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Hepatitis B virus variants with lamivudine-related mutations in the DNA polymerase and the 'a' epitope of the surface antigen are sensitive to ganciclovir

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Abstract

Lamivudine is a new antiviral agent effective against hepatitis B viral (HBV) infections but can result in virus–drug resistance associated with mutations in the conserved '$YM_{552}DD$' motif of the HBV DNA polymerase. Due to their overlapping coding regions in the HBV genome, mutations in the DNA polymerase may result in substitutions in the hepatitis B surface antigen (HBsAg), albeit outside the antigenic 'a' epitope. Here we report the identification of a novel type of lamivudine-related mutations located in both the polymerase ($YM_{552}DD \rightarrow YI_{552}DD$) and the 'a' epitope of HBsAg ($Gly_{130} \rightarrow Asp_{130}$). The same virus carried a HBsAg $Gly_{145} \rightarrow Arg_{145}$ mutation prior to therapy. Both the wild type HBV and lamivudine-related mutants with the $Gly_{145} \rightarrow Arg_{145}$ HBsAg mutation were suppressed following ganciclovir treatment, indicating a beneficial additive effect of both drugs against different forms of HBV mutants.

Keywords: Hepatitis B virus; DNA polymerase; HBsAg; Lamivudine-related mutations; Ganciclovir

1. Introduction

Lamivudine, the negative enantiomer of 2′-dideoxy-3′-thiacytidine or 3TC, is a reverse transcriptase inhibitor, active against both human immunodeficiency virus (HIV) and human hepatitis B virus (HBV) (Dienstag et al., 1995). Its clinical use has resulted in a reduction of serum HBV DNA in chronic carriers (Lai et al., 1997) and, in the case of liver transplantation, has prevented allograft infection and the carrier state in

Abbreviations: HBV, hepatitis B virus; 3TC, (-)2′-deoxy-3′-thiacytidine (lamivudine); HBsAg, hepatitis B surface antigen; HBV Pol, hepatitis B viral polymerase; HIV, human immunodeficiency virus; YMDD, Tyr–Met–Asp–Asp; PCR, polymerase chain reaction; kb, kilo base.

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recipients who were previously positive for HBsAg (Honkoop et al., 1997).

Although the vaccine-based immunoprophylaxis has resulted in a decrease of incidence of HBV infection, an increasing number of vaccine-escape mutants has been reported (Oon et al., 1995; Carman et al., 1997). These HBV variants carrying mutations in the immunogenic loop (amino acid 99 to 160) (Carman et al., 1997), in particular the major antigenic 'a' epitope (amino acid 124 to 147) of hepatitis B surface antigen (HBsAg), are capable of escaping neutralizing antibodies. Some of these HBV variants have been associated with liver diseases (Carman et al., 1995; Oon and Chen, 1998). Anti-HBV therapy with new nucleoside analogues, such as lamivudine, offers an alternative approach in preventing further deterioration of liver function.

However, long-term lamivudine treatment has led to the emergence of drug-resistant HBV strains, indicated by a rise of serum HBV DNA during prolonged therapy (Bartholomew et al., 1997). Mutations in the catalytic region of HBV DNA polymerase, in particular $Met_{552} \rightarrow Ile_{552}$ or $Met_{552} \rightarrow Val_{552}$ in the conserved 'Tyr–Met–Asp–Asp' ('YMDD') motif of domain C (Poch et al., 1989), have been associated with the loss of inhibitory activity of lamivudine (Locarnini, 1998). On the viral genome, the major antigenic 'a' epitope on HBsAg overlaps with the variable linker region between the two conserved domains A (amino acid 410 to 426) and B (amino acid 498 to 524) of the DNA polymerase (Poch et al., 1989).

We present a case report of a patient carrying novel HBV mutants following prolonged lamivudine therapy. While displaying an altered 'a' epitope of HBsAg, in addition to the 'YIDD' motif that is associated with resistance to lamivudine, these lamivudine-related mutants could be suppressed by ganciclovir treatment.

