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Progress in the application of high sensitivity HBV-DNA technology in the detection and analysis of HBV

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Abstract: Chronic hepatitis B is one of the most prominent global public health issues, posing a serious threat to human health. Despite the effective reduction in the incidence of hepatitis B due to the use of hepatitis B vaccines in recent years, 10 to 30 million people still become infected with the hepatitis B virus (HBV) annually, and approximately 1 million people die from HBV infection and its complications. To effectively control the spread of HBV and ensure timely treatment for HBV-infected individuals, efficient detection of HBV is particularly important. Nucleic acid detection techniques targeting HBV DNA have been widely applied in diagnosing, monitoring, evaluating prognosis, and screening for blood transfusions due to their advantages of high sensitivity and strong specificity. However, there remain some HBV-infected individuals in clinical practice who harbor low concentrations of HBV DNA that are difficult to detect with existing methods. This not only increases the risk of developing chronic hepatitis, cirrhosis, and primary liver cancer but may also result in HBV infections during blood transfusions and liver transplant procedures. Additionally, the detection and monitoring of antiviral drug resistance mutations in hepatitis B can help prevent treatment failures. Due to the presence of "quasi-species," some patients have extremely low levels of HBV resistant strains that are hard to detect. In certain circumstances, such as liver transplantation and co-infection with hepatitis C virus (HCV), these undetected low-level HBV resistant strains may lead to severe clinical consequences (such as HBV infection and liver failure). Therefore, developing a high-sensitivity detection technology for HBV DNA and resistance mutations holds significant practical importance for the prevention and treatment of hepatitis B. Based on this, this article focuses on exploring the application of high-sensitivity HBV DNA technology in the detection and analysis of the hepatitis B virus, as well as the predictive value for the long-term prognosis of hepatitis B virus patients.

1. Introduction

Hepatitis B virus (HBV) infection is currently one of the most severe global public health issues. It is reported that approximately 2 billion people worldwide (around one-third of the global population) have been infected with the hepatitis B virus, with about 240 million (or 6% of the

world population) being chronic HBV carriers[1,2]. Each year, about 620,000 patients die from HBV-related diseases, primarily due to end-stage liver diseases such as HBV-related cirrhosis and liver cancer. Although HBV infection is globally distributed, there are significant regional differences in the prevalence of HBV infections, particularly severe in the Asia-Pacific and Western Pacific regions[3,4]. Statistics from 2016 indicate that 80% of HBsAg carriers worldwide are concentrated in 21 countries, with 57% of the infected individuals residing in China, India, Nigeria, Indonesia, and the Philippines[5]. China currently has the highest number of HBsAg carriers in the world (approximately 90 million), with around 20 million chronic hepatitis B patients[6]. Approximately 300,000 people die each year from HBV-related cirrhosis and liver cancer, making hepatitis B a major threat to public health in China.

Widespread vaccination programs and potentially effective antiviral treatments have led to a significant decline in the incidence of new hepatitis B infections. However, the number of HBV-related deaths due to cirrhosis and/or hepatocellular carcinoma (HCC) continues to rise. Among healthy adults infected with the hepatitis B virus, nearly 5% will develop chronic infection, which may then progress. If cirrhosis develops, the likelihood of developing liver cancer significantly increases.

Conventional quantitative PCR testing for hepatitis B suffers from low sensitivity and a broad range of negative detection, often resulting in false-negative results for patients with low viral loads, especially those with occult HBV infection, leading to missed or misdiagnoses. High-sensitivity HBV DNA testing can accurately measure the viral load in this patient group, enabling early and precise diagnosis and initiation of antiviral treatment. However, studies on peripheral blood HBV DNA levels in patients with low viral loads of chronic hepatitis B are currently limited due to methodological constraints. The lower limit of detection for conventional HBV DNA assays is 500 IU/ml, which hinders early assessment and continuous monitoring of the disease, ultimately affecting treatment and long-term prognosis. Therefore, enhancing the application of high-sensitivity HBV DNA testing in patients with chronic hepatitis B, particularly those with post-hepatitis B cirrhosis, should be prioritized in clinical practice.

2. Hepatitis B virus drug resistance

During the replication process of HBV, there is a lack of proofreading activity in the reverse transcription of RNA, which prevents the correction of mispaired bases, ultimately leading to the emergence of mutations[7]. Compared to other DNA viruses, the mutation rate of HBV is approximately ten times higher than that of other viruses[8]. The high variability of the hepatitis B virus (HBV) allows it to form a quasispecies virus population within the infected individual, dominated by advantageous variants, which is beneficial for the virus's survival in adverse environments. The development of HBV resistance is closely related to this inherent high variability. Additionally, the emergence of HBV resistant strains is also linked to the long-term use of antiviral medications.

