Hepatitis B virus (HBV) is a major cause of hepatocellular carcinoma (HCC). However, very little is known about the replication of HBV in HCC tissues. We analyzed viral and cellular parameters in HCC (T) and nontumor liver (NT) samples from 99 hepatitis B surface antigen (HBsAg)-positive, virologically suppressed patients treated by tumor resection or liver transplantation. We examined total HBV DNA and RNA as well as covalently closed circular DNA (cccDNA) and pregenomic RNA (pgRNA), which are considered as markers of active HBV replication. Total HBV DNA and RNA were detected in both T and NT samples in a majority of cases, but only a subset of tumors harbored detectable levels of HBV cccDNA and pgRNA (39% and 67%) compared to NT livers (66% and 90%; P < 0.01). Further evidence for HBV replication in tumor tissues was provided by sequencing of the X gene derived from episomal forms, showing that HBV genotypes differed between T and matched NT samples in 11 cases. The detection of pgRNA and cccDNA in tumors was correlated to the absence of tumorous microvascular invasion and to better patient survival. Analysis of gene expression profiles by Agilent microarrays revealed that pgRNA-positive HCCs were characterized by low levels of cell cycle and DNA repair markers and expression of the HBV receptor, sodium taurocholate cotransporting polypeptide, indicating well-differentiated tumors. Conclusion: HCC replicating HBV represents a subtype of weakly invasive HCC with a transcriptomic signature. pgRNA originating from nonintegrated, complete HBV genomes is a sensitive marker for viral replication and prognosis. (HEPATOLOGY 2018;67:86-96).

Chronic infection with hepatitis B virus (HBV) is a major cause of hepatocellular carcinoma (HCC). In the nucleus of hepatocytes, the relaxed circular double-stranded HBV-DNA genome (RC) is converted into covalently closed circular DNA (cccDNA), which is the template for viral RNA transcription. Among HBV mRNAs, 3.5-kb preC and pregenomic RNAs (pgRNA) originate from HBV cccDNA, and pgRNA is reverse transcribed to enable the synthesis of HBV DNA.

In the absence of a dominant oncogene encoded by the HBV genome, indirect roles have been proposed, including the insertional activation of cancer-related genes through the integration of HBV-DNA sequences, (1) the induction of genetic instability by viral integration or expression of the hepatitis B X protein,...
(HBx) regulatory protein. Despite their high degree of heterogeneity, HBV-related HCC cases display distinctive profiles, involving frequent chromosomal alterations and a low frequency of beta-catenin mutations. Moreover, significant differences in expression profiles and genetic alterations were noted between HCC associated with high versus low HBV-DNA copy numbers.

In hepatitis B surface antigen (HBsAg) carriers, the risk of HCC is closely linked to the level of viral replication. Virological suppression is easily achieved at present, and drugs are given to HBsAg-positive patients with HCC. Using immunohistochemical (IHC) and in situ hybridization techniques, studies have suggested that tumor cells no longer allow viral replication, and most tumor tissues contain only integrated viral DNA. Although extensive studies have characterized integrated HBV sequences, which are fragmented and cannot support viral replication and the transcription of pgRNA, little is known about the presence of episomal HBV cccDNA in HCC cells and the expression of 3.5-kb pgRNA, which obligatorily arises from RC or cccDNA. However, cccDNA and pgRNA may be present in HCC cells, evoking viral replication in tumor tissues. It has also been suggested that HBV replication in tumor cells might contribute to a recurrence of HBV after liver transplantation (LT). Here, we have analyzed the viral and cellular parameters of HBV-related HCC in order to determine whether HBV can replicate in tumor tissue, and to better characterize HBV-replicating HCC.

Materials and Methods

STUDY POPULATION

The study population was extracted from the French Liver Biobanks network -INCa, BB-0033_00085. We collected frozen samples of HCC and matched livers obtained after tumor resection or LT from 99 HBsAg-positive patients, together with their prospective clinical and histological annotations. These patients had given their informed consent regarding the use of their samples for biological studies. This study was approved by the ethics committees of the respective medical centers.

