Deep sequencing analysis of naturally occurring mutations associated with resistance to HCV NS3 protease inhibitors

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Hepatitis C virus quasispecies

Flavivirus: enveloped, positive strand, RNA viruses

- HCV replication is extremely robust
  - 1 trillion viral particles produced per day
- HCV, like HIV, circulates as a quasispecies in infected individuals
  - quasispecies = closely related but genetically diverse sequences
  - error-prone polymerase (NS5B)

HCV drug resistance

• Both mathematical models and observations from clinical trials suggest that mutations conferring resistance to DAA pre-exist within HCV populations

• Given the quasispecies nature of circulating HCV, the viral swarms may harbor mutations at low frequencies not readily detectable by conventional sequencing methods

• Drug resistant variants can occur naturally and circulate as dominant quasispecies, and can persist long-term *in vivo* (e.g. R155K)

• Lessons from HIV: presence of pre-existing drug resistant mutations is associated with treatment failure

• HCV replication produces large and complex populations, and naturally occurring drug-resistant variants within viral quasispecies may impact therapy using DAA
Study Aim

• The abundance and the temporal dynamics of drug resistance variants, especially the low abundance variants, have not been investigated in detail
• We examined the prevalence, abundance, and temporal dynamics of pre-existing mutations associated protease resistance

Approach

• Ultra-deep, high-throughput sequencing (the Roche/454 method) of partial NS3 gene fragments
• Comparison with population sequencing, and molecular clonal sequencing
Subjects and samples

- Cross-sectional
  - 9 subjects with chronic HCV
    - 6 G1a and 3 G1b

- Longitudinal:
  - 13 subjects with chronic HCV who underwent liver transplantation
    - 10 G1a and 3 G1b
    - For each subject
      - 2 pre-transplant samples
      - 2-3 post-transplant samples
    - 53 transplant samples

- Technical control: in vitro transcript of the NS3 gene from H77C sequence cloned in a plasmid
Methods

- All RNA templates were quantified by qPCR
- RT-PCR generated ~650 base pair NS3 gene segment
- Each PCR sample is tagged with a unique barcode
  - Allows pooling of samples for pyrosequencing runs
- Queried 10 positions associated with protease resistance
  - Amplicon A: amino acid 1-91
    - V36A/M/L, Q41R, F43S, T54A, V55A, Q80R/K
  - Amplicon B: amino acid 94-173
Sequence processing pipeline

• Exact match to the barcode and primer sequences
• > 290 bases in sequence length
• No ambiguous base (N’s)
• Multiple sequence alignment
• Quality control - manual inspection
• Deep sequencing amplified products from *in vitro* transcripts of known sequences to estimate technical error rates from RT-PCR and pyrosequencing

Pyrosequencing results

• A total of ~735,000 high-quality sequence reads available for analysis
• Good representation of all barcodes/samples
Summary of drug resistance alleles: population sequencing

• Population sequencing of NS3 in 22 subjects with chronic HCV:
  – 15 subjects (68%) with no major PI mutations
  – 5 subjects with Q80K
  – 1 subject with Q80K/V55A
  – 1 subject with Q80K/V36L

• Consistent with previous observation based on population sequencing analysis that the prevalence of high-level naturally occurring protease-inhibitor-resistant variants is low
Summary of drug resistance alleles detected by deep sequencing

Genotype 1a  
(n=16)

Genotype 1b  
(n=6)

* drug resistance alleles detected by population sequencing

Summary of drug resistance alleles detected: longitudinal samples from liver transplant

G1a (n=9)

G1b (n=3)

Subjects 1 2 3 4 5 6 7 8 9 10 11 12

Time
Evolution of the dominant viral quasispecies

Selective sweep

Selective sweep by new sequences:
A minor quasispecies in the earlier time points becomes the major variant post-transplant
Evolution of the dominant viral quasispecies

No selective sweep

No selective sweep:
A major quasispecies remains the dominant variant post-transplant

Liver transplantation
Conclusions

• Naturally occurring variants harboring mutations associated with protease resistance are more common than previously reported

• Most resistant variants circulate at low frequencies (except for Q80K) not readily detectable by standard population sequencing – these minor variants can be detected by deep sequencing

• Variants conferring high-level resistance to protease inhibitors were uncommon

• Drug resistant variants are highly dynamic in vivo, which may be related to immune suppression associated with liver transplantation

• The biological relevance of low-frequency, drug resistant quasispecies should be further investigated, and their temporal stability characterized
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