**Hepatitis E virus mutations associated with ribavirin treatment failure result
in altered viral fitness and ribavirin sensitivity**

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List of abbreviations in order of appearance: HEV, hepatitis E virus; RBV, ribavirin; ALT, alanine aminotransferase; IU, international units; γ GT, γ -glutamyltransferase; CLL, chronic lymphoid leukemia; NGS, next-generation sequencing; RdRp, RNA-dependent RNA polymerase; HVR, hypervariable region; ORF, open reading frame; IMPDH, inosine 5'-monophosphate dehydrogenase; MPA, mycophenolic acid; LOD, limit of detection; RLU, relative light units; FBS, fetal bovine serum; HS, human serum; WT, wild-type; VC, virus control.

Keywords: hepatitis E virus; chronic hepatitis E; ribavirin; resistance; G1634R; fidelity; RNA-dependent RNA polymerase; hypervariable region.

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Abstract

Background & aims: Ribavirin monotherapy is the preferred treatment for chronic hepatitis E, although occasional treatment failure occurs. We present a patient with chronic hepatitis E experiencing ribavirin treatment failure with a completely resistant phenotype. We aimed to identify viral mutations associated with treatment failure and explore the underlying mechanisms.

Methods: Viral genomes were deep-sequenced at different time points and the role of identified mutations was assessed *in vitro* using mutant replicons, antiviral assays, cell culture of patient-derived virus and deep-sequencing.

Results: Ribavirin resistance was associated with Y1320H, K1383N and G1634R mutations in the viral polymerase, but also an insertion in the hypervariable region comprising a duplication and a polymerase-derived fragment. Analysis of these genome alterations *in vitro* revealed replication-increasing roles for Y1320H and G1634R mutations and the hypervariable region insertion. In contrast, the K1383N mutation in the polymerase F1-motif suppressed viral replication and increased the *in vitro* sensitivity to ribavirin, contrary to the clinical phenotype. Analysis of the replication of mutant full-length virus and *in vitro* culturing of patient-derived virus confirmed that sensitivity to ribavirin was retained. Finally, deep-sequencing of hepatitis E virus genomes revealed that ribavirin is mutagenic to viral replication *in vitro* and *in vivo*.

Conclusions: Mutations Y1320H, G1634R and the hypervariable region insertion compensated for K1383N-associated replication defects. The specific role of the K1383N mutation remains enigmatic, but it appears to be of importance for the ribavirin resistant phenotype in this patient.

Abstract word count: 236

Lay summary: Ribavirin is the most common treatment for chronic hepatitis E and is mostly effective, although some cases of ribavirin treatment failure have been described. Here, we report on a particular case of ribavirin resistance and investigate the underlying causes of treatment failure. Mutations in the viral polymerase, an essential enzyme for viral replication, appear to be responsible.

Introduction

Chronic hepatitis E is a persistent infection of the liver by the hepatitis E virus (HEV).[1,2] Although HEV infections are often asymptomatic and resolve spontaneously in healthy people, infections may evolve to chronicity in immunocompromised patients such as solid-organ transplant recipients, HIV-infected individuals and patients with hematological malignancies undergoing chemotherapy.[3] Chronic hepatitis E is now recognized as a major complication in the transplant setting, where an increasing number of cases are reported.[4]

Treatment options for chronic hepatitis E are limited. Reducing immunosuppression is the first intervention for transplant recipients and results in viral clearance in 30% of patients.[5] The other established options are pegylated interferon alpha or ribavirin (RBV).[6,7] Interferon cannot be used in most transplant patients due to the risk for acute rejection, making RBV the drug of choice in most cases. RBV is usually administered as a monotherapy for at least 3 months and its efficacy against acute and chronic hepatitis E has been confirmed.[7,8] However, RBV treatment results in several side effects, most prominently anemia, which may necessitate dose reductions and even blood transfusions.[7] RBV treatment failure has been reported, often linked to dose reductions.[8,9] We recently analyzed the viral genomes in two such cases and identified the G1634R mutation associated with treatment failure.[9]

Recent advances in HEV cell culturing, such as the isolation of the cultivable Kernow-C1 p6 strain and several HEV replicons,[10,11] have allowed to explore the molecular virology of HEV and assess its *in vitro* susceptibility to antiviral drugs (e.g. [12,13]). These systems also enabled us to study the mechanism of G1634R in RBV treatment failure. This mutation does not alter RBV susceptibility *in vitro*, but increases viral replication which may explain the apparent clinical resistance to RBV.[9]

Here we describe a particularly robust case of seemingly RBV-resistant chronic hepatitis E. The onset of RBV failure was associated with multiple mutations and insertions in the viral genome. The role of these alterations was explored using the currently available *in vitro* tools.

Materials and methods

See supplementary file.

