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Short communication

Prevalence of baseline polymorphisms for potential resistance to NS5A inhibitors in drug-naive individuals infected with hepatitis C genotypes 1–4

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Abstract

Background: The non-structural 5A (NS5A) protein of HCV is a multifunctional phosphoprotein involved in regulation of viral replication and virion assembly. NS5A inhibitors targeting domain I of NS5A protein have demonstrated high potency and pan-genotypic antiviral activity, however they possess a low genetic barrier to resistance. At present, only genotype 1, the most prevalent HCV genotype has been studied in detail for resistant variants.

Methods: Utilising a panel of genotypic-specific resistance assays, population sequencing was performed on plasma derived viral RNA isolated from 138 patients infected with HCV genotypes 1-4 and not treated with directly acting anti-viral agents (DAAs). Amino acid changes in HCV NS5A domain I at codon positions 28, 30, 31, 32 and 93, reported to confer reduced susceptibility to certain NS5A inhibitors were examined. Additionally, genotypic outcome based on NS5A sequences were compared with LiPA and Abbott® real time.

Results: Amino acid substitutions associated with moderate to high level resistance to NS5A inhibitors were detected in 2/42 (4.76%) HCV-1a, 3/23 (13.04%) HCV-1b, 4/26 (15.38%) HCV-2, 1/24 (4.17%) HCV-3 and 1/23 (4.35%) HCV-4 infected patients who had not been treated with NS5A inhibitors. Genotype prediction based on NS5A sequences were concordant with LiPA and/or Abbott® real-time for 97.10% of cases.

Conclusions: Primary resistance mutations associated with resistance to first generation NS5A inhibitors such as Daclatasvir (DCV) were observed in all genotypes, albeit at low frequencies. An excellent correlation based on NS5A genotyping and LiPA or Abbott® real-time was achieved.

Introduction

A number of direct-acting antiviral agents (DAAs) targeting protease or polymerase enzymes encoded by hepatitis C virus (HCV) are under development or in clinical trials for the treatment of HCV. NS5A, a membrane-associated phosphoprotein of HCV, plays a crucial role in regulating viral replication and host cell interactions [1,2]. NS5A inhibitors targeting domain I of NS5A protein are promising with their pan-genotypic antiviral activity and once daily dosing. Their resistance profiles do not overlap with other DAAs, and therefore can provide IFN-free treatment options in combination with other DAAs.

NS5A inhibitor monotherapy rapidly leads to emergence of resistant associated variants [3,4]. The efficacy of DAAs can be genotype/subtype dependent, mainly due to HCV diverse genetic variability, mainly due to the presence of natural polymorphisms associated with resistance. Polymorphisms in the N-terminus domain I of NS5A, with amino acid changes at positions M28T, Q30E/H/R, L31M/V, P32L, Y93C/H/N and L31F/V and Y93H/N for genotypes 1a and 1b respectively confer resistance to NS5A inhibitors based on *in vitro* and *in vivo* data [4–7].

As the majority of NS5A resistance data is based primarily from patients infected with genotype 1, the aim of this study was to assess the prevalence of NS5A domain I resistance associated polymorphisms in NS5A DAA naïve HCV patients infected with genotypes 1-4. Secondly, we evaluate a panel of NS5A resistance assays as potential genotypic predictor tools.

Methods

HCV RNA was quantified using Abbott[®] real-time assay and genotyped using Abbott® real-time genotyping assay based on 5' UTR sequences, with genotype 1 further subtyped into 1a or 1b by NS5B sequencing. Prior to 2010, genotyping was performed using VERSANT® HCV genotype 2.0 assay (LIPA) (Siemens Healthcare, Surrey, UK), targeting core and 5' UTR.

The entire 213 amino acids of NS5A domain I were amplified from plasma derived HCV viral RNA, using Qiagen[®] one-step RT-PCR system (Qiagen, Crawley, UK), and nested PCR using HotStarTaq PCR (Qiagen, Crawley, UK), incorporating HCV genotype/subtype specific primers (Table 1). Purified PCR amplicons were sequenced using ABI PRISM 3730 Genetic Analyser (Applied Biosystem, Paisley, UK). Consensus sequences were aligned against HCV reference sequences using SeqScape v3.0 (Applied Biosystem, Paisley, UK), and translated into amino acid using Bioedit version 7.2.3 to determine the prevalence of resistant-associated natural polymorphisms. HCV genotypes based on NS5A sequences were compared with those obtained either by LiPA or Abbott[®] assay. Core/E1 and NS5B sequencing was performed on discrepant specimens for confirmation purposes and to identify potential recombination events [8].

Results

PCR amplicons covering the entire HCV NS5A domain I of genotypes 1-4 were successfully generated for 138 HCV patients. Table 2 depicts the prevalence of amino acid polymorphisms associated with resistance to NS5A inhibitors. Amino acid substitutions associated with NS5A inhibitor resistance at codons 28, 30, 31 and 93, existed but were relatively rare events across genotype.

