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Rapid Turnover of cccDNA in Chronic Hepatitis B Patients Who Have Failed Nucleoside Treatment Due to Emerging Resistance

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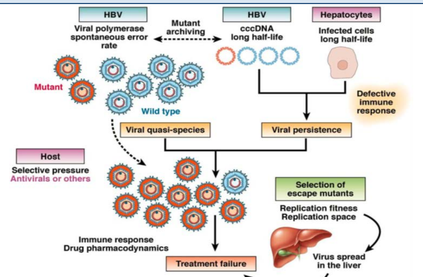
Background

Chronic Hepatitis B (CHB) infection relies on the stability and functionality of the viral covalently closed circular DNA (cccDNA). The failure of current standard of care therapy is believed to be due to an inability to eliminate cccDNA pools. It remains unclear whether cccDNA persistence is due to a long half-life or efficient replenishment by either *de novo* infection or intracellular amplification[1,2]. If cccDNA has a limited half-life, then therapy blocking future cccDNA establishment may lead to a clinical cure.

We monitored the emergence and disappearance of nucleos(t)ide resistance (Nuc^R) mutations as genetic markers of cccDNA turnover and evolution in patients failing Nuc treatment in two clinical studies. This ongoing cccDNA study provides initial results that address the following key questions:

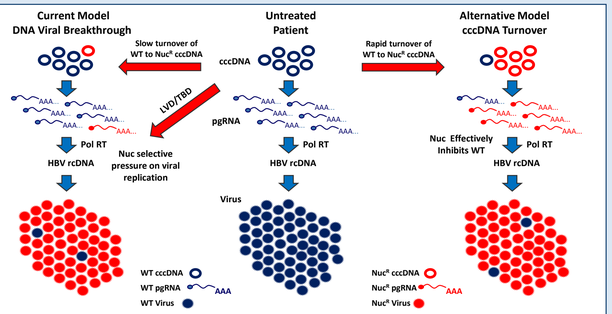
- Do serum HBV DNA sequences accurately reflect serum pgRNA and nuclear cccDNA pools?
- What is the rate of cccDNA wild-type (WT) to mutant turnover in CHB patients experiencing viral breakthrough on Nuc therapy?
- What are the kinetics of Nuc^R cccDNA to wild-type reversion after ending Nuc treatment?
- Do cccDNA pools consist of both active and inactive populations?

Current Model of cccDNA Biogenesis



- The current model[2] assumes that cccDNA pools have a very long half-life (~14 yr) 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

Alternative Model of cccDNA Biogenesis



- Longitudinal DNA, pgRNA and cccDNA samples from serum and biopsies obtained from patients on LVD or TBD therapy were sequenced to better understand the emergence and disappearance of Nuc^R signature mutations

References

1. Zoulim, F. et al. Hepatitis B virus resistance to nucleos(t)ide analogues. Gastroenterology. (2009)
2. Werle-Lapostolle, B. et al. Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. Gastroenterology. (2004)
3. Sun, J. et al. The 104-week efficacy and safety of Telbivudine-based optimization strategy in chronic hepatitis B patients: A randomized control study. Hepatology. (2014)
4. Zhou, B. et al. Composition and interactions of hepatitis B virus quasispecies defined the virological response during Telbivudine therapy. Scientific Reports. (2015)
5. Sun, J. et al. Randomised clinical trial: Efficacy of peginterferon alfa-2a in HBeAg positive chronic hepatitis B patients with Lamivudine resistance. Alimentary Pharmacology & Therapeutics. (2011)
6. Bhogal, R. et al. Isolation of primary human hepatocytes from normal and diseased liver tissue: A one hundred liver experience. Plos ONE. (2011)
7. Cai, D. et al. A southern blot assay for detection of hepatitis B virus covalently closed circular DNA from cell cultures. Methods in Molecular Biology. (2013)
8. Lai, C. et al. Reduction of covalently closed circular DNA with long-term nucleos(t)ide analogue treatment in chronic hepatitis B. Journal of Hepatology. (2017)

Materials and Methods

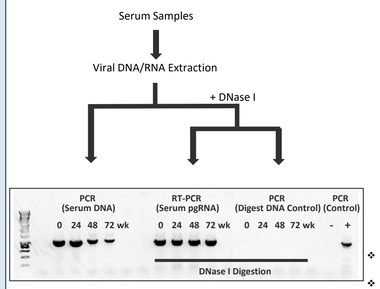
Patients Samples: Stored serum and liver biopsy samples collected from CHB patients who experienced virologic breakthrough on lamivudine (LVD) or telbivudine (TBD) therapy in ML18376[5] and EFFORT[3,4] clinical studies were evaluated.

HBV DNA and pgRNA PCR/RT-PCR Assays: HBV DNA and pgRNA were extracted from patient sera 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 calculated by population sequencing or clonal sequencing following TA-cloning.