Results

Patient

A 68-year-old man was admitted to a hepatology unit for acute hepatic cytolysis with alanine aminotransferase (ALT) levels of 1681 international units (IU)/mL and γ -glutamyltransferase (γ GT) levels of 379 IU/mL (figure 1A). Medical history was marked by grade C (Binet classification) chronic lymphoid leukemia (CLL) for which he had received several lines of treatment. At the time of admission, he was treated with a Bruton's tyrosine kinase inhibitor in the context of a clinical trial, allowing control of the hematological disease. Specific investigations revealed acute hepatitis E, based on detection of HEV RNA (genotype 3f) in plasma. At the time of diagnosis, the viral load was $5.52 \log_{10}$ copies/mL. HEV serology was negative for IgM and IgG because of hypogammaglobulinemia secondary to CLL. The patient was negative for other viruses and there was no evidence of associated vascular, metabolic or autoimmune diseases. Introduction of RBV (800 mg per day) was decided to control viral replication. The evolution was initially favorable and HEV RNA undetectability was obtained after 10 weeks. RBV was stopped after 1 month of undetectability. Eight weeks after RBV cessation, the patient presented a second episode of transaminase flare (ALT = 395 IU/mL) and relapse was diagnosed with $6.6 \log_{10}$ copies/mL HEV RNA in the serum. RBV was re-introduced but HEV undetectability could not be achieved: viral loads remained $>6 \log_{10}$ copies/mL during the first 6 months following RBV re-introduction. RBV dosage was progressively increased to 1200 mg per day with pharmacological assessments showing correct plasma exposure (residual concentrations >2 mg/L). To date, 58 weeks after RBV re-introduction, HEV could not be eradicated despite a modest decrease in viral load ($4.83 \log_{10}$ copies/mL), a weak improvement of liver function (ALT = 189 IU/mL) and the CLL-treatment showing efficacy (lymphocyte count 5.43 G/L versus >300 G/L one year before).

Viral genome sequencing

To identify mutations associated with RBV failure, the HEV genome was sequenced by shotgun next-generation sequencing (NGS) at different time points: before RBV introduction (HEV_{preRBVres}, day 0), at the time of relapse (HEV_{relapse}, day 197) and during RBV reintroduction (HEV_{postRBVres}, day 241). Comparison of HEV_{preRBVres} and HEV_{postRBVres} revealed 3 substitutions in the RNA-dependent RNA polymerase (RdRp), resulting in Y1320H, K1383N and G1634R (figure 1A-C). These substitutions were nearly undetectable in HEV_{preRBVres} (<0.5%), detected at low levels in HEV_{relapse} (1.8%, 2.9% and 6.5%, respectively), but were highly predominant in HEV_{postRBVres} (95.0%, 99.5% and 95.3%, respectively). Sanger sequencing confirmed the continued presence of these mutations at day 600 (data not shown). We previously found G1634R in RBV treatment failure.[9] The Y1320H mutation is located in the N-terminus of the presumed HEV RdRp,[14] whereas K1383 is part of the F1-motif and is highly conserved among positive-sense RNA viruses (figure 1C, supplementary figure 1).[15]

An additional mutation was found in the hypervariable region (HVR), resulting in A723V. This substitution was present in 99% of aligned reads in HEV_{postRBVres} and could already be detected in HEV_{preRBVres} and HEV_{relapse} at frequencies of 17% and 19.2%, respectively. Furthermore, a large in-frame 282bp-insertion in the HVR was found in HEV_{postRBVres}, consisting of a duplicated fragment of 258bp from the HVR and a 24bp RdRp-derived fragment (figure 1B, supplementary figure 2). The recombination event had already occurred before the first introduction of RBV but mutants bearing the insert represented only a minority at that time (supplementary figure 3). During the second RBV treatment, only the insert-containing HVR was detected.

Finally, alignment of consensus sequences HEV_{preRBVres} and HEV_{relapse} revealed the occurrence of another substitution at position 647 in the HVR in HEV_{relapse}: A647T (figure 1B). However, this mutation was not withheld during the second RBV treatment (reversion to A in HEV_{postRBVres}). No changes in the sequences of ORF2 and ORF3 were observed.

Impact of RdRp mutations on *in vitro* p6/luc replication and RBV sensitivity

To assess the role of the RdRp mutations, the Y1320H, K1383N and G1634R mutations were introduced into the genotype 3 p6/luc replicon. The replication capacity and RBV sensitivity of all combinations were assessed in Huh7 cells. As reported before,[9] 1634R resulted in significantly increased viral replication (1.5-fold, supplementary figure 4A). The same applied for Y1320H (1.7-fold) and when both mutations were combined, an additive effect on the increase in viral replication (2.7-fold) was noted. Conversely, the K1383N mutation reduced luminescence to levels comparable to mock-transfection, abrogating viral replication. Combining K1383N with Y1320H and G1634R rescued viral replication to some extent (4.7-fold increase versus K1383N alone, $p=0.006$), but luminescence still only reached 23% of wild-type signal. Next, RBV sensitivity was assessed (supplementary figures 4B-C). Y1320H and G1634R did not alter RBV susceptibility. Surprisingly, K1383N-bearing constructs had a significantly increased RBV sensitivity with ± 2 -fold lower EC_{50} -values. Although these results contradict the clinical findings, they indicate that the K1383N mutation may play an important role in the antiviral effect of RBV.