2. Materials and methods

2.1. Patient

A Chinese male aged 36 years with Pugh Child's A grade cirrhosis and spider naevi on his chest was tested positive for both HBsAg and HBeAg in April 1993 (Fig. 1), and had a HBV DNA level of 247.3 pg/ml (Genostics assay, Abbott Laboratories, Chicago, IL). His other serological markers included elevated levels of serum alanine aminotransferase (ALT) 105 IU/l (normal range < 50 IU/l), and aspartate transaminase (AST) 94 IU/l (normal range < 50 IU/l). Ultrasonography showed a coarse liver with attenuated blood vessels and normal spleen. He was treated with lymphoblastoid α-interferon that resulted in a decrease of serum HBV DNA (18.5 pg/ml). This treatment was, however, discontinued because of intolerance to α-interferon, and symptomatic treatment was given instead since 1993.

In October 1995 (Fig. 1), the patient had a 'flare up' of hepatitis B and became jaundiced (Bilirubin 9 mg/dl), his ALT and AST levels were at 45 and 90 IU/l, respectively, and the HBV DNA level was at 22 pg/ml. He was treated daily with 150 mg of lamivudine. This resulted in an improvement both clinically and biochemically, and his HBV DNA was undetectable by the Genostics assay in September 1996. Subsequently, his HBV DNA level rose to 142.8 pg/ml and HBeAg became again positive in October 1997 (Fig. 1) while on continuous lamivudine therapy. His ALT level rose to 59 IU/l and one month

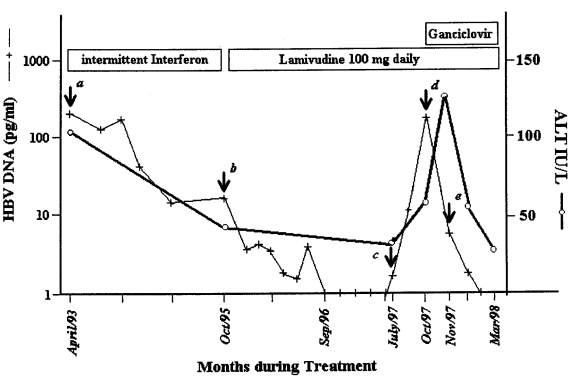

Fig. 1. Serum HBV DNA and ALT levels during combined therapy with lymphoblastoid α-interferon, lamivudine and ganciclovir. HBV DNA level was measured as pg/ml (Genostics, Abbott Laboratories). Viral DNA was extracted at different time points indicated by arrows. Serum ALT level was measured as IU/l.

later to 133 IU/l. Oral ganciclovir at 500 mg three times daily was then added, while lamivudine therapy was continued. By 7 March 1998 (Fig. 1), the patient became negative for both HBV DNA (Genostics assay) and HBeAg, positive for anti-Hbe, and his ALT level had fallen to 33 IU/l. His liver condition remained stable with undetectable HBV DNA level (by Genostics assay and by PCR) and a normal level of ALT as of December 1998, while still on both drugs.

2.2. HBV DNA analysis

Sera HBV DNA concentrations were determined by solution hybridization assay (Abbott Laboratories). Samples of HBV DNA selected throughout the lamivudine therapy (Fig. 1) were isolated by treating 100 μl of serum with proteinase K, followed by phenol–chloroform extraction, and ethanol precipitation. Segment of the viral genome was amplified using the polymerase chain reaction (PCR) with a 5′ primer (5′-TCTAGACTCGTGGTGGACTTCT-3′) located from position 248 to 270, and a 3′ primer (5′-TCACGGTGGTCGCCATGCAACGT) located from position 1625 to 1603 on the HBV wild type genome. The blunt-end DNA fragment of 1.35 kilo bases (kb), amplified from 35 cycles of PCR using *Pfu* polymerase (Stratagene), was then cloned into Zero-Blunt plasmid (InvitroGen). Nucleotide sequence was determined on plasmid DNA template by Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham Life Sci.), using specific internal primers. Sequence analysis was carried out using both DNAStar and Omiga packages (Oxford Mol. Group).

3. Results

The changes in serum HBV DNA and ALT levels of the above patient, treated by lymphoblastoid α-interferon, lamivudine and ganciclovir, are shown in Fig. 1. The lamivudine treatment resulted in an undetectable HBV after twelve months. However, twenty months after the start of lamivudine therapy, there was a rise in both the HBV DNA and ALT levels with mild hyperbilirubinemia (Bilirubin 3.9 mg/dl) but without significant symptoms.

