

The precore mutation is associated with severity of liver damage in Brazilian patients with chronic hepatitis B

Rosamar E.F. Rezende^{a,*}, Benedito A.L. Fonseca^a, Leandra N.Z. Ramalho^b, Sérgio Zucoloto^b, João Renato R. Pinho^c, Dennis A. Bertolini^c, Ana L.C. Martinelli^a

^a Department of Medicine, School of Medicine of Ribeirão Preto, University of São Paulo, FMRP-USP, 14048-900, Ribeirão Preto, SP, Brazil

^b Department of Pathology, School of Medicine of Ribeirão Preto, University of São Paulo, FMRP-USP, Ribeirão Preto, Brazil

^c Virology Branch, Institute Adolfo Lutz, São Paulo, Brazil

Received in revised form 1 August 2004; accepted 1 August 2004

Abstract

Background: The clinical relevance of the G1896A precore mutation in chronic hepatitis B is still poorly understood.

Objectives: To assess the frequency of G1896A precore mutation in Brazilian patients with chronic hepatitis B, as well as its relation to the viral genotype, serum HBV–DNA levels and liver damage.

Study design: Fifty chronic hepatitis B patients (29 HBeAg-negative and 21 HBeAg-positive) were studied. HBV–DNA was quantified by the Amplicor HBV Monitor test and precore region and S gene were amplified and submitted to automatic sequencing. The histological activity index (HAI), degrees of hepatic fibrosis and distribution of core antigen (HBcAg) in hepatocytes were determined.

Results: Precore mutation occurred in 1/21 (4.8%) HBeAg-positive patients and in 17/29 (58.6%) HBeAg-negative ($p < 0.0001$). Genotype D was identified in 56.5%, genotype A in 41.3%, and genotype F in 2.2%. The frequency of genotypes D and A, as well as serum levels of ALT and HBV–DNA were similar in patients infected with wild type and with precore mutant. Patients infected with precore mutant presented a higher frequency of moderate/severe HAI ($p: 0.03$) and moderate/severe fibrosis and cirrhosis ($p: 0.03$) than those infected with wild type. There was no association between G1896A mutation and cytoplasmic expression of HBcAg.

Conclusions: Precore mutation was frequent among Brazilian subjects with chronic hepatitis B and its presence was associated with greater severity of liver disease.

© 2004 Elsevier B.V. All rights reserved.

Keywords: Hepatitis B virus; HBV genotypes; G1896A precore mutation; Severity of liver disease; Chronic hepatitis

1. Introduction

The integrity of the host immunological system, viral replication and probably the genetic heterogeneity of the hepatitis B virus (HBV), including genotypes and mutations, play an important role in the determination of the natural

history of hepatitis B (Fattovich et al., 1991; Lindh et al., 2000).

The major mutation of the precore region of the HBV is that of codon 28, position of the 1896 nucleotide (G1896A), resulting from the exchange of a guanine to adenine, which promotes a TAG stop codon leading to premature interruption of the translation of the HBe antigen (Brunetto et al., 1989; Carman et al., 1989).

The prevalence of the precore mutation is variable, being high in the Mediterranean region and in the Far East and rare in the US and northern Europe (Hadziyannis and Vassilopoulos, 2001). In Brazil, the prevalence of this mu-

Abbreviations: ALT, alanine aminotransferase; HBV, hepatitis B virus; HBcAg, core antigen; HCV, hepatitis C virus; HAI, histological activity index; HIV, human immunodeficiency virus; PCR, polymerase chain reaction

* Corresponding author. Tel.: +55 16 602 2456; fax: +55 16 633 1144.

E-mail address: rosamarrezende@uol.com.br (R.E.F. Rezende).

tation is still unknown. A previous study in which hepatitis B virus DNA was detected by the polymerase chain reaction (PCR) in anti-HBe positive Brazilian patients with chronic hepatitis B suggested that infection by viral variant precore mutations could occur in our country (Pinho et al., 1993).

The variability of infection with the G1896A precore mutant of HBV in different geographic areas is related to the distribution of HBV genotypes, being more frequent in patients infected with the B, C, D and E genotypes and rare in patients infected with the A genotype (Hadziyannis and Vassilopoulos, 2001; Li et al., 1993; Lindh et al., 1995; Lok et al., 1994).

