Naturally occurring dominant drug resistance mutations occur infrequently in the setting of recently acquired hepatitis C

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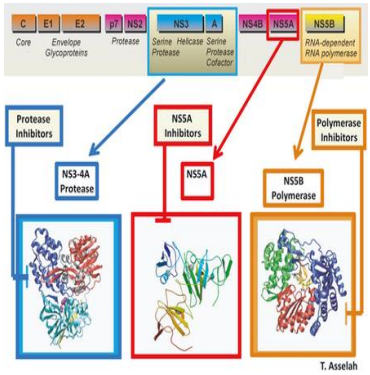
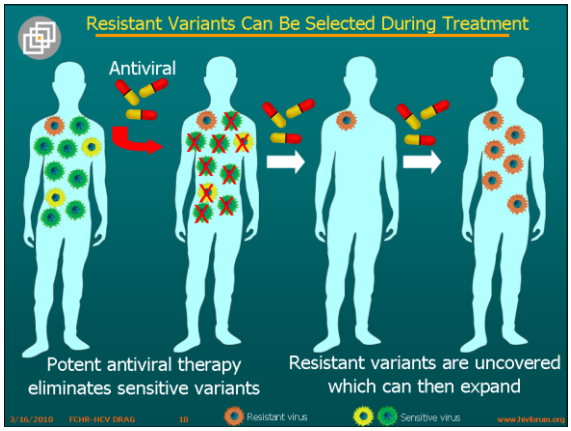
Direct-acting antiviral (DAA) drugs for HCV infection



DAAs limited by presence of amino acid substitutions within targeted proteins

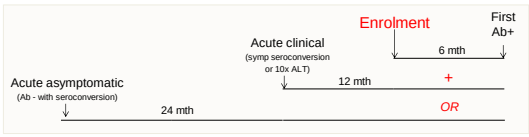


Pre-existing drug resistant variants

- Rapid replication and low fidelity of RNA-dependent polymerase results in HCV quasispecies (1 mutation per 10^3-10^5 bases per replication cycle)
- Frequency of strains change over time within host due to selective (pressures)
  - replication efficiency; immune response (HLA, KIR, IFN); drugs
- Pre-existing DAA resistance associated variations (RAVs) identified in treatment naïve chronic-infected subjects (sanger-based technology) but not in the context of recently acquired hepatitis C infection
- **Use of next-generation sequencing technology to determine frequency of RAVs in ATAHC cohort**
  - **circulating viruses in high-risk exposure populations**
  - **compensatory mutations**
  - **influence of non-drug selection pressures (immune response early in infection)**

Subject characteristics

Australian Trial in Acute Hepatitis C (ATAHC, 2004-2007)



	n (%)
Total	66
Genotype 1a	50 (76)
Genotype 3a	14 (21)
Genotype 1b	2
HIV+	29 (44)

Stored screening or pre-treatment sample available, with an HCV RNA> 1000 IU/ml were included

Pre-existing drug resistant variants (sanger)

	1a			1b			3a		
NS3 protease (wt/RAV)		Chronic (n=205)	Acute (n=67)		Chronic (n=54)	Acute (n=3)		Chronic (n=146)	Acute (n=49)
V36A/M	V/L/M	1.8	1.8	V	0	0	L	0	0
Q41R	Q/H	0.8	6.9	Q	0	0	Q	0	0
F43C/S	F	0	0	F	0	0	F	0	0
T54A/S	T/S	4.4	0	T	0	0	T/S	0.9	2.1
V55A	V/A/I	6.9	6.8	V	0	0	V	0	0
Q80K/R	**Q/K/L**	**18.1**	**10**	**Q**	**0**	**0**	**Q/K**	**0.8**	**0**
S138T	S	0	0	S	0	0	S	0	0
R155K/Q/T	R/T	0.6	0	R	0	0	R	0	0
A156S/T/V	A	0	0	A	0	0	A	0	0
D168A/T/V	D/E	1.3	0	D	0	0	Q/R/K	1.7	2.1
V170A/T	I/V	5.8	13.8	V/I	9.4	0	I/V	6	6.4

Australian subjects Q80K
Chronic 9.1% K (n=77)
ATAHC 5.6% K (n=53)

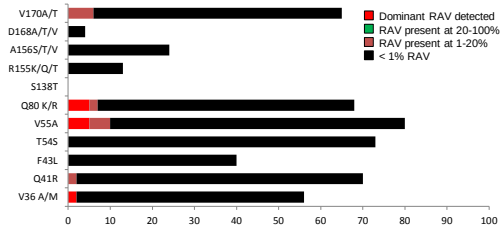
Gaudieri et al Hepatology 2009
Applegate et al Antiviral Ther 2014

Method: FLX 454 Analysis (NGS)

