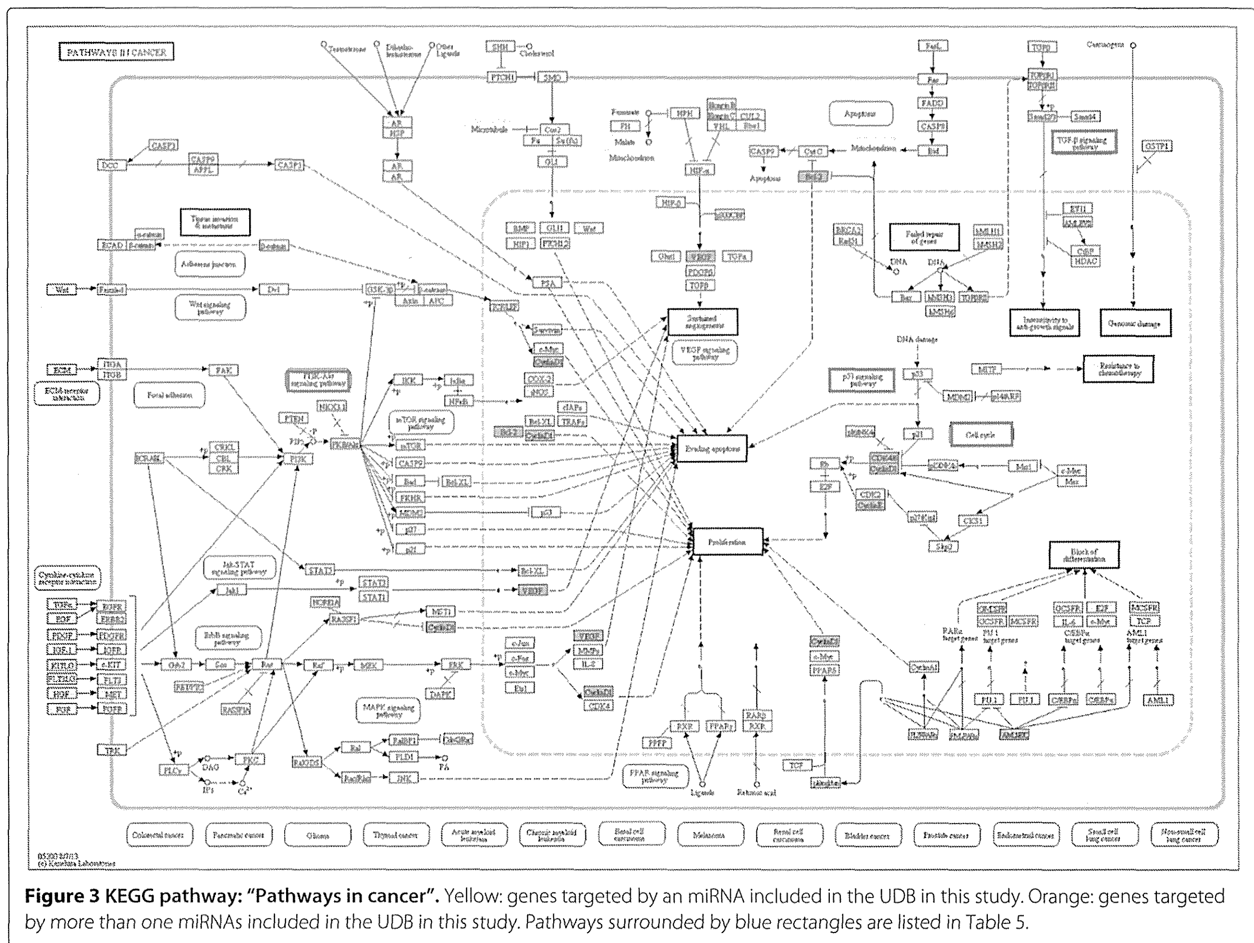

Figure 3 KEGG pathway: "Pathways in cancer". Yellow: genes targeted by an miRNA included in the UDB in this study. Orange: genes targeted by more than one miRNAs included in the UDB in this study. Pathways surrounded by blue rectangles are listed in Table 5.

as is usual. λ_i and $\boldsymbol{u}_i$ represent the eigenvalue and vector, respectively.

$$x_{ik} \equiv \sum_j u_{kj} x_{ij}$$

gives the principal component score (PCS) of ith miRNA. Using the obtained $x_{ik}, k = 1, \ldots, D(< M)$, miRNAs were determined to be embedded into low D dimensional space.

Multiplying $\mathbf{X}$ on both sides, the following is obtained:

$$\frac{1}{N}\left(\mathbf{X}\mathbf{X}^T\right)(\mathbf{X}\boldsymbol{u}_k) = \lambda_k\left(\mathbf{X}\boldsymbol{u}_k\right), (k = 1, \ldots, M)$$

where $\boldsymbol{v}_k = \mathbf{X}\boldsymbol{u}_k$ can be regarded as an eigenvector. Then,

$$x_{kj} \equiv \sum_i v_{ki} x_{ij}$$

gives the PCS of the jth sample. Using the obtained $x_{kj}, k = 1, \ldots, D(< M)$, samples were regarded to be embedded into low D dimensional space.

PCA-based unsupervised FE selects outlier miRNAs in low $K(< M)$ dimensional embedding space,

$$r_{Ki} > \Delta$$

where

$$r_{Ki}^2 \equiv \sum_{k=1}^{K} x_{ik}^2$$

Typically K is taken to be two. Since these outliers could have a major contribution to $\boldsymbol{u}_k$'s by definition, if there are a limited number of well-defined outliers, the exclusion of miRNAs other than outliers does not alter $\boldsymbol{u}_k$'s. Since $\boldsymbol{v}_k$ is a linear transformation of $\boldsymbol{u}_k$ as shown above, the exclusion of miRNAs other than outliers does not alter $\boldsymbol{v}_k$. Thus, retaining only outlier miRNAs may also preserve lower dimensional embeddings of samples that are important for disease diagnosis, e.g., discrimination between patients and healthy controls. Although this is only hypothetical, it explains why PCA-based unsupervised FE is expected to function well. Currently, there are no well-defined criteria for the selection of Δ. Although

Δ was decided to include sufficient numbers (majority) of outliers, these were selected by the visual inspection of two-dimensional embedding of miRNAs. Singular decomposition-based interpretation is also available as Additional file 3: Text S1.

Discriminatory analyses between patients and healthy controls with cross-validations

Three discriminant analyses were performed in this study as follows. The first, a PCA-based LDA, a discriminant counterpart of the partial least square (PLS), is defined as discrimination using the first k PCSs (i.e., from the first to the kth PCSs). First, PCA was applied to all samples. Then, PCA-based LDA was performed using only PCSs in the training set. Since the learning process includes unlabeled information of the test set, it is semi-supervised learning. Samples in the test set were predicted using trained PCA-based LDA. LDA was performed using `lda` functions in R [31] and the prediction of samples in the test set was performed by `predict.lda` functions in R. Optimal k was determined using cross-validations. The second analysis used an SVM trained with training set samples using `svm` function included in the `e1071` R package with default settings (e.g., with the usage of Gaussian kernel), other than `class.weight` argument that was set to attribute equal weights to sets of normal controls and patients when the number of samples in normal controls differed from that of patients. Then, samples in the test set were predicted using `predict.svm` function in R. Third, lasso was used for a discrimination study. Lasso was performed using the `lars` function included in `lars` R package, attributing 1 and 2 to healthy controls and patients, respectively, and using the setting `type=`'lasso'. Then, samples in the test set were predicted using `predict.lars` function in R for $s = n/100, n = 0, \ldots, 100$ with mode='fraction'. Samples with predicted values larger (less) than 1.5 were regarded to be patients (healthy controls). Optimal s was selected by cross-validation. For all cases, leave one out cross-validation (LOOCV) was employed.

