HBV Cure?

The impact of HBV DNA Integration?
evolution of rocks

4.5 billion years ago

present day
Integration of HBV DNA is a key mechanism of HBV persistence

Expression of HBsAg from integrated HBV DNA sequences has been well demonstrated:

- Conservation of HBsAg-encoding sequences in most HBV DNA integrants
- Expression of HBsAg from cloned integrated HBV DNA genomes
Characterization of integrated hepatitis B viral DNA cloned from a human hepatoma and the hepatoma-derived cell line PLC/PRF/5

ANNE DEJEAN, CHRISTIAN BRECHOT, PIERRE TIOLLAIS, AND SIMON WAIN-HOBSON


HBsAg expression upon transfection of HBV DNA integrated sequences
The genetic organization of integrated hepatitis B virus DNA in the human hepatoma cell line PLC/PRF/5

Stefan Koch, Arndt Freytag von Loringhoven, Regine Kahmann¹, Peter Hans Hofschneider and Rajen Koshy²

HBsAg expression upon transfection of HBV DNA integrated sequences
FURTHER STUDIES ON PRODUCTION AND CHARACTERIZATION
OF HBsAg
DERIVED FROM A HUMAN HEPATOMA CELL LINE (PLC/PRF/5)

F. Barin 1, A. Goudeau 1, C. Brechot 2,
J. Romet-Lemonne 1, Camille Sureau 1 and G. Lesage 1

HEPATITIS-B SURFACE ANTIGEN IN TUMOUR TISSUE AND NON-TUMOROUS LIVER IN BLACK PATIENTS WITH HEPATOCELLULAR CARCINOMA

M. C. KEW, M. B. RAY, V. J. DESMET AND J. DESMYTER
RNAi-based treatment of chronically infected patients and chimpanzees reveals that integrated hepatitis B virus DNA is a source of HBsAg

**HBsAg reduction in ETV naive patients** (single 4 mg dose (cohort 7))

![Graph showing HBsAg reduction in ETV naive patients](image1)

**HBsAg reduction in Chimpanzees**

![Graph showing HBsAg reduction in Chimpanzees](image2)

Wooddell et al., Sci. Transl. Med. 9, 27 September 2017

**Courtesy: Fabien Zoulim**
RNAi-based treatment of chronically infected patients and chimpanzees reveals that integrated hepatitis B virus DNA is a source of HBsAg

C.I.Woodell et al
Science Translational Medicine 2017
HBV DNA integration into cellular DNA is an early event and is associated to clonal hepatocyte expansion

- Integration occurs very early during HBV infection: Acute in vitro and in vivo infection

- A very high percentage of Woodchucks hepatocytes is infected during acute infection

- Liver cells containing integrated HBV DNA sequences undergo clonal expansion from acute to chronic stages of the viral infection
State of hepatitis B virus DNA in hepatocytes of patients with hepatitis B surface antigen-positive and -negative liver diseases

(recombinant DNA/hepatocellular carcinoma/chronic and acute hepatitis/Southern blot technique/immunofluorescence)

CHRISTIAN BRÉCHOT*, MICHELLE HADCHOUEL†, JACQUES SCOTTO†, MICHELLE FONCK*, FRANÇOIS POTET‡, GIRISH N. VYAS§, AND PIERRE TIOLLAIS**
HBV DNA Integration and Clonal Hepatocyte Expansion in Chronic Hepatitis B Patients Considered Immune Tolerant

Inverse PCR for detection of clonal expansion of hepatocytes
William S. Mason, et al
Gastroenterology
2016

Around 5x10^-6 HBV integrants/liver
Clonal expansion of hepatocytes with a selective advantage occurs during all stages of chronic hepatitis B virus infection
T. Tu et al ; J Viral Hepatitis 2015

Detection of Clonally Expanded Hepatocytes in Chimpanzees and Woodchucks with Chronic HBV and WHV infections

Cloned Hepatocyte size independent of sites of HBV DNA integration and HBsAg expression?

Clonal expansion in histologically normal hepatocytes

Mechanisms; molecular bases?
HBV DNA integration is a dynamic process during chronic infection

- Novel integration sites generated through ongoing replication of HBV DNA
  
  Double stranded linear DNA (dsI DNA):
  
  Mispriming events
  
  Splicing?

