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Divergent quasispecies evolution in de novo hepatitis C virus infection associated with bone marrow transplantation

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ABSTRACT

Quasispecies is a remarkable characteristic of hepatitis C virus (HCV) and has profound roles in HCV biology and clinical practice. The understanding of HCV quasispecies behavior, in particular in acute HCV infection, is valuable for vaccine development and therapeutic interference. However, acute HCV infection is seldom encountered in clinic practice due to its silent onset. In the present study, we reported a unique case of de novo HCV infection associated with the transplantation of bone marrow from a HCV-positive donor. HCV quasispecies diversity was determined in both the donor and the recipient over a 4-year follow-up, accompanied with simultaneous measurement of HCV neutralizing antibody. Detailed genetic and phylogenetic analyses revealed a divergent quasispecies evolution, which was not related to dynamic changes of HCV neutralizing antibody. Instead, our data suggested an essential role of the fitness adaptation of founder viral population in driving such an evolutionary pattern.

1. Introduction

Hepatitis C virus (HCV) is an enveloped single-stranded, positive-sense RNA virus classified into the Flaviviridae family and has been well documented as the major etiological agent responsible for most post-transfusional and community-acquired hepatitis [1]. Currently nearly 200 million cases of HCV infection worldwide represent a major public concern. A remarkable feature for HCV is high genetic variability, which is responsible for the inability to develop an efficient preventative vaccine and clinically limited success of antiviral therapy.

Phylogenetic analyses reveal the existence of at least six major genotypes and more than 100 subtypes [2]. Moreover, within an infected individual, HCV circulates as a group of different but genetically closely related variants, referred to as viral quasispecies, a characteristic shared by most RNA viruses [3]. Although the variability has been well documented across the entire HCV genome [4], the most variable regions are located on envelope domains. In particular, the 5′ end of the second envelope sequence, an 81 bp domain, has been proved to be extremely variable, named hypervariable region 1 (HVR1) [5].

Due to the lack of reliable HCV cell culture and appropriate small animal models, biological roles of HCV quasispecies have been mainly studied in various clinical settings. Of specific interest is acute HCV infection. As HCV establishes persistent infection in most infected individuals, the understanding of HCV quasispecies behavior immediately after infection should provide valuable insight for vaccine development and therapeutic interference. However, acute HCV infection is seldom encountered in clinic practice due to its silent onset. In the present study, we reported a unique case in which the subject obtained HCV infection from a HCV-positive bone marrow donor. HCV quasispecies diversity was monitored for its dynamic changes over a 4-year period. The role of HCV neutralizing antibody on quasispecies evolution was also estimated.

2. Materials and methods

2.1. Patient and samples

The subject was a 50-year-old male who received allogeneic bone marrow transplantation due to acute myelogenous leukemia. The bone marrow donor, his brother, was known to be seropositive for both anti-HCV and HCV-RNA. The transplantation was performed because of a high degree human leukocyte antigen (HLA) match and a life-saving situation. The patient developed acute hepatitis 30 days after bone marrow transplantation. Other etio-

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logical factors for acute hepatitis were excluded based on the negative detection of IgM of anti-hepatitis A virus (HAV), hepatitis B virus (HBV) surface antigen, HBV core antibody IgM, IgG & IgM of anti-cytomegalovirus (CMV) and CMV antigenemia. The patient was also negative for anti-human immunodeficiency virus (HIV) and anti-nuclear antibody (ANA).

Informed consent and institutional review board approval were obtained prior to the study. Serum samples were collected from both the donor at the time of bone marrow transplantation and the recipient at 13 time points over 4-year follow-up. Samples were stored at −80 °C until use.

2.2. Laboratory examination

Serum samples were analyzed for HCV-RNA level by bDNA assay (Bayer VERSANT HCV 3.0), anti-HCV by second generation enzyme immunoassay (EIA) (Abbott), anti-E2 by EIA (Chiron in-house assay) and alanine aminotransferase (ALT). The patient also received liver biopsies at 26 weeks and 16 months after the transplantation.