Data normalization

Since this study is a meta-analysis using data sets collected from various independent studies employing distinct measuring methods, we normalized data sets individually by distinct methods (Table 6). Data from multiple studies were treated identically and compared. In addition, some miRNAs with abnormally large values were excluded from the analysis. Excluded miRNAs were hsa-miR-486-5p (AD), hsa-miR-223 and hsa-miR-338 (CAD), and hsa-miR-451 (NPC).

Stability test

On LOOCV FE, selected features (miRNAs) are listed. For lasso, miRNAs with non-zero βs were listed by setting `type=`'coefficients' for `predict.lars` function with estimated optimal s. Because of LOOCV, FE was performed by M(=the number of samples) times. Then stability was defined as

$$S \equiv \frac{1}{\hat{N}} \sum_{i \in \{i | F_i \neq 0\}} \frac{F_i}{M}$$

where F_i is the number of times that ith miRNA was selected within M times FE. Summation was performed for miRNAs that were non-zero F_i (i.e., selected at least once in FEs) and $\hat{N}$ is the number of miRNAs included in the summation. Larger S, $\left(\frac{1}{M} \leq S \leq 1\right)$ indicates more stable FEs.

P-values computation for significant difference between healthy controls and patients

P-values computed for significant differences between healthy controls and patients of each disease were determined using t-test for each miRNA. Computed P-values were adjusted by BH-criterion [32] and miRNAs with

Table 6 Details of data normalization

GEO ID	Disease	Data set names/ Data retrieval methods	Data normalization timing	Data normalization methods
GSE46579	AD	GSE46579_AD_ngs_data_summarized.xls.gz	before FE	zero mean/variance is one
GSE37472	carcinoma	getGEO	before FE	zero mean/variance is one
GSE49823	CAD	getGEO	after FE	zero mean/variance is one*
GSE43329	NPC	getGEO	before FE	zero mean/variance is one+
GSE50013	HCC	getGEO	before FE#	zero mean/variance is one*
GSE41922	BC	GSE41922_series_matrix.txt.gz	after FE	zero mean/variance is one*
GSE49665	AML	getGEO	after FE	zero mean/variance is one*

*no normalization for SVM/lasso, +no normalization for SVM with PCA-based FE, #after FE for PCA-based LDA with universal features. All the sample normalizations were sample-based; i.e., each sample was normalized to have both zero mean and unit variance. AD, Alzheimer disease; CAD, coronary artery disease; NPC, nasopharyngeal carcinoma; HCC, hepatocellular carcinoma; BC, breast cancer; AML, acute myeloid leukemia. Data retrieval methods/data set names were used to name files and for analysis. getGEO indicates that individual sample profiles whose files names started with "GEO" were downloaded by the getGEO command in R.

adjusted *P*-values less than 0.05 were regarded to have significantly different expression between normal controls and patients.

KEGG pathway analysis of UDB using DIANA-mirpath

DIANA-mirpath [25] was employed to investigate KEGG pathways enriched by miRNA target genes. Twelve genes were uploaded to DIANA-mirpath with the following settings: "Species" was "Human", "FDR" correction was "yes", "P-value threshold" was 0.05, and "Select the way to merge results" was "pathway union" (direct link to DIANA-mirpath and full list of KEGG pathways are shown in Additional file 3: Text S2 and Additional file 1: Table S5).

Additional files

Additional file 1: Supporting Tables.
Additional file 2: Supporting Figures.
Additional file 3: Supporting Texts.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
YHT and YM planned all the projects. YHT performed analyses and wrote the paper. All authors read and approved the final manuscript.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Role of hepatitis B virus DNA integration in human hepatocarcinogenesis

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Abstract

Liver cancer ranks sixth in cancer incidence, and is the third leading cause of cancer-related deaths worldwide. Hepatocellular carcinoma (HCC) is the most common type of liver cancer, which arises from hepatocytes and accounts for approximately 70%-85% of cases. Hepatitis B virus (HBV) frequently causes liver inflammation, hepatic damage and subsequent cirrhosis. Integrated viral DNA is found in 85%-90% of HBV-related HCCs. Its presence in tumors from non-cirrhotic livers of children or young adults further supports the role of viral DNA integration in hepatocarcinogenesis. Integration of subgenomic HBV DNA fragments into different locations within the host DNA is a significant feature of chronic HBV infection. Integration has two potential consequences: (1) the host genome becomes altered ("*cis*" effect); and (2) the HBV genome becomes altered ("*trans*" effect). The *cis* effect includes insertional mutagenesis, which can potentially disrupt host gene function or alter host gene regulation. Tumor progression is frequently associated with rearrangement and partial gain or loss of both viral and host sequences. However, the role of integrated HBV DNA in hepatocarcinogenesis remains controversial. Modern technology has provided a new paradigm to further our understanding of disease mechanisms. This review summarizes the role of HBV DNA integration in human carcinogenesis.

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Key words: Hepatitis B virus; Integration; Hepatocarcinogenesis; *Cis* effect; *Trans* effect; Whole genome sequencing

Core tip: A high viral load is associated with an elevated risk of hepatocellular carcinoma (HCC), and the risk remains increased in hepatitis B surface antigen-negative hepatitis B virus (HBV) and occult infections. The ability of HBV to integrate into the infected host's hepatocyte genome is one of the most important direct pro-oncogenic properties. The recent development of efficient tools for genome-wide analysis of gene expression and genetic defects has allowed a comprehensive overview of the changes occurring with HCC. Specific HBV features, including the integration of viral DNA into host chromosomes, may trigger increased genetic instability.

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INTRODUCTION

Approximately two billion people worldwide have been infected with hepatitis B virus (HBV). With more than 350 million chronic HBV carriers, this virus is one of the most common human pathogens and is a significant public health issue[1].

Liver cancer is the sixth most common cancer, and the third leading cause of cancer-related deaths[2,3]. Hepa-

tocellular carcinoma (HCC) is the most common type of liver cancer, accounting for approximately 70%-85% of cases[4]. In recent studies conducted in Asia and Northern America, the estimated risk of developing HCC was observed to increase by 25-37-fold in hepatitis B surface antigen (HBsAg) carriers compared with non-infected patients[5,6]. HBV frequently causes liver inflammation, hepatic damage and subsequent cirrhosis. The development of liver cirrhosis is recognized as a major step in HCC pathogenesis because it occurs in 80%-90% of HCCs[7]. A high viral load is associated with an elevated risk of HCC[8], and the risk remains higher in HBsAg-negative HBV and occult infections[9-11]. HBV replication has unique characteristics[1]. HBV is classified as a pararetrovirus because of its similarity to retroviruses. In fact, HBV replicates through reverse transcription of pregenomic RNA that is an intermediate replicative molecule[12]. The ability of HBV to integrate into the infected host's hepatocyte genome is one of the most important aspects of its direct pro-oncogenic properties[13-15]. Unlike retroviruses, genomic integration has no role in HBV replication and does not produce integrase enzymatic activity protein, meaning that the integrative process is likely mediated by cellular topoisomerase I activity[16].

Integrated viral DNA is found in 85%-90% of HBV-related HCCs and its presence in tumors from non-cirrhotic livers of children or young adults further supports the role of viral DNA integration in hepatocarcinogenesis[17,18]. A significant feature of chronic HBV infection is that HBV DNA fragments are integrated into different locations within the host DNA[19-23]. Tumor progression is often associated with rearrangement and partial gain or loss of both viral and cellular sequences[24-26]. Various small-scale isolated studies have suggested that HBV integration into the host genome is a random event[25]; however, integration has been observed at chromosomal fragile sites, scaffold/matrix attachment regions, and repeat/satellite sequence-rich regions[19]. Therefore, the role of integrated HBV DNA in hepatocarcinogenesis remains controversial. This review summarizes the role of HBV DNA integration in human carcinogenesis.

