Review

Development of HBV S gene mutants in chronic hepatitis B patients receiving nucleotide/nucleoside analogue therapy

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Structurally modified nucleotide/nucleoside analogues can exert potent inhibitory effect on HBV polymerase activities. Some of these agents have been approved for the treatment of chronic hepatitis B. Because of a high risk of reactivation upon drug withdrawal, continuous long-term therapy is recommended to maintain maximal viral suppression. Consequently, drug resistance has developed in a significant proportion of patients. During long-term therapy, mutations occur not only in the polymerase gene but also in the S gene, resulting in the emergence of surface protein mutants. Two types of surface protein mutants are recognized. The first type arises as a result of amino acid substitutions caused by primary and compensatory resistance mutations in the polymerase gene, which concomitantly generate S gene mutations owing to overlapping S and polymerase genes. The second type occurs because of prolonged viral suppression leading to seroclearance of HBV surface antigen, where vaccine-escape-like mutants might be selected. The second type of mutants does not possess primary resistance mutations in the polymerase gene. Some drug-related S gene mutations are nonsense mutations, leading to truncation of the surface proteins. Among them, the rtA181T/sW172* mutant has a dominant negative secretion effect as well as an increased oncogenic potential. The clinical consequences of infection by these S gene mutants demand further clarification. Judicious selection of the antiviral agents and vigilant monitoring of viral mutants during the course of therapy are advised.

Introduction

Following the successful development of lamivudine (3TC), several oral antiviral agents have subsequently been approved for treatment of chronic hepatitis B virus (HBV) infection, including adefovir dipivoxil (ADV), entecavir (ETV), telbivudine (LdT) and tenofovir disoproxil fumarate (TDF) [1–5]. These antiviral agents are nucleotide or nucleoside analogues that competitively bind to the HBV polymerase to inhibit its activities [6,7]. The inhibitory effect of these agents is so potent that serum HBV DNA levels can be suppressed by 4 to 7 log10 [8]. However, owing to the persistence of covalently closed circular DNA in hepatocytes, serum hepatitis B e antigen (HBeAg) remains positive in approximately 70% of patients even after 5 years of continuous therapy [8,9]. A high risk of relapse was observed upon withdrawal of oral antiviral agents, when patients were still in the HBeAg-positive stage [10]; therefore, maintained long-term viral suppression is now recommended as the major therapeutic strategy for chronic HBV infection. Unfortunately, long-term usage of oral antiviral agents lead to viral resistance [11]. At present, only ETV has been found to confer a low resistance rate when it is given to treatment-naive patients. Only 1.5% of treatment-naive patients developed viral resistance after 5 years of ETV therapy [12]. TDF might be another drug capable of achieving low resistance; however, only 1-year treatment results are published to date [13]. For the other oral antiviral agents, significant proportions of patients develop viral resistance and the proportions of resistance increase with the duration of therapy. 3TC is one of the oral antiviral agents that confer a high risk of viral resistance. During long-term therapy, 3TC resistance developed in 24%, 42%, 53%, 70% and 75% of patients after 1–5 years of therapy, respectively [14]. Despite high drug resistance risk, 3TC remains the first-line oral antiviral agent for the treatment of chronic HBV infection in many parts of the world, largely attributed to its low cost, absence of side effects and oral route of intake. It can be expected that treatment of 3TC-resistant HBV
infection will continue to be a medical challenge for hepatologists worldwide in the next decade.

On the basis of findings from in vitro experiments, 3TC-resistant HBV was replication incompetent [15]; thus, 3TC-resistant HBV was initially believed to be a less virulent strain compared with wild-type HBV. Additionally, clinical data indicated that median serum alanine aminotransferase and HBV DNA levels remained lower than baseline after resistant viruses had emerged [16]; however, subsequent studies indicated that severe exacerbations could occur after the development of drug resistance and that histology activity index scores worsened after long-term therapy [17–19]. Moreover, sequential viral sequencing data indicated that additional viral mutations different from the major resistant mutations occurred, which helped to restore the impaired replication efficiency [20]. The latter type of mutations was named secondary or compensatory mutations. Currently, there are several major or primary mutations that confer drug resistance for each oral antiviral agent. For example, rtM204I/V or rtA181T confers 3TC resistance, rtA181T/V or rtN236T confers ADV resistance, rtM204I/V confers LdT resistance and rtM204I/V+rtL180M (in combination with one of rtT184, rtS202 or rtM250 mutations) confer ETV resistance [21]. Additionally, there are other mutations, such as rtV173L and rtL80V/I, which are capable of restoring replication efficiency in mutants harbouring primary resistant mutations [22,23].