There are many doubts about the clinical significance of the G1896A precore mutation, with reports of its association with severe chronic hepatitis (Brunetto et al., 1990) and fulminant hepatitis (Bahn et al., 1995; Liang et al., 1991; Omata et al., 1991; Terazawa et al., 1991), and of its occurrence in inactive HBV carriers (Niitsuma et al., 1995; Okamoto et al., 1990; Tur-Kaspa et al., 1992).

The objectives of the present study were to assess the frequency of the G1896A precore mutation in Brazilian patients with chronic hepatitis B, to investigate its effect on the severity of liver damage and its relation to different HBV genotypes and serum HBV–DNA levels.

2. Patients and methods

2.1. Patient series

The study was conducted prospectively on 50 Brazilian patients followed up at the Hepatitis Outpatient Clinic of the University Hospital, Faculty of Medicine of Ribeirão Preto, University of São Paulo (HC-FMRP-USP), with chronic hepatitis B, i.e. with serum positivity for HBsAg for a period of more than 6 months and with evidence of liver damage determined by a liver biopsy showing chronic hepatitis or by persistent or intermittent elevation in serum ALT levels. The patients were selected at random and studied before antiviral treatment during the period from March 1999 to March 2002. All patients were white and came from the Southeast region of the country. Only two patients had a history of decompensated liver disease, due to upper digestive hemorrhage secondary to the rupture of esophageal varices associated with ascites in one, and to ascites complicated by spontaneous bacterial peritonitis in the other. No patient had hepatocellular carcinoma. Serum collected before antiviral treatment and stored at -20°C was available for all patients. Patients with hepatitis C virus (HCV) or human immunodeficiency virus (HIV) co-infection, with a history of alcohol ingestion of more than 40 g ethanol/day, with autoimmune hepatitis or drug-induced hepatitis were excluded. The study was conducted according to the ethical norms of the 1975 Helsinki Declaration and was analyzed and approved by the Ethics Committee of the institution. The patients included in the study signed a term of informed consent.

2.2. Clinical evaluation and determination of alanine aminotransferase (ALT)

Data concerning age, sex and risk factors for HBV infection were obtained and the time of HBV infection was estimated for patients with risk factors of blood transfusion and vertical transmission. Mean serum ALT levels were calculated after measurements on at least three occasions during a 6 month period preceding the liver biopsy (47 patients) or starting from the beginning of clinical follow-up for patients who were not submitted to a biopsy (three patients).

2.3. Serologic tests

A search for serologic markers of HBV (HBsAg, anti-HBc total, HBeAg, and anti-HBe), hepatitis C virus (anti-HCV) and human immunodeficiency virus type 1 and 2 (anti-HIV) was carried out by enzyme immunoassay (ELISA, Abbott Laboratories, Chicago, IL, USA).

2.4. Quantification of serum HBV–DNA

Serum HBV–DNA was quantified in 48/50 patients using the Amplicor HBV Monitor Test kit (Roche Diagnostic Systems, Branchburg, NJ, USA). The detection limits of this method range from 4×10^2 to 4×10^7 copies/ml.

2.5. HBV genotyping and detection of mutations in the precore region

HBV–DNA was extracted using the commercial QIAamp blood kit (Qiagen, Hilden, Germany) and a 200 μl serum aliquot according to manufacturer instructions. The S gene was amplified by nested PCR and HBV genotypes were determined by automatic sequencing (ABI Prism 377 Genetic Analyzer–PE Applied Biosystem, Foster City, CA, USA) as previously described (Sitnik et al., 2000). Mutation of codon 28 and genetic variability of the nucleotide at position 1858 (codon 15) of the precore region were determined by nested PCR according to a previously described method (Kramvis et al., 1998). Fragments of 204 base pairs (bp) were obtained by nested PCR and visualized on 1.5% agarose gel, corresponding to the position of nucleotides between 1763 and 1966. Automatic sequencing was carried out (ABI Prism 310 Genetic Analyzer–PE Applied Biosystem, Foster City, CA, USA) using the reagents of the ABI PRISM Big DyeTM Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA, USA). All PCR procedures were accompanied by a negative control. The EditSeg and Megalign programs of the Lasergene software (DNASTar, Inc., Madison, WI, USA) and the PolyPhred software version 4.0 (University of Washington, Nickerson Group, USA) were used for analysis of the data obtained by automatic sequencing and the sequences obtained were compared to the HBV sequences of GenBank.

