Clinical utility of complex mutations in the core promoter and proximal precore regions of the hepatitis B virus genome

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Abstract

The core promoter and proximal precore regions are the most complex portions of the hepatitis B virus (HBV) genome. These regions cooperatively regulate viral replication and differentially regulate the synthesis of the viral proteins E, core, and X. Multiple mutations in these regions are associated with the persistence of viral infection and the development of cirrhosis and hepatocellular carcinoma (HCC). In South Korea, nearly all HBVs are classified as HBV genotype C2; the majority of these viruses have the basal core promoter double mutation, a precore stop mutation, or both. These mutations may play a role in the alteration of viral and clinical features, and abundant and complex mutations are particularly prevalent in the core promoter and proximal precore regions. We previously demonstrated that the accumulation of ≥ 6 mutations at eight key nucleotides located in these regions (G1613A, C1653T, T1753V, A1762T, G1764A, A1846T, G1896A, and G1899A) is a useful marker to predict the development of HCC regardless of advanced liver disease. In addition, certain mutation combinations were predominant in cases with ≥ 4 mutations. In cases with ≤ 5 mutations, a low Hepatitis B e antigen titer (< 35 signal to noise ratio) was indicative of HCC risk. Viral mutation data of the single HBV genotype C2 suggest that the combined effect of the number and pattern of mutations in the core promoter and proximal precore regions is helpful in predicting HCC risk.

Key words: Hepatitis B virus; Point mutation; Hepatitis B virus X protein; Hepatocellular carcinoma; Cancer screening

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Core tip: Multiple mutations in the core promoter and proximal precore regions of the hepatitis B virus (HBV) genome are associated with hepatocellular carcinoma (HCC), but mutations predictive of outcome in chronic HBV carriers have not been distinguished. In the Korean HBV genotype C2, the number of mutations at eight key nucleotides located in these regions (G1613A, C1653T, T1753V, A1762T, G1764A, A1846T, G1896A, and G1899A) is positively correlated with HCC. In addition, some selected mutation combinations among individuals with ≥ 4 mutations are predominant in the HCC group.
OUTCOME OF CHRONIC HBV INFECTION

Chronic hepatitis B virus (HBV) infection increases the risk of developing liver cirrhosis and hepatocellular carcinoma (HCC) \([1-5]\). The natural course of HBV infection involves three clinical phases: immune tolerance, immune eradication, and recovery. The phase of HBV infection is classified based on serum aminotransferase levels and HBV DNA titer, which represent hepatitis and viral replication, respectively \([1-4]\). When the HBV DNA titer is greater than 2000 IU/mL, host immune mechanisms trigger the flare-up of hepatitis and regulate hepatitis activities that bridge the gap between the virus replication phase and the development of liver cirrhosis or HCC. The majority of hepatitis patients are anicteric, and the process tends to repeat until HBV loses the capability to replicate in hepatocytes \([5,6]\). When serum viral loads are persistently less than 350 IU/mL, the progressive loss of HBV genomic activity and the inability to stimulate a host immune response are observed, indicating that the carrier is in the recovery phase \([7]\). Throughout chronic HBV infection, sex and age are important host factors for predicting HCC risk \([8]\).

HISTOLOGICAL VIEWPOINT OF THE OUTCOME OF CHRONIC HBV INFECTION

Hepatitis is graded based on histological analysis of necro-inflammation and fibrosis, and severe hepatitis simultaneously promotes hepatic fibrogenesis and carcinogenesis \([6]\). For example, serial liver biopsy data indicate that chronic hepatitis B patients with severe necro-inflammation exhibited significantly poorer morbidity and mortality compared with patients with mild necro-inflammation \([6]\). The histological findings are occasionally paradoxical and indicate healing or aggravation of fibrosis. In cases of chronic hepatitis B with bridging hepatic necrosis, a feature of aggressive hepatitis, patients frequently recover after the flare-up, but fibrosis or cirrhosis remains \([6]\). These findings suggest that the degree of fibrosis, also referred to as the fibrosis stage, potentially reflects the sequential changes associated with progressive chronic liver disease and is a more efficient indicator of prognosis than the ongoing inflammation \([6,7,10]\).