This sharp increase of serum HBV DNA level during lamivudine therapy suggested the emergence of HBV variants. HBV DNA was therefore examined from the patient's serum at five time points during the periods of treatment (Fig. 1). Two of these time points were chosen during lymphoblastoid α-interferon treatment, at the initial stage when serum HBV DNA level was 247 pg/ml (a, Fig. 1) and at the end, but prior to lamivudine treatment when serum HBV DNA was 18.5 pg/ml (b, Fig. 1). Three other time points were selected during lamivudine therapy: with moderate DNA rebound (2.2 pg/ml of serum HBV DNA) (c, Fig. 1), with the highest rebound DNA level (142 pg/ml) (d, Fig. 1), and with reduced DNA level (6.7 pg/ml) (e, Fig. 1). Polymerase chain reaction (PCR) using the viral DNA extracted at different time points as template was carried out. The amplified products covered the coding region that encompassed conserved motifs B and C (with most lamivudine-induced mutations identified) of the catalytic domain of HBV DNA polymerase as well as the major antigenic 'a' epitope of HBsAg.

Direct sequencing of the amplified products from time point *a* (Fig. 1) indicated the existence of mixture of virus in the serum. In addition to wild type HBV, there was $\sim 50\%$ of an HBV variant that carried a GGA to AGA mutation leading to the $Gly_{145} \rightarrow Arg_{145}$ substitution in HBsAg. The same mixture was observed at time points b and c. The conserved '$YM_{552}DD$' motif was found in all the three time points. New mutations were detected at time point d, when the HBV DNA reached its highest level. In the coding region covering the conserved '$YM_{552}DD$' motif, there was a mixture of mutations consisting of ATG to ATA and ATG to ATC leading both to the $Met_{552} \rightarrow Ile_{552}$ change at the amino acid level. There was also a $Leu_{568} \rightarrow Val_{568}$ change in the DNA polymerase. Conversely, analysis of the coding sequence covering the 'a' epitope indicated a mixture of wild-type and mutant sequences, in which a novel mutation GGC to GAC leading to $Gly_{130} \rightarrow Asp_{130}$ (GGC → GAC) in HBsAg was detected.

Table 1
Sequence characteristics of lamivudine-induced HBV variant with mutations in both DNA polymerase and 'a' epitope of HBsAg

Clone	1	2	3	4	5	6	7	8	9
Codon for Met_{552} in DNA polymerase	ATC	ATC	ATC	ATC	ATC	ATA	ATA	ATC	ATC
'YM'$_{552}$DD' motif	YIDD	YIDD	YIDD	YIDD	YIDD	YIDD	YIDD	YIDD	YIDD
Position Trp196 in HBsAg	Ser	Ser	Ser	Ser	Stop	Stop	Ser	Ser	Ser
Codon for Leu_{568} in DNA polymerase	CTT	CTT	CTT	CTT	CTT	GTT	GTT	CTT	CTT
Position Leu_{568} in DNA polymerase	Leu	Leu	Leu	Leu	Leu	Val	Val	Leu	Leu
Position Gly_{130} in HBsAg	Gly	Gly	Gly	Gly	Gly	Asp	Asp	Asp	Asp
Position Gly_{145} in HBsAg	Gly	Gly	Gly	Gly	Gly	Arg	Arg	Arg	Arg

To determine whether the lamivudine-related mutations were located in the same virus, the amplified products were cloned into a plasmid and sequenced. Analysis of nine individual clones indicated that five of them carried an ATG→ATC mutation resulting in the 'YI$_{552}$DD' mutant motif, but wild type sequence at both amino acids 130 and 145 of HBsAg (clones 1–5, Table 1). The mutated 'YI$_{552}$DD' in turn caused a Trp_{196}→Ser_{196} change in HBsAg in all the five clones. Among the four remaining clones, two carried an ATG→ATA mutation (clones 6 and 7, Table 1), while two others carried an ATG→ATC mutation (clones 8 and 9, Table 1), leading to the same 'YI$_{552}$DD' mutant motif; but all the four clones now had the Gly_{145}→Arg_{145} and Gly_{130}→Asp_{130} changes in HBsAg. The mutation ATG→ATA detected in clones 6 and 7 also led to a stop codon at amino acid 196 of HBsAg. In contrast, for mutation Gly_{130}→Asp_{130} in HBsAg no mutation was detected in the corresponding position in the DNA polymerase. Two clones also carried the Leu_{56}→Val_{568} mutation in the DNA polymerase (clones 6 and 7, Table 1).

