Single-Variant Sequencing Revealed Rapid HCV Evolution in HIV Immune Reconstitution

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Background/Aims

Hepatitis C virus (HCV) associated liver disease has become the leading cause of death in patients infected with human immunodeficiency virus (HIV) [Sulkowski 2003]. HIV infection, on the other hand, leads to decreased sustained virological response (SVR) to anti-HCV treatment [Chung 2004]. HCV dynamics and evolution in HIV co-infected patients with regard to host immune status and antiretroviral therapy are poorly defined. Like other hypervariable viruses, HCV replicates as a pool of closely related but distinct variants, namely quasispecies [Martell 1992]. The study of viral quasispecies has been largely hampered by accurate and efficient approaches to authentically detect minor variants and represent their frequencies in the viral population. Linkages among mutations are frequently broken with the use of short amplicons. We address these questions by investigating viral dynamics and evolution in HIV-HCV co-infected patients using a novel approach that tags single variant followed by next-generation sequencing, namely single-variant sequencing (SVS).

Patients and Methods

Subjects

Study subjects and samples were obtained from the University of Pennsylvania Center for AIDS Research (CFAR) Cohort. All subjects were chronically infected with HCV and co-infected with HIV, and had been followed up for up to 13 years (Fig. 1).

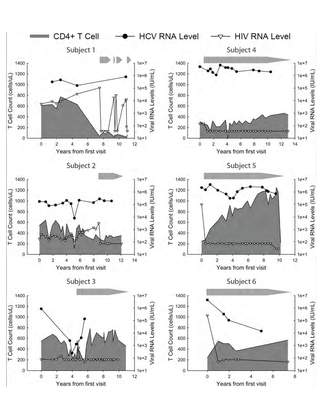


Fig. 1 Clinical profiles of study subjects. Study subjects were divided into either immune decline or immune restoration groups based on their CD4+ T cell count. The dynamic changes of HCV and HIV RNA as well as CD4+ T cell counts were shown. Durations of anti-retroviral therapy (ART) for each subject were indicated using arrow bars

Single-variant sequencing (SVS)

We modified a previously reported primer-ID method [Jabara 2011] to create SVS (Fig.2). By tagging the RNA templates, several critical issues related to quasispecies study have been resolved, including biases related to template re-sampling [Liu 1996], PCR and sequencing introduced errors, and preferential amplification biases caused by primers. The single-stranded cDNA was column purified using a PCR clean-up gel extraction kit (Macherey-Nagel). Primer removal was verified by electropherogram analysis using both RNA-Nano and RNA-Pico microfluidic chips on an Agilent 2100 bioanalyzer (Agilent).

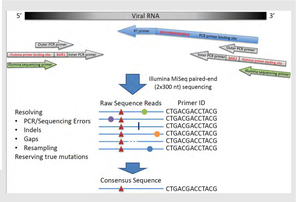


Fig. 2 Schematic diagram for SVS. CDNA was synthesized through a separate RT step which employs a tagging RT primer. With consecutive degenerate bases (shown as multiple "N"s) flanking the binding nucleotides, this primer randomly assign a unique "tag" to each RNA template. In-house deep sequencing was performed using a MiSeq Benchtop Sequencer (Illumina) initiated as a 2 x 300 bases of paired-end sequencing. CDNA tags were used to merge reads into consensus sequences after sequencing

Next-generation sequencing and bioinformatics analysis

A consensus sequence was called when at least three sequences contain an identical tag. Sequencing reads were filtered using a series of stringent criteria (Fig. 3). Results generated using SVS were compared to those generated using raw reads (Fig. 4, 5). Further analyses were conducted using MEGA (phylogenetic analysis), VarPlot (sliding window analysis) and VisSPA (sequence logo analysis) as previously described [Liu 2010, 2012].

Results

Section I: SVS successfully eliminates PCR/sequencing introduced errors and corrects PCR amplification biases.

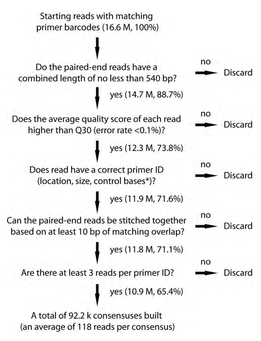


Fig. 3, Workflow of sequencing reads filtration. Reads that do not satisfy the filtration criteria were discarded at each step. Consensus sequences were built based on at least 3 reads carrying identical tags.