- **Step 1**
  - Raw sequence data collected as .fna and .qal files that contain the fasta sequences and quality scores using Roche 454 image and signal processing software
  - Image data kept for 3 months
  - .fna and .qual files kept for 3 months unless special arrangements made
  - .fna and .qual files are provided to all clients with the reports as requested
- **Step 2**
  - Sort reads by MID (barcode)
  - *Use "sequence classifier by Id" tool (In house tool)*
- **Step 3**
  - Trim Primer Sequences
  - *Use "Stripper" tool. (Only sequences in .fna file are used i.e only high quality reads used) (In house tool)*
- **Step 4**
  - Align Sequences using Reference sequence
  - *Use the tool "Sequence aligner". (In house tool)*
- **Step 5**
  - If required seperate by Amplicons
  - *Use "Select reads covering regions" (In house tool)*
- **Step 6**
  - Re- do the alignments for each amplicon if required
  - *Use the tool "Sequence aligner". (In house tool)*
- **Step 6**
  - Generate a SNP report
  - *Use "Codon usage" or "SNP analyser" tool (In house tool)*

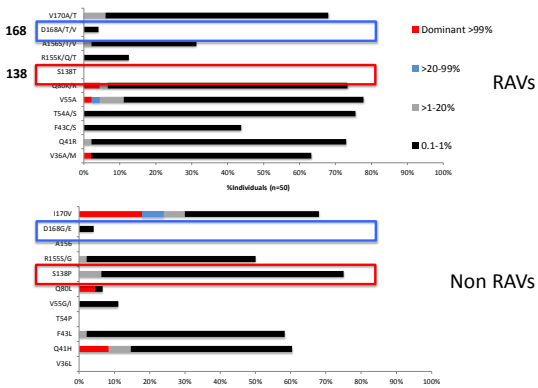
Results: Protease gene (n = 50 GT1a)

Coverage 3918-5239 reads



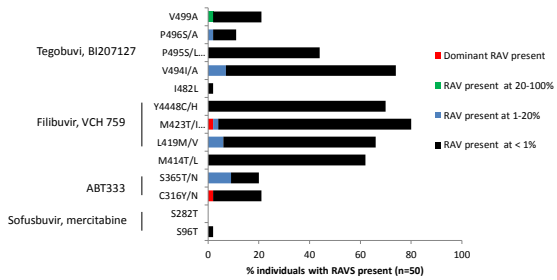
% individuals with RAVs present (n=50)

No association found between RAVs (frequency or number within subject) and HIV status or duration

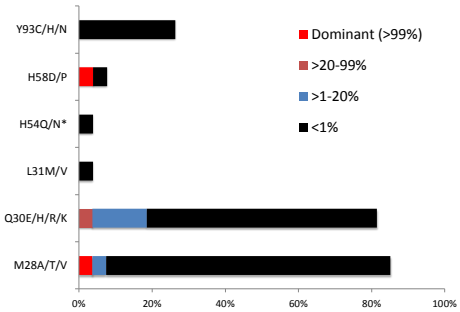


Results: Polymerase gene (n = 50)

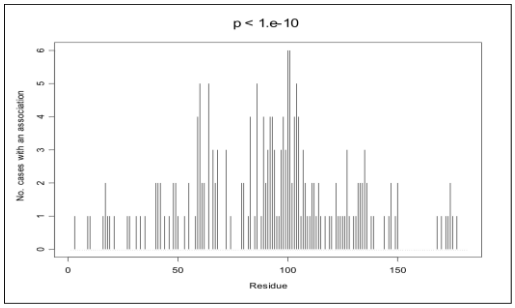
Coverage 2012-6722 reads



Results: NS5A (n=28)

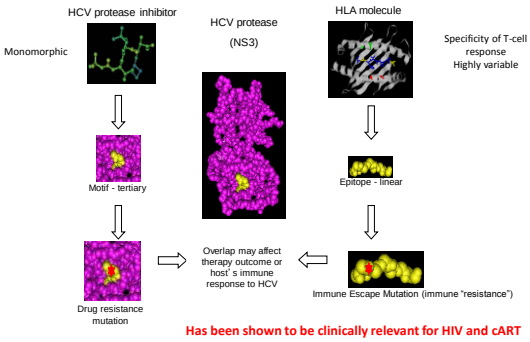


Limited evidence for compensatory mutations – NS3



For most DAA resistance associated sites no evidence of co-variation in more than one subject

Overlap between drug and immune pressure



Has been shown to be clinically relevant for HIV and cART

(John et al Antiviral Ther 2005)

Limited evidence of effect of immune pressure on frequency of RAVs – NS3

- HLA-A2-restricted epitope CINGVCWTV includes T54 and V55
  - **3/8 HLA-A2 positive >1% RAV at V55** and 2/17 HLA-A2- have RAV >1% at V55
- HLA-A24-restricted epitope MYTNVDQDL includes Q80.
  - **1/4 HLA-A24+ dominant K** and 2/13 HLA-A24- have different dominant amino acid
- HLA-A2-restricted epitope HAVGIFRAA includes 155 and 156
  - **1/8 with HLA-A2 RAV 14.7% at 155**. No change >1% within HLA-A2-

Limited evidence of effect of immune pressure on frequency of RAVs – NS5B

- HLA-A3-restricted epitope SLTPPHSAK includes 96
  - **0/4 HLA-A3+ but no RAVs >1%**
- NS5B HLA-B27-restricted epitope ARMILMTHF includes 423
  - **0/2 HLA-B27+ no RAV >1%**, no RAV >1% for HLA-B27-
- NS5B HLA-A1-restricted epitope QLEQALDCEIY includes 448
  - **0/9 HLA-A1+ with RAV >1%**, no RAV >1% for HLA-A1-