Impact of RdRp mutations on chimeric p6/RdRp_{preRBVres}/luc

The clinical isolates and p6/luc replicon are both genotype 3, but still differ to some extent: 19 amino acid differences (4%) were observed between RdRp sequences when comparing the Kernow-C1 p6 with the clinical isolate HEV_{preRBVres}. To test whether these differences affect the impact of the mutations, the patient-derived HEV_{preRBVres} RdRp sequence was swapped into p6/luc, generating p6/RdRp_{preRBVres}/luc. This chimeric replicon had a comparable RBV sensitivity as p6/luc (figure 2A), but replicated significantly better (figure 2B). Next, Y1320H, K1383N and G1634R were introduced into p6/RdRp_{preRBVres}/luc. The Y1320H and G1634R mutations increased replication 1.7- and 2.0-fold respectively and 2.8-fold when combined (figure 2C), in line with p6/luc results (supplementary figure 4A). Similarly, K1383N virtually abrogated viral replication which could be partially restored by combining all three mutations (up to 12% of wild-type, i.e. a 5.4-fold increase over K1383N single

mutant). The antiviral data followed the same pattern as observed for p6/luc with K1383N increasing RBV sensitivity and 1320H and 1634R not affecting antiviral susceptibility (figure 2D).

Next, we sought to confirm these results in an alternative cell line. Analysis of the mutants in HepG2/C3A cells yielded similar results as in Huh7 cells, although differences were more pronounced. The Y1320H and G1634R mutations increased replication 8- and 10-fold respectively and 15-fold when combined (figure 2E). Combining both mutations with K1383N rescued viral replication to 61% of wild-type. The K1383N mutation also increased RBV sensitivity in HepG2/C3A cells, but to a much larger extent, with ± 50 -fold shifts in EC_{50} -values (figure 2F).

K1383N decreases GTP usage efficiency by the RdRp

We previously established that the *in vitro* mechanism of action of RBV against HEV depends on the depletion of intracellular GTP pools through inhibition of inosine 5'-monophosphate dehydrogenase (IMPDH) and the antiviral effect of RBV can be reversed by adding guanosine to the culture medium, thus replenishing GTP pools.[12] In addition, the F1-motif (which encompasses residue K1383 for the HEV RdRp) has been implicated in GTP binding for Japanese encephalitis virus.[16] To test whether the K1383N mutation results in altered GTP use by the HEV polymerase, we assessed the susceptibility of wild-type and triple mutant p6/RdRp_{preRBVres}/luc to mycophenolic acid (MPA), a specific IMPDH inhibitor, in Huh7 cells.[17] The mutant replicon showed increased sensitivity to MPA with a significantly reduced EC_{50} -value (0.41 vs 0.26 μ M for wild-type and mutant respectively, $p=0.02$, figure 2G), which indeed suggests less efficient GTP usage by the K1383N-containing RdRp. To corroborate this finding, the guanosine concentration required to salvage viral replication was determined. Replicon-transfected Huh7 cells were treated with a fixed RBV concentration and different concentrations of guanosine; the fold-induction in luminescence signal over control cells treated with RBV without guanosine is depicted in figure 2H. Guanosine was significantly more effective in rescuing the replication of the wild-type compared to the triple mutant replicon; overall, a 2-fold higher concentration of guanosine was required for the mutant replicon to reach the same

fold induction as the wild-type. Taken together, these data suggest that the K1383N mutation leads to less efficient binding of GTP and consequently a decreased replication and increased sensitivity to the GTP-depleting activity of RBV.

***In vitro* culture of full-length p6 and patient-derived HEV**

Next, we introduced 1320H, 1383N and 1634R into the full-length Kernow-C1 p6 clone and compared viral replication in Huh7 cells in the presence or absence of RBV. Viral yields in the culture medium after 20 days are presented in figure 3A. Triple mutant p6 replicates considerably less well than its wild-type counterpart, but retained RBV sensitivity, confirming the results obtained with the replicon.

To rule out that the lack of RBV resistance is a mere artifact of the Kernow-C1 p6 strain which has been adapted to cell culture to some extent,[10] we attempted to culture HEV directly from several serum and plasma samples from this patient. We attempted infection of HepG2/C3A cells using fetal bovine serum-supplemented medium, but also tried to infect Huh7 cells in the presence of human serum, given its stimulatory effects on hepatitis C virus replication.[18] We were unable to detect robust shedding of progeny virus into the culture medium for both cell lines, confirming other reports that noted great difficulty in culturing wild-type HEV.[19,20] Nevertheless, quantifiable amounts of viral RNA were detected intracellularly in several cultures infected with samples taken after onset of treatment failure (figure 3B). Parallel cultures simultaneously infected with the same serum/plasma sample and treated with RBV at 25 μ M yielded approximately 10-fold lower viral RNA titers than their untreated counterparts. This indicates that even following treatment failure, the virus is still inherently sensitive to RBV.