2.6. Liver histology and immunohistochemistry for the detection of HBcAg

Forty-seven patients were submitted to an ultrasound-guided percutaneous liver biopsy with a 14G Tru-Cut needle under local anesthesia. Analysis of liver histology and of immunohistochemistry was carried out by a single experienced pathologist of our service in a blinded fashion. The degree of fibrosis and the histological activity index (HAI) were assessed according to the classification of Knodell et al. (1981) modified by Desmet et al. (1994). Anti-HBc mouse primary monoclonal antibodies (DAKO A/S, Glostrup, Denmark) diluted 1:100 were used for the detection of the core antigen in liver. Standard light microscopy at 40× magnification was used to determine the localization of HBc antigen positivity in the nucleus and cytoplasm of the hepatocytes, defined as N: nucleus, C: cytoplasm, and NC: nucleus and cytoplasm.

2.7. Statistical analysis

Proportions were compared by the exact Fisher test or by the chi-square test when recommended (expected frequency higher than five). Quantitative variables were analyzed by the nonparametric Mann–Whitney test. Values of 4×10^2 and 4×10^7 copies/ml were attributed when the serum levels of HBV–DNA were below or above the detection limit of the method employed, respectively. The level of significance was set at $p < 0.05$.

3. Results

3.1. Demographic and epidemiological characteristics of the patients

The patients were first classified into two groups according to the results of the HBeAg/anti-HBe serologic markers. During an observation period of at least 6 months, without receiving specific antiviral treatment, 21 patients presented HBeAg-positive/anti-HBe-negative serologic markers and 29 patients presented HBeAg-negative/anti-HBe-positive markers. The

Table 2

Distribution of patients infected with the wild type and the G1896A precore mutant variant according to HBV genotype

Genotype	Wild	G1896A precore mutant	<i>p</i> -value
A	14/29 (48.3%)	5/17 (29.4%)	0.3
D	15/29 (51.7%)	11/17 (64.7%)	0.3
F	–	1/17 (5.9%)	–

two groups were similar in terms of age, sex, risk factors, and estimated time of HBV infection (Table 1).

3.2. Frequency of mutations in the precore region

All 50 patients in the study presented amplification of the precore region by nested PCR. No false-positive PCR result was observed in the negative controls. The precore mutation of codon 28, G1896A, was detected in 18/50 (36%) patients and was more frequent in HBeAg-negative patients ($p < 0.0001$). The G1896A mutation occurred in 1/21 (4.8%) patients of the HBeAg-positive group (mixed sequence of wild type + precore mutant) and in 17/29 (58.6%) patients of the HBeAg-negative group (11/29 (37.9%) with mixed sequence of wild type + precore mutant and 6/29 (20.7%) with pure sequence precore mutant).

3.3. HBV genotypes and precore G1896A mutation

In four patients (three infected with the wild type and one infected with the precore mutant variant), identification of the HBV genotype was not possible because of difficulty in S gene amplification. The following genotypes were detected: genotype D in 26/46 (56.5%) cases, genotype A in 19/46 (41.3%) cases, and genotype F in 1/46 (2.2%). The frequency of genotype A and D distribution was similar for patients infected with the wild type and patients infected with the G1896A precore mutant variant (Table 2).

The presence of the thymine nitrogen base in the position of nucleotide 1858 was more frequent in genotype D ($p: 0.0005$). Thymine was observed in the position of nucleotide 1858 in 22/26 (84.6%) cases and cytosine in 4/26 (15.4%) cases of genotype D. In genotype A, thymine was

Table 1

General characteristic of patients with chronic hepatitis B distributed according to the presence or absence of HBe antigen in serum

	HBeAg (+)	HBeAg (–)	<i>p</i> -value
No of patients	21	29	
Age (years) Mean ± S.D. (range)	33.2 ± 13.2 (14–62)	34.1 ± 11.6 (15–59)	0.7
Sex (M/F)	16/5	25/4	0.4
Risk factors:			
Vertical	6 (28.6%)	12 (41.4%)	0.2
Transfusion	3 (14.3%)	1 (3.4%)	0.2
Sexual promiscuity*	4 (19%)	5 (17.2%)	1.0
Unknown	8 (38.1%)	11 (38%)	1.0
Time** of infection (years) (median)	25	27	0.8

* Defined as sex relations with more than three partners during a period of 6 months.

