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Characterization of Naturally Occurring and Lamivudine-Induced Surface Gene Mutants of Hepatitis B Virus in Patients with Chronic Hepatitis B in India

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Key Words
Antiviral therapy · Hepatitis B virus · Lamivudine · Mutations · Resistance, lamivudine

Abstract
Background: Besides vaccine escape or immune escape hepatitis B virus (HBV) mutants, naturally occurring and drug-induced mutations have been reported in the surface gene (S-gene) of HBV. ***Aim:*** To investigate the frequency and profile of naturally occurring S-gene mutants and the influence of long-term lamivudine therapy in patients with chronic hepatitis B (CHB). ***Materials and Methods:*** 57 patients with histologically proven CHB, on lamivudine 100 mg/day for more than 24 months, were included. Viral DNA was extracted at baseline and from on-therapy serum samples. The region encoding the complete major hydrophilic region (MHR) and flanking regions (nucleotides 425–840) of major S-gene that overlapped with the viral polymerase was PCR amplified and sequenced. End-of-therapy response (ETR) was assessed. ***Results:*** Two (3.5%) patients had naturally occurring HBV mutants, sP127S and sS143L seen in the 'a' determinant of the S-gene. Following lamivudine therapy, 14 of 57 (24.5%) patients developed 16 types of S-gene mutations (sP120S, sA128V, sS143L, sW182St., sT189I, sV190A, sS193L, sI195M, sW196L, sW196St., sS207R, sI208T, sS210E, sF219S, sF220L and sC221G). Thirteen (81.2%) of these mutations emerged downstream to the MHR. Nine of 16 types of S-gene mutations observed with lamivudine therapy were also associated with the corresponding changes in the polymerase gene. Baseline viral DNA was significantly higher (2,093 vs. 336 pg/ml; $p < 0.05$) among patients developing S-gene mutants and the ETR in them was significantly lower [3 of 16 (18.8%) vs. 17 of 41 (41.5%); $p < 0.05$]. ***Conclusions:*** Naturally occurring S-gene mutations are uncommon and are restricted to the 'a' determinant region. Mutations develop in about a quarter of the patients on lamivudine therapy, mostly downstream to the MHR. They may contribute to non-response to the antiviral therapy.

Introduction

HBV is a hepadnavirus with a genome of 3.2-kb size. The genome of HBV has four overlapping open reading frames (ORFs: S-, C-, P- and X-ORFs) where surface gene overlaps with the catalytic domains of the polymerase (P) gene [1]. Three envelope proteins [large (L), middle (M)

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and small (S 'or' HBsAg)] are synthesized from three in-frame start codons (ATG) of the pre-S1, pre-S2 and S genes of S-ORF [2], that are involved in receptor binding and viral assembly, and are important targets for immune-mediated virus elimination.

The major B-cell epitope cluster of S-protein or HBsAg is referred to as the 'a' determinant spanning from amino acids (aa) 124 to 147, but recent evidence from observed natural variation and the complex arrangements of epitopes suggests that this epitope cluster could be extended up- and downstream to include the entire major hydrophilic region (MHR, aa 100–160).

The humoral immune responses to HBV surface (S) protein contributes to the clearance of circulating HBV particles, whereas cellular immune responses are responsible for the elimination of infected hepatocytes [3]. Thus, the emergence of variant viruses that escape from humoral or cellular immunity may be implicated in the persistence or progression of HBV infection [4–6].

Mutations in the entire surface gene could arise in the natural course of HBV infection. The prevalence of these naturally occurring mutations remains unknown because of the lack of comprehensive studies. The surface gene of HBV also contains putative HLA class I-restricted cytotoxic T lymphocyte (CTL) epitopes [3]. In general, viral mutations in CTL epitopes could evade cellular immunity and lead to viral persistence [7, 8].

Specific mutations in the catalytic domains of the polymerase gene, especially in the YMDD motif of the domain C, often appear in relation to the breakthrough during antiviral therapy with nucleoside analogues such as lamivudine or famciclovir [9–11]. S-gene mutants can also be found accompanying mutations of the HBV polymerase gene, in patients receiving lamivudine treatment [12]. It is however, not known whether the emergence of the S-gene mutants could alter the response to antiviral therapy.