HCC MECHANISMS

The are three major molecular mechanisms of hepatocarcinogenesis caused by HBV infection[27]. First, the expression of viral proteins, particularly hepatitis B virus X protein (HBx), promotes cell proliferation and viability. Second, the integration of HBV DNA into the host genome alters the expression and function of endogenous genes and induces chromosomal instability. Finally, genetic damage accumulates as a result of inflammation and ongoing hepatocyte division to replace cells killed by virus-specific T cells.

Genetic alteration plays a crucial role in cancer initiation and progression. The recent development of efficient tools for genome-wide analysis of gene expression and genetic defects has allowed a comprehensive overview of the changes occurring with HCC[28,29]. Specific HBV features, including HBV DNA integration into host genome, may trigger increased genetic instability.

Table 1 Main integration sites in human genome and in hepatitis B virus DNA

Integration sites in host genome	HBV DNA
hTERT	3' end of HBx
MLL	Pre-S2/S
RAR-b	
CCNE1	
Cyclin A2	
FN1	
ROCK1	
SENP5	
ANGPT1	
PDGF receptor	
Calcium signaling-related genes	
Ribosomal protein genes	
Epidermal growth factor receptor	
Mevalonate kinase	
Carboxypeptidase	
Platelet growth factor receptor	

HBV: Hepatitis B virus; HBx: Hepatitis B virus X protein.

ROLE OF HBV DNA INTEGRATION IN HUMAN HEPATOCARCINOGENESIS

The association between HBV DNA integration into the host genome and HCCs was first reported in the early 1980s[13,23,30]. Subsequently, many studies were performed to further investigate this association (Table 1).

The integration of HBV DNA into host cellular DNA during HBV chronic infection disrupts or promotes cellular gene expression that is important for cellular growth and differentiation. Furthermore, the expression of HBV proteins may have a direct effect on cellular functions, and may promote malignant transformation. Integration events are thought to precede tumor development because they are found in chronic hepatitis patients and during the acute infection stage[31].

Technological limitations of PCR and Southern blot-based methods restricted previous studies that attempted to characterize the most common HBV integrant(s) in a small number of patients[15,32]. HBV has a large number of mutations at both the nucleotide and structural levels, and the lack of prior knowledge of HBV sequences in each sample may lead to PCR failure and false-negative results. This occurs when the primers are designed for deleted or polymorphic sites on the HBV genome. Recently, two studies reported "short-read" whole genome DNA paired-end sequencing of four and eighty-eight HCC patients[33,34]. Integration sites could only be inferred from paired-end reads containing both human and viral sequences, because of the limitations of the short reads generated using these platforms. Indirect roles have been proposed because the lack of identification of a dominant oncogene encoded by HBV, including insertional

activation of cancer-related genes from HBV integration, induction of genetic instability by viral integration or HBx, and long-term effects of viral proteins that enhance immune-mediated liver disease.

Integration has two potential consequences: (1) the host genome becomes altered ("*cis*" effect); and (2) the HBV genome becomes altered ("*trans*" effect). The cis effect includes insertional mutagenesis, which can potentially disrupt host gene function or alter host gene regulation [*e.g.*, telomerase reverse transcriptase (TERT)][35]. Despite drastic rearrangements, the coding regions of PreS2 and HBx were generally conserved and could be transcribed[36]. Hence, these two HBV proteins may have a *trans* role in hepatocarcinogenesis[37-39].

CIS EFFECT

The main integration sites in the human genome and the preferred integrating region within the HBV genome have been researched extensively.

HBV DNA integration occurs randomly within human genomes, and may involve multiple sites in different chromosomes[25]. Thus, HBV behaves like an insertional, non-selective mutagenic agent. The important host genome rearrangements associated with viral integration suggest that the main oncogenic effect is from the induction of higher genomic instability[40]. Most reported integration events occur near or within fragile sites or other repetitive regions, such as the Alu sequences and microsatellites that are prone to instability, tumor development, and progression[22]. Integration of HBV DNA sequences begins in the early stages of acute infections, and multiple integrations have been detected in chronic hepatitis tissues. Clonal integrated HBV sequences have been observed in approximately 80% of HBV-related HCCs[41]. Viral insertion sites have been mapped in multiple regions on virtually all chromosomes, suggesting a random distribution throughout the host genome. HBV insertions are commonly associated with large genetic alterations that may lead to the abrogation of control mechanisms that safeguard chromosomal integrity[42-45]. Similar to retroviral proviruses, HBV DNA targets actively transcribed chromosomal regions within genes or in the immediate vicinity. Sequence analysis of multiple viral-host junctions have identified cellular coding regions within several kbps in 90% of cases, with frequent targeting of gene families involved in cell survival, proliferation and immortalization including: hTERT, the PDGF receptor, MLL, calcium signaling-related genes and ribosomal protein genes[15]. These findings favor the view that viral insertion induces the first genetic alteration in tumor development. Target genes may play a role in hepatocarcinogenesis, which was previously shown for HBV insertions into the retinoic acid receptor b (RAR-b) and the cyclin A2 genes[46,47].

Among the numerous viral integration sites described, some notable regions include the tyrosine-protein-kinase domain of the epidermal growth factor receptor gene[48], the mevalonate kinase gene[49,50], the carboxypeptidase gene[51], platelet growth factor receptor genes[15] and *hTERT*.

The HBx gene in the HBV genome tends to be the most common region, but the most common integration sites in the human genome are not fully identified. Several integration sites in the human genome such as *TERT*, *MLL4*, *CCNE1*, *FN1*, *ROCK1* and *SENP5* have been reported[33-52]. *TERT* encodes a telomerase reverse transcriptase, which plays an essential role in overriding cellular senescence. Its dysregulation in somatic cells is linked to carcinogenesis[53]. *MLL4* encodes a histone methyltransferase that plays a critical role in gene expression and epigenetics in cancer cells. The translocation breakpoint of the intron 3 region of *MLL4* is one of the preferential targets for HBV DNA integration and may be involved in liver oncogenesis[54]. *CCNE1* encodes cyclin E1, which is required for cell cycle G1/S transition. *FN1* encodes fibronectin, a component of the extracellular matrix that is involved in cell adhesion and migration processes. The protein encoded by *ROCK1* can activate LIM kinase, and inhibits actin-depolymerizing activity by phosphorylating cofilin. *SENP5* encodes a protease specific for SUMO proteins, and is required for numerous biological processes. All of these genes are upregulated in malignant tissues[34]. Hence, HBV integration into these genes may cause HCC.