As summarized in Table 1, all 99 patients had been treated for HBV-related HCC by HCC resection (n = 60) or LT (n = 39). All were anti-hepatitis C virus, anti-delta, and human immunodeficiency virus negative. Most were suffering from compensated cirrhosis (n = 72 of 99), and none had a normal liver. Histological examination confirmed HCC in all cases. Tumor size was defined by its largest dimension. Eighty-two patients (82%) had received antiviral therapy (AVT) for more than 6 months before surgery and the others had low viremia. Accordingly, viremia was undetectable in 85 patients and at a mean value of 200 IU/mL (up to 680,000 IU) in the others. Ethnicity and sexual orientation of the patients were not available following the French regulation.

QUANTIFICATION OF HBV DNA, cccDNA, RNA, AND pgRNA IN LIVER TISSUE

All tissue samples were stored at −80°C until use. Nontumor (NT) liver samples were collected at distance from the tumor.

Preliminary Southern and northern blotting experiments on 10 tumor (T) and NT samples did not evidence any replicative forms of HBV and only showed a high molecular integrated signal. For the analysis of cccDNA and total HBV DNA in liver biopsy samples, we therefore used real-time PCR assays as described. To enhance the specificity of cccDNA detection, DNA samples were treated with Plasmid-safe DNase to
degrade the cellular DNA and viral RC form. Efficient treatment was assessed by an absence of β-globin detection. After DNase treatment, β-globin copy number decreased >100-fold, from 10^4 copies to a mean of 10 copies, indicating the effectiveness of DNase treatment. Quantification of total HBV RNA and of pgRNA is described in the Supporting Methods and Data. It should be noted that the primers used to amplify pgRNA also amplified the longer preC RNA also transcribed from the cccDNA. To ensure that no HBV DNA was measured, the RNA samples were analyzed in parallel using qPCR without reverse transcription. The lower limit of quantification for all these PCR procedures was determined at one HBV complementary DNA or DNA copy per 10,000 cells. Above this dilution, the reproducibility of PCR was poor. A quantification <1 in 10^4 cells was considered to be negative.

SEQUENCING OF EPISOMAL HBx SEQUENCES

The full-length HBV genome (3.2 kb) was amplified using a nested-PCR assay as described in the Supporting Methods. Samples in which the internal primer set was unable to amplify the X region without preliminary amplification of the whole genome were further analyzed because the X sequence originated from the complete (i.e., nonintegrated) HBV-DNA genome. Cloning and sequencing of episomal HBx sequences are described in the Supporting Methods.

HISTOLOGY AND IMMUNOSTAINING OF LIVER T AND NT SAMPLES

Tumor grades were scored using the modified nuclear grading scheme outlined by Edmondson and Steiner. Grades I and II were defined as well differentiated, grade III as moderately differentiated, and grade IV as poorly differentiated. Microvascular or macrovascular invasion, and the presence of a capsule or fibrous stroma, were determined in all cases. For the immunostaining of fixed sections, we used a three-step streptavidin-biotin technique, according to the manufacturer's instructions (Menarini Diagnostics, Rungis, France). The primary monoclonal antibodies were anti-HBsAg (Glostrup, Denmark) and anti-HBcAg (hepatitis B core antigen; Dako, Glostrup, Denmark).

MICROARRAY AND TRANSCRIPTOMIC ANALYSIS

The quality of the total RNA samples was determined by their RNA integrity number (RIN) on the Agilent 2100 Bioanalyzer. Only samples with RIN ≥6 were analyzed on AgilentSurePrint G3 Human GE 8 × 60K v2 Microarrays as described in the Supporting Methods.

STATISTICAL ANALYSIS

Contingency tables, together with tests of independence and measures of association, were obtained using R software with the X-squared test (group size >5) with Yates’ continuity correction or Fisher’s exact test (group size ≤5), and the Kruskal-Wallis rank-sum test. Variables associated with overall survival were identified using the Cox proportional hazard regression models (log-rank test), and Kaplan-Meier plots were used to describe survival rates. Nonparametric Spearman or Mann-Whitney U tests were used when appropriate (R package). All reported P values were
two-tailed, and differences were considered to be significant when <0.05.

Results

COMPARISONS OF HBV MARKERS BETWEEN T AND NT COMPARTMENTS

Total HBV DNA was detected in T samples in 84 of 99 of cases (84%) and in NT livers of 90 of 99 patients (91%; not significant [NS]). HBV cccDNA was detected more frequently in NT than in T samples (66 of 99 vs. 39 of 99; *P* = 0.001). When detectable in both compartments, the mean level of total HBV DNA was slightly higher in T than in NT samples, whereas the levels of HBV cccDNA were similar in both compartments.