The aims of the present study were to determine the profile and frequency of naturally occurring S-gene mutations and to investigate the frequency and significance of S-gene mutants emerging due to long-term lamivudine therapy in patients with chronic hepatitis B.

Patients and Methods

Fifty-seven patients with histologically proven chronic liver disease due to HBV infection, fulfilling the following criteria were included: (i) HBsAg positivity >6 months, (ii) elevated ALT (>1.5 times upper limit of normal), (iii) HBV DNA positive by a quantitative DNA assay, (iv) lamivudine therapy for ≥ 24 months. *Exclusion criteria*: (i) infection with hepatitis C virus (anti-HCV and/or HCV RNA positive), hepatitis D virus (total anti-δ positive), or HIV (HIV I and II positive), (ii) history of HBV vaccination, (iii) associated hepatocellular carcinoma or renal failure and (iv) recipient of hyperimmune γ-globulin.

Collection of Serum Samples

Serum samples from the patients were collected at baseline, month 1 and once at every 1–3 months (irregular intervals) subsequently. Sera were stored for molecular studies at –70°C.

Lamivudine Therapy

Lamivudine was given in a dose of 100 mg/day for >24 months. The patients were followed up regularly throughout the antiviral therapy.

DNA Extraction from Serum Samples

The DNA was extracted from 100 μl of patient serum with the help of standard phenol/chloroform extraction method. Briefly, 100-μl serum samples were treated with 5 μl proteinase K (15 mg/ml), 10 μl of 10 × serum lysis buffer and 5 μl of 20% SDS at 37°C for 3 h. Subsequently, the supernatant obtained after phenol, chloroform and chloroform iso-amyl alcohol (24:1) steps was left for overnight DNA precipitation in the presence of 3 *M* NaOAc, pH 5.2, and absolute alcohol. After centrifugation, the obtained pellet was washed with 70% alcohol, dried, and, dissolved in 30 μl of TE (10 m*M* Tris and 1 m*M* EDTA) buffer.

Nested Polymerase Chain Reaction Amplification of HBV DNA

The region encoding the complete MHR along with the flanking regions of surface protein (HBsAg) that overlapped with catalytic domains of P-ORF (nucleotide position, nt., 425–840 of the HBV genome) was amplified by nested polymerase chain reaction (PCR). Three microliters of extracted DNA from serum samples were amplified in a 25-μl reaction mixture containing dNTP, $MgCl_2$, PCR buffer and Taq DNA polymerase in the appropriate quantity. The amplification step was performed for 30 cycles with the outer primers SA1: 5′-ATCGC,TGGAT, GTGTC, TGCGG-3′ and SA2: 5′-GGCAA, CGGGG, TAAAG, GTTCA-3′ (position 369–388 and 1136–1155, respectively) in the HBV genome. Ten microliters of amplified product were analyzed on 1.2% agarose gel in 1 × TBE. Samples that did not show amplification were directly used for nested amplification. Where a 787-bp fragment was generated, nested PCR was done after appropriate dilution. Three microliters of the first round PCR product (directly or after dilution depending on the band intensity visualized on the gel) were used as a template in a second amplification step performed for 30 cycles with inner primers SB1: 5′-TTAGG, GTTTA, AATGT, ATACC, C-3′ (position 822–842) and SB2: 5′-CATCT, TCTTG, TTGGT, TCTTC, TG-3′ (position 427–448). PCR was carried out using denaturation at 94°C for 1 min, annealing at 55°C for 1 min and primer extension at 72°C for 2 min for a total of 30 cycles and finally a single cycle for primer extension was carried out at 72°C for 7 min. Ten microliters of nested PCR product (416 bp) were analyzed by the agarose gel electrophoresis on 1.2% gel in 1 × TBE buffer. In addition, all negative controls from the first round of amplification were included in the second amplification step. Purified HBV DNA from the recombinant vector pcf 80 (pBR322 having full-length HBV genome cloned at its *Eco*RI site) was used as the positive control.

DNA extracted from serum samples of healthy controls and water were used as negative control.