The resistant mutations of the hepatitis B virus primarily occur in the P region of the polymerase gene, especially in the reverse transcriptase (RT) coding region. Mutations in the RT coding region lead to changes in the sequence of specific amino acids, altering the conformation of the RT region, which prevents nucleoside analog drugs from binding, resulting in drug resistance[9]. Moreover, since there is some overlap between the RT gene and the HBsAg gene, mutations in this region can simultaneously cause changes in HBsAg, thereby affecting the antigenicity, immune recognition, replication capability, and virulence of the hepatitis B virus[10-12]. Consequently, under the selective pressure of medication, the wild-type HBV, which cannot replicate, is suppressed, allowing the drug-resistant mutant HBV to gradually proliferate and eventually replace the wild

type, leading to hepatitis B resistance.

Since existing hepatitis B treatments cannot eradicate the virus from patients' bodies, the majority of chronic hepatitis B (CHB) patients require long-term antiviral therapy, and the emergence of drug resistance can severely affect treatment outcomes. Research indicates that once HBV develops resistance, it may lead to rapid viral replication, resulting in the failure of antiviral therapy, particularly increasing the risk of progression to hepatocellular carcinoma (HCC) in patients with decompensated cirrhosis[13]. Previous studies have shown that among 312 patients with decompensated cirrhosis followed for two years, 198 received antiviral therapy with nucleoside analogs (NAs), of which 162 achieved complete virological response (CVR), while 36 developed resistance. The cumulative incidence of HCC in the resistance group over two years was 30.6%, significantly higher than that of the CVR group (4.3%) and the control group (10.3%). In the resistant group, 9 patients experienced treatment failure, and among them, 5 (55.6%) developed HCC[14].

According to the ease with which HBV develops resistance to nucleoside analogs, these drugs can be divided into two categories: those with a low resistance barrier, such as lamivudine (LAM), adefovir dipivoxil (ADV), and telbivudine (TBV), and those with a high resistance barrier, such as entecavir (ETV) and tenofovir disoproxil fumarate (TDF). The rates of resistance development vary between drugs: the five-year cumulative resistance rate for LAM is approximately 70%, for ADV it is around 29%, and the three-year resistance rate for TBV is about 34%[15]. In contrast, ETV has a relatively low rate of resistance during treatment, with only a 1.2% resistance rate after six years[16], and TDF shows a resistance rate of less than 1% after two years of treatment[17]. Due to their lower rates of resistance, entecavir and tenofovir have become the preferred treatments for hepatitis B in clinical recommendations.

Among the various resistance mutation sites in HBV, the mutation at the rt204M site was first observed in HBV strains resistant to lamivudine (LAM). Additionally, the rtM204V/I mutations are among the most frequently occurring spontaneous mutations associated with HBV resistance; in untreated patients, the mutation rate can reach 5%[18]. Furthermore, the rtM204V/I mutations are also linked to resistance against ETV. A study conducted in Europe found that rtM204V/I is the most commonly occurring primary mutation, detectable in 49% of patients undergoing antiviral treatment. Furthermore, the naturally occurring mutation at the rtM204 site has been confirmed to be closely related to the occurrence of hepatocellular carcinoma (HCC)[19].

3. The Importance of Highly Sensitive Detection of Hepatitis B Drug Resistance Mutations

In the treatment of hepatitis B, antiviral drugs can control serum HBV DNA at lower levels. However, long-term antiviral therapy not only reduces hepatitis B virus (HBV) load but can also lead to the emergence of drug-resistant strains in patients. A study monitoring resistance during early treatment with lamivudine (LAM) found that after approximately 2 months of LAM therapy, serum HBV DNA levels fell below the limit of detection for quantitative PCR (qPCR) [20]. By 4 months, drug-resistant YMDD mutations were detected, and after continuing treatment for 10 months, a viral breakthrough occurred. During the 4 to 8-month window period before the breakthrough, viral load remained undetectable. Using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to detect HBV drug resistance mutations indicated that YMDD resistant strains were present even when qPCR did not detect HBV DNA, suggesting that low-concentration drug-resistant strains emerge prior to a viral breakthrough. If these mutations are detected in a timely manner and antiviral therapy is adjusted accordingly, it may be possible to prevent viral outbreaks. However, clinical practice primarily relies on monitoring changes in serum HBV DNA levels to predict the emergence of drug resistance. While in most

cases, the emergence of low-proportion resistant mutations does not worsen the patient's condition, severe liver failure can still occur in certain situations, such as after liver transplantation[21]. Therefore, utilizing highly sensitive detection techniques for HBV drug resistance mutations for early identification can aid in formulating more appropriate hepatitis B treatment strategies, thereby reducing the risks associated with drug resistance.

