Whole genome analysis of hepatitis B virus before and during long-term therapy in chronic infected patients: Molecular characterization, impact on treatment and liver disease progression

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Hepatitis B virus (HBV) infection remains a serious public health concern worldwide despite the availability of an efficient vaccine and the major improvements in antiviral treatments. The aim of the present study is to analyze the mutational profile of the HBV whole genome in ETV non-responder chronic HBV patients, in order to investigate antiviral drug resistance, immune escape, and liver disease progression to Liver Cirrhosis (LC) or Hepatocellular Carcinoma (HCC). Blood samples were collected from five chronic hepatitis B patients. For each patient, two plasma samples were collected, before and during the treatment. Whole genome sequencing was performed using Sanger technology. Phylogenetic analysis comparing the studied sequences with reference ones was used for genotyping. The mutational profile was analyzed by comparison with the reference sequence M32138. Genotyping showed that the studied strains belong to subgenotypes D1, D7, and D8. In particular, cG1764A, cC1766G/T, cT1768A, and cC1773T in the BCP; cG1896A and cG1899A in the C gene. The mutational analysis showed high genetic variability. In the RT region of the polymerase gene, 28 amino acid (aa) mutations were detected. The most significant mutations were the pattern rtL180M + rtS202G + rtM204V, which confer treatment resistance. In the S gene, 35 mutations were detected namely sP120T, sT126S, sG130R, sY134F, sS193L, sI195M, and sL216stop were previously described to lead to vaccine, immunotherapy, and/or diagnosis escape. In the C gene, 34 mutations were found. In particular, cG1764A, cC1766G/T, cT1768A, and cC1773T in the BCP; cG1896A and cG1899A in...
the precore region and cT125, cE64D, cA80T, and cP130Q in the core region were associated with disease progression to LC and/or HCC. Other mutations were associated with viral replication increase including cT1753V, cG1764A/T, cC1766G/T, cT1768A, and cC1788G in the BCP as well as cG1896A and cG1899A in the precore region. In the X gene, 30 aa substitutions were detected, of which substitutions xT36D, xP46S, xA47T, xI88F, xA102V, xI127T, xK130M, xV131I, and xF132Y were previously described to lead to LC and/or HCC disease progression. In conclusion, our results show high genetic variability in the long-term treatment of chronic HBV patients causing several effects. This could contribute to guiding national efforts to optimize relevant HBV treatment management in order to achieve the global hepatitis elimination goal by 2030.

**KEYWORDS**

HBV, antiviral resistance, liver cirrhosis, PCR, whole genome, Sanger sequencing, hepatocellular carcinoma

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**Introduction**

Hepatitis B virus (HBV) infection remains a serious public health concern worldwide despite the availability of an efficient vaccine and the major improvements in antiviral treatments. The World Health Organization (WHO) estimates that, in 2021, approximately 296 million persons are chronic HBV carriers. Among them, 820,000 represent a high risk of mortality caused by developing progressive liver diseases including hepatocellular carcinoma (HCC) and liver cirrhosis (LC) (WHO, 2021).

The genome of HBV is a circular DNA partially double-stranded of 3.2 kb and classified into 10 genotypes from A to J (Sunbul, 2014). It is organized into four main open overlapped reading frames (ORFs; pre-S1/pre-S2/S, pre-C/C, P, and X), encoding several proteins including the surface proteins S, M, and L holding the HBs antigen (HBsAg), the precore/core proteins holding HBeAg and HBcAg antigens, the polymerase (P), and the X protein holding the antigen HBxAg. Thus, mutations that occur in one gene can result in significant changes in the other overlapping genes.

Long-term treatment of HBV chronic patients with the available antiviral molecules can lead to the emergence of mutations throughout the whole genome. Mutations that occur within the reverse transcriptase (RT) domain of the P gene, target of antiviral treatment, may lead to treatment failure (Locarnini and Mason, 2006). Potential resistance-related mutations are grouped into 4 categories, primary mutations (category 1) could reduce antiviral susceptibility and HBV replication fitness. Secondary/compensatory mutations (category 2) developed subsequently and could restore functional defects in the RT activity of HBV caused by primary mutations. Putative antiviral resistance mutations (category 3) were reported as possible drug-resistant mutations but not verified experimentally and may be related to prolonged treatment or replication compensation. Pre-treatment mutations (category 4) could be found among treatment-naive patients but their role in antiviral treatment resistance has not been elucidated (Liu et al., 2010; Ciftci et al., 2014).

Moreover, mutations that emerge throughout a prolonged therapy could affect not only the RT region (Locarnini and Mason, 2006) but also the different overlapping genes. Therefore, such variations might result in hepatitis B immunoglobulin (HBIG) therapy escape, vaccine escape, misdiagnosis, and immune escape. They also could enhance viral replication capacity and viral persistence leading to the progression of severe liver diseases such as HCC or LC (Sheldon et al., 2006; Sheldon, 2008; Rajoriya et al., 2017).

On the other hand, it has been found that the presence of pre-existing naturally occurring mutations in treatment-naive patients may influence the efficacy of antiviral treatments. Therefore, knowledge of the mutational profile by whole genome sequencing of the HBV genome, for chronically infected patients, is of great interest for a complete diagnosis toward an efficient therapy scheme.

For HBV chronic patients in Tunisia, the national therapeutic schema is based on Entecavir (ETV) as a first-line of HBV treatment, and it is fully covered by the National Health Insurance Fund (NHIF), in case of resistance, Tenofovir disoproxil fumarate.
(TDF), is recommended alone or combined to ETV. However, the TDF is not covered by the NHIF. The aim of the present study is to analyze the mutational profile through the HBV whole genome in ETV non-responder chronic HBV patients, in order to investigate antiviral drug resistance, immune escape, and liver disease progression to LC or HCC.

Materials and methods

Patients and samples

HBV chronic patients with quantifiable viral load and suspected to be ETV non-responders after viral breakthrough were included in the study. Blood samples were collected from five chronic hepatitis B patients investigated during the routine diagnostic activity of the Laboratory of Clinical Virology in Pasteur Institute of Tunis. For each included patient two plasma samples were collected: one before treatment as part of the pre-treatment diagnostic, and one during the treatment upon request of the treating physician. The period separating the second sample from the date of treatment beginning ranging between 14 and 72 months depending on the time of the viral breakthrough for each patient. Virological and clinical data are shown in Table 1.

Methods

DNA extraction, amplification, and sequencing

DNA was extracted from 200 μl of plasma using the Qiagen QIAamp® DNA extraction kit (QIAGEN® Inc., Hilden, Germany) according to the manufacturer’s instructions. Three pairs of primers previously described by Chekaraou et al. (2010) were used to amplify 3 overlapping amplicons of 1,228-bp (nt 2,817–863), 1,253 bp (nt 448–1,701), and 1,653 bp (nt 1,609–80) covering the whole HBV genome as shown in Figure 1.

PCR reactions were performed in 50 μl of reaction mixture containing 1X polymerase buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.2 μM of each primer, 1.25 U of Taq Core MP® (Applied Biosystems) and nuclease-free water. The amount of DNA extract added varied between 10 to 35 μl depending on the viral load. PCR cycling was as follows: 94°C for 5 min, 40 cycles (94°C for 1 min, 56°C/57°C/62.5°C for regions 1, 2, and 3, respectively, 72°C for 1 min) with a final extension step at 72°C for 10 min. PCR products were analyzed by electrophoresis on 1% agarose gels stained with 1,25X of Red gel™ dye Nucleic Acid (Biotium®) and visualized by UV transilluminator.