Whole genome sequencing (WGS) of a large cohort has provided an opportunity to identify novel recurrent integrations. In addition to the confirmation of recurrent HBV integration into the *MLL4* (n = 9) and *TERT* (n = 18) loci accompanied by upregulation of gene expression, recurrent integration events were observed at the *CCNE1* (n = 4), *SENP5* (n = 3), and *ROCK1* (n = 2) loci[34]. *CCNE1* expression was, on average, 30-fold higher in tumors with HBV integration compared to the normal controls. Cyclins are mainly involved in regulating the cell cycle in eukaryotic cells, and are major targets for oncogenic signals. HBV integration at the *CCNE1* locus has provided at least one molecular mechanism driving aberrant cell cycle control leading to HCC. Currently, three genome-sequencing studies have been published that analyzed HBV integration events. Genome sequencing of four HCC patients identified 255 HBV integration sites in the three HBV-positive patients including the *MLL4* locus in one sample and the *ANGPT1* locus in another[33]. RNA sequencing revealed a distinct transcriptional impact of viral integration. HBV DNA integration into the third exon of *MLL4* resulted in a human-viral fusion transcript, and a 20-fold increase in *MLL4* transcription in comparison to the adjacent normal liver tissue. For the *ANGPT1* gene, HBV DNA was inserted into 10-kb upstream of the promoter region, leading to a greater than eightfold elevation in *ANGPT1* expression. In a genome sequencing study of 27 HCCs, including 11 HBV-associated HCC, 14 HCV-associated HCC, and two cases that were unrelated to viral infection, the average proportion of the *TERT* integration sites (41%) was higher than that of other integration sites. These findings are consistent with previous reports of recurrent HBV integration at the *TERT* locus[55].

Preferential HBV integration into gene promoters ($P < 0.001$), and significant enrichment of integration into chromosome 10 ($P < 0.01$) was observed in the tumors. Integration into chromosome 10 was significantly associated with poorly differentiated tumors ($P < 0.05$). In particular, recurrent integration into the *TERT* promoter was correlated with increased TERT expression[56].

We found that HBV DNA integration enhanced host chromosomal instability leading to large inverted duplications, deletions and chromosomal translocations[32]. Many of these chromosomal segments contain genes encoding key factors in liver carcinogenesis, such as p53, Rb, Wnt/b-catenin, cyclins A and D1, TGFb, and Ras[57].

TRANS EFFECT

Integrated viral sequences may contribute "*in trans*" to tumorigenesis through the production of truncated and mutated HBx or preS2/S proteins, though they cause defective replication. These proteins may impact HCC development by disrupting cellular gene expression control or by activating oncogenic signaling pathways.

The HBx protein is a multifunctional regulator of viral and cellular genes that interferes with viral replication and proliferation. HBx and Pre-S2/S regulatory proteins that are generated from integrated viral sequences are involved in hepatocyte transformation. Moreover, HBx and truncated Pre-S2/S have been shown to be effective transactivators of cellular and viral genes and are involved in signal transduction pathways, cell cycle control and transcriptional regulation[36,58].

The C-terminal region of HBx, produced by HBx truncation, contributes to HCC development. It has been suggested that the C-terminal region is required for reactive oxygen species (ROS) production and 8-oxoguanine (8-oxoG) formation, which are biomarkers of oxidative stress. Oxidative stress and mitochondrial DNA damage play an important role in the development of HCC[59]. Other studies have found that HBx C-terminal truncation, particularly involving 24 amino acids, plays a role in enhancing cell invasiveness and metastasis in HCC by activating MMP10 through C-Jun signaling[60]. Also, HBx C-terminal truncation was closely related to the overexpression of centromere protein A in HCC[61]. In addition, HBx C-terminal truncation directly regulates miRNA transcription and promotes hepatocellular proliferation[62].

Most HBV-related HCCs have integrated viral genomic sequences, including the HBx gene. Although the integrated forms of HBx are frequently rearranged and show numerous point mutations, deletions or truncation, integrated HBx may encode functionally active proteins with transactivating ability[31,41]. Characterization of HBx expression in malignant hepatocytes and infected liver tissues has been often hampered by the difficulty in obtaining valid high-affinity anti-HBX antibodies for immunodetection[63]. Despite this, the expression of HBx is maintained through multistage hepatocarcinogenesis from pre-neoplastic nodules or foci of transformed hepatocytes to HCC[64,65].

Evidence of transcriptional activity at integrated X sequences has been demonstrated in tumors and chronically infected livers[66,67] and may be correlated with the detection of the X protein in human HCCs[68]. It was suggested that downstream cellular sequences contribute to activated expression and/or enhanced transactivating capacities of the integrated HBV sequences[58,69]. The X gene product transactivates homologous and heterologous transcriptional enhancers and promoter sequences. In the meantime, expression of cellular genes is activated "*in trans*" from increased X gene products. Many clones preserved transactivation activity in spite of the truncation at the 3' end of the X ORF[67]. The cDNA structure of X mRNA from integrated HBV DNA suggested X-cell fusion mRNA.

The preferred region within the HBV genome involved in integration and viral structural alteration is located at nucleotides 1600-1900 around the 3'-end of HBx and the 5'-end of the Precore/Core genes, where viral replication and transcription is initiated. Upon integration, the 3'-end of HBx is frequently deleted and HBx-human chimeric transcripts, which can be expressed as chimeric proteins, are commonly observed[56]. The 3'-end of the HBx gene is the preferred region for human genome integration[34,52,70], leading to the C-terminal truncated form of HBx, and is an important mechanism in HBV-related hepatocarcinogenesis.

Recently, WGS was performed on a large cohort of HCC patients with 81 HBV-positive, seven HBV-negative HCC samples and adjacent normal tissues to survey HBV integration in liver cancer genomes[34]. A systematic and in-depth bioinformatics analysis was performed to study HBV integration. The 399 detected HBV integration events occurred more frequently in tumors (344 events) than the normal controls (55 events), and represented a 6.3-fold increase. The HBV genome break points were also examined, and 40% of the break points were restricted to an 1800-bp region of the HBV genome where the viral enhancer, the X gene and the core gene are located. This viral breakpoint may facilitate the formation of human-viral fusion proteins and create cis-regulatory effects on expression of downstream genes that disturb the host gene regulatory network.

Some HCC patients do not have detectable hepatitis B surface antigen in their serum, but have low levels of serum HBV DNA and fragments of HBV DNA in their genomic cellular DNA (occult HBV infections). The prevalence and molecular status of occult HBV in HCC patients has been investigated in many studies in patients from different regions worldwide[10,71,72]. In HBsAg-negative HCC patients, HBV DNA was detected in neoplastic and/or adjacent non-neoplastic liver tissue in almost half of patients, some of which were anti-HCV positive[73]. In some patients, positivity for anti-HBc antibodies was the only marker of HBV infection. Covalently closed circular HBV DNA may be detected in the liver of some patients, indicating persistence of the viral genome template for transcription and replication. An observational cohort study showed that HCC develops more commonly in oc-

cult HBV patients among HBsAg-negative patients with chronic hepatitis C.

In addition to genetic and genomic perturbations, HBV integration is also associated with various clinical parameters including disease occurrence at younger age, higher levels of AFP and poor overall survival[34]. This suggests an association between viral DNA integration and a more aggressive pathogenesis of HCC.

Beside genomic alterations, epigenetic factors, such as methylation-associated gene silencing, have been shown to be involved in the deregulation of cellular function in HCC. The HBV genome is almost completely unmethylated in the early stages of carcinogenesis, from chronic active hepatitis to hepatic cirrhosis, while it becomes more methylated in the established liver tumors, both in patients and in cultured cancer cell lines[74].

CONCLUSION

The multistep development of liver cancer is associated with the accumulation of genetic and epigenetic changes. The long latency of HCC development following primary HBV infection reflects an indirect oncogenic pathway. Evidence of multiple cooperative mechanisms during neoplastic transformation is increasing. Genetic instability, which is particularly high in HBV-related HCCs, may be related to HBV integration.

The integration of HBV has the primary *cis* effect of altering gene regulation. Sequence variations and structural alterations of the HBV genome that modify viral protein structure, function and integration events generate novel HBx-human chimeric proteins that may exert a *trans* effect by facilitating host immune surveillance evasion and/or that contribute to tumorigenesis.