** Estimated time of HBV infection for patients whose risk factors were blood transfusion and vertical transmission.

observed in the position of nucleotide 1858 in 6/19 (31.6%) cases and cytosine in 13/19 (68.4%). The only case of genotype F coursed with thymine in the position of nucleotide 1858.

There was association between the presence of the G1896A mutation and the presence of the thymine nitrogen base in the position of nucleotide 1858 of the precore region ($p < 0.0001$). Sequencing of the precore region of all patients infected with the G1896A precore mutant variant revealed thymine in the position of nucleotide 1858. All patients infected with the G1896A precore mutant variant, genotype A, presented the association of a second mutation, i.e. an exchange of cytosine with thymine, in the position of nucleotide 1858, codon 15 (C1858T). In the wild type, cytosine was detected in 19/32 (59.4%) patients and thymine in 13/32 (40.6%) patients in the position of nucleotide 1858.

3.4. Serum ALT and HBV–DNA levels and G1896A precore mutation

Patients infected with the wild type and the G1896A precore mutant variant presented similar median ALT values (Fig. 1). Quantification of serum HBV–DNA was not possible in two patients (one from the group infected with the wild type and the other from the group infected with the G1896A mutant precore variant). There was no difference in median serum HBV–DNA levels between patients infected with the wild type and patients infected with the G1896A precore mutant variant (Fig. 2).

3.5. Liver histopathology and G1896A precore mutation

Patients infected with the G1896A mutant variant presented a higher frequency of moderate/severe HAI ($p: 0.03$) and of moderate/severe fibrosis/cirrhosis ($p: 0.03$) than patients infected with the wild type (Table 3). Immunohistochemistry for the detection of the HBc antigen was positive in 24/29 (82.7%) patients infected with the wild type and in 14/18 (77.8%) patients infected with the G1896A pre-

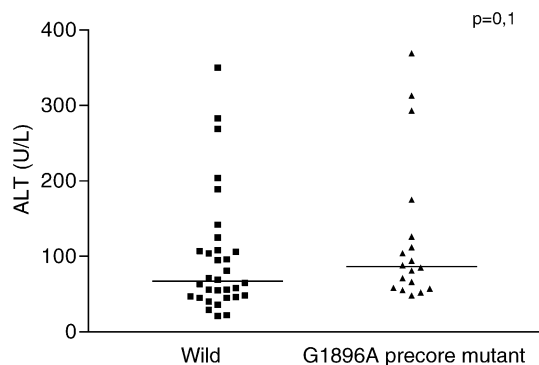


Fig. 1. Serum ALT levels in patients infected with the wild type and the mutant G1896A precore variant of HBV. median (—). Reference values: ALT: 10–44 U/L.

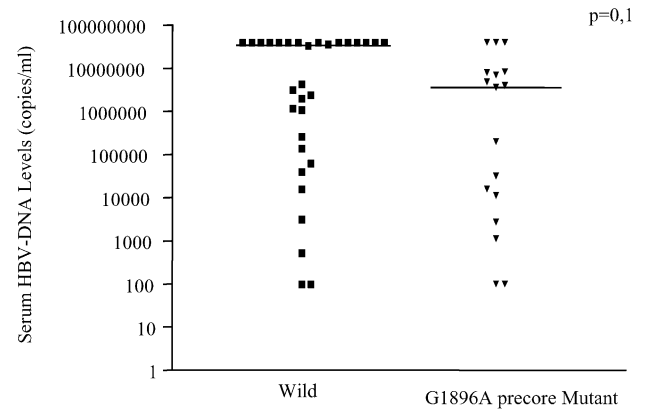


Fig. 2. Serum HBV–DNA levels in patients infected with the wild type and the mutant G1896A precore variant of HBV. Median: (—).