The purified template DNA was sequenced using a BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems) using the same primers pairs on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems).

HBV genome assembly, genotyping, and subtyping

The obtained overlapping sequences were then assembled using BLAST multiple sequences software by comparison with a reference sequence (M32138). The generated final sequences were submitted to Genbank under accession numbers: MT591274-MT591281 and OP121186.

Sequence alignment was performed with MAFFT online server using default parameters by comparing the obtained
genomic sequences with 58 reference sequences representing the 10 HBV genotypes (A–J) and their corresponding subgenotypes. The resulting alignment was used to build a maximum likelihood phylogenetic tree using the IQ-TREE web server, supported by 1,000 bootstrap replicates. The phylogenetic tree was then visualized using Figtree software. The tree was rooted using the midpoint rooting method. Genotypes were also confirmed by the National Center for Biotechnology Information’s (NCBI) E-genotype online software. HBV subtypes were inferred from sequences of the S gene by identifying amino acids (aa) at positions 122, 160, 127, 140, and 159 according to an algorithm previously described (Purdy et al., 2006).

Mutation analysis

Mutational profiles of the nucleotide or amino acid sequences were determined by comparing each gene (P, S, C, and X) before and during treatment with the corresponding reference sequence using Mega 7.026 (Kumar et al., 2016). Mutations’ impacts on treatment, immune response, and liver disease progression were analyzed based on the literature.

Results

HBV whole genome assembly

Whole genome sequences were obtained before and during treatment for 3 patients (1, 2, and 3). For the two remaining patients (4 and 5) we succeeded to obtain the whole genome before treatment. During treatment, the obtained sequence of patient 4 was lacking 408 bp (from nucleotide 45 to nt 453) and for
patient 5 we could not be able to amplify the HBV genome which could be due to the low viral load.

**HBV genotyping and subtyping**

Phylogenetic analysis (Figure 2) showed that all the sequences belong to genotype D. Subgenotyping showed that patients 1 and 4 were infected with subgenotype D7; patients 2 and 3 with D1 and patient 5 with D8, supported by high bootstrap values: 100, 100, and 85, respectively.

Subtyping showed that the studied HBV strains belonged to the ayw2 subtype based on Arg122, Lys160, Pro127, Tyr140, and Gly159 positions.

**Genetic variability in the P, S, C, and X genes**

**Mutational profile of the RT region in the polymerase gene**

The mutational analysis of the RT region revealed a total of 28 aa substitutions ranging between 7 and 12 per patient, among them, several potential resistance-related mutations were detected. Primary mutations (category 1), rtS202G and rtM204V, occurred in patients 2 and 3 during treatment. Secondary/compensatory mutations (category 2), were found in 8 aa replacements; 5 were detected before treatment (rtL91I and rtT128N in patient 2; rtQ149K and rtP237T in patients 1, 4, and 5; rtQ267H in patients 4 and 5) and 3 changes emerged during treatment (rtL180M in patients 2 and 3; rtQ215S and rtF221Y in patient 2).

Three putative antiviral resistance mutations (category 3) were detected: rtR153W in treatment-naïve patients 1, 4, and 5 as well as rtD134E and rtC256S during treatment in patients 3 and 1, respectively. Three pre-treatment mutations (category 4) were also found: rtR110G and rtI266R in patient 1 and rtD263E in patient 5.

Mutations that did not fit categories 1 to 4 were classified into “novel amino acid substitutions” and were observed in 12 aa positions. Six variations namely rtE11D, rtH54Y, rtW257Y, rtD263E, rtQ267Y, and rtE271D were found in treatment-naïve patients and six variations namely rtL145M, rtL260F, rtQ267R, rtK270R, rtM309K, and rtN337T occurred during treatment. The aa changes detected in the RT region of the P gene are mentioned in Table 2; Figure 3.

**Mutational analysis of the pre-S/S coding regions**

A total of 35 aa substitutions were observed in the whole S gene ranging between 3 and 18 mutations per patient, most of them (n = 16) were located in the S region. In the pre-S1 and pre-S2 regions, n = 9 and n = 10 substitutions were observed, respectively. Mutations detected in the S gene are summarized in Table 3; Figure 3. Out of the 16 aa changes in the S region, 10 were clustered in HBsAg epitopes including B-cells epitopes (aa100-160) as follows: 6 (sN3T, sL42R, sL49R, sT57I, sC76Y, and sQ101H) in HBs1 (upstream of aa120); 1 (sP120T) in HBs2 (aa120–123) and 3 (sT126S, sG130R and sY134F) in HBs3 (aa124–137). Furthermore, the major hydrophilic region (MHR, aa99–169) of the S region had accumulated 5 aa variations of which three were within the HBsAg “a” determinant region (aa124–147).

In addition, nine mutations out of the 16 substitutions in the S gene occurred in different CD4 and CD8 recognition epitopes with the following distribution: 6 aa changes (sL42R, sL49R, sT57I, sI193L, sI195M, and sL216) in T-helper CD4 epitopes (aa21–65/aa186–197/aa215–223) and 3 (sS207R, sP211R, sL213I) within cytotoxic T lymphocyte CD8 epitopes (aa206–215).

Immune escape mutations (sQ101H, sG130R, sY134F, sS207R, and sL213I) were detected in patients 2 and 5, HBsAg vaccine escape mutations (sP120T, sT126S, sI193L, and sI195M) were found in patients 2 and/or 3, HBIG immunotherapy escape mutations (sP120T and sT126S) were detected in patients 2 and 3, respectively, and misdiagnosis mutations (sP120T, sT126S, sI195M, and sL216stop) were observed in patients 2 and/or 3. Other mutations such as sN3T, sL42R, sC76Y, and sP211R are either not reported or with unknown impacts are also detected in our study.

**Mutational analysis of the basal core promoter BCP, precore, and core coding regions**

The analysis of the 9 HBV genomic sequences bearing the BCP, pre-C, and Core regions is summarized in Table 4. In the BCP region, 14 different aa mutations were identified over 10 sites ranging between 2 and 8 per patient. Nucleotide mutation G1757A was detected in patients 2, 3, 4, and 5; A1762T, G1764A, and T1753V were detected in patients 1, 3, and 5; C1773T in patients 2 and 3, G1764T/C1766G in patient 2 and C1766T/T1768A in patient 3. The double mutation G1764A/A1762T was found in patients 1, 3, and 5.

In the precore region, 2 mutations were identified: G1896A (patient 2) and G1899A (patients 1, 2, and 3). Whereas, 22 mutations were observed in the core region ranging between 2 and 8 per patient. Among them, several amino acid substitutions were found within different antigen immunogenic epitopes. In particular, 4 aa changes were detected in the T-helper CD4 epitopes (aa35–45 and 48–69; cE40D/Q, cE64D, cT67N, cA69G), 5 in the B-cell epitopes (aa76–89, 105–116, 130–135; cP79Q, cA80T/S, cV85I, cI116V and cP130Q) and 7 in the CTL CD8 epitopes (aa18–27, 50–69, 74–83, 141–151; cS21T, cE64D, cT67N, cA69G, cV74S/G, cA80T/S, and cR151Q). The detected nucleotide/aa mutations found in the C gene are presented in Table 4; Figure 3.