- Rearrangements of integrated HBV DNA sequences

- Novel integration events from previously integrated sequences (translocation etc..)
Interrupted Replication of Hepatitis B Virus in Liver Tissue of HBsAg Carriers with Hepatocellular Carcinoma

GIOVANNI RAIMONDO,* ROBERT D. BURK,t+ HARVEY M. LIEBERMAN,* JOSEPH MUSCHEL,t STEPHANOS J. HADZIYANNIS,§ HANS WILL,” MICHAEL C. KEW,II GEOFFREY M. DUSHEIKO,IT AND DAVID A. SHAFRITZ
VIROLOGY 166, 103-112 (1988)

- Blocked virus assembly/secretion and accumulation of unencapsidated HBV DNA replicative intermediates in the liver cell.

- Accumulation of such HBV DNA molecular forms in the liver may lead to an increased propensity for HBV DNA to integrate into the host genome
Direct and indirect effects of HBV chronic infection

Host immune response
Inflammation; oxidative stress; fibrosis

Liver carcinogenesis

Integration of HBV DNA: Translocations, chromosomal rearrangements
Insertional mutagenesis of cellular genes

Prolonged expression of viral genes
HBx, COOH-terminally deleted HBx
PreS1/PreS2/S: LHBs
Modulation of liver cell proliferation and viability

Genetic instability
Activation or inactivation of cellular genes
HBV DNA integration: which impact?

- Mechanisms of HBV DNA integration:
  - Random integration followed by selection of clonally expanding cells?
  - Family of preferential sites for insertion?
    - Fragile sites
    - Transcriptionally active genes (chromatin remodelling)

- Insertion into cellular genes: cause or consequence of liver cell proliferation?
  - Insertion in key driver genes for carcinogenesis
  - Insertional mutagenesis of the target gene
HBV insertion targets a variety of genes that regulate key cellular pathways

Unique target genes:
- RAR beta
- Cyclin A
- Meval. kinase
- MCM
- SERCA1
- TRAP150
- FR7
- EMX2-like
- MAPK1
- IRAK2
- TRUP
- NRTK2
- Ras-REBP-1
- Calmodulin 1

Recurrent target genes:
- hTERT
- IP3R
- MLL2

Genome-wide survey of recurrent HBV integration in hepatocellular carcinoma

Massively parallel sequencing of 81 HBV-positive and 7 HBV-negative hepatocellular carcinomas (HCCs) and adjacent normal tissues.

HBV integration observed more frequently in the tumors (86.4%) than in adjacent liver tissues (30.7%).

Recurrent HBV integration events validated by RNA and Sanger sequencing at the known and putative cancer-related TERT, MLL4 and CCNE1 genes, which showed upregulated gene expression in tumor versus normal tissue.

Sung et al Nature 2013
In total: 1377 Viral Integration Sites (VIS) within close proximity to protein coding genes and 767 with noncoding genes, respectively.

23.1% of protein-coding genes and 24.7% of long noncoding RNAs (lncRNA) targeted more than two times.

Only 4.8% of Viral Integration Sites common between HCC and non-tumor tissues. Total number of VIS in tumorous tissues: 2-fold higher than non-tumorous tissue.

The number of integration sites on each chromosome correlated with the number of fragile sites in non-tumorous tissue but not in HCC tissue. Enrichment of cancer-related gene at or in close proximity to HBV integration sites in HCC tissue.
HBV DNA Integration into cellular DNA is a dynamic process

- Novel integration sites generated through ongoing replication of HBV DNA

- Rearrangements of integrated HBV DNA sequences

- Novel integration events from previously integrated sequences (translocation etc..)

- Novel integrations generated through spliced HBV RNAs?
**dHBV particles and HBSP protein**

**2.2 Kb Alternative single splicing**

**Integration?**

**Defective HBV particles**

- **Soussan et al. JCI. 2000**
- **Soussan et al. J.Hepatol. 2003**
- **Kremsdorf et al. Oncogene. 2006**
- **Mancini-Bourgine et al. J. Virol. 2007**
- **Soussan et al. JID. 2008**
- **Asrir et al. Frontiers in bioscience. 2010**
- **Bayard et al. J.Viral.Hepat. 2012**
- **Pol et al. Faseb J. 2015**
HBSP contributes to limit liver immune recruitment

Patrick Soussan and Dina Kremsdorf

Anti-HBSP: 40% of HBV chronic carriers

Tg HBSP + CCl4

Liver fibrosis-related cytokine/chemokine genes expression

Duriez et al
Journal of Hepatology 2017 vol. 67 j 687–699
Novel pre-mRNA splicing of intronically integrated HBV generates oncogenic chimera in hepatocellular carcinoma Yung-Tuen Chiu et al; J Hepatol 2016

Fig. 1. HBV-human chimeric junctions at splice sites. (A) Abundance of chimeric junctions on HBV genome. Note that the chimeric junctions were found more frequently at site of ~1800 nt and 460 nt of the HBV genome. (B) Chimeric junctions limited to human splice sites. Note that multiple peaks were found at 292 nt, 458 nt and 2448 nt. The locations of HBV genes were annotated as S gene, polymerase gene, X gene, and pre-core gene, respectively. The blue bars indicate the number of reads detected at the position. (C) List of genes detected with HBV-human junction at human splice sites. Only tumor-specific events are listed. (This figure appears in colour on the web.)