Next generation sequencing technology has provided a new paradigm for understanding disease mechanisms. WGS and whole exome sequencing efforts have led to the discovery of previously unknown somatic variations in HCC, such as point mutations in chromatin remodeling genes and recurrent HBV integrations. A large number of data sets from genome wide association studies may need further investigation. Additional research into the development and treatment of resistant HBV strains is warranted.

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HEPATOLOGY

Prospective long-term study of hepatitis B virus reactivation in patients with hematologic malignancy

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Abstract

Background and Aim: To elucidate the clinical characteristics of hepatitis B virus reactivation (HBV-R), we performed a prospective long-term study of patients with hematologic malignancy, including both hepatitis B virus (HBV) carriers and those with resolved HBV infection.
Methods: Twenty-one patients with hematopoietic stem-cell transplants (HSCT) and 36 patients given rituximab-based chemotherapy were enrolled. Entecavir was administered prophylactically to eight patients with HBV surface antigen (HBsAg). HBV-DNA was measured every month in 49 patients with resolved HBV infection, and preemptive therapy was given to eight patients with HBV-R.
Results: HBV-R developed in five (26%) of 19 patients with HSCT and three (10%) of 30 patients given rituximab-based chemotherapy. HBV-R occurred a median of 3 months (range: 2–10) after the end of rituximab-based chemotherapy and 22 months (range: 9–36) after HSCT. HBV-R did not develop in patients with an antibodies against HBsAg (anti-HBs) titer exceeding 200 mIU/mL at baseline. Mutations in the "a" determinant region with amino acid replacement were detected in four of the eight patients with HBV-R. Preemptive therapy prevented severe hepatitis related to HBV-R. Entecavir treatment was stopped in four patients with HBV-R. Since the withdrawal of entecavir, HBV-DNA has not been detected in two patients persistently positive for anti-HBs. No patient had fatal hepatitis.
Conclusions: Proper management of patients with HBsAg or resolved HBV infection prevented fatal hepatitis related to HBV-R in patients who received immunosuppressive or cytotoxic therapy. Entecavir could be safely discontinued in patients with HBV-R who had acquired anti-HBs.

Introduction

Hepatitis B virus (HBV), a circled DNA virus with approximately 3000 bases, causes liver disease in humans, including acute hepatitis, chronic hepatitis, cirrhosis, and hepatocellular carcinoma.[1,2] HBV viral loads in serum are regulated by both viral replication and host immunity. It is well known that HBV reactivation (HBV-R) occurs in patients who receive immunosuppressive or cytotoxic therapy and in patients after transplantation, particular in those who receive allogeneic or autologous hematopoietic stem-cell transplants (HSCT).[3–7] HBV-R is generally defined as a consecutive more than 1-log increase in the serum HBV-DNA level in patients with previously inactive or resolved HBV infection.[8,9] In patients with HBV-R, hepatitis flare can occur, leading to hepatic failure.[10] *De novo* hepatitis is defined as a hepatitis flare caused by HBV-R in patients negative for hepatitis B virus surface antigen (HBsAg).

Screening and prophylaxis for HBV-R in patients who receive cytotoxic therapy have been recommended by several groups of hepatologists.[11–13] However, it remains unclear how long such patients should be observed. A meta-analysis has suggested that prophylactic treatment with lamivudine, a nucleoside analogue with very potent anti-HBV replication activity, might reduce the risk of HBV-R and HBV-associated morbidity and mortality.[14] As compared with entecavir, prolonged treatment with lamivudine has a higher risk of viral breakthrough owing to the emergence of viral variants with reduced sensitivity to the drug, resulting from mutations in the YMDD locus of the HBV polymerase gene. Entecavir is speculated to be more suitable for patients with HBV-R who require more than 1 year of treatment. Recently, retrospective

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studies have shown that entecavir is more effective than lamivudine as prophylactic therapy for HBV-R.[15,16]

To our knowledge, there were few prospective long-term studies that have evaluated outcomes after prophylactic therapy in patients with HBsAg or preemptive therapy in patients without HBsAg. In particular, the endpoint of the nucleoside analogue therapy remains uncertain in patients with HBV-R. We performed this prospective study to elucidate the prevalence of HBV-R on regular screening and the characteristics of patients with HBV-R. We also evaluated the effectiveness of entecavir for HBV-R and assessed the risk of HBV reappearance after the end of entecavir treatment.

Patients and methods

Enrolled patients and management. In 2007, we organized a project team to perform a prospective study of HBV-R in patients with hematologic malignancy in Osaka City University Hospital. Before the start of rituximab-based chemotherapy or HSCT, HBsAg, antibodies against hepatitis B virus core antigen (anti-HBc), and antibodies against HBsAg (anti-HBs) in sera of the patients were tested by chemiluminescent enzyme immunoassay (CLEIA; Fujirebio Inc., Tokyo, Japan). Patients positive for one or more HBV serum marker were enrolled in the study. After enrollment, HBV-DNA was measured by a real-time polymerase chain reaction (real-time PCR)-based method (COBAS *TaqMan PCR*, Roche Diagnostics, Tokyo, Japan).[17] The quantified range of the real-time PCR assay was between 2.1 and 8.8 log copies/mL. Patients with hepatitis C virus, alcoholic liver disease, primary biliary cirrhosis, or autoimmune liver disease were excluded. HBV-DNA was regularly measured every month, from the start of chemotherapy or the time of HSCT to 1 year after the end of therapy. After monthly screening, HBV-DNA was measured once every 3 months. In patients in whom HBV-DNA was detected, HBV genotype was identified by enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies to type-specific epitopes in the preS2-region (Institute of Immunology, Tokyo, Japan), as described elsewhere.[18] Prophylactic or preemptive treatment against HBV-R-associated hepatitis was given to patients with a serum HBV-DNA level exceeding 2.1 log copies/mL; such patients received 0.5 mg entecavir per day. Treatment with entecavir was discontinued after more than 6 months had elapsed from the disappearance of both HBsAg and HBV-DNA in serum. In the present study, HBV-R was defined as more than a 1-log increase in the serum HBV-DNA level as compared with the value at enrollment or as a serum HBV-DNA level higher than 2.1 log copies/mL.

Fifty-seven patients (23 women and 34 men) were enrolled from November 2007 to January 2013. The mean age was 60 years (range, 23–82). Eight patients were positive for both HBsAg and anti-HBc, 43 were positive for both anti-HBs and anti-HBc, and 6 were positive for only anti-HBc (Table 1). No patient had a history of HBV vaccination. The mean follow up was 16 months (range, 4–63). Seven patients died within less than 1 year because of progression of hematologic malignancy or infection without HBV.

Sequencing of HBV-DNA. In patients with HBV-R, the nucleotide sequences of HBV polymerase coding area were determined by the direct sequencing method after nested PCR amplification.[19] Briefly, HBV-DNA was extracted from 200 μL of serum and was amplified as two overlapping fragments, A (nucleotide [nt] 271–1941) and B (nt 1679–335), with the use of an Expand High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany). To amplify fragment A, primers HBMF1 (5′-YCCTG CTGGTGGCTCCAGTTC-3′) and 1972R (5′-AAAGAATTCAG AAGGCAAAAAAGA-3′) were used for the first-round PCR, and primers HBMF2 (5′-GTCTAGACTCGTGGTGGACTTCTCTC-3′) and n1941R (5′ -CAGAAGCTCCAAATTCTTTATA-3′) were used for the second-round PCR. To amplify fragment B, primers 1653F (5′-CATAAGAGGACTCTTGGACT-3′) and HBMR2 (5′-AAGCCAXACARTGGGGGAAAGC-3′) were used for the first-round PCR, and primers 1679F (5′-AATGTCAACGACCG ACCTTG-3′) and 335R (5′-TGAYTGGAGRTTKGGGACT-3′) were used for the second-round PCR. Each PCR product was purified and sequenced directly by the dideoxy chain termination method, using a BigDye Terminator v1.1 Cycle Sequencing Kit and an ABI PRISM 3100 DNA Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Ethical considerations. This study protocol complied with the ethical guidelines of the Declaration of Helsinki 1975 (2008 revision) and was approved by the Ethics Committee of Osaka City University Graduate School of Medicine (UMIN Clinical Trials Registry, UMIN000009491). Written informed consent was obtained from all enrolled patients.

