REVIEW ARTICLE

Newer Diagnostic Virological Markers for Hepatitis B Virus Infection

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Abstract

Chronic Hepatitis B (CHB) remains a major public health problem, leading to various complications such as liver fibrosis, cirrhosis, and hepatocellular carcinoma. The existing diagnostic markers for Hepatitis B virus (HBV) are limited in distinguishing different CHB phases and intra-hepatic viral replication activity. In the past few years, several non-invasive potential blood markers that reflect viral intra-hepatic replicative state more accurately have been in progress and are gaining importance. Despite substantial efforts, the clinical utility of these new markers in CHB management is limited and unexplored. Therefore, in this review, we will discuss some of the newer HBV markers, their potential role in the diagnosis and monitoring of CHB patients.

Keywords: Chronic Hepatitis B, covalently closed circular DNA, Hepatitis B virus, Hepatitis B core-related antigen, Hepatitis B virus DNA, Hepatitis B virus RNA, Hepatitis B surface antigen, Integrated DNA.

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Introduction

With approximately 254 million cases reported, Chronic Hepatitis B (CHB) is a major public health problem, leading to complications including liver cirrhosis, fibrosis, and hepatocellular carcinoma (HCC).[1] Currently, available treatment including nucleos(t)ide analogues (NAs), and pegylated interferon (PEG-IFN) are effective in suppressing viral replication in blood. However, complete cure (complete viral eradication from the liver) has not been achieved due to Hepatitis B virus (HBV) persistence as covalently closed circular DNA (cccDNA) or integrated DNA inside the liver cell. The widely used traditional markers viz Hepatitis B surface antigen (HBsAg), Hepatitis B e antigen (HBeAg), antibody to HBeAg (anti-HBe), antibody to HBsAg (anti-HBs), antibody to Hepatitis B core antigen (anti-HBc) and HBV DNA have certain limitations in predicting clinical outcome of HBV infection.[2] Quantification of cccDNA truly reflects HBV intra-hepatic transcriptional activity but is not routinely preferred as it requires liver biopsy, which is an invasive procedure. Therefore, non-invasive biomarkers predicting disease progression, outcome, and determining therapy endpoint is need of the hour. In the last decade, many non-invasive potential blood markers [viz HBsAg quantitative, HBsAg isoforms, HBV RNA, Hepatitis B core-related antigen, integrated HBV DNA] that accurately reflect intra-hepatic virus replicative state have been a robust area of research.[3–5] In this review, we will discuss these newer diagnostic markers for HBV and their potential role in diagnosis and monitoring.

HBV Genome Organization

HBV belongs to *Hepadnaviridae* family and genus *Orthohepadnavirus*. The HBV envelope is composed of a host lipid bilayer and small, middle, and large HBsAg (S-HBsAg, M-HBsAg, and L-HBsAg).[6] The virion DNA is approximately 3.2 kb in length which is partially double-stranded relaxed-circular DNA (rcDNA). The genome is composed of four overlapping open reading frames (ORFs): Surface (preS/S), core (PreC/C), polymerase (P) and X; of these, P is the largest ORF that overlaps the remaining three ORFs (Fig. 1). The regulation of HBV replication and transcription relies on specific regulatory elements. This include enhancers, various promoters (S1, S2, pre-core, core, and X), as well as signals responsible for encapsidation (ε), polyadenylation, and replication [termed as direct repeats 1 (DR1) and direct repeats 2 (DR2)]. Since it has overlapping genes, mutations in one region can likely lead to changes in other genes. Some of these changes may lead to functional changes in the subsequent translational products.[7,8]

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Replication of HBV

HBV enters the hepatocyte by binding to its surface receptors – heparan sulfate proteoglycans (HSPGs) and sodium-taurocholate cotransporting polypeptide (NTCP). Further, nucleocapsid is uncoated, and viral DNA is released into the nucleus. In the nucleus, rcDNA is converted into cccDNA; and is maintained as "episome" or "mini chromosome" (Fig. 2). The cccDNA further acts as a template and uses host RNA polymerase to transcribe HBV transcripts of varied lengths (0.7, 2.1, 2.4 and two 3.5 kb). In the cytoplasm, 2.4 and 2.1 kb transcripts are translated into L-, M-, and S- HBsAg, while 0.7 kb transcript into Hepatitis B X (HBx) protein, a multifunctional trans-activator.[8] The synthesis of surface proteins leads to the formation of non-infectious sub-viral (spherical and filamentous) and complete viral particles (Dane particle); all of which are secreted into the circulation.[9] The 3.5 kb pre-core RNA (pcRNA) is translated into HBeAg. Another 3.5 kb pre-genomic RNA