Fig. 2. Graphical illustration of CCNA2-HBV chimeric fusion transcript (A2S) sequence alignment. Reads alignment to the human CCNA2 and HBV are shown. Note that HCC gene (282 nt to 458 nt) was integrated into intron 1 of CCNA2. Original data are shown in Supplementary Fig. 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Full name</th>
<th>HBV splice site (nt.)</th>
<th>Human splice site</th>
<th>Affected samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCNA2</td>
<td>Cyclin A2</td>
<td>282, 458</td>
<td>Intron 2</td>
<td>6/48 HCC vs 0/22 Non-HCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2448</td>
<td>Intron 2</td>
<td></td>
</tr>
<tr>
<td>NR3C2</td>
<td>Nuclear receptor subfamily 3, group C, member 2</td>
<td>458</td>
<td>Intron 2</td>
<td>308T</td>
</tr>
<tr>
<td>TERT</td>
<td>Telomerase reverse transcriptase</td>
<td>458</td>
<td>Intron 2</td>
<td>383T</td>
</tr>
<tr>
<td>MLL4</td>
<td>Mixed-lineage leukemia 4</td>
<td>458</td>
<td>Intron 3</td>
<td>228T</td>
</tr>
</tbody>
</table>

26
The actual impact of HBV DNA persistence after serum HBsAg negativation?

- "signature" of the HBV infection with no functional consequences?

- Potential persistent risk?:
  - Reactivation of HBV multiplication?
  - HCC development?
Twenty-eight studies, involving 34,952 patients with HBsAg seroclearance.

The overall pooled proportion suggested that 2.29% (95% CI: 1.19–4.37%) CHB patients would develop HCC despite HBsAg seroclearance.
Decreased cumulative risk of HCC after HBsAg loss

Yuen et al, Gastroenterology 2008
The impact of occult HBV infections? A debated issue

Statements from the Taormina expert meeting on occult hepatitis B virus infections


Persistence of intrahepatic replication in the liver of HBsAG negative patients

Persistence of Hepatitis B and Hepatitis C Viral Genomes in Primary Liver Cancers from HBsAg-Negative Patients: A Study of a Low-endemic Area

Patrizia Paterlini,1,6 Françoise Driss,3 Bertrand Nalpas,2 Emilio Pisi,6 Dominique Franco,6 Pierre Berthelot,2 and Christian Bréchot1,2,4

(HEPATOLOGY 1993;17:20-29.)
HBV insertion targets a variety of genes that regulate key cellular pathways

**Unique target genes:**
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- MAPK1
- IRAK2
- TRUP
- NRTK2
- Ras-REBP-1
- Calmodulin 1

**Recurrent target genes:**
- hTERT
- IP3R
- MLL2

Hepatitis B virus (HBV) DNA integration in patients with occult HBV infection and hepatocellular carcinoma

Carlo Saitta et al
Integrated HBV DNA persistence

HCV
Alcohol
Metabolic Disorders (Diabetes, Obesity)
Iron overload
Chemicals

Low grade inflammation
Direct effects

HCC
Occult and previous hepatitis B virus infection are not associated with hepatocellular carcinoma in United States patients with chronic hepatitis C


Hepatology. 2011 Aug;54(2)
Risk of HCC in HBsAg negative, HBV DNA positive patients

Prospective studies

HBV DNA +

vs

HBV DNA –

8.25 fold increased risk of HCC

Ikeda J Viral Hepatitis

2009; 16: 437

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<table>
<thead>
<tr>
<th>Factors</th>
<th>Category</th>
<th>Hazard ratio (95% confidence interval)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Women</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Men</td>
<td>15.4 (2.24–111.1)</td>
<td>0.005</td>
</tr>
<tr>
<td>Serum HBV DNA</td>
<td>Negative</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>8.25 (2.01–33.93)</td>
<td>0.003</td>
</tr>
<tr>
<td>Total alcohol</td>
<td>≥500 kg</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>intake</td>
<td>&lt;500 kg</td>
<td>7.19 (1.98–26.32)</td>
<td>0.003</td>
</tr>
<tr>
<td>Age</td>
<td>&lt;60 years</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥60 years</td>
<td>3.98 (1.10–14.42)</td>
<td>0.035</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>No</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>3.89 (1.22–12.47)</td>
<td>0.022</td>
</tr>
</tbody>
</table>

*Positive HBV DNA: positive for both HBe DNA and HBx DNA.
Intrahepatic hepatitis B virus replication and liver histology in subjects with occult hepatitis B infection

D. K.-H. Wong et al  Clin Microbiol Infect 2015;
TRANSMISSION OF HEPATITIS B FROM HEPATITIS-B-SERONEGATIVE SUBJECTS
V. THIERs et al Lancet

Impact on infectiosity?
Persistence of HBV DNA in extra-hepatic sites?


