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Characterization of Naturally Occurring Resistance to HCV NS5A Inhibitors

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BACKGROUND

- Clinical trials with some NS5A inhibitor-containing regimens have resulted in less favorable responses among individuals with GT1a compared to GT1b HCV.
- Previous studies have identified mutations in NS5A that emerge under NS5A inhibitor pressure and confer reduced inhibitor susceptibility.
- To assess whether quantitative or qualitative differences in naturally occurring resistant associated variants (RAVs) may explain the discordant response rates, we used deep sequencing and recombinant replicons to determine the prevalence and resistance profiles of NS5A inhibitor resistant variants in HCV from DAA treatment-naïve individuals.

METHODS

- The NS5A coding region of 109 genotype 1 viruses were amplified from patient plasma and cloned into a subgenomic HCV 1b replicon (Con1).
- Libraries for deep sequencing on the Illumina MiSeq were prepared using the Nextera XT library preparation kit.
- Analysis of deep sequencing reads were performed using Monogram's deep sequencing pipeline. Positions 28, 30, 31, 32, and 93[1,2] were evaluated for RAVs.
- NS5A nucleotide sequences were also determined using conventional dideoxy chain-termination chemistry and compared to NGS results.
- Susceptibility of the recombinant replicons (IC50 fold-change relative to the Con1 reference replicon (FC)) or site-directed mutants (SDMs) to NS5A inhibitors and interferon was measured using a cell-based replicon assay.

RESULTS

- The NS5A region of 109 HCV genotype 1 (1a=71, 1b=38) clinical isolates were evaluated. Analysis of predicted NS5A amino acid sequences identified 35 viruses (32%; 1a=27 (38%),1b=8 (21%))

Table 1: Ultra-deep Sequencing Analysis of NS5A Sequences from 109 HCV Infected Individuals

Sample Number	Mutation List	Mean depth	Min Depth	Subtype	M28T Frequency Mutant Reads/ Total Reads	M28V Frequency Mutant Reads/ Total Reads	Q30H Frequency Mutant Reads/ Total Reads	L31M Frequency Mutant Reads/ Total Reads	P58S Frequency Mutant Reads/ Total Reads	Y93C Frequency Mutant Reads/ Total Reads	Y93H Frequency Mutant Reads/ Total Reads	Y93N Frequency Mutant Reads/ Total Reads	Drug 1 IC95 FC	Drug 2 IC95 FC
1	M28V	73628	36314	1a	-	***0.9938*** ***64,928/65,333***	-	-	-	-	-	-	3.8	4.5
5	M28T	64904	48661	1a	***0.9924*** ***75,438/76,016***	-	-	-	-	-	-	-	max	max
15	M28T+Q30H	57587	27448	1a	0.0069 534/77,448	-	***0.9059*** ***69,235/76,427***	-	-	-	-	-	max	max
18	M28V+Q30H+Y93H	41641	21750	1a	-	0.0065 378/58,102	***0.9898*** ***56,390/56,971***	-	-	-	***0.983*** ***27,146/27,616***	-	max	max
19	M28V	92175	37482	1a	-	***0.8092*** ***72,795/89,959***	-	-	-	-	-	-	3.1	2.3
23	M28T+Q30H+Y93H+Y93N	58300	38145	1a	0.0066 379/57,389	-	***0.4545*** ***27,318/60,105***	-	-	-	***0.3226*** ***18,854/58,444***	***0.4373*** ***25,558/58,444***	max	max
46	M28V	85554	54754	1a	-	***0.9932*** ***68,136/68,602***	-	-	-	-	-	-	3.4	1.9
49	M28V+Q30H	60331	35191	1a	-	0.007 488/69,696	***0.9786*** ***67,790/69,273***	-	-	-	-	-	max	max
50	L31M	102665	50344	1a	-	-	-	***0.1355*** ***9,376/69,196***	-	-	-	-	max	67.4
56	M28V+Y93H+Y93N	97440	58017	1a	-	0.141 13,448/95,347	-	-	-	-	0.0189 2,292/121,296	0.0299 3,628/121,296	3.7	4.4
4	M28V	108881	66219	1a	-	0.0077 712/92,429	-	-	-	-	-	-	2.5	2.9
8	M28V	109952	55732	1a	-	0.0198 2,658/134,230	-	-	-	-	-	-	2.3	2.3
9	M28V	37798	24802	1a	-	0.0054 226/42,328	-	-	-	-	-	-	2.8	3.2
12	M28V+L31M	45305	13815	1a	-	0.0073 321/43,909	-	0.0094 445/47,311	-	-	-	-	2.9	2.2
21	M28V	68454	39624	1a	-	0.0064 367/57,282	-	-	-	-	-	-	2.7	2.1
25	Y93H	73863	51111	1a	-	-	-	-	-	-	0.0092 672/73,049	-	3.8	4.3
26	M28V	52469	39879	1a	-	0.0069 442/64,017	-	-	-	-	-	-	3.1	4.2
29	M28V	74278	37605	1a	-	0.0079 388/49,052	-	-	-	-	-	-	3.5	3.4
33	M28V	34999	28818	1a	-	0.009 333/37,040	-	-	-	-	-	-	3.3	2.6
41	M28V	63727	45584	1a	-	0.0209 1,271/60,791	-	-	-	-	-	-	3.0	2.4
43	Y93C	49299	33998	1a	-	-	-	-	-	0.0227 1,225/53,946	-	-	3.2	4.1
47	M28V	94476	46621	1a	-	0.0096 623/64,906	-	-	-	-	-	-	1.6	1.6
48	M28V+L31M+Y93N	88555	54290	1a	-	0.0056 484/86,504	-	0.0107 924/86,357	-	-	-	0.0311 3,081/99,072	2.0	2.1
51	M28V	107280	56163	1a	-	0.0089 962/107,711	-	-	-	-	-	-	3.1	3.0
59	M28V	75454	37298	1a	-	0.0114	-	-	-	-	-	-	2.9	2.5
60	M28V	97293	50176	1a	-									
62	M28V	16067	10993	1a	-									
H77	-	113408	18736	1a	-									