**Mutational analysis in the X coding region**

In total, 30 aa substitutions were found in the X gene region ranging between 4 and 14 aa substitutions per patient of which 29 were before treatment beginning and only 1 was during it. Among
them 10 variations were detected in the B-cell epitope (aa 29–48) namely: xL34I, xT36D/G, xS38P, xS39P, xP40S, xL42P, xS43P, xP46S and xA47T. Four mutations; xK95N, xL98I, xA102, and xT105M; were detected within the T-helper CD4 epitope (aa 91–105) and 2 substitutions (xD119N and xL123W) were detected in CTL CD8 epitope (aa115–123).

As BCP overlaps partially with the HBx coding sequence, mutations at nucleotide positions T1753C/A/G, T1762T, G1764A/T, and C1788G; induce amino acid changes xI127T/D/G, xK130M, xV131I/L and xH139D near the C-terminus of the HBx protein, respectively.

The amino acid substitutions detected in the HBV X gene and their impact are summarized in Table 5; Figure 3.

**Discussion**

In the present study, we have succeeded to generate the whole HBV genome by amplifying 3 overlapping PCR products covering the entire genome (3.2 kb) using Sanger technology. This technology remains of great importance despite the transition of most laboratories to Next generation sequencing (NGS) technologies. In fact, for small genomes, such as HBV, the Sanger technology is cost effective and more efficient for low viral loads < 10^3 IU/ml.

The whole genome was assembled for the 5 patients before the treatment and for 4 patients during the treatment. HBV genome was used for genotyping as well as to study the mutational profile.
TABLE 2. Amino acid substitutions detected within the RT region sequences of the five HBV Chronic infected patients with their reported antiviral resistance.

| Amino acid substitution | Mutation category | Patients | Drug resistance | Change in overlapping genes | References |
|-------------------------|-------------------|----------|-----------------|-----------------------------|------------|
| E11D                    | Novel mutation    | P4       | –               | Unknown                     | N.C        |
| H54Y                    | Novel mutation    | P4/P5    | P3              | Unknown                     | N.C        |
| N76D                    | Novel mutation    | –        | P2              | Clinical failure of famciclovir | N.C       |
| L91I                    | Secondary/        | P2       | P2              | LMV ETV                     | N.C        |
|                         | compensatory      |          |                 |                              |            |
| R110G                   | Pre-treatment     | P1       | P1              | Potential resistance        | N.C        |
| T128N                   | Secondary/        | P2       | P2              | LMV                         | sP120T     |
|                         | compensatory      |          |                 |                              |            |
| D134E                   | Putative          | –        | P3              | TDF                         | sT126S     |
| L145M                   | Novel mutation    | –        | P4              | Unknown                     | N.C        |
| Q149K                   | Secondary/        | P1/P4/P5 | P1/P4           | Unknown                     | N.C        |
|                         | compensatory      |          |                 |                              |            |
| R153W                   | Putative          | P1/P4/P5 | P1/P4           | TDF                         | N.C        |
| L180M                   | Secondary/        | –        | P2/P3           | LMV, ETV, LdT, TDF          | N.C        |
|                         | compensatory      |          |                 |                              |            |
| S202G                   | Primary           | –        | P2/P3           | LMV, ETV                    | sS193L     |
| M204V                   | Primary           | –        | P2/P3           | LMV, LdT, ETV, TDF          | sL195M     |
| Q21SS                   | Secondary/        | –        | P2              | LMV, ADV                     | sS207R     |
|                         | compensatory      |          |                 |                              |            |
| F221Y                   | Secondary/        | –        | P2              | ADV                         | sL213I     |
|                         | compensatory      | P1/P4/P5 | P1/P4           | ADV                         | N.A        |
| C256S                   | Putative          | –        | P1              | LMV, TDF                    | N.A        |
| W257Y                   | Novel mutation    | P2/P3    | P2/P3           | Unknown                     | N.A        |
| L260F                   | Novel mutation    | –        | P4              | Unknown                     | N.A        |
| D263E                   | Pre-treatment     | P5       | –               | Potential partial resistance to TDF | N.A        |
| I266R                   | Pre-treatment     | P1       | P1              | Unknown                     | N.A        |
| Q267H/R/Y               | Secondary/        | P4/P5    | P4              | H: LMV, LdT                  | N.A        |
|                         | compensatory      |          |                 |                              |            |
| K276R                   | Not reported      | P1       | P1              | Unknown                     | N.A        |
| E271D                   | Novel mutation    | P4       | P4              | Unknown                     | N.A        |
| M309K                   | Novel mutation    | –        | P3              | Unknown                     | N.A        |
| N337R                   | Not reported      | –        | P2              | unknown                     | N.A        |

P1–P5 = patients 1–5. NC, no change; mutation is silent in the surface antigen reading frame. NA, not applicable; polymerase substitution is downstream of the surface antigen reading frame. ADV, Adefovir dipivoxil; ETV, Estecavir; LdT, Telbivudine; LMV, Lamivudine; TDF, Tenofovir disoproxil fumarate.
in all the genes (S, P, X, and C) in order to give scientific proof of antiviral treatment resistance.

Genotyping showed that genotype D was detected in the 5 studied patients. This genotype was previously described as a predominant HBV genotype in Tunisia and the Maghreb region as well as in the Middle East with a low co-circulation rate of genotype E (Ayed et al., 2007; Ezzikouri et al., 2008; Ouneissa et al., 2013).

Subgenotypes D1 and D7, found in the present study, were previously described as the most prevalent subgenotypes circulating in Tunisia (Meldal et al., 2009). However, subgenotype D8 is to our knowledge detected for the first time in Tunisia. This subgenotype has been firstly detected in Niger and has been described as a recombinant strain between genotypes D and E (Chekaraou et al., 2010). The recombination analysis of the detected D8 strain, using the NCBI viral genotyping tool, was in line with the previous findings. Further studies are needed on larger population size to estimate the prevalence of this subgenotype in Tunisia.

In the second part of the present study, we have analyzed the mutational profile of all HBV genes P, S, C, and X.

The mutational profile of the RT region in the P gene showed high genetic variability with 28 different mutations. Before the treatment, 14 aa mutations were detected of which patient 2 had already 2 secondary/compensatory substitutions: rtL91I and T128N, described to be a resistance mutation to ETV and/or to LMV, respectively (Torresi et al., 2002a; Mahabadi et al., 2013; Ziaee et al., 2016). For the remaining patients, four mutations were detected and reported to be resistant to at least one of the following antivirals: rtQ267H in patients 4 and 5 to LMV and LdT; rtP237T in patients 1, 4, and 5 to ADV; rtR153W in patients 1, 4, and 5 in addition to rtD263E in patient 5 potentially to TDF (Pollicino et al., 2009; Qin et al., 2013b; Bakhshizadeh et al., 2015; Mokaya et al., 2020). The eight remaining substitutions were not previously described to have an impact on antiviral treatment.

