

Detection of hepatitis B virus variants resistant to lamivudine and famciclovir among randomly selected chronic carriers from Spain

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BACKGROUND. Treatment for chronic hepatitis B with lamivudine is often hampered by the emergence of point mutations in the YMDD motif of the HBV DNA polymerase gene that confer drug resistance. This usually occurs after several months of therapy, but early detection of lamivudine-resistant mutants has been reported among patients in South Korea. Data from Japan and France suggest that naturally occurring, lamivudine-resistant hepatitis B virus (HBV) variants can be found among chronic carriers who have never received lamivudine treatment. Famciclovir can be used as an alternative when lamivudine-resistant variants emerge, though the substitute treatment may also give rise to the emergence and selection of drug-resistant variants.

METHODS. The presence of mutations related with lamivudine and famciclovir resistance was studied in serum samples from 79 randomly selected Spanish HBV carriers, using a line probe assay (LiPA) on HBV genome fragments amplified by polymerase chain reaction. Data concerning antiviral therapy prior to sampling were available for these patients.

RESULTS. Mutations related with resistance to either drug were detected in ten patients. Three of them (3.8% of the 79 carriers studied) had no record of prior lamivudine or famciclovir treatment at the time of sampling. Wild-type strains together with either the rtM204I (M552I) or rtV207I (V555I) point mutation were found in two of these cases, and the rtV207I mutation alone was detected in the third.

CONCLUSIONS. These findings seem to indicate that lamivudine and famciclovir-resistant variants circulate among Spanish HBV carriers. Since it is expected that antiviral therapy will be ineffective when drug-resistant variants are present before the beginning of treatment, it could be beneficial to test for these variants as an additional routine procedure when designing antiviral therapy on an individual basis.

Key words: Hepatitis B virus. Hepatitis B virus variants. Chronic hepatitis B. Antiviral therapy. Lamivudine. Famciclovir. Line probe assay.

Detección de variantes del virus de la hepatitis B resistentes a lamivudina y famciclovir en portadores crónicos seleccionados aleatoriamente en España

INTRODUCCIÓN. El tratamiento de la hepatitis B crónica con lamivudina se ve frecuentemente afectado por la emergencia de mutaciones en el motivo YMDDV de la ADN polimerasa viral que confieren resistencia a la droga. Este fenómeno acostumbra a aparecer tras varios meses de tratamiento, pero a veces sucede en momentos tempranos del mismo. Este hecho, junto con otros hallazgos realizados en Japón y Francia, sugiere que las variantes resistentes circulan entre la población y originan infecciones en las que existe resistencia primaria al tratamiento.

MÉTODOS. Se utilizó una prueba de hibridación reversa en tira (*line probe assay*, LiPA) para estudiar la presencia de mutaciones asociadas a resistencia a lamivudina o famciclovir en fragmentos del genoma del virus de la hepatitis B (VHB) amplificados del suero de 79 portadores crónicos mediante la reacción en cadena de la polimerasa. Las muestras fueron remitidas desde diferentes centros sanitarios españoles para detección y cuantificación del ADN viral entre enero de 2001 y mayo de 2002, incluyéndose en el estudio una muestra, elegida al azar, de las que resultaron positivas en ese período. Se recogieron datos sobre tratamiento antiviral previo al momento de la toma de la muestra en pacientes seleccionados.

RESULTADOS. Se detectó alguna de las mutaciones estudiadas en muestras procedentes de 10 pacientes. Tras revisar los datos disponibles y solicitar información adicional, se encontró que tres de ellos (3,8% de los 79 pacientes estudiados) carecían de antecedente de tratamiento antiviral. En 2 casos, se halló mezcla de secuencias salvajes y de las mutaciones rtM204I (M552I) y rtV207I (V555I), respectivamente. En el restante, se detectó la mutación rtV207I en ausencia de niveles detectables de la secuencia salvaje.