Of forty-two HCV-1a infected patients, only two (4.76%) harboured mutations associated with loss of activity for Daclatasvir (DCV), a first generation NS5A inhibitor. One patient harboured a Q30H/Q and Y93H/Y mixture, the presence of Q30H and Y93H on the same viral genome confers 93,136 fold resistance to DCV [9]. A second patient contained M28V and Q30R, reported to confer 350 fold resistance to DCV [9], whilst in two patients, the M28M/V mixture existed alone, conferring minimal DCV resistance (1.3 fold change) [6]. Additionally, only three HCV-1a patients had the baseline variant E62D, which on its own does not confer DCV resistance [10].

Three (13%) of the 23 HCV 1b patients contained amino acid substitutions involved in NS5A resistance. One patient contained Y93H, conferring 19-24 fold resistance compared with wild type [6,9] whilst a second patient harboured Y93H/Y mixture. L31M was observed in the third patient, which confers 3-fold DCV resistance [6].

Of 26 HCV-2 infected patients, four (15.38%) contained L31M. The NS5A M31 is a common genotype 2 variant [>50%; 11] and was observed in all four HCV-2a patients genotyped, conferring 350 fold DCV resistance. Additionally, thirteen HCV-2b patients harboured S58P polymorphism, and one HCV-2c patient contained K30R polymorphism, both variants conferring minimal DCV resistance effects.

Only one of 24 HCV-3 patients harboured an amino acid substitution implicated in DCV resistance, at codon 30 (A30K). The A30K polymorphism has been reported to confer 44-62 fold resistance and a replicative capacity of 66% compared to wild type in a genotype 3 replicon model [12,13]. Both L31F and Y93H polymorphisms associated with DCV resistance *in vitro* for genotype 3 were not observed in this cohort [13].

Finally, of 23 HCV-4 patients, a single patient harboured a serine at codon 30, instead of an arginine (14/23) or leucine (8/23) at this position, and associated with 167 fold resistance to DCV based on genotype 4 *in vitro* replicon model [15]. Additionally, the majority of NS5A sequences 21/23 (91.30%) harboured L31M, also reported elsewhere in HCV-4, with unknown effects [14]. Interestingly, two patients who harboured a leucine instead of methionine at codon 31 (L31), genotyped as HCV-4r based on NS5A, NS5B and Core sequences. Overall, HCV genotyping based on NS5A sequences were concordant with LiPA or Abbott® real-time for 134/138 (97.10%) of HCV patients. Among the four discordants, two specimens genotyped as HCV-1b by LiPA were subsequently genotyped as HCV-1a on NS5A, Core/E1 and NS5B sequencing. One specimen that could not be genotyped by LiPA (designated as either HCV-1 or HCV-4) and another specimen

originally genotyped as HCV-4 by Abbot real-time, genotyped as HCV-4r when NS5A, Core/E1 and NS5B nucleotide sequences were submitted to http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Discussion

NS5A inhibitors have shown to have pan-genotypic activity; however, there is limited data on the prevalence of natural polymorphisms associated with resistance to NS5A inhibitors, other than genotype 1. NS5A resistance variants frequently pre-exist among HCV patients and thus there is currently a need for genotypic predictions prior to combination therapy to establish optimal use of NS5A inhibitors.

Overlapping resistance signatures are common among NS5A inhibitors in development [3,14]. This study provides the first data on the prevalence of polymorphisms in HCV NS5A domain I for genotypes 1 to 4. In this cohort of 138 patients we were able to detect natural polymorphisms in HCV NS5A domain I, reported to confer moderate to high level resistance to NS5A inhibitors in all genotypes investigated. The prevalence of these signature polymorphisms differed according to genotype/subtype in that NS5A resistance associated polymorphisms were detected in 4.76% HCV-1a; 13.04% HCV-1b; 15.38% HCV-2; 4.17% HCV-3 and 4.35% in HCV-4. These findings are in contrast to the data by Plaza *et al*, 2012 [14], where resistance-associated polymorphisms were only detected in HCV-1b and HCV-4 and absent in HCV-1a and HCV-3, with no available data for HCV-2.

The level of resistance conferred by these natural polymorphisms varies considerably depending upon the amino acid residue and the genetic backbone [6]. In our HCV-1a cohort, the most common resistant-associated variant was at codon 30 (Q30H or Q30R), followed by Y93H, similar to the frequency reported in the European HCV database. The major resistant variant observed in two HCV-1b patients was at codon 93 (Y93H), which predominated over L31M, detected in only a single patient, and supports findings by others [16]. In HCV-2, NS5A L31M polymorphism was observed at a prevalence of 15.38%, which confers 75 fold resistance to NS5A inhibitor, IDX719 [11] and 350 fold DCV resistance. Polymorphisms conferring minimal DCV resistance effects were also observed in HCV-2. For HCV-3, which was least polymorphic, only NS5A A30K resistant variant was observed in a single patient. For HCV-4 NS5A sequences, only one patient harboured the S30 resistant variant, and has been implicated as an important site for DCV resistance *in vitro* [15].

Although available clinical data indicates that baseline polymorphisms or heterogeneity of NS5A sequencing in HCV infected patients has minimal impact on NS5A inhibitor potency, it may significantly affect signature of resistance that emerges [11]. From our observations and others, baseline resistance testing for HCV-2 may be warranted.

