Absence of mutations in the HBsAg “a” determinant during REP 2139 therapy validates serum HBsAg reductions observed in the REP 102 protocol

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INTRODUCTION

Infection with hepatitis B virus (HBV) may lead to acute or chronic hepatitis. Approximately 5% of infected adults develop a chronic HBV infection that may progress to liver cirrhosis or hepatocellular carcinoma.

Globally, 240 million people are chronically infected with HBV, and approximately 1 million people die annually from HBV-related diseases

Nucleic acid polymers (NAPs) clear HBsAg from the blood by blocking its release from infected hepatocytes. In the REP 102 protocol (NCT02646189) monotherapy of patients with chronic Hepatitis B with the NAP REP 2139 achieved 2–7 log reductions of serum HBsAg accompanied by 3–9 log reductions in serum HBV DNA and the appearance of anti-HBs (Al-Mahtab et al., 2016 PLOS one).

HBsAg is the primary diagnostic target of serological diagnostic in HBV infection. The detection of HBsAg depends on the “a” determinant region (124 - 147aa).

Mutations within this region may impair detection of HBsAg and allow HBV to escape vaccine induced immunity or passive immunoglobulin therapy

AIMS

1. Sequence analysis of a 376 bp fragment of HBsAg was performed to determine haplotypes in patients at different phases of REP 2139 therapy in the REP 102 protocol to better understand its outcome.
2. Investigate possible sequence differences among the haplotypes between the responder and non-responder patients, which could help understand mechanism of the drug action.
3. Investigate to what extent the accumulation of mutations in HBsAg region may be responsible for speed of response to NAPs.
4. Explore and identify the mutations within the “a”- determinant region can result in reduced detection of HBsAg by standard diagnostic assays.

METHODS

- Serum samples of 12 patients obtained throughout therapy and during follow-up were used for analysis. Sample were selected up to the lowest possible HBsAg result (highlighted in green in Table 1) containing sufficient HBV DNA for sequencing analysis (see Table 1.)
- PCR products for direct and deep sequencing were prepared by single or semi nested PCR of a HBsAg fragment of HBV DNA.
- Deep sequencing targeted the major hydrophilic region (MHR) (including the “a” determinant) of HBsAg.
- Direct sequencing was performed by Supremerun® and analyzed with Geneious® software.
- NGS analysis was performed on Illumina® data from all 12 REP 102 patients.
- The NGS pipeline involved read filtering (Cutadapt), assembly (BWA), variant calling (GATK) and haplotype reconstruction (QuasiRecomb) to detect variants within the samples.

RESULTS

- Of 12 patients treated (1 gtA, 4 gtD, 7 gtC), 9 responders (with HBsAg reduction) and 3 non responders (1 log or less HBsAg reduction) were identified (Al-Mahtab et al., 2016, see table 1)
- No mutations evolved in the “a” determinant region during REP 2139 therapy in all 12 patients (Figure 1, Figure 3). Haplotypes either decreased or remained unchanged during treatment (Figure 2).
- Among the 3 non-responders, a total of 4 mutations were observed occurring outside the “a” determinant region include G76C, L88P, V118G and P120T (Figure 3).
- In the 9 responder patients, 24 different mutated positions were observed occurring outside the “a” determinant region which include G76C, F85C, L88P, L98V, D99G, Q101R, Q101K, M103I, V106F, L109P, L109Q, G112R, T115N, V118G, G119R, P120T, R122K, P153T, A159V, F161Y, V168A, F170S, Q181R V184A, P188L, P188H, and T189I (Figure 3).
- Within the “a”-determinant, mutations were observed in some haplotypes I126S (Patient 3) and I126T (Patient 12) with frequencies of 18% and 13% respectively, which disappeared as REP 2139 therapy continued (Figure 3).
- Additionally G130R (Patient 8), G145R (Patient 6) and D144E (Patient 9) mutations were reported with a very low frequencies (3.4%, 6% and 2.89% respectively), which also disappeared as REP 2139 therapy continued (Figure 3).

Table 1. Sampling points used for sequencing analysis.