VIROLOGICAL VIEWPOINT OF THE OUTCOME OF CHRONIC HBV INFECTION

During the active hepatitis phase, the immune response significantly inhibits viral replication while simultaneously inducing mutation of the HBV genome, including so-called “escape mutations”. HBV DNA titers in serum and hepatocytes have been associated with a less favorable course due to either poor clearance of the virus or increased virus production, whereas the long-term prognosis of patients with a low viral load is generally good \([5,11-14]\). However, HBV DNA titers are dynamic and depend on the type of mutation and anti-viral immunity, and are intricately connected to changes in hepatitis B surface antigen and hepatitis B e antigen (HBeAg) levels \([5,15,16]\). In particular, serum HBeAg and HBV DNA levels are closely associated with A1762T and G1764A, which are known as the basal core promoter (BCP) double mutation (A1762T/G1764A), and G1896A, the precore stop mutation; both A1762T/G1764A and G1896A are associated with e-suppressive phenotypes as well as decreased HBV genome replication \([5,15,16,19,20]\). The precore stop mutation synergistically modulates the influence of the BCP double mutation on HBV replication \([20]\). These mutations tend to increase HBV persistence \([19,20]\). The relationship between viral loads and hepatitis flare-ups in the immune eradication phase is not clear, and persistent infection by mutant HBV may influence the progression of chronic hepatitis \([2,12-15,20,24]\), prompting interest in the identification of viral mutations that affect the outcome of chronic HBV infection.

HBV GENOTYPES, THE BCP DOUBLE MUTATION, AND THE PRECORE STOP MUTATION

Eight distinct genotypes of HBV have been reported (denoted A-H); each genotype includes variants with less than 8% divergence among their DNA sequences \([20,28]\). HBV genotypes B and C are more closely associated with the development of HCC than other genotypes \([5,10,12,15,16]\), and are characterized by a higher prevalence of the BCP double mutation and the precore stop mutation \([20,25,27]\). Thus, genotypes B and C are apparently aggressive with respect to the development of HCC. However, in South Korea, nearly all HBV cases are genotype C2 (C) \([22,33]\). Although highly prevalent in HCC (86%), the prevalence of the precore stop mutation does not differ significantly among chronic HBV carriers with or without HCC \([22,33]\). Most isolates of HBV genotype C2 in South Korea carry the T1858 mutation \([32,33]\), which attenuates the stability of the secondary structure of the pregenome encapsidation signal (epsilon signal). In contrast, C1858 prevents the formation of G1896A \([20,25,27]\). The BCP double mutation is also not a significant factor because it is present in the majority of HBV genotype C2 strains in South Korea. For instance, the BCP double mutation is identified in 93.5% of HBeAg-negative bDNA-positive patients, 94.9% of HBeAg-negative bDNA-negative patients, and 74% of HBeAg-positive patients \([32]\). Despite the high prevalence of G1896A and BCP double mutations, the single C2 genotype of South Korea represents an intriguing model system in which to identify viral mutations with prognostic utility. Complex mutations

NATURAL HISTORY OF CHRONIC HEPATITIS B VIRUS INFECTION

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in the core promoter and precore regions of the HBV genome are of particular interest.

**CLINICAL FEATURES OF WILD TYPE HBV GENOTYPE C2**

Because the literature regarding the clinical features of wild-type HBV genotype C2 is lacking, we analyzed this genotype in comparison with three mutation types using our published raw data ($n = 442$). The selected 109 patients consisted of four groups: wild-type ($I, n = 29$), precore stop mutation alone ($II, n = 14$), BCP double mutation only ($III, n = 44$), and the A1762T, G1764A and G1896A triple mutation ($IV, n = 22$). The proportion of patients classified as group I decreased dramatically among those over 40 years of age, whereas the other groups experienced a relative increase in the proportion of individuals over 40 years of age. The proportions of HBeAg-negative patients and patients with serum HBV DNA levels < 15000 IU/mL (or 6 log copies/mL) were reduced in groups I and III compared with groups II and IV (HBeAg negative, 3.4% and 13.5% vs 57.1% and 50%, respectively; HBV DNA < 15000 IU/mL, 7.1% and 16.2% vs 33.3% and 36.4%, respectively). These results suggest that the precore stop mutation is more closely associated with the attenuation of self-replication and HBeAg production than the BCP double mutation. In group I, active hepatitis, advanced liver disease, and HCC were uncommon regardless of age compared with groups II-IV. In addition, half of the cases remained inactive for a long period (i.e., greater than 5 years). In groups I-IV, active hepatitis was noted in 44.8%, 57.1%, 72.7%, and 54.5% of patients, respectively. Advanced liver disease was noted in 6.9%, 28.6%, 22.7%, and 18.2% of patients, respectively. HCC was reported in 3.4%, 14.3%, 13.6%, and 13.6% of patients, respectively. In groups II-IV, most of the patients with advanced liver disease and/or HCC were over the age of 40. Thus, the clinical features of wild-type HBV genotype C2 conversely reflect the aggressiveness and persistence of the mutant type, and the BCP double mutation is associated with the initiation of HCC regardless of age. Nevertheless, these three types of mutations are insufficient as viral markers for outcome prediction because their capacity to discriminate between high and low risk of HCC is minimal. However, other mutations are likely important, particularly among the BCP mutant type HBVs, and the potential combinations of mutations are abundant and complex.