**Antiviral-drug-associated S gene mutants**

During antiviral therapy, it has been observed that not only polymerase gene mutations but also S gene mutations are selected [24–26]. These S gene mutations sometimes lead to severe alteration of the antigenicity of hepatitis B surface antigen (HBsAg) to an extent that it cannot be recognized by many monoclonal antibodies [25,27,28]. As a result, these mutants are very similar to vaccine-escape mutants, which have been selected by HBV vaccination.

At present, two types of antiviral-drug-associated S gene mutants (ADASMs) can be recognized. The first type of ADASMs arises as a result of amino acid substitutions caused by mutations in the overlapping HBV S and polymerase gene. Because the HBV S gene is overlapped completely by the polymerase gene, whenever a nucleotide mutation occurs in the polymerase gene, there is a concomitant nucleotide mutation developed in the S gene. As a result, all the primary and secondary drug-resistant mutations selected in the polymerase gene (in the area overlapped by the S open reading frame) lead to the generation of concomitant nucleotide substitutions in the S gene. These S gene nucleotide mutations can lead to three different consequences in the pre-S/S reading frame: amino acid substitution mutations in the pre-S/S proteins (including large, middle and small S proteins), nonsense mutations leading to truncation of the pre-S/S proteins and silent mutations (where no amino acid change in the pre-S/S proteins has occurred). For example, rtF166L, rtI69T, rtV173L, rtA181V, rtS184G, rtV207I, rtA204V and rtM204I could respectively lead to sF158Y, sF161L, sE164D, sL173F, sL/V176G, sV194F, sI195M and sW196S/L (dependent on codon usage) substitution mutations in the S gene. By contrast, rtA181T, rtV191I, rtM204I and rtV207I lead to sW172*, sW182*, sW196* (dependent on codon usage) and sW199* nonsense mutations in the S gene. Finally, rtL180M, rtA194T and rtN236T do not change the surface proteins.

In several surface protein mutants carrying amino acid substitutions, altered antigenicity of HBsAg has been characterized, including decreased sensitivity of recognition by monoclonal antibodies developed against wild-type HBsAg and decreased ability of the mutant surface proteins to compete with wild-type HBsAg for binding of polyclonal antibodies against HBsAg (anti-HBs) [27,28]. Viral fitness has also been examined and decreased replication efficiency has been recognized. Altered secretion efficiency of the large, middle and small surface proteins was demonstrated in several mutants harbouring S gene mutations [29]. Additionally, the ability to support in vitro generation of hepatitis D virus as well as its subsequent infectivity was altered in these mutants [29]. Taken together, in the antiviral era, hepatologists are facing the challenge of atypical chronic HBV infection caused by mutants with altered antigenicity and viral fitness. Conceivably, there will be problems in monitoring the therapeutic efficacy during antiviral therapy by use of enzyme-immuno assays. The clinical presentation of mutant-associated HBV might also be different from that of the wild-type virus.

A second type of ADASMs has often been overlooked. In vaccine-escape mutants, the most well-characterized mutation is the sG145R substitution located in the second loop of the ‘a’ determinant [30]. However, there is another frequently encountered mutant located in the first loop, the sP120A substitution [31]. Interestingly, in a liver transplant patient receiving oral antiviral agents as well as hepatitis B immunoglobulin treatment, a complex mutant harbouring the sP120A mutation was selected, supposedly through coselection by the oral antiviral agent and immunoglobulin treatment [32]. In another study, HBsAg seroclearance was observed in 11 patients treated by long-term usage of 3TC, but these patients remained HBV-DNA-positive in their sera [33]. Sequence analysis revealed several S gene mutations and a mutational hotspot, sP120A, was identified in 6 of the 11 patients. In vitro experiments demonstrated that the sP120A substitution in
the surface proteins led to impaired recognition by monoclonal antibodies against HBsAg. Interestingly, these patients did not carry primary polymerase resistance mutations and in four of the six patients, anti-HBs was positive in their sera. For all the S gene mutations reported in this study, the corresponding polymerase gene mutations were either silent or have resulted in amino acid substitutions in the polymerase gene located far upstream of domains B and C [33]. Such S gene mutants cannot be explained by the gene overlapping theory, whereby the polymerase gene mutations were selected because of the need to confer drug resistance as a result of which the S gene mutations resulted. A possible scenario is that after long-term and potent viral suppression, the amount of serum HBsAg decreases to a level either equal to or smaller than the level of serum anti-HBs, thus creating a selection pressure to a level either equal to or smaller than the level of suppression, the amount of serum HBsAg decreases.