Table 1 Clinical characteristics of the enrolled patients

	Age	Gender	Anti-HB marker	Disease	Treatment
HBsAg-positive					
n = 8	62 (53–79)	Male: 7	Anti-HBs positive: 7	ML: 7	CHOP-R: 6
		Female: 1	Anti-HBc positive: 8	Leukemia: 1	HSCT: 2
HBsAg-negative					
n = 49	60 (23–82)	Male: 27	Anti-HBs positive: 43	ML: 29	CHOP-R: 28
		Female: 22	Anti-HBc positive: 49	Leukemia: 14	HSCT: 19
				MDS: 6	R-Hyper CVAD: 2

CHOP-R, combination chemotherapy with cyclophosphamide, doxorubicin, vincristine, prednisolone, and rituximab; HSCT, hematopoietic stem-cell transplantation; MDS, myelodysplastic syndromes; ML, malignant lymphoma; R-Hyper CVAD, combination chemotherapy with cyclophosphamide, vincristine, doxorubicin, dexamethasone, and rituximab.

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Results

Prophylactic therapy for patients with HBsAg. In the eight patients with HBsAg, prophylactic treatment with entecavir was started before cytotoxic therapy (Table 2). All eight patients were infected with HBV genotype C. In response to entecavir, the HBV-DNA load decreased to under 3 log copies/mL in all patients and fell to undetectable levels in all but one patient with HBeAg (case 32). Four of the eight patients died because of progression of hematologic malignancy or infection. Hepatic failure did not occur in any of the patients with HBsAg. Entecavir treatment has continuously prevented HBV-R in the other four patients.

Preemptive therapy for patients with HBV resolution. The clinical backgrounds of the 49 HBsAg-negative patients are shown in Table 1. At enrollment, HBV-DNA was not detected in patients without HBsAg. At the end of follow up, HBV-R has occurred in five (26%) of 19 patients who received HSCT and three (10%) of 30 patients who received rituximab-based chemotherapy. HBV-R occurred a median of 3 months (range, 2–10) after the end of rituximab-based chemotherapy. On the other hand, HBV-R occurred a median of 22 months (range: 9–36) after HSCT.

As compared with patients without HBV-R, anti-HBs titers at enrollment were slightly but not significantly lower in patients with HBV-R ($P = 0.085$). Among patients given rituximab-based chemotherapy, the anti-HBc titer was significantly higher in the presence of HBV-R ($P = 0.02$, Table 3). HBV-R occurred in one (17%) of six patients without anti-HBs. Reactivation occurred in six (26%) of 23 patients with anti-HBs titers below 50 mIU/mL, one (13%) of eight patients with anti-HBs titers between 50 and 200 mIU/mL, and none of 12 patients with anti-HBs titers exceeding 200 mIU/mL. During the screening period, anti-HBs titers gradually decreased in six patients with HBV-R. Anti-HBs titers became negative at the time of HBV-R in seven patients. Anti-HBs titers remained persistently positive in 36 patients without HBV-R.

Alanine aminotransferase (ALT) levels increased to more than five times the upper limit of normal in three of eight patients with HBV-R (Table 4). In one patient (case 4) who had received rituximab-based chemotherapy, the ALT level rose to 452 IU/L after entecavir treatment (Fig. 1). At that time, HBV-DNA decreased to below 2.1 log copies/mL. It was speculated that HBV-R was not directly related to ALT flare in this patient. Two other patients who underwent HSCT discontinued regular screening for HBV-DNA on their own initiative. Briefly, case 30 dropped out of regular screening 15 months after enrollment, and ALT levels rose to 362 IU/L with an increase in HBV viral load at month 22. Another patient (case 205) dropped out of the study 25 months after enrollment, and ALT levels elevated to 1642 IU/L with a concurrent increase in HBV viral load at month 36. Although HBV-R-related hepatitis occurred in these patients, treatment with entecavir fortunately prevented hepatic failure. With the exception of these two patients, preemptive therapy prevented hepatitis related to HBV-R. Treatments for hematologic diseases were completed without hepatic failure in all of the enrolled patients without HBsAg. One patient with HBV-R died of infection 43 months after HSCT. At the last follow up, HBV-DNA was not detected on real-time PCR. Among the seven survivors with HBV-R, four patients discontinued treatment with entecavir. After the withdrawal of entecavir, HBV-DNA was detected again in two patients without anti-HBs. One of the two patients required

Table 2 Baseline characteristics and outcomes of HBsAg-positive patients

No.	Gender	Age	Hematologic disease	Treatment	HBeAg	Anti-HBe (% inh)	HBV-DNA (log/mL)	ALT (IU/L)	Observation period (month)	Outcome
32	M	79	ML	CHOP-R	1600	—	8.5	78	26	Dead
66	M	63	ML	CHOP-R	—	100	ND	10	37	Alive
77	M	57	ML	CHOP-R	—	97	2.8	22	40	Alive
87	M	62	ML	HSCT	419	—	3.6	10	16	Dead
80	M	62	ML	CHOP-R	—	100	4	106	5	Dead
120	M	53	AML	HSCT	—	89	2.3	155	3	Dead
141	M	58	ML	CHOP-R	—	100	3.7	18	26	Alive
211	F	58	ML	CHOP-R	—	100	4	106	5	Alive

AML, acute myeloid leukemia; ML, malignant lymphoma; ND, data no available.

Table 3 Comparison between patients with or without HBV reactivation in the HBsAg-negative group

	All patients (*n* = 49)		Patients with HSCT (*n* = 19)		Patients with chemotherapy (*n* = 30)	
	With reactivation	Without reactivation	With reactivation	Without reactivation	With reactivation	Without reactivation
Age	55 (44–64)	64 (23–82)	55 (44–60)	49 (23–66)	60 (53–64)	67 (49–82)
Gender; M/F	2/6	21/20	2/3	8/6	3/0	13/14
Anti-HBs	35 ± 48	243 ± 366	41 ± 63	151 ± 210	25 ± 5	295 ± 420
Anti-HBc	77 ± 33	63 ± 38	80 ± 13	67 ± 36	99 ± 1*	69 ± 36*
Observation period	37 (24–63)	12 (4–61)	41 (32–52)	9 (4–55)	32 (24–63)	13 (4–61)

*$P = 0.02$, There were no differences in anti-HBs between the two groups.
Data were shown mean ± SD.

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Table 4 Clinical characteristics of patients with HBV reactivation

No.	Gender	Age	Hematological Disease	Treatment	Anti-HBs/Anti-HBc at the enrollment	At the time of HBV reactivation (month)	HBV-DNA at reactivation (Log/mL)	HBsAg at or after reactivation (IU/mL)	ALT peak after reactivation (IU/L)	Outcome
4	M	53	ML	CHOP-R	19.6/98.4	2	5.4	1047	452	Alive
30	M	59	Chronic leukemia	HSCT	30.2/70	22†	6.6	2000	362	Death
37	M	60	ML	CHOP-R	28.5/97.9	10	3.6	negative	28	Alive
68	F	46	MDS	HSCT	ND/97.4	10	4.1	45.7	49	Alive
121	M	55	Acute leukemia	HSCT	151.7/71	22	2.8	negative	58	Alive
128	M	64	ML	R-Hyper CVAD	26.9/99.2	3	3.1	negative	45	Alive
150	F	60	MDS	HSCT	14/ND	9	5.4	63.4	22	Alive
205	M	44	MDS	HSCT	7.4/81.5	36†	5.4	145	1642	Alive

†Two patients with HSCT dropped out of regular screening for HBV-DNA 1 year after enrollment. In another patient who had received rituximab-based chemotherapy, ALT increased to 452 IU/L during entecavir treatment.
ALT flare occurred in three patients with HBV reactivation.