Sample Number	Mutation List	Mean depth	Min Depth	Subtype	M28T Frequency Mutant Reads/ Total Reads	M28V Frequency Mutant Reads/ Total Reads	R30Q Frequency Mutant Reads/ Total Reads	L31M Frequency Mutant Reads/ Total Reads	P58S Frequency Mutant Reads/ Total Reads	Y93C Frequency Mutant Reads/ Total Reads	Y93H Frequency Mutant Reads/ Total Reads	Y93N Frequency Mutant Reads/ Total Reads	Drug 1 IC95 FC	Drug 2 IC95 FC
73	R30Q	79049	38586	1b			***0.7433*** ***49838/67135***						0.7	0.8
78	L31M+Y93H	78365	43131	1b	-	-	-	***0.9882*** ***70,432/71,273***	-	-	***0.2072*** ***19,942/96,246***	-	max	max
109	Y93H	86715	53814	1b	-	-	-	-	-	-	***0.4924*** ***41,224/83,721***	-	max	max
90	P58S+Y93H	40926	27562	1b	-	-	-	-	0.0117 518/44,275	-	0.0057 257/45,129	-	1.3	1.2
92	Y93H	71202	51613	1b	-	-	-	-	-	-	0.0058 423/72,896	-	N.T.	N.T.
98	Y93H	73501	47536	1b	-	-	-	-	-	-	0.0054 445/82,397	-	1.2	0.9
99	Y93H	73650	50094	1b	-	-	-	-	-	-	0.0107 786/73,484	-	1.0	0.7
108	Y93H	80568	49918	1b	-	-	-	-	-	-	0.0072 674/93,573	-	1.1	1.2
Con1		171366	86980	1b	-	-	-	-	-	-	-	-	1.0	1.0

Bold indicates mutants were also detected by Sanger sequencing

Table 2: Summary of Resistance Associated Variants Detected Using Ultra-deep Sequencing

Subtype	N (%)	M28T	M28V	Q30H (1a) R30Q (1b)	L31M (1a) L31M/F (1b)	Y93C	Y93H	Y93N	Total Samples with Primary Positions above 0.5%
1a	71 (100)	3 (4.2)	21 (29.6)	4 (5.6)	3 (4.2)	1 (1.4)	4 (5.6)	3 (4.2)	27 (38.0)
1b	38 (100)	-	-	1 (2.6)	1 (2.5)	-	7 (17.9)	-	8 (21.0)