During the treatment, 14 additional aa substitutions occurred. The most significant ones were rtM204V, rtL180M, and rtS202G detected in patients 2 and 3. Indeed, it has been described that the rtM204V substitution is usually associated with the compensatory mutation rtL180M, which restores the replication capacity of rtM204V mutants (Tenney et al., 2004). Thus, the pattern rtL180M, rtS202G, and rtM204V act synergistically not only to increase viral load but also to reduce treatment susceptibility and confer cross-resistance to ETV, TDF, LMV, and LdT (Kamiya, 2003; Li et al., 2005; He et al., 2015; Mokaya et al., 2020). Other emerged aa variations have been detected in our patients and previously described as resistance mutations that reduce the affinity and susceptibility to antiviral drugs namely rtQ215S and rtD214S.

FIGURE 3
Hepatitis B genome map with summary of the most significant mutations and their clinical impact detected in 5 Tunisian HBV patients. A representation of the four overlapping genes encoding the polymerase P, the surface gene S, the capsid gene C, and X gene. The detected significant mutations are grouped depending on their clinical impact based on the literature. BCP, Basal core Promotor; C, core; PreC, precore; S, surface; RT, reverse transcriptase; HBIG, Immunoglobulin.
### TABLE 3 Amino acid substitutions within the HBV surface gene sequences from the studied patients with their impact.

| Region       | Cell subsets | Amino acid substitution | Patients Treatment | During treatment | Effects                                                                 | References |
|--------------|--------------|-------------------------|--------------------|------------------|------------------------------------------------------------------------|------------|
| Pre S1 region|              | A28T                    | P1/P5              | P1               | Unknown                                                                | Taghiabadi et al. (2019) |
|              |              | A28N                    | P3                 | P3               | Unknown                                                                | Feeney et al. (2013)   |
|              |              | T40P                    | –                  | –                | Unknown                                                                | Pourkarim et al. (2014) |
|              |              | H60D                    | P3                 | –                | Unknown                                                                | Not reported           |
|              |              | P78T                    | S85C               | –                | –                                                                      |                        |
|              |              | IT4L                    | P2/P3              | P2/P3            | Unknown                                                                | Mondal et al. (2015)   |
|              |              | S90L                    | P1                 | P1               | Unknown                                                                | Not reported           |
|              |              | N103D                   | P5                 | P2               | Unknown                                                                | Mondal et al. (2015)   |
|              |              | T11N                    | P3                 | P3               | Unknown                                                                | Pourkarim et al. (2014) |
|              |              | R16K                    | P2                 | –                | Unknown                                                                | Pourkarim et al. (2014) |
|              |              | R18K                    | P1                 | P1/P2            | Unknown                                                                | Lago et al. (2014)     |
|              |              | F22L                    | P1                 | P1/P2            | Association with HCC progression                                      | Gopalakrishnan (2013); Chaudhuri et al. (2004) |
|              |              | N33D                    | –                  | P2               | Unknown                                                                | Kim et al. (2013)      |
|              |              | A93V                    | P2/P3/P4           | P2/P3            | Unknown                                                                | Pollicino et al. (2007) |
|              |              | P41H                    | P2/P3/P5           | P2/P3            | Unknown                                                                | Pollicino et al. (2007) |
|              |              | I42T                    | P3                 | P2/P3            | Unknown                                                                | Kim et al. (2010)      |
|              |              | F46S                    | P2                 | –                | Unknown                                                                | Olinger et al. (2007)  |
|              |              | P52L                    | P3                 | –                | Unknown                                                                | Pollicino et al. (2007) |
| S region     | Other        | N3T                     | P4                 | –                | Unknown                                                                | Not reported           |
|              | T-helper (CD4) epitope (aa21-65) | L42R                  | –                  | P2               | Unknown                                                                | Chaouch et al. (2016)  |
|              |              | L49R                    | P3                 | –                | Association with LC progression                                       | Chaouch et al. (2016)  |
|              |              | T57I                    | P5                 | –                | Reduced HBsAg antigenicity                                            | Duda (2020)            |
|              | Other        | C76Y                    | P5                 | –                | Unknown                                                                | Wei et al. (2011)      |
|              | B-cell epitope (aa 100–160) | Q101H                  | P2                 | P2               | - Immune escape                                                       | Amini-Bavil-Olyaee et al. (2010); Bahramali et al. (2008) |
|              |              | P120T                   | P2                 | P2               | - HBIG therapy escape                                                |                        |
|              |              |                         |                    |                  | - Misiagnosis                                                         |                        |
|              |              |                         |                    |                  | - Vaccine escape                                                       |                        |
|              |              |                         |                    |                  | - Reduced HBsAg secretion                                             |                        |
|              |              |                         |                    |                  | - HBIG therapy escape                                                |                        |
|              |              |                         |                    |                  | - Vaccine escape                                                       |                        |
|              |              |                         |                    |                  | - Misiagnosis                                                         |                        |
|              |              |                         |                    |                  | - Reduced in vitro affinity to anti-HBs antibodies.                   |                        |
|              |              | T126S                   | –                  | P3               | - Immune escape                                                       | Moerman et al. (2004); Sitnik et al. (2004) |
|              |              | G130R                   | P5                 | –                | - Immune escape                                                       | Kwei et al. (2013); Tokgoz et al. (2018) |
|              |              | Y134F                   | P2                 | –                | - Immune escape                                                       | Chaouch et al. (2016); Coppola, (2015) |
|              | T-helper (CD4) epitope (aa 186–197) | S193L                  | –                  | P2               | - Vaccine escape                                                       | Aydin et al. (2019); Sunkur et al. (2019) |
|              |              | I195M                   | –                  | P2/P3            | - Misiagnosis                                                         | Colagrossi et al. (2018); Torresi et al. (2002b); Araujo et al. (2008) |
|              | CTL (CD8) epitope (aa 206–215) | S207R                  | –                  | P2               | - Immune escape                                                       | Hosseini et al. (2019) |
|              |              | P211R                   | –                  | P4               | Unknown                                                               | Choga et al. (2020)    |
|              |              | L213J                   | –                  | P2               | - Immune escape                                                       | Hosseini et al. (2019); Datta et al. (2014) |
|              | T-helper (CD4) epitope (aa 215–223) | L216stop               | –                  | P3               | - Truncated HbsAg protein                                             | Araujo et al. (2008); Hosseini et al. (2019) |