Direct DNA Sequencing of the PCR Product

Purified 416-bp fragment (with the help of PCR purification kit, Qiagen) was sequenced with both forward and reverse primers (SB1 and SB2, respectively) in an automated DNA sequencer (ABI 377-18 sequencing system, Perkin Elmer). Briefly, 200–250 ng of purified template in 5 μl of volume were mixed with 4 μl of sequencing reaction mixture supplied by Perkin-Elmer and 1 μl each of the SB1 and SB2 primers of 5 pmol/μl concentration in two different sets of reactions. Sequencing reaction was carried out under the cyclic conditions of 96°C for 30 s, 55°C for 30 s and 60°C for 4 min. After completion of 30 cycles, the sequencing reaction product was precipitated by adding 90 μl H_2O, 10 μl 3 *M* NaOAc (pH 4.6) and 250 μl absolute alcohol at room temperature for 10 min, and centrifuged at 12,000 rpm for 15 min. Subsequently, 70% alcohol wash was given to the pellet at the same speed for 10 min, and at 37°C the dried pellet was dissolved in 3.5 μl of gel-loading dye (formamide and dextran blue). Samples were denatured for 10 min at 90°C, and then quenched on ice for 5 min, prior to loading on polyacrylamide gel in the automated DNA sequencer. Obtained electrophorograms were carefully read to repair the appearance of 'N' in the sequence and to ensure if the laser reader has correctly read the peaks in the electrophorograms. Obtained sequences were translated to amino acid sequences using the software ExPasy tool. Consensus amino acid sequences of known subgenotypes A1 and D2 from India [13] were aligned with amino acid sequences of patients' isolates using the 'Multalin' software.

Mutational Analysis

Presence of amino acids at a particular locus from the isolates of patients of this study, which was not found in the consensus amino acid sequences of Indian subgenotypes (A1 and D2) at that locus, was considered as the mutation/variation.

Viral DNA Quantitation

HBV DNA was quantified using a liquid hybridization commercial assay (Digene; lower limit of detection <0.5 pg/ml of serum) in the serum obtained at baseline, month 1 and subsequently every 1–3 months.

Response Assessment [14]

End of Therapy Response (ETR). In HBeAg-positive patients, it was defined as loss of HBeAg, loss of viral DNA (<0.05 pg/ml) and normalization of ALT. In HBeAg-negative/anti-HBe-positive patients, however, it was defined as loss of viral DNA accompanied with the normalization of ALT.

Sustained Viral Response (SVR). Persistence of the status of ETR for 6 months after stopping the therapy.

Non-Response. Persistence of HBeAg and/or detectable levels of HBV DNA at the end of therapy/follow-up.

Drug Resistance. Mutational analysis was done by direct DNA sequencing in the baseline and on-therapy samples. Mutations were also studied whenever the patients showed any biochemical derangement or sudden rise in viral DNA. Drug resistance was diagnosed only when rtYM204I/VDD substitution within the viral polymerase was detected by direct DNA sequencing, irrespective of the viral DNA or ALT profile at that time.

Table 1. Demographic profile and baseline characteristics of the patients

Parameters	
Patients	57
Males:females	51:6
Mean age, years	
Males	37.5 ± 16.7
Females	39.2 ± 12.8
HBsAg positive	57
HBeAg positive	32
HBeAg negative/anti-HBe positive	25
Dose of lamivudine, mg/day	100; orally
Mean HBV DNA, pg/ml	
HBeAg positive patients	1,478 (range 9.5–9000)
HBeAg negative patients	827 (range 2.4–9166)
Mean ALT, IU/l	
HBeAg positive	80.4 ± 28
HBeAg negative	107 ± 36

Results

Baseline Characteristics

All the 57 patients were positive for HBsAg, 32 were positive for HBeAg and 25 were HBeAg negative/anti-HBe positive at baseline. In the 32 HBeAg-positive and in 25 anti-HBe-positive patients, the baseline HBV DNA was 1,478 (range, 9.5–9,000 pg/ml) and 827 pg/ml (range, 2.4–9,166 pg/ml), respectively. Mean baseline ALT in HBeAg-positive and anti-HBe-positive patients was 80.4 ± 28 and 107 ± 36 IU/l, respectively (table 1).