(pgRNA) is translated into viral polymerase and core protein. The pgRNA also acts as a reverse transcription template to produce HBV DNA minus strand, leading to the formation of rcDNA, or a replication intermediate, i.e., double-stranded linear DNA (dslDNA). This rcDNA enveloped by surface proteins is further released into the circulation (Dane particle) or re-enters the nucleus to replenish cccDNA pool. Similar to rcDNA, dslDNA can also re-enter the nucleus to serve as a dominant substrate for integration with the host genome or can be released into the circulation as enveloped virions. Virions released extracellularly infect neighboring cells, leading to sustained infection.[8,10]

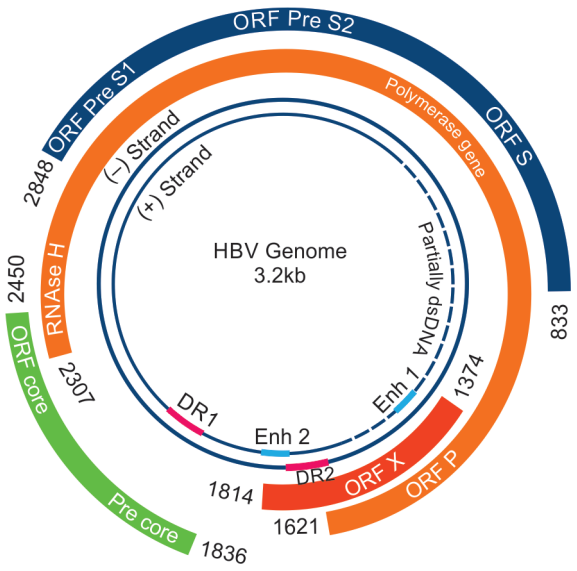

Fig.1: HBV genome organization

Conventional HBV Markers

To effectively screen, diagnose, and subsequently treat individuals infected with HBV, it is essential to have a comprehensive understanding of the conventional diagnostic makers. Some markers like HBsAg and anti-HB core total are useful in diagnosis while others like HBeAg, anti-HBe, anti-HBs, and HBV DNA are useful in defining the different phases of CHB and monitoring patients.[3] In recent years, HBV diagnostic assays with enhanced detection limits and reduced turn-around time (TAT) have been developed and used across laboratories, which is briefly discussed in the subsequent section.

HBsAg is a hallmark for diagnosis and screening of HBV infection and its detection include both qualitative and quantitative methods. It is secreted by both cccDNA and integrated DNA, therefore representing intra-hepatic viral burden. The currently available assays cannot differentiate different HBsAg forms and their origin.[11] Qualitative detection of HBsAg is routinely done using rapid card tests (RDT), enzyme linked immunosorbent assay (ELISA) or chemiluminescent assay (CLIAs). Due to the limited accessibility and affordability of laboratory-based methods, WHO recommend RDTs as a potential alternative for HBV diagnosis offering improved linkage to care and treatment.[12] Only a few RDTs have met WHO pre-qualification criteria for diagnostic purposes, i.e., ≥99% sensitivity, ≥98% specificity with analytical sensitivity ≤4 IU/mL.[13] A false-negative result with RDTs is possible which may be associated with low HBsAg levels, various HBsAg mutants and HBV genotypes/subtypes. With recent advances in technologies, newer RDTs have overcome these limitations. A recently launched "Determine HBsAg 2" RDT has an improved limit of detection (0.1 IU/mL) and ability