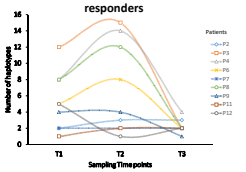
Responder?	Patient (GT)	Sampling point	Treatment timeline	HBV DNA (cpm)	HBsAg (IU / mL)
NO	Patient 1 (C)	1	Baseline	9.98E+08	1.68E+05
		2	27 weeks REP 2139	7.18E+08	2.39E+04
		3	40 weeks REP 2139	5.90E+08	3.19E+04
	Patient 5 (D)	1	2 weeks REP 2139*	3.15E+07	7.25E+03
		2	22 weeks REP 2139	5.47E+05	8.25E+03
		3	34 weeks REP 2139	1.11E+07	8.07E+03
	Patient 10 (C)	1	Baseline	9.88E+08	> 1.25E+05
		2	11 weeks REP 2139	9.89E+08	> 1.25E+05
		3	24 weeks REP 2139	9.88E+08	> 1.25E+05
YES	Patient 2 (D)	1	Baseline	6.23E+08	5.23E+04
		2	20 weeks REP 2139	1.05E+03	4.60E-01
		3	29 weeks REP 2139 12 weeks REP 2139 / Thymosin alpha 1	2.48E+03	3.00E-02
	Patient 3 (C)	1	Baseline	1.01E+08	1.30E+04
		2	6 weeks REP 2139	1.04E+07	1.44E+03
		3	17 weeks REP 2139	2.18E+04	9.03E-02
	Patient 4 (A)	1	Baseline	1.18E+08	3.61E+03
		2	27 weeks REP 2139	8.71E+03	2.59E+00
		3	37 weeks REP 2139 13 weeks REP 2139 / Thymosin alpha 1 6 weeks REP 2139 / peg IFN alpha	3.75E+03	1.90E+01
	Patient 6 (C)	1	Baseline***	1.29E+08	6.78E+04
		2	12 weeks REP 2139	6.96E+05	3.13E+03
		3	20 weeks REP 2139	1.18E+03	6.68E-01
	Patient 7 (C)	1	7 weeks REP 2139*	9.89E+08	1.68E+06
		2	11 weeks REP 2139 6 weeks REP 2139 / thymosin alpha 1****	5.74E+06	2.50E+02
		3	Followup: 18 weeks no therapy	4.57E+03	1.76E+00
	Patient 8 (D)	1	Baseline	9.89E+08	9.99E+05
		2	11 weeks REP 2139	8.38E+04	4.23E+04
		3	20 weeks REP 2139	2.75E+03	2.01E+03
		4	28 weeks REP 2139 7 weeks REP 2139 / peg IFN alpha	8.37E+03	3.00E-01
	Patient 9 (D)	1	2 Weeks REP 2139*	7.25E+05	2.29E+04
		2	16 weeks REP 2139	1.28E+04	9.63E+00
		3	24 weeks REP 2139 24 weeks REP 2139 / peg IFN alpha	2.80E+03	1.33E+00
	Patient 11 (C)	1	Baseline	9.89E+08	8.57E+06
		2	6 weeks REP 2139	3.55E+07	1.60E+02
		3	13 weeks REP 2139	~1.00E+04	3.00E-02
	Patient 12 (C)	1	Baseline**	9.09E+05	1.29E+03
		2	20 weeks of REP 2139 + 12 weeks of REP 2139 / peg IFN	~1.00E+03	3.00E-02
		3	Followup: 63 weeks no therapy	2.15E+03	7.26E+00

* No baseline serum sample available.
** TND: target not detected (reported result 0.00 IU / mL)
*** Different baseline sample was used than published in Al-Mahtab et al., 2016.
**** REP 2139 therapy had been withheld in this patient due to unrelated infectious gastroenteritis for 2 weeks at this sampling point.

Table 2. Common MHR mutations shared across the responders

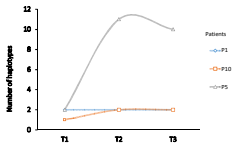
Amino acid position	Amino acid substitution	Responder Patients
76	G->C	2, 5, 6, 7, 8, 9, 11, 12
	P->L	3
109	L->Q	4
	L->Q	8
115	N->T	3
	T->N	8
126	I->S	3
	I->T	12
148	P->L	9

Figure 2 (A): MHR haplotype complexity during treatment in responders



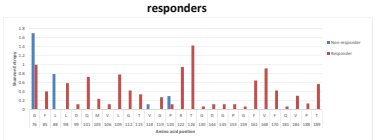
The number of haplotypes for 4 / 9 responder patients increased in the middle of the treatment, followed by asharp decline at end of the treatment. Haplotypes in the remaining 5/9 responder patients did not undergo any clear changes in abundance.