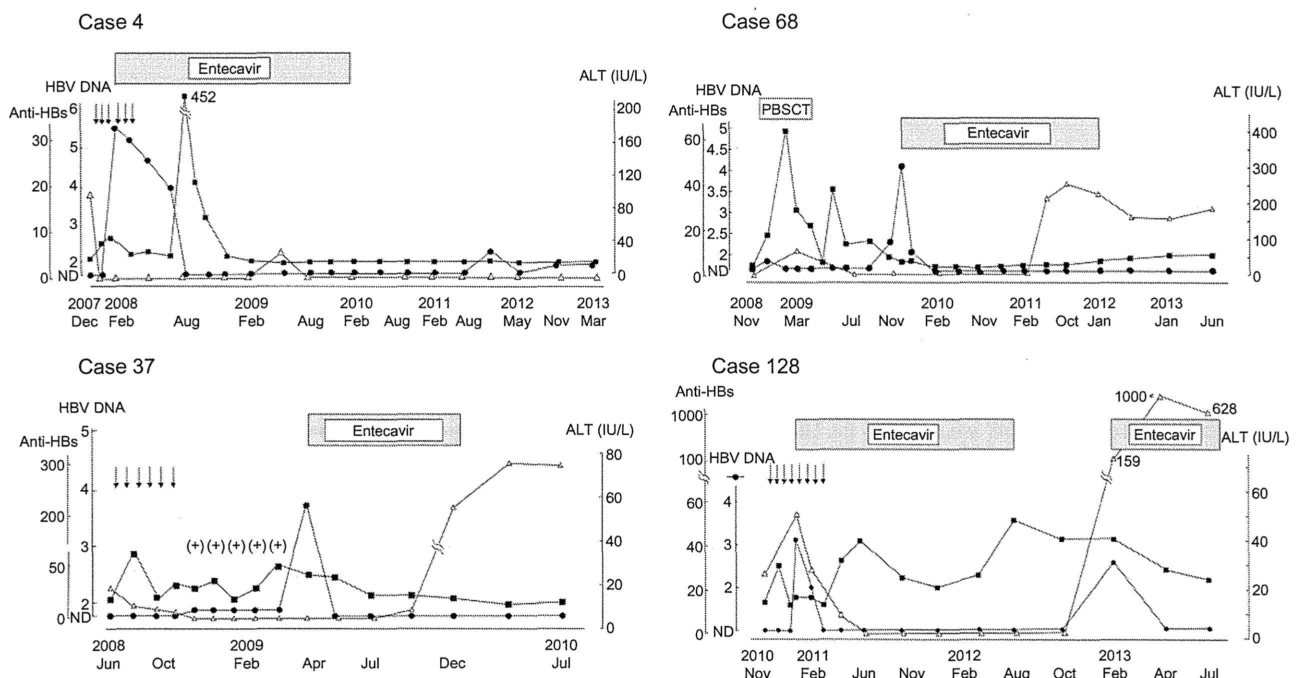

Figure 1 Clinical course of four patients with hepatitis B virus (HBV) reactivation in whom entecavir was withdrawn. After entecavir treatment, HBV-DNA was detected again in patients 4 and 128. On the other hand, HBV-DNA has not been detected in patients 37 and 68, in whom antibodies against HBsAg (anti-HBs) remains above 20 mIU/mL. CHOP-R: combination chemotherapy with cyclophosphamide, doxorubicin, vincristine, prednisolone, and rituximab, PBSCT: peripheral blood stem-cell transplantation. ↓, CHOP-R; -●-, HBV-DNA; -△-, Anti-HBs; -■-, ALT (IU/L).

retreatment with entecavir. On the other hand, HBV-DNA has not been detected in two other patients who were persistently positive for anti-HBs (Fig. 1).

DNA sequence of reactivated HBV. All reactivated HBV was genotype C. Sequence analysis showed that reactivated HBV did not have mutations associated with resistance to nucleos(t)ide analogues in the reverse transcriptase region.

Four of eight reactivated HBVs had mutations in the "a" determinant region of the S gene region with amino acid replacement (Fig. 2). In detail, case 121 had two mutations: 113 threonine to serine and 143 serine to threonine. In case 128, two mutations were detected (129 glutamine to arginine and 130 glycine to asparagine), and anti-HBs was positive at HBV-R (Fig. 1, case 128). An amino acid replacement of 145 glycine to arginine was detected in cases 150 and 205. In both cases, anti-HBs were negative at the time of HBV-R. At the time of HBV-R, HBsAg was

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            111               120                  130                 140                150
HBV DNA ;   P G T S T T S T G P C K T C T I/T P A Q G T S M F P S C C C T K L S D G N C T C I P I P S S W
case 4 ;    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
case 30 ;   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
case 37 ;   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
case 68 ;   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
                113                                                         143
case 121 ;  - - S - - - - - - - - - - - - - - - - - - - - - - - - - - - - - T - - - - - - - - - - - -
                                                      130
case 128 ;  - - - - - - - - - - - - - - - - - - R N - - - - - - - - - - - - - - - - - - - - - - - - -
                                                                                145
case 150 ;  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - R - - - - - - - - - - -
                                                                                145
case 205 ;  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - R - - - - - - - - - - -
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Figure 2 Alignment of amino acids codes from the 111th to 156th amino acids of hepatitis B (HB) surface antigen, the "a" determinant region. Comparison of the modified hepatitis B virus (HBV) ADR[20] and the eight reactivated HBV revealed several point mutations in "a" determinant region. Point mutations with amino acid replacement were detected in cases 121, 128, 150, and 205.

not detectable in two (cases 121 and 128) of four patients with HBV mutated in the "a" determinant region.

Discussion

In the present prospective study, the rates of HBV-R in patients with resolved HBV infection were 26% after HSCT and 10% after rituximab-based chemotherapy. Previous studies reported that HBV-R occurred in 12–20% of patients who had undergone HCST[6,7,21–23] and 4.1–17.9% of those who had received rituximab-based chemotherapy for malignant lymphoma.[4,24–26] The rate of HBV-R in our study is consistent with these previous finding. In retrospective studies of patients who underwent HSCT, HBV-R was defined as seroreversion in HBsAg-negative patients.[6,7,21] This is quite a difference from the present study, which used real-time PCR to measure HBV-DNA. During follow up, HBV-DNA was detected earlier than HBsAg. In addition, HBsAg did not turn positive in three of the eight patients with HBV-R. Two of the five patients in whom HBsAg was consistently negative had mutations in the S determinant region of HBV-DNA. Our data confirmed that detection of the viral genome was the most specific and sensitive screening tool for HBV-R, particularly as compared with serological tests. A recent large-scale prospective study using HBV-DNA test showed that HBV-R occurred in 17 (11.3%) of 150 HBV resolved patients who had received rituximab-based chemotherapy.[27]

In our patients with resolved HBV infection, HBV-R occurred within 1 year after the end of rituximab-based chemotherapy and more than 1 year after HSCT. Although HBV-R rarely occurs more than 3 years after HSCT,[28,29] the longest reported period to HBV-R after HSCT was 47 months.[23] In the two patients in the present study who discontinued HBV monitoring more than 15 months after enrollment, HBV-R-associated ALT flare occurred. These results might be useful for establishing follow-up periods for HBV-R according to treatment. Recently, careful monitoring for HBV-R has been broadly recommended for anti-HBc-positive patients who receive immunosuppressive or cytotoxic therapy. However, the incidence and timing of reactivation might differ according to the details of treatment, such as the drugs used or procedures performed. Cost–benefit analyses should be performed according to specific diseases and treatments to assess the value of screening for HBV-R.