The HVR insertion increases viral replication with a crucial role for the RdRp-derived fragment

RBV non-responsiveness in this patient was associated with a duplication, a point mutation (A723V), and the insertion of a short RdRp-derived fragment in the HVR (figure 1B). To assess the role of this specific HVR, we replaced the HVR in the p6/luc replicon with the patient-derived HVR obtained after

onset of RBV failure (HVR_{postRBVres}) (figure 4A). This removed the S17 insert,[10] which drastically decreased replication (figure 4B), as could be expected given the important role of the S17 insert in HEV cell culture.[10] To test whether the HVR_{postRBVres} affects fitness or RBV sensitivity of the RdRp_{postRBVres} (triple mutant), we constructed and studied the phenotype of p6/HVR+RdRp_{postRBVres}/luc. This double chimera combined the replication handicaps of p6/HVR_{postRBVres}/luc and triple mutant p6/RdRp_{postRBVres}/luc (figure 4B). Subsequently, the effect on RBV sensitivity was determined: p6/luc and p6/HVR_{postRBVres}/luc displayed a similar susceptibility to RBV with EC₅₀-values of 4.7±2.7 and 4.8±3.7 µM respectively (figure 4C), while p6/HVR+RdRp_{postRBVres}/luc retained the slightly increased RBV sensitivity of the triple mutant p6/RdRp_{postRBVres}/luc with EC₅₀-values of 2.5±0.5 and 1.5±0.6 µM respectively. These data argue against a specific interaction between HVR and RdRp in the context of RBV non-responsiveness and suggest that the HVR is not directly involved in the antiviral activity of RBV, as was anticipated. Next, we assessed the impact of the HVR alterations on viral fitness (figure 4D): p6/HVR_{postRBVres}/luc replicated significantly better than p6/HVR_{preRBVres}/luc with 1.5-fold and 2.7-fold increases in luminescence signal in Huh7 and HepG2/C3A cells respectively (figure 4E-F), suggesting that the HVR modifications may also be compensatory for the fitness loss associated with the K1383N mutation.

Next, to gain insight in the increased replication in p6/HVR_{postRBVres}/luc, we determined the individual contributions of the A723V mutation, the HVR duplication and the RdRp-derived insertion by constructing p6/HVR_{preRBVres}/luc with A723V, p6/HVR_{postRBVres}/luc without RdRp-derived insert and p6/HVR_{preRBVres}/luc with RdRp-derived insert downstream of position 792 (figure 4D). Whereas A723V did not influence viral replication, removal of the RdRp-derived 8aa-insert resulted in a strong reduction in viral replication, i.e. to only 45% and 31% of p6/HVR_{postRBVres}/luc replication in Huh7 and HepG2/C3A cells respectively (figure 4E-F), which highlights the importance of the RdRp-derived insertion in this case. Surprisingly, the addition of the RdRp-derived insert into p6/HVR_{preRBVres}/luc completely abrogated viral replication. Taken together, these data suggest that HVR-insertions may

in general be beneficial for HEV during chronic infection by increasing viral replication, but that specific requirements may apply regarding sequence, length and/or context of these insertions.

RBV is mutagenic to HEV replication *in vitro* and *in vivo*

Despite the deleterious effect of K1383N on HEV replication capacity, this virus population still retains the mutation and compensates replication defects with Y1320H, G1634R and HVR insertions. This suggests that the K1383N mutation is nonetheless advantageous during prolonged RBV treatment in another way than by increasing viral fitness or antiviral resistance. RBV acts as a RNA virus mutagen *in vitro*[21,22] and increased mutation rates have been observed in chronic hepatitis C patients treated with RBV,[23,24] whereas *in vitro* RBV resistance depends on mutations increasing RdRp fidelity.[25,26] The K1383 mutation is located in the F1-motif which is believed to bind the incoming nucleotide-triphosphate in the RdRp and may therefore be important for selecting the correct nucleotide.[15] In fact, the nearby F3-motif has been associated with fidelity[27] and a resistance mutation to T705, another putative antiviral mutagen,[28] was identified in the RdRp of T705-resistant Chikungunya virus at the exact position (K291R) that corresponds to HEV K1383N.[29] This led us to the hypothesis that RBV may be mutagenic to HEV during extended treatment and that K1383N may alter RdRp fidelity, possibly reducing RBV incorporation into the viral genome.

To test this hypothesis *in vitro*, deep-sequencing was performed on wild-type and triple mutant HEV p6, cultured with or without RBV for 20 days (figure 3A). No mutations were found with a quasispecies prevalence >5% when wild-type HEV was cultured without RBV, whereas 2.47 mutations per kb were found when wild-type HEV was cultured in the presence of 25 μ M RBV (figure 5A-B, supplementary table 1). Mainly G-to-A and C-to-U mutations were detected, which are the preferred mutations induced by RBV.[21] No known mutations (such as G1634R) were detected and no specific hot-spots for mutations were identified (figure 5B). Surprisingly, when the triple-mutant was cultured in the absence of RBV, 0.80 novel mutations per kb were identified, whereas Y1320H, K1383N and G1634R were not reversed (figure 5C). This increase in the number of mutations compared to wild-