P1–P5 = Patients 1–5. HCC, hepatocellular carcinoma; LC, liver cirrhosis.
| Region            | Cell subsets | Substitution | Amino acid | Nucleotide | Patients | Treatment during treatment | Effects                                                                 | References                                                                                   |
|-------------------|--------------|--------------|------------|------------|----------|---------------------------|--------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|
| Basal core promoter | naïve        | A1752G       | P3         | P3         | - Low viral replication capacity | Quarleri (2014); Ng et al. (2005)                                                                 |
|                   | During treatment | T1753V (C/A/G) | P1/P5 (C) | P3 (G)     | - Increase viral replication | Caliguri et al. (2016); Parekh et al. (2003)                                              |
|                   |              | G1757A       | P2/P3/P4/P5 | P2/P3/P4   | - Reduction in HBeAg synthesis | Poustchi et al. (2008); Ducancelle et al. (2013); Mohamadkhani et al. (2011)             |
|                   |              | A1762T       | P1/P3/P5   | P1/P3      | - Protection from liver disease progression to LC and/or HCC |                                                                                              |
|                   |              | G1764A       | P1/P3/P5   | P1/P3      | - Reduction in HBeAg synthesis | Quarleri (2014); Chen et al. (2005); Leng et al. (2015); Yan et al. (2015); Fang et al. (2008) |
|                   |              | G1764T       | P2          | P2          | - Increase viral replication | Sendi et al. (2005); Poustchi et al. (2008)                                                |
|                   |              | C1766G       | P2          | P2          | - Increase core promoter activity | Sendi et al. (2005); Poustchi et al. (2008); Salarneia et al. (2016)                        |
|                   |              | C1766T       | P3          | P3          | - Increase viral replication | Tong et al. (2013); Kitab et al. (2012); Nishizawa et al. (2016)                           |
|                   |              | T1768A       | P3          | P3          | - Reduction in HBeAg synthesis |                                                                                              |
|                   |              | C1773T       | P2/P3       | P2/P3      | - Increase viral replication | Yin et al. (2011); Jammeh et al. (2008); Huang et al. (2011)                               |
|                   |              | C1788G       | –           | P4          | - Increase viral replication |                                                                                              |
|                   |              | C1799G       | P2          | P2          | - Inversely associated with HCC and significantly associated with LC | Chen et al. (2005); Yin et al. (2011)                                                      |
| Precore           |              | W28stop      | –           | P2          | - Inhibition of HBeAg synthesis | Kargar Kheirabad et al. (2017); Al-Qahtani et al. (2018); Tong et al. (2007)               |
|                   |              | G1896A       | –           | P2          | - Immune escape to anti-HBe |                                                                                              |
|                   |              | G1899A       | P1/P2/P3    | P1/P3      | - Increase viral replication |                                                                                              |
|                   |              |              |            |            | - Association with liver progression to LC and HCC |                                                                                              |

(Continued)
| Region | Cell subsets | Substitution | Patients | Effects | References |
|---|---|---|---|---|---|
| Core | Other | T12S | A1934T | P1/P1 | Association with disease severity | Datta et al. (2014); Saha et al. (2014) |
| CTL (CD8) epitope (aa 18–27) | Other | S21T | T1961A | P1/P1 | Unknown | Sominskaya et al. (2011) |
| Other | D29H | G1985C | P5 | - | Unknown | Not reported |
| T-helper (CD4) epitope (aa 35–45) | Other | E40D | A2020T | P1/P4/P5 | Unknown | Pollicino et al. (2007) |
| CTL (CD8) epitope (aa 50–69) | Other | E64D | A2092C | P3/P4 | P2/P4 | Pollicino et al. (2007); Al-Qahtani et al. (2018); Homs et al. (2011) |
| Other | D29H | G1985C | P5 | - | Unknown | Not reported |
| CTL (CD8) epitope (aa 74–83) | Other | A69G | C2106G | P4/P4 | Same effects as E64D | Datta et al. (2014); Saha et al. (2014); Pollicino et al. (2007); Homs et al. (2011) |
| Other | V74G | T2121G | P2/P3 | P3 | Reduction in HBc antigenicity | Sominskaya et al. (2011) |
| B-cell epitope (aa 76–89) | Other | V74S | G2138A | P2/P3 | P2/P3 | Pollicino et al. (2007); Homs et al. (2012) |
| B-cell epitope (aa 105–116) | Other | A80S | G2138T | P4 | - | Not reported |
| Other | M93V | A2177G | P3 | - | Unknown | Al-Qahtani et al. (2018) |
| Other | I116V | A2246G | P2/P3 | - | Association with disease progression to HCC or LC | Datta et al. (2014); Pollicino et al. (2007) |
| Other | P156S | C2366T | P2/P3 | - | Unknown | Not reported |
| Other | R166P | C2366T | P4 | - | Unknown | Not reported |

P1–P5 = Patients 1–5. HCC, Hepatocellular carcinoma; LC, liver cirrhosis.
TABLE 5 Amino acid/nucleotide substitutions detected within the X gene sequences of the patients with their reported effects.

| Cell subsets | Aa substitution | Nucleotide mutation | Treatment naïve | During treatment | Effects | References |
|--------------|----------------|---------------------|-----------------|------------------|---------|------------|
| Other        | C26S           | T1449A              | P2              | P2               | Unknown | Not reported |
|              | C26R           | T1449C              | P3              | P3               | Unknown | Pollicino et al. (2007) |
| B-cell epitope (aa 29–48) | L34I          | C1473A              | P5              | –                | Unknown | Not reported |
|              | T36D           | A1479G C1480A       | P1/P4           | P1/P4            | - Association with HCC progression | Pollicino et al. (2007); Sominskaya et al. (2011); Javannard et al. (2020) |
|              | T36G           | A1479G C1480G       | P5              | –                | Unknown | Not reported |
| Other        | S38P           | T1485C              | P1              | P1               | Unknown | Mani et al. (2019) |
|              | S39P           | T1488C              | P5              | –                | Unknown | Pollicino et al. (2007) |
| Other        | P40S           | C1491T              | P1/P4           | P1/P4            | Unknown | León et al. (2005) |
|              | S41P           | T1494C              | P5              | –                | Unknown | Xu et al. (2007) |
| T-helper (CD4) epitope (aa 91–105) | L42P          | T1498C              | P2/P3           | P2/P3            | Unknown | Not reported |
|              | S43P           | T1500C              | P1/P4/P5        | P1/P4            | - Immune escape (B-cell epitope affected) | Pollicino et al. (2007); Li et al. (2018) |
| Other        | T82S           | A1617T              | P2              | P2               | Unknown | Not reported |
|              | H86R           | A1630G              | P5              | –                | Unknown | Huang et al. (2014) |
| Other        | I88F           | A1635T              | P2              | P3               | - Association with HCC progression | Javannard et al. (2020); Pollicino et al. (2007) |
|              | I88C           | A1635T T1636G       | P3              | P2               | Unknown | Abdel Hamid and Salama (2018) |
| CTL (CD8) epitope (aa 115–123) | T105M         | C1687T              | P3              | P3               | Unknown | Mani et al. (2019) |
|              | D119N          | G1728A              | P3              | –                | Unknown | Zhu et al. (2008) |
| Other        | L123W          | T1741G              | P3              | –                | - Association with HCC progression | Al-Qahtani et al. (2017); Artarini et al. (2016) |
|              | I127T          | T1753C              | P1/P5           | P1               | - Promote transactivation and increase anti-proliferative activity | Artarini et al. (2016); Lin et al. (2005); Elkady et al. (2008) |
| Other        | I127D/G        | T1753A/G            | P3 (D)          | P3 (G)           | Unknown | Not reported |
|              | K130M          | A1762T              | P1/P3/P5        | P1/P3            | - Increase viral replication and cell invasion | Mani et al. (2019); Lin et al. (2005); Yuan et al. (2009) |

(Continued)
TABLE 5 (Continued)

| Cell subsets | Nucleotide mutation | Patients | Treatment naïve | During treatment | Effects | References |
|--------------|---------------------|---------|----------------|-----------------|---------|-----------|
| V131I        | G1764A              | P1/P3/P5| P1             | - Increase viral replication and cell invasion |
|              |                     |         |                | - Decrease the expression of HBeAg |
|              |                     |         |                | - Association with HCC progression |
| V131L        | G1764T              | P2      | P2             | Unknown |
| F132Y        | T1768A              | P3      | P3             | - Increase viral replication and cell invasion |
| H139D        | C1788G              |         | P4             | Unknown |

P1–P5 = Patients 1–5. HCC, Hepatocellular carcinoma.

rtC256S to LMV; rtQ215S and rtF221Y to ADV; rtD134E, rtQ215S, and rtC256S to TDF (Moriconi et al., 2007; Amini-Bavil-Olyaee et al., 2009; Liu et al., 2009; Pollicino et al., 2009; Ciftci et al., 2014; Park et al., 2019; Mokaya et al., 2020).