T and NT HBV RNA were tested in 66 cases, where total RNA had an RIN score ≥6. The amplification of Roth2 mRNA (which is transcribed at a low level in liver tissue) was possible in 60 T and 61 NT samples. Total HBV RNA was detected in 58 of 60 T samples (97%) and in 60 of 61 (98%) NT samples (NS). When detectable in both compartments, the mean HBV RNA level was slightly higher in NT than in T samples. pgRNA was detectable more frequently in NT (55 of 61; 90%) than in T samples (40 of 60 [67%]; *P* < 0.01). When detectable in both compartments, the mean level of total pgRNA was slightly higher in NT than in T samples (Fig. 1).

HBV DNA, HBV cccDNA, HBV RNA, and HBV pgRNA were highly correlated between T and matched NT samples (Supporting Fig. S1).

The mean HBV DNA level was 2.3 log (±1.5) higher than that of HBV cccDNA in the T samples and 1.73 log (±1.1) higher in NT samples (NS). The

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**FIG. 1.** Comparisons of HBV markers between T and NT compartments. (A) Dot plots of HBV DNA, cccDNA, RNA, and pgRNA. (B) Qualitative detection of HBV markers. (C) Quantification of HBV markers taking account of only positive results: HBV-RNA levels were lower in T than in NT. P and N mean the detection of more or less than 1 copy/10⁴ cells. Log(copies) means number of copies/cells.
mean level of HBV RNA was 2.5 log (±1.7) higher than the mean of pgRNA in T samples and 2.3 log (±1.4) higher in NT samples (NS; Fig. 2). In both compartments, cccDNA and pgRNA thus represented only a small percentage of total HBV DNA or RNA. In both T and NT, the levels of HBV cccDNA were correlated to those of total HBV DNA, and those of HBV pgRNA were correlated to those of total HBV RNA (Supporting Fig. S2). Finally, as expected, pgRNA levels were highly correlated to those of HBV cccDNA in T and NT samples. In multivariate analysis, and in each compartment, the correlation between pgRNA and cccDNA was independent of total HBV DNA and HBV RNA (data not shown). Moreover, total HBV RNA levels were not correlated to those of HBV cccDNA in the T compartment, suggesting that most HBV transcripts were transcribed from integrated rather than episomal HBV DNA (Supporting Fig. S3).

**IHC ANALYSIS OF HBsAg**

IHC analyses of HBsAg and HBcAg were available for 45 T/NT pairs. HBsAg expression was less frequently positive in T (18 of 45) than in NT (32 of 45) samples ($P = 0.0002$). HBcAg was observed at very low levels in both T and NT samples from 3 patients. HBsAg levels in T and NT samples measured as a percentage of infected hepatocytes were correlated to one another and to the levels of HBV RNA in T ($P = 0.02$) and NT ($P = 0.003$). The mean number of positive hepatocytes was lower in T than in NT samples (Supporting Fig. S4).

**COMPARTMENTALISATION OF HBV GENOTYPES IN HCC**

To characterize episomal HBV DNA, we amplified viral DNA in T and NT samples using nested PCR. P1 and P2 primers were used as external primers to amplify the full-length HBV DNA, followed by PCR amplification of the X gene region. Because the full-length viral genome has never been found in integrated HBV sequences, the detection of HBV DNA
dependent on P1 and P2 primers reflected the presence of complete RC DNA or cccDNA. As a control, single-round PCR amplification using internal X gene primers was negative in all samples, implying that the final HBx amplification originated from the previous amplification of RC or cccDNA. Among the 63 paired T and NT samples in which the X gene could be amplified from complete HBV DNA, cloning-sequencing revealed genotypic differences between T and NT in 11 patients (17%; Fig. 3; Supporting Table S1). This is the first evidence that episomal HBV DNA can be compartmentalized in HCC at the genotypic level, reinforcing the notion that HCC can replicate and propagate HBV. Among the 11 patients with dual infection, plasma was available in 6 and HBV DNA could be amplified in 3 by using a nested PCR amplifying HBx. Because the viral load was extremely low, we did not perform cloning sequencing, but we directly sequenced the HBx region. In 1 patient (#85), the plasma viral sequence had more than 99% of homology with the main tumoral sequence whereas in the 2 others, the plasma strain was similar to the main NT viral sequence (Fig. 3).