4. Clinical Applications of Highly Sensitive HBV-DNA Testing

HBV-DNA primarily exists within Dane particles in the blood, and the virus must be lysed prior to testing. HBV-DNA is a direct marker of viral replication and infectivity. Quantitative detection of HBV-DNA is significant for assessing the replication level of the hepatitis B virus, the degree of infectivity, and evaluating the efficacy of antiviral treatment. Once the hepatitis B virus enters liver cells, it begins its replication process. HBV-DNA enters the nucleus to form covalently closed circular DNA (cccDNA), which then serves as a template for synthesizing pregenomic mRNA. This mRNA is subsequently used to synthesize negative-strand DNA, which in turn is used to synthesize positive-strand DNA, resulting in the formation of complete HBV-DNA[22]. Throughout the entire process of HBV replication, stable cccDNA exists in the nucleus, which undergoes a reverse transcription process. Since this reverse transcription process cannot be blocked by nucleoside analogs, cccDNA within liver cells is difficult to eliminate completely, making it challenging to eradicate the virus from hepatitis B patients clinically.

Currently, the most commonly used testing method for HBV is serological testing, with key indicators including HBsAg, HBeAg, anti-HBs, anti-HBe, and anti-HBc, collectively referred to as the five hepatitis B markers. These are detected using immunological methods such as enzyme-linked immunosorbent assay (ELISA). However, clinical scenarios often present challenges such as the infection window period, antigen epitope mutations, and serological conversion. Additionally, the sensitivity of immunological testing methods is relatively low, making it ineffective in detecting HBV infections in certain cases[23].

With the development and enhancement of HBV-DNA technology, HBV-DNA positivity has still been detected in the serum or liver tissue of some HBsAg-negative hepatitis patients. Consequently, scholars have proposed the concept of Occult HBV Infection (OBI), indicating that OBI is a hidden form of chronic HBV infection that can persist in the body for a long time. Typically, when immune function is normal, patients may exhibit no clinical symptoms or signs. However, in the presence of other conditions that may exacerbate virus-induced liver tissue pathological damage, such as hepatic steatosis, co-infection with HCV or HIV, chronic hematological diseases, immune dysfunction, obesity, and excessive alcohol consumption, liver function abnormalities may arise, manifested by elevated serum ALT levels. This is particularly relevant for patients receiving nucleos(t)ide analog (NAs) treatment, as it may be related to immune damage associated with OBI[24,25].

Yu Juanchun [26] conducted a study on 20 patients diagnosed with occult hepatitis B, measuring HBV DNA levels. Among these, 14 patients had HBV DNA levels below 500 copies/ml, indicating that a significant proportion of this population has low-level positive HBV DNA despite being HBsAg negative. High-sensitivity HBV DNA testing demonstrates both high sensitivity and specificity, playing an important role in the monitoring and treatment of hepatitis B patients. Therefore, the authors recommend paying close attention to this group of patients and continuously monitoring other serological and virological indicators to avoid missed diagnoses.

Lee et al. [27] conducted a study on 49 patients with chronic hepatitis B who achieved HBeAg seroconversion after receiving lamivudine treatment. They continued lamivudine (LAM) therapy for another 12 months to determine whether extending LAM treatment could reduce the risk of

virological relapse after treatment in patients with chronic hepatitis B. Based on the duration of extended LAM treatment, the patients were divided into two groups: Group 1 (6 months, n=23) and Group 2 (12 months, n=26). During the study, HBeAg seroconversion and HBV DNA levels at the end of treatment were measured. The results showed that the relapse rate after two years was 37% in patients with HBV DNA levels below 200 copies/ml, whereas the rate was 73% among patients with HBV DNA levels ≥ 10^3 copies/ml. Therefore, the researchers concluded that extending lamivudine treatment (from 6 to 12 months) did not reduce the virological relapse rate in patients with chronic hepatitis B. However, continuous monitoring of serum HBV DNA levels using quantitative PCR could be useful for assessing disease progression and virological relapse.