Several studies have suggested that decreased levels or loss of anti-HBs is a predictor of HBV-R in anti-HBs-positive patients.[23,30] In our study, anti-HBs had become negative at the time of HBV-R in seven of eight patients. However, the other patient (case 128) was positive for anti-HBs at HBV-R. A case report has documented the development of fatal hepatitis in a patient with HBV-R who had a high titer of anti-HBs.[31] It is well known that HBV vaccination provides no protection against HBV with mutations in the HBsAg coding region (i.e. "escape mutant HBV"). Consequently, escape mutant HBV can increase in anti-HBs-positive patients. In our patient who was positive for anti-HBs at the time of HBV-R, two mutations in the "a" determinant region of the S gene were detected. Borentain *et al.* showed that reactivated HBV is associated with several mutations in the "a" determinant region of the S gene.[22] Interestingly, four reactivated HBVs in our study had mutations with amino acid replacement in "a" determinant region. This finding suggests that the mutated HBV might persist in some patients who have HBV-R without serum HBsAg and/or that such HBV might preferentially increase during immunosuppressive or cytotoxic therapy. Taken together, although patients with low anti-HBs titers might have an increased risk of HBV-R, assessment of anti-HBs alone without screening for HBV-DNA may fail to identify some patients at high risk for HBV-R.

Our study showed that prophylactic therapy in HBsAg-positive patients and preemptive therapy in HBV-resolved patients could prevent hepatic failure related to HBV-R associated with cytotoxic or immunosuppressive therapy for hematologic malignancies. Specifically, entecavir reduced HBV viral load in both patients with HBsAg and eight patients with HBV-R and maintained it below 2.1 log copies/mL for more than 6 months; the duration of entecavir treatment ranged from 3 to 35 months. The emergence of lamivudine-resistant HBV mutants has been reported in patients who received prophylactic treatment for HBV-R.[16,32] No entecavir-resistant mutants emerged in our study, suggesting that entecavir

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might be better suited for patients who require longer periods of prophylactic or preemptive treatment.

In a recent randomized controlled study of HBV-resolved patients with lymphoma, prophylactic entecavir treatment before rituximab-based chemotherapy prevented HBV-R in all but one (2.4%) of 41.[26] As compared with preemptive treatment at the time of HBV-R, prophylactic treatment with entecavir more effectively prevented HBsAg reverse seroconversion. However, ALT levels increased to above 100 IU/mL in each patient who received prophylactic or preemptive treatment. Fatal hepatitis did not occur in that trial. Our study also showed that preemptive therapy prevented fatal hepatitis in patients with HBV-R who continued to undergo regular screening. Further studies are needed to establish whether prophylactic therapy should be started before cytotoxic or immunosuppressive treatment in all patients with resolved HBV infection.

Another important issue is whether entecavir treatment can be safely discontinued in patients with HBV-R. Fatal hepatic failure has been reported after the withdrawal of prophylactic lamivudine therapy in HBsAg-positive patients with HSCT.[33] In general, nucleot(s)ide analogue treatment should be continued in HBsAg-positive patients. However, there are no firm recommendations for patients who have HBV-R without HBsAg. We withdrew entecavir after more than 6 months after the disappearance of both HBV-DNA and HBsAg in four patients with HBV-R who had received preemptive therapy. After the withdrawal of entecavir, HBV-DNA was detectable in two patients without anti-HBs. On the other hand, HBV-R has not occurred in the other patients whose anti-HBs turned positive after preemptive therapy. Our findings suggest that entecavir can be safely discontinued in patients with HBV-R after anti-HBs has become consistently positive. To confirm our speculations, longer-term studies in larger groups of patients are necessary.

In conclusion, this prospective study confirmed that current recommendations for patients with HBsAg and those with resolved HBV infection can prevent fatal hepatitis related to HBV-R in patients who receive immunosuppressive or cytotoxic therapy. To improve cost–benefit ratios, future studies should attempt to find other reliable markers and to establish optimal screening periods for HBV-R according to specific diseases or treatments. Finally, we speculated that entecavir can be safely discontinued in patients with HBV-R who have acquired anti-HBs.

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RESEARCH

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Clinical and pathological features of five-year survivors after pancreatectomy for pancreatic adenocarcinoma

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Abstract

Background: Clinical factors determining short-term survival after pancreatectomy have been well studied, but factors predicting long-term survival with curative resection are poorly understood in pancreatic carcinoma. Our objective was to identify clinical and pathological features of five-year disease-free survivors after surgical resection of pancreatic adenocarcinoma.

Methods: The clinical and pathological data from 147 patients who underwent a potentially curative resection for pancreatic adenocarcinoma at our institution between 1988 and 2012 were retrospectively analyzed.

Results: Of 147 patients, 18 survived for more than five years after surgery without disease recurrence. A univariate analyses demonstrated that: two or fewer lymph node metastases ($P = 0.014$), a preoperative serum carbohydrate antigen 19-9 (CA19-9) level of 40 U/mL or less ($P = 0.0018$), an absence of intrapancreatic nerve invasion ($P = 0.028$), and undergoing an R0 resection ($P = 0.011$) were significantly associated with five-year survival. A logistic regression model identified the following independent cancer-related predictors of five-year survivors: having two or fewer lymph node metastases (odds ratio (OR): 6.02; 95% confidence interval (CI): 1.08 to 112.98; $P = 0.0385$), a preoperative serum CA19-9 level of 40 U/mL or less (OR: 5.02; 95% CI: 1.68 to 16.48; $P = 0.0036$), and undergoing an R0 resection (OR: 3.63; 95% CI: 1.12 to 14.28; $P = 0.0316$).

Conclusions: We conclude that number of lymph node metastases being two or less, a preoperative serum CA19-9 level of 40 U/mL or less, and undergoing an R0 resection may be independent predictive factors to identify actual five-year survivors after pancreatectomy for pancreatic adenocarcinoma.

Keywords: Pancreatic carcinoma, Five-year survivors, CA19-9, R0, Lymph node metastasis

Background

Pancreatic carcinoma is the fourth leading cause of death from cancer and is responsible for 43,000 deaths per year in the United States [1]. The prevalence of pancreatic cancer in Japan has also increased in the last decade to become the fifth leading cause of cancer death in men, and the sixth in women [2]. This malignancy is devastating, with a five-year overall survival rate of approximately 5% [1]. The only potentially curative treatment for pancreatic cancer is surgical resection. However, only a small number of patients (between 15 and 20%) present with a resectable tumor at the time of diagnosis [3]. Moreover, the prognosis even after potentially curative resection is considered to be poor. The following characteristics have been reported to be significant prognostic factors for patient survival after tumor resection: age [4], tumor size [4-6], lymph node metastasis [4-6], surgical margin status [7-9], preoperative serum CA19-9 level [9-11], and tumor grade [7]. Clinical factors determining short-term survival after pancreatectomy have been well studied, but prognostic factors predicting long-term survival with curative resection are poorly understood [12-14].

In the earlier studies, it was difficult to discuss factors related to five-year survival because of the high

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