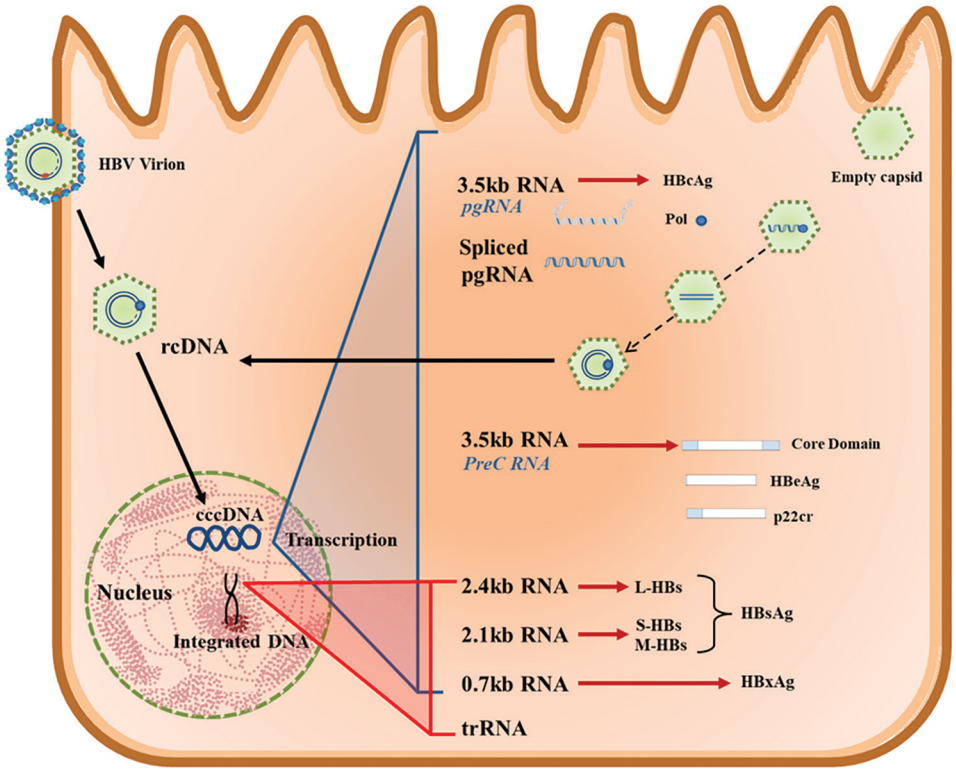

Fig. 2: Schematic representation of HBV replication

to detect major vaccine escape mutants.[14] Recently, new version of CLIA-based assay like HBsAg Next assay (Abbott Diagnostics, IL, USA) is now available with an enhanced detection of very low HBsAg levels (up to 0.005 IU/mL, earlier 0.05 IU/mL). The improved analytical sensitivity will help in ascertaining HBsAg loss following treatment or spontaneous clearance to assess functional cure, diagnosing occult Hepatitis B infection (OBI) and screening in blood bank settings.[15,16]

HBV DNA is indicative of active replication and correlates with disease progression. Quantitation of HBV DNA is essential for guiding treatment decisions, monitoring treatment response and may indicate the emergence of resistance variants. Serum HBV DNA levels have also been shown to predict the risk of cirrhosis and HCC.[17,18] Real-time quantitative PCR-based assays are the most commonly used for HBV DNA detection and expressed as WHO-standardized IU/mL. The bottleneck in any molecular test is cost, time, trained personnel, and infrastructure demanding. Fully automated assays with extraction followed by detection/quantitation are commercially available and include Cobas AmpliPrep/Cobas TaqMan HBV and Abbott Real Time HBV assay. These assays offer lower limit of quantitation as low as 10–20 IU/mL and high sensitivity (99%) and specificity (≥95%). However, their utility in resource-limited settings is restricted due to the high cost and need for specialized instruments.[19] Point of care (POC)/near POC molecular assay (Xpert® HBV Viral Load) for HBV DNA quantitation is also available which is user-friendly and obviates the need for batch testing with shorter TAT.[20] As opposed to batch testing dependent systems, random access systems allow testing of a single sample also even if other analyses are in progress further causing a reduction in TAT. One such assay is NeuMoDx™ 96 Molecular System (QIAGEN) which has recently been evaluated for HBV DNA and has shown promising results.[21,22]

New HBV Markers

Currently, available HBV markers are insufficient in predicting the clinical outcome of HBV. Therefore, new potential non-invasive biomarkers which in part, can predict early disease progression and aid in better monitoring and response to antiviral therapy are essential. Several studies have evaluated new biomarkers such as quantitative HBsAg, HBV RNA, and HBV core-related antigen (HBcrAg) for their clinical utility and response to treatment. Here, we endeavor to summarize a few emerging HBV biomarkers and their clinical relevance.