Hepatitis B virus infection of peripheral blood mononuclear cells is common in acute and chronic hepatitis. Pasquinelli et al J Med Virol 1990

Detection of mononuclear cells expressing hepatitis B virus in peripheral blood from HBsAg positive and negative patients by in situ hybridization. Hadchouel et al J Med Virol 1988

Hepatitis B Virus DNA in Peripheral-blood Mononuclear Cells in Chronic Hepatitis B after HBsAg Clearance A Mason et al Hepatology 1992

Gene copy number variations in the leukocyte genome of hepatocellular carcinoma patients with integrated hepatitis B virus DNA; Y Pang et al Oncotarget 2016

Whole-genome comparative genomic hybridization (CGH) chip array analyses to screen gene copy number variations (CNV) in the leukocyte genome,

The results were confirmed by quantitative polymerase chain reaction (qPCR).
Can we eliminate integrated HBV DNA sequences?

- Progressive elimination of hepatocytes containing integrated HBV in the absence of ongoing HBV DNA replication?: Hepatocyte turnover?:

But:
- clonal proliferation of hepatocytes containing integrated HBV DNA
- Frequent persistence of low rate intra-hepatic HBV DNA replication despite undetectable serum viremia.
- Reinfection events from one hepatocyte to the other
How to get rid of integrated HBV DNA sequences?
Combining different approaches

- **Blocking HBV genome replication**
  cccDNA: inhibited expression? or elimination?

- **Eliminating infected hepatocytes:**
  Immunotherapy-based approaches

- **Blocking expression of integrated HBV DNA:**
  Ex: HBV RNA specific siRNA

- **Eliminating integrated HBV DNA sequences?**
RNAi-based treatment of chronically infected patients and chimpanzees reveals that integrated hepatitis B virus DNA is a source of HBsAg.
C.I.Woodell et al Science Translational Medicine 2017
The CRISPR/ Cas9 system facilitates clearance of the intrahepatic HBV templates In Vivo.

Suppression of hepatitis B virus DNA accumulation in chronically infected cells using a bacterial CRISPR/CasRNA-guided DNA endonuclease
Virology 2015; 476: 196–205

Complete Spectrum of CRISPR/Cas9-induced Mutations on HBV cccDNA
Christoph Seeger and Ji A Sohn
Molecular Therapy vol. 24 no. 7, 1258–1266 jul. 2016
Removal of Integrated Hepatitis B Virus DNA using CRISPR-Cas9

Hao Li, et al
Frontiers in Cellular and Infection Microbiology March 2017; Volume 7

Hazards?
« Off targets » ?:
Novel rearrangement of integrated HBV DNA?
Mutations in cellular genome?

Challenges:
Which sequences to be used for targeting Cas9?
« personalized » ??
CRISPR-Cas9: lessons from HIV

CRISPR-Cas9 Can Inhibit HIV-1 Replication but Non Homologous end-joining (HEJ) Repair Facilitates Virus Escape
Wang et al Molecular Therapy 2016

CRISPR/Cas9-Derived Mutations Both Inhibit HIV-1 Replication and Accelerate Viral Escape
Zhen Wang et al Cell Reports 2016
Impact of Integrated HBV DNA?

➢ Should negative serum HBsAg be an endpoint marker of « functional cure »? Not the only one

➢ Do we need to eliminate integrated sequences? Yes

➢ Is it feasible to eliminate integrated HBV DNA: « real cure »?: ??

A Research Agenda for Curing Chronic Hepatitis B Virus Infection. Alter et al Hepatology 2017

Hepatitis B cure: from discovery to regulatory approval: Anna Lock, Fabien Zoulim, Geoffrey Dusheiko and Marc G Ghany. Hepatology 2017


Antiviral strategies to eliminate hepatitis B virus covalently closed circular DNA (cccDNA). Revill, Locarnini; Current Opin Pharmacol 2016 Oct;30:144
Progress in science depends on new techniques, new discoveries and new ideas, probably in that order.

Sydney Brenner