With the detection of a large number of OBI patients, increasing research indicates that HBV DNA is a direct marker of viral replication and infectiousness[28]. HBV DNA can be detected in the early stages of HBV infection (one month post-infection)[29]. Due to the high sensitivity and timeliness of nucleic acid testing for HBV DNA, its detection has gradually been applied to the diagnosis of hepatitis B virus. From 2004 to 2008, the global implementation of nucleic acid amplification tests (NAT) for HBV DNA significantly reduced the risk of HBV transmission through blood transfusion[30]. Compared to serological testing techniques, NAT can shorten the window period for detecting HBsAg seroconversion and improve the detection rate of OBI[31]. Furthermore, quantitative detection of HBV DNA is of great significance in assessing the replication level of the hepatitis B virus, the degree of infectiousness, and evaluating antiviral efficacy [32].

However, some HBV-infected patients still harbor low concentrations of HBV DNA that cannot be detected by existing methods. Studies have shown that low levels of HBV DNA are commonly found in the blood or liver tissue of HCC patients, low-replicative hepatitis B virus carriers, normal hepatitis B virus carriers, some hepatitis C patients, and patients co-infected with HCV and HBV. Low concentrations of HBV DNA are also present in patients undergoing long-term antiviral treatment, with viral loads even below the detection limits of current testing methods[33].

A study on HBV DNA in HCC patients with HCV infection found that HBV DNA was detected in 50% of the cancerous tissues of HCC patients, with only 12.5% of patients being detected by hybridization methods, and some HCC patients' liver tissue cells contained only single copies of HBV DNA. Previous studies have suggested that the serum HBV DNA load in chronic hepatitis B patients fluctuates over the long course of the disease. HBV infection can be divided into four different phases: immune tolerance phase, immune clearance phase, inactive or low-replicative phase, and reactivation phase. Chronic hepatitis B patients in different infection phases exhibit significant differences in HBV DNA concentration, with the HBV DNA levels in inactive or low-replicative phase patients often falling below the detection limits of conventional testing methods. During the long-term treatment of chronic hepatitis B, existing hepatitis B treatments can effectively suppress the replication of the hepatitis B virus in patients, controlling the viral load at lower levels. Research shows that after one year of nucleoside analog treatment, up to 21-76% of patients had serum HBV DNA levels drop below the clinical detection range. However, current antiviral medications cannot eradicate HBV from patients' bodies, and hepatitis B recurrence often occurs after discontinuation of treatment. Extremely low concentrations of HBV DNA have been found in some CHB patients who have met the criteria for stopping treatment[34]. After antiviral therapy, most hepatitis B patients are unable to completely eliminate HBV from their bodies, and low concentrations of viremia persist.

Patients with occult hepatitis B virus (HBV) infection typically have negative serum HBsAg, yet positive HBV DNA in serum and/or liver tissue, along with clinical manifestations of chronic hepatitis B. It is estimated that 20% of patients with occult chronic hepatitis B have negative HBV serological markers. An epidemiological study on occult HBV infection (OBI) found that some OBI

patients had extremely low concentrations of HBV DNA in their serum (with a maximum of only 14.1 IU/mL), and even lower levels of HBV DNA in their liver tissue (with a maximum of only 6.21 IU/mL). In OBI patients, the low concentration of HBV DNA and its fluctuations severely impact the diagnosis of OBI.

Furthermore, studies have indicated that using a lower HBV DNA level as a withdrawal indicator for antiviral treatment can reduce the rate of hepatitis B recurrence after treatment cessation. In a study examining the correlation between the virological recurrence of chronic hepatitis B after stopping nucleos(t)ide analogs (NAs) and HBV DNA levels at the time of withdrawal, researchers found that patients with HBV DNA levels below and above 20 IU/mL at treatment cessation had recurrence rates of hepatitis B one year after withdrawal of 56.4% (62/110) and 76.8% (73/95), respectively. Similarly, in another study investigating the critical HBV DNA threshold for stopping antiviral treatment and the recurrence of hepatitis B, it was found that patients with residual HBV DNA below and above 2.24 IU/mL had recurrence rates of 50% and 86.7%, respectively[34]. Additionally, for the treatment of certain special patients, due to the higher risk of disease progression, highly sensitive HBV DNA diagnostics can directly affect the timeliness of antiviral treatment. For example, inactive HBV carriers have a higher risk of developing hepatocellular carcinoma (HCC) and liver disease-related deaths compared to uninfected individuals. Both the American Association for the Study of Liver Diseases and the European Association for the Study of the Liver recommend the use of highly sensitive quantitative PCR assays to detect HBV DNA levels in patients' serum, to guide the clinical management of chronic hepatitis B patients. The 2015 edition of China's chronic hepatitis B prevention and treatment guidelines also recommends using HBV DNA quantitative detection reagents with higher sensitivity and accuracy for relevant testing. In patients with decompensated liver cirrhosis, initiation of treatment is necessary as soon as HBV DNA is detected; for patients with severe chronic hepatitis B recurrence and elevated ALT, detection of HBV DNA also requires prompt initiation of treatment.