Table 3

Frequency of the different degrees of liver damage (HAI and fibrosis scores) and of intracellular HBc antigen expression in patients with chronic HBV infection with the wild type and the mutant G1896A precore variant of HBV

	Wild	G1896A precore mutant	<i>p</i> -value
Liver HAI score			0.03*
Minimal/mild	18/29 (62.1%)	6/18 (33.3%)	
Moderate/severe	11/29 (37.9)	12/18 (66.7)	
Liver fibrosis			0.03**
Absent/mild	18/29 (62.1%)	6/18 (33.3%)	
Moderate/severe	10/29 (34.5%)	11/18 (61.1%)	
Cirrhosis	1/29 (3.4%)	1/18 (5.6%)	
Positive HBcAg			1.0
Nucleus	13/24 (54.2%)	8/14 (57.1%)	
Cytoplasm	–	2/14 (14.3%)	
Nucleus/cytoplasm	11/24 (45.8%)	4/14 (28.6%)	

* Minimal/mild HAI vs. moderate/severe HAI.

** Absent/mild fibrosis vs. moderate/severe fibrosis and cirrhosis.

core mutant variant ($p: 0.7$). There was no difference in HBc expression predominantly in the nucleus or predominantly in the cytoplasm and nucleus/cytoplasm between patients infected with the wild type and patients infected with the G1896A precore mutant variant (Table 3).

4. Discussion

In the 1980 decade, infection with the precore mutant variant was believed to be limited to the Mediterranean area and to the Far East, with a frequency of 50% to 80% in Greece, Israel and Italy and of 40% to 55% in Hong Kong, Taiwan and Japan (Hadziyannis, 1995). However, different studies have demonstrated a wide geographic distribution of this variant, which has also been reported in the United Kingdom (Ballard and Boxall, 2000) and Germany (Knoll et al., 1999).

In the present study, the G1896A precore mutation occurred in 36% of Brazilian patients with chronic hepatitis B. In agreement with other authors (Grandjacques et al., 2000;

Lok et al., 1994; Mangia et al., 1996), the frequency of the G1896A mutation was significantly higher in the HBeAg-negative group, demonstrating a higher prevalence of this mutation during a later phase of infection, after HBeAg/anti-HBe seroconversion, thus permitting the persistence of viral replication and the consequent maintenance of the hepatic necro-inflammatory activity. The *e* antigen is an important target for cell-mediated and antibody-mediated immune responses, so that the loss of *e* antigen production by precore mutants may help the virus to evade the host immune response (Zuckerman and Zuckerman, 1999). Hence the occurrence of the precore mutation seems to be a strategy of viral selection secondary to the immunological pressure against HBV (Hadziyannis and Vassilopoulos, 2001).

Few data are available in Brazil about the prevalence of the HBV genotypes. In the present study, in agreement with previous observations about the genotypic distribution of HBV in HBV-infected Brazilian patients (Moraes et al., 1996; Teles et al., 1999), the most frequent genotypes were genotype D (56.5%) and genotype A (41.3%), whereas genotype F was rare (2.2%).

The G1896A precore mutation was more frequent in the group of patients with genotype D than in the patients with genotype A. However, possibly because of the small size of the sample studied, no difference was observed in the frequency of genotype distribution when the patients groups with wild type infection and precore mutant infection were compared. The relation between the G1896A precore mutation and the HBV genotypes is due to the base pairing of the stem loop structure of the encapsidation sequence of pregenome RNA. Nucleotide 1896 (codon 28) present in the descending part of the lower stem of pregenome RNA pairs with nucleotide 1858 (codon 15) located in the ascending part of this same stem. When the G1896A mutation is present, if nucleotide 1858 is composed of the thymine–nitrogen base, there is better stabilization of the lower stem, and the opposite occurs when cytosine is present at position 1858 (Li et al., 1993; Lindh et al., 1999; Lok et al., 1994). In the present study, all patients with the G1896A mutation presented thymine at position 1858 and all patients with HBV genotype A and with the G1896A mutation presented the C1858T mutation in association. These results support previous observations about the association between nucleotide 1858 consisting of the thymine–nitrogen base and the G1896A mutation precore mutation (Hunt et al., 2000; Lok et al., 1994; Lindh et al., 1995; Rodriguez-Frias et al., 1995).

