厚生労働科学研究費補助金

医薬品・医療機器等レギュラトリーサイエンス総合研究事業

# 献血者でのHBV-DNA陽性血におけるデルタ肝炎ウイルス感染の実態

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研究代表者

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# 献血者でのHBV-DNA陽性血におけるデルタ肝炎ウイルス感染の実態

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研究要旨　日赤NAT検査でHBV-DNA陽性であった141検体を用いてHDV感染状況を検討した。HDV抗体は1検体（0.7%）に検出された。HDV-RNAの検出は、3名の研究者が測定をおこない、陽性検体は、7検体（4.9%）、7検体（4.9%）、1検体（0.7%）であったが、測定結果に乖離が認められた。献血者を対象とした場合、NAT検査でHBV-DNA陽性検体の中にHDV重複感染例が少なからず存在する可能性が高いも、その頻度を論じるには、さらなる検討が必要である。

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## A．研究目的

わが国の献血システムでは、安全な輸血をおこなうために献血者血液は数種類の血液由来感染ウイルスのスクリーニング検査がおこなわれている。一方、献血者を対象としたスクリーニング検査によって新たなB型肝炎ウイルス（HBV）感染者が診断され本人に告知されているところであるが、最近、本来日本には存在しない欧米型B型肝炎（HBV遺伝子型Ae型）感染者が献血者において増加している点が注目され問題視されるようになった。欧米型B型肝炎感染の特徴は、成人初感染例でも約10%が持続感染に移行し、将来的には、慢性肝炎、肝硬変、肝癌への移行が危惧されている。

一方デルタ肝炎ウイルス（HDV）感染は、HBVをヘルパーウイルスとして増殖する特異な肝炎ウイルスである。HDVキャリアはHBs抗原陽性でなければならない。欧米に比してわが国ではHDV感染率は低頻度であり、HBs抗原陽性者の0.6%と報告されてきた。しかしながら、この0.6%の頻度は、本来日本に存在するHBVキャリア（HBV遺伝子型C型ないしB型）での感染率であり、欧米型B型肝炎例での検討はおこなわれておらず、その感染実態は不明である。

本研究の目的は、わが国の献血者を対象としてB型肝炎感染者におけるデルタ肝炎ウイルス感染実態を明らかにすることである。

## B．研究方法

2005年以後、日赤での献血システムでのNAT検査でHBV-DNA陽性結果が判明した血液を用いて、HBV遺伝子型を明らかにするとともに、HDV感染マーカーとしてHDV抗体（日赤測定）とHDV-RNA（日赤と長崎医療センターでの2重測定）の測定をおこなう。HDV-RNA陽性例では、遺伝子配列を明らかにした上で、分子系統樹を作成し、HDV遺伝子型を明らかにする。

C．研究結果

今回の検討では日赤NAT検査で、HBV-DNA陽性であった141検体を用いて検討した。141検体のHBV遺伝子型の内訳は、HBV遺伝子型A型は50検体、HBV遺伝子型B型は26検体、HBV遺伝子型C型60検体、HBV遺伝子型D型3検体、HBV遺伝子型E型1検体、HBV遺伝子型H型1検体である。

HDV抗体（EIA法）の測定では141検体中1検体（HBV遺伝子型B型）が抗体陽性で、0.7%の陽性率であった。

HDV-RNA陽性に関して、日赤での測定では7検体（4.9%）にHDV-RNAが検出された。その内訳は、HBV遺伝子型A型5検体、HBV遺伝子型B型1検体、HBV遺伝子型C型1検体であった。7検体では塩基配列の決定が可能で7例ともHDV-I型であった。

長崎医療センターでの測定は、2名の研究者（研究者a、研究者b）がHDV-RNAの検出をおこなった。研究者aは、7検体（4.9%）においてHDV-RNAを検出した。その内訳は、HBV遺伝子型A型2検体、HBV遺伝子型B型2検体、HBV遺伝子型C型3検体であった。研究者bは1検体（0.7%）においてHDV-RNAを検出したが、その検体のHBV遺伝子型はB型であった。研究者bが、長崎医療センターでの測定でHDV-RNA陽性となった検体を用いて塩基配列の決定を試みるも、HDVに特異的な配列は増幅できなかった。