1 type could indicate that RdRp fidelity is altered in the triple mutant, but this could also be a
2 consequence of cell-culture adaptation of the mutant construct. Unfortunately, replication of the
3 triple mutant in the presence of RBV was too limited to yield any reliable sequencing results, making
4 it impossible to assess the mutation rate under these conditions.
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10 Finally, deep-sequencing was performed on viral populations in patient sera at different time points
11 to explore a potential mutagenic effect of RBV on *in vivo* HEV replication. Comparison of the number
12 of mutations present in >0.5% of reads at days 4, 197, 263 and 686 post-diagnosis indicated a limited
13 number of mutations (63.0 mutations/kb) before the first RBV treatment (day 4) and at the time of
14 relapse (day 197, 37.3 mutations/kb) (figure 6A). In contrast, a large number of mutations (212.9
15 mutations/kb) were detected 66 days after the start of RBV retreatment (day 263). Most of these
16 mutations were either G-to-A or C-to-U mutations (90.5%, figures 6A-B), corroborating the
17 mutagenic effect of RBV on HEV replication observed *in vitro*. Finally, deep sequencing of a late
18 sample (day 686, still under RBV treatment) showed no change in the total number of mutations,
19 while the number of new mutations was strongly reduced (from 186.9 to 63.2 mutations/kb).
20 Moreover, the proportion of G-to-A and C-to-U mutations was reduced as well (56.1%). This suggests
21 that the mutations may indeed alter RdRp fidelity and reduce the number of RBV incorporation
22 events and consequently the number of mutations.
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Discussion

RBV treatment of chronic hepatitis E in immunosuppressed patients has shown efficiency in most cases. Nevertheless, treatment failure has been described.[8,9] We report on a robust case of RBV failure in a chronically HEV-infected patient: after a first successful RBV regimen, this patient relapsed and developed complete resistance during retreatment. Sequencing of viral genomes revealed three mutations in the viral RdRp and a HVR-insertion coinciding with onset of treatment failure. A summary of our findings is given in figure 7.

We recently described the G1634R mutation in two patients experiencing RBV treatment failure and proposed that increased viral replication contributes to RBV failure.[9] In these cases, no additional mutations were observed. In the case described here, we believe that G1634R has an adaptive role, compensating for the fitness loss resulting from K1383N.

Amino acid Y1320 is located at the N-terminus of the proposed RdRp sequence,[14] upstream of the F1-motif, but the function of this part of the RdRp is unknown. Contrary to the common occurrence of G1634R in circulating HEV strains,[9] Y1320 is highly conserved in human/swine HEV. Only two sequences with other residues at this position were found: N1320 in the swMN06-C1056 strain isolated from Mongolian pigs[30] (GenBank accession number BAF65208) and H1320 in the CNSH448-08 isolate from a Chinese patient[31] (AGE83345), the latter mutation was also found in the postRBVres sequence in the present case. Y1320H increased HEV replication without affecting RBV sensitivity and may also be a compensatory mutation for the K1383N-induced fitness loss.

The K1383N substitution is most intriguing since it strongly decreased viral replication and even increased *in vitro* RBV sensitivity, contrary to what would be expected based on the clinical phenotype. Position 1383 is located in the RdRp F1-motif and this lysine is highly conserved, also in other families of positive-sense RNA viruses (supplementary figure 1). The selection of K1383N at such a conserved position, despite a strong impact on viral fitness, and the persistence of this mutant during more than a year of retreatment argues for a crucial role in the observed resistance

phenotype. Based on its localization in the F1-motif, we hypothesized that K1383N may affect RdRp fidelity which could be beneficial in the setting of prolonged RBV treatment. RBV is known to be mutagenic to certain RNA viruses [21,22] and this was also the case for HEV replication *in vitro*. Unfortunately, the currently available tools did not allow studying the impact of the K1383N mutation on HEV RdRp fidelity: in the presence of RBV, viral replication was too limited to yield reliable deep-sequencing data. Improvements in HEV cell culture systems should allow overcoming this hurdle in the future.

Indeed, despite recent advances, HEV research remains challenging due to the poor *in vitro* replication, especially for HEV strains derived from patient material, as also observed in this study. The limited viral replication in culture also hampers the study of the effect of mutations or other interventions that decrease HEV replication to some extent, as is the case for the full-length triple mutant when treated with RBV. No robust enzymatic RdRp assay is available and attempts to set up a HEV polymerase assay using isolated crude replication complexes were not successful. Despite these limitations, we clarified the role of several mutations in the context of RBV treatment failure and provided clues on the possible underlying mechanisms. The replication-enhancing properties of Y1320H and G1634R, especially when combined, as well as the increased replication of the p6/RdRp_{preRBVres}/luc chimera may also be useful for other studies requiring robust HEV replication *in vitro*.