2.3. Molecular cloning of partial HCV envelope domain spanning HVR1

Amplification and cloning of the partial HCV E1E2 domain (1.38 kb) were essentially the same as what was described previously [6]. In brief, serum RNA was reverse transcribed with 200 U of M-MLV reverse transcriptase (Promega), followed by nested PCR with Taq DNA polymerase (Applied Biosystems). The PCR product was gel purified by using QIAEX II Gel Extraction Kit (Qiagen) and ligated into the pTOPO-TA cloning vector (Invitrogen). *Escherichia coli* TOP-10 cells (Invitrogen) were used for transformation and recovery of recombinant clones. Approximately 4–10 clones for each sample were sequenced with ABI PRISM dye terminator cycle sequencing ready reaction kit using an ABI 373A automated sequencer (Applied Biosystems). It should be noted only partial domain spanning HVR1 was sequenced with primer 6BR1, 5′-tctgggcatccggacgagttg-3′.

2.4. Genetic analysis

Raw sequences were edited with the programs ClustalW [7] and BioEdit [8] in which HCV H77 strain (AF009606) served as the reference sequence. Primer sequences were removed prior to the genetic analysis. Inter- or intra-group genetic distance (d) was calculated with the maximum composite likelihood method (all sites) in the Molecular Evolutionary Genetics Analysis software package (MEGA, version 4.0) [9]. Similarly the number of synonymous substitutions per synonymous site (dS), the number of non-synonymous substitutions per non-synonymous site (dN) and dN/dS values were measured with Nei-Gojobori method implemented in MEGA. It should be mentioned that the inter-group calculation of all genetic parameters was based on the comparison with the donor. The phylogenetic tree was constructed using the Neighbor-Joining method [10] with a bootstrap test in MEGA.

2.5. Bayesian Tip-Significance testing (BaTS)

BaTS analysis was used to determine if the phylogenetic tree showed a time-dependent topology [11]. In doing so, the best-fit nucleotide substitution model was first estimated through a hierarchical likelihood ratio test (hLRT) with Modeltest [12]. Bayesian Markov chain Monte Carlo (MCMC) phylogenetic trees were simulated in BEAST package under the best-fit nucleotide substitution model as well as additional parameter settings, including a relaxed molecular clock (uncorrelated, lognormal), a Bayesian skyline coalescent prior, and a total run of 50 million generations to reach relevant parameter convergence as estimated by Tracer [13]. The inferred MCMC trees then served as the input to estimate the strength of HCV clone clustering in terms of sample dates in program BaTS with 1000 replications and the removal of the first 10% trees as burn-in [11]. Both the association index (AI) [14] and the parsimony score (PS) [15] were computed to see whether or not sampling dates are more strongly associated with the underlying phylogeny than expected by chance alone.

2.6. HCV neutralization assay

Titers of HCV neutralizing antibodies (nAb) were determined using the HCV pseudoparticles (HCVpp) approach [16,17]. Human embryonic kidney (HEK) 293 T cells (CRL-1573) and Hep3B cells (HB-8064) were purchased from the American Type Culture Collection (ATCC) and were maintained in Dulbecca's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin. Plasmids pNL4.3Luc.R$^-$E$^-$ and pcDNA3.1 HCV E1E2 were a gift from Dr. Charles M. Rice (The Rockefeller University) and Dr. Jane McKeating (University of Birmingham). The former is an envelope-deficient HIV proviral genome with an expression cassette of luciferase reporter gene. Plasmid pcDNA3.1 HCV E1E2 expresses the full length HCV envelope protein of strain H77 (GenBank accession number: AF009606).

Briefly, HCVpp were generated by co-transfection of 293T cells (ATCC) with equal amounts of pNL4.3Luc.R$^-$E$^-$ and pcDNA3.1HCVE1E2 using FuGENE6 (Roche Applied Science, Indianapolis, IN). The supernatant, collected 72 h post-transfection, was filtered through 0.45 μm pore size filter and stored in aliquots at −80 °C. HIV p24 antigen content was determined by HIV-1 p24 antigen capture assay (Advanced BioScience Lab., Kensington, MD). For neutralization experiments, Hep3B cells were seeded in 96-well plate (8×10^3/well) 24 h before infection. Serum samples were 2-fold diluted, inactivated at 56 °C for 1 h and incubated with equal volume of HCVpp in 3% fetal bovine serum (FBS)/DMEM plus 4 μg/ml polybrene at 37 °C for 1 h. After the infection with HCVpp, cells were incubated at 37 °C for 6 h followed by complete media replacement. Seventy-two hours after incubation, cells were washed in PBS once, and lysed with 40 μl per well of 1× cell culture lysis buffer (Promega, Madison, WI). The lysates were used to measure luciferase activity in Glomax 96 Microplate luminometer (Promega). HCVpp infectivity based on luciferase activity was determined in terms of relative light units (RLUs) in the presence of test serum (RLU test) versus average infection in the presence of 3 HCV-negative human serum samples (RLU control). Percent neutralization was calculated as 100× [1 − (RLU test/RLU