**KEY MUTATIONS IN THE CORE PROMOTER AND PROXIMAL PRECORE REGIONS OF THE HBV GENOME**

The core promoter overlaps the distal part of the X gene, and the proximal precore includes the epsilon signal. These two genetically distinct regions are the most complex portion of the HBV genome, which includes various functional gene clusters, such as enhancer II, the basal core promoter, the X-termination signal, two pregenomic RNA start points, the poly A signal, epsilon, and other important sequences. These regions differentially regulate the synthesis of pregenomic and pre-C mRNAs of HBV and the production of HBeAg and hepatitis B core antigen (HBCAg), and cooperatively regulate viral replication. Any single mutation can induce some form of inherent change that affects viral loads and the levels of HBeAg in serum and HBcAg and X protein in hepatocytes. These effects can subsequently modulate the immune response to viral antigens and enhance the carcinogenic effects of altered X proteins. In Far East Asia, HBV genotypes B and C are predominant, and five mutations are prominent in the core promoter and proximal precore regions: G1613A, C1653T, T1753V, A1846T, and G1899A. These select mutations are associated with the development of HCC when combined with the BCP double mutation. Many additional mutants have been reported in the literature, but most of these mutations are sporadic. Our data indicate that these mutations, together with A1762T, G1764A, and G1896A, are the most important frequent mutations in HBV genotype C2. Considering the accumulation of mutations with time and the age of HCC patients, analyses must focus on the complexity of mutations associated with HCC risk, particularly in chronic HBV carriers greater than 40 years of age.

**TRIPLE OR QUADRUPLE MUTATIONS INCLUDING THE BCP DOUBLE MUTATION**

Although single G1613A or G1896A mutations are commonly noted in HBV genotype C2 in South Korea, single C1653T, T1753V, A1846T or G1899A mutations are rarely identified. Most of these mutations occur in combination with the BCP double mutation. These results suggest that the BCP double mutation (A1762T/G1764A) may function as a starting point for the generation of viral variants harboring C1653T, T1753V, A1846T, and G1899A mutations. Therefore, it is not surprising that T1753V is more frequently linked to HBV genotype C than genotype B (19.2% vs 1.9%; $P = 0.013$). G1899A combined with the BCP double mutation is the single risk factor indicating HCC risk in Thailand and Tunisia, but the linkages between mutations are less clear in South Korea. Our data indicate that while G1896A increases steadily with time, the accumulation of A1846T begins to increase during the quadruple phase of mutations. In contrast, the other mutations begin to accumulate at the triple phase. Various specific quadruple mutations are superior to the BCP double mutation for determining HCC risk, whereas any individual triple mutation is not superior. The combination of G1613A and C1653T is associated with HCC in HBV genotype C patients, whereas the combination of C1653T and T1753V is associated with...
HCC in HBV genotype B patients\(^{33}\). Among 15 different quadruple mutations containing the BCP double mutation, our analyses indicate that only five types are predominant \([74.2\% (72/97)]\), including the combinations (G1613A + C1653T), (C1653T + T1753V), (C1653T + G1896A), (T1753V + G1896A), and (A1846T + G1896A). These mutations account for 94.4\% (34/36) of HCC cases that develop in the context of quadruple mutations. When exclusively compared with the BCP double mutation \(\text{[HCC, 13.6\% (6/44)]}\), the prevalence of HCC among these five quadruple mutations was 46.2\% (6/13, \(P = 0.02\)), 40\% (2/5, \(P = 0.1821\)), 27.3\% (6/20, \(P = 0.168\)), 66.7\% (14/21, \(P = 0.00003\)) and 46.2\% (6/13, \(P = 0.02\)), respectively. The respective odds ratios for five specific combinations were 5.4286 (95\%CI: 1.353-21.7821), 4.2222 (95\%CI: 0.5797-30.7518), 2.7143 (95\%CI: 0.7495-9.8293), 12.6667 (95\%CI: 3.6262-44.2465) and 5.4286 (95\%CI: 1.353-21.7821). The combination of C1766T and T1768A appears to enhance the carcinogenic effects of the X protein, but these mutations are rarely identified in South Korea\(^{33,50}\). Multivariate analyses of variables in relation to HCC indicate that mutation number is the only significantly independent viral factor\(^{33}\). These data indicate that complex mutations should be systematically evaluated as a function of the number of mutations.