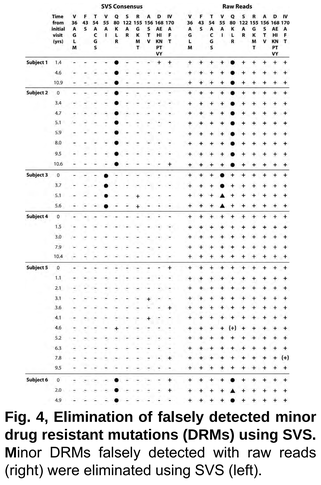


Fig. 4, Elimination of falsely detected minor drug resistant mutations (DRMs) using SVS. Minor DRMs falsely detected with raw reads (right) were eliminated using SVS (left).

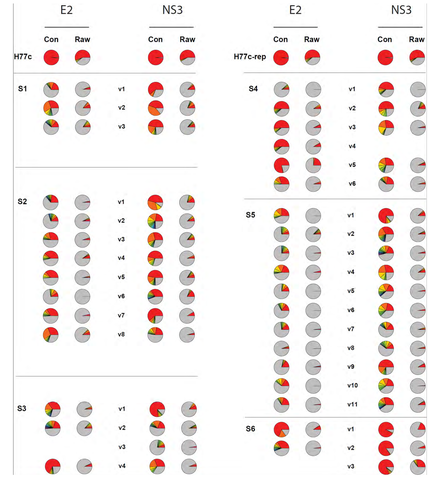


Fig. 5, HCV quasispecies distribution reconstructed using either SVS (left) or raw sequences (right). Proportions of different variants in each sample/amplified region were depicted, with rainbow color for major variants and grey for minor variants (1% as threshold). SVS unmasked the true structure of HCV quasispecies due to elimination of PCR/sequencing introduced errors.

Section II: Rapid HCV evolution in immune restoring subjects vs. dormant evolution in those with immune decline.

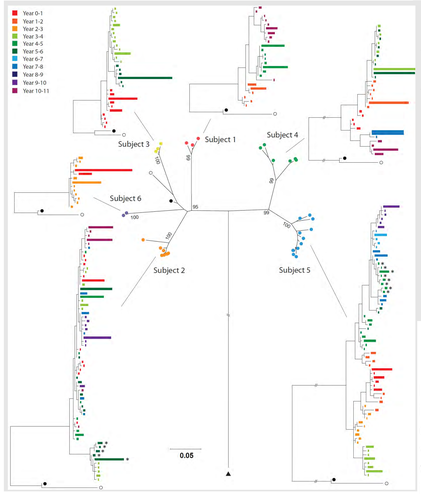


Fig 6. Maximum likelihood (ML) phylogeny of E1E2 sequences. ML trees were built using major E1E2 sequences (>=1%) for each subject, with a centered master tree built using only dominant sequences. Color code is provided for individual trees. For each variant/sequence, the length of the bar represents its proportion in the viral population. In case multiple visits fall within the same year, variants from the later visit are marked with asterisks. Reference sequences are Bole1a (solid circle, [Munshaw 2012]), H77 (empty circle) and Con1 (subtype 1b, solid triangle).

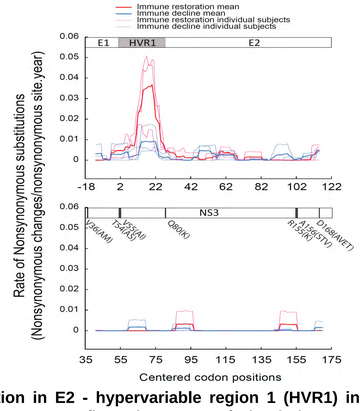


Fig. 7, Rapid nonsynonymous evolution in E2 - hypervariable region 1 (HVR1) in immune restoring subjects (red lines, upper panel) vs. dormant evolution in immune declining subjects (blue lines, upper panel) or in NS3 region (lower panel). Mean values (thick lines) as well as individual values (thin lines) comparing first and last visits were used in sliding window analysis. Window size is 10 codons with one-codon increments. Locations of common drug-resistant mutations in NS3 region are indicated.

Section II *(continued)*

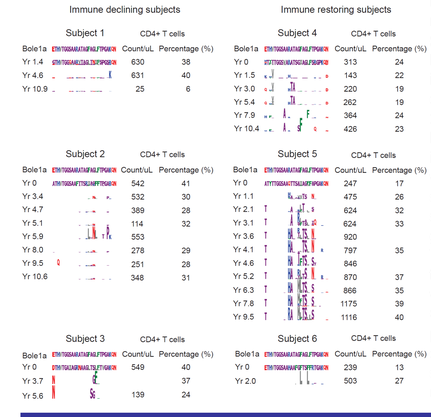


Fig. 8, Prominent vs. neglectable amino acid (aa.) changes in E2-HVR1 region in immune restoring vs. declining subjects over 10 years of follow-up. HVR1 aa. sequences of initial visits (type 1 logo) were queried using sequences from subsequent visits to generate type 2 logos. Time from an initial visit to each following visit was shown on the left. CD4+ T cell count and percentage were shown on the right. HVR1 aa. sequence of bole1a was used as reference.

