B virus genome in patients with genotype C chronic hepatitis in relation to HBeAg and anti-HBe. *J. Med. Virol.* 79(6), 683–693 (2007).

10.2217/fvl-2020-0104

《Background & aim》

B 型肝炎ウイルス(HBV)は急性肝炎、慢性肝炎、肝硬変、肝細胞癌などさまざまな肝疾患の原因となるウイルスである。慢性 B 型肝炎患者において、経過中に血清 ALT 値と HBV-DNA の上昇を認めることがあり、HBV 急性増悪や再活性化やウイルス学的ブレイクスルーなどと呼ばれている。免疫抑制剤による治療を受けた際に HBV 再活性化が起きる可能性があることは知られているが、特に誘因がなくとも生じることもある。また核酸アナログによる治療を受けていても、薬剤耐性株の出現によってウイルス学的ブレイクスルーが生じうる。このほか、加齢や肝線維化の進行や他ウイルス感染の併存等も急性増悪の誘因となりうる。これらの病態は重症化する場合があり、慢性 B 型肝炎患者の生命を脅かす脅威の一つである。本研究では、急性増悪を来した慢性 B 型肝炎患者血清より分離抽出した HBV-DNA について全長ゲノム解析を行い、急性増悪の機序について検討することを目的とした。

《Materials & method》

＊再活性化とウイルス学的ブレイクスルーの定義について

急性増悪は、血清アミノトランスフェラーゼ値が正常上限の 5 倍超であり前値の 2 倍超の状態が断続的に認められる状態とした。再活性化は非活動性キャリアの状態から肝逸脱酵素の上昇を伴ってウイルス増殖が再開した状態とした。ウイルス学的ブレイクスルーは、核酸アナログによる治療を 1 ヶ月以上継続した状態で、HBV-DNA が低下した水準より 1 LIU/mL 以上の再上昇を認めた状態とした。

＊患者について

コントロール症例として慢性 B 型肝炎患者 3 例(C1, C2, C3)、特に誘因なく急性増悪を来した 1 例(Case1)、核酸アナログ投与中にウイルス学的ブレイクスルーが生じた 1 例(Case2)の計 5 症例を用いた。すべて Genotype C 型である。

＊血清学的マーカー

HBs 抗原、HBe 抗原、抗 HBc 抗体の測定には CLEIA 法、ECLIA 法を用いた。血清 HBV-DNA 値の測定には Taqman PCR を用いた。Genotype は ELISA 法で決定した。

＊DNA 回収と PCR による増幅

各採取検体は測定までの間、-80℃で保存した。採取検体より QIAmp DNA Blood Mini Kit(Qiagen, Hilden, Germany)を用いて DNA 回収を行い、下記の方法でシングルステップ PCR を行った(94℃ 120 秒、98℃ 10 秒-50℃ 10 秒-68℃ 120 秒を 45 サイクル、4℃)。プライマーは既報を参考にし、HBV ゲノム X 領域を中心に作成した。Taq polymerase は KOD FX Neo(Toyobo Life Science, Osaka, Japan)を用いた。PCR エラーは PCR40 サイクルで 100,000 塩基あたり約 10 塩基程度とされる。PCR 産物は 1%アガロースゲルを用いて電気泳動を行った。

＊サンガー法によるダイレクトシークエンス

PCR 産物を QIA quick Spin Kit(Qiagen)を用いて精製した。シークエンスプライマーは既報を参考に設計し、BigDye Terminator version3.1 Cycle Sequencing Kit(Thermo Fisher Scientific, Tokyo, Japan)を用いてダイレクトシークエンスを行った。得られた核酸配列情報は DNA Data Bank of Japan へ登録し、GENETYX 10(GENETYX Corp., Tokyo, Japan)を利用し解析した。得られた塩基配列は、GenBank に登録されている AB014376(HBV Genotype C)と比較した。

＊統計解析

カイ二乗検定と、必要に応じスチューデント t 検定を行った。

《Results》

＊患者の特徴

C1 は核酸アナログを投与されていない HBe 抗原陽性の無症候性キャリアである。C2 はエンテカビルを投与されている HBe 抗原陽性の慢性肝炎例、C3 はエンテカビルを投与されている HBe 抗原陰性の慢性肝炎例である。いずれの症例も経過中に血清 ALT 値の異常上昇は認めていない。Case1 は核酸アナログの投与は受けおらず、特に誘因なく急性増悪を来した症例である。Case2 はラミブジン、エンテカビルの投与歴があり、エンテカビルの投与下でウイルス学的ブレイクスルーを起こした症例である。