**THE UTILITY OF THE NUMBER OF MUTATIONS OF EIGHT KEY NUCLEOTIDES IN THE PREDICTION OF HCC**

Although the development of HCC correlates with the accumulation of mutations, most studies have examined combinations of four mutations or less\(^{33,50}\). We analyzed the cumulative effects of complex mutations through a stratified analysis based on mutation number. The HCC rate in chronic HBV carriers increased linearly from wild-type to eight mutations as follows: 3.4\% (1/29), 8.7\% (2/23), 14.5\% (8/55), 21.2\% (21/99), 35.6\% (36/101), 31.8\% (21/66), 52.4\% (22/42), 78.9\% (15/19), and 75\% (6/8), respectively (\(Y = 0.0917\times X, r^2 = 0.9199\)). Quadruple mutations were the most prevalent among the study subjects; double and triple mutations were most common in the non-HCC group, whereas multiple mutations, including more than four mutations, were predominant in the HCC group\(^{33}\). Compared with the BCP double mutation, the odds ratios (95\%CI, \(P\)-value) of three to eight mutations were 1.7561 (0.6131-5.0301, 0.3250), 3.4286 (1.2623-9.3127, 0.0513), 2.7143 (0.9333-7.8939, 0.0820), 4.8571 (1.5527-15.1942, 0.0079), 30.0000 (5.3429-168.4491, 0.0000), and 24.0000 (2.4104-238.9646, 0.0023), respectively\(^{33}\). Based on these findings, we hypothesize that the number of mutations is a more sensitive predictor of HCC risk than any specific mutation\(^{33}\). In particular, cases with \(\geq 6\) mutations were associated with HCC with the greatest accuracy; the sensitivity, specificity, positive predictive value and negative predictive value were 44.0\%, 97.3\%, 94.3\%, and 63.5\%, respectively. The diagnostic efficiency of \(\geq 6\) mutations was comparable to that of alpha-fetoprotein (AFP), a specific biomarker for HCC diagnosis. The AUC was 0.824 (95\%CI: 0.759-0.890) for \(\geq 6\) mutations and 0.869 (95\%CI: 0.812-0.925) for AFP\(^{33}\).

**SPECIFIC MUTATION COMBINATIONS ASSOCIATED WITH HCC**

In a longitudinal cohort of 25 patients with serial serum samples spanning the years before and after HCC diagnosis, most of the patients with HCC (24/25, 96.0\%) exhibited \(\geq 4\) mutations including the BCP double mutation years prior to HCC development; these patients also exhibited an equal or increasing number of mutations until HCC development\(^{33}\). In particular, some mutation combinations were specifically associated with HCC, and the core mutations differed little among combinations of four, six, and seven mutations. Although the (G1613A + C1653T), (C1653T + T1753V), (C1653T + G1896A), (T1753V + G1896A), and (A1846T + G1896A) double mutations were prominent in HCC patients with quadruple mutations, the addition of any single mutation did not improve the combined effect of an existing quadruple mutation\(^{37}\). With regard to six mutations, the (G1613A + C1653T + A1846T + G1896A) and (G1613A + C1653T + A1846T + G1899A) combinations were observed in half of the HCC patients, whereas additional mutations were sporadic\(^{37}\). Compared with the BCP double mutation alone, the prevalence and odds ratios were 71.4\% (5/7, \(P = 0.0032\)) and 15.8333 (95\%CI: 2.4843-100.9110) for the (G1613A + C1653T + A1846T + G1896A), respectively, and 83.3\% (5/6, \(P = 0.0012\)) and 31.6667 (95\%CI: 3.1331-320.0591) for the (G1613A + C1653T + A1846T + G1899A), respectively\(^{37}\). With regard to seven mutations, the combinations (G1613A + C1653T + T1753V + A1846T + G1896A) and (G1613A + C1653T + A1846T + G1896A + G1899A) were observed in 86.7\% of the HCC group; the rate of HCC was 100\% (6/6) for the former combination and 85.7\% (6/7) for the latter\(^{37}\). These data suggest that the acquisition of a new mutation is not incidental; however, the new mutation potentially follows the rules of association and linkage between a mutation and an existing mutation combination.