One special group of the antiviral-drug-related mutants is the surface truncation mutants. In this group of mutants, the rtA181T mutant was selected and responsible for drug resistance. Interestingly, in a study adopting the add-on strategy, 12% of cirrhotic patients receiving such therapy still developed hepatocellular carcinoma [37].

In vitro experiments indicated that the rtA181T/sW172* mutant conferred a dominant negative effect on the secretion of HBsAg and viral particles [46]. Clinical observation in a hepatoma patient infected with the rtA181T/sW172* mutant was consistent with this result [47]. This patient had a phenotype of negative serum HBsAg and positive serum HBeAg, although immunohistochemical examination revealed retention of HBsAg in the hepatocytes. A potentially hazardous effect of this mutant is that intracellular retention of HBsAg could generate significant endoplasmic reticulum (ER) stress leading to continuous liver cell damage and regeneration.

Previously, surface truncation mutations have been identified in HBV genomes that had been integrated into the chromosome of hepatoma cells [48]. Subsequently, a transactivity-on-region has been characterized, located between codons 122 and 139 in the S region [49]. When nonsense mutations emerged in this region, the resulting middle surface truncation mutants were capable of transactivating several oncogene promoters. In a recent study, serum and tissue samples from eight patients, in whom hepatoma developed despite 3TC therapy, were subjected to pre-S/S gene sequence analysis and several surface truncation mutations (including sW172*) located in the borders of the transactivity-on-region were recognized [50]. Different from previous studies focusing on integrated forms of HBV, these mutations were found to occur in freely replicative forms of HBV. When expressed in NIH3T3 cells, some of them conferred tumourigenicity in nude mice. Furthermore, the sW172* mutant has increased tumourigenicity compared with the wild type. In this study, however, the ER stress, monitored by measuring XBP-1 splicing, was not observed. Although these studies suggested a role of the rtA181T/sW172* mutant in the development of hepatomas, clinical evidence is still lacking in patients undergoing long-term 3TC therapy, rtM204V/I mutation developed initially, but was later replaced by the rtA181T mutant in 17% of patients who developed drug resistance [20]. Furthermore, in woodchucks, the major drug-resistant mutant that developed after 3TC therapy was the one that corresponded to rtA181T in human HBV [43,44]. This woodchuck HBV mutant also possessed the corresponding sW172* mutation. Clinically, after the approval of ADV, the consensus of most hepatologists in treating 3TC-resistant patients is to add on ADV [45]. Several studies indicated that this add-on therapy resulted in a small risk of cross-resistance after 3–4 years of treatment [37,38]. When resistance developed, the rtA181T mutant was selected and responsible for drug resistance. Interestingly, in a study adopting the add-on strategy, 12% of cirrhotic patients receiving such therapy still developed hepatocellular carcinoma [37].

**Altered oncogenic potential in the surface truncation mutants**

One special group of the antiviral-drug-related mutants that catch much attention of molecular virologists are the surface truncation mutants. In this group of mutants, the rtA181T/sW172* mutant is of particular importance because it is resistant to both 3TC and ADV [37–42]. This mutant can also be selected during 3TC monotherapy [37,38]. In a study conducted in Taiwan, in
clarifying whether patients carrying this mutant suffer a higher risk of liver cancer. Presumably, oncogenicity of virus-related hepatoma is dependent on multiple factors, including viral load, oncogenic potential of viral proteins and host factors. Therefore, a more oncogenic mutant with a lower viral load could be less carcinogenic compared with the wild-type virus with a much higher viral load. In patients with liver cirrhosis, it remains unclear whether resistant mutants are more oncogenic than the wild-type virus given the same viral load and host conditions.

In conclusion, long-term oral antiviral therapy for chronic HBV infection not only leads to the emergence of drug-resistant polymerase mutants, but also leads to the development of S gene mutants (ADASMs). Two types of ADASMs are now recognized, one generated because of the overlapping S and polymerase genes and the other possibly generated because of a lower or reverse ratio of HBsAg to anti-HBs. These mutants have altered antigenicity, viral fitness and oncogenic potential. Because of the similarity between ADASMs and vaccine-escape mutants, it is highly suspected that these mutants may spread among individuals with or without previous HBV vaccination. The altered oncogenicity in ADASMs requires further attention because it calls for significant modification of our current strategy to treat patients with liver cirrhosis.

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