＊核酸配列とアミノ酸配列の推定

X 領域について、(130M/131I)の二重変異と(123S、22E)変異は Case1 と Case2 のみで認めた。Core 領

域について、Case1 と Case2 含め、HBe 抗原陰性例で全て Precore 領域にストップコドンを認めた。アミノ酸変異では、(97L)変異が Case1 と Case2 に共通であり、コントロール症例には認められない変異であった。また(74G、84A、100I、113D、130T、149I)変異は Case1 でのみ、(87G、180G)変異は Case2 のみで認められた。S 領域について pre-S1 領域(10K)、pre-S2 領域(1T、22L、23Del、45T)、S 領域(19Y、47A、122R、190A、203R)変異が Case2 のみで認められた。

＊Polymerase 領域の核酸アナログ耐性変異

ラミブジン耐性である(L180M､M204V)変異を C2 と Case2 で認めた｡エンテカビル耐性である(L180M、M204V、T184I、S202G)は Case2 のみで認められた。

《Discussion》

HBV 急性増悪は急性肝不全に至ることもあり予後不良である。免疫抑制剤の使用や、核酸アナログ耐性変異のほか、HBs 抗原陽性例、高齢、高度肝線維化、HBV-DNA 高値などが危険因子として考えられている。Genome-wide Association study より B 型肝炎の病態と HLA-DP などの関連が報告されており、HLA-DP や HLA-DQ 等も急性増悪と関連している可能性がある。HBV 急性増悪の前後では、凝固能の障害に伴い PT 時間や PT-INR の延長がみられる。また、IL-6 や IL-8、TNF-alpha、IFN-beta などが上昇することも報告されている。そこで HBV 急性増悪症例やウイルス学的ブレイクスルー症例の HBV-DNA ゲノム解析を行い、その特徴を検討した。これらの症例では Core 領域のヌクレオチド変異、S 領域のアミノ酸変異が多い傾向にあった。また Basal core promoter 領域の二重変異と Precore 領域のストップコドン変異を Case1 と Case2 の共通変異として認めた。一般に、Precore 領域のストップコドン変異により、HBe 抗原の産生が障害され、HBe 抗原陰性となるが、本症例においても同様の結果が得られた。核酸アナログ製剤未使用例における同薬剤耐性変異出現頻度は、ラミブジンでは 1 年 23%、3 年 44%、5 年 80%、エンテカビルでは 1 年 1%未満、3 年 1%未満、5 年 1.2%、テノホビル 1 年 0%、3 年 0%、5 年 0%と報告されている。S 領域は Polymerase 領域とオーバーラップしており、核酸アナログ投与により Polymerase 領域に耐性変異が入ることで、S 領域にも変異が生じうる。そのため核酸アナログ投与によって S 領域にワクチンエスケープ変異が生じることがあり、同変異が Case2 に認められた。Core 蛋白や S 蛋白は細胞障害性 T 細胞の標的となり得るため、その変異は免疫から逃れる機構の可能性がある。ウイルス学的ブレイクスルーが生じた時点の検体解析において、pre-S2 領域、S 領域のほかに Polymerase 領域内の Spacer 領域にも変異の集積を認めた。

《Conclusion & future perspective》

HBV 急性増悪を来した症例について、血清より HBV-DNA を抽出し、PCR 増幅産物を用いて HBV ゲノム解析を行った。Precore 領域、Core 領域、pre-S1 領域, pre-S2 領域, S 領域の変異が急性増悪と関連している可能性があると考える。ウイルス学的ブレイクスルー症例において、特に pre-S1、pre-S2、S 領域の変異が集積しており、これらの変異が核酸アナログ製剤使用時の急性増悪予知マーカーとなる可能性がある。また同症例において、経時的にゲノム変異をみとめ、Precore 領域について 28W から stop コドンへの変化を認めている。本研究の新規性は、核酸アナログ耐性 Break through 症例で Polymerase 領域の核酸アナログ耐性変異のほかに、Pre-S1、Pre-S2、S 領域にそれぞれ変異を見出したことが挙げられる。しかし今回の結果を一般化するには症例数が少ないため、今後も症例を蓄積し、これらの機能解析を行うなど検討を重ねていき、臨床医学に貢献したい。