Quantitative Hepatitis B surface antigen (qHBsAg)

In the past few years, quantitative HBsAg (qHBsAg) has emerged as a crucial marker, correlating with HBV DNA levels. The quantification is done on CLIA-based platforms, including Architect HBsAg assay (Abbott Diagnostics, IL, USA), Elecsys HBsAg II quant assay (Roche Diagnostics, Indianapolis, USA), and DiaSorin Liaison XL (DiaSorin, Saluggia, Italy), all with an analytical sensitivity of 0.05 IU/mL.[23–25] Recently, a novel HBsAg quantitative assay, Lumipulse G HBsAg is launched with improved analytical sensitivity of 0.005 IU/mL.[26] The qHBsAg is increasingly used to monitor disease progression and treatment response to NA therapy.[27,28] In particular, higher levels of qHBsAg have been observed among HBeAg-positive than that of HBeAg-negative patients. Along with HBV DNA, qHBsAg levels may be a relevant marker to predict the risk of progression to HCC and viral relapse, if any.[29] The available qHBsAg assays are cost-effective with high throughput; therefore, can be widely implemented. However, the correlation of HBsAg titers with levels of HBV DNA and cccDNA needs more probing with clinical implications.

Hepatitis B surface antigen isoforms

A single ORF encodes all three isoforms of HBsAg, i.e., S-, M-, and L-HBsAg. S-HBsAg constitutes the predominant fraction in HBV-virions and sub-viral particles.[6] Recently, it has demonstrated that different HBsAg forms level vary significantly during different CHB stages.[30] Studies demonstrated that M- and L-HBsAg decline rapidly before S-HBsAg loss, suggesting their potential role in predicting functional cures of HBV.[31,32] However, due to limited studies evaluating the correlation of HBsAg isoforms with CHB phases and treatment response, overall findings are still debatable. Therefore, extensive studies are required to determine association between HBsAg isoforms and other markers like qHBsAg and HBV DNA.

Quantitative Hepatitis B core antibodies (qHBcAb)

Anti-HBc is a traditional serological marker for diagnosis and includes IgM and IgG. IgM type is seen in acute phase and also during reactivation whereas IgG can be seen for year's post-HBV infection.[33] Estimating anti-HBc IgM levels effectively differentiates acute HBV infection from reactivation.[34] The current immunoassays available for quantification of anti-HBc are based on ELISA (Wantai Biological, Beijing, China), CLIA (Fujirebio, Tokyo, Japan), or lateral flow. The role of qHBcAb in differentiating CHB stages is debatable.[33] A good correlation has been documented between qHBcAb and ALT levels.[35] As opposed to ALT, qHBcAb is HBV specific indicator for hepatocyte damage and hence serves a better predictor of significant liver inflammation even in subjects with normal to near normal ALT levels.[36] It can also help in predicting the chances of HBeAg seroconversion among both treated and untreated individuals.[37,38] Since qHBcAb indicates the immune response, baseline level may also help to predict response to anti-viral therapies.[37,39,40] Additionally, qHBcAb levels >1,000 IU/mL at the time of discontinuation of NA were associated with a lower relapse risk.[41] Early identification of HBV reactivation following immunosuppressive therapy is also possible in patients with lymphoma and resolved HBV infection.[42]

Quantitative Hepatitis B e antigen

HBeAg can be measured in immunoassay including CLIA, ELISA, or lateral flow based.[24,43,44] They are often used to assess the treatment response in CHB patients who are HBeAg positive and initiated on antiviral treatment.[19] WHO proposed first international standard for HBeAg (100 IU/mL) in 2013.[45] Presently, no commercial kits are available for quantitation of HBeAg. As a result, due to a lack of standardization and heterogeneity among the reference standards and immunoassays, the findings of the studies could not be compared well. However, few studies to determine the association between HBeAg levels with seroconversion, HBV DNA levels and response to anti-viral therapy were conducted and showed promising results.[43,46,47] Further prospective studies evaluating potential role of HBeAg levels, in immune-modulation and disease progression, in conjunction with other HBV markers are required to delineate HBV pathogenesis better.

