THU-216

Rapid Turnover of HBV cccDNA in Chronic Hepatitis B Patients During Drug Resistance Emergence and Breakthrough

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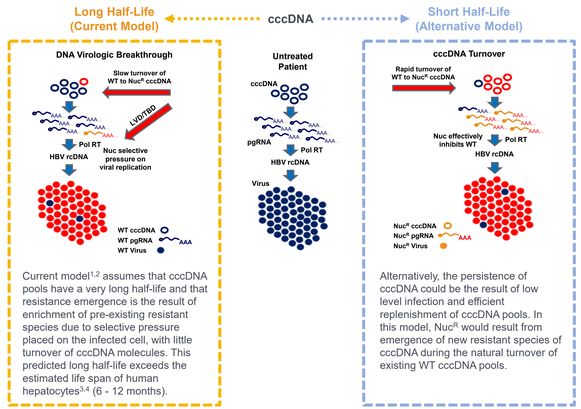
INTRODUCTION

To maintain chronicity, the Hepatitis B Virus (HBV) relies on both the generation of and maintenance of cccDNA populations. Current standard of care (SOC), nucleos(t)ide (Nuc) therapy, is able to suppress viremia in patients with chronic HBV (CHB), but very few patients maintain a "sustained viral response" after stopping Nuc therapy, or achieve a "functional cure" (including loss of detectable HBV S antigen) while on Nuc therapy. Initial mathematical modeling, built on the premise that Nucs such as ADV and ETV effectively blocked new cccDNA formation, estimated that it would take approximately 14 years to clear intrahepatic cccDNA from a chronically HBV infected patient. We now know that Nucs do not completely inhibit HBV replication and are ineffective inhibitors of cccDNA formation. In this continuing study, cccDNA biogenesis was revisited using a molecular genetics approach. By monitoring the emergence and disappearance of Nuc resistance (Nuc^R) mutations in patient liver and serum samples as a genetic marker for cccDNA, we are able to evaluate the evolution of cccDNA pools over time. These studies had four primary objectives: 1) Develop methodologies to enable distinct analysis of cccDNA, pgRNA and viral DNA populations from patient serum and liver biopsy samples; 2) Establish the relationship between genetic sequence in pgRNA and cccDNA; 3) Measure the turnover rate of cccDNA pools; and 4) Determine if inactive subpopulations of cccDNA exist.

MODELS OF cccDNA BIOGENESIS



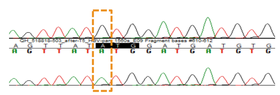
Current model[1,2] assumes that cccDNA pools have a very long half-life and that resistance emergence is the result of enrichment of pre-existing resistant species due to selective pressure placed on the infected cell, with little turnover of cccDNA molecules. This predicted long half-life exceeds the estimated life span of human hepatocytes[3,4] (6 - 12 months).

Alternatively, the persistence of cccDNA could be the result of low level infection and efficient replenishment of cccDNA pools. In this model, Nuc^R would result from emergence of new resistant species of cccDNA during the natural turnover of existing WT cccDNA pools.

METHODS

- **Strategy:** HBV DNA, pgRNA and cccDNA from longitudinal serum and paired biopsies of LVD or TBD virologic breakthrough patients were examined by population sequencing to better understand the dynamics of resistance emergence and cccDNA biogenesis.
- **Serum HBV DNA and pgRNA Extraction, Amplification and Sequencing:** HBV DNA and pgRNA were extracted from patient serum using a QIAamp MinElute Virus kit (Qiagen). Aliquots were digested by DNase I (ThermoFisher) and used as a template for RT-PCR. A pair of pan-genotype primers were designed to amplify the HBV reverse transcriptase (RT) gene. Sanger sequencing results of PCR and RT-PCR fragments were analyzed using Sequencher™ software (Gene Codes) and the percentage of resistant mutations was calculated using population sequencing[5].
- **Intrahepatic HBV DNA, RNA and cccDNA Extraction, Amplification and Sequencing:** Protein-free relaxed circular DNA (rcDNA), HBV RNA and cccDNA from snap-frozen liver biopsies were extracted by a modified Hirt method[9,10], digested with T5 exonuclease, amplified by PCR or RT-PCR and analyzed by population sequencing. Spiking experiments (with Nuc^R M204V rcDNA) demonstrated that cccDNA was free of rcDNA contamination after T5 digestion (shown below).