In summary, three distinct types of lamivudine-related HBV mutants were detected in the serum during prolonged lamivudine therapy. Amino acid sequence alignments of these mutants are summarized in Fig. 2. Mutants in type I (clones 1–5, Table 1) had a mutated 'YI$_{552}$DD' motif in their DNA polymerase (Fig. 2(A)). Type II mutants (clones 8 and 9, Table 1) had the 'YI$_{552}$DD' motif in their DNA polymerase (Fig. 2(A)), as well as the Gly_{130}→Asp_{130} and Gly_{145}→Asp_{145} mutations in their HBsAg (Fig. 2(B)). In addition to the changes in type II mutants, type III mutants (clones 6 and 7, Table 1) also had a Leu_{568}→Val_{568} change in their DNA polymerase (Fig. 2(A)). Furthermore, the mutations identified in all the three types appeared to be stable as they remained detectable following ganciclovir treatment (time point e).

4. Discussion

This report reveals the following findings. Firstly, the pre-existing Gly_{145}→Arg_{145} HBsAg mutant was not cleared by interferon and lamivudine treatment. Secondly, no mutation was detected in the HBV DNA polymerase following the interferon treatment. Thirdly, novel lamivudine-related HBV mutants were detected upon prolonged lamivudine therapy. These mutants carried independent mutations in the major antigenic 'a' epitope of HBsAg (Gly_{130}→Asp_{130}) in addition to the commonly found mutation in the catalytic domain of HBV DNA polymerase ($YM_{552}DD$→$YI_{552}DD$) of the same virus.

An additional mutation in the HBV DNA polymerase (Leu_{568}→Val_{568}) (type III mutants, Table 1), not previously reported, was detected in the lamivudine-related HBV variants. Its functional significance remains to be clarified.

The mutated 'YI$_{552}$DD' in HBV DNA polymerase led to either W_{196}→Ser_{196} or W_{196}→ stop mutations of HBsAg due to their overlapping coding regions (Table 1). The lamivudine-related mutation (Gly_{130}→Asp_{130}) in the most antigenic 'a' epitope of HBsAg has, however, not been previously reported. Despite the co-existence of both wild-type and HBsAg mutant (Gly_{145}→