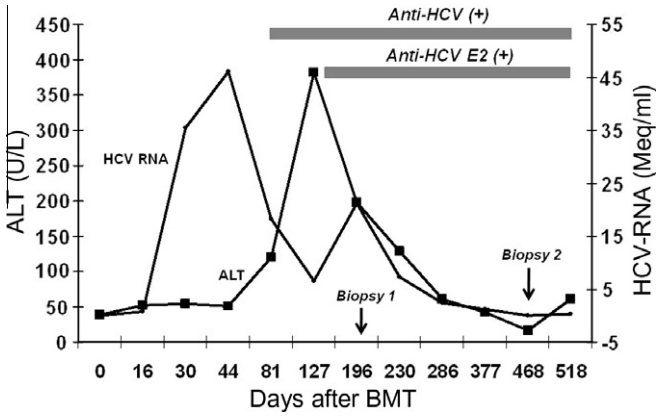

Fig. 1. Monitoring of serum ALT and HCV-RNA levels after bone marrow transplantation (BMT). Positive detection of both anti-HCV and anti-HCV E2 are shown as grey rectangles. Time points for liver biopsies are also indicated.

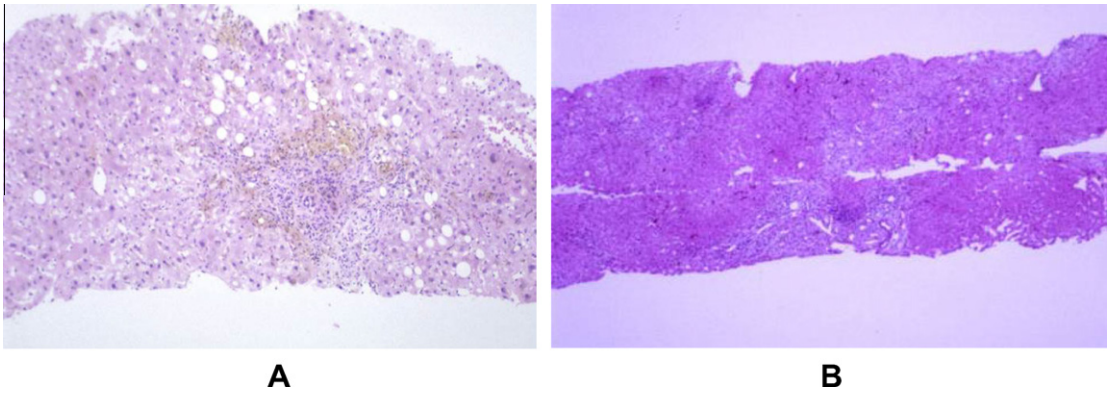

Fig. 2. Liver biopsy examination at 26 weeks (A) and 16 months after bone marrow transplantation. At 26 weeks, the liver showed iron overload and steatohepatitis, featured by inflammation with concurrent fat accumulation (A). A repeat liver biopsy at 16 months after the transplantation presented significantly less lobular ballooning, steatosis and necroinflammatory activity. However there had been the progression of fibrosis and architectural distortion (B).

control)]. Results are reported as 50% inhibitory dilution (ID50), defined as the serum dilution that causes a 50% reduction in luciferase activity compared to the control.

2.7. GenBank accession numbers

HCV sequences generated in the study were deposited in GenBank with assigned accession numbers JN613022 through JN613111.

3. Results

3.1. Clinical outcome after bone marrow transplantation

The subject survived successfully bone marrow transplantation and was followed closely with the measurement of serum ALT, anti-HCV, anti-HCVE2 and HCV-RNA levels up to 510 days after