After effective antiviral treatment, liver tissue pathology in patients with chronic hepatitis B can show improvement and normalization, and tissue damage in some patients with liver fibrosis and early cirrhosis can even be reversed. With the continuous advancement of antiviral medications (such as interferons and nucleotide analogs), the treatment goals for chronic hepatitis B patients have gradually elevated to higher standards. Theoretically, the goal of antiviral treatment should be a long-term and sustained virological response, aiming for the eventual complete clearance of HBV DNA. Global treatment guidelines for hepatitis B vary in their objectives. In 2015, the American Association for the Study of Liver Diseases (AASLD) proposed a definition of "immunological cure" for chronic hepatitis B treatment: HBsAg clearance with sustained HBV DNA suppression; virological cure refers to the complete elimination of the virus, including the cccDNA form. The 2015 version of the Chinese "Guidelines for the Prevention and Treatment of Chronic Hepatitis B" stated that the criteria for clinical cure include achieving a sustained virological response after discontinuation of treatment, along with HBsAg seroconversion or the presence of anti-HBs, normal ALT levels, and mild or no liver tissue lesions. In 2017, the European Association for the Study of the Liver (EASL) emphasized the treatment goal of "functional cure": the clearance of HBsAg, which is considered the optimal treatment endpoint. Additionally, the 2015 version of the guidelines for the prevention and treatment of chronic hepatitis B clearly recommended using quantitative detection reagents with higher sensitivity and accuracy for HBV DNA quantification. Some studies also recommended using highly sensitive detection reagents produced by Roche.

5. Shortcomings and prospects

Currently, the existing methods for detecting drug resistance mutations in the hepatitis B virus

(HBV) include direct sequencing, amplification refractory mutation system PCR (ARMS-PCR), mismatch PCR, restriction fragment length polymorphism, gene chips, INNO-Lipa, MALDI-TOF MS, and pyrosequencing[35]. Among these, direct DNA sequencing is considered the gold standard for detecting gene mutations in clinical practice. This method allows for the direct acquisition of the gene sequence in the HBV drug resistance mutation region, enabling the simultaneous analysis of multiple drug resistance mutation sites while obtaining information on both known and unknown resistance mutation sites. It is currently the most widely used detection method in clinical practice. However, this method is time-consuming and labor-intensive, requiring professional personnel to analyze the results, making it unsuitable for high-throughput screening of mutations. Additionally, it has relatively low sensitivity, as it can only detect mutations when the mutant strain exceeds 20% of the total HBV population, leading to missed detections of low-frequency drug resistance mutations and hindering early detection of resistance mutations[36]. Other detection methods for drug resistance mutations are not suitable for clinical application due to complex sample preprocessing, high instrument requirements, and high costs. Currently, qPCR-based YMDD drug resistance mutation detection technology is relatively mature and has higher sensitivity than direct sequencing; however, it cannot accurately determine results for samples with low viral loads or Ct values close to the threshold. Therefore, there is an urgent need for methods that can sensitively detect HBV DNA and drug resistance mutations to monitor viral replication status and treatment efficacy, allowing for timely adjustments to clinical treatment plans.

6. Conclusion

Currently, there are still some HBV-infected individuals in clinical settings who have low concentrations of HBV DNA that are difficult to detect with existing methods. This not only increases the risk of developing chronic hepatitis, cirrhosis, and primary liver cancer, but may also lead to HBV infections during blood transfusions and liver transplantation processes. Additionally, some patients harbor extremely low levels of HBV resistant mutant strains that are challenging to detect; in certain cases, these resistant strains may result in serious clinical consequences (such as HBV infection and liver failure). This article explores the application of high-sensitivity HBV DNA technology in the detection and analysis of hepatitis B virus, as well as its predictive value for the long-term prognosis of HBV patients. It presents the current status of high-sensitivity HBV DNA detection, highlights some shortcomings, and suggests directions for future development, with the hope of providing insights for research on high-sensitivity detection of the hepatitis B virus.

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