HDV抗体陽性例でのHDV-RNA検出は、日赤、長崎医療センターともに陰性であった。

D．考察

わが国でのHDV感染は、極めて稀であると報告されているが、世界的には、地中海や南米においてHDV感染が報告されている。HDV遺伝子型は以下の3種類に分類される。HDV-I型：世界中に分布。活動性高く、高率に肝硬変、肝癌に進展する。HDV-II型：東アジア（沖縄）に分布し、非活動性が多い（Wu et al., 1995年）。HDV-III型：南米に分布し、劇症化しやすい（Casey et al., 1993年）。わが国のHDV感染は、沖縄地方を中心にHDV-II型の報告が多数を占め、比較的予後がよいと言われてきたが、2003年、我々がJournal of General Virology（2003年）に報告した長崎のHDV感染者2名は、HDV-I型であり、かつこの2名は、HBV遺伝子型A型とBa型であった。この2名はHDV遺伝子型、HBV遺伝子型ともに外国由来のウイルス株であったことから、今回、献血者HBV-DNA陽性で外国由来のHBV遺伝子型の対象者の中にもHDV重複感染者が存在するのか、存在するならば、その頻度をどの程度かなのかを明らかにする目的で検討した。

今回、日赤での献血システムでのNAT検査でHBV-DNA陽性結果が判明した血液を用いて、HDV感染の有無を検討したが、HDV抗体は141検体中1検体（0.7%）において検出され、HDV-RNAは、日赤の測定では141検体中7検体（4.9%）、長崎医療センターでは、研究者aの測定では141検体中7検体（4.9%）、研究者bの測定では1検体（0.7%）が陽性であった。HDV抗体陽性検体ではHDV-RNAは検出されなかった。

日赤での検出結果からは、わが国の献血者を対象にして、NAT検査でHBV-DNA陽性例の4.9%にHDV感染例が存在し、その中でもHBV遺伝子型A型例では、50例中5例（10%）がHDVとHBVの重複感染例で、ともにウイルス遺伝子型は外国由来の肝炎ウイルス株であったと考えられる。

HDV-RNAの検出およびHDV塩基配列の測定結果に関して、日赤での測定と長崎医療センターの間には乖離が認められた。その理由として、検体中のHDV-RNA量が少ないことが考察される。

今回測定に用いた検体とは、RPHA法でHBs抗原陰性の検体であることから、HBVとHDVの同時感染例の感染初期の検体と考えられ、またHDVの増殖にはHBs抗原が必要なことも考え合わせると、HDVの増殖が不十分な時期の検体であったと思われる。

いずれにしても、施設間、測定者間で測定結果が完全に一致していないことから、今回の測定結果をもって、わが国の献血者でのデ

ルタ肝炎ウイルス感染の頻度を一律に論じることはできない。いずれかの施設でHDV-RNA陽性検体となった例では、その後の時期に採取された検体を用いてHDV-RNA検出ないしHDV抗体の測定をおこない、複数の方法で陽性を確認する必要があると考えられた。

E．結論

日赤NAT検査でHBV-DNA陽性であった141検体を用いてHDV感染状況を検討した。HDV抗体は1検体（0.7%）に検出された。HDV-RNAの検出は、3名の研究者が測定をおこない、陽性検体は、7検体（4.9%）、7検体（4.9%）、1検体（0.7%）であったが、測定結果に乖離が認められた。献血者を対象とした場合、NAT検査でHBV-DNA陽性検体の中にHDV重複感染例が少なからず存在する可能性が高いも、その頻度を論じるには、さらなる検討が必要である。

F．健康危険情報

なし。

G．研究発表

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2．学会発表

なし。

H．知的財産権の出願・登録状況

なし。

研究成果の刊行に関する一覧表

雑誌

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HEPATOLOGY

# Detection of HBV core promoter and precore mutations helps distinguish flares of chronic hepatitis from acute hepatitis B

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**Key words**
acute hepatitis, chronic hepatitis, hepatitis B e antigen, hepatitis B surface antigen, hepatitis B virus.

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## Abstract

**Background and Aim:** Acute exacerbation of chronic hepatitis B has to be distinguished from acute hepatitis, because treatment strategies differ between them.