Response to Therapy

Of the 32 HBeAg-positive patients, loss of HBV DNA was achieved in 13 (41%) and loss of HBeAg and normalization of ALT along with undetectable HBV DNA was observed in 8 (25%) patients. Thus, ETR was achieved in 8 (25%) patients. SVR was achieved in 7 (22%) patients. One patient relapsed with HBeAg reappearance. Of the 25 anti-HBe-positive patients, the ETR was seen in 12 (48%) patients, and 10 (40%) patients maintained SVR at 6 months of follow-up. None of the 57 patients lost HBsAg during therapy or follow-up.

Naturally Occurring S-Gene Mutations

Only 2 of 57 (3.5%) patients had two different mutations in their baseline samples. Both mutations were present within the MHR of the S-gene: one at amino acid position 127 (sP127S) and another at 143 (sS143L) in an HBeAg-negative and an HBeAg-positive patient, respec-

Table 2. Naturally occurring surface and overlapping polymerase gene mutations

Patient code	Nucleotide change	Amino acid substitution (HBsAg)	Corresponding change in polymerase	HBeAg	HBV subgenotype	Diagnosis	Response to therapy
A27	C533T	P127S	S135F	–	A1	DC	no
A20	C582T	S143 L	F151F	+	D2	CHB	no

CHB = Chronic hepatitis B; DC = decompensated cirrhosis.