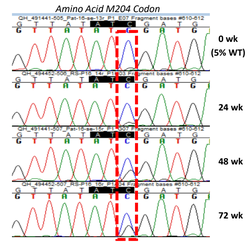
Sequencing of Intrahepatic HBV DNA, RNA and cccDNA: Protein-free relaxed circular DNA (rcDNA), HBV RNA and cccDNA from snap-frozen liver biopsies were extracted by a modified Hirt method[7,8], digested with T5 exonuclease, amplified by PCR or RT-PCR and analyzed by population sequencing. Spike-in experiments were used to confirm that purified cccDNA was free of rcDNA.

Detection of Nuc^R Variants in Patient Samples

Purification and Amplification of Serum HBV DNA and pgRNA



Detection of Nuc^R Variant in Patient 16 Serum pgRNA by Population Sequencing

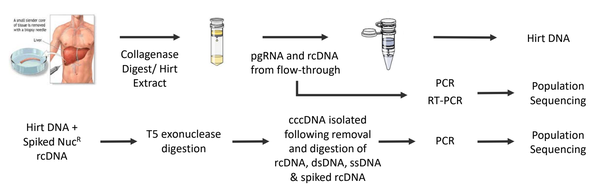


- Population sequencing able to detect codon variants as low as 5% by calculating peak ratios (5% WT at 0 wk)
- Results confirmed by clonal analysis (3% WT at 0 wk, data not shown)

- Nucleic acid extracted from patient serum samples was digested with DNase I and analyzed by RT-PCR and PCR
- Only HBV serum pgRNA was amplified by RT-PCR, confirming that the ability to distinguish between viral DNA and RNA species

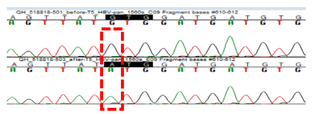
cccDNA, rcDNA and RNA from Liver Biopsies

Co-Purification and Amplification of Liver HBV DNA, pgRNA and cccDNA



Hirt Extracted cccDNA from Liver Biopsy Free of Spiked Nuc^R rcDNA

M204 Amino Acid Codon



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

After T5 digestion, Nuc^R rcDNA not detected

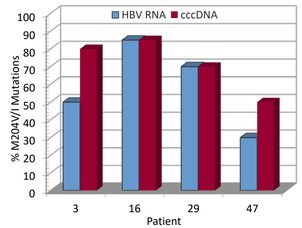
- CHB patient liver biopsy samples were digested with collagenase and nuclear Hirt DNA was extracted and cccDNA isolated after further digestion with T5 exonuclease
- Spike in experiments (with Nuc^R M204V rcDNA) were performed to ensure that cccDNA was free of rcDNA contamination (shown above)
- This methodology allowed us to closely monitor emergence of Nuc^R variants in cccDNA

Intra-Hepatic RNA Reflects cccDNA Population

Study ML18376

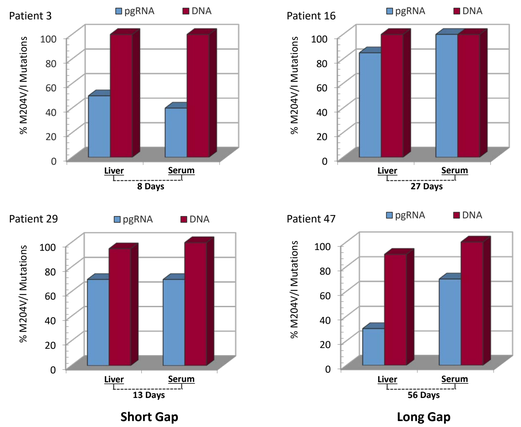
ADV Switch Arm		
Patient ID	Biological Metric	Baseline Serum
3	Alt	131
	Viral titer (log_{10})	5.0
	DNA	100% Nuc^R
	pgRNA	60% Nuc^R
16	Alt	51
	Viral titer (log_{10})	6.9
	DNA	100% Nuc^R
	pgRNA	95% Nuc^R
29	Alt	42
	Viral titer (log_{10})	6.0
	DNA	100% Nuc^R
	pgRNA	70% Nuc^R
47	Alt	54
	Viral titer (log_{10})	7.4
	DNA	100% Nuc^R
	pgRNA	95% Nuc^R

Baseline M204V/I Substitutions in Liver Biopsies



- Baseline serum pgRNA from Patients 3 and 29 is <100% Nuc^R, indicating cccDNA turnover lagged behind viral DNA breakthrough
- Population sequencing of cccDNA and HBV RNA found no significant subpopulation of inactive cccDNA molecules in liver biopsy samples
- Clonal sequencing of both serum HBV DNA and pgRNA (Patients 3 and 16, 24–120 clones of each sample) highly correlated with and confirmed population sequencing (data not shown)