Hepatitis B core-related antigen (HBcrAg)

With recent developments in diagnostic assays, HBcrAg is being increasingly recognized as a potential non-invasive surrogate marker reflecting intra-hepatic transcriptional activity. It comprises of three proteins: HBcAg, HBeAg, and a 22 kDa truncated core-related protein (p22cr), all encoded from pre-core/core region

and share identical 149 amino acid sequences.[48] The commercial assays available for quantifying HBcrAg levels are CLIA-based: Lumipulse G HBcrAg assay (Fujirebio Europe) and ultrasensitive iTACT-HBcrAg assay (Fujirebio Inc, Tokyo).[49,50] HBcrAg level varies significantly during the different stages of CHB and is reported to be a better marker than qHBsAg for differentiation. Previous reports showed that HBcrAg levels were higher in HBeAg-positive than in HBeAg-negative patients. Among HBeAg positives, higher levels were observed in chronic infection than in chronic hepatitis.[51,52] A good correlation of HBcrAg with HBV DNA, intrahepatic pgRNA and cccDNA had been documented.[48] Reduced intra-hepatic transcriptional activity with a lower amount of cccDNA and reduced fibrosis and necro-inflammatory scores were observed in patients with HBcrAg levels less than 3 log U/mL.[53] Hence, HBcrAg may reflect better transcriptional activity of cccDNA compared with qHBsAg.[54,55] HBcrAg also serves a useful marker in predicting HBsAg and HBeAg serconversion.[52,56] In contrast to HBV DNA, HBcrAg declines in similar fashion as cccDNA level among those patients receiving anti-HBV therapy.[55,57,58] In addition to various other markers, HBcrAg levels may also serves to be useful tool to identify patients at relapse risk following cessation of therapy.[59,60] The risk of HBV reactivation following immunosuppressive therapy can also be predicted by measurement of HBcrAg levels.[61] More recently, HBcrAg proven to be superior to HBV DNA in predicting HCC occurrence among both treatment naive and treatment-experienced.[62,63] Additionally, it also serves a very important tool to predict HCC reoccurrence after curative surgical treatment.[64]

Hepatitis B virus RNA (HBV RNA)

Circulating HBV RNA has emerged as a promising surrogate marker of cccDNA, reflecting intra-hepatic transcriptional activity. HBV RNA circulates primarily in the form of virus-like particles (including virions and capsids) in the blood. As concluded from recent understanding, circulating HBV RNA is heterogeneous in length, and has been known to be primarily pgRNA consisting of full-length, 3′ terminally truncated (poly A-free), and spliced pgRNA.[65] In treatment-naive individuals, generally, HBV RNA is present in lower levels (approximately 1–2 $\log_{10}$ copies/mL) compared with HBV DNA.[66] Studies have shown HBV RNA positively correlated with HBV DNA and HBsAg in HBeAg positive, unlike HBeAg negative cases in which HBV RNA positively correlated with HBV DNA only.[67] Serum HBV RNA level varies during different CHB phases, with the highest level recorded in HBeAg-positive chronic infection followed by HBeAg-positive chronic hepatitis.[68] Recent studies have demonstrated HBV RNA as a better predictor for HBeAg seroconversion compared with HBsAg and HBV DNA in CHB cases receiving NAs.[69] HBV RNA has also shown promising results in predicting virological and clinical relapse after NA discontinuation with a lower rate of relapse with negative/low HBV RNA at the end of the therapy.[70] Circulating HBV RNA levels have also been correlated with the chances of HCC development among CHB.[71]

Several techniques with different amplification targets for HBV RNA quantitation are available viz. real-time PCR-based assays, droplet digital PCR (ddPCR), rapid amplification of cDNA ends (RACE)-based methods, simultaneous amplification testing (SAT), hybridization-based and via branched DNA signal amplification technology (Quant Gene assays).[2] Recently, commercial HBV RNA assays, like Abbott m2000 RNA (Abbott Laboratories, Abbott Park, IL, USA), Roche HBV RNA (Roche Diagnostics, Pleasanton, CA, USA) and Rendu Biotechnology HBV-SAT (Rendu Biotechnology, Shanghai, China), have been developed and validated.[72,73] The performance of the available methods has been evaluated, yet no standardized HBV RNA detection method and no widely accepted RNA standard are available so far. Apart from varying sensitivity, interference by HBV DNA during RNA detection is also one of the major challenges. Hence, the development of convenient and reproducible methods and further comprehensive exploration of HBV RNA is the need of the hour.