RBV resistance was also associated with an HVR insertion, consisting of HVR- and RdRp-derived fragments. Insertions of host RNA segments or duplicated viral sequences in the HVR have been described and seem relatively common in chronic hepatitis E.[32,33] The exact function of the HVR remains unknown, but insertions may be involved in virus adaptation to the host. Interestingly, similar insertions comprising HVR- and RdRp-derived sequences have been reported before.[33,34] In our patient, selection of this insert was probably not directly related to RBV treatment, but its replication-enhancing properties may have facilitated the emergence of the K1383N mutant,

1 together with Y1320H and G1634R. We tested several combinations of inserts and found that the
2 RdRp-derived insertion was crucial for increasing viral replication, but its mere insertion into
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4 HVR_{preRBVres} abrogated replication. This suggests that particular inserts require a specific context
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6 sequence to be beneficial for the virus.
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10 Surprisingly, viremia in the patient remained high whereas we observed a strong decrease in viral
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12 replication for the triple mutant constructs. Several hypotheses can be proposed to explain this
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14 discrepancy. First, the host genetic background and/or environmental factors could have contributed
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16 to the high viral titers in the patient. When comparing the effect of the different mutations in Huh7
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18 and HepG2/C3A cells, large differences in the impact of the mutations on viral replication (figures 2C
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20 and E) were observed. One could hypothesize that specific host factors may have allowed efficient
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22 replication of the mutant strain in this particular patient. This also suggests that host factors play an
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24 important role in the underlying mechanisms. Comparison of different cell lines may provide an
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26 attractive translational model to study differences between patients, e.g. susceptibility to chronic
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28 hepatitis E, response to RBV and the role of specific mutations. A second potential explanation can
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30 be found in the fact that p6/luc, and even the chimeric constructs, have a different background
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32 compared to the patient strain. Differences in non-coding regions or non-structural proteins may
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34 explain the different replication efficacies observed between both strains. Finally, the *in vitro*
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36 situation is not entirely comparable to the *in vivo* situation: although the virus replicates efficiently in
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38 patients with acute and chronic hepatitis, culturing HEV in cells is very challenging. The factors
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40 allowing efficient *in vivo* replication that are missing *in vitro* are still unknown, but they may have
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42 contributed to the high viral titers observed after onset of ribavirin resistance.
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51 The observed mutagenic effect of RBV, both *in vitro* and *in vivo*, has interesting implications for the
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53 proposed mechanism of action against HEV. Earlier, we found that RBV's anti-HEV effect was
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55 mediated by depletion of intracellular GTP.[12] These phenomena do not need to be mutually
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57 exclusive: by depleting intracellular GTP, RBV-triphosphates no longer need to compete with high
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1 concentrations of GTP and have a higher chance of being incorporated into the viral genome. The
2 relative importance of either mechanism *in vivo* is unclear.
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5 Relapse following a 3-month course of RBV occurs in a minority of chronically HEV-infected patients.
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7 However, subsequent retreatment for a prolonged period leads to viral clearance in most cases,
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9 suggesting that the emergence of complex mutants with complete RBV resistance only occurs in a
10
11 limited number of patients with specific characteristics. An interesting question is whether the kinase
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13 inhibitor used for CLL treatment contributed to the clinical phenotype and viral evolution. Therefore,
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15 a detailed investigation of the effect of different treatments for hematological malignancies on *in*
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17 *vitro* HEV replication and on the clinical course of chronic hepatitis E is warranted.
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23 RBV-resistant hepatitis E is particularly challenging from a clinical point-of-view, given the lack of
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25 other effective therapies. Assessing the role of mutations in other cases of RBV failure will be crucial
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27 to completely understand the underlying mechanisms and susceptibilities. The experimental use of
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29 sofosbuvir, which inhibits *in vitro* HEV replication, may be an alternative therapeutic option worth
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31 exploring in these patients.[35]
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Figure legends

Fig. 1. RBV treatment failure associated with Y1320H, K1383N and G1634R mutations and a HVR insertion. (A) Clinical course and NGS for a chronic hepatitis-E-patient experiencing RBV treatment failure. The frequency of mutations in the quasispecies at the indicated positions is represented by pie charts. * The extended HVR with duplication and RdRp-derived insertion was only detected as a minor variant at these time points (supplementary figure 2). (B-C) Alignment of sequences before (day 0) and after onset of RBV treatment failure (day 241) and during relapse (day 197). For the HVR (B), the duplicated region is underlined, point mutations are indicated in bold and with *, while the RdRp-derived insert is marked with a box. For the RdRp (C), point mutations are indicated with *, while the F1-motif and the origin of the RdRp-derived insert in the HVR are marked with boxes. Numbering is according to NCBI reference sequence NP_056779. ALT, alanine transaminase; γ GT, gamma-glutamyl transferase; HVR, hypervariable region; IU, international units; LOD, limit of detection for HEV RT-qPCR assay; RBV, ribavirin; RdRp, RNA-dependent RNA polymerase.