**ASSOCIATION BETWEEN LOW-TITER HBsAg AND A NUMBER OF KEY MUTATIONS**

In the data analyses, we arbitrarily defined low-titer HBsAg as a signal-to-noise ratio of less than 35 as measured by ELISA (Abbott Laboratories, Diagnostic Division, Abbott Park, IL 60064, United States)\(^{37}\). Of 442 cases, 57.2\% (\(n = 253\)) were HBsAg-positive. Of 132 HCC cases, 47\% (\(n = 62\)) were classified as HBsAg-
Although extracting useful data regarding HBV muta-
tions in South Korea has been difficult, the present analy-
theses demonstrate that HBV genotype C2 is a good model to investigate the significance of viral mutations. Based on our previous two studies, we proposed the following hypothesis: the presence of ≥ 6 mutations is the most important viral factor in predictions of HCC risk in chronic HBV carriers infected by the BCP mutant virus (Figure 1). The number of mutations is positively correlated not only with advanced liver disease, but also with HCC independent of advanced liver disease (Figure 2). Although the eight key mutations can occur in various combinations, specific mutation combinations

Table 1  Hepatocellular carcinoma rate of specific mutation combinations and odds ratio in comparison with basal core promoter double mutation only

| Specific mutation combinations in combination with BCP2 double mutations | HCC1 rate | OR | 95%CI | P-value |
|---------------------------------------------------------------|---------|----|-------|--------|
| Wild-type                                                      | 3.4% (1/29) | 0.23 | 0.0048-2.0622 | 0.2391 |
| BCP double mutations only [(A1762T + G1764A)]2                  | 13.6% (6/44) | 1 |  |  |
| Dominant quadruple mutations                                   |         |    |       |  |
| (G1613A + C1653T)                                              | 46.2% (6/13) | 5.4286 | 5.4286-1.3530 | 0.0200 |
| (C1653T + G1896A)                                              | 27.3% (6/20) | 2.7143 | 2.7143-0.7495 | 0.1821 |
| (C1653T + G1896A)                                              | 66.7% (14/21) | 12.6667 | 12.6667-3.6262 | 0.0000 |
| (A1846T + G1896A)                                              | 46.2% (6/13) | 5.4286 | 5.4286-1.3530 | 0.0200 |
| Dominant combinations in sextuplet mutations:                  |         |    |       |  |
| (G1613A + C1653T + A1846T + G1896A)                           | 71.4% (5/7) | 14.5142 | 1.8869-185.1359 | 0.0033 |
| (G1613A + C1653T + A1846T + G1896A)                           | 83.3% (5/6) | 28.2555 | 2.5885-1517.9673 | 0.0012 |
| Dominant combinations in septuplet mutations:                  |         |    |       |  |
| (G1613A + C1653T + T1753V + A1846T + G1896A + G1899A)          | 100% (6/6) | Infinity | 5.4236-infinity | 0.0001 |
| (G1613A + C1653T + T1753V + A1846T + G1896A + G1899A)          | 85.7% (6/7) | 39.3553 | 4.0487-2018.0433 | 0.0001 |

1Hepatocellular carcinoma; 2Basal core promoter; 3Reference of odds ratio analyses; P-value was calculated by Fisher’s exact test for count data. BCP: Basal core promoter; HCC: Hepatocellular carcinoma.
are predominant in the HCC group (Table 1). However, a low titer HBeAg (< 35 signal-to-noise ratio) is indicative of HCC risk for viruses containing ≤ 5 mutations or the BCP double mutation only (Table 2). Therefore, viral mutations and clinical features are complementary in the prediction of HCC risk.

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