Thus, our results support the need to introduce HBV genome sequencing as a pre-treatment diagnosis to predict potential resistance to available antiviral molecules, as well as to monitor the evolution of treatment response.

In addition, we have studied the mutational profile in the preS1, preS2, and S genes. As the coding sequence of the HBsAg is completely overlapped with the RT domain of the HBV polymerase, some mutations occurring in the RT region may lead to the emergence of escape mutants in the S region and vice versa. Thus, rtT128N, rtD134E, rtS202G, rtM204V, rtT128N, rtD134E, rtS202G, rtM204V, and rtF221Y substitutions observed in the RT region result in sP120T, sR229Q, and sG29D, respectively, leading to the production of a truncated precore protein and then the abolition of HBeAg expression (Kobayashi et al., 2003; Thompson et al., 2010; Ducancelle et al., 2016). These variations are the major immune escape mutants of HBV as HBeAg is the main target for both cellular and humoral immune responses leading to a higher risk of liver HCC and LC progression (Tong et al., 2005; Liao et al., 2012; Suppiah et al., 2015; Pahal et al., 2016). In addition, precore mutants impose serious consequences on the treatment and enhance viral replication (Ouneissa et al., 2012; Kargar Kheirabad et al., 2017; Boyd et al., 2018).

Concerning the core mutations, cT67N within the T-helper CD4 epitope might be able to escape the host immune response (Datta et al., 2014; Saha et al., 2014). Moreover, cV74G, cP79Q, and cA80T mutations are known to reduce both HBe and HBc antigenicity (Pollicino et al., 2007; Huang et al., 2014). In addition, cA80T has resulted in the production of altered and truncated HBcAg protein leading potentially to abnormal immune reaction and negativity of anti-HBc (Bajpai et al., 2017).

In the last part of this study, we studied the mutational profile in the X gene. Substitutions xS43P and xP46S located in the B-cell epitope were detected in our study and have been suggested to be related with immune escape (Putri et al., 2019). Mutations xP46S, xA47T, xI88F, xA102V, xL127T, xK130M, xV131I, and xF132Y, were previously reported as significant HCC-related HBx mutants alone or combined such as (iL127T + K130M + V131I) in patients 1, 3, and 5 and (xK130M + xV131I + xF132Y) in patient 3 (Pollicino et al., 2007; Ghosh et al., 2012; Ali et al., 2014; Al-Qahtani et al., 2017). Moreover, the double mutant xK130M + xV131I has been suggested to exacerbate the host’s immune response, increase viral replication, and lead to a truncated HBx protein (Wungu et al., 2019). In addition, it is associated with the activation of proto-oncogenes and inactivation of the tumor suppressor gene reported in Tunisian studies with an occurrence alone or in association (Triki et al., 2000; Bahri et al., 2006; Ayed et al., 2007; Poustchi et al., 2008; Ouneissa et al., 2012). These mutants result in a stop codon at position W28* and a substitution at position G29D, respectively, leading to the production of a truncated precore protein and then the abolition of HBeAg expression (Ouneissa et al., 2012; Kargar Kheirabad et al., 2017; Boyd et al., 2018).
Several mutations previously reported to be significantly associated with an increased risk of severe liver disease progression to HCC and/or LC progression were also detected in other genes namely (rtD134E/rtF221Y/rtM204V/rtM309k) in the RT region; (sF22L) in the preS2 region; (sL149R, sL213I and sL216*) in the S region; (C1766T/T1768A double mutant, C1773T, C1799G, and C1766G) in the BCP region; and (ctT12S/ctE64D/ctT67N/ctA80T/ctP130Q) in the core region of the C gene. These HCC-related mutations could be used as markers of HCC evolution in particular rtF221Y mutant which has been indicated as an independent risk factor for poor overall survival (Jammeh et al., 2008; Yin et al., 2011; Kitab et al., 2012; Zheng et al., 2012; Gopalakrishnan, 2013; Tong et al., 2013; Datta et al., 2014; Chaouch et al., 2016; Nishizawa et al., 2016; Kim et al., 2017; Li et al., 2017; Al-Qahtani et al., 2018; Choi et al., 2018; Hosseini et al., 2019). In contrast, the early development of G1757A in the BCP reduces the oncogenic potential of HBV suggesting that it might be a protective biomarker in chronic hepatitis B (Poustchi et al., 2008; Mohamadkhani et al., 2011; Ducancelle et al., 2013).

In addition to the commonly mentioned substitutions in all genes (P, S, C, and X), several nucleotide/amino acid substitutions have been detected in our patients (see Tables 2–5) but have never been reported previously or have been reported with unknown impact. Therefore, further studies are necessary to better understand and elucidate the effect of these mutations on HBV treatment, antigenicity, and disease evolution.

### Conclusion

In conclusion, we would propose the whole genome sequencing as a pre-treatment diagnosis to predict potential resistance to available antiviral molecules, as well as to monitor the evolution of treatment response and prevent progression to cirrhosis or hepatocellular carcinoma. Thus, this could contribute to guiding national efforts to optimize relevant HBV treatment management in order to achieve the global hepatitis elimination goal by 2030.

### Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article supplementary material.

### References

Abdel Hamid, M., and Salama, A. (2018). X Gene variability and genotyping of hepatitis B virus (HBV) in sudanese patients with liver diseases, Khartoum, Sudan. Available at: https://www.researchgate.net/publication/325477946_X_GENE_VARIABILITY_AND_GENOTYPING_OF_HEPATITIS_B_VIRUS_HBV_IN_SUDANESE_PATIENTS_WITH_LIVER_DISEASES_KHARTOUM_SUDAN (Accessed October 22, 2020).

Al-Qahtani, A. A., Al-Anazi, M. R., Nazir, N., Ghai, R., Abdo, A. A., Sanai, F. M., et al. (2017). Hepatitis B virus (HBV) X gene mutations and their association with liver disease progression in HBV infected patients. Oncotarget 8, 105115–105125.

Ali, A., Abdel-Hafiz, H., Sohail, M., Al-Marz, A., Zakaria, M. K., Fatima, K., et al. (2014). Hepatitis B virus, HBx mutants and their role in hepatocellular carcinoma. World J. Gastroenterol. 20, 10238–10248. doi: 10.3748/wjg.v20.i30.10238

### Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

### Author contributions

ZB, AC, HTI, MG, SA, LH, and MM: conceptualization. ZB, HTI, AS, WH, WK, and LY: methodology. ZB, AC, and HTI: validation. ZB and AC: formal analysis. ZB, AC, HTI, AS, WH, WK, and LY: investigation. ZB, AC, MG, SA, LH, and MM: data curation. ZB and KA: writing—original draft preparation. AC and HTI: editing and reviewing. All authors contributed to the article and approved the submitted version.

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### Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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recombination between genotypes D and E, is circulating in Niger along with hepatitis B virus infection in chronic liver disease: full-length genome and analysis of patients.