Figure 2 (B): MHR haplotype complexity in non-responders



The number of haplotypes remain stationary throughout the treatment for two of the non-responders, However, one non-responder (Patient 5) shows a dramatic increase following slight decline towards the end of the treatment.

Figure 1: MHR amino acid variability in responders and non-responders



Shannon entropy was calculated for both responder and non-responder haplotype sequences. A total of 28 different amino acid positions experienced changes in responders and non-responders.
25 amino acid substitutions occurred in responder patients while 4 substitutions were observed among the non-responders.
Among the changes, the most noted was C76G occurring in all responder and non-responder patients.
However, most of the amino acid substitutions were independent for each of the patients.

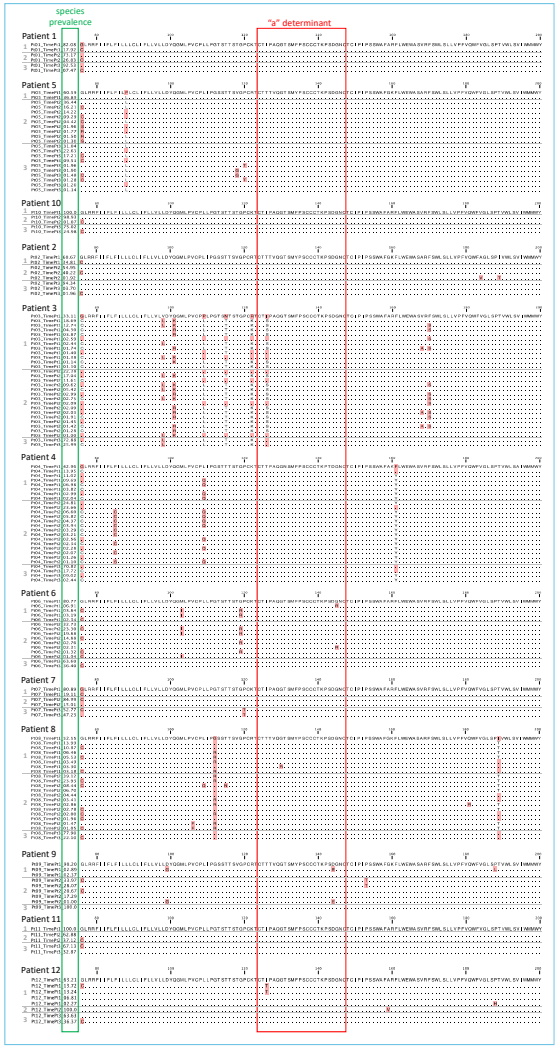


Figure 3. Deep sequencing analysis.
HBsAg quasispecies pevalance in the major hydrophilic region around the “a”-determinant during exposure to REP 2139 is presented. Quasispecies with > 1% prevalence are presented. The location of the “a”-determinant within the sequence is identified by the red box. Time points for quasispecies listings are identified on the left. Individual species prevalence is identified inside the green box.

CONCLUSIONS

- **Mutation of the “a”-determinant region of HBsAg or alteration in MHR quasispecies does not occur with REP 2139 therapy.**
- **Evolution of HBsAg variants escaping detection by HBsAg assays does not occur with chronic exposure to REP 2139.**
- **No relationship between haplotypes present within the MHR of HBsAg and response to REP 2139 therapy were observed.**
- **Treatment with REP 2139 does not appear to induce any selection pressure on the MHR.**
- **Circulating virus species persisting at later stages of REP 2139 treatment may not be recognized by an impaired host immune response.**

ACKNOWLEDGEMENTS

This work was supported by DAAD (German Academic Exchange Service), Replicor Inc. and TUM.

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DISCLOSURES

MB and AV are employees of and shareholders in Replicor Inc. MR is a member of the scientific advisory board of Replicor. The other authors have nothing to disclose.

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