Fig. 2. Replication and antiviral sensitivity of chimeric p6/RdRp_{preRBVres}/luc and impact of Y1320H, K1383N and G1634R. Comparison of RBV sensitivity (A) and replication (B) of p6/luc and p6/RdRp_{preRBVres}/luc. Effect of mutations on p6/RdRp_{preRBVres}/luc replication in Huh7 (C) and HepG2/C3A (E), RBV sensitivity in Huh7 (D) and HepG2/C3A (F) and MPA sensitivity in Huh7 (G). (H) Fold-induction of p6/RdRp_{preRBVres}/luc replication under RBV treatment (30 μ M) by different concentrations of guanosine in Huh7 cells. MPA, mycophenolic acid; RBV, ribavirin; RLU, relative light units. Values represent mean $\pm$ standard deviation (SD) for 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 3. *In vitro* culture of full-length p6 and patient-derived HEV. (A) Huh7 cells transfected with full-length capped p6 RNA (wild-type or Y1320H-K1383N-G1634R triple mutant) were cultured with or

without RBV (25 μ M). RNA copies in culture medium at day 20 post transfection are depicted. Values represent mean $\pm$ SD for 3 independent experiments. (B) HepG2/C3A cells cultured with 10% FBS and Huh7 cells cultured with 2% human serum were infected with day 263 serum (one data point for Huh7) or day 318 plasma (both data points for HepG2/C3A and one for Huh7), intracellular viral RNA was quantified after 20 days. Values represent individual data points. FBS, fetal bovine serum; HS, human serum; RBV, ribavirin; WT, wild-type.

Fig. 4. Impact of the HVR and modifications on viral replication and RBV sensitivity. Chimeric p6/luc with HVR_{postRBVres} and/or RdRp_{postRBVres} were constructed (A) and their replication capacity (B) and RBV sensitivity (C) were assessed in Huh7 cells. The patient-derived HVR is depicted in green with a grey box for the RdRp-derived insertion; the patient-derived RdRp is depicted in grey with red lines representing the Y132H, K1383N and G1634R mutations. Comparison of the replication of p6/HVR_{preRBVres}/luc with and without A732V (red line), p6/HVR_{postRBVres}/luc, p6/HVR_{postRBVres}/luc without RdRp-derived insert (grey box) and p6/HVR_{preRBVres}/luc with RdRp-derived insert (D), in Huh7 (E) and HepG2/C3A (F) cells. RBV, ribavirin; RLU, relative light units. Values represent mean $\pm$ SD for 3 independent experiments. $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 5. RBV is mutagenic *in vitro* to wild-type HEV replication, whereas triple-mutant HEV accumulates mutations even in the absence of RBV. (A) The number of mutations per kb present above 5% in the quasispecies of HEV p6 wild-type or triple RdRp-mutant cultured with or without RBV (25 μ M) was determined by deep-sequencing. Values are combined from 3 independent experiments. (B-C) Position and frequency of mutations present above 5% of the quasispecies for wild-type in the absence or presence of RBV (B) or triple mutant (C) for each individual experiment. Areas in grey were successfully deep-sequenced. Red bars represent the mutations introduced into the triple mutant RdRp, which were conserved over the course of all experiments. Mut, Y1320H-K1383N-G1634R triple mutant; RBV, ribavirin; VC, virus control; WT, wild-type.

Fig. 6. RBV is mutagenic to *in vivo* HEV replication. (A) The number of mutations per kb present above 0.5% in the quasispecies of patient serum-derived HEV was determined by deep-sequencing at different times post diagnosis. (B) The number of new mutations (i.e. not detected at any of the previous time points) per kb present above 0.5% in the quasispecies of patient serum-derived HEV was determined in the same way.

Fig. 7. HEV genome organization with mutations and their impact. HEV genome organization is depicted with the location of the mutations and insertion. Their impact on *in vitro* HEV replication and RBV sensitivity is given together with the resulting hypothesis of the present study. HVR, hypervariable region; MTase, methyltransferase; ORF, open reading frame; Pro, protease; RBV, ribavirin; RdRp, RNA-dependent RNA polymerase; UTR, untranslated region; X, macrodomain; Y, Y-domain.

Figure 1

A

B

Hypervariable region

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preRBVres 593  TFDDSRQAMGAGPHNLTYELVPAQLQVKISSNGLDCTAIFPPGAAPSAAPSEVA*AFCSAL
relapse 593    TFDDSRQAMGAGPHNLTYELVPAQLQVKISSNGLDCTAIFPPGAAPSAAPSEVA*TFCSAL
postRBVres 593  TFDDSRQAMGAGPHNLTYELVPAQLQVKISSNGLDCTAIFPPGAAPSAAPSEVA*AFCSAL

preRBVres 653  YRYNKFTQRHSLTGGLWLYPEGLLGLFPFSPGHLWESANPFCGESTLYTRTWSTSGFSS
relapse 653    YRYNKFTQRHSLTGGLWLYPEGLLGLFPFSPGHLWESANPFCGESTLYTRTWSTSGFSS
postRBVres 653  YRYNKFTQRHSLTGGLWLYPEGLLGLFPFSPGHLWESANPFCGESTLYTRTWSTSGFSS

preRBVres 713  D*FSPP*EAAHA*APVPDVGLPSGAPSSASDVWVFPFPPSEGSAIVPPPETPVSKPANPPCSIT
relapse 713    D*FSPP*EAAHA*APVPDVGLPSGAPSSASDVWVFPFPPSEGSAIVPPPETPVSKPANPPCSIT
postRBVres 713  D*FSPP*EAAHA*APVPDVGLPSGAPSSASDVWVFPFPPSEGSAIVPPPETPVSKPANPPCSIT

preRBVres 773  PRPPVRKPPTPPPARTRRL-----
relapse 773    PRPPVRKPPTPPPARTRRL-----
postRBVres 773  PRPPVRKPPTPPPARTRRL[SLKGFWKK]SGFSSD*FSPP*EAAHA*APVPDVGLPSGAPSSA

preRBVres 793  -----YMY
relapse 793    -----YMY
postRBVres 833  SDVWVFPFPPSEGSAIVPPPETPVSKPANPPCSITPRPPVRKPPTPPPARTRRLYMY