**CLINICAL CORRELATIONS**

The detection of HBV DNA, cccDNA, and pgRNA in the T compartment was strongly correlated to the absence of tumorous microvascular invasion (MVI) and to the levels of these markers in the corresponding NT compartment (Fig. 4).

Under univariate analysis, patient survival was significantly better in LT patients than in others (Fig. 5) and in those without tumorous vascular invasion. The presence of HBV pgRNA and HBV cccDNA, but not of total HBV DNA or HBV RNA, in T samples was predictive of survival under univariate analysis. Multivariate Cox model analysis showed that the presence of pgRNA was the only significant predictive factor (Supporting Table S2).
Tumoral compartmentalization of HBV genotypes was not correlated to any other clinical and biological variables, including duration of therapy and viremia.

**GENE EXPRESSION PROFILES, CLINICAL, AND HBV VARIABLES**

The preparation of high-quality RNA enabled accurate transcriptional analysis in 66 T/NT pairs. For each gene, we established the distance ratio between matched T and NT samples. Using the Significance Analysis Microarray (SAM) algorithm, three variables influencing survival were analyzed: vascular invasion; HBV cccDNA; and HBV pgRNA.

Most genes were up-regulated in the event of vascular invasion (Supporting Fig. S5). Genes involved in collagen organization, programmed death, and blood vessel development had high Z-scores (Supporting Table S3) after enrichment on the Gene Ontology Biological Process database. A large number of genes were implicated in functionalities, such as the cell cycle, cell proliferation, and viral processes. Sodium taurocholate cotransporting polypeptide (NTCP), which is the HBV receptor, was one of the few down-regulated genes in the event of MVI. The up-regulated genes revealed using the SAM approach were then analyzed by means of PCA. In this model, tumours were clustered according to the presence of microvascular invasion (Supporting Fig. S5; \( P < 0.000001 \)).

**FIG. 4.** HBV markers and tumorous MVI. cccDNA and pgRNA were less frequently detected in case of MVI in the T, but not in the NT, liver. When detected, the levels of the four HBV markers tended to be lower in case of MVI in the NT liver, but not in the T liver.
The different families were more heterogeneous (Supporting Table S3). When the genes revealed by SAM were analyzed using principal component analysis (PCA), the clustering of HCC according to the presence of MVI was also significant ($P < 0.03$; Supporting Fig. S6).

SAM analysis revealed that the presence of pgRNA in tumor samples was associated with the down-regulation of 40 genes (Fig. 6; Supporting Table S3). These genes are mainly implicated in the cell cycle (such as cyclin B1) and in DNA damage. Conversely, pgRNA was positively correlated to NTCP (Supporting Fig. S7). PCA analysis performed using the 40 aforementioned genes revealed strong clustering according to the existence of MVI ($P = 0.0009$; Fig. 6). In summary, the presence in HCC of episomal DNA or RNA forms of HBV was related to both the absence of vascular invasion and the down-regulation of signals related to cell proliferation and progression in the cell cycle.

**Discussion**

The data presented here show that HCC in HBsAg-positive patients treated with active antiviral drugs can express episomal forms of HBV DNA in 40% or pgRNA in 66% of cases. HCC patients were sometimes infected by HBV genotypes not found in the NT liver, demonstrating that HBV may chronically replicate in HCC tissues. The presence of HBV cccDNA, and more significantly of 3.5-kb HBV RNA (pgRNA) in HCC tissue, was linked to a low incidence of microvascular tumor invasion and hence to better survival. Finally, transcriptomic analysis revealed signatures such as up-regulation of the HBV receptor NTCP, and down-regulation of the genes involved in mitosis, the cell cycle, and DNA repair in HCC patients positive for HBV cccDNA or pgRNA.

HBV RC as well as cccDNA play a pivotal role in viral replication, but their detection in tumor tissue does not demonstrate *per se* the replication of HBV.
Detection of pgRNA reflects the transcription from HBV cccDNA. Conversely, the detection of specific HBV strains infecting HCC, but not adjacent liver tissues, has demonstrated that HBV chronically replicates and propagates in tumorous liver. It was recently shown(15) that T and NT liver may harbor HBV-DNA sequences from different genotypes, but the mutations observed were mainly those of an inactivating type, leading to nonreplicative, probably integrated forms of the viral DNA. In the present work, the HBx sequences were retrieved from nonintegrated, episomal HBV DNA.