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CONCLUSIONES. Los hallazgos sugieren que un porcentaje significativo de los portadores crónicos de VHB que existen en nuestro medio sufren infecciones que involucran variantes potencialmente resistentes al tratamiento con lamivudina famciclovir. Toda vez que cabe esperar que el tratamiento con esos fármacos sea ineficaz en presencia de variantes resistentes, estas observaciones llevarían a tomar en consideración la posibilidad de estudiar rutinariamente la presencia de tales mutaciones antes de seleccionar la pauta terapéutica más adecuada para cada paciente.

Palabras clave: Virus de la hepatitis B. Variantes del virus de la hepatitis B. Hepatitis B crónica. Terapia antiviral. Lamivudina. Famciclovir. Hibridación reversa en tira.

Introduction

Lamivudine is an antiviral drug approved for the treatment of chronic hepatitis B. Lamivudine therapy is well tolerated and is effective in reducing hepatitis B virus (HBV) replication and inducing normalization of serum ALT levels. Nevertheless, selection of mutant viral strains resistant to the drug is common, occurring at a rate of around 20% per year of treatment among immunocompetent patients¹⁻³. When such mutants emerge during treatment, famciclovir and other drugs (adefovir, tenofovir) can be used as therapeutic alternatives¹. Point mutations related with lamivudine resistance occur within the P open reading frame (ORF) of the HBV genome and affect domains B and C of the HBV reverse transcriptase (RT), leading to various aminoacid substitutions (fig. 1). Aminoacid transitions in positions 528 (L528M) and 552 (M552I, M552V) of the HBV DNA polymerase have been associated with lamivudine resistance. Another point mutation, leading to an aminoacid transition in position 555 (V555I), has been associated with famciclovir resistance. These positions change in the various HBV genotypes, so a genotype-independent nomenclature has been proposed for the mutations, starting with the highly-conserved EDWGPCDEHG motif at the beginning

of the HBV RT domain⁴. The unified denominations are as follows: rtL180M (previously L528M), rtM204I (M552I), rtM204V (M552V) and rtV207I (V555I).

Once mutations are produced, selection of resistant mutants occurs under pressure of the drug: wild-type strains are progressively eliminated by the treatment and the viral population gradually becomes enriched with the resistant mutants. This selection reduces the efficacy of the treatment and may be a reason for stopping it. When lamivudine is discontinued, replication of residual wild-type strains is again possible, and this sometimes leads to a rapid increase in viral replication, with sudden episodes of hepatocyte destruction that may produce severe liver necrosis and death^{5,6}. Since the replicative competence of lamivudine-resistant HBV mutants is usually lower than that of the wild-type strains, it is recommended to maintain the treatment for some time after mutant selection has taken place, in order to avoid these serious complications.

Substitution of wild-type strains by drug-resistant mutants takes place over several months of treatment and seems to be more frequent when specific HBV genotypes are involved in the chronic infection^{7,8}. Nonetheless, cases of early emergence of mutants have been described, suggesting that mutations conferring lamivudine resistance were already present in the liver of these patients before starting therapy⁹. This would mean that these mutant strains circulate among the population, so that a certain proportion of HBV carriers from a given geographical area might have any of them. The emergence of lamivudine-resistant mutants seems to be more frequent in treated patients carrying genotype A HBV strains⁷. Thus, the prevalence of naturally occurring resistant strains is expected to be higher in countries such as Spain, where genotype A is the most prevalent among the general population. Research aimed at searching for these naturally occurring variants among untreated carriers has not yet been reported from European countries, but a single case of acute, primary HBV infection involving one of these variants has been found in France¹⁰. Data from Japan suggest that the prevalence of lamivudine-resistant strains among naive HBV carriers