Summary

1. Single-variant sequencing (SVS) successfully eliminates PCR/sequencing introduced errors, lowering systemic error rate over 40 times comparing to raw reads (3.5×10^{-5} vs. 1.5×10^{-3}). It also unmasks the authentic HCV quasispecies structure from artificial errors and amplification biases, evidenced by significantly increased proportions of major variants (Fig. 4) and largely uneven reads distribution in consensus, i.e. most consensus sequences were built with 3 to 10 reads whereas some were constructed using over 10,000 reads.
2. SVS accurately identified and quantified DRMs in NS3 gene at low frequencies (as low as 0.05%). Notably, a specific DRM, aaVI170T, was detected as a minor variant (<1%) in 4 of 6 subjects over multiple visits (data not shown). In contrast, minor DRMs were observed unexceptionally across all DRM sites based on raw reads, reflecting influences of PCR/sequencing introduced errors ($P<10^{-32}$, Fig. 5).
3. Subjects with restoring immunity demonstrate rapid HCV evolution comparing to immune decliners in envelope gene, mostly hypervariable region 1 (HVR1), but not NS3 gene, indicating increasing humoral but not cellular immunity against HCV (Fig. 6-8). Sequence changes are more likely predicted by changes in CD4+ T cell count rather than the absolute temporal T cell number.

Conclusion

1. Single-Variant Sequencing (SVS) accurately identifies both major and minor HCV variants with their respective population proportions.
2. Immune reconstitution in HIV/HCV co-infection is associated with rapid sequence evolution in the envelope but not NS3 gene, likely driven by increased immune pressure from enhanced humoral responses during antiretroviral therapy.
3. Drug resistant mutations in NS3 can occur naturally at low frequencies in HCV-HIV co-infected individuals, which can be accurately identified and quantified using SVS.

Abstract

Human immunodeficiency virus (HIV) infection accelerates the progression of liver disease in Hepatitis C virus (HCV) co-infected individuals, raising the question of how immune status in co-infected individuals affects HCV quasispecies populations. We hypothesize that the structure and evolution of HCV populations in individuals with immune reconstitution differ from that in those with immune decline in HCV-HIV co-infections. To precisely define and quantify individual HCV variants, we developed a single-variant sequencing (SVS) approach by combining random nucleotide tagging with an optimized Illumina MiSeq deep sequencing strategy. We applied this method to examine HCV quasispecies structure and evolution in envelope and NS3 genes in HCV-HIV co-infected subjects, and assess the abundance and dynamics of naturally occurring drug resistant mutations (DRMs) in NS3. We sequenced 35 serum samples collected across 10 years of follow-up from 6 HCV-HIV co-infected subjects. Three subjects who received ART early during follow-up experienced immune reconstitution. The other three subjects showed a gradual decline in CD4 count with delayed ART. A total of >16 million paired-end reads were generated to build 92,226 SVS consensus sequences based on unique sequence tags. Each consensus sequence represents an individual HCV variant. We showed that the SVS approach (1) corrected PCR/sequencing errors as well as resampling and amplification bias from PCR, and (2) provided an accurate representation of HCV population structure *in vivo*. Rapid non-synonymous sequence evolution was observed in hypervariable region 1 (HVR1) of E2, but not NS3, in subjects with immune reconstitution compared to subjects with immune decline. Notably, using SVS consensus, a naturally occurring DRM in NS3, aaVI170T, was detected as a minor variant (<1%) in 4 of 6 subjects over multiple visits. In contrast, an even distribution of minor DRMs across all DRM sites was observed based on raw reads ($P<10^{-32}$). Single-Variant Sequencing accurately defines HCV quasispecies population structure and evolution *in vivo*. Immune reconstitution in HIV/HCV co-infection is associated with rapid sequence evolution in the envelope but not NS3, likely driven by increased immune pressure from enhanced humoral responses during antiretroviral therapy. Drug resistant mutations in NS3 can occur naturally at low frequencies in HCV-HIV co-infected individuals, which can be accurately identified and quantified using SVS.

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