There was no significant difference in ALT or HBV–DNA levels between patients infected with the G1896A precore mutant and patients infected with the wild type. However, 14 patients infected with the wild type and only three patients infected with the G1896A precore mutant presented serum levels of HBV–DNA higher than 4×10^7 copies/ml. We wish to point out that the upper limit of detection of serum HBV–DNA levels by the Amplicor HBV Monitor test is 4×10^7 copies/ml. It is possible that, if the real value of

HBV viremia had been available to us, we might have observed differences in serum HBV–DNA levels between the group of patients infected with the wild type and the group of patients infected with the G1896A precore mutant, with these levels being higher in the group of patients infected with the wild type. Grandjacques et al. (2000) studied 85 HBeAg-positive patients and 66 anti-HBe-positive patients and observed results similar to those obtained here with respect to ALT levels and the presence of the precore mutation. The same authors, however, observed lower HBV–DNA levels in patients infected with the mutant precore variant than in patients infected with the wild type. Based on *in vitro* studies, it has been suggested that precore mutant variant has better replicating ability than the wild type (Guidotti et al., 1996; Lamberts et al., 1993). A difference in viral replication between the mutant G1896A variant and the wild type has not been demonstrated using *in vivo* animal models (Chen et al., 1992).

The impact of the G1896A precore mutation on the natural history of hepatitis B, and its role in the pathogenesis of liver disease are still unknown. Acute infection with the precore mutant variant has been associated with a higher incidence of fulminant hepatitis (Bahn et al., 1995; Kosaka et al., 1991; Liang et al., 1991; Omata et al., 1991; Shafritz, 1991). In chronic infection, the emergence of the precore mutant variant during the immune clearance phase or after HBeAg/anti-HBe seroconversion, causing this to be the predominant variant in relation to the wild type, may be associated with episode of exacerbation of liver disease (Laskus et al., 1994) and with a lower response to antiviral treatment compared to the wild type (Rizzetto et al., 2000). However, the precore mutant variant has also been observed in inactive carriers (Niitsuma et al., 1995; Tur-Kaspa et al., 1992). Thus, variable results have been reported concerning the precore mutation and liver damage. In the present study, we observed an association between the presence of the precore mutation and severity of liver damage. Histopathological analysis of the liver revealed that patients infected with the G1896A precore mutant presented more severe liver damage, with a higher frequency of moderate/severe HAI scores (67%) and severe fibrosis (61%) compared to patients infected with the wild type. In the present study, positivity of the HBc antigen in the hepatocytes was similar for patients infected with the wild type and patients infected with the G1896A precore mutant. It has been reported that a predominantly cytoplasmic distribution of the core antigen (HBc) in the hepatocytes is related to greater liver aggression (Chu et al., 1995; Franca et al., 1989; Ramalho et al., 1988). An association between the presence of the G1896A precore mutation and the cytoplasmic expression of HBcAg has been recently reported (Grandjacques et al., 2000; Park et al., 1999). In contrast, in the present study no relation was observed between G1896A precore mutation and cytoplasmic expression of the core antigen, although the small number of cases evaluated should be considered. It is possible that that other, still no elucidated, mechanisms associated with the precore mutation which do

not depend on cytoplasmic expression of the HBc antigen participate in liver aggression.

We conclude that infection with the G1896A precore variant was frequent in the Brazilian population studied and that the presence of the G1896A precore mutation was associated with greater severity of liver damage.

Acknowledgements

The authors are grateful to Marie Secaf and Sandra S. Rodrigues for excellent technical assistance. This research was partly supported by FAPESP.