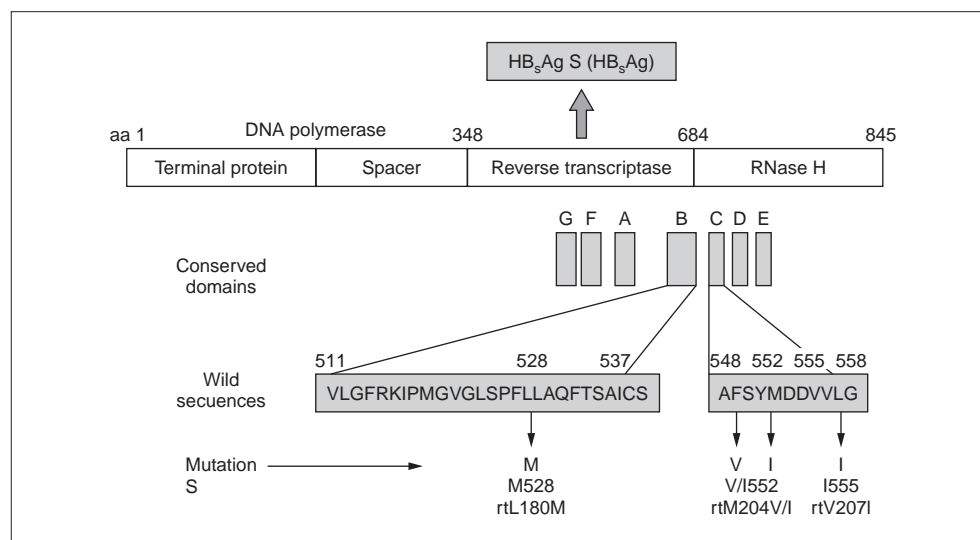


Figure 1. Aminoacid transitions in the HBV DNA polymerase conferring resistance to lamivudine (rtL180M, rtM204V/I) or famciclovir (rtV207I). Such transitions arise from point mutations within the B or C conserved domains from the reverse transcriptase region of the P gene.

in that country could be as high as 28%¹¹, although the series of carriers studied was limited.

The presence of a drug-resistant HBV variant before treatment could be a reason for selecting an alternative drug for therapy. Thus, routine testing for mutations involved in antiviral drug resistance might be useful for candidate patients. To judge whether this activity would be appropriate, however, prior knowledge of the prevalence of these variants among the HBV carrier population is required. This retrospective study was performed to obtain preliminary data on the prevalence of lamivudine-resistant HBV mutants among Spanish carriers.

Methods

From January 2001 to May 2002, serum samples from 588 HBsAg carriers were sent to a Spanish reference laboratory for detection and quantification of HBV DNA. Samples were tested by both quantitative molecular hybridization and nested-polymerase chain reaction tests (see below); HBV DNA-positive samples from 229 patients were identified, and 79 patients were randomly selected for the study. After performing the tests for detection of drug-resistant mutants, additional samples from some of these patients, obtained before January 2001, were recovered and included in the study.

HBV DNA was quantified by molecular hybridization, using a commercial assay (Digene Hybrid Capture II, HBV DNA Test, Digene Corp., Gaithersburg, MD, USA) and a fragment of the viral genome was amplified by a nested-polymerase chain reaction (n-PCR) in-house developed test, targeted on the P region of the HBV genome. Outer primers HBPr134 and HBPr135 (5'-TGC TGC TAT GCC TCA TCT TC-3' and 5'-CA(A/G) AGA CAA AAG AAA ATT GG-3', respectively) were used in the first reaction to obtain a fragment that was amplified again in a second reaction using nested primers HBPr75 and HBPr94 (5'-CAA GGT ATG TTG CCC GTT TGT CC-3' and 5'-GGT A(A/T)A AAG GGA CTC A(A/C)G ATG-3', respectively)¹². A final 341 base-pair fragment, corresponding to aminoacids 465 to 562 of the HBV DNA polymerase gene, was obtained and studied by agarose gel electrophoresis. Since the nested primers were biotinylated, the final amplification product was biotin-labeled, enabling direct testing for the presence of point mutations related with lamivudine or famciclovir resistance by a commercial line probe assay test (INNO-LiPA HBV DR, Innogenetics N.V., Ghent, Belgium)¹². The performance of this assay for the detection of the mutations studied as compared to sequence analysis has been found satisfactory¹³. HBV DNA tests were performed when samples arrived at the laboratory, whereas LiPA assays were done retrospectively on biotin-labeled amplification products stored at -20 °C.

