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Short communication

Prevalence of baseline HCV NS5A resistance associated substitutions in genotype 1a, 1b and 3 infection in Australia

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ABSTRACT

Background: Direct-acting antivirals (DAA) have revolutionised hepatitis C virus (HCV) treatment, and most regimens include an NS5A inhibitor. Certain amino-acid substitutions confer resistance to NS5A inhibitors, termed resistance-associated substitutions (RAS). If present at baseline, they can reduce virological response rates. Population-based sequencing (PBS) is generally used for baseline sequencing, however next generation sequencing (NGS) reduces the threshold for detection of sequences encoding RAS from 20% to 5%. We determined the prevalence of NS5A RAS at baseline amongst Australian chronically infected with genotype (GT)1a, GT1b and GT3 HCV, using both PBS and NGS.

Methods: Samples from DAA-naïve individuals were received at the Victorian Infectious Disease Reference Laboratory between June 2016 and December 2018. All samples were analysed for NS5A RAS using PBS. A subset of GT1 HCV samples were processed using NGS technology (Vela Diagnostics, Singapore) to determine the improvement in sensitivity.

Results: In total, 672 samples were analysed using PBS. The baseline prevalence of NS5A RAS was 7.6% for GT1a (n = 25/329), 15.7% for GT1b (n = 8/51) and 15.1% for GT3 (n = 44/292). NGS only marginally increased sensitivity for NS5A RAS at baseline in GT1a (16% vs 17%) and GT1b (29% vs 36%).

Conclusion: The prevalence of NS5A RAS in GT1a HCV in Australia was low compared with international data, and was similar to other reported international prevalence for GT1b and GT3 infection. NGS at baseline only marginally increased sensitivity for the detection of NS5A RAS in patients with GT1 HCV and cannot be recommended for routine use at baseline in clinical practice.

1. Background

It is estimated that hepatitis C virus (HCV) infection affects 71 million people globally [1]. Direct-acting antiviral (DAA) therapy targeting the NS3, NS5A and the NS5B proteins has revolutionised HCV treatment, and cure rates exceed 90% in real world settings. Amongst those infected, the poor fidelity of the HCV viral polymerase generates a range of genetic variants, some which result in amino-acid polymorphisms. These can reduce viral susceptibility to DAAs and are known as resistance-associated substitutions (RAS); they have been detected at baseline and virological relapse.

The presence of HCV NS5A RAS at baseline can affect treatment outcomes and certain NS5A RAS have been implicated in diminished sustained virological response rates (SVR) [2–4]. As such, guidelines recommend HCV NS5A RAS testing at treatment baseline for particular regimens, including elbasvir/grazoprevir; for this regimen, the detection of specific NS5A RAS requires extension of treatment duration and addition of ribavirin [5]. The prevalence of baseline HCV RAS varies among geographical regions. In Australia there are limited data regarding the prevalence of baseline genotype (GT)1a and GT1b NS5A RAS [6]. Recent data has demonstrated a baseline GT1a NS5A RAS prevalence of 11.9% in one Australian region [7], however there are no Australian prevalence data regarding baseline HCV GT3 or GT1b NS5A RAS, nor data evaluating the utility of next generation sequencing (NGS) at baseline.

Population-based sequencing (PBS) for detection of NS5A RAS has a threshold limit of approximately 20%. The added yield of NGS, as well as the clinical relevance of low frequency variants remains unclear [8].

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In this context, we determined the frequency of NS5A RAS in DAA-naïve HCV GT1a, 1b and 3 in Australia, using both PBS and NGS.

2. Objectives

We tested for NS5A RAS in a large population of DAA-naïve individuals who had a baseline serum sample sent for sequencing studies prior to DAA treatment in Victoria, Australia. We restricted analysis to individuals infected with HCV GT1a, GT1b or GT3 infection. All subjects were tested for NS5A RAS using PBS. A subset of participants with GT1a and GT1b HCV were also tested for NS5A RAS using NGS.

3. Study design

3.1. Clinical samples

All serum samples received at the Victorian Infectious Disease Reference Laboratory between June 2016 and December 2018 were investigated. HCV genotype was previously determined with the Abbott RealTime genotyping II assay or the Versant HCV Genotype 2.0 assay (Siemens Healthcare Diagnostics, Surrey, UK). All participants were verified as DAA-naïve from their medical record. RNA was extracted from 200 µL of patient serum and eluted to 100 µL by an automated method, using the Abbott m2000 instrument (Abbott MolecularAbbott Park, IL) and the Sample Preparation System$_{RNA}$ kit (AbbottMolecular).