**Methods:** Mutations in the core promoter and precore region of hepatitis B virus (HBV) were determined in 36 patients with acute exacerbation of chronic hepatitis B, in whom alanine aminotransferase (ALT) increased above 500 IU/L, as well as the 36 patients with acute hepatitis.

**Results:** Mutations in the core promoter (A1762T/G1764A) and precore region (G1896A) were more frequent in patients with acute exacerbation of chronic hepatitis than acute hepatitis (81% *vs* 19%; $P < 0.0001$ and 58% *vs* 6%; $P < 0.0001$, respectively). Of the 19 patients with mutations in both the core promoter and precore region, 17 (89%) had acute exacerbation of chronic hepatitis. In contrast, among the 32 patients with the wild-type for both the core promoter and precore region, 29 (89%) developed acute hepatitis. By multivariate analysis, the double mutation in the core promoter was predictive of acute exacerbation in chronic hepatitis with the highest odds ratio at 26.4.

**Conclusions:** In patients with hepatitis B having ALT levels >500 IU/L, mutations in the core promoter and precore region are useful in distinguishing acute exacerbation of chronic from acute HBV infection. Detection of these mutations would be useful for commencing prompt antiviral treatments on patients with acute exacerbation of chronic hepatitis for a better prognosis.

## Introduction

There are two clinical entities of acute liver disease induced by hepatitis B virus (HBV).[1] Acute hepatitis is induced by immune responses of hosts for eliminating HBV. Most cases of acute hepatitis clear hepatitis B surface antigen (HBsAg) from serum and resolve infection within 6 months after the onset. Acute exacerbation of hepatitis, by contrast, occurs in individuals chronically infected with HBV. They have been infected perinatally or in an early infancy and are tolerant to HBV. Later in their lives, however, the tolerance to HBV is terminated, and immune responses are elicited in them. As a result, severe hepatitis can develop along with subjective symptoms and abnormalities in liver function tests. It is therefore difficult to distinguish acute hepatitis from acute-on-chronic hepatitis. The antibody to hepatitis B core antigen (anti-HBc) of the IgM class is used to distinguish acute from chronic HBV infection. However, IgM anti-HBc develops in some patients with chronic hepatitis during acute exacerbation, in titers overlapping with those of acute hepatitis.[2,3] Hence, high-titred anti-HBc can not always differentiate between acute and acute-on-chronic hepatitis B.

HBV is a small, partially double-stranded DNA virus made of approximately 3200 nucleotides (nt). Since its replication involves the reverse transcription of pregenome RNA,[4] mutations occur more frequently in HBV than in other DNA viruses.[5] Individuals persistently infected with HBV have hepatitis B e antigen (HBeAg) in serum initially. Later in their lives, they lose HBeAg and develop antibodies to HBeAg (anti-HBe). The seroconversion is induced by mutations in two different domains of HBV-DNA. The double mutations in core promoter (A1762T/G1764A) interfere with the transcription of precore RNA and reduce the expression of HBeAg precursor.[6] G-to-A mutation at nt 1896 in the precore region converts codon 28 for tryptophan (TGG) to a stop codon (TAG), and terminates the translation of HBeAg precursor.[7,8] These mutations in the core promoter and precore region are reported in patients with fulminant hepatitis,[9–11] as well as in those with chronic active hepatitis.[12]

In the present study, core promoter and precore mutations were determined in patients with acute exacerbation of chronic hepatitis and those with acute hepatitis. The results obtained indicate that these mutations would be useful for distinguishing acute-on-chronic from acute hepatitis B.



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## Methods

### Patients

During a 5-year period from 2000 through 2004, 36 patients with acute hepatitis B were admitted to National Hospital Organization Nagasaki Medical Center. The diagnosis of acute hepatitis B was made for patients who presented with signs and symptoms suggestive of acute hepatitis (nausea, jaundice, fever, abdominal pain, and enlarged liver) and who were positive for HBsAg and/or IgM anti-HBc, negative for anti-HCV as well as IgM anti-HAV, and had alanine aminotransferase (ALT) values exceeding five-times the upper limit of normal (40 U/L). The loss of HBsAg from serum within 6 months after onset was confirmed in all patients. Infectious sources of acute hepatitis B were sexual contacts in 25 patients, illicit intravenous drugs in four, and unknown in the remaining seven patients.