After identifying samples with significant mutations, the corresponding clinical records were reviewed and the clinician responsible for the management of each patient studied was contacted, in order to confirm the data regarding the patient's status with respect to lamivudine or famciclovir treatment at the time of sampling.

Results

Point mutations related with drug resistance were detected in ten patients (tables 1 and 2). The rtM204I mutant was the most frequent (6 cases), either alone or associated with rtM204V or rtL180M. Single rtM204V or rtV207I mutants were found in two cases, respectively. The rtL180M mutant was found together with rtM204I in one case. Wild-type strains were associated with mutant strains in three patients.

Seven of the ten patients had a record of lamivudine therapy. Mixtures of mutants in a single position

(rtM204V + rtM204I) or in two positions (rtL180M + rtM204I) were found in three of them, and single mutants (rtM204I or rtM204V) were detected in the remaining four (table 1). All these mutations would predict resistance to lamivudine. Follow-up samples, taken over a period of 14 months, were available from four patients. On the initial sample, before or at the beginning of therapy, wild type sequences alone were found in two of them, and a mixture of wild type and rtM204I mutant was found in Patient 1. The wild-type strains cleared later on and emerged again at the end of follow-up. This patient had human immunodeficiency virus (HIV) coinfection and received highly active antiretroviral therapy (HAART) together with lamivudine treatment. The patient's compliance with therapy was poor; lamivudine treatment was interrupted during August and September 1999, and started again in October. Compliance with therapy continued to be poor and finally, both HAART and lamivudine treatment were considered unsuccessful and were withdrawn in January 2002. All patients were still undergoing treatment at the time of follow-up sampling.

There was no record of treatment with lamivudine or famciclovir in the remaining three patients, 3.8% of the 79 carriers studied. We consider that these cases are likely to reflect natural infections by strains resistant to lamivudine (one case) or famciclovir (two cases) among Spanish HBV carriers. All three patients were men, 32 to

TABLE 1. Patients with HBV variants undergoing lamivudine treatment at the time of sampling

Patient	Lamivudine since (month/year)	Sampling date (month/year)	HBV DNA (pg/mL)	LiPA HBV DR
1	Jan/99	Oct/99	2.250	WT + rtM204I
		Jan/00	510	WT + rtM204I
		Sep/00	120	rtM204I
		Jan/01	5.850	WT + rtM204I
2	Sep/00	Aug/00	3.120	WT
		Nov/01	5.850	rtM204I + rtM204V
3	May/01	May/01	600	WT
		Oct/01	Negative	-
		Mar/02	40	rtM204I
4	Jun/00	Jun/01	900	rtM204I + rtM204V
5	Sep/00	Sep/01	3,2	rtL180M + rtM204I
		May/02	5,0	rtL180M + rtM204I
6	Sep/00	Oct/01	490	rtM204V
7	Jan/01	Dec/01	5.910	rtM204V

WT: wild type HBV strains.

TABLE 2. Patients with HBV variants and no record of lamivudine or famciclovir treatment before or at the time of earliest sampling

Patient	Sampling date (month/year)	HBV DNA (pg/mL)	LiPA HBV DR
1	Feb/01	250	WT + rtV207I
	May/01	250	WT + rtV207I
	Apr/02	3,4	rtV207I
2	Mar/01	290	WT + rtM204I
	Nov/01	30	WT
3	Nov/01	75	rtV207I

WT: wild type HBV strains.