3.2. Population sequencing approach

The NS5A region (639 nt) was amplified in a nested PCR using primers with Superscript III one-step RT-PCR system with Platinum Taq (Invitrogen, Waltham, MA) and Taq DNA Polymerase (Qiagen, Hilden, Germany). Purified PCR product was sequenced using ABI-Prism 3730 Genetic Analyser (Applied Biosystems, Life Technologies, Ltd, Paisley, UK). Using the ABI sequence analysis program SeqScape version 2.1.1 (Applied Biosystems), a consensus sequence was generated and aligned to a stored GenBank HCV reference sequence (Subtype 1a H77, subtype 1b Con 1, and subtype 3a HPCK3A).

3.3. Next generation sequencing approach

A subset of HCV GT1 samples were also tested using the NGS platform *Sentosa*® SQ HCV Genotyping assay (Vela Diagnostics, Singapore). With an initial input of 530 µL of serum, RNA was isolated from serum samples by an automated method using the Vela Diagnostics *Sentosa*® SX101 instrument. The PCR amplification step was performed off-board and the plate returned to the *Sentosa*® SX101 instrument for normalisation, enzymatic shearing, purification, adapter ligation and library pooling. After emulsion PCR and enrichment of the ion sphere particles, the amplicons were then subjected to NGS on the *Sentosa*® SQ301 Sequencer, with the data analysed by the *Sentosa*® SQ Suite software. The threshold for detection of this platform is 5%.

3.4. Definition of NS5A resistance associated substitutions

HCV NS5A RAS were defined as polymorphisms encoded by the NS5A gene associated with at least two-fold reduced susceptibility to available NS5A inhibitors in *in-vitro* cell-based replicon assays [9–11]. All changes at positions 24, 28, 30, 31, 58, 92 and 93 were investigated.

3.5. Ethical considerations

This study was approved by the St Vincent's Hospital Melbourne Human Research Ethics committee (HREC/17/SVHM/143).

Table 1
Frequency of NS5A RAS at treatment baseline amongst subjects chronically infected with GT1a, 1b and 3 HCV using a population sequencing approach.

Frequency of baseline HCV RAS, N (%)	NS5A RAS	GT1a	GT1b	GT3
Samples with NS5A RAS identified		25/329 (7.6)	8/51 (15.7)	44/292 (15.1)
Single RASs at NS5A position:				
28	M28L	1 (0.3)		
	M28T	2 (0.6)		
	M28V	9 (2.7)		
30	A30K			15 (5.1)
	A30S			4 (1.4)
	A30V			1 (0.3)
	Q30H	1 (0.3)		
	Q30R	1 (0.3)		
31	L31M	6 (1.8)	3 (5.9)	
	L31V	1 (0.3)		
92	A92T		1 (2.0)	
93	Y93H	1 (0.3)	4 (7.8)	18 (6.2)
	Y93N	1 (0.3)		
	Y93S	1 (0.3)		
Dual RAS at NS5A position				
28, 58	M28 V, H58Q	1 (0.3)		
30, 31	A30 K, L31M			2 (0.7)
93, 30	Y93H, A30K			2 (0.7)
	Y93H, A30T			2 (0.7)

Prevalence of NS5A RAS detected via population sequencing at baseline, presented by genotype, as frequencies and (percentages). Abbreviations: GT, genotype, RAS, resistance-associated substitution, HCV hepatitis C Virus.

4. Results

4.1. Prevalence of NS5A RAS using PBS

In total, 672 samples were analysed. There were 329 HCV GT1a samples, and 26 NS5A RAS were identified in 25 patient samples (7.6%, n = 25/329) (Table 1). The most frequent GT1a NS5A RAS were M28 V (2.7%, n = 9/329) and L31 M (1.9%, n = 6/329). Dual NS5A RAS were identified in one sample (Table 1).

There were 51 HCV GT1b samples. NS5A RAS were identified in 15.7% (8/51) (Table 1). Y93H was most frequently identified (7.8%, n = 4/51), then L31 M (5.9%, n = 3/51) and A92 T (2.0%, n = 1/51).

There were 292 GT3 HCV samples (subtype 3a accounts for almost all GT3 infection in Australia [12]) and 47 NS5A RAS were identified in 44 patient samples (15.1%, n = 44/292)(Table 1). The most prevalent single RAS included Y93H (6.2%, n = 18/292) and A30 K (5.1%, n = 15/292). Dual RAS were identified in six samples(Table 1).

4.2. Benefit of NGS at baseline in GT1a and 1b samples

Eighty-nine GT1a and 14 GT1b samples were also analysed using NGS, of which 75 and 10 respectively had no NS5A RAS detected by PBS. NGS confirmed that the RAS detected by PBS were the dominant quasispecies (Table 2). NGS detected additional low frequency variants in 2/89 GT1a samples (in one of which no RAS was detected by PBS) and 1/14 GT1b samples, Table 2).

5. Discussion

This is the first Australian data describing the frequency of baseline HCV GT1b and GT3 NS5A RAS amongst DAA-naïve individuals with chronic HCV infection, and contributes to recent data regarding HCV GT1a RAS [7].