Among 261 patients with chronic hepatitis B who had been followed up during the same period, acute exacerbation developed in 36 (14%), and 30 of them (83%) reported a family history of HBV infection. HBsAg had persisted for 1 year or longer and ALT increased to >500 IU/L in them all. All the 36 patients with acute-on-chronic hepatitis B underwent a liver biopsy. Fibrosis stages were F0 in 1, F1 in 4, F2 in 16, F3 in 8, and F4 in 7, and activity grades were A1 in 3, A2 in 9, and A3 in 24. The five patients in mild fibrosis stages (F0 or F1) had been infected with HBV for longer than 6 months before they suffered from acute exacerbation, thereby excluding the possibility of acute HBV infection.

Fulminant hepatitis was diagnosed by prothrombin time <40% and hepatic encepharopathy of grade II or higher, and acute severe hepatitis by prothrombin time ≥40% and encephalopathy of grade I or less. Among the 36 patients with acute hepatitis, one developed fulminant hepatitis and five came down with severe hepatitis. Among the 36 patients with acute exacerbation of chronic hepatitis B, one developed fulminant hepatitis and one had severe hepatitis. The two patients with fulminant hepatitis died of advanced hepatic failure; their family members did not agree with liver transplantation.

Mutations in the core promoter and precore region were determined in sera from patients obtained when they presented with acute hepatitis or acute exacerbation of chronic hepatitis.

Informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a *priori* approval by the institution's human research committee.

### Determination of mutations in precore region and core promoter

HBV-DNA was recovered from serum (50 μL) with use of the SMITEX-R&D extraction kit (Medical Biological Laboratories [MBL], Nagoya, Japan). The stop-codon mutation in precore region (G1896A) was determined by enzyme-linked mini-sequence assay (ELMA) with a commercial kit (MBL).[13,14] HBV-DNA solution (50 μL) was mixed with ELMA solution (50 μL), and subjected to polymerase chain reaction (PCR). Amplification products were delivered to wells in a microtiter plate that had been coated with probes for the wild-type or mutant; they had G or A at the position 1896. Reaction was determined by colorimetry, and an optical density >0.100 was judged positive, while that of ≤1.00 was regarded negative. Positive reading on the well for wild-type only was recorded as the wild-type; that on mutant well alone as the mutant type; and positive readings both on wild-type and mutant wells were classified as the mixed type.

Mutations in core promoter were determined by enzyme-linked specific probe assay (ELSPA) with commercial kits (MBL).[13,14] HBV-DNA solution (50 μL) was mixed with ELMA solution (50 μL), and subjected to PCR. Amplicons were transferred to three wells in a microtiter plate which had been coated with different probes. One of wells was coated with probe for the wild-type with A1962/G1764 and another with that for the mutant type with T1762/A1764, and the third with a highly preserved HBV-DNA sequence for guaranteeing successful amplification by PCR. Determination was possible when optical density of the control well exceeded 0.800 and that of the well for the wild-type or mutant was higher than 0.400, in accordance with the decision table in package inserts of the kit.

The sensitivity and specificity of the ELSPA and MBL kits were examined on cloned wild-type and mutant-type HBV-DNA. Reproducible results were obtained with a sensitivity of 100 copies/100 μL of HBV-DNA.[13,14]

### Statistical analysis

Categorical variables were compared between groups by the $\chi^2$-test and Fisher's exact test, and continuous variables by the Student's *t*-test. Influence of various factors on the manifestation of disease was evaluated by logistic regression in univariate and multivariate analyses. Analyses were performed with SAS software (SAS Institute Japan, Tokyo, Japan), and differences were considered significant when the *P*-value exceeded 0.05.