T5 Exonuclease Specifically Digests and Eliminates HBV rcDNA

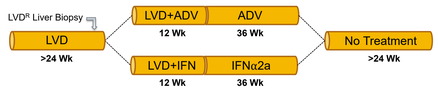


Before T5 digestion, population sequencing detected Nuc^R rcDNA (M204V) added at 40x excess

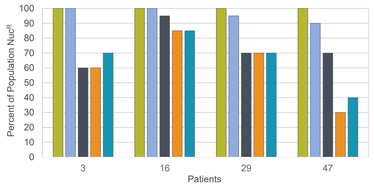
After T5 digestion, Nuc^R rcDNA was not detected, showing effectiveness of removing any existing rcDNA

HBV RNA REFLECTS cccDNA

Study ML 18376

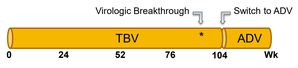


Patients from Study ML 18376

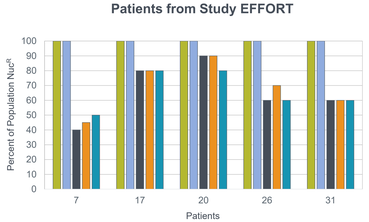


- Intrahepatic HBV DNA, RNA and cccDNA were sequentially extracted from biopsy samples of four LVD^R CHB patients before initiation of rescue treatment[6]
- Time between biopsy and serum sampling was 8 - 56 days
- Nuc^R variants were found in 90-100% of HBV DNA samples, and at lower percentages in RNA and cccDNA samples at the time of virologic breakthrough
- Comparing the composition of HBV DNA, RNA and cccDNA sequences shows that RNA and cccDNA resistance percentages correlated well, indicating that viral RNA, rather than viral DNA, more accurately reflects sequences in cccDNA pools

Study EFFORT: Mono Group Arm

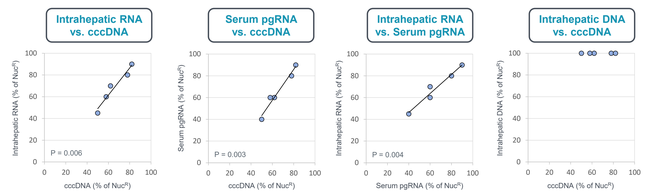


Patients from Study EFFORT



- Paired liver biopsy and serum samples (Baseline and Week 104) collected from five CHB patients who experienced virologic breakthrough on TBD therapy were evaluated[7]
- Nuc^R M204V/I was identified in 100% of the HBV DNA populations, but at lower percentages (40-80%) in HBV RNA and cccDNA populations
- No Nuc^R M204V/I variants were detected in Baseline samples (data not shown)
- Data from these patients shows a strong correlation between serum and intrahepatic RNA with cccDNA populations

Predictive Relationships (Study EFFORT)

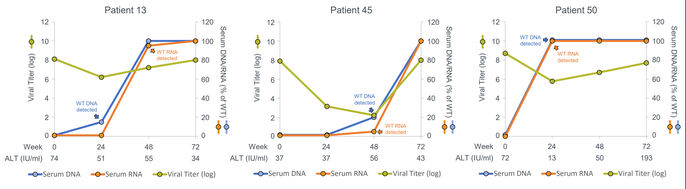


- Data from paired serum and liver biopsies demonstrated that serum pgRNA correlated well with intrahepatic RNA and cccDNA, which validated that serum pgRNA accurately reflects the genetic composition of cccDNA
- The strong correlation between sequence results obtained from cccDNA and HBV RNA in biopsy samples also indicates an absence of any significant portion of inactive WT cccDNA during virologic breakthrough
- The establishment of serum pgRNA as a cccDNA genetic marker allows monitoring of cccDNA biogenesis using longitudinal serum samples when biopsy samples are unavailable

HBV RNA populations from both serum and intrahepatic samples are highly predictive of cccDNA populations and genetic makeup