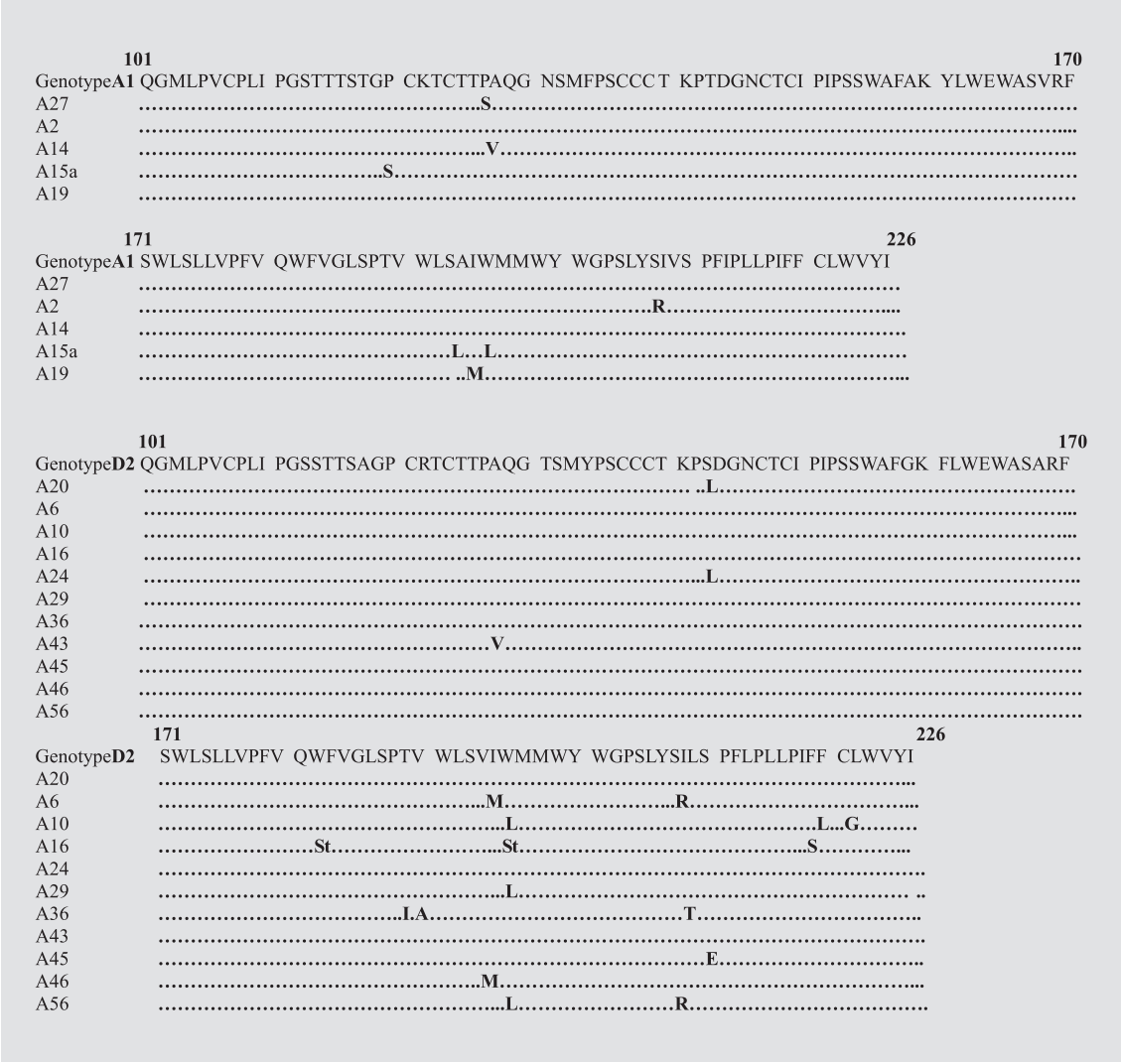

Fig. 1. Alignment of amino acid sequences from 101 till the last amino acid of HBsAg, i.e. aa 226, with the consensus sequences of respective Indian subgenotypes A1 and D2 [13] among 16 patients harbouring S-gene mutations in this study. Patients A20 and A27 had naturally occurring S-gene mutations and the remainder (14 patients) had lamivudine-associated S-gene mutations.

Table 3. Lamivudine-associated surface and overlapping polymerase gene mutations

Patient code	Nucleotide change	Amino acid substitution (HBsAg)	Corresponding change in polymerase	HBeAg	Time of emergence month	HBV subgenotype	Response to therapy
A15a	C512T	P120S	T138I	+	12	A1	no
A14	C537T	A128V	C136C	+	12	A1	yes
A43				+	12	D2	no
A24	C582T	S143L	F151F	+	12	D2	yes
A16	G700A	W182Stop	V191I	+	16	D2	no
A36	C720T	T189I	H197H	–	23	D2	no
A36	T723C	V190A	C198C	–	23	D2	no
A15a	C732T	S193L	F201F	+	13	A1	no
A19	A739G	I195M	M204V	+	18	A1	no
A46				+	30	D2	no
A6				–	26	D2	no
A10	G741T	W196L	M204I	–	20	D2	no
A15a				+	13	A1	no
A29				–	34	D2	no
A56				+	10	D2	no
A16	G741A	W196Stop	M204I	+	16	D2	no
A2	CA772,773TC	S207R	E215S.	–	8	A1	yes
A6				–	26	D2	no
A56				+	10	D2	no
A36	T777C	I208T	H216H	–	23	D2	no
A45	GT783,784AG	S210E	S219A	–	33	D2	no
A16	T810C	F219S	F227F	+	16	D2	no
A10	TT814,815GG	F220L & C221G	L229G	–	20	D2	no

tively (table 2). Both patients remained non-responsive to therapy. One HBeAg-negative patient harbouring a sP127S mutation developed decompensated cirrhosis. Corresponding amino acid substitution for sP127S was rtS135F, whereas sS143L substitution did not affect the overlapping polymerase sequence (table 2). Amino acid sequence alignment for both these mutations is shown in figure 1 with their respective subgenotype.

Lamivudine-Induced S-Gene Mutations

Upon analysis of the mutational profile in samples obtained during therapy, 16 different types of mutations were found emerging at varying time points in 14 of 57 (24.5%) patients (tables 3, 4). The S-gene mutations included sP120S, sA128V, sS143L, sW182St., sT189I, sV190A, sS193L, sI195M, sW196L, sW196St., sS207R, sI208T, sS210E, sF219S, sF220L and sC221G (table 3). Of the 16 types of mutations associated with lamivudine, only 3 (18.7%; sP120S, sA128V and sS143L) were noticed within the MHR. The remainder (13 of 16; 81.2%) of the mutations emerged downstream to the MHR, significantly more often in the region compared to the naturally occurring mutations ($p < 0.05$). All the S-gene mutations associated with lamivudine have been listed in table 3 along with the corresponding changes in polymerase.