Aydin, M., Tekin, S., Sayan, M., and Akhan, S. (2019). Molecular characterization of Hepatitis B virus strains isolated from chronic Hepatitis B patients in southeastern region of Turkey. Viral Hepat. 25, 40–44. doi: 10.1042/ vhd.galenos.2019.2019.0015

Ayed, K., Gorg, Y., Ayed-Jendoubi, S., Aouadi, H., Star, N., Najjar, T., et al. (2007). Hepatitis B virus genotypes and precore/core promoter mutations in Tunisian patients with chronic hepatitis B virus infection. J. Infect. 54, 291–297. doi: 10.1016/j. jinf.2006.05.013

Azarkan, Z., Ziaeie, M., Ebrahimzadeh, A., Sharifzadeh, G., and Javannard, D. (2018). Epidemiology, risk factors, and molecular characterization of occult hepatitis B infection in patients with anti-HBc positive-anti core antigen alone subjects. J. Med. Virol. 91.

Brahmatals, G., Sadeghzadeh, M., Amini-Bavil-Olyaee, S., Alvaian, S. M., Behzad-Behbahani, A., Adeli, A., et al. (2008). Clinical, virologic and phylogenetic features of Hepatitis B virus infection in Iranian patients. World J. Gastroenterol. 14, 5448–5453. doi: 10.3748/wjg.14.5448

Bahr, O., Cheikh, I., Haji, N., Djebbi, A., Maamouri, N., Sadraou, A., et al. (2006). Hepatitis B virus genotypes, precore and core promoter mutations circulating in Tunisia. J. Med. Virol. 82, 1055–1067. doi: 10.1002/jmv.20766

Baiap, V., Gupta, E., Kundra, S., Sharma, S., and Shashtry, S. M. (2017). Hepatitis B core antibody negativity in a chronic hepatitis B infected patient: report of an unusual serological pattern. J. Clin. Diag. Res. 11, DD04–DD06. doi: 10.1186/ JCDR/2017/26821.10498

Bakhshezhadeh, F., Hekmat, S., Keshviri, M., Alavian, S. M., Mostafavi, E., Kervani, H., et al. (2015). Efficacy of tenofovir disoproxil fumarate therapy in patients with chronic hepatitis B virus infection who are antiviral treatment-naive in Iran. J. Gastroenterol. Hepatol. 30, 15. doi: 10.1111/1440-1746.127574

Biswas, A., Panigrahi, R., Chandra, P. K., Banerjee, A., Datta, S., Pal, M., et al. (2013). Characterization of the occult hepatitis B virus variants circulating among the blood donors from eastern India. Sci. World J. doi: 10.1100/2013.221704

Boyd, A., Kouraie, M. G., Houghtaling, M., Loh, R., Gabbillard, D., Maylin, S., et al. (2019). Hepatitis B virus activity in untreated hepatitis B antigen-negative human immunodeficiency virus/hepatitis B virus co-infected patients from sub-Saharan Africa. J. Infect. Dis. 219, 437–445.

Boyd, A., Moh, R., Maylin, S., Abdou Chekourau, M., Mahjoub, N., Gabbillard, D., et al. (2018). Precore G1896A mutation is associated with reduced rates of HBAg seroclearance in treated Hepatitis B virus co-infected patients from Western Africa. J. Viral Hepat. 25, 1211–1212. doi: 10.1111/jhj.12914

Caliguri, P., Cerruto, R., Icardi, G., and Bruszone, B. (2016). Overview of hepatitis B virus mutations and their implications in the management of infection. World J. Gastroenterol. 22, 145–154. doi: 10.3748/wjg.v22.i2.1145

Chaouche, H., Taffon, S., Villain, U., Equestre, M., Bruni, L., Belhadj, M., et al. (2016). Naturally occurring surface antigen variants of Hepatitis B virus in Tunisian Africa. Virol. J. 10.5812/vj.2016.01.1015

Chaouche, H., Taffon, S., Villain, U., Equestre, M., Bruni, L., Belhadj, M., et al. (2016). Naturally occurring surface antigen variants of Hepatitis B virus in Tunisian Africa. Virol. J. 10.5812/vj.2016.01.1015

Chaudhuri, V., Tayal, R., Nayak, B., Acharya, S. K., and Panda, S. K. (2004). Occult hepatitis B virus infection in HIV-infected cytotoxic lymphoma therapy in an anti-HBc negative patient. World J. Gastroenterol. 10.3748/wjg.v10.i27.54579

Chen, C. H., Lee, C. M., Lu, S. N., Changchien, C. S., Eng, H. L., Huang, C. M., et al. (2008). Genotype determination in Moroccan hepatitis B chronic carriers. J. Med. Virol. 80, 353–357. doi: 10.1002/jmv.20318

Cheng, H. C., Lee, C. M., Lu, S. N., Changchien, C. S., Eng, H. L., Huang, C. M., et al. (2005). Clinical significance of hepatitis B virus genotypes and precore/core promoter mutations affecting HBc e antigen expression in Taiwan. J. Clin. Microbiol. 43, 6000–6006. doi: 10.1128/JCM.43.12.6000-6006.2005
Worldwide distribution of HBV subgenotype D1. Circulating in the northern coast of the Persian Gulf and its comparison with patients with hepatocellular carcinoma.

Molecular and functional analysis of occult hepatitis B virus isolates from untreated and lamivudine-resistant chronic hepatitis B patients.

Precore mutations and their relatedness to genotypes and disease pathogenesis.

Method for Hepatitis B Virus Genotyping.

A systematic literature review and structural analysis of drug resistance mechanisms.

Hepatitis B virus resistance to tenofovir: fact or fiction? A critical region where the hepatitis B virus makes decisions.

Hepatitis B virus: Time for an individualised approach?

Hepatitis B virus resistance to tenofovir: fact or fiction? A synthesis of recent evidence.

Hepatitis B virus resistance to tenofovir: fact or fiction? A meta-analysis of recent studies.

Hepatitis B virus: fact or fiction? A review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A meta-analysis of recent studies.

Hepatitis B virus: fact or fiction? A synthesis of recent evidence.

Hepatitis B virus: fact or fiction? A critical review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

Hepatitis B virus: fact or fiction? A comprehensive review of recent studies.

Hepatitis B virus: fact or fiction? A systematic review of recent literature.

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Thompson, A. J. V., Nguyen, T., Iser, D., Ayres, A., Jackson, K., Littlejohn, M., et al. (2010). Serum hepatitis B surface antigen and hepatitis B e antigen titers: Disease phase influences correlations with viral load and intrahepatic hepatitis B virus markers. *Hepatology* 51, 1933–1944. doi: 10.1002/hep.23571

Tokgoz, Y., Terlemez, S., Sayan, M., and Kirdar, S. (2018). Investigation of antiviral resistance and escape mutations in children with naive chronic hepatitis B patients and their parents. *Turk. J. Pediatr.* 60, 514–519. doi: 10.24953/turkped2018.05.007

Tong, M. J., Blatt, L. M., Kao, J. H., Cheng, J. T., and Corey, W. G. (2007). Basal core promoter T1762A/T1764 and precore A1896 gene mutations in hepatitis B surface antigen–positive hepatocellular carcinoma: a comparison with chronic carriers. *Liver. Int.* 27, 1356–1363. doi: 10.1111/j.1478-3231.2007.01585.x

Tong, S., Kim, H. K., Chante, C., Wands, J., and Li, J. (2005). Hepatitis B virus e antigen variants. *J. Med. Sci.* 2, 2–7. doi: 10.7150/jms.2.2

Tong, S., Li, J., Wands, J. R., and Wen, Y. (2013). Hepatitis B virus genetic variants: biological properties and clinical implications. *Emerg. Microbes Infect.* 2, 1–11.