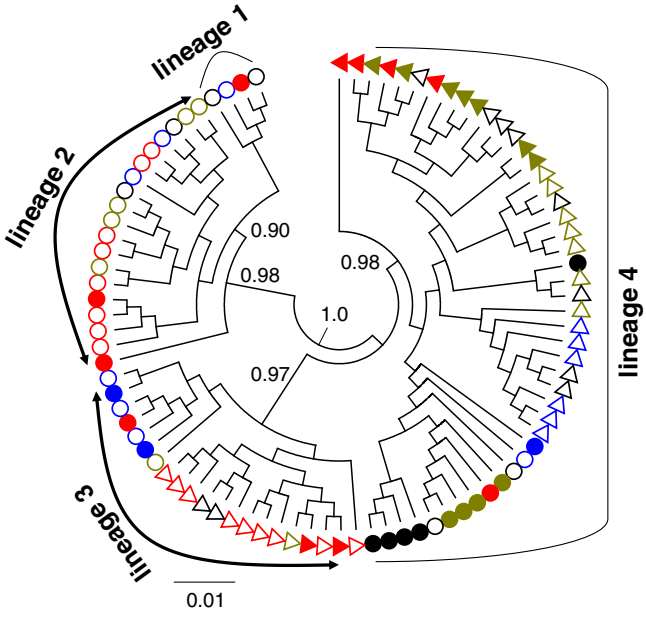

Fig. 3. Maximum clade credibility tree of 90 HCV partial envelope sequences, including 8 from the donor and 82 from the recipient over 13 sequential sampling dates. Sequences on the tree are represented by open circles (red, the donor; dark yellow, day 16; blue, day 30; black, day 81), filled circles (red, day 127; dark yellow, day 196; blue, day 286; black, year 1), open triangles (red, year 1.4; dark yellow, year 1.7; blue, year 2; black, year 2.5) and filled triangles (red, year 3; dark yellow, dark yellow). Posterior probability values are shown on major branches. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

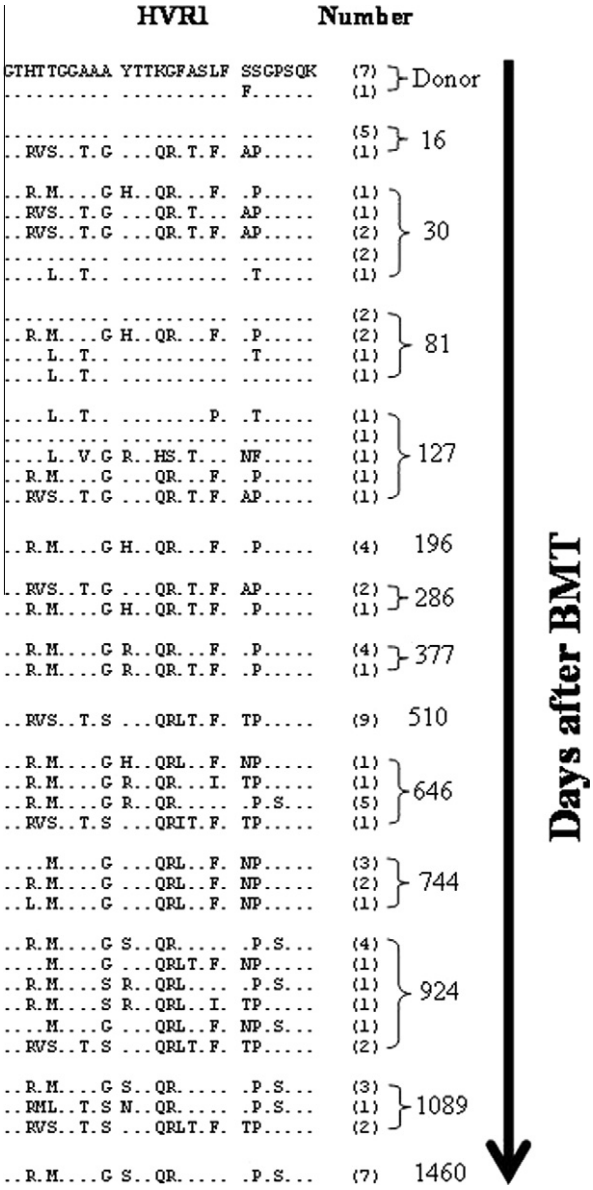

Fig. 4. The alignment of HCV HVR1 variants derived from the donor and the recipient over 13 sampling dates. At a given time point, the number of each distinct HVR1 variant is shown on the right of sequence alignment. Dots indicate the identity.