Of the 57 patients, 8 developed a rtYM204I/VDD lamivudine-resistant polymerase mutant that in turn was associated with the surface gene mutants as sW196Stop, sI195M and sW196L in 1, 3 and 4 patients, respectively. In 1 of the 8 lamivudine-resistant patients, a unique and novel change of G to A at nt. 741 resulted in a non-sense mutation (sW196Stop) in the surface gene (fig. 1). Interestingly, the same patient had another stop codon at aa 182 (sW182St.) in the surface gene (fig. 1, table 4), which was due to rtV191I substitution in the polymerase gene. This patient also developed hepatocellular carcinoma and died eventually (table 4).

In a patient with decompensated liver disease, a cluster of mutations (sT189I, sV190A and sI208T) was found emerging due to prolonged lamivudine therapy. Interestingly, none of these three mutations affected the polymerase reading frame (table 3). The patient was HBeAg negative at baseline and did not respond to lamivudine with raised viral DNA level till his last visit.

Table 4. Accumulation of lamivudine-associated S-gene mutations in each individual along with HBeAg status, diagnosis and response to therapy

Patient No.	Patient code	HBeAg	Diagnosis	HBV subgenotype	Response to therapy	Mutations
1	A2	–	CHB	A1	yes	S207R
2	A6	–	CHB	D2	no	I195M; S207R
3	A10	–	CHB	D2	no	W196L; F220L; C221G
4	A14	+	CHB	A1	yes	A128V
5	A15a	+	HCC (death)	A1	no	P120S; S193L; W196L
6	A16	+	HCC (death)	D2	no	W182Stop; W196Stop; F219S
7	A19	+	CHB	A1	no	I195M
8	A24	+	CHB	D2	yes	S143L
9	A29	–	CHB	D2	no	W196L
10	A36	–	DC	D2	no	T189I; V190A; I208T
11	A43	+	CHB	D2	no	A128V
12	A45	–	CHB	D2	no	S210E
13	A46	+	CHB	D2	no	I195M
14	A56	+	DC	D2	no	W196L; S207R
Total: 14 patients		HBeAg positive: 8 HBeAg negative: 6			types of mutations: 16	

CHB = Chronic hepatitis B; DC = decompensated cirrhosis; HCC = hepatocellular carcinoma.

Three types of double mutations emerged due to two consecutive nucleotide substitutions. One double mutation at nt. positions 772 and 773 (CA to TC) resulted in change of glutamine to serine at aa 215 in the polymerase gene which, in turn, affected the surface gene as sS207R substitution. In 2 of the 3 patients harbouring the sS207R mutation, it emerged in association with lamivudine-resistant mutant (table 4). Another double mutation led to rtS219A change in the polymerase gene, which in turn resulted in a substitution of sS210E in the surface gene in an HBeAg-negative patient who never responded to the 2-year course of lamivudine therapy. Mutations at nt. 814 and 815 (TT to GG) were also found in a lamivudine-resistant patient affecting both polymerase (rtL229G) and surface genes (sF220L and sC221G; tables 3, 4).

A change of T to C at nucleotide position 810 resulted in sF219S substitution in a lamivudine-resistant patient, but this substitution was not due to any change in the polymerase gene (table 3).

sW196L, sI195M, sS207R and sA128V were present in 4, 3, 3 and 2 patients, respectively, while the other lamivudine-associated mutations were found once in each patient (table 3).

Overall, of the 16 types of S-gene mutations observed with lamivudine therapy, 9 (sP120S, sW182St., sI195M, sW196L, sW196St., sS207R, sS210E, sF220L and sC221G) mutations were associated with the changes occurring in the polymerase gene, too (table 3).

sS143L mutation was the only mutation which was noticed in the baseline sample of a patient (table 2), and it also emerged in response to lamivudine therapy in another patient (table 3). All the other mutations were either present in the baseline sample or emerged after lamivudine therapy.