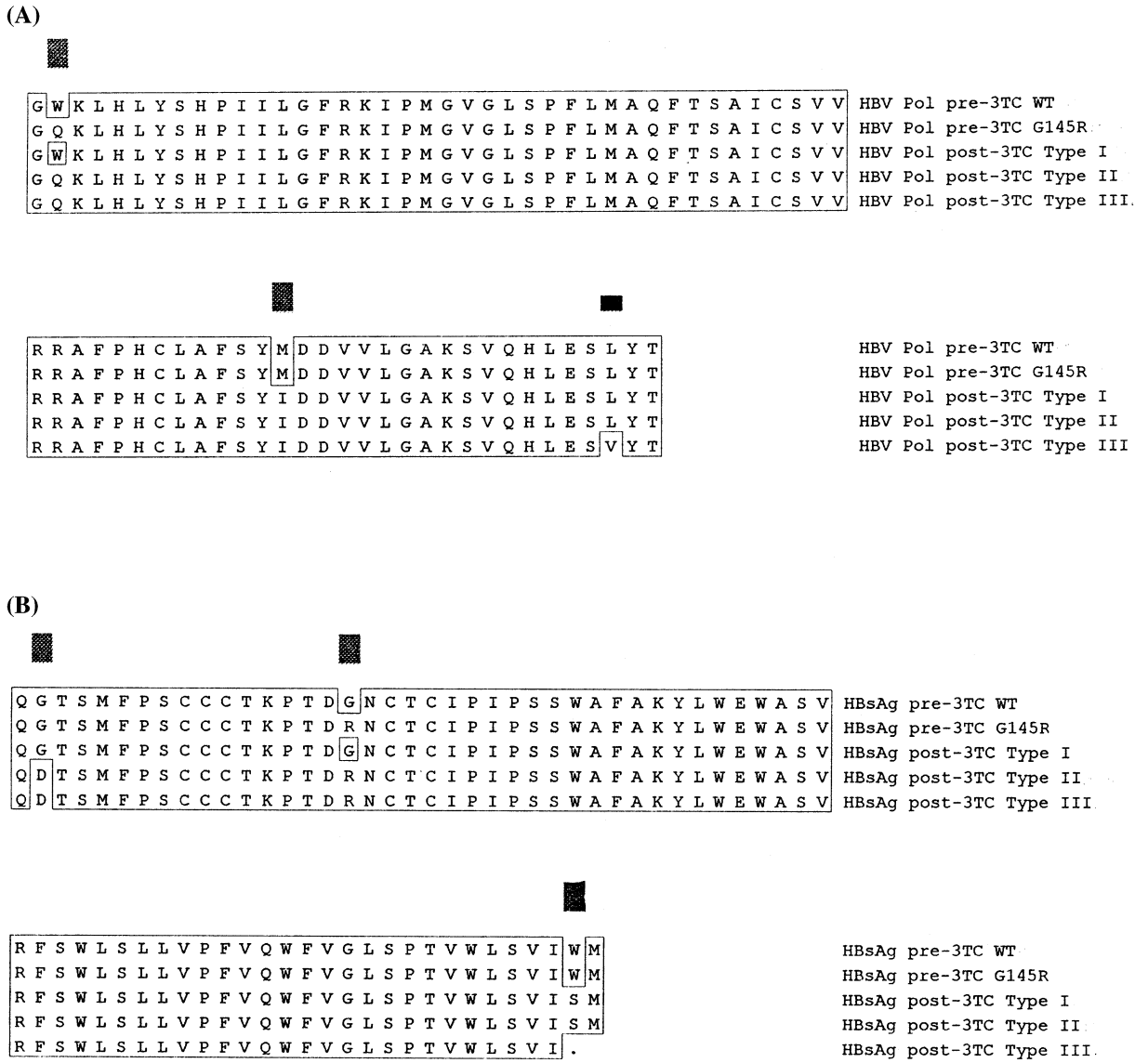

Fig. 2. Sequence alignment of HBV DNA polymerase and HBsAg before and after HBV recurrence during combined therapy. (A) Partial amino acid sequence of HBV DNA polymerase from residue 490 to 570 is shown. HBV Pol pre-3TC WT: sequence before HBV recurrence with wild-type HBsAg. HBV Pol pre-3TC G145R: sequence before HBV recurrence with mutated $G_{145} \rightarrow Arg_{145}$ HBsAg leading to the $W_{491} \rightarrow Q_{491}$ change in DNA polymerase. HBV Pol post-3TC Type I, II and III: sequences of the three HBV mutants emerged after viral recurrence (Table 1). Mutations are indicated by black boxes above the sequences. The $W_{491} \rightarrow Q_{491}$ change (seen in pre-3TC G145R and HBV Pol post-3TC Class II, III) coincides with $G_{145} \rightarrow Arg_{145}$ in HBsAg. Lamivudine-related mutations were $M_{552} \rightarrow I_{552}$ (types I, II and III) and $L_{568} \rightarrow V_{568}$ (type III); (B) Partial amino acid sequence of HBsAg from residue 129 to 197 is shown. HBsAg pre-3TC WT: wild-type HBsAg before HBV recurrence. HBsAg pre-3TC G145R: $G_{145} \rightarrow Arg_{145}$ mutated HBsAg before HBV recurrence. HBsAg post-3TC Type I, II and III: sequences of HBsAg of the three HBV mutants that emerged after viral recurrence (Table 1). Mutations are indicated by black boxes above the sequences. The lamivudine-related $Gly_{130} \rightarrow Asp_{130}$ change was seen in type II and III mutants. The $W_{196} \rightarrow Ser_{196}$ change seen in type I and II mutants was due to the lamivudine-related YIDD mutation in the HBV DNA polymerase (Table 1). The $W_{196} \rightarrow$ stop change seen in HBsAg post-3TC Type III was also due to the lamivudine-related YIDD mutation in HBV DNA polymerase (Table 1), and the stop codon is indicated by a dot (.).

Arg_{145}) in the serum before the rise of HBV DNA during lamivudine therapy, the $Gly_{130} \rightarrow Asp_{130}$ mutation was detected only in the $Gly_{145} \rightarrow Arg_{145}$ HBsAg mutant and not the wild type (Table 1). Epidemiological data suggest that the $Gly_{130} \rightarrow Asp_{130}$ HBsAg mutant is rare globally, and occurred in only 0.08% of a local population of 2538 individuals randomly surveyed in 1997. The pathological significance of this mutant will need to be monitored closely.

The novel forms of lamivudine-related HBV mutants displaying independent mutations in both the DNA polymerase and HBsAg, responded favorably to the addition of ganciclovir. The additive effect of these two drugs have resulted in the suppression of both lamivudine-resistant and wild-type HBV strains. This may lead to additional alternatives in anti-HBV therapy.

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