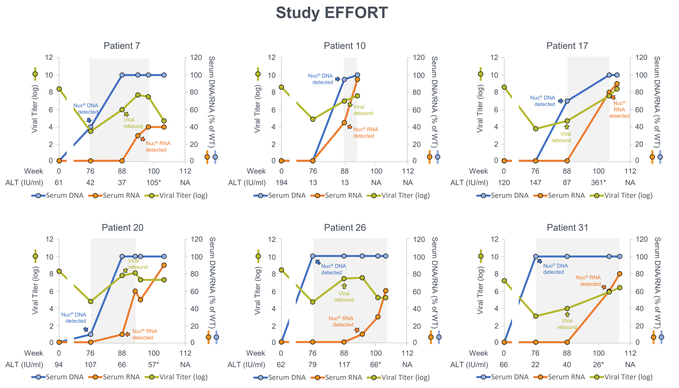
RAPID TURNOVER OF cccDNA

Study ML 18376



- Five LVD patients experiencing virologic breakthrough harbored a high percentage of Nuc^R mutations in their serum DNA and pgRNA populations at Study Baseline
- When these LVD^R patients (non- or weak-responders) switched to IFN, they showed a rapid reversion to WT M204 in both serum DNA and pgRNA within 3 - 5 months
- The rapid kinetics of Nuc^R pgRNA (cccDNA surrogate marker) replacement by WT pgRNA is likely accelerated by the favorable replicative fitness of low level residual WT virus not detected by population sequencing

Study EFFORT

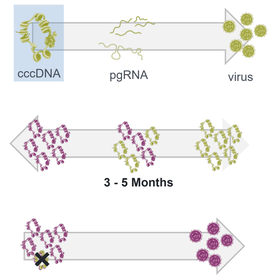


*ALT level measured at Week 104

- HBV DNA and RNA analyzed from longitudinal serum samples of six TBV patients experiencing virologic breakthrough
- Nuc^R variants emerged rapidly during virologic breakthrough in serum DNA, then gradually enriched in pgRNA populations with at least 50% of pgRNA molecules converted from WT to resistant variants within 5 - 28 weeks (shaded area)
- During the initial virologic breakthrough stage, Nuc^R variants were detected at a high percentage in serum DNA, but not in pgRNA. However, Nuc^R variants were also found at a high percentage in serum pgRNA pools during viral rebound
- The change of pgRNA composition indicates rapid establishment of new species of Nuc^R cccDNA and decay of pre-existing WT cccDNA
- Clonal linkage studies suggested that additional changes (L91I and A222T) were linked to M204I (data not shown), indicating that compensatory changes likely enhance viral replicative fitness and impact the rate of Nuc^R emergence
- ALT levels fluctuated during treatment in some patients, however, they did not appear to impact the composition or rate of pgRNA turnover

cccDNA populations appear to turnover in weeks-months rather than years!

SUMMARY



Genetic source of resistance shown to be cccDNA

Turnover of cccDNA from sensitive to resistant and from resistant to sensitive occurs in 3 - 5 months, suggesting relatively rapid turnover of both pgRNA and cccDNA pools of infected cells

No evidence of an inactive subpopulation of cccDNA, as resistance mutations were observed in the entire cccDNA population

CONCLUSIONS

- Direct comparison of HBV DNA, RNA and cccDNA in nine patients established that serum pgRNA composition accurately reflects the intrahepatic RNA and cccDNA pools, allowing cccDNA biosynthesis to be monitored by pgRNA composition and turnover
- Biopsy results from two clinical studies provide little evidence for existence of significant pools of inactive cccDNA (complete turnover of population sequences), or that Nuc^R pgRNA are generated by only a subpopulation of active cccDNA molecules
- All five IFN non-responders in Study ML18376 showed reversion of serum HBV DNA and pgRNA populations from Nuc^R to WT in as few as 12 weeks when Nuc selection pressure was withdrawn
- In six HBV patients with virologic breakthrough on TBV therapy, pgRNA and cccDNA sequencing demonstrated rapid establishment of newly formed cccDNA molecules harboring Nuc^R mutations
- Turnover of WT pgRNA molecules within a few months suggests that existing cccDNA may decay faster than previously predicted
- The data generated in this analysis support the proposed "alternative" model of cccDNA biogenesis
- This study suggests that cccDNA has a limited half-life, indicating that therapies which fully inhibit establishment of new cccDNA may lead to higher rates of cure for patients with CHB

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