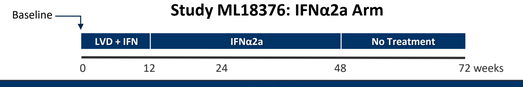
HBV RNA Population Continues to Evolve Under Selective Pressure



- Study ML18376 biopsy results found intrahepatic HBV RNA and serum pgRNA to be a better correlate of cccDNA than intrahepatic and serum HBV DNA
- Level of Nuc^R in cccDNA is somewhat higher than in intrahepatic HBV RNA; likely due to RNaseH degradation of Nuc^R pgRNA under Nuc selective pressure
- Patient 47 showed serum pgRNA continued to undergo significant evolution, suggesting that cccDNA was rapidly changing over a period of 56 days
- The strong correlation between sequence results obtained from cccDNA and HBV RNA in biopsy samples suggests an absence of any significant pool of inactivated WT cccDNA during pgRNA turnover
- The dynamic kinetics of WT cccDNA replacement by Nuc^R cccDNA will likely be impacted by drug potency and the replicative fitness of resistant variants, aspects which are currently under investigation

Turnover of cccDNA Populations

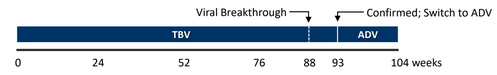
Study ML18376: IFNα2a Arm



IFNα2a Arm					
Patient ID	Biological Measure	0	24	48	72
13	Alt	74	51	55	34
	Viral titer (log_{10})	8.1	6.2	7.2	8.0
	DNA	100% Nuc^R	85% Nuc^R	WT	WT
	pgRNA	100% Nuc^R	NA	<5% Nuc^R	WT
20	Alt	37	37	56	43
	Viral titer (log_{10})	8.1	5.7	7.4	8.7
	DNA	100% Nuc^R	90% Nuc^R	WT	WT
	pgRNA	100% Nuc^R	NA	<5% Nuc^R	NA
50	Alt	72	13	50	193
	Viral titer (log_{10})	8.7	5.77	6.7	7.7
	DNA	100% Nuc^R	WT	WT	WT
	pgRNA	100% Nuc^R	WT	WT	WT
53	Alt	72	18	81	28
	Viral titer (log_{10})	7.0	6.9	7.4	5.9
	DNA	100% Nuc^R	100% Nuc^R	5% Nuc^R	WT
	pgRNA	100% Nuc^R	100% Nuc^R	50% Nuc^R	WT
194	Alt	18	44	49	47
	Viral titer (log_{10})	7.3	7.2	6.1	8.1
	DNA	100% Nuc^R	100% Nuc^R	40% Nuc^R	WT
	pgRNA	100% Nuc^R	100% Nuc^R	23% Nuc^R	WT

- LVD-treated patients experiencing viral breakthrough harbored a high percentage of Nuc^R mutations in their serum DNA and pgRNA populations at Baseline
- LVD resistant patients who switched to IFN showed reversion back to WT M204 in both their serum DNA and pgRNA populations in 12–60 weeks
- The kinetics of Nuc^R pgRNA (cccDNA surrogate marker) replacement by WT is likely impacted by the replicative fitness of residual (low level) WT virus, which was not detected by population sequencing

Study EFFORT: Mono Group Arm



Patient ID	TBV Treatment Week	0	24	52	76	88	93
10	Alt	194	15	19	13	13	NA
	Viral titer (log_{10})	8.6	5.3	4.4	4.9	7.0	7.6
	DNA	WT	WT	WT	WT	95% Nuc^R	100% Nuc^R
	pgRNA	WT	WT	WT	WT	45% Nuc^R	95% Nuc^R

- Nuc^R variants emerged rapidly (Week 76 to 93) in both serum DNA and pgRNA, with the vast majority of pgRNA molecules converted from WT to resistant genotypes within 17 weeks
- Clonal linkage studies suggested that two additional substitutions (L91I and A222T) were linked to M204I (data not shown), indicating that compensatory changes may enhance viral replicative fitness and impact the rate of emergence of Nuc^R variants

Conclusions

- Data suggests that pgRNA composition accurately reflects the cccDNA population
- Results provide little evidence for existence of significant pools of inactive cccDNA or that Nuc^R pgRNA are generated by only a subpopulation of active cccDNA molecules
- Serum HBV DNA and pgRNA populations can revert from Nuc^R to WT in as few as 12 weeks
- In HBV patients with breakthrough on Nuc therapy, pgRNA and cccDNA sequencing demonstrated rapid establishment of newly formed cccDNA molecules harboring Nuc^R mutations
- Turnover of WT pgRNA molecules within 17 weeks suggests that existing cccDNA may decay faster than previously predicted
- The initial data generated in this analysis support the proposed alternative model of cccDNA biogenesis
- This study suggests that cccDNA has a limited half-life, leading to the belief that therapies inhibiting establishment of new cccDNA may lead to a higher cure rate