S-Gene Mutations and Response to Therapy

Sixteen of 57 (28%) CHB patients receiving lamivudine had surface and/or overlapping polymerase gene mutations, 2 (3.5%) patients had mutations at the baseline while 14 (24.5%) developed these during the course of therapy. The remaining 41 (72%) patients always had wild-type surface and overlapping polymerase gene sequences. Seventeen of the 41 (41.5%) patients with wild-type sequences responded to the lamivudine therapy, whereas only 3 of 16 (18.8%) patients harbouring S-/Pol-gene mutation/s responded to lamivudine therapy ($p < 0.05$; table 5). The baseline viral DNA was significantly higher in the patients who developed S-gene mutations (n = 16) compared with the other patients (n = 41; 2,093 vs. 336 pg/ml; table 5). Patients with polymerase gene

Table 5. Comparing the viral load, ALT and response rate between patients with and without S-gene mutations

	Patients with S-gene mutations (n = 16)	Patients without S-gene mutations (n = 41)	p value
DNA (baseline)	2,093 pg/ml	336 pg/ml	<0.05
ALT (baseline)	99 ± 22 IU/l	93 ± 19 IU/l	NS
Response rate (ETR)	3 of 16 (18.8%)	17 of 41 (41.5%)	<0.05

mutations (n = 11) also had higher baseline viral DNA compared to those with wild-type polymerase gene (n = 46; 2,205 vs. 337 pg/ml, $p < 0.05$). The aminotransferase levels were however comparable between the patients with and without S-gene mutations (table 5).

Discussion

Therapy with lamivudine results in several mutations in the polymerase gene, some of which are associated with alterations in the 'a' determinant of HBsAg [15]. These drug-resistant isolates may have the potential to become vaccine escape mutants. Following lamivudine therapy, a series of S-gene mutations were seen to emerge at varying time points in the present study. Fourteen of the 57 (24.5%) patients on long-term lamivudine showed lamivudine-associated mutations. Since the ORF encoding HBsAg overlaps with that of the polymerase, mutations within the former may affect the latter or vice versa, with knock-on effect on viral replication [16]. The interesting feature of mutations associated with prolonged lamivudine therapy was their emergence downstream to the MHR. Only sP120S substitution emerged within MHR, but upstream to the 'a' determinant. This mutation has been found earlier in an immunized infant born to a chronic HBsAg-positive mother. Consistently, a decreased binding to the 'a' determinant-specific monoclonal antibody was demonstrated for the HBsAg variant sP120S [17].

Recent reports show that even mutations emerging outside the MHR also lead to decreased antibody binding. It has been found that the sF183C mutation had decreased binding of the HBsAg to the 'a' determinant-specific antibody [17]. In the light of these reports, emergence of mutations outside the MHR due to long-term lamivudine therapy is a matter of serious concern, as significantly more mutations were found to develop downstream to the MHR in patients of the present study.

Few lamivudine-selected mutants occurring downstream to the MHR have been studied earlier. For instance, sE164D, sW196S and sM198I showed reduced binding of HBsAg with the antibody. Moreover, sI195M has been shown to have moderately reduced binding of HBsAg with anti-HBs [18]. In Indian patients undergoing lamivudine therapy, most frequently drug-selected surface gene mutants are at aa 195 and 196. In agreement with earlier findings, isoleucine at aa 195 was substituted with methionine, which may be responsible for moderately reduced affinity to bind anti-HBs in the process of Ag-Ab neutralization. The sI195M mutation was selected in 3 of our patients. Whereas, unlike the substitution of tryptophan with serine studied by Torresi et al. [18], in our patients, it was replaced with leucine in 4 patients and with a stop codon in 1 patient. We do not know the effect of the sW196L substitution on HBsAg binding with anti-HBs. However, in view of HBsAg conformation stability, it is certain that any type of amino acid substitution within aa 192–200 may alter the HBsAg-antiHBs binding affinity. At this conformational region, the HBsAg protein consists of two short α- helices separated by a disulphide bridge forming a turn [19]. Sequence changes within this region may alter the structural integrity of the protein and may alter binding to anti-HBs antibody without affecting the assembly or secretion of the virus [20]. In one of our patients within this region, tryptophan turned to be stop codon resulting in premature termination of the HBsAg. In the same patient, another stop codon mutation leading to premature termination of the HBsAg, 13 amino acids upstream, was also seen. Till now there are only a few reports regarding the emergence of lamivudine-induced mutations which lead to premature termination of HBsAg. In an in vitro cell culture system, Yeh et al. [12] reported the coexistence of a stop codon (at aa 172) in the surface gene, severely impairing the secretion of HBsAg. Tillmann et al. [21] reported the emergence of rtA181T polymerase mutant due to famciclovir therapy. With famciclovir therapy, a similar mutation at rtV207I in the 'C' domain of viral polymerase leading to a stop