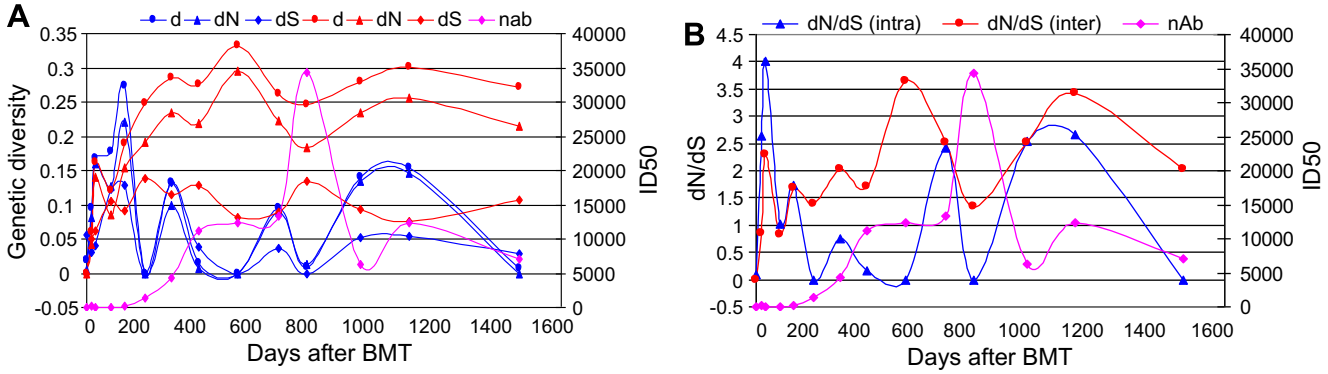

Fig. 5. Dynamic changes of intra- (blue) and inter-group (red) HCV quasispecies diversity, including genetic distance (*d*), the number of synonymous substitutions per synonymous site (dS), the number of non-synonymous substitutions per non-synonymous site (dN) (A) and dN/dS ratios (B). The measurement of HCV nAb was also shown over 13 time points (pink). BMT, bone marrow transplantation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the transplantation. Serum ALT was gradually elevated with a peak value (382 U/L) about 4 months after the transplantation. HCV-RNA level showed a similar dynamic curve with the peak value ahead of the highest ALT measurement, followed by low-level fluctuation as seen commonly in chronic HCV infection (Fig. 1). The earliest positive detection of anti-HCV was at 81 days after the transplantation while anti-HCV E2 was not developed until 127 days after the transplantation (Fig. 1), giving a reasonable lag period as the antigens used for anti-HCV detection kit contain highly immunogenic HCV non-structure protein [20]. These laboratory evaluations showed the typical dynamic curves of viral parameters documented in acute HCV infection [21], indicating a successful transmission of HCV from the HCV-positive bone marrow donor. Further evidence came from pathological examination of liver biopsies sampled at 26 weeks and 16 months after the transplantation. The former showed steatohepatitis and marked siderosis (iron stain 3+), characterized in acute HCV infection (Fig. 2a). The biopsy at 16 months confirmed a persistent HCV infection that resulted in advanced hepatic fibrosis (stage 3) (Fig. 2b).

3.2. Transmission and evolution of HCV quasispecies after bone marrow transplantation

A total of 90 clones were derived from the donor and the recipient at 13 time points over the 4-year follow-up. Initial phylogenetic analysis (Neighbor-Joining method) with the sequenced domain, 333 bp in length spanning HVR1, revealed the infection of HCV genotype 1b (data not shown). Dynamic changes of HCV quasispecies were examined in three settings, i.e., quasispecies lineage, intra- and inter-group genetic diversity. Overall there were 4 major quasispecies lineages (Fig. 3, Fig. 4). Lineage 2, presented in the donor, was transmitted into the recipient and gradually replaced by lineages 3 and 4, which were alternatively dominated at subsequent sampling dates. Lineage 1 seemed "extinct", under a detectable level since 4 months after the transplantation.

There were a couple of observations with regard to genetic diversity. First, both intra- and inter-group genetic diversity (d, dN, and dS) showed an increase within the first six months after transplantation (Fig. 5a). After that the intra-group genetic diversity fluctuated at low levels while the inter-group genetic diversity maintained higher values (Fig. 5a). Second, dN/dS values were much higher in the inter-group (dN/dS > 1) than the intra-group genetic diversity (Fig. 5b), indicating a progressive positive selection of HCV quasispecies after the transplantation. Furthermore, BaTS analysis revealed a date-dependent topology in MCMC trees constructed with all 90 HCV clones (AI = 5.35, $p < 0.01$; PS = 42, $p < 0.01$) (Fig. 3). Taken together, these data suggested a divergent evolution of HCV after the transmission into the recipient.