Due to a large proportion of HCV quasispecies pre-existing in infected individuals, variants that confer resistance to anti-viral agents can be rapidly enriched and selected during anti-viral therapy [3]. There is a direct correlation between resistant variants emerging *in vivo* with DCV therapy and variants observed *in vitro*, with certain variants persisting months after treatment therapy [17].

Analysis of emergent resistant variants during virological failure is warranted to determine an association with baseline NS5A polymorphisms and to guide future treatment options.

In this study, we obtained excellent concordance between LiPA, Abbott® real time assay and NS5A sequencing as a genotype predictor tool for 97.10% of HCV sequences. In addition, based on NS5A sequencing we were able to genotype four discordant specimens that were either misclassified or unable to be genotyped by commercial assays. Our observation of two cases of HCV-1a misclassified as HCV-1b by LiPA has been published elsewhere [8], and attributed to the low heterogeneity of 5'UTR.

A limitation of our study is the small data set of non-genotype 1 HCV infected patients and the use of population sequencing which detects variants at 20% compared with ultra-deep sequencing detecting minority variants below 1%. Future work on a larger cohort including NS5A DAA treated patients and studying the entire NS5A gene (domains I, II and III) will be informative.

Disclosure Statement:

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Table 1: Genotypic specific oligonucleotides used for PCR amplification of HCV NS5A domain 1

HCV genotype/ subtype	Primer name	5'-3' nucleotide sequence	Position based on H77 locus	Primer T_m
1a	**RT PCR:** Out forward 1a Out reverse 1a **Nested PCR:** In forward 1a In reverse 1a	5' <u>GTTGGCCCGGGCGAGG</u>3' 5' <u>CGCCCATCTCCTGCCTCCA</u>3' 5' <u>TCCCCCACGCACTAYGTG</u>3' 5' <u>GTGCAAGTTGCCTTGAGAG</u>3'	6057-6072 7038-7056 6129-6146 6970-6988	58°C 58°C
1b	**RT PCR:** Out forward 1b Out reverse 1b **Nested PCR:** In forward 1b In reverse 1b	5' <u>TGGATGAACCGGCTGATAG</u>3' 5' <u>CCACAGGAGRTTGGCCTC</u>3' 5' <u>TCCCCCACGCACTAYGTG</u>3' 5' <u>CTRGCYGARGAGCTGGCC</u>3'	6084-6102 7023-7040 6129-6146 6935-6952	57°C 57°C
2	**RT PCR:** Out forward 2 Out reverse 2 **Nested PCR:** In forward 2 In reverse 2	5' GCGGTCCAGTGGATGAACAG3' 5' ACTCRATCCGGGTCACRTC3' 5' CACTACGTGRCRGAGTCTG3' 5' AGCTGGCTYGCTGARGAGC3'	6075-6094 7059-7077 6138-6156 6940-6958	57°C 57°C
3	**RT PCR:** Out forward 3 Out reverse 3 **Nested PCR:** In forward 3 In reverse 3	5' TGGATGAACAGGCTCATYGC3' 5' ATGTTRCTGCCCATCTCTTG3' 5' GTYCCCGAGAGCGATGCYGC3' 5'AGTTGGCTGGRGAYGAGCTYGC3'	6084-6103 7044-7063 6144-6163 6936-6958	55°C 57°C
4	**RT PCR:** Out forward 4 Out reverse 4	5' GATCATGAGYGGCGAG3' 5' GAYGGRTCTGTCARCATGG3'	5942-5957 6859-6877	55°C

Primers underlined have been modified with degeneracies and published elsewhere [14]. RT-PCR and Nested PCR conditions were performed according to the manufacturer. Primer annealing temperatures performed in RT-PCR and nested PCR are shown.

Table 2: Prevalence of NS5A domain I amino acid polymorphisms associated with resistance to NS5A inhibitor in HCV infected patients.

Genotype/ Subtype	Number Patients genotyped	NS5A inhibitor resistance associated polymorphisms	DCV fold resistance	Reference
1a	42	Q30H/Q+Y93H/Y (1) M28V+Q30R (1)	93,136 350	[9] [9]
1b	23	Y93H (1) Y93H/Y (1) L31M (1) M28+Q30*(2)	19-24 3	[6,9] [6]
2a	4	L31M (4)	75‡ 350	[11] www.geno2pheno.org
2b	13	S58P (13)	5	www.geno2pheno.org
2c	6	K30R (1)	3	www.geno2pheno.org
2i	3	none	-	-
3	24	K30 (1)	44-62	[12,13]
4	23	S30 (1) L31** (2)	167	[15]

Amino acid residues at codon positions associated with Daclatasvir (DCV) resistance are shown in bold type. Number of patients are shown in brackets. ‡ Fold resistance to IDX719 NS5A drug inhibitor. *Patients genotyped as 1b by VERSANT LiPA 2.0 subsequently genotyped as 1a based on NS5A, Core/E1 and NS5B sequence data. ** Patients genotyped as 4r based on NS5A, Core/E1 and NS5B data.