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RdRp

C

RNA-dependent RNA polymerase

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preRBVres 1207 GGEVGHHRPSTIPRGNDQNLDTLRAFPSCQISAYHQLAEELGHRPAPVAAVLPCCPEL
relapse 1207    GGEVGHHRPSTIPRGNDQNLDTLRAFPSCQISAYHQLAEELGHRPAPVAAVLPCCPEL
postRBVres 1207 GGEVGHHRPSTIPRGNDQNLDTLRAFPSCQISAYHQLAEELGHRPAPVAAVLPCCPEL

preRBVres 1267 EQGLLYMPQELTVSDSVLIFELTDIVHCRMAAPSQRKAVLSTLVGRYGRRTKLYEAAHSD
relapse 1267    EQGLLYMPQELTVSDSVLIFELTDIVHCRMAAPSQRKAVLSTLVGRYGRRTKLYEAAHSD
postRBVres 1267 EQGLLYMPQELTVSDSVLIFELTDIVHCRMAAPSQRKAVLSTLVGRYGRRTKLYEAAHSD*

preRBVres 1327 VRESLGRFIPTIGPVQATTCELYELVEAMVEKQDGSVALELDLCSRDSVRITFFKDCN
relapse 1327    VRESLGRFIPTIGPVQATTCELYELVEAMVEKQDGSVALELDLCSRDSVRITFFKDCN
postRBVres 1327 VRESLGRFIPTIGPVQATTCELYELVEAMVEKQDGSVALELDLCSRDSVRITFFKDCN*

preRBVres 1387 KFTTGETIAHGKVGQGISAWSKTF*CALFGPWFAIEKEILALLPPNIFYGDAYEETVFS
relapse 1387    KFTTGETIAHGKVGQGISAWSKTF*CALFGPWFAIEKEILALLPPNIFYGDAYEETVFS
postRBVres 1387 KFTTGETIAHGKVGQGISAWSKTF*CALFGPWFAIEKEILALLPPNIFYGDAYEETVFS

preRBVres 1447 AISGAGSSMVFENDFSEFSTQNNFSLGLECVIMEECGMPQWLIRLYHLVRSAWTLQAPK
relapse 1447    AISGAGSSMVFENDFSEFSTQNNFSLGLECVIMEECGMPQWLIRLYHLVRSAWTLQAPK
postRBVres 1447 AISGAGSSMVFENDFSEFSTQNNFSLGLECVIMEECGMPQWLIRLYHLVRSAWTLQAPK

preRBVres 1507 E*SLKGF*WKKHSGEPGTLNWTWNMAIAHCYFRDLRVA*AFKGGDSVVLCS*SDYRQSRNA
relapse 1507    E*SLKGF*WKKHSGEPGTLNWTWNMAIAHCYFRDLRVA*AFKGGDSVVLCS*SDYRQSRNA
postRBVres 1507 E*SLKGF*WKKHSGEPGTLNWTWNMAIAHCYFRDLRVA*AFKGGDSVVLCS*SDYRQSRNA

preRBVres 1567 ATLIAGCGLKLV*DYRPIGLYAGVVVAPGLGALPDVVRFAGRLSEKNWGPGERAEQLRL
relapse 1567    ATLIAGCGLKLV*DYRPIGLYAGVVVAPGLGALPDVVRFAGRLSEKNWGPGERAEQLRL
postRBVres 1567 ATLIAGCGLKLV*DYRPIGLYAGVVVAPGLGALPDVVRFAGRLSEKNWGPGERAEQLRL

preRBVres 1627 AVCDFLRGLTNVAQVCVDVVSRYGVSPGLVHNLIGMLQTIADGKAHFTETVKPVLDTLN
relapse 1627    AVCDFLRGLTNVAQVCVDVVSRYGVSPGLVHNLIGMLQTIADGKAHFTETVKPVLDTLN
postRBVres 1627 AVCDFLRGLTNVAQVCVDVVSRYGVSPGLVHNLIGMLQTIADGKAHFTETVKPVLDTLN*

preRBVres 1687 SIIQRLE
relapse 1687    SIIQRLE
postRBVres 1687 SIIQRLE

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F1 motif

HVR insert origin

Figure 2

Figure 3

A

B

Figure 4

A

B

C

D

E

F

Figure 5

A

B

C

Figure 6

A **B**

Figure 7

Hypothesis:

- Y1320H, G1634R and the HVR insertion compensate for the K1383N-associated replication defect
- K1383N may play a crucial role in RBV resistance by altering RdRp fidelity

Position

1320

1383

1634

HVR

Original*

Original*

Duplication +
insert < RdRp