Our data support the theory that two types of HCC can be distinguished as a function of their competence for HBV replication. The expression of HBV at the transcriptional and protein levels was clearly lower in HCC than in NT liver, indicating that tumorous tissues are less competent for the replication and transcription of HBV. One major finding was that HCC in which HBV cccDNA was detected, and far more significantly those expressing pgRNA, were associated with a low incidence of vascular invasion and hence with better survival. In NT, the levels of HBV RNA or DNA were also lower in patients with an MVI than in others, suggesting that replication in the NT liver may influence the tumoral microenvironment. This latter finding is an apparent contradiction with a recent article.(16) However, in our work, the virosuppression was strong given that 93% of our patients had an indetectable viremia whereas most patients of Wang et al.’s article had been treated for a short course of time preceding surgery and 50% had detectable viremia. In this view, we evidenced, for all HBV markers, a strong positive correlation between T and NT compartments, suggesting that T and NT HBV were similarly sensitive to antiviral drugs.

HCC affording HBV replication, and more significantly the transcription of 3.5-kb HBV pgRNA, might...
be specifically differentiated. A relationship between large HCC and low levels of HBV cccDNA in the tumor has been reported recently.\(^{16}\) HCC with intravascular invasion presented down-regulated liver functions: cytochrome p450; fatty and amino acid metabolism; complement and coagulation; and bile acid metabolism. The transcriptional inhibition of NTCP expression during cell-cycle progression is mediated by cyclin D1,\(^{17}\) and we found a down-regulation of NCTP and cyclin D1 in HCCs that did not express pgRNA. This suggests that tissue differentiation might provide an explanation for the intratumor replication of HBV. Conversely, an absence of HBV cccDNA was related to enhanced mitosis, whereas an absence of pgRNA was related to up-regulation of the cell cycle and DNA repair. Cell proliferation may antagonize viral replication, as suggested by a study in the primary Tupaia hepatocytes model\(^ {18}\) or in a cell line replicating HBV.\(^ {19}\) Zucman-Rossi et al. recently stated\(^ {20}\) that HCC can be classified into two types, according to cell proliferation. Taken together, these findings suggest that HBV replication in HCC may depend on low levels of cell proliferation combined with cellular differentiation.

Among quantitative HBV markers, HBV RNA or DNA were not discriminant. The detection of cccDNA or pgRNA, both representing a small proportion of viral nucleic acids in the present study, was far more informative than the detection of whole DNA or RNA. The detection of 3.5-kb pgRNA was also more discriminant than that of cccDNA in selecting molecular HCC subtypes associated with vascular invasion and also with survival. The detection of pgRNA is probably more sensitive than that of cccDNA in reflecting viral replication. Multiple pgRNAs are transcribed from each cccDNA molecule explaining that quantities of HBV RNA were 10-fold higher than that of HBV DNA. Furthermore, the use of DNase together with the circular constrained structure of cccDNA explain the low sensitivity of cccDNA assays. Whereas most studies on the replication of HBV in liver tissues have focused on cccDNA, pgRNA has scarcely been used as a marker of HBV replication, or at least of transcription from a complete viral DNA template. Furthermore, given that cccDNA is the subject of epigenetic regulation, the presence of cccDNA does not mean viral expression or replication. Accordingly, in this study, pgRNA could not be detected in some samples despite the presence of HBV DNA. For all these reasons, the detection of pgRNA is probably a relevant marker of the expression and replication of HBV. A theoretical drawback of all PCR methods used to detect pgRNA should be mRNA transcribed from integrated viral DNA, compatible with HBV primers and quantified as pgRNA. This potential artifact was not significant in this study because the correlation between pgRNA and cccDNA did not depend on total HBV RNA.

In conclusion, our findings show that HCC expressing the episomal form of HBV DNA or pgRNA is a particular HCC subtype that is well differentiated, nonproliferative, and noninvasive. Future studies should focus on the development of high-throughput methods for the detection of HBV variants as tumor markers.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.29463/suppinfo.