Summary

- **Next generation sequencing identifies low frequency RAVs in most individuals but typically <1%**
  - **Relevance of low frequency variants in DAA treated subjects unknown**
  - **Presence of compensatory mutations will be investigated within a longitudinal cohort + boceprevir**
- **No obvious association between RAV frequency or number with HIV status, duration of infection or adaptive immune response**
- **Future use of primer ID adaptations/3rd gen sequencing technologies can eliminate amplification bias**

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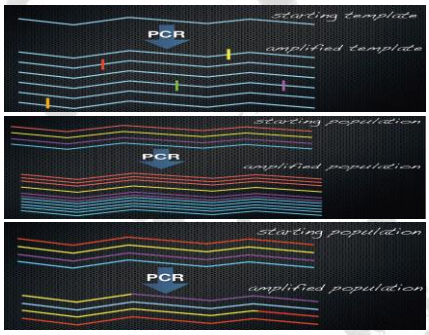
	1a			1b			3a		
NS5A (wt/RAV)		Chronic (n=160)	Acute (n=58)		Chronic (n=34)	Acute (n=12)		Chronic (n=90)	Acute (n=16)
M28A/T/V	M/V/T	5.6	1.7	L	0	0	M	0	0
Q30E/H/R/K	Q/H	0.6	0	R/Q	12.9	28.6	A/K/S/M	8	0
L31M/V	L/M	0.6	0	L/M	6.5	0	L	0	0
Q54H/N	H/Y	0.7	0	Q/H/Y/N	31.3	42.9	S/T	6.7	20
H58D/P	H/P/R/L/Y	6	8.6	P/S	3.2	0	P/A/S	3.4	0
Y93C/H/N	Y	0	0	Y	0	8.3	Y/H	1.2	0

	1a			1b			3a		
NS5B polymerase (wtRAV)		Chronic (n=205)	Acute (n=64)	□	Chronic (n=54)	Acute (n=13)	□	Chronic (n=146)	Acute (n=50)
S96T	S	0	0	S	0	0	S	0	0
S282T	S/N	0.6	0	S/G	2.3	0	S/N	1.9	0
C316Y/N	C	0	0	C/N	11.6	38.5	C	0	0
S365T/N	S	0	0	S	0	0	S	0.8	0
M414T/L	M	0	0	M	0	0	M	0	0
L419M/V	L	0	0	L	0	0	L/I	0	2.3
M423T/I/V	M/I/A/V	2.4	1.7	M	0	0	M/I/A/V	0	0
Y448C/H	Y	0	0	Y	0	0	Y	0	0
I482L	I	0	0	I	0	0	I	0	0
V494I/A	V/I	1.4	0	V	0	0	V/I	2.1	0
P495S/L/A/T	P	0	0	P	0	0	P	0	0
P496S/A	P	0	0	P	0	0	P	0	0
V499A	A/T	5.6	0	V/T/A	30	66.7	A/T/V	2.2	2.5

□

PCR artefacts and errors



Misincorporation

Differential Amplification ("PCR fitness")

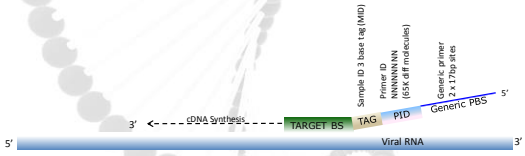
Recombination (Especially at high [Template] and # cycles)

Image courtesy of Dr Cass Jabara UNC

The Primer ID method was first used in HIV variant sequencing and relies on a special primer in the RT step

Molecular tagging of viral cDNA using a PID

Unique tagging during RT reaction



8 random bases gives 4^8=65,536 unique combinations of PID

Manufacture N8 critical
Critical that the ratio of template to #PID is kept low

Adapted from Jabara CB, et al. Accurate sampling and deep sequencing of the HIV-1 protease gene using a Primer ID. Proc Natl Acad Sci U S A. 2011 108:20166-71

This figure summarises the PID process

Considerations

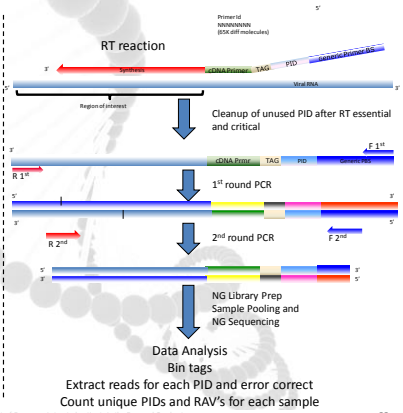
RT
Binding site polymorphisms
Possible interference (TAG & PID)
Incomplete cleanup

PCR
Pr binding site conservation
Amplicon not too long

PCR efficiency of each seq
PCR error

Sequencing
Clonal amplification errors
Sequencing errors

PID consesus correctable



We used this approach to identify RAVs in a subset of Boceprevir treated patients in the Respond-2 cohort