HCV NS5A RAS were detected in 7.6% of people with GT1a HCV

Table 2
Positive NS5A RAS results among a subset of GT1a and GT1b HCV, including the increased sensitivity of NGS over PBS methods.

Sample code	Population based sequencing	Next Generation sequencing
GT1a		
8	M28V	M28V (52.3%)
11	M28V	M28V (91.1%)
15	L31M	L31M (99.65%)
21	Y93H	Y93H (16.4%)
36	L31M	L31M (28.3%)
37	L31M	L31M (99.54%)
39	M28T	M28T (99.78%)
45	M28V	M28V (96.7%) Q30R (5.6%)
50	Q30H	Q30H (99.7%)
53	M28T	M28T (17.0%)
62	M28V	M28V (99.1%)
83	L31M	L31M (45.2%)
86	No RAS detected	L31M (5.4%)
87	L31M	L31M (98.9%)
88	M28V	M28V (66.4%)
GT1b		
1	L31M	L31M (79.6%)
2	L31M	L31M (99.5%)
3	L31M	L31M (99.1%)
9	No RAS detected	Y93H (8.2%)
14	Y93H	Y93H (68.3%)

Comparison between the frequency of detection of NS5A RAS between PBS and NGS methodologies. GT1a samples 45 and 86 and GT1b sample 9 (highlighted in blue) represent serum samples where a single or dual NS5A RAS were identified by NGS (including the percentage of the prevalent virus) which was not detected by PBS.

infection. The most prevalent GT1a NS5A RAS were M28 V and L31 M. Certain baseline NS5A RAS at positions M28, Q30, L31 and Y93 are relevant for GT1a patients treated with elbasvir and grazoprevir, and pre-treatment testing is recommended when this regimen is utilised in certain regions. Our data demonstrates a low prevalence of these substitutionsin HCV infected Australian patients. The prevalence of GT1a NS5A RAS in this study was lower than recent Australian data and may be partly explained by the exclusion of secondary variants such as at residue H58, when identified in isolation [7]. Large international DAA registration trials have demonstrated a higher prevalence of 11–23% in GT1a HCV [3,13,14], and 16% in a subset from Oceania [6]. Smaller overseas studies have also reported higher GT1a NS5A RAS frequencies of 10–17% (15–17). Our lower prevalence supports Australian guidelines that baseline PBS to detect pre-existing RAS is not indicated [18].

The frequency of RAS among GT1b and GT3 HCV was similar to other international studies. Our analysis demonstrates baseline GT1b NS5A RAS frequency of 15.7%, similar to international data(16–33%) [3,6,14–16,19,20]. The prominent GT1b NS5A RAS identified included Y93H (7.8%) and L31 M (5.9%), concordant with other geographical regions.

The baseline frequency of GT3 NS5A RAS was similar to that in North America and Europe (12–19%) [3,14,16,21], however lower compared to other countries in the Asian-Pacific region. This includes China (> 50%), reflecting their high prevalence of GT3b infection and

likelihood of baseline dual A30 K/L31 M RAS [20]. Y93H was the most prevalent GT3 NS5A RAS (7.5% including where present as a dual substitution) conferring significant resistance to all NS5A inhibitors in vitro, and reducing virological outcomes in GT3 patients treated with sofosbuvir/velpatasvir [3]. A30 K was identified in 6.5% of samples, including those with dual NS5A RAS. A30Khas been associated with reduced SVR12 in non-cirrhotic GT3 infected patients treated with glecaprevir/pibrentasvir for 8 weeks, a regimen used commonly in Australia [22]. Sequencing for GT1b and GT3 RAS at baseline is not currently recommended in treatment naïve individuals.

We evaluated 103 GT1 HCV samples using both NGS and PBS. The increased diagnostic yield of NGS was low, and only a small number of additional NS5A RAS at low frequencies were identified. North American and European guidelines designate that only RAS with a prevalence of > 15% are clinically significant [23,24], greater than the prevalence of the additional RAS identified via NGS. Given the overall low frequency of NS5A RAS in GT1 participants in Australia at baseline, and the minimal improvement in sensitivity, our data suggests that NGS does not have a role in routine pre-treatment assessment in DAA-naïve patients.

In summary, amongst DAA-naïve patients, baseline GT1a NS5A RAS were lower compared to international data, and comparable amongst GT1b and GT3 populations. Detection of HCV NS5A RAS by NGS at baseline only marginally increased diagnostic sensitivity and therefore has limited clinical applicability.

Author contribution statement

All authors contributed substantially to either the conception, design, acquisition, analysis or interpretation of the data and the drafting or revising the intellectual content and approved the final version being considered for publication. All authors agree to be accountable for all aspect of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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