3.3. Roles of HCV neutralizing antibodies in quasispecies evolution

Compared to the anti-HCV and anti-HCV E2, delayed HCV nAb detection was observed, which is consistent with previous reports [20,22]. Using HCVpp approach HCV nAb was first detected at week 28 and then increased gradually with a peak titer at 2 years after the transplantation (Fig. 5). By the end of follow-up, i.e., 4 years after the transplantation, HCV nAb was still detectable (Fig. 5). However, its role in HCV quasispecies genetic diversity was not apparent. The intra-group genetic diversity continued at fluctuating low levels after the positive detection of HCV nAb (Fig. 5a). Similar levels of the inter-group genetic diversity were observed before and after the appearance of HCV nAb (Fig. 5a). Moreover, at the peak titer of HCV nAb, both intra- and inter-group genetic diversity were located in the valley of curves (Fig. 5). Thus, in this case, HCV nAb may play an only minor role in terms of quasispecies evolution.

4. Discussion

Quasispecies nature has profound role in HCV biology and is a single independent factor modulating clinical outcomes of HCV infection, such as disease progress and antiviral therapy [18,19]. It is therefore important to understand the evolutionary patterns of HCV quasispecies, especially the early dynamics immediately after viral transmission. Due to the lack of suitable animal models, these issues have been explored mostly in the settings of liver transplantation and drug injectors for which "de novo" HCV infection is easily documented [23–25]. However, in liver transplantation, there is no change with regard to host environment. Acute HCV infection in drug injectors may have an immunological background due to previous HCV infection [26]. Thus the case reported here represents an authentic model of natural HCV infection, acute phrase followed by persistent infection as documented by both laboratory and pathological examinations (Figs. 1 and 2).

In general, our data did not deviate from previous observation [27]. The HCV strain, after transmittal from the donor, showed a divergent evolution over the 4-year follow-up, as revealed by a time-dependent tree topology in BaTS analysis and a high level of inter-group dN/ds values (Figs. 3 and 5b). Beyond the identification of evolutionary patterns, it is more important to understand the underlying force that drives such an evolutionary pattern. In this setting, immune pressure is often considered as a major

player. However, our data suggest that this dogma is not a full explanation, at least in the case presented here. First, the intra- and inter-group HCV quasispecies diversity were maintained at higher levels up to 6 months after the transplantation, ahead of the detection of HCV antibodies, especially HCVnAb (Fig. 5). Second, the appearance of HCV nAb was not associated with quasispecies extinction (lineages 1 and 2) or continuous survival (lineages 3 and 4). Similarly, dynamic curves of both intra- and inter-group quasispecies diversity were not altered after the detection HCV nAb (Fig. 5). The highest titer of HCV nAb actually corresponded to lower values of both intra- and inter-group genetic diversity. Taken together, these data suggest that HCV nAb is not a major force in driving viral evolution. The dominant factor responsible for highly dynamic changes may be the competition among various HCV quasispecies variants in adaption into a new replication host. This conclusion is consistent with two recent reports that underlined the importance of viral replicative fitness in viral transmission in the setting of liver transplantation [25,28].

It should be noted that HCV neutralization titers were estimated using HCVpp constructed with heterogeneous HCV envelope genes (strain H77, genotype 1a) while the subject was infected with HCV genotype 1b. Since HCV nAb is believed to recognize discontinuous epitopes that are conserved across genotypes, the use of HCVpp with heterogeneous envelope genes should not bias the data generation and interpretation [29,30]. However, it should be emphasized that the role of HCV nAb in HCV quasispecies evolution cannot be fully excluded, in particular when taking into account the HCV isolate-specific nAb, which was not measured in the present study. In fact, while HCV quasispecies lineages 3 and 4 survived over the follow-up period, there were subtle substitutions of amino acids in the HVR1 domain, suggesting possible roles of either cross-genotype or isolate-specific HCV nAb.

In summary, a divergent evolution of HCV quasispecies was found in this well characterized de novo HCV infection resulting from the transplantation of HCV-positive bone marrow. The fitness adaptation of the founder viral population may play an essential role in driving HCV quasispecies evolution, in particular in the acute phase. HCV nAb seems an additional but not dominant factor in shaping such an evolutionary pattern. Its exact role in modulating HCV infection at quasispecies level deserves further investigation.

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