References

- Bahn A, Hilbert K, Martiné U, Westedt J, Weizsäcker FV, Wirt S. Selection of a precore mutant after vertical transmission of different hepatitis B virus variants is correlated with fulminant hepatitis in infants. *J Med Virol* 1995;47:336–41.
- Ballard AL, Boxall EH. Epidemiology of precore mutants of hepatitis B in the United Kingdom. *J Med Virol* 2000;62:463–70.
- Brunetto MR, Stemler M, Schodel F, Will H, Ottobrelli A, Rizzetto M, et al. Identification of HBV variants which cannot produce precore derived HBeAg and may be responsible for severe hepatitis. *Ital J Gastroenterol Hepatol* 1989;21:151–4.
- Brunetto MR, Stemler M, Bonino F, Schodel F, Oliveri F, Rizzetto M, et al. A new hepatitis B virus strain in patients with severe anti-HBe positive chronic hepatitis B. *J Hepatol* 1990;10:258–61.
- Carman WF, Jacyna MR, Hadziyannis S, Karayiannis P, McGarvey MJ, Makris A, et al. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet* 1989;2:588–91.
- Chen HS, Kew MC, Hornbuckle WE, Tennant BC, Cote PJ, Gerin JL, et al. The precore gene of the woodchuck hepatitis virus genome is not essential for viral replication in the natural host. *J Virol* 1992;66:5682–4.
- Chu C-M, Yeh C-T, Sheen I-S, Liaw Y-F. Subcellular localization of hepatitis B core antigen in relation to hepatocyte regeneration in chronic hepatitis B. *Gastroenterology* 1995;109:1926–32.
- Desmet V, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994;19:1513–20.
- Fattovich G, Brollo L, Giustina G, Noventa F, Pontisso P, Alberti A, et al. Natural history and prognostic factors for chronic hepatitis type B. *Gut* 1991;32:294–8.
- Franca ST, Kiyosawa K, Imai Y, Sodeyama T, Hayata T, Nakano Y, et al. Change of intrahepatic expression of hepatitis B core antigen during the clinical course of type-B chronic hepatitis. *Scand J Gastroenterol* 1989;24:454–60.
- Grandjacques C, Pradat P, Stuyver L, Chevallier M, Chevallier P, Pichoud C, et al. Rapid detection of genotypes and mutations in the pre-core promoter and the pre-core region of hepatitis B virus genome: correlation with viral persistence and disease severity. *J Hepatol* 2000;33:430–9.
- Guidotti LG, Matzke B, Pasquinelli C, Shoenberger JM, Rogler CE, Chisari FV. The hepatitis B virus (HBV) precore protein inhibits HBV replication in transgenic mice. *J Virol* 1996;70:7056–61.
- Hadziyannis SJ. Hepatitis B e antigen negative chronic hepatitis B: from clinical recognition to pathogenesis and treatment. *Viral Hepat Rev* 1995;1:7–36.
- Hadziyannis SJ, Vassilopoulos D. Hepatitis B e antigen-negative chronic hepatitis B. *Hepatology* 2001;34:617–24.
- Hunt CM, McGill JM, Allen MJ, Condreay LD. Clinical relevance of hepatitis B viral mutations. *Hepatology* 2000;31:1037–44.
- Knodell RG, Ishak KG, Black WC, Chen TS, Craig R, Kaplowitz N, et al. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology* 1981;1:431–5.
- Knoll A, Rohrhofer A, Kochanowski B, Wurm EM, Jilg W. Prevalence of precore mutants in anti-HBe positive hepatitis B virus carriers in Germany. *J Med Virol* 1999;59:14–8.
- Kosaka Y, Takase K, Kojima M, Shimizu M, Inoue K, Yoshida M, et al. Fulminant hepatitis B: induction by hepatitis B virus mutants defective in the precore region and incapable of encoding e antigen. *Gastroenterology* 1991;100:1087–94.
- Kramvis A, Kew MC, Bukofzer S. Hepatitis B virus precore mutants in serum and liver of southern african blacks with hepatocellular carcinoma. *J Hepatol* 1998;28:132–41.
- Lamberts C, Nassal M, Velhagen I, Zentgraf H, Schroder CH. Precore-mediated inhibition of hepatitis virus progeny DNA synthesis. *J Virol* 1993;67:3756–62.
- Laskus T, Rakela J, Tong MJ, Persing DH. Nucleotide sequence analysis of the precore region in patients with spontaneous reactivation of chronic hepatitis B. *Dig Dis Sci* 1994;39:2000–6.
- Li J-S, Tong S-P, Wen Y-M, Vitvitski L, Zhang G, Trépo C. Hepatitis B virus genotype A rarely circulates as an HBe-minus mutant: possible contribution of a single nucleotide in the precore region. *J Virol* 1993;67:5402–10.
- Liang TJ, Hasegawa K, Rimon N, Wands JR, Ben-Porath E. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. *N Engl J Med* 1991;324:1705–9.
- Lindh M, Furuta Y, Vahlne A, Norrkans G, Horal P. Emergence of precore TAG mutation during hepatitis B e seroconversion and its dependence on pregenomic base pairing between nucleotides 1858 of 1896. *J Infect Dis* 1995;172:1343–7.
- Lindh M, Hannoun C, Dhillon AP, Norrkans G, Horal P. Core promoter mutations and genotypes in relation to viral replication and liver damage in East Asian hepatitis B virus carriers. *J Infect Dis* 1999;179:775–82.
- Lindh M, Horal P, Dhillon AP, Norrkans G. Hepatitis B virus DNA levels, precore mutations, genotypes and histology activity in chronic hepatitis B. *J Viral Hepat* 2000;7:258–67.
- Lok ASF, Akarca U, Greene S. Mutation in the pre-core region of hepatitis B virus serve to enhance the stability of the secondary structure of the pre-genome encapsidation signal. *Proc Natl Acad Sci USA* 1994;91:4077–81.
- Mangia A, Chung YH, Hoofnagle JH, Birkenmeyer L, Mushahwar I, Di Bisceglie AM. Pathogenesis of chronic liver disease in patients with chronic hepatitis B virus infection without serum HBeAg. *Dig Dis Sci* 1996;41:2447–52.
- Moraes MT, Gomes AS, Niel C. Sequence analysis of pre-S/S gene of hepatitis B virus strains of genotypes A, D, and F isolated in Brasil. *Arch Virol* 1996;141:1767–73.
- Niitsuma H, Ishii M, Saito Y, Miura M, Kobayashi K, Hitoshi O, et al. Prevalence of precore-defective mutant of hepatitis B virus in HBV carriers. *J Med Virol* 1995;46:397–402.
- Okamoto H, Yotsumoto S, Akahane Y, Yamanaka T, Miyazaki Y, Sugai Y, et al. Hepatitis B viruses with precore region defects prevail in persistently infected host along with seroconversion to the antibody against e antigen. *J Virol* 1990;64:1298–303.
- 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* 1991;324:1699–703.
- Park YN, Han KH, Kim KS, Chung JP, Kim S, Park C. Cytoplasmic expression of hepatitis B core antigen in chronic hepatitis B virus infection: role of precore stop mutants. *Liver* 1999;19:199–205.
- Pinho JRR, Santos CA, Gonzalez CLM, Bassit L, Barreto CC, Saez-Alquezar A, et al. Detection of hepatitis B virus DNA by the poly-