## Results

### Comparison of patients with acute exacerbation of chronic hepatitis and acute hepatitis

Table 1 compares clinical and virological characteristics between patients with acute exacerbation of chronic hepatitis and acute hepatitis. Men predominated (86% *vs* 58%; $P < 0.01$) and platelets counts were lower ($177 \pm 56$ *vs* $238 \pm 60 \times 10^3/mm^3$; $P < 0.0001$), while IgM anti-HBc was less frequent (58% *vs* 97%; $P < 0.0001$) in patients with acute-on-chronic than acute hepatitis. Distribution of HBV genotypes was no different between patients with acute-on-chronic and acute hepatitis, and genotype C accounted for ~90% and genotype B for only 8% in them both.

### Mutations in the core promoter and precore region

The double mutation in core promoter (A1762T/G1764A) and precore mutation (G1896A) were more frequent in patients with acute-on-chronic than acute hepatitis (81% *vs* 19% and 58% *vs* 6%, respectively; $P < 0.0001$ for each). Of the five patients with fulminant or severe acute hepatitis, four (80%) possessed mutations in the core promoter and/or precore region. Table 2 summarizes mutations in the core promoter and precore region in

**Table 1** Clinical and virological characteristics of patients with acute exacerbation of chronic hepatitis and those with acute hepatitis

| Features | Acute exacerbation of chronic hepatitis ($n = 36$) | Acute hepatitis ($n = 36$) | Differences |
|---|---|---|---|
| Men | 31 (86%) | 21 (58%) | $P = 0.009$ |
| Age (years) | 36 ± 13 (16–62) | 38 ± 19 (16–87) | NS |
| Albumin (g/dL) | 4.1 ± 0.4 (3.1–5.0) | 4.0 ± 0.5 (2.4–5.1) | NS |
| ALT (IU/L) | 1499 ± 577 (808–2740) | 1792 ± 785 (209–2990) | NS |
| Total bilirubin (mg/dL) | 4.0 ± 4.2 (0.4–17.2) | 5.6 ± 5.3 (0.8–21) | NS |
| Platelets (x $10^3$/mm$^3$) | 177 ± 56 (72–313) | 238 ± 60 (75–356) | $P < 0.0001$ |
| Prothrombin time (%) | 71 ± 21 (36–114) | 77 ± 27 (5–120) | NS |
| IgM anti-HBc | 21 (58%) | 35 (97%) | $P < 0.0001$ |
| HBV genotypes | | | |
| B | 3 (8%) | 3 (8%) | NS |
| C | 33 (92%) | 32 (89%) | NS |

ALT, alanine aminotransferase; anti-HBc, antibody to hepatitis B core antigen; HBV, hepatitis B virus; NS, not significant.

**Table 2** Clinical manifestation of hepatitis B virus of the wild-type or with mutations in the core promoter and/or precore region

| Core promoter (nt 1762/1764) | Precore region (nt 1896) | Acute exacerbation of chronic hepatitis | Acute hepatitis |
|---|---|---|---|
| Wild | Wild ($n = 32$) | 3 (9%) | 29 (91%) |
| Wild | Mutant ($n = 17$) | 12 (71%) | 5 (29%) |
| Mutant | Wild ($n = 7$) | 4 (100%) | 0 |
| Mutant | Mutant ($n = 19$) | 17 (89%) | 2 (11%) |

the patients with acute exacerbation of chronic hepatitis and those with acute hepatitis. Of the 19 patients with the mutant type both for core promoter and precore region, 17 (89%) were those with chronic hepatitis who had developed acute exacerbation. Of the 32 patients infected with the wild-type both for core promoter and precore region, in contrast, 29 (91%) had been diagnosed with acute hepatitis.

### Factors contributing to the differentiation of acute exacerbation of chronic hepatitis from acute hepatitis

Univariate and multivariate analyses were performed for sorting out factors predictive of acute exacerbation in patients with chronic hepatitis B (Table 3). In univariate analysis, male gender, low platelet counts, negative IgM anti-HBc, and mutations in the core promoter, as well as the precore region, predicted the acute exacerbation of chronic hepatitis. In multivariate analysis, only male gender, negative IgM anti-HBc, and the double mutation in the core promoter were predictive of acute exacerbation of chronic hepatitis. Among these three parameters, the core promoter mutation had the highest odds ratio at 26.4.