42 years old. None of them were HIV coinfecting, a fact that makes unnoticed prior treatment with lamivudine unlikely. The patients had the rtM204I or rtV207I mutation; variant mixtures or strains with multiple mutations were not found (table 2). Wild-type strains together with the drug-resistant variants (rtM204I or rtV207I) were found in two cases. Serum clearance of the wild-type strains apparently occurred in Patient 1, nearly one year after they were last detected. The patient had liver cirrhosis and was diagnosed with chronic hepatitis B in 1992. In Patient 2, the rtM204I variant was not detected on follow-up testing. Clearance of the variant was observed to coincide with exacerbation of liver disease, with increased aminotransferase levels, anti-HBc IgM production, and a decrease in viremia from 98 to 30 pg/mL of viral DNA. No follow-up samples could be obtained from Patient 3.

Discussion

Data supporting the natural occurrence of lamivudine-resistant strains among the population of chronic HBV carriers have been reported recently from Japan and France^{10,11}. Such natural occurrence might also explain the early emergence of resistant mutants observed in South Korea among some patients receiving lamivudine therapy⁹. On the basis of these observations, routine testing for lamivudine-resistant variants might be useful in chronic HBV carriers scheduled for antiviral therapy, particularly in countries where genotype A strains are prevalent^{7,8}. Since famciclovir is often used as replacement therapy in cases of emergence of lamivudine-resistant variants, research into mutations conferring primary resistance to this alternative drug would also be of interest. In order to assess the appropriateness of optimizing routine testing for HBV drug resistance in a given country, the prevalence of naturally occurring variants among randomly selected, chronic HBV carriers should be investigated.

The results from the present study indicate that HBV variants associated with drug resistance circulate in the Spanish population and suggest a prevalence among chronic HBV Spanish carriers of nearly 4%. Since the replication fitness of these variants is low, it is likely that these infections result from direct transmission from an external source rather than from natural emergence during chronic infection. The 4% incidence found in this study is much lower than the 28% reported from Japan¹¹, but could be significant enough to support the performance of prospective, population-based studies to establish the true prevalence of these variants in Spain and to assess the need for routine screening for lamivudine resistant mutants prior to therapy.

Lamivudine therapy was approved for the treatment of chronic hepatitis B in Spain in 2001. Before receiving this approval, it was used only in very selected cases, except in the case of HIV-infected patients. Therefore, it seems unlikely that the finding of HBV strains with mutations conferring resistance to lamivudine or famciclovir among samples from three HIV-negative patients lacking records of prior antiviral therapy after exhaustive investigation would respond to former, unnoticed treatment with these

drugs. It is much more likely to reflect the circulation of these variants within the general population.

Mutations in the YMDD motif from the HBV RT have been shown to produce other effects besides the emergence of lamivudine resistance. Association of the rtL180M mutation with changes in position 204, either rtM204I or rtM204V, restores the replicative competence of rtM204I/V mutants and induces resistance to other antiviral drugs that may be useful in treating chronic hepatitis B¹². Changes in the "a" determinant of the HBsAg have also been reported in association with various mutations in the YMDD motif¹³. In vitro experiments have shown that these changes enhance the replication of resistant mutants in the presence of the drug¹³. It is possible that modifications in such an important region of the HBsAg may lead to other relevant effects. Thus, testing for these mutations might provide further interesting information on the viral strains infecting a patient, in addition to the specific information about lamivudine resistance.

Routine testing for HBV drug resistance requires robust, reliable molecular assays. The INNO-LiPA HBV DR reverse hybridization test¹⁴ used in the present study has correlated well with sequence analysis for the detection and identification of YMDD mutations¹⁵. We have found it to be a user-friendly, clean method that can be almost fully automated using the AUTO-LiPA unit. Our experience over the last decade with routine use of LiPA technology for genotyping hepatitis C virus strains has shown that obtaining genome fragments by nested PCR improves the quality of the results, probably because of the high concentration of target material required to obtain clean hybridization patterns. The use of biotinylated primers for the diagnostic PCR nested reaction provides a material that can be safely stored at -20 °C and directly tested by LiPA when indicated, without a significant increase in cost.

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