- merase chain reaction in anti-HBe positive chronic hepatitis B patients. *Rev Inst Med Trop São Paulo* 1993;35:515–20.
- Ramalho F, Bunetto MR, Rocca G, Piccari GG, Batista A, Chiaberge E, et al. Serum markers of hepatitis B virus replication, liver histology and intrahepatic expression of hepatitis B core antigen. *J Hepatol* 1988;7:14–20.
- Rizzetto M, Volpes R, Smedile A. Response of pre-core mutant chronic hepatitis B infection to lamivudine. *J Med Virol* 2000;61:398–402.
- Rodriguez-Frias F, Buti M, Jardi R, Cotrina M, Viladomiu L, Esteban R, et al. Hepatitis B virus infection: precore mutants and its relation to viral genotypes and core mutations. *Hepatology* 1995;22:1641–7.
- Shafritz DA. Variants of hepatitis B virus associated with fulminant liver disease. *N Engl J Med* 1991;324:1737–9.
- Sitnik R, Pinho JRR, Da Silva LC, Fonseca LEP, Carrilho FJ, Bernardini AP. Hepatitis B virus genotypes and pre core mutants in chronic hepatitis B patients from São Paulo cityBrazil. *Hepatology* 2000;32:587A abstract.
- Teles SA, Martins RMB, Vanderborgh B, Stuyver L, Gaspar AMC, Yoshida CFT. Hepatitis B virus: genotypes and subtypes in Brazilian hemodialysis patients. *Artif Organs* 1999;23:1074–8.
- Terazawa S, Kojima M, Yamanaka T, Yotsumoto S, Okamoto H, Tsuda F, et al. Hepatitis B virus mutants with precore-region defects in two babies with fulminant hepatitis and their mothers positive for antibody to hepatitis B e antigen. *Pediatr Res* 1991;29:5–9.
- Tur-Kaspa R, Klein A, Aharonson S. Hepatitis B virus precore mutants are identical in carriers from various ethnic origins and are associated with a range of liver disease severity. *Hepatology* 1992;16:1338–42.
- Zuckerman AJ, Zuckerman JN. Molecular epidemiology of hepatitis B virus mutants. *J Med Virol* 1999;58:193–5.