Torresi, J., Earnest-Suliveira, L., Civitico, G., Walters, T. E., Lewin, S. R., Fyfe, J., et al. (2002a). Restoration of replication phenotype of lamivudine-resistant hepatitis B virus mutants by compensatory changes in the “fingers” subdomain of the viral polymerase gene that are selected by lamivudine therapy. *Virology* 293, 305–313. doi: 10.1006/viro.2001.1246

Triki, H., Ben Slimane, S., Ben Mami, N., Sakka, T., Ben Ammar, A., and Delliga, K. (2000). High circulation of hepatitis B virus (HBV) precore mutants in Tunisia. *North. Africa. Epidemiol. Infect.* 125, 169–174. doi: 10.1017/S0950268899003921

Villet, S., Ollivert, A., Pichoud, C., Barraud, I., Villeneuve, J. P., Trépo, C., et al. (2007). Stepwise process for the development of entecavir resistance in a chronic hepatitis B virus infected patient. *J. Hepatol.* 46, 531–538. doi: 10.1016/j.jhep.2006.11.016

Wang, L., Han, F., Duan, H., Ji, F., Yan, X., Fan, Y., et al. (2017). Hepatitis B virus pre-existing drug resistant mutation is related to the genotype and disease progression. *J. Infect. Dev. Ctries.* 11, 727–732. doi: 10.1016/j.jidc.2017.05.001

Wang, Y., Zeng, L., and Chen, W. (2016). HBV X gene point mutations are associated with the risk of hepatocellular carcinoma: A systematic review and meta-analysis. *Met. Clin. Oncol.* 4, 1045–1051. doi: 10.3895/mco.2016.847

Wang, Q., Zhang, T., Ye, L., Wang, W., and Zhang, X. (2012). Analysis of hepatitis B virus X gene (HBx) mutants in tissues of patients suffered from chronic hepatitis B: a comprehensive review. *Biomarkers Prev.* 7, 1–11. doi: 10.11718/bmp.2012.05.01

We, C., Yu-tian, C., Ji-zi, W., Yong-wei, L., and Gang, L. (2011). Characterization of hepatitis virus B isolated from a multi-drug refractory patient. *Virus Res.* 155, 254–258. doi: 10.1016/j.virusres.2010.08.018

Westland, C. (2003). Week 48 resistance surveillance in two phase 3 clinical studies of adefovir dipivoxil for chronic hepatitis B. *Hepatology* 38, 96–103. doi: 10.1053/jhep.2003.50288

WHO (2021). Hepatitis B WHO guidelines. Available at: https://www.who.int/news-room/fact-sheets/detail/hepatitis-b (Accessed April 25, 2022).

Wu, Y., Gan, Y., Gao, F., Zhao, Z., Jin, Y., Zhu, Y., et al. (2014). Novel natural mutations in the Hepatitis B virus reverse transcriptase domain associated with hepatocellular carcinoma. *PMR* 9, 10.1371/journal.pone.0115141

Wu, J. F., Ni, Y. H., Chen, H. L., Hou, H. Y., and Chang, M. H. (2014). The impact of hepatitis B virus precore/core gene carboxyl terminal mutations on viral biosynthesis and the host immune response. *J. Infect. Dis.* 209, 1374–1381. doi: 10.1093/infdis/jiu638

Wungo, C. D. K., Amin, M., Ruslan, S. E. N., Purwono, P. B., Kholid, U., Maimunah, U., et al. (2019). Association between host TNF-α, TGF-β1, p53 polymorphisms, hbv x gene mutation, hbv viral load and the progression of hbv-associated chronic liver disease in indonesian patients. *Biomed. Res. Rep.* 11, 145–153. doi: 10.3892/br.2019.1239

Xu, R., Zhang, X., Zhang, W., Fang, Y., Zheng, S., and Yu, X. F. (2007). Association of human APOBEC3 cytidine deaminases with the generation of hepatitis virus B x antigen mutants and hepatocellular carcinoma. *Hepatology* 46, 1810–1820. doi: 10.1002/hep.21893

Yamani, L. N., Yano, Y., Utsumi, T., Wastuyutti, W., Rinonce, H. T., Widasari, D. I., et al. (2017). Profile of mutations in the reverse transcriptase and overlapping surface genes of hepatitis B virus (HBV) in treatment-naïve indonesian HBV carriers. *Jpn. J. Infect. Dis.* 70, 647–655. doi: 10.7883/yoken.JJID.2017.078

Yang, L., Zhang, H., Ma, H., Liu, D., Li, W., Kang, Y., et al. (2015). Deep sequencing of hepatitis B virus basal core promoter and precore mutants in HBsAg-positive chronic hepatitis B patients. *Sci. Rep.* 59.5

Yang, H., Qi, X., Sabogal, A., Miller, M., Xiong, S., and Delaney, W. E. (2005). Cross-resistance testing of next-generation nucleoside and nucleotide analogues against lamivudine-resistant HBV. *Antivir. Ther.* 10.539

Yang, H., Westland, C. E., Delaney, W. E., Heathcote, E. J., Ho, V., Fry, J., et al. (2002). Resistance surveillance in chronic hepatitis B patients treated with adefovir dipivoxil for up to 60 weeks. *Hepatology* 36, 464–473. doi: 10.1053/jhep.2002.34740

Yin, J., Xie, J., Liu, S., Zhang, H., Han, L., Lu, W., et al. (2011). Association between the various mutations in viral core promoter region to different stages of hepatitis B, ranging of asymptomatic carrier state to hepatocellular carcinoma. *Am. J. Gastroenterol.* 106, 81–92. doi: 10.1038/ajg.2010.159

Yuan, J. M., Ambinder, A., Fan, Y., Gao, Y. T., Yu, M. C., and Groopman, J. D. (2009). Prospective evaluation of hepatitis B 1762 T/1764 a mutations on hepatocellular carcinoma development in Shanghai, China. *Cancer Epidemiol. Biomarkers Prev.* 18, 590–594. doi: 10.1158/1055-9966.EPI-08-9966

Zheng, J., Zeng, Z., Zhang, D., Yu, Y., Wang, F., and Pan, C. Q. (2012). Prevalence and significance of Hepatitis B reverse transcriptase mutants in different disease stages of untreated patients. *Liver. Int.* 32, 1535–1542. doi: 10.1111/j.1478-3231.2012.02859.x

Zhu, R., Zhang, H. P., Yu, H., Li, H., Ling, Y. Q., Hu, X. Q., et al. (2008). Hepatitis B virus mutations associated with in situ expression of hepatitis B core antigen, viral load and prognosis in chronic hepatitis B patients. *Pathol. Res. Pract.* 204, 731–742. doi: 10.1016/j.prp.2008.05.001

Ziaee, M., Javanmard, D., Shariﬁzadeh, G., Namazi, M. H., and Azarkar, G. (2016). Genotyping and mutation pattern in the overlapping MHR region of HBV isolates in southern khorasan, eastern Iran. *Hepat. Mon.* 16, 37806. doi: 10.5812/hepatmon.37806.