Figure 1: Phylogenetic Tree

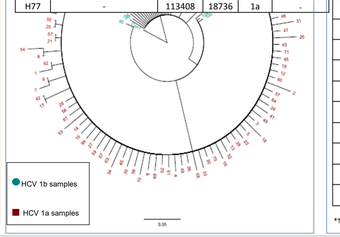


Figure 2: Susceptibility of Site-Directed Mutants to an NS5A Inhibitor

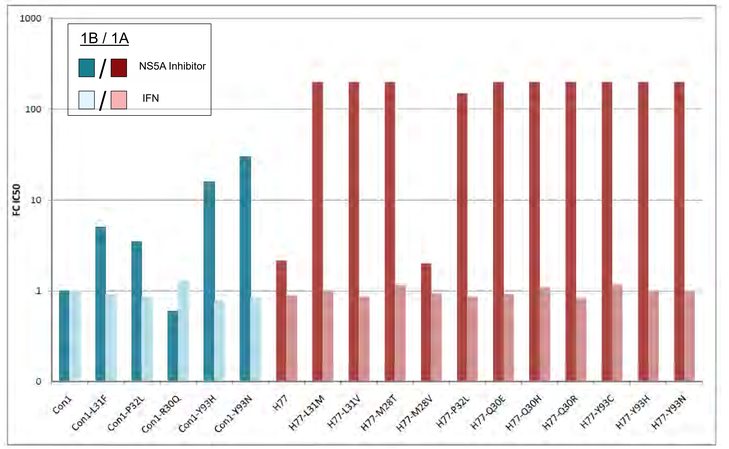


Table 3: Reproducibility of NGS results

Sample Number	Subtype	Mean Depth	Min Depth	Replicate	Mutation 1	Frequency	Mutation 2	Frequency	Mutation 3	Frequency
12	1a	21340	7924	1	L31M	0.0095	M28V	0.0057		
		45305	13815	2	L31M	0.0094	M28V	0.0073		
15	1a	45124	22234	1	Q30H	0.9188	M28T	0.0065		
		57587	27448	2	Q30H	0.9059	M28T	0.0069		
18	1a	41641	21750	1	Q30H	0.9898	Y93H	0.983	M28V	0.0065
		47200	22008	2	Q30H	0.9924	Y93H	0.9854	M28V	0.0061
48	1a	88555	54290	1	L31M	0.0107	Y93H	0.0311	M28V	0.0056
		31399	22952	2	L31M	0.0107	Y93N	0.0307	M28V	0.0058
49	1a	60331	35191	1	Q30H	0.9786	M28V	0.007		
		135405	79044	2	Q30H	0.9859	M28V	0.0072		
56	1a	97440	58017	1	M28V	0.141	Y93H	0.0189	Y93N	0.0299
		24903	17698	2	M28V	0.1513	Y93H	0.0169	Y93N	0.028
60*	1a	97293	50176	1	M28V	0.0051				
		66193	43754	2	M28V	0.0045				
73	1b	79049	38586	1	R30Q	0.7433				
		104109	76594	2	R30Q	0.7499				
78	1b	78365	43131	1	L31M	0.9882	Y93H	0.2072		
		111079	68055	2	L31M	0.9905	Y93H	0.2193		
92	1b	64418	45261	1	Y93H	0.0065				
		71202	51613	2	Y93H	0.0058				
98	1b	73501	47536	1	Y93H	0.0054				
		68703	46532	2	Y93H	0.0054				
99	1b	66996	51613	1	Y93H	0.0107				
		73650	50094	2	Y93H	0.0107				

*The second replicate of sample 60 was not above the 0.5% threshold

RESULTS (continued)

containing amino acid substitutions at resistance-associated positions to NS5A inhibitors at 0.5% or greater (**Table 1 and 2**).

- The RAVs detected by NGS in GT1a viruses included M28T=3, M28V=21, Q30H=4, L31M=3, Y93C/H/N=6. RAVs detected in GT1b viruses included R30Q=1, L31M=1, Y93H=7 (**Table 2).**
- 7/27 (26%) GT1a viruses contained ≥ 2 RAVs; 2/8 GT1b viruses contained ≥ 2 RAVs (25%).
- Eleven (1a=8, 1b=3) of the 35 samples with mutations were detected by population sequencing (**Table 1**), and were present at >10% (range:13.6-99.2%) of reads.
- The reproducibility of the NGS results was robust with a median FC between runs of 1.02 **(Table 3)**.
- Of the 11 samples with RAVs detected by population sequencing, eight viruses had marked reductions in NS5A inhibitor susceptibility to the NS5A inhibitors tested **(Table1)**.
- Analysis of SDMs indicated the reductions in susceptibility to NS5A inhibitor were more pronounced in the replicons containing 1a NS5A sequence (FC>150) than the replicons containing 1b NS5A sequence (FC<20). All SDM replicons exhibited similar interferon susceptibilities (**Figure 2**).

CONCLUSIONS

- The prevalence of resistant variants was slightly higher in GT1a versus GT1b isolates.
- NS5A RAVs were more diverse among GT1a viruses compared to GT1b.
- The combination of multiple resistance pathways, lower genetic and resistance barriers may provide advantages for GT1a viruses to escape NS5A inhibition.

REFERENCES

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