## Discussion

Acute HBV infection in adulthood is mostly self-limited, and rarely becomes chronic.[15] Acute exacerbation can emerge in chronic hepatitis, however, making it difficult to differentiate from acute self-limited hepatitis. The prognosis is more severe for acute-on-chronic than acute hepatitis B; it can transit swiftly to decompensation and cirrhosis.[16] Recently, many antiviral drugs have been introduced, including lamivudine, adefovir-dipivoxyl, and entecavir, and they can prevent the development of decompensation and cirrhosis in patients with chronic hepatitis B.[17,18] Hence it is necessary to diagnose the acute exacerbation in patients with chronic hepatitis B in order to start treatment with antiviral drugs immediately.[19–24]

In persistent HBV infection, mutations in the core promoter and/or precore region accumulate with time, as hosts seroconvert from HBeAg to anti-HBe. In the present series of 36 patients with the exacerbation of chronic hepatitis, core promoter and precore mutations were found more frequently than in the 36 patients with acute hepatitis (81% *vs* 19%; $P < 0.0001$ and 58% *vs* 6%; $P < 0.0001$, respectively). Of the 19 patients with mutations both in the core promoter and precore region, in particular, 17 (89%) had developed acute exacerbation of chronic hepatitis. In remarkable contrast, of the 32 patients with the wild-type both for the core promoter and precore region, 29 (91%) had acute HBV infection. By multivariate analysis, core promoter mutations were predictive of chronic HBV infection with the highest odds ratio at 26.4.

For acute hepatitis B, the wild-types both for the core promoter and precore region had positive and negative predictive values of 90% (29/32) and 81% (29/36), respectively. For acute exacerbation of chronic hepatitis B, mutation in either the core promoter or precore region had positive and negative predictive values of 83% (33/40) and 92% (33/36), respectively. Taken altogether, determination of mutations in the core promoter and precore region would be helpful in distinguishing between chronic and acute HBV infections in the patients who present themselves with serum HBsAg and ALT levels exceeding 500 IU/L. HBV genotypes can influence the development of core promoter and precore mutations.[25] They would have made little difference in patients in this study; the majority of them were infected with HBV genotype C.

Patients with acute HBV infection possess IgM anti-HBc in high titers, which can differentiate them from those with acute-on-chronic heaptitis.[26,27] IgM anti-HBc appears in considerably high titers in sera of some patients with chronic hepatitis undergoing acute exacerbation.[2,3] It is therefore difficult to differentially diagnose acute from chronic infection by IgM-anti-HBc alone. Based on the results obtained in this study, mutations in the core promoter and precore region would improve the diagnosis of acute exacerbation in chronic hepatitis.



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**Table 3** Odds ratio for the acute exacerbation of chronic hepatitis

| Factors | Univariate analysis (95% confidence interval) | Multivariate analysis (95% confidence interval) |
|---|---|---|
| Male gender | 4.4 (1.4–14.0); $P = 0.0115$ | 9.0 (1.0–76.6); $P = 0.0455$ |
| Platelets < 100 × $10^3$/mm$^3$ | 6.7 (2.4–19.0); $P = 0.0003$ | 4.0 (0.7–23.1); $P = 0.1275$ |
| Negative IgM anti-HBc | 25.0 (3.1–203.2); $P = 0.0026$ | 21.6 (1.7–267.5); $P = 0.0167$ |
| Core promoter mutations | 17.2 (5.3–55.2); $P < 0.0001$ | 26.4 (3.6–192.6); $P = 0.0013$ |
| Precore mutation | 23.8 (5.0–114.7); $P < 0.0001$ | 5.0 (0.7–37.2); $P = 0.1138$ |

anti-HBc, antibody to hepatitis B core antigen.

As we have reported previously, however, these mutations are frequent in patients with fulminant or severe acute hepatitis.[13] In this study, also, four of the five (80%) patients with fulminant or severe acute hepatitis possessed mutations in the core promoter and/or precore region. This would have to be taken into consideration when using these mutations to differentiate between acute-on-chronic and acute hepatitis.

It is hoped that our findings indicating the usefulness of the core promoter and precore mutations, obtained in limited numbers of patients with acute and chronic HBV infection, would be extended in further studies for prompting antiviral treatment in patients with chronic hepatitis who develop acute exacerbation.

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