codon at aa 199 in overlapping surface protein was reported [22]. In all these patients, antiviral resistance and impaired secretion of HBsAg was shown in the in vitro culture system. In the present study, similar mutations were observed, but at novel loci, which resulted in the premature termination of HBsAg. Additionally, both the mutations (sW182Stop and sW196Stop) were in the same patient. The significance of finding the HBsAg prematurely terminated at two different loci at an interval of 12 aa is unclear. It is possible that these two mutations might be present in different viral populations. However, it is not clear why they emerged simultaneously. HBsAg positivity in this patient may be due to the mixed population of the viruses having intact surface reading frame or due to the nuclear covalently closed circular DNA that remained wild type.

Insight into viral mutations has also come by exploring the regions of HLA class II-restricted epitopes within the entire HBsAg. Four class II-restricted epitopes within HBsAg have been described till date [23]. Two of these lie downstream to the proposed MHR, and variations in these epitopes have been established to influence the T-cell proliferation, antiHBc and IFN-γ production in in vitro systems [23].

Within the recently proposed fourth epitope (aa 213–226) also, three types of amino acid substitutions emerged in 2 of our patients, both of them received long-term lamivudine therapy but remained non-responders throughout. sF219S emerged at month 16 in a patient and sF220L and sC221G emerged simultaneously at month 20 in another patient. The sC221G mutant may be important as substitutions involving replacement or introduction of glycine or proline residues, which are α-helix breakers, may seriously affect secondary and tertiary structures of the HBsAg and impair the T-cell response [23]. The increasing use of antiviral agents, directed against the polymerase, could lead to new mutations in the molecule and in HBsAg, as sequences of these two molecules are partially identical [22, 24, 25].

During lamivudine therapy, 9 of the 16 mutations in the surface gene (sP120S, sW182St., sI195M, sW196L, sW196St., sS207R, sS210E, sF220L and sC221G) emerged due to changes occurring in the polymerase gene (table 3). The other 7 mutations (sA128V, sS143L, sT189I, sV190A, sS193L, sI208T and sF219S), though developed in patients who were on long-term lamivudine therapy, did not have associated changes in the polymerase reading frame.

These mutations are significantly associated with a decreased response rate as only 3 of the 16 (18.8%) S-gene mutant patients responded to therapy compared to 17 of 41 (41.5%) patients with completely wild-type S-gene sequence at every point of time ($p < 0.05$).

In summary, lamivudine therapy led to emergence of significantly more S-gene mutants compared to the naturally occurring S-gene mutations ($p < 0.05$). Naturally occurring mutations are restricted to the 'a'-determinant region, whereas drug-associated mutations generally occur downstream to the MHR. Seven types of surface mutations, which emerged during prolonged antiviral therapy, did not affect the polymerase region at all. Whether they emerged in response to therapy or under the immune selection pressure needs to be further investigated. Additionally, whether the changes arising outside the MHR of HBsAg are contributing to the formation of antigenic epitopes of varying specificity and if they are also affecting the reactivity with anti-HBs is of considerable interest. Mutations (sW182St. and sW196St.) resulting in premature S-gene termination should also be studied further as they may affect the processes involved in virion assembly and surface antigen formation. Careful studies of the molecular epidemiology of hepatitis B variants arising due to prolonged usage of nucleoside and nucleotide analogues should extensively be done before their widespread usage.

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