Integrated HBV DNA

HBV DNA integration is an early event in HBV infection seen in all the phases of CHB in cases with or without HCC. Integrated DNA represents a source of HBsAg and not new viral particles due to intact and functional promoters of S ORF only.[10] The exact mechanism behind association of integrated DNA with HCC development is largely inconclusive. It has been shown that integration led to prolonged secretion of HBsAg, HBx, or mutated HBsAg/HBx proteins which further favor viral persistence, causing liver damage via multiple mechanisms, ultimately leading to HCC development.[74] A high frequency of integrated DNA is seen among HBeAg positive compared with HBeAg negative patients. It has been hypothesized that during HBeAg seroconversion, HBV-specific T cells selectively kill hepatocytes undergoing HBV replication, which lead to clonal selection and expansion of HBV-integrated hepatocytes as they bypass the immune response and further contributes to HCC development.[75]

Integrated DNA can be detected in both serum and liver biopsy of HBV-infected cases. Several detection methods have been evolved with time including Southern Blot assays, *in situ* hybridization, Inverse PCR (inv PCR), Alu PCR, all being technically challenged and further limiting their clinical utility. With advances in the genomics era, next-generation sequencing (NGS) platforms are increasingly recognized as useful tools which include whole-genome sequencing (WGS), whole-exome sequencing (WES), and RNA sequencing (RNA-seq).[74] Overall molecular biology of integrated DNA has been vastly explored recently, however, their role in liver disease progression, HCC development and impact on achieving functional cure still remain the question of interest for better HBV biology understandings.

Hepatic Covalently Closed Circular DNA (cccDNA)

The persistence of intra-hepatic cccDNA is a key obstacle in achieving the complete cure for HBV as currently available treatment does not eliminate cccDNA. Novel anti-viral agents targeting replication-competent cccDNA are thus required for a complete cure. The cccDNA persistence is also associated with HCC development due to the production of HBx and HBsAg protein with oncogenic potential by cccDNA.[76] Barriers to cccDNA measurement include the requirement of invasive procedure for liver biopsy collection and the lack of standardize techniques allowing specific cccDNA quantitation due to heterogeneity of HBV DNA in infected hepatocytes.[77] Researchers have demonstrated cccDNA in the blood which possibly originates due to damage of infected hepatocytes, thus reflecting intrahepatic cccDNA activity but require further validation.[78] Several direct approaches to quantitate cccDNA have been in progress over the past few years. The traditional non-PCR based methods such as Southern blot, *in situ* hybridization, and invader assays are time-consuming and low-throughput. Next, different types of quantitative PCR-based assays like competitive PCR, nested and semi-nested PCR, rolling

cell mechanism PCR, magnetic capture hybridization PCR, cccDNA inversion quantitative PCR, ddPCR have been used to measure cccDNA. With technological advancements, ddPCR has emerged as one of the sensitive method for detection of low level of cccDNA in the liver.[79] The highest level of cccDNA has been observed among HBeAg positive compared with HBeAg negative cases.[80] Very limited studies concluding the kinetics of cccDNA in cases receiving HBV treatment have been conducted.[81–83] Considering the technical challenges in absolute measurement, recently non-invasive markers reflecting transcriptional cccDNA activity like HBcrAg and HBV RNA had emerged as surrogate markers and have been the area of interest in the past few years.

Conclusion

In conclusion, the exploration of new biomarkers for HBV represents a significant advancement. Emerging biomarkers offer promising avenues for improving the diagnosis, monitoring, and treatment of HBV infection. These novel markers enhance our understanding of disease progression, predict treatment response, and identify individuals at risk of developing complications when combined with traditional ones. Integrating these biomarkers into clinical practice has the potential to personalize and optimize therapeutic strategies, ultimately improving patient outcomes. Continued research and validation are crucial to ensure these biomarkers' reliability